University of Strathclyde
Faculty of Engineering
Department of Bioengineering

The Investigation of Di-(2-ethylhexyl) Phthalate (DEHP) Plasticiser Migration and Extraction from Medical Grade Poly Vinyl Chloride by *In Vitro* and *Ex Vivo* methods.

By
David Charles Horne MEng

A thesis submitted in fulfilment of the requirements for the degree of an Engineering Doctorate in Medical Devices

May 2011

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: *Date:*

ABSTRACT

Plasticised poly (vinyl) chloride (PPVC) is one of the most commonly utilised plastics in clinical practice. However, its has recently come under much scrutiny due to perceived adverse affects associated with migration of the plasticiser di (2-ethylhexyl) phthalate (DEHP) on contact of the PPVC with various biological solutions. This migratory behaviour, combined with DEHP being shown to be a toxin and an inflammatory mediator in animal and human models, has lead to further studies into its effects during extracorporeal interventions.

In this thesis the migration and extraction of DEHP from PPVC was investigated using carbon-14 labelled DEHP. The study included the development of a biomaterial test cell for use *in vitro* and in a novel *ex vivo* rat perfusion model.

In addition, the present study investigated DEHP migration in the clinical setting showing that plasticiser migration is a serious issue in pre-primed ECMO storage. We demonstrated the effectiveness of the radiolabel for migration studies, with DEHP concentration values that compared favourably with previous *in vitro* studies, also showing that the make up of the fluid exerts an influence on DEHP extraction with methanol (0.741±0.154mg/ml at 60 min; 2.21±0.02.16mg/ml at 300 min), blood (0.1067±0.0636mg/ml at 300 min) and plasma (0.0142±0.001652 mg/ml at 300 min) having differing values.

The *ex vivo* studies showed that DEHP or its major metabolite MEHP is located in various tissues post procedure. The plasticiser tissue levels depended on the means of exposure, with gavage and perfusion models producing different deposition profiles that favoured the filtering and lipid rich organs respectively. Critically, the perfusion studies also revealed substantial DEHP levels in the brain.

Although this thesis reports that carbon-14 is a suitable method of accurately detecting DEHP migration from PPVC, it is not the answer to this assay problem due to the logistical problems associated with radiation work. The *ex vivo* results also indicate the extrapolation of possible DEHP effects from perfusion interventions cannot be deduced from gavage studies due to the differences in deposition profiles.

ACKNOWLEDGEMENTS

I would like to firstly thank my supervisors, Dr J.D.S. Gaylor and Professor T. Gourlay, for their great support and belief in this project. Their continual encouragement and expertise were invaluable in the completion of this thesis.

I would also like to thank Liz Smith, for her help with the experimental aspects of the project as well putting up with the mountains of regulatory paperwork that were generated by the radiation experiments, it was appreciated greatly.

Thanks are also made to Professor Jim Courtney, who was instrumental in developing the project in its early days and for the constant encouragement when things started to go wrong.

Thanks are also due to Dr Jason Leadbitter and Mr Ross Law from INEOS Chlorovinyls Ltd, whose kind sponsorship and backing to the project was greatly appreciated.

I would also like to thank Dr Thomas Modine and Professor Serdar Gunaydin and everyone involved at their institutions in France and Turkey whose interest and support in this work greatly helped in the completion of some tricky experiments.

Thanks to all my friends, family and colleagues who have put up with my various rants during this project. The support was greatly appreciated.

Finally, a Big Thank you to my girlfriend, Laura, who has put up with me during the many highs and lows I experienced during this work and your support and encouragement was a constant source of energy and drive which I couldn’t have done without.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADP	Adenosine Diphosphate
ARC	American Radio-chemicals
ATBC	Acetyl Tributyl Citrate
ATEC	Acetyl Tri-2-ethylhexyl Citrate
ASTSDR	Agency for Toxic Substances and Disease Registry
BCD	Beta Cyclodextran
BSP	Sulfobromophthalein
BTHC	Butyl Tri-n-Hexyl Citrate
CERHR	Centre for the Evaluation of Reproductive Risks in Humans
CFD	Computational Fluid Dynamics
CHRU	Service de Chirugie Cardiovasculaire, Hôpital Cardiologique
CNC	Computer Numerical Control
CPB	Cardio-Pulmonary Bypass
CSTEE	Scientific Committee on Toxicity, Ecotoxicity and the Environment
DEHA	Di-2-ethylhexyl Adipate
DEHP	Di(2-ethylhexyl) Phthalate
DEN	N-nitrosodiethylamine
DIDP	Diisodecyl Phthalate
DINP	Diisononyl Phthalate
DNA	Deoxyribonucleic Acid
DOA	Di-octyl Adipate
DOP	Di-octyl Phthalate
DPM	Disintegrations Per Minute
EC	European Commission
ECB	European Chemical Board
ECMO	Extra-corporeal Membrane Oxygenation
ECPI	The European Council for Plasticisers and Intermediates
EDTA	Ethylene di-aminetetraacetic Acid
EPA	US Environmental Protection Agency
ESBO	Expoxsidsed Soybean Oil
FDA	Food and Drug Administration
HCl	Hydrochloric Acid

HMWK	High Molecular Weight Kinogen
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IL	Interleukin
IV	Intravenous
LOAEL	Lowest observable adverse effects levels
LSC	Liquid Scintillation Counting
MAC	Membrane Attack Complex
MEHP	Mono(2-ethylhexyl) Phthalate
MODS	Multiple Organ Dysfunction
MRSA	Methicillin-resistance Staphylococcus Aureus
NHS	National Health Service
NMR	Nuclear Magnetic Resonance
NOAEL	No observable adverse effects levels
OVA	Immunogen Ovalbumin
PE	Poly Ethylene
PEG	Polyethylene Glycol
PPARα	Peroxisome Proliferator Activated Receptor αlpha
PPVC	Plasticised Poly Vinyl Chloride
PVC	Poly Vinyl Chloride
RBC	Red Blood Cell
RTV	Room Temperature Vulcanising
SCENHIR	Scientific Committee on Emerging and Newly- Identified Health Risks
SIRS	Systemic Inflammatory Response Syndrome
SD	Sprauge Dawley
TEHTM	Tri-2-ethylhexyl trimellitate
TF	Tissue Factor
Tg	Glass Transition Temperature
TOTM	Trioctyl Trimellitate
TPN	Total Parenteral Nutrition
TXA_2	Thromboxane
UV	UltraViolet
VCM	Vinyl Chloride Monomer
WBC	White Blood Cell

1. Introduction

1.1. Medical Devices and Modern Medicine

Medicine is a vocation which is continually changing. Physicians constantly have to update their knowledge to provide the best possible care to their patients. Part of this changing face of medicine has been the incorporation of technology into the healthcare arena. The integration of this technology has greatly increased the capacity of the services that clinicians can offer to their patients which in turn has led to an increase in the level of care that is now available to the public.

Whilst many of the advances reported in the media have focused on new pharmaceuticals, the importance of mechanical, electrical and passive devices being integrated into the healthcare system should not be overlooked. The range of these devices is enormous; from large MRI imaging machines to the smallest computer controlled instruments used in neurosurgery.

The advances in devices are not just limited to mechanical or electrical components. The development in materials used in medicine has had just as great an impact as the development of such devices as MRI Scanners or heart pacemakers. One of the most important material developments over the last 50 years was the introduction of plasticised polyvinyl chloride (PPVC). Its utilisation as a replacement for the glass blood storage containers of the day was a revolutionary concept at the time. It soon became the blood storage container of choice and still remains so today (Figure 1.1).

The introduction of PPVC into the medical field soon led to its utilisation in a wide range of medical related devices. It has now become the most commonly found plastic within the hospital environment and it was estimated that in 2004 around 40,000 tonnes of PPVC was used in medical products throughout Europe. (Zhao & Courtney, 2009).

Today, the utilisation of modern biomaterials in medical devices is a multi-billion dollar business worldwide and research into development of new materials is continually ongoing.

1.2. Biomaterials and their Importance

The increase in new medical technologies and techniques has led to a massive increase in demand for suitable materials for use in these applications. The reliance on artificial materials to fulfil many different roles is now commonplace. The time for taking a material off the shelf and using it because it seemed the best option has now passed and many materials are now carefully tailored for the role that they play in the medical device. So what is a biomaterial you may ask?

A consensus definition of a biomaterial is: "A material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body" (2nd Biomaterials Consensus Conference, 1986, Chester, UK).
The development of biomaterials has in part helped in the development of many lifesaving procedures such as Renal Dialysis, Cardiopulmonary Bypass (CPB) and Extracorporeal Membrane Oxygenation (ECMO).

Whilst biomaterials play a substantial role in the large devices used in the above procedures, they also play major roles in much smaller devices, e.g. artificial heart valves (Figure 1.2), coronary stents and vascular prostheses (Figure 1.3). They are also involved in many devices which are utilised to improve the quality of life of the patient. The longevity of the current population has led to an increase in debilitating diseases such as arthritis and general wear and tear of joints reducing the quality of life of the sufferer. Joint replacement is an example of an area of medicine that has benefited greatly from the development of biomaterials (Figure 1.4).

Most of the materials used in the aforementioned areas are well known materials like PPVC, Poly ethylene (PE), stainless steel and titanium, but modern materials like silicone hydrogels are now becoming just as common as more and more uses for them become identified such as in soft contact lenses.

Biomaterials and their derivatives are therefore seen as an important part of modern medicine and their future development will continue to see further advances in medical devices and techniques.

Figure 1.1: Examples of PVC blood bags and collection tubing

Figure 1.2: St Jude Medical ® Inc mechanical heart valve

Figure 1.3: Terumo Vascutek Gelweave® aortic arch prosthesis.

Figure 1.4: Examples of artificial knee replacements produced by Stryker Healthcare

1.3. P-PVC: The DEHP controversy

As stated above, one of the most prevalent biomaterial polymers in use in medical devices is plasticised polyvinyl chloride. PPVC has been used in medical products for over 50 years. In addition to its major application in blood and blood product storage bags PPVC is utilised in IV infusion sets, catheters, parenteral feeding devices and inflatable splints. These diverse applications are in part due to the excellent material properties of PPVC (e.g. clarity, mechanical strength, flexibility) and also to its ability to be easily tailored to suit many different applications. Combining these properties with its cost effectiveness and proven track record, ensures that PPVC remains one of the most attractive biomaterials to manufacturers of medical products.

However, whilst PPVC has been used successfully for the last 50 years or so there are now a number of issues that have been brought to the publics attention regarding the safety of this material. The manufacture and disposal of PPVC involves the release of harmful chemicals such as dioxins which have been identified by the Environmental Protection Agency (EPA) as probable carcinogens and possible endocrine disruptors (ATSDR 1993).

Whilst this environmental problem is of a serious nature it is not the main issue surrounding PPVC. The problem of most concern surrounds the use of phthalates especially the plasticiser Di-2-ethylhexyl phthalate (DEHP) in the PVC.

DEHP is used to impart flexibility into an otherwise rigid polymer and can be up to 40% of the total polymer weight (Jaeger & Rubin, 1973). DEHP is known to leach out of the PVC into liquids that contact the material and, in the medical context, patients may be exposed to the plasticiser. Many government agencies such as the Food and Drug Administration (FDA) in 2001 and Health Canada in 2002 have issued warnings regarding the use of DEHP with certain patient groups who are possibly at a higher risk of developing adverse problems associated with DEHP.

The controversy surrounds the validity of these known problems in humans as the majority of the adverse effects that have been shown in animals are so far not

translated into humans or primates. It is the issue of cross species barrier and also the levels of DEHP used in the studies which are not relevant to many of the procedures humans undergo, which has led to the scientific controversy over DEHP.

Does the use of DEHP in medical devices actually increase the risk of the patient developing an adverse response? Or have the adverse effects seen in animals been blown out of proportion?

The answer to these questions is something that needs to be addressed sooner rather than later as the possible ramifications could be extremely wide reaching both clinically and industrially. Denmark has already banned the use of DEHP-plasticised materials in medical devices and Sweden is following suit, but is it really justified to ban a product that has been used for over 50 years just on suspicion? (Hansen, 2006)

1.4. Current Detection Methods

The controversy over DEHP and phthalate usage in medical PPVC has led to numerous studies into not only the toxicology and adverse effects of the chemical but also into the migratory nature of the chemical. Numerous ways have also been investigated to try and retard the migration of the DEHP in an attempt to negate the possible adverse effects associated with the DEHP.

Detection of the DEHP chemical is in part a complicated process due to the small size of the molecule especially when it is to be detected in biological fluids such as blood. The review of the literature yielded a number of techniques that have been used successfully over the years to detect DEHP and its metabolites. Namely,

- UV Spectroscopy (Zhao, 1999; French, 2001; Gourlay et al, 2001b; Horne et al, 2009)
- High performance Liquid Chromatography (Koch et al, 2005; Takatori et al, 2008)
- ^{14}C Radio-labelling (Waddel et al, 1976; Friocourt et al, 1979; Lhuguenot et al, 1985)
- Mass Spectrometry (Haishima et al, 2004; Takatori et al, 2004)

- Gas Chromatography (Karle et al, 1997; Ito et al 2005; Burkhart et al, 2007)

The lack of a reliable and accurate method of detecting DEHP in a wide variety of scenarios has hindered the progress in developing a full understanding of the mechanisms of migration of the chemical and its journey post exposure. The development of a technique that could allow for easy and rapid detection of DEHP would be of considerable benefit to researchers in this field.

1.5. Project Aims

The investigation of DEHP migration has been studied extensively, but is in no way complete. Most of the work has centred on static, *In vitro* experimentation. Many of these experiments have involved the incubation of the test material in various serums and solvents and then determining the DEHP content in these solutions. This, however, does not portray the type of conditions that the material would be exposed to in the clinical environment. The explorations of these conditions are an important part of the development into a more complete understanding of the migration of DEHP from medical plastics.

The aims of this research project are as follows:

- To investigate if plasticiser migration is an actual clinical problem
- To investigate whether the use of a radiolabel could be a possible standard method of determining plasticiser migration from PPVC
- To further the understanding of DEHP plasticiser migration from medical grade PPVC using *In vitro* experiments and a novel *Ex vivo* model.
- To determine the fate of migrated DEHP after exposure in a novel rat perfusion model and after gavages

2. P-PVC as a Bio-material

2.1. The History of Poly-vinyl Chloride

Poly Vinyl Chloride or PVC in its abbreviated form was accidentally discovered by different chemists on two separate occasions during the 19th Century. The first occurred in 1835, when Henri Victor Regnault, a French Chemist, first noticed the appearance of a white solid in flasks of Vinyl-Chloride that had been left in sunlight. This phenomenon was also reported by the German Chemist, Eugene Baumann, in 1872. These events are the first known production of Polyvinyl Chloride, but it was never clear if Baumann understood the polymeric nature of the compound that he and his peers had discovered at this early time.

In 1912 and 1913, two scientists by the name of Ostromislensky and Klatte both gained patents for the polymerisation processes for Vinyl-chloride, but due to the high decomposition at the processing temperatures neither of the scientists was ever able to successfully commercialise it.

In 1926, the Grisheim Elektron Company, relinquished Klattes patent freeing up the research field for other companies and it was in this year that the first useful form of PVC was developed by Waldo Semon, whilst working for B.F. Goodrich in the United States of America. He developed a method of plasticising the PVC by blending it with other additives to make it stable and resistant to degradation due to heat and light.

Mass production of PVC then began in earnest in Europe over the following two decades and during World War Two. Its first main use was as a rubber substitute for coating electrical wires. Since then, it has replaced rubber as an insulate and found many more uses to the extent that it is now the second most used plastic in the world today with in the region of 26 million tonnes being produced each year.

The vast majority of the P-PVC produced in modern society is used in the medical field. Since its revolutionary introduction in the 1950s by Walter and Murphy as an alternative blood storage bag to the glass storage containers of the day, it has gone from strength to strength and has become one of the most utilised blood contacting bio-materials to-date.

2.2. Poly-Vinyl Chloride: Structure and Production

Poly Vinyl Chloride production is a two stage process. The first stage is in itself a two part process. It firstly involves the actual production of the Vinyl-Chloride monomer and secondly the polymerisation of this monomer into long chains of PVC which is in a powder form. The second stage is the blending of the PVC powder with additives to produce a wide range of grades that are suitable for many different applications. Both stages will be out lined below.

2.2.1. Stage 1: Production of the Vinyl Chloride Monomer

The initial stage involves the use of salt, 57%, and oil, 43% as the raw ingredients. The salt is dissolved in water and is then decomposed using the process of Electrolysis to produce Hydrogen, Chlorine and Caustic Soda. The oil is treated separately from the salt and it is refined and by using a process called “cracking” the compound ethylene is produced. The next stage is to combine the ethylene and the chlorine. This then produces Ethylene Dichloride which can be seen in the following equation.

$CH_2=CH_2 + Cl_2 \rightarrow CH_2Cl\text{-}CH_2Cl$ (1.1)

The Ethylene Dichloride is then subjected to further “cracking” to produce the Vinyl Chloride monomer (VCM).

$CH_2Cl\text{-}CH_2Cl \rightarrow CH_2=CHCl$ (1.2)

It is this monomer that is the building block for the actual PVC. The PVC is formed by an addition polymerisation of the VCM monomer.

Modern techniques also apply an oxy-chlorination technique that uses recycled HCl to produce more Ethylene Dichloride. This is known as a balanced process which is seen to be more cost efficient.

The polymerisation of this VCM into long chains of PVC can be achieved through three different methods,

- Suspension Polymerisation
- Emulsion Polymerisation
- Mass or Bulk Polymerisation

Each of which are defined and their various differences outlined below.

2.2.2. Bulk Polymerisation

Bulk Polymerisation, also known as Block or Mass polymerisation, is a polymerisation process that does not contain any form of solvent. It involves only the monomer, polymer and an initiator. The polymer is soluble in the monomer and the viscosity of the solution increases as the polymerisation reaction progresses until all the stock monomer is used up. The main advantage of this polymerisation technique is the absence of a solvent, which would be required to be removed before the final processing stage of the polymer. The removal process can be a difficult and time consuming process.

2.2.3. Suspension Polymerisation

Suspension Polymerisation, also known as pearl polymerisation, is a process which involves the dispersion of water insoluble monomer droplets with diameters of between 0.1- 1mm in a liquid phase, which is usually water, by mechanical agitation whilst in the presence of water-soluble suspending agents. The main advantage of this technique is the easy removal of heat from the process by the low viscosity aqueous suspension medium. Hence, it is a popular industrial method for the vinyl chloride polymerisation process, which is an extremely exothermic reaction. The disadvantage of this method is the need for the removal of the suspending additives from the polymer beads. A schematic of the suspension polymerisation process is shown in Figure 2.1.

2.2.4. Emulsion Polymerisation

Emulsion Polymerisation is a process that has similarities to suspension polymerisation yet has major differences as well. Both reactions involve the polymerisation of monomers that are insoluble in water in an aqueous dispersion, but the emulsion reaction involves the use of a monomer insoluble initiator. It is the difference in this initiator that causes the change in the mechanisms and kinetics compared to the other forms of polymerisation. As well as having a different type of initiator, there are also other differences that set emulsion polymerisation apart from the other processes. The first is the size of the final particles; they are in the order of 1μm compared to the 0.1-1mm size of the suspension particles. The second difference is that the suspension agent is now called an emulsifying agent and it is present in a much larger quantity due to the much larger surface area of the particles.

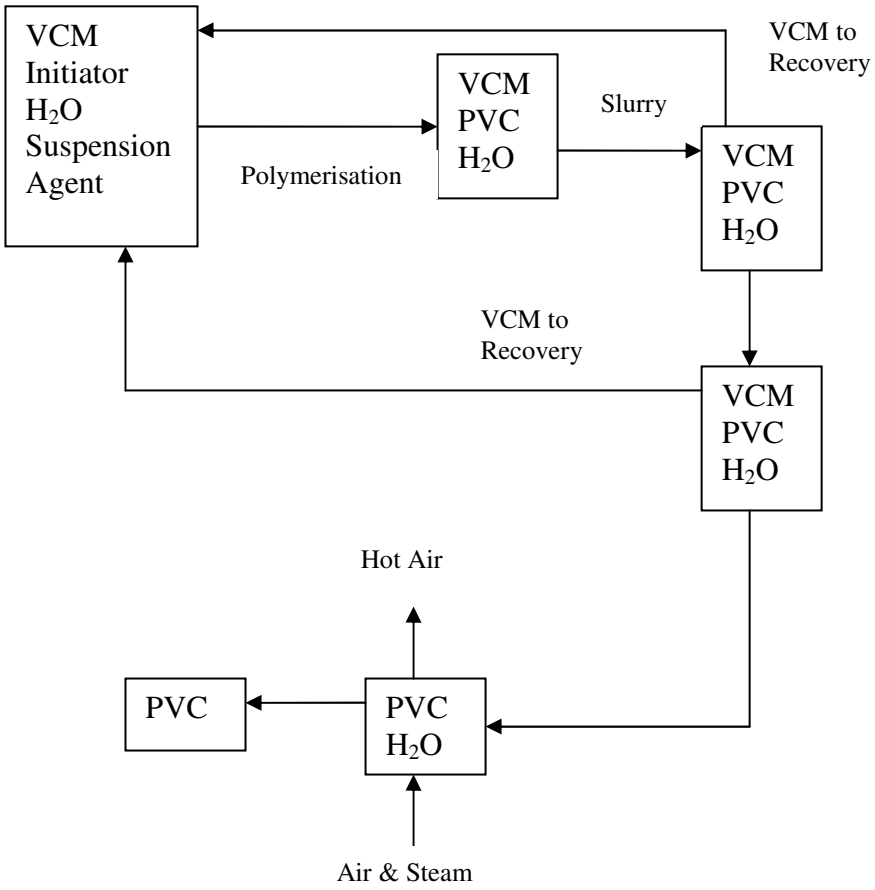


Figure 2.1: Diagram of Suspension Polymerisation Process (adapted from Whelan & Craft, 1977).

2.2.5. P-PVC Additives

The end product from these polymerisation processes is a thermoplastic which takes on the appearance of a white powdery substance. This is the PVC in a form that is not entirely useful. To make it useful the second production stage must be completed. This involves the blending of the PVC powder with various other ingredients to produce different formulations for a wide range of uses. Below is a list of additives which are routinely incorporated into PVC to make it a more useful product.

- Plasticiser
- Stabiliser
- Colorants
- Flame retardants
- Filler materials
- Pigments

The make up of the Plasticised PVC (PPVC) depends on the function it is being produced for. In the case of Medical products, a typical formulation for sheets used in blood bags and extruded tubes can be seen in Table 2.1 and Table 2.2 respectfully.

PVC Medical Grade Sheets and Films	Parts Per Hundred
PVC Resin	**100**
Plasticiser	**40-66**
Mineral Filler	**5-15**
Stabilizer	**1.5**
ESO (epoxidised Soybean oil)	**3**
Pigments	**X**

Table 2.1: Typical formulations for Medical Grade Sheets and Films (Provided by Ross Law of INEOS Chlorovinyls)

PVC Medical Grade IV & Blood Bags	Parts Per Hundred
PVC Resin	**100**
Plasticiser	**50**
Stabilizer (CaZn)	**0.6**
Lubricant (Calcium Stearate)	**0.3**
ESO (epoxidised Soybean Oil)	**3**

Table 2.2: Typical Formulation for Medical Grade I.V. Tubing and Blood Bags (Provided by Ross Law of INEOS Chlorovinyls)

There are various reasons behind the addition of some many other products into the PPVC but each different additive is used for a particular reason. An example of why each additive is used will be given in the following sub-sections to demonstrate how each affects the properties of the final PVC product.

2.2.6. Plasticisers

The first and most abundant additive is that of the plasticiser. This compound is used to impart flexibility to the otherwise rigid PVC. The more flexible the PVC is required to be the higher the amount of plasticiser will be present. There are many different kinds of plasticisers used in PPVC but the main plasticiser group that is used in Medical grade PPVC is the Phthalate group. Much has been debated about the safety of these plasticisers, especially Di(2-ethylhexyl) phthalate or DEHP, which is the most used of all the plasticisers in Medical PVC but as of yet it is the only plasticiser that has been certified safe to use in Medical products (SCENHIR, 2008). More on the phthalate plasticisers will be discussed in later chapters.

2.2.7. Mineral Fillers

Mineral fillers are used for a number of reasons in PPVC. These compounds are cheaper than PVC resin and hence their use as a replacement instead of the more costly PPVC resin reduces the overall cost of the product. The material properties of the filler also provide improvements in both processing and bulk material properties of the final PPVC. Some of the advantages that can be gained from the addition of fillers are:

- Better Impact Strength & increased Stiffness

- Less Sticking to metals

Examples of popular Fillers are:

- Calcium Carbonate
- Dolomite
- Kaolin
- Chalk

2.2.8. Heat Stabiliser

Another additive that is found in all PVC grades is a Heat Stabiliser. These are used as PVC resin is not stable at the temperatures used during the processing techniques. A heat stabiliser is therefore needed to stop the PVC decomposing during production. An example of a stabilizer is the Calcium/Zinc mix. This is used as it has a low toxicity level as well as having good heat and light stability. It also works well with its organic co-stabiliser the Epoxsidised Soybean Oil, providing good heat stabilising effects as well as acting as a secondary plasticiser.

2.2.9. Lubricants

The aim of the lubricant added to the PPVC is to provide both internal and external lubrication properties to the material. The internal lubrication is to help reduce the friction between the PVC particles during the compounding process, where as the external is to help control the friction and/or the release of the compounded PPVC material from hot metal compounding rollers used in its manufacture. A further advantage is the provision "Anti-Block" effect for end products. An example of this would be to stop PVC films sticking to each other, if they were stacked on-top of each other.

2.2.10. Pigments

These are used in PPVC to colour the plastic to whatever shade it is desired to be. For Medical Purposes the PPVC is generally required to be translucent and this is achieved by adding an aquamarine pigment.

2.3. Uses of Plasticised Poly-Vinyl Chloride

2.3.1. Introduction

Plasticised Poly-vinyl chloride (PPVC) is one of the most widely used plastics in modern society. Its ability to be produced in many different formulations with a wide range of physical properties has allowed it to find a use in nearly every aspect of modern life. The following list shows a number of the areas in which P-PVC can be found:

- Building and Construction
- Toys
- Transport
- Consumer Goods
- Cables
- Packaging
- Medical Applications

This list shows that everyone will come into contact with some form of PPVC at some point within their everyday activities, whether it is opening a pack of sandwiches or playing with a child's toy. The largest use of PPVC in modern society is the last entry on the list, that of Medical Applications.

It is by far the most common polymer used in medical products and has been used in this way for over 50 years due to its ability to adapt to the changing needs of this constantly advancing field. It first came to prominence by replacing glass and rubber in single use pre-sterilised components in the likes of blood bags and IV systems (Figure 2.2). A number of its uses as external communication medical products are illustrated in Figure 2.3. Its popularity is due in-part to its ease of processing and fabrication but also to its chemical stability and its perceived inertness, which provides the basis for a safe product that can be used within the body without causing harm. The advantages of using PPVC in this field are numerous, but the main reasons will be discussed in a later section. PPVC is not only found in Blood

contacting devices but also many other products. PPVC is the most abundantly used plastic in medical applications with over 25% of all devices having some sort of PVC component (http://www.pvc.org/How-is-PVC-Used/PVC-for-Health).

The following table (Table 2.3) that has been adapted from Zhao and Courtney 2009, highlights a number of the medical products that PPVC is found in.

Applications of PVC-P biomaterial in the Clinical Environment	
Pharmaceutical solution packaging or delivery sets	Intravenous solution pack, IV sets, Peritoneal dialysis solution packs, Endotracheal tubes, Connectors
Medical disposables	Gloves, syringes, Drainage tubing or bags, Urinary bags and tubing Other surgical products
Medical building products	Waterproof mattress sheets, Wall-coverings, floor-coverings, Electrical systems Appliances and furnishings, Oxygen tents
Tissue-contacting biomaterials	Burn dressings, Artificial skin, Other surgical dressings
Biosensor or enzyme electrodes	Glucose biosensors, Protamine-sensitive polymer membrane electrode, Ion-sensors
Drug-delivery system	Prostaglandin-releasing polymers, Fungicidal and bactericidal additive-releasing PVC

Table 2.3: PPVC biomaterial applications that are found in the clinical environment other than the standard blood contacting applications.

2.3.2. Alternative Healthcare Applications of P-PVC

PVC is not only used in medical products that directly interact with the patient but is also used in products that indirectly interact with the patient in the surroundings. The above table details a few of the alternative applications for PPVC in the hospital environment.

Even small areas like those mentioned above do have a large effect on the overall healthcare environment. Today, infection control is one of the largest areas of hospitals expenditure and any products that can help reduce or eliminate the chance of cross contamination between patients will be embraced by the healthcare sector.

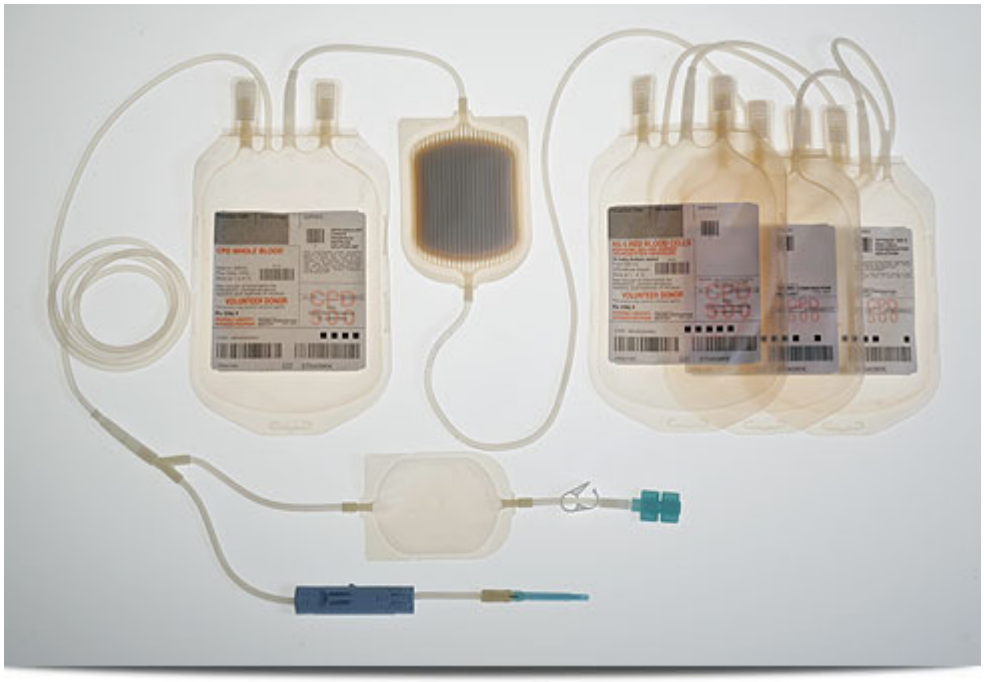


Figure 2.2: Terumo IMUFLEX WB-RP Blood bag system with leukocyte reduction filter. ©Terumo Medical Corporation. All rights reserved.

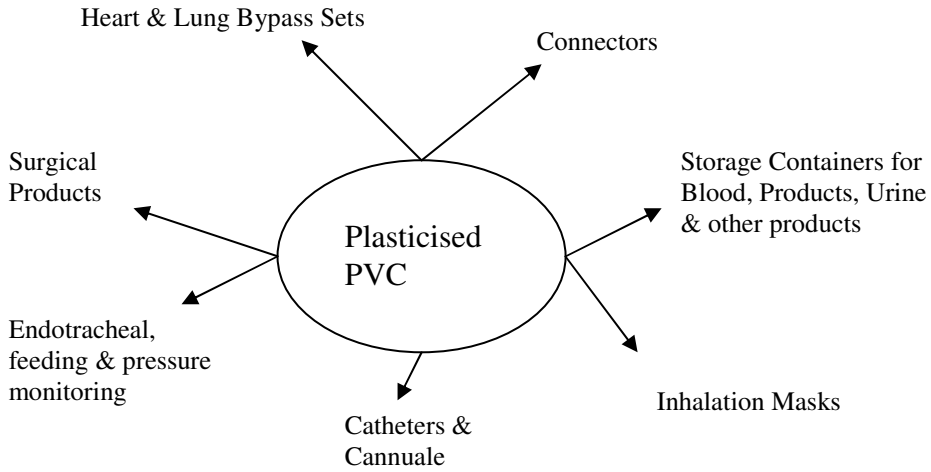


Figure 2.3: Uses of PPVC in Medical Applications

2.3.3. Advantages of P-PVC in the Use of Medical Products

There are many advantages for the use of PPVC but there are key properties which represent the main reasons for the popularity of the PPVC within this field, these include:

- Bio-compatibility
- Cost
- Safety
- Flexibility and Durability
- Sterilisation

2.3.3.1. Sterilisation

This is an extremely important facet in modern healthcare especially with the onset of multi-drug resistant *staphylococcus aureus* bacterium related infections (MRSA) in hospitals. The utilisation of materials in medical devices that allow for quick and easy sterilisation is an important aspect in reducing the impact of these new hospital based infections. PVC products provide an excellent material choice for this category as they can be sterilised by a range of different methods such as steam, ethylene oxide without changing any of their properties.

2.3.3.2. Flexibility and Durability

Many of the modern devices used in healthcare require the materials to provide both a great deal in flexibility whilst also providing good durability over a range of temperatures. An example of this is Dialysis or Cardio-Pulmonary Bypass Tubing, IV sets and blood bags. PVC has been used for many years in each of these products as it flexible enough to be produced in different grades that fulfil the requirements of all current applications, yet it still has the strength and durability which is demanded of a modern day medical product.

2.3.3.3. Safety

All components used in medical devices and the materials that are used to make them must undergo stringent testing, so as to fully understand their toxicological effect on the human body before they are deemed suitable to be used as a medical product.

The gaining of a CE Mark or FDA approval is a must if the device or material is to be accepted by the medical fraternity. All of the current knowledge and expertise in the healthcare sector deems that PVC is safe to use in medical devices (SCENHIR, 2008).

2.3.3.4. Cost

The current healthcare climate is one in which technology plays a substantial role. The cost of performing a Coronary Bypass Operation for the NHS can range from £7000-£9,000 per operation according to the NHS procedure costs data sheet for 2010-2011 and with around 29,000 of these operations being carried out every year in the UK (www.heartstats.org) this equates to a large investment by the NHS.

PPVC is a relatively low cost material, and despite its key role in many complex medical appliances, it contributes little to the overall costs in the healthcare sector. Indeed, the low price movement in PVC cost over the years suggests some degree of cost maintenance in healthcare where PVC is a key component. The alternatives to PVC for most of these applications would be prohibitively expensive and may impact negatively on healthcare provision.

2.3.3.5. Biocompatibility

The area of Bio-compatibility is probably the most important aspect of any artificial material that is to come in contact with human tissue and fluids. It is a field that has been interesting scientists and engineers for years and will continue to do so until a material can be created that produces no response at all from the body's immune system. Until this development arises, the best situation is to use a material that has a high biocompatibility level and therefore produces as low an immune response as possible which is important if the material is in long term contact with the tissue or fluid.

PVC is one such material that is said to have a reasonable level of biocompatibility in its own right. However, the chemical composition of PPVC allows for particularly easy modification of the base material in an attempt to improve its overall biocompatibility by incorporating other molecules on and into its surface. Hence it

has been the subject of a number of studies involving the incorporation of such compounds as Heparin (Kim et al, 1976; Svenmarker et al, 1997; Weber et al, 2002; Belboul et al, 2000), BetaCycla Dextran, BCD, (Sreenivasan 1996; Zhao 1999; French 2001; George 2007; Zhao & Courtney 2007) to try an improve the biocompatibility of the PPVC.

Overall, PVC is seen within the medical fraternity as a material that can fulfil the many different roles asked of it whilst still being able to meet all of the performance and safety criteria deemed necessary for a medical product at the minimum cost.

2.4. Biocompatibility and Blood Compatibility

PVC is widely recognised as having excellent biocompatibility - a characteristic that has made it a preferred material for blood contacting medical devices over the last 50 years.

One of the main areas within the biocompatibility section is the interaction between the blood and the material or blood compatibility of the material. It is commonly known that the plasticiser in the PVC is leached from the surface of the material by the contacting medium and therefore it is convenient to review its compatibility in terms of interactions, factors that influence blood response and evaluation procedures (Courtney et al, 1994). This would allow for greater understanding of the changes to the blood that are caused by the contact with the artificial biomaterial which would allow not only for a better utilisation of the current biomaterial but also for improved biomaterials in the future (Figure 2.4). (Courtney et al, 1994)

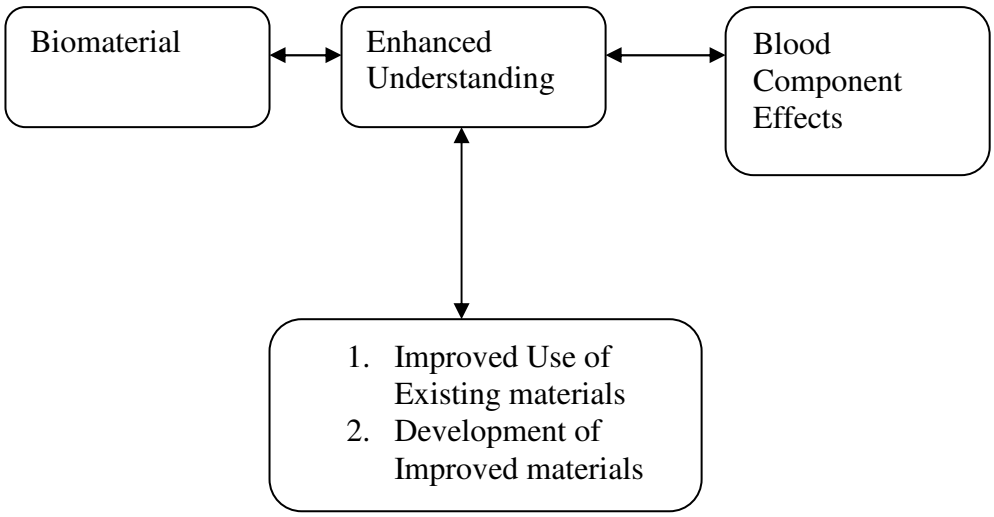


Figure 2.4: Biomaterial-Blood Interaction Investigation Objectives

It is important to define what a blood-biomaterial interaction actually is. One such definition is as follows:

"Any interaction between a biomaterial (device) and blood or any component of blood, resulting in effects on the biomaterial (device), or on the blood, or any organ or tissue. Such effects may or may not have clinically significant or undesirable consequences." (ISO/TC 194, 1991; Missirlis, 1992; Zhao, 1999)

The reaction that occurs when blood comes into contact with the artificial material is "a highly complex set of separate interactions which involves the rapid onset of a sequence of processes that partly occur simultaneously". This hypothesis by Courtney et al (1989), has been generally accepted and is demonstrated in Figure 2.5.

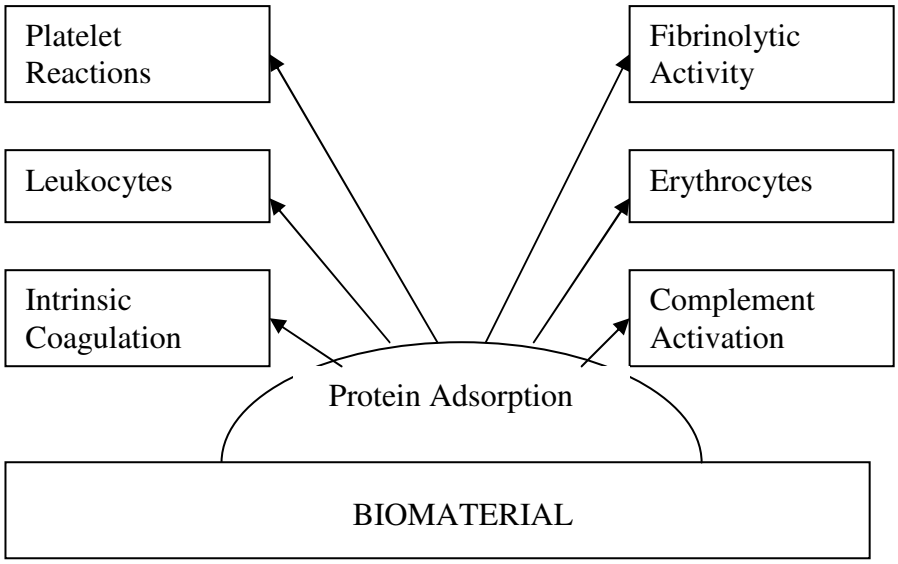


Figure 2.5: Blood biomaterial response reactions that occur when biological fluids come in contact with artificial materials (adapted from Courtney et al, 1994)

2.4.1. Plasma Protein Adsorption

The first observable event at the interface between an artificial surface and the blood is adsorption of the plasma proteins onto the surface (Courtney et al 1994a) and occurs immediately after the blood coming in contact with the material (Sawyer & Pate, 1953; Sharma, 1981).

The nature of the protein layer depends on a number of factors such as contact duration and the surface characteristics of the material and it is this initial protein layer that affects the subsequent reactions of the blood with the biomaterial surface. Vroman in 1969 showed that the proteins, that have high concentration levels in the blood, adhere themselves to the artificial surface in the first instance. These initial proteins are then partially replaced by less diffusible trace proteins that have a stronger affinity to the surface and this in turn influences the inflammatory response of the artificial surface. This initial adhesion and replacement of proteins is commonly known as the "Vroman effect" and an example of this is the replacement of adsorbed fibrinogen by high molecular weight kininogen (HMWK). (Vroman &

Adams, 1969). Table 2.4 lists the various proteins (and their concentrations) that are present in human plasma.

Protein	**Concentration mg/100ml Plasma**
Albumin	3500-5500
IgG	800-1800
Fibrinogen	200-450
α_1-Lipoproten	290-770
α_1-Antitrypsin	200-400
β-Lipoprotein	200-650
Transferrin	200-400
IgA	90-450
IgM	60-250
IgE	~300
α_2-Macroglobulin	160-370
Hapatogloblin 1-1 type	100-220
2-1 type	160-300
2-2 type	120-260
> 100 known minor plasma proteins	<100

Table 2.4: Protein composition of human plasma (adapted from Weiss, 1983).

There has been a substantial amount of work performed in this field as it is these adsorbed proteins that are able to trigger defence mechanisms such as the complement activation and the coagulation cascade in blood when they undergo certain changes on contact with the artificial surface (Lassen & Malmsten, 1997).

Much of the work conducted into protein adsorption has focused on albumin (Lee & Kim, 1979) and fibrinogen (Packam et al, 1969; Zucker & Vroman, 1979). Albumin that has been adsorbed onto the surface of the material has been associated with the reduction of biological reactions such as the inhibition of thrombus formation and reduced platelet and leukocyte adhesion, all of which are desirable properties in blood contacting biomaterials. Fibrinogen has been studied because of its close relationship with platelets and its role in the coagulation cascade. There have also been studies of other proteins such as γ-globulin, and its role in the promotion of platelet and leukocyte adhesion, fibronectin and its influence on leukocyte adhesion

and plasminogen and its role in surface mediated fibrinolytic activity (Courtney et al, 1994a).

Fibrinogen has been found to promote the adhesion of cultured human monocytes and platelets to artificial materials such polystyrene, stainless steel and a stainless steel with a diamond like carbon coating (Shen & Horbett, 2001; Santin et al, 2004; Ratner, 2007). Fibrinogen also plays an important physiological role in the mechanism of haemostasis by forming a dense fibrin network in the presence of thrombin as part of both the intrinsic and extrinsic coagulation cascades (Mosesson, 1990).

Fibrinogen has also been shown to mediate an acute inflammatory response to implanted materials in both animals and also in humans by attracting neutrophils and macrophages. (Tang & Eaton,1993; Zdolsek et al,2007). One of the most studied areas is that of the interactions of fibrinogen and platelets and in particular the promotion of platelet reactivity by absorbed fibrinogen (Courtney et al 1994c).

Research on albumin has focused on the ability of this protein to reduce the adhesion of both platelets and also leukocytes to artificial surfaces as well inhibiting thrombus formation, all of which are desirable in a biomaterial and hence attempts have been made to incorporate albumin into the biomaterial to try and improve blood compatibility. (Courtney et al, 1994a; Piskin, 1992)

2.4.2. Platelet Adhesion

Platelets are anuclear disc- shaped cells with a diameter of 2-4μm and their main role is to close any apertures in the vascular system by forming a platelet plug. They are derived in the bone marrow from megakaryocytes and they circulate around the vascular system with an average concentration in healthy subjects of around 350,000/μl. (Martini 2006a)

Platelet adhesion onto an artificial surface is controlled by the initial process of protein adsorption and the degree to which it adheres to the surface is dependent on

the type of protein that has been adsorbed, e.g. a higher degree of adhesion would be seen with fibrinogen compared with albumin. (Pitt, 1985; Courtney,1994b; Ratner, 2007)

There are other factors that affect the adhesion of platelets to artificial surfaces such as shear force and shear rate, contact time as well as red cell concentration and von Willebrand factor. (Courtney et al, 1995; Baumgartner et al, 1980; Walker et al, 1983)

The platelets adhere to the surface of the material in either a monolayer or aggregate formation. Morphological studies have shown that the first layer of platelets display the characteristics of activated platelets, highlighted in the following section, as they adhere as flattened and degranulated cells as well as possessing pseudopods. (Baumgartner et al, 1976)

2.4.3. Platelet Activation

Once the platelets have adhered to the surface of the material they produce a response that is referred to as the "platelet release reaction". These reactions are known to be heavily involved in starting and maintaining the subsequent reactions.
A minimal amount of stimulus is all that is required to activate platelets. This is achieved when the platelet surface comes into contact with any thrombogenic surface such as an artificial material (Gorbet, 2004).

The activation of platelets is known to result in five physiologic responses, (Gorbet et al, 2004)

1. A platelet release reaction occurs in which biologically active compounds such as platelet factor 4, thrombospondin, ADP and serotonin stored are secreted into the micro-environment from the platelet.
2. The cell surface glycoprotein, P-selectin is released and expressed on the platelet membrane after α granule secretion. This plays an important role in controlling the adhesion of the activated platelets to leukocytes. (Gorbet, 2004)

3. The platelet eicosanoid pathway is initiated, which releases arachidonic acid from platelet phospholipids and in turn causes the synthesis and release of prostaglandins and thromboxane B2.
4. A significant change in shape occurs, promoting better platelet to platelet contact and adhesion.
5. Platelet activation results in the formation of micro particles, which are formed from the surface membrane by exocytotic budding. These micro particles can bind and adhere to fibrinogen and fibrin as well as producing co-aggregation with other platelets.

2.4.4. Platelet Aggregation

Platelet aggregation is a build-up of platelets in an interaction between the activated platelets that are already adhered to the artificial surface and newly arriving platelets. The aggregation process is a two stage process. The first stage is the interaction of single platelets to form small aggregates that have loose platelet attachments. The reaction occurs in the presence of low concentrations of aggregating agents such as adenosine diphosphate (ADP) or Thromboxane (TXA_2). This initial stage is reversible but if the ADP or TXA_2 are present in higher concentrations then the second irreversible stage of aggregation is initiated. This produces much larger and tightly bound aggregates. Over time, the aggregates become associated with fibrin and other cells like leukocytes and proliferation of these thrombi can lead to separation from the artificial material, which can lead to blockages of blood vessels and cardiovascular problems (Courtney & Forbes, 1994).

2.5. Fibrinolysis

The fibrinolytic system removes unwanted fibrin deposits to aid the healing process after injury and inflammation by the fragmentation of fibrin into soluble parts. The levels of activation of the fibrinolytic system from artificial material contact can be studied by the analysis of the levels of fibrin degradation products present post procedure (Walker 1983; Forbes & Courtney, 1994; Coleman et al, 1994).

The process involves the protease plasmin which is created by a reaction involving its precursor plasminogen and an activator. The plasmin then degrades the fibrin over time to produce well characterised fibrin degradation products. (Walker, 1983) A simplified schematic can be seen in Figure 2.6. There are many activators for this reaction such as factors XIIa and XIa as well as kallikrein all of which are produced by blood/material contact (Courtney et al, 1994a).

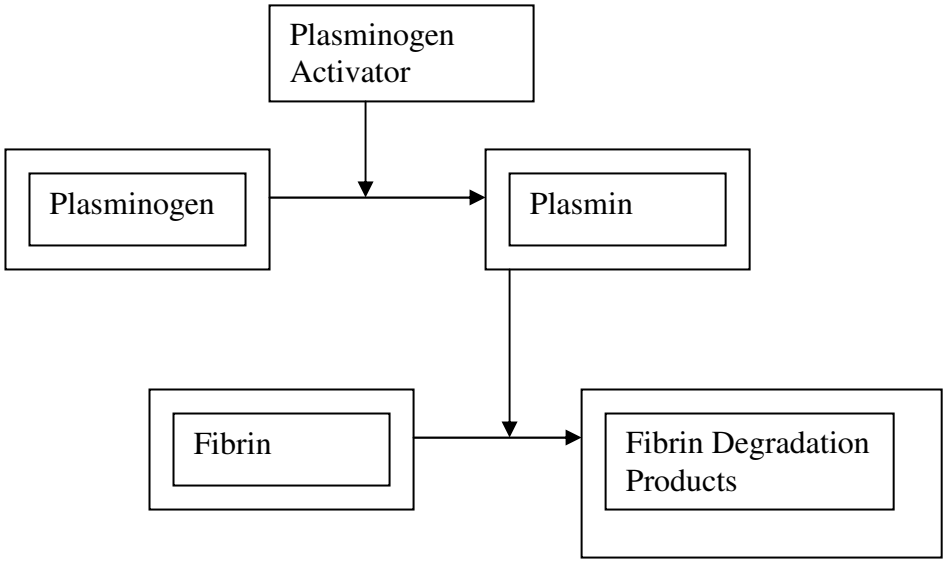


Figure 2.6: Schematic outlining the fibrinolytic sequence (adapted from Jones, 1989)

2.6. Cellular Components

2.6.1. Erythrocytes (Red Blood Cells)

The role of erythrocytes in blood/biomaterial interaction is one of the least discussed areas of the blood interactions, even though these cells constitute around 96% of the total cell volume in the blood. The "activation" of the erythrocytes is believed to happen in a localised response to the biomaterial. It is thought that there is a release of certain lipids and fatty acids from the cell membrane which causes adhesion of the erythrocytes as well as a significant change in the cell membrane metabolism and even haemolysis (Buck et al, 1969).

The haemolysis of the red cells causes the release of adenosine diphosphate (ADP) and haemoglobin, with the ADP increasing the stimulation of platelet aggregation whilst the haemoglobin is adsorbed onto the surface of the artificial material (Brash & Lyman, 1969).

Further work by Mohandras et al. in 1974 has provided a better understanding of the adhesion of the red blood cells (RBCs) to the material surface. They showed that the adhesion of the RBCs is strongly influenced by the material surface morphology and chemical structure and also by the local fluid dynamic conditions (e.g. the shear rate at the blood-material interface.) (Mohandras et al, 1974).

2.6.2. Leukocytes (White Blood Cells)

Leukocytes are the main line of defence for the human body against anything that enters into its environment, be it a material or a virus. The circulating medium consists of the three main leukocyte groups, namely, granulocytes, monocytes and lymphocytes (Martini 2006b) It is these cells that mediate the body's inflammatory response to foreign materials and pathogens.

The interactions of these cells with an artificial material is controlled by the initial protein layer that adheres to the materials surface and also depends greatly on which of the three groups of cells attaches itself to this layer. It has been suggested that

within blood contacting environments, the leukocyte response is linked to the relationship between the leukocytes and also complement activation (Courtney et al, 1994a), with the adhesion to artificial materials appearing to be mediated by the complement product C3b (Gorbet et al, 2004).

The three groups of leukocytes are produced in the bone marrow and lyphoid tissue and are transported from these sites to the target areas by the circulation soon after their creation due to their short lifespan.

The different groups of cells react in different ways. For example, granulocytes are known to de-granulate in the presence of inflammatory stimuli in an attempt to destroy the invading pathogen. On de-granulation, the granulocyte releases other chemical agents such as leukotrienes which attract more and more of these cells which in turn causes the pathogen to remain trapped.

Neutrophils are by far the most abundant of the leukocytes with a percentage of around 50-70% of the total circulating amount of white blood cells (WBCs). Even though they have an extremely short lifespan of 8-20 h in blood, after an inflammatory stimulus this lifespan can increase by three fold (Gorbet et al, 2004) as well as increasing their activity.

Monocytes react in a different way to granulocytes as they undergo morphological and physiological changes to become macrophages. These cells then have the ability to adhere to the damaged site, activate other cells and also perform phagocytosis.

Monocytes make up around only 2-8% of the total number of white blood cells with a circulating concentration of 0.2×10^6 -1.0×10^6 per ml (Gorbet et al, 2004). The lifespan of such cells in the circulation under normal circumstances is fairly short (24 h), after which they migrate from the circulatory system into tissues where they develop and change into macrophages (Gorbet et al, 2004).

2.7. Coagulation Cascade

The coagulation cascade is a biological process that involves as series of reactions which concludes with the formation of a fibrin clot (Gorbet et al, 2004). Thrombin is formed by a cascade of events in one of two pathways, the intrinsic or extrinsic, each of which join a common pathway where pro-thrombin is converted to thrombin. Thrombin is then used to cleave fibrinogen to produce fibrin. The fibrin clot is then stabilised by Factor XIII, which has been activated by the thrombin, by converting it to an insoluble fibrin gel.

The coagulation cascade can be seen in Figure 2.7.

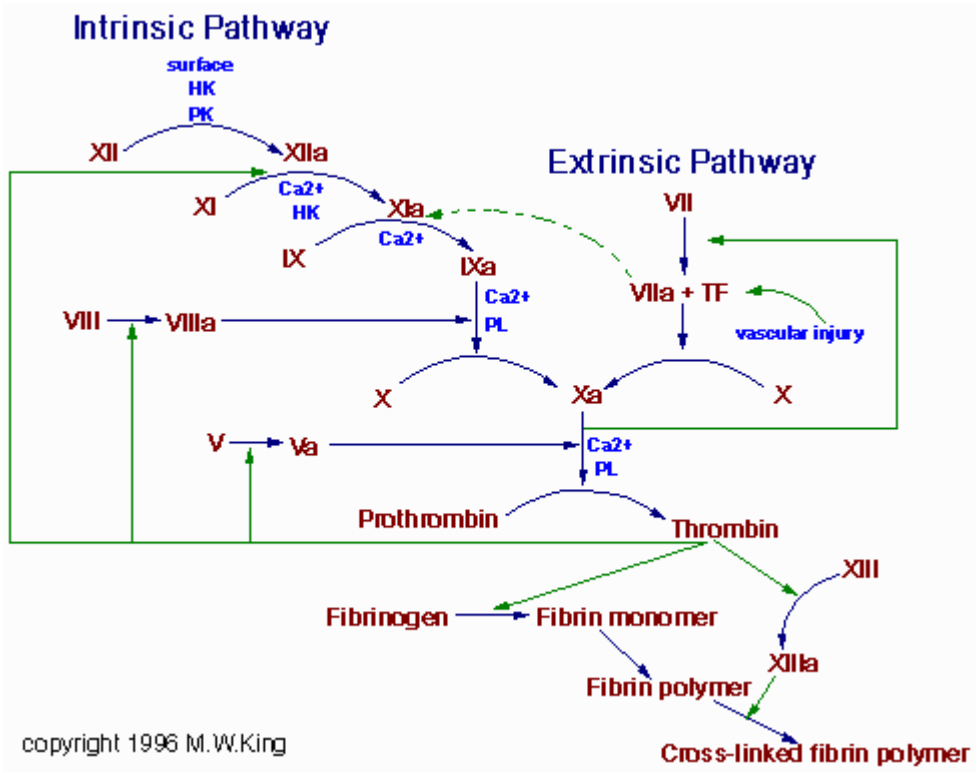


Figure 2.7: Coagulation Cascade (http://www.med.unibs.it/~marchesi/blood.html)

The intrinsic pathway can be seen on the left-hand side on the above diagram. It is also known as the "contact activation" pathway as it is activated by contact with artificial materials as well as exposed endothelial cell surfaces.

The chain reaction is started by the activation of the Hageman Factor (or Factor XII), by high molecular weight kininogen (HMWK) which itself has been activated by

contact with the foreign material. The newly activated Factor XIIa then activates Factor XI to Factor XIa. This activated factor is then involved in the activation of Factor IX to IXa. It is the reactions of this activated factor and the intrinsic tenase complex, (Factor VIIIa) combined with certain co-factors that then cause the cleavage of Factor X to the activated Factor Xa, which acts in the common pathway along with other cofactors such as Ca^{2+} and platelets, to cleave the pro-thrombin to thrombin.

The monitoring of the amount of Factor XIIa in the blood is used as a method to detect that contact activation has occurred. Even though this part of the cascade system has been explored greatly, there remains doubt over its importance in the *in vivo* environment. This is because of the low availability of a negatively charged surface to activate the HMWK, pre-kallikrien and Factor XII, *in vivo*.

The extrinsic pathway is initiated by exposure of the blood to tissue factor (TF) caused by vascular injury. The plasma factor VII binds to the TF on the cell membranes and is then activated by one of the following compounds, thrombin, Factor IXa, Factor Xa and TF-VIIa. The activated complex, TF-VIIa, then cleaves Factor X to its activated form, Factor Xa, in the presence of calcium as well as playing a secondary role in activation of Factor IX. The activated Factor Xa then moves into the common pathway and continues the cascade as previously mentioned. The two pathways do not operate independently. When the coagulation is initiated by a TF- pathway, the intrinsic tenase continues to have an important role, since the production of FXa by FIXa by FIXa-FVIIIa complex has been shown to significantly contribute to thrombin generation (Lawson et al, 1994). Further to this it has been shown that both the intrinsic and extrinsic tenases play important roles in the onset and propagation of coagulation respectively (Rand et al, 1995).

2.8. Complement Activation: The Intrinsic and Extrinsic Pathways

The complement system is made up of 11 plasma proteins that interact with one another to "complement" the action of antibodies.

These activated proteins play a major role in the hosts defence mechanism against infection and "foreign body" invasion. They facilitate processes such as phagocytosis, regulate the hosts immune response as well as mediating the acute inflammatory response (Martini 2006). Due to the ability of the complement system to cause major disruption to the hosts haemostasis, it is carefully regulated to prevent unwanted problems arising.

It is generally accepted that there are two main pathways involved in the complement activation phase. These are the classical and the alternative pathways. Each are activated in different ways, with the classical being activated by antibody-antigen complexes, whereas the alternative pathway is activated when a previously activated complement component attaches to the surface of a pathogen (Gorbet, 2004).

Figure 2.8, shows a basic flow diagram of the two main pathways to complement activation and the results of activating this system.

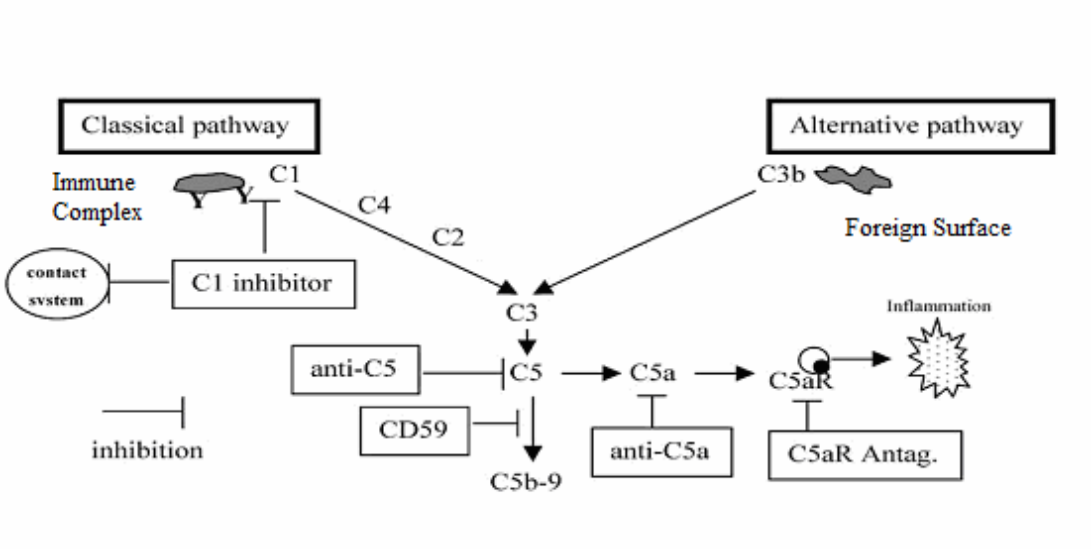


Figure 2.8: The classical and alternative pathways of the complement system are shown along with various natural and synthetic inhibitors and their points of action for inhibiting downstream portions (C3 and C5) of the complement cascades; only C1 inhibitor is shown as being effective at a level prior to the formation of the C3 or C5 convertases (Gorbet, 2004). Reproduced with Copyright permission from Elsevier.

2.8.1. Classical pathway

This pathway involves antigen-antibody complexes or occasionally direct contact with the surface of a pathogen that bind the various C1 complex with the C1 component. This in turn then cleaves the C1 component to produce an active serum protease which cleaves the C4 to C4a and C4b in the first branch of the complement cascade (Favoreel et al, 2003).

2.8.2. Alternative pathway

The alternative pathway is the default process and is important in the defence against bacteria (Martini 2006c). It consists of spontaneous breakdown of the complement protein C3 in serum by hydrolysis, to produce fragments of C3b and C3a. The C3b fragments are then indiscriminately deposited onto the surfaces of host cells or foreign particles (Favoreel et al, 2003). Once this has been initiated, the complement cascade will continue until it has been down regulated by specific mechanisms such as C3b in-activator enzymes.

Once one of the two above pathways has been activated, the complement cascade continues with the cleavage of C3 which is then followed by the cleavage of the C5 protein which results in the production of several other activated components such as C3a and C5a. The effects of these molecules are to increase the recruitment of antibody, complement and leukocytes to the infection site.

The production of these anaphylatoxins is part of the terminal pathway that leads to the production of membrane attack complexes which are incorporated into the lipid bilayer of the cell and result in osmotic disruption of the affected cell (Martini 2006c). It is this pathway that is the one that is activated the most by biomaterials.

2.9. Summary of Biomaterial Interactions

The interaction of blood and biomaterials is an extremely complicated process. A complex balancing act is quickly established between activating and inhibiting processes of the blood response systems to the detection of a foreign material.

The magnitude of the response generated determines the overall biocompatibility of the material. The smaller the magnitude of the biological response, the better the biocompatibility of the material and vice versa. An example of this is the complex nature of leukocyte interactions. They are known to interact in the intrinsic coagulation cascade, be involved in the fibrinolysis process and also the complement activation. If this was not enough, it as been shown that leukocyte adhesion is mediated by certain complement proteins such as C3b (Marchant, 1984). This highlights the extremely complex nature of the blood-biomaterial response and the importance of having a biocompatible material.

Presently, there is no truly biocompatible material available. Each material evokes a varying degree of biological response. Materials such as stainless steel and polyethylene glycol (PEG), generate small responses and hence are classified as being highly biocompatible. PPVC, is one of the most biocompatible plastics used in blood contacting procedures, but studies have shown that there are blood contact-mediated complications associated with its use in certain extracorporeal procedures.

Courtney et al (1994c) have shown that the activation of leukocytes as mentioned before has an important bearing on the efficacy in extracorporeal blood contacting applications. Zhao and Courtney showed that the nature and structure of key surface protein deposits, such as albumin and fibrinogen, was affected significantly when surface DEHP levels were controlled in PPVC blood contacting materials (Zhao et al. 2003). Gourlay et al also found that the presence of DEHP on the surface of blood contacting PPVC materials incorporated into a rat recirculation test apparatus promoted the observed inflammatory response, which was identified by an increase in the expression of the CD11b MAC 1 receptor which is a commonly used marker

for leukocyte activation (Gourlay et al, 2001, 2002). This was furthered in a study by the same authors who described the pro-inflammatory nature of DEHP per se, both in rat and human blood products (Gourlay et al. 2003). The inflammatory response, or activation of leukocytes, associated with the use of DEHP-plasticised PVC may be of little significance in single exposure procedures such as cardiopulmonary bypass (CPB). However, it may be of considerable significance in multi-exposure applications, such as haemodialysis, or in applications where a proportionately large surface area of PPVC is employed, such as paediatric ECMO.

Many different forms of surface and bulk chemistries and surface treatments have been investigated over the years to improve the biocompatibility of PPVC with varying success. However the more successful methods greatly increase the cost of the material and do not present a good cost to benefit ratio and hence are not attractive to the medical community.

2.10. Improving Biocompatibility

Improving the biocompatibility of artificial materials, especially medical polymers, is an area that has long been explored by engineers and material scientists. One of the most highly studied materials is that of medical grade PVC. Although introduced in the early 1950s and despite its widespread application there are still many issues regarding its use as a blood contacting material. The latter aspect has been the subject of numerous investigations.

Courtney et al have proposed many factors that relate to the biocompatibility of the PVC material. One of the main questions that was answered was what component of the PVC actually affected the blood response the greatest, was it the PVC itself or the plasticiser DEHP?

Previous studies had indicated that the blood response is not only influenced by the nature of the plasticisers involved (Jones et al, 1989; Zhao et al, 1997) but also by the concentration of the plasticiser that was used (Kicheva et al, 1995).

Zhao (1999) reported that the surface of the PVC was composed of 69% DEHP plasticiser (Figure 2.9). Further work by Zhao (2003) and also by Gourlay et al (2000), showed that washing of the PVC surface with methanol removes the DEHP and this produced a reduction on the influence of the PVC on the blood components.

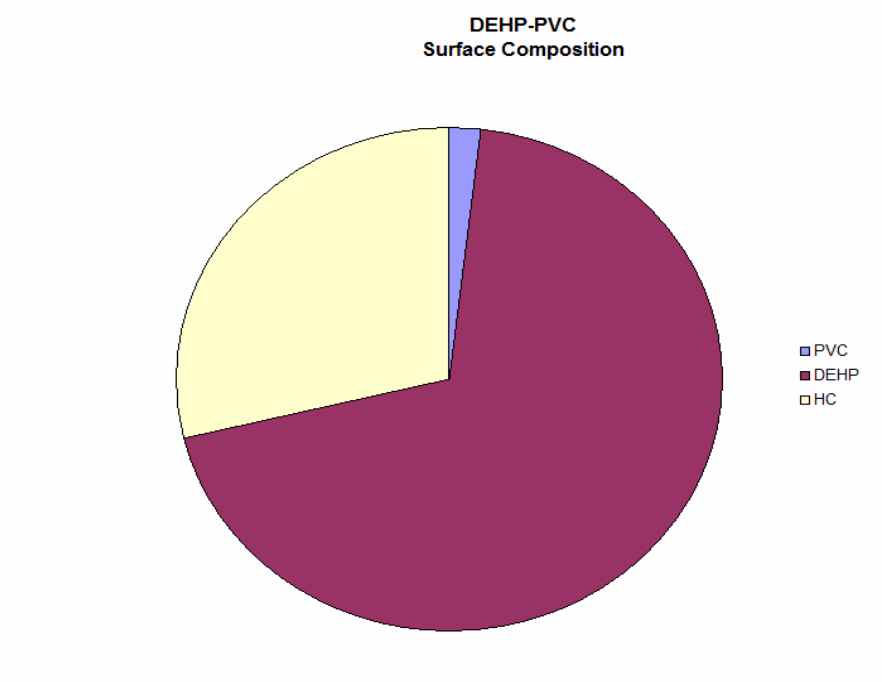


Figure 2.9: Surface composition of DEHP PPVC (taken with permission of the author from Courtney et al, 2008)

The goal of reducing the effects that surface has on the blood components is a major one and subsequently there have been many approaches reported. They all fall broadly into the following categories:

- Surface Modification
    - Surface cross-linking and incorporation of other molecules
    - Modification of surface hydrophilicity/lipophilicity
    - Surface coating

- Alternative Plasticisers
- Alternative Polymers

One of the most common forms of modification is the cross-linking of polymer surface molecules. This produces a polymer surface that acts as a barrier to interfacial mass transport of plasticizer molecules (Rahman et al, 2004).
An example was the technique developed by Courtney et al (2003), involving the incorporation of a complex formed with cyclodextrin and a low molecular weight copolymer. This had two objectives, firstly to improve the compatibility of the polymer and secondly to reduce the plasticiser migration.

Another popular area of investigation (e.g. Gouzy et al, 2004; Lee et al, 2007) is the incorporation of an anticoagulant such as heparin, onto the surface of the polymer.

2.10.1. Molecule Incorporation

Gouzy et al (2004) reported the surface immobilization of a benzamidine derivative as it has been shown to be a potent inhibitor that acts directly on the active site of serine proteases (i.e. trypsin and thrombin) which helps to reduce the coagulation activities. A significant increase in the short term biocompatibility of the material was found. The results also showed that the incorporation of a polyethylene-glycol spacer between the thrombin inhibitor and the surface helped to reduce the thrombogenicity even further.

Zhao et al (2008) also performed studies in this area and found that the covalent attachment of heparin to the surface of the polymer produced the following effects:

- Improved blood compatibility
- Reduced DEHP surface concentration
- Reduced DEHP extraction when the material was in contact with blood

The heparin coating of materials was first described by Gott et al in 1963, but only since the late 1980's have such materials become commercially available. Heparin coating has been applied to a range of medical devices, for example, vascular stents and extracorporeal bypass circuits.

Lee et al (2007) showed that a heparin-coated drug eluting stent improved haemocompatibility by substantially reducing the platelet adhesion to the surface of the stent. The heparinised top coat polymer also produced a diffusion barrier that allowed the controlled release of a drug which was contained in a further polymer in a sustained manner by creating a network of channels that slowed the progress of the drug from the undercoat polymer. This shows another possible benefit of the incorporation of heparin into the medical plastics

Svenmarker et al (1997), performed a study into the effects of heparin coated cardio-pulmonary bypass circuitry on brain function parameters and general patient outcome. The results showed several favourable outcomes regarding general patient well being.

- Reduced hospital stay
- Shorter time on post-operation ventilator support
- Reduced chest drain blood loss at 8h and 24h post-operation.

In a similar application, Belboul et al (2000) showed that the inflammatory response was markedly reduced during open heart surgery with heparin-coated circuitry. As well as the improvement in biocompatibility, the results also suggested that the heparin coating was associated with less myocardial ischemic damage compared to patients treated with non-coated circuitry. This was due in part to the reduction of the neutrophil response leading to a decrease in lipid peroxidation and subsequent reduction in myocardial damage.

Weber et al (2001) investigated the haemocompatibility of heparin coated materials and the role of selective plasma protein adsorption. They showed that there was a distinct difference in the absorption patterns of proteins onto the surface of those materials coated with heparin and those without, notably for fibronectin, fibrinogen, C3 and high molecular weight kininogen and that the improved compatibility of the heparin-coated materials was due in part to this selective uptake of plasma proteins.

2.10.2. Surface Coating

The coating of the surface of the polymer with another polymer has also been explored by a number of authors. Lakshmi et al (1998) modified the surface of PVC by grafting on a coating of polyethylene glycol (PEG). This produced favourable results by reducing the amount of DEHP that migrated and also increased the biocompatibility of the PVC.

A novel technique was reported by Ito et al (2005), in which the external surface of PVC tubing was modified by exposing it to UV irradiation. The DEHP release from the treated surface was reduced by almost 50%. This reduction only occurred on the side that was irradiated as when tubing was tested the inside of the tubing had no reduction in the amount released. The analysis work performed by the group showed that the UV irradiation had a definite effect on the structure of the surface, by increasing the cross linking of the polymer on the irradiated side, creating a barrier to the migration of the DEHP plasticiser and this is what has reduced its release.

Jaykrishnan and Lakshmi (1998), produced a method that worked on the basis of the relative biocompatibility of the PVC itself. They developed a sulfonation process that immobilized the plasticiser used in the manufacture of the PPVC hence limiting its migration from the material. The process alters the surface structure of PPVC by creating bonds between sulphur groups that replace the surface chloride ions. These covalent bonds between sulphur groups create a barrier along the surface of the PVC sufficient to impede the mobility of DEHP molecules.

Gourlay et al (2009) confirmed the findings of Jayakrishnan and co-workers and also showed that the lower level of surface DEHP was associated with a significant reduction in contact mediated activation of inflammatory and coagulation processes.

2.10.3. Alternative Plasticisers

As the DEHP plasticiser in PVC has been thought to cause many of the toxicity problems discovered in animals, such as liver cancer and reproductive anomalies in males, numerous attempts have been reported to replace it with a safer plasticiser.

Courtney and Zhao (2003) replaced the DEHP with tri-(2-ethylhexyl) trimellitate (TEHTM), a compound with a very similar structure as DEHP, but of higher molecular weight. It was reasoned that the higher molecular weight would reduce the extraction when in contact with blood. The results were not favourable as it produced a higher blood response compared to that of the DEHP plasticised tubing. This was attributed to the fact that there was more TEHTM in the polymer and more migrated to the surface causing the heightened response.

This work was explored further by Kambia et al (2001) who performed extraction experiments on different blends of tubing used in haemodialysis procedures. They used a normal DEHP plasticised tube and compared the results with a co-extruded tri-octyl trimellitate (TOTM) and DEHP tube. The normal DEHP tube, leached around 122.95 mg after the 4h dialysis procedure compared to the tri-octyl trimellitate, TOTM, TOTM/DEHP tube which leached only 41.8 mg of DEHP and 75.11 mg of TOTM. As less TOTM plasticiser was leached from the TOTM/DEHP plasticised tube the investigators concluded that TOTM would be a superior choice for medical devices due to its perceived lower leachability compared to the fully DEHP plasticised tube.

Zhao and Courtney (2003) suggested the possibility of using a citrate based plasticiser (Butyryl tri-n-hexyl citrate, BTHC) which is bio-based and also bio-degradable, to replace the DEHP, within PPVC. They performed experiments utilising single solute fibrinogen adsorption using BTHC plasticised PPVC sheeting to determine the initial reactivity to blood components. The results showed that fibrinogen adsorption behaviour was dependent on the plasticiser type, surface level of plasticiser and the adsorption conditions such as contact time and solution concentration. They demonstrated that this citrate based plasticiser had a reduced effect on the blood components, by showing the lowest adsorption capacity compared to DEHP and TEHTM and a reduced migration pattern on blood contact compared to the other tested plasticisers. These results produced initial data that

would support the use of citrate plasticisers as a possible replacement for the DEHP plasticiser in medical grade PPVC

Many companies have since produced products containing these plasticisers, such as the Citroflex A6 and B6 plasticisers by Morflex. Even though the initial toxicology tests considered citrate based plasticisers safe to use, certain animal work has shown that they do produce adverse effects on the nervous system such as the introduction of a reversible nerve blocking action and also a dose related reduction in blood pressure (Rahman and Brazel, 2004). Due to the unknown long term toxicological exposure effects and also the increased cost they have not been so popular in the market place.

2.10.4. Current Situation

The quest to improve the biocompatibility of PPVC has in many cases led to discovering various techniques that stop the DEHP plasticiser reaching the surface of the material as it is the DEHP which has been shown by Zhao (1999,2003) and Gourlay (2000) to be the greatest factor in the materials' response. Others have focused on replacing the DEHP with other plasticisers in an attempt to improve its blood compatibility and this has led to a number of commercially available products that utilise alternative plasticisers such as TOTM and DOA as well as heparin-coated tubing sets.

However, whilst many of these techniques have ended up with alternative products in the commercial market place, DEHP plasticised products are still the primary choice in many medical centres around the world. The new alternative products so far cannot compete with DEHP products on cost and until this gap is bridged DEHP plasticised products will continue to dominate the market place, despite the concerns relating to its safety. An understanding of the different types of plasticisers and the role they play in PPVC as well the other constituents that make up PPVC may lead to developments that would give non-DEHP PPVC a stronger footing in the medical market place.

3. Plasticisers and DEHP

3.1. Introduction

The properties that make P-PVC the premier choice material for such a diverse range of applications is primarily due to the formulation of additives added to the PVC resin. The additive that has largest proportion is the plasticiser. The plasticiser is an organic compound which is blended into the PVC in such a way that it mixes with the PVC but does not form any covalent bonds; it instead forms an intimate bond with a portion of the PVC, whilst the remainder is held within the polymer matrix. This is demonstrated in Figure 3.1. In extremely soft P-PVC the amount of plasticiser can be as much as 50% of the total plastic weight (Zhao, 1999).

Plasticisers were originally used as a mechanism to help reduce the softening temperatures and in-turn reduce the processing temperatures required for industrial use of PVC, but are now exclusively used to impart flexibility into the final PVC blend (Matthews, 1996).

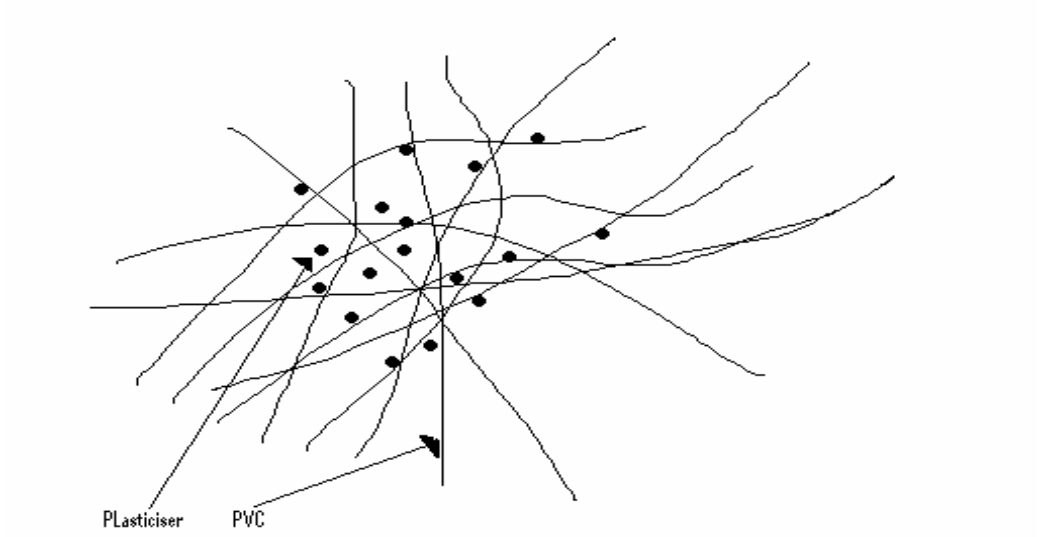


Figure 3.1: Diagram of the relationship of a Plasticiser (black dots) in a PVC polymer demonstrating the random intermixing of the plasticiser without bonding of the plasticiser in the PVC polymer chains (adapted from Zhao, 1999).

3.2. Plasticiser Theory

The plasticisation of PVC is an area that has undergone the best part of four decades of research, but this has still yet to produce a comprehensively encapsulating theory on the mechanics of plasticisation. The process of plasticisation is the interaction of a plasticiser with the PVC resin to impart flexibility to what would otherwise be a rigid material, thus increasing its usefulness. Currently, there are three main theories on plasticisation that have been proposed and accepted by the PVC fraternity. These are:

- Lubricity Theory
- Gel Theory
- Free Volume Theory

3.2.1. Lubricity Theory

As its name suggests this is a theory that is based around the plasticiser acting as a lubricant between the polymer molecules, similar to the way oil works in an engine. This then allows the polymer resin molecules to move more freely over each other. The action of plasticisation for the lubricity theory is best described in The Technology of Plasticizers by Sears and Darby who suggest that when a polymer is flexed, the macromolecules must work back and forth over each other, and that a plasticizer lubricates these internal glide planes. This can be seen in Figure 3.2.

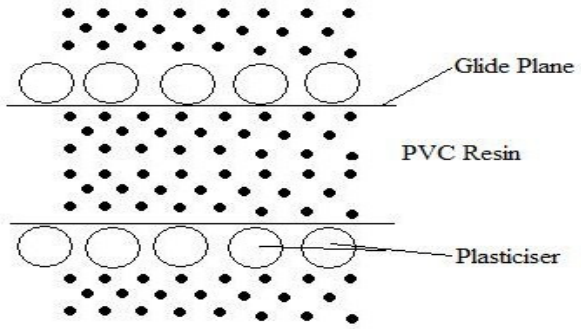


Figure 3.2: Schematic of the lubricity theory showing the glide planes

This is a very simple theory that also assumes that there no bonding between the polymer molecules and only weak bonding between the plasticiser and the polymer molecules. This theory is rarely applied on its own and is more often found being used in conjunction with the other theories. One such area in which this theory is useful is at higher temperatures where plasticiser action can be related to the coefficients of friction. (Nakamura, 1975)

3.2.2. Gel Theory

This theory states that the rigidity of the polymer is caused by a three dimensional honeycomb network which is due to loose attachment points at points along the polymer molecules producing a gel like structure. In this current state the polymer is a stiff and brittle structure which does not accommodate deformation well. The plasticiser works by acting on the polymer resins that have many points of attachment. It then acts by breaking these attachments and masking centres of force that have held these polymer chains together.

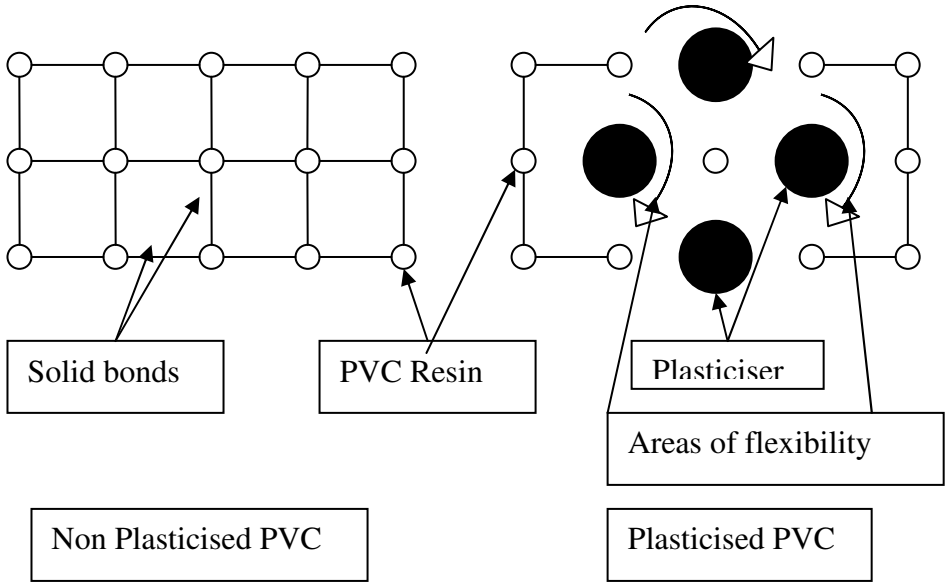


Figure 3.3: Schematic showing the Gel Theory of plasticisation.

The plasticiser masks these centres of force by selectively solvating the polymer chains at these points. (Sears and Darby, 1982a). This then produces a situation which is similar to having a fewer number of starting attachment points. Not all of

the plasticiser molecules are attached in this way, many are only indirectly attached to the polymer by being attached to other plasticiser molecules. These are seen to be "swelling" the gel structure so as to allow easier movement between the polymer molecules which in turn improves the flexibility of the polymer (Figure 3.3).

3.2.3. Free Volume Theory

The Free Volume Theory was the slowest of the main theories to be developed due to it not being based on easily visible structures or simple analogies. It instead grew from less obvious characteristics of crystals, glasses and liquids and relies on mathematical corroboration for its strength. (Sears and Darby, 1982b).

The theory is based around the glass transition temperatures (Tg) of polymers. It is defined as;

"The difference between the specific volume at the temperature under consideration and the specific volume at absolute zero." (Matthews, 1996)

The main action of the plasticiser in this theory is that it helps to reduce the glass transition temperature of the polymer. It achieves this because of the relation that free volume increases allowing a larger space for the polymer molecules to move. The increased space allows for the side-chains and end groups to move more freely in the polymer thus making it more flexible. The increasing of the free volume is achieved by the following processes, adapted from Sears and Darby, 1982b

- Increasing the number of end groups
- Increasing the number of side chains
- Increasing the chance of main polymer chain movement by including low polarity compounds.
- Inclusion of low molecular weight compounds that achieve all of the above processes

Plasticiser addition helps to increase the free volume of the polymer due to the plasticiser molecules being of a much smaller size than the polymer, this then substantially increases the number of side and end chains present in the polymer,

which subsequently reduces the glass transition temperature (Tg) of the polymer which making it softer and more flexible (Figure 3.4).

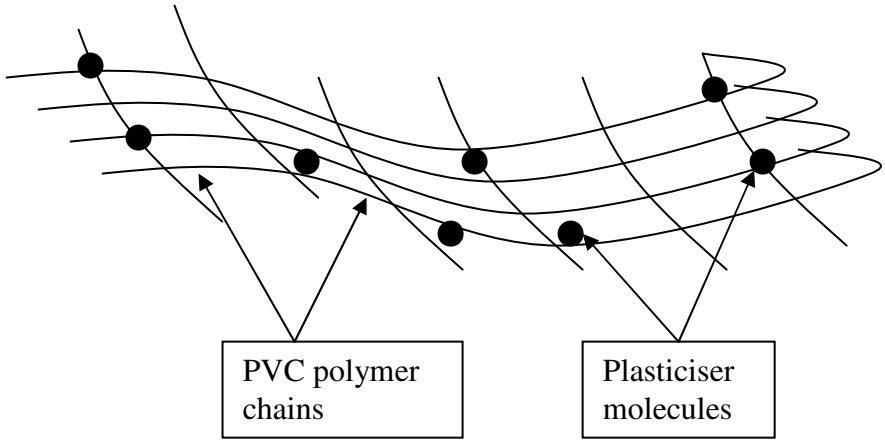


Figure 3.4: Schematic of the Free Volume Theory of Plasticisation showing the increased polymer chains, side and end groups.

3.3. Plasticiser Types

The range of plasticisers currently available on the commercial market is substantial. There are around 300 known plasticisers, with around 100 of these being commercially available. For PVC, the most commonly used plasticisers are those derived from the Phthalate family of compounds with around 95% being phthalate esters (ECPI, 1996).

Plasticisers used in plastics can be divided into two separate groups, Primary and Secondary, depending on how well they interact with the polymer resin. These groups can then be subdivided according to the method of derivation. Primary plasticisers are seen to have a high level of interaction with the resin over the whole range of compositions and conditions and are deemed the most important group. Secondary Plasticisers exhibit lower level of interaction over only a partial range of temperatures and conditions and must be used in-conjunction with a primary plasticiser to increase their overall effectiveness, which in turn improves certain properties of the polymer.

Most plasticisers are derived from the petroleum industry but there are also a few which are derived from other natural means such as vegetable oils. The following diagram (Figure 3.5), adapted from Zhao, 1999, shows the different types of plasticisers that are used predominately in the plasticisation of PVC.

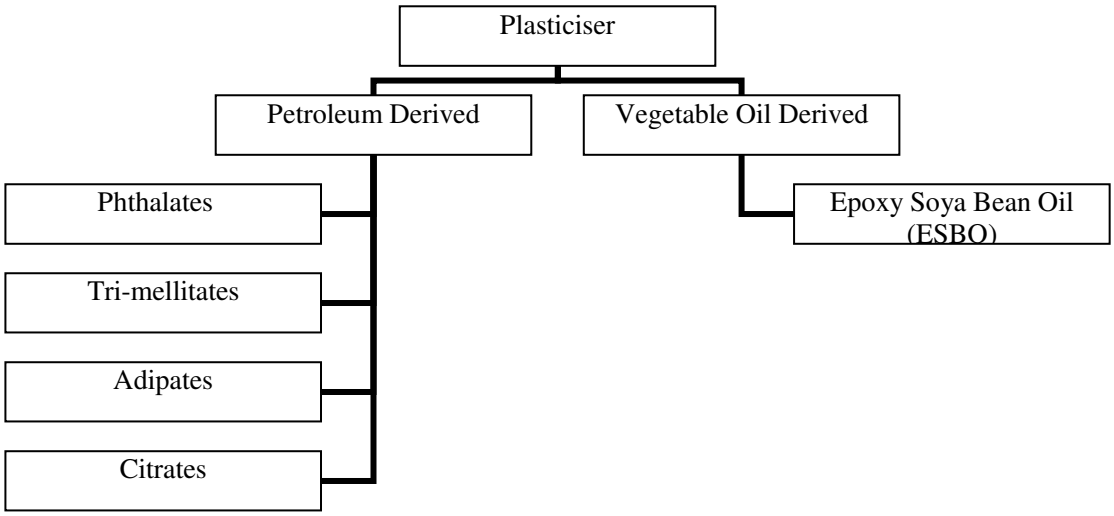


Figure 3.5: Grouping of PVC Plasticisers by methods of Derivation. (Adapted from Zhao, 1999)

3.4. Phthalates

The Phthalate plasticiser family are produced by the estrification of phthalic anhydride with an appropriate alcohol. It is by far the most commonly produced plasticiser with around 100 million tonnes being produced in Western Europe each year. Of this around 90% are used solely in the production of P-PVC. The remaining 10% is used in the production of other commodities such as ink pigments, sealants and adhesives. There are many different types of phthalates produced, but the market is dominated by three main compounds who account for around 85% of the total phthalate production (http://www.pvc.org/What-is-PVC/How-is-PVC-made/PVC-Additives/Plasticisers) these are namely,

- DEHP- Di(2-ethylhexyl) Phthalate
- DINP- Diisononyl Phthalate
- DIDP- Diisodecyl Phthalate

3.4.1. DEHP

Di(2-ethylhexyl) Phthalate is a 2-ethylhexanol diester of phthalic acid. It has very simple chemical structure that is illustrated in Figure 3.6. It has an appearance similar to clear vegetable oil and has a similar viscosity. It is also known to have good solubility in substances with high lipid contents such as oil and plasma but is insoluble in water. (http://www.dehp-facts.com/DEHP)

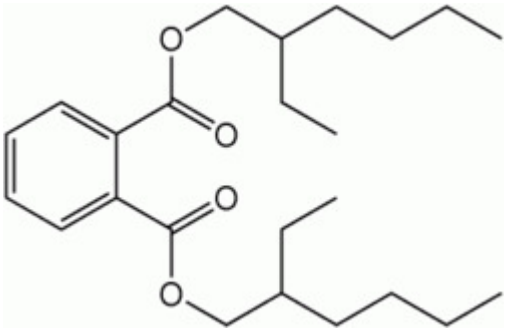


Figure 3.6: Di-2ethylhexyl phthalate chemical structure (www.chemblink.com/products/117-81-7.htm)

It has been and still is the first- choice plasticiser for use in all P-PVC medical and surgical products since it first appeared over 50 years ago. It is the only plasticiser that has been approved for use in Medical Devices by the European Pharmacopoeia. The wide spread use of this plasticiser which accounts for around 20% of all phthalates produced, is testament to its good all-round performance. DEHP has a high plasticising efficiency, a good compatibility with PVC as well as good low temperature characteristics in that it maintains its ability to provide flexibility over a wide range of temperatures and also a low volatility. This therefore allows it to be used successfully in many general purpose applications (http://www.phthalates.com/index.asp?page=7).

3.4.2. DINP

Diisononyl Phthalate or DINP (Figure 3.7), is produced by the estrification of phthalic anhydride with an “oxo” alcohol with an average chain length of between 9 and 11 carbon atoms. It is characterised by having two alkyl chains that are eight-ten

carbon atoms in length but with the majority having chains of nine atoms long illustrated in Figure 8. Its chemical formula is $C_{26}H_{42}O_4$ and is used mainly in P-PVC with around 95% of it being used for this purpose. It is probably the third of the main three plasticisers with around 107, 000 tonnes of it being produced in Western Europe in 1994 (http://www.greenfacts.org/en/dinp-didp/index.htm#il1), but this figure will have been expected to increase steadily over the years. As with DEHP it has an oily liquid appearance with low water solubility as well as being soluble in saliva and plasma. It is a general purpose plasticiser that is used in many products, such as garden hoses, shower curtains and PVC flooring. It main use is found in PVC toys. Until recently, it had been deemed safe for all toys, but as of January 2007, it has been banned from use in plastic toys and articles that are designed to be put in the mouth of toddlers and other children under the age of three (SCENHIR 2008). This is due to the increased risk of plasticiser exposure due to the increased migration of the DINP into the saliva. It has been cleared for use in all products that are not designed for this application. This will no doubt affect the usage of the plasticiser in the toy market and may see it dropping out of the top three most used plasticisers.

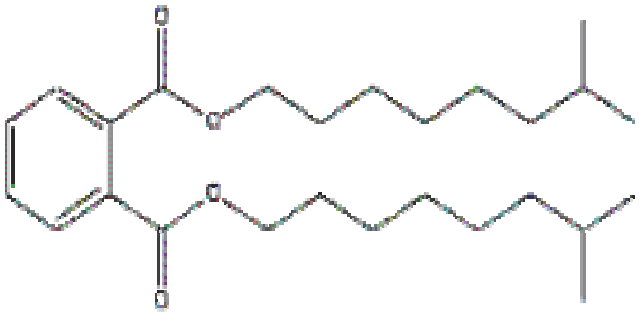


Figure 3.7: Diisononyl Phthalate Structure (www.chemblink.com/product/28553-12-0.htm)

3.4.3. DIDP

The third of the main three phthalate plasticisers is Di-isodectyl Phthalate. It is produced in the same way as DINP but is different in the fact that its alkyl chains have a length of between nine and eleven carbon atoms, with the average length being ten atoms. It has a chemical formula of $C_{28}H_{46}O_4$ and its structure is shown in Figure 3.8 and shares the same properties as DINP with regard to volatility and solubility. It is regarded as the second of the top three phthalates with around 200,

000 tonnes being produced throughout Western Europe in 1994. Again, that figure is expected to be much larger in today's time frame. DIDP can be found in a smaller array of P-PVC products such as heat resistant electrical cords, PVC flooring and leather for car interiors. A small percentage of DIDP is used in anti-corrosion and fouling paints and sealing compounds.

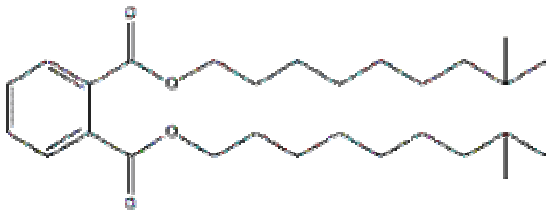


Figure 3.8: Di-isodoctyl Phthalate structure (www.chemblink.com/products/117-84-0.htm)

3.5. Adipates

This family of plasticisers are produced from alcohols of similar length to those used in the production of Phthalates. The main difference is that they are estrified with adipic acid leading to the production of the adipate plasticisers. The structure of a common adipate plasticiser, Di-2-ethylhexyl Adipate, DEHA, can be seen in Figure 3.9.

Adipate plasticisers provide different characteristics which are required for certain applications of PPVC. Adipates, tend to produce PPVC that has much better low temperature performance compared to those using Phthalates. They also have a much lower viscosity compared to the Phthalate group which produces much lower viscosities in plastisol (liquid plastic) applications. Recently, adipates have been seen as an alternative to DEHP PPVC in medical products like tubing. This is because of the lower propensity of the plasticiser to migrate from the PPVC when in contact with biological fluids.

Some of the disadvantages of Adipates are found to increase with lengthening of the carbon chains, which tends to cause incompatibility issues. This combined with their

higher costs mean that it is quite common to find them being mixed with other plasticiser types in applications.

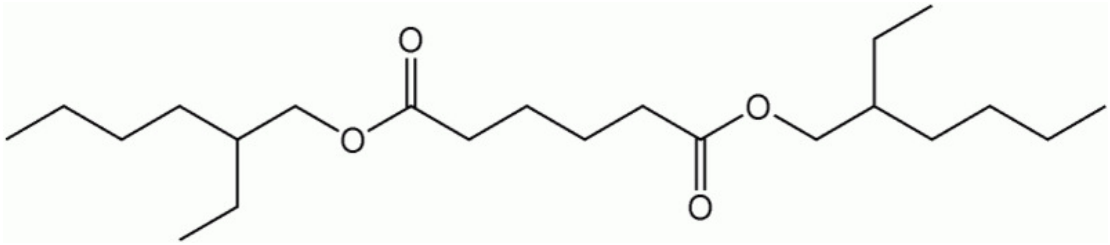


Figure 3.9:Structure of Di-2-ethylhexyl Adipate plasticiser (www.chemblink.com/products/103-23-1.htm)

3.6. Trimellitates

The Trimellitates are produced by the estrification of alcohols with another compound called Trimellitic Anhydride (TMA). The structure of a common Trimellitate plasticiser called tri-2-ethylhexyl trimellitate (TEHTM) is illustrated in Figure 3.10.

This range of plasticisers was developed to provide a good all round plasticiser with the added advantage of low volatility. It also has a much higher resistance to migration and also extraction than the phthalate family, hence has been touted as a possible replacement to DEHP in PPVC medical applications. Currently, the vast majority of this plasticiser is used in high specification electrical cables and insulates. Another application for this plasticiser is in the use of vehicle interiors to prevent window fogging. It is the low volatility loss property that makes this plasticiser particularly attractive for this market as the fogging on the car windscreen is due in part to a build up of plasticiser that has migrated from the plastic interior components.

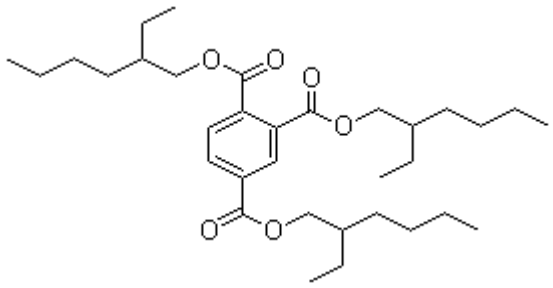


Figure 3.10: Trimellitate plasticiser structure (www.chemblink.com/products/3319-31-1.htm)..

3.7. Citrates

This group of plasticisers are derived from the natural feed stock Citric Acid. They are generally much more expensive than the Phthalate compounds and yet this extra cost does not produce any great advances in physical properties in the P-PVC that contains them. The main advantages of these plasticisers are their low toxicity. The uses of this plasticiser group are based around its low toxicity property with its main uses in food wrap and also medical products such as blood bags. There are many different citrate plasticisers available on the current market but the main three commercial ones are;

- Acetyl Tributyl Citrate (ATBC)
- Butyryl Tri-n-Hexyl Citrate (BTHC)
- Acetyl Tri-2-ethylhexyl Citrate (ATEC)

With regard to the thesis subject matter the most relevant Citrate plasticiser is Butyryl Tri-n-Hexyl Citrate (BTHC) as it has been seen as a possible replacement for the Phthalate, DEHP for use in non-phthalate P-PVC medical devices and blood contacting products. (Zhao and Courtney, 2003)

The structure of BTHC is illustrated in Figure 3.11.

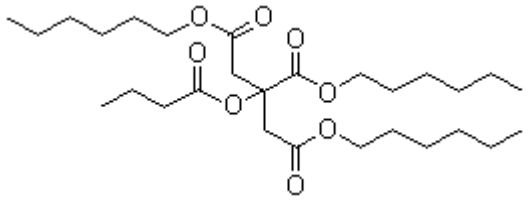


Figure 3.11: BTHC Chemical Structure (www.chemblink.com/products/82469-79-2.htm))

3.8. Vegetable Oil derived Plasticisers

This group of plasticisers are different from the previous types discussed as they are not derived from petroleum. Instead they are produced from natural plant resources such as linseed and Soybean. The most abundantly used member of this group is the soybean derived product, Epoxidised Soybean Oil or ESBO. This particular compound accounts for around 43% of the total non petroleum based plasticiser market. (Zhao 2003) The structure for ESBO can be seen in Figure 3.12.

These plasticisers are classed as being "secondary" plasticisers and therefore are used in conjunction with a primary plasticiser to increase their interaction with the PVC resin to provide further flexibility to the product. It finds itself creating an opening in the plasticiser market due to its biodegradability properties which are now becoming more and more desirable due to recycling and global warming issues.

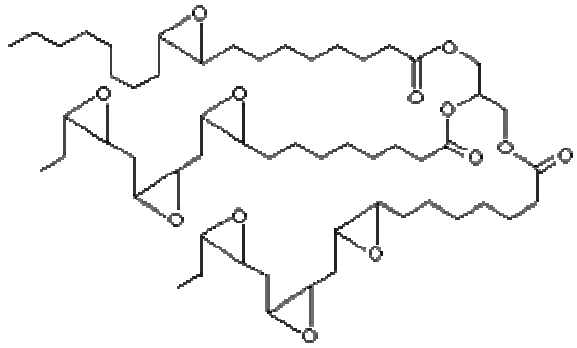


Figure 3.12: Chemical Structure of ESBO (taken from www.chemicalland21.com)

3.9. Plasticisers and Medical Devices

The main plasticiser used in medical products is DEHP. There are a number of reasons for this, but low cost and the very good mechanical properties associated with DEHP use are certainly two of these. However, bearing in mind the potential for adverse effects associated with the deployment of DEHP, which is a reproductive and developmental toxic substance in animals and also a suspected endocrine disruptor in humans, there are a number of clinical interventions in which there may be real cause for concern. More on these adverse effects are discussed later in section 3.11. Principal amongst these clinical interventions are the various extracorporeal applications, haemodialysis, CPB and in particular ECMO where the PVC surface area to body mass relationship and duration of exposure is at its most extreme. The knowledge of the possible adverse effects of DEHP, has led many PVC manufacturers to investigate possible alternative plasticisers and other means of reducing the migration of the DEHP plasticiser from PVC tubing. Currently, these products are not cost effective especially when the adverse effects are not clinically defined. So DEHP is at present here to stay and so the question of associated risk from DEHP exposure during medical interventions still remains.

3.10. Metabolism of DEHP

The fate of DEHP that has entered mammals depends on a number of factors such as dosage, route of administration and host type. There are two possible outcomes for DEHP in the host, the first is that it is excreted in its whole form. This applies to a small portion of the chemical dose. The second is that the DEHP is metabolised into more easily excretable compounds (Koch et al., 2006).

Figure 3.12 shows the various metabolism pathways that DEHP can experience in mammals. (Adapted from Koch et al, 2006) There are around seventeen different metabolites that can be produced on the break-up of DEHP, but the interest lies with the five most prevalent metabolites found after metabolism occurs.

- Mono (2-ethylhexyl) phthalate ,MEHP
- Mono (2-ethyl-5-hydroxyhexyl) phthalate,5OH-MEHP

- Mono (2-ethyl-5-oxy-hexyl) phthalate,5OXO-MEHP
- Mono (2-ethyl)-5-carboxypentyl phthalate,5CX-MEHP
- Mono (2-carboxymethyl)hexyl phthalate,2CX-MMHP

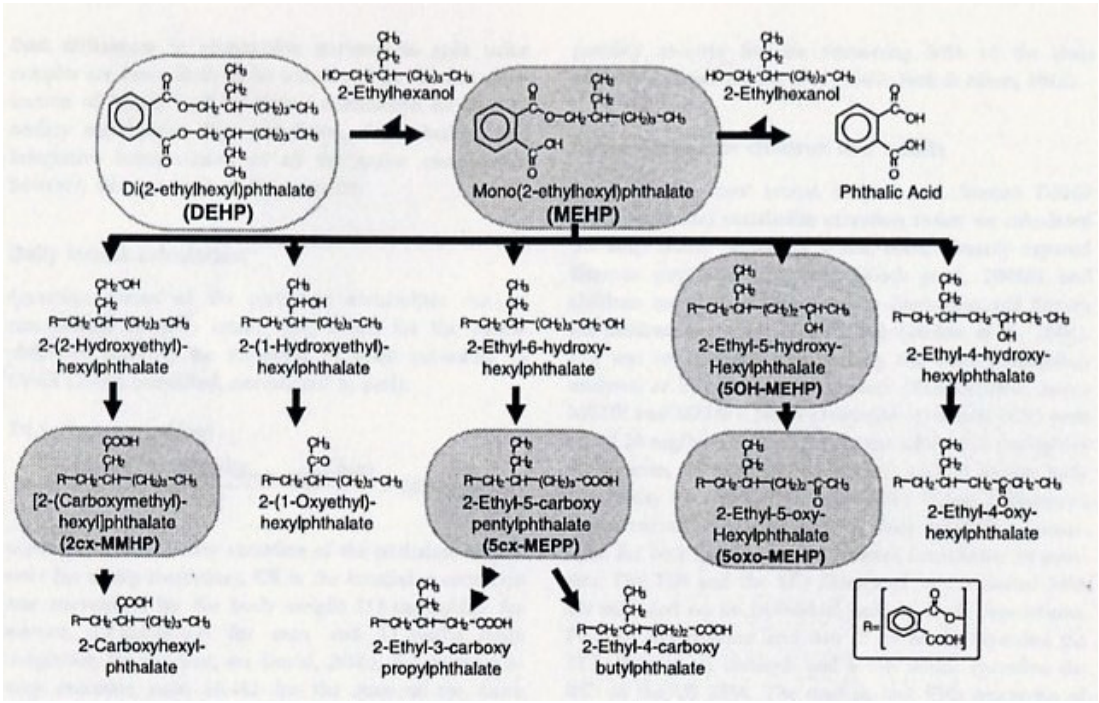


Figure 3.13: Metabolism of di(2-ethylhexyl) phthalate (DEHP). (taken from Koch et al 2006 and reproduced with kind permission from the publisher John Wiley & sons Inc).

The initial process in the metabolism of the DEHP is the rapid hydrolytic cleavage to Mono-2-ethylhexyl Phthalate or MEHP, the main metabolite. The MEHP monoester is then extensively further metabolised by different oxidation reactions (Albro et al, 1982; Schmid & Schlatter, 1985).

The enzymes (non-specific lipase, E.C.-11) that cause this initial metabolism are found mainly in the intestinal tract (Albro et al, 1982; Albro and Thomas 1973) but are also present in the mouth so metabolism can occur right at the site of oral ingestion (Nino et al, 2001, 2006). Because of this evidence, it has been hypothesised that the majority of the DEHP will be absorbed rapidly in the form of MEHP in the gut after exposure by an oral dose.

However, whilst the metabolism of DEHP to MEHP remains similar between species, the rate of metabolism differs substantially. Larger mammals such as primates or humans have a much less efficient means of metabolism compared to smaller animals of the rodent family. Even then, there is differentiation between the rodent species, in relation to types of metabolites that are excreted in urine. Rats primarily excrete un-conjugated metabolites whereas mice excrete glucuronide conjugate versions of the metabolites (Fredrikson et al, 2007). The metabolic distribution of the various secondary metabolites also varies substantially between rats and mice as well as with age of the animals (Heindel et al, 1992; Albro 1986, 1983; Gollamudi et al, 1983).

The findings of the various animal studies concluded that DEHP is systematically broken down *in vivo* regardless of animal species. However, the effectiveness of the metabolism process depends on the host species, with rodents being more effective than the larger humans.

3.10.1. Human Metabolism of DEHP

The data generated from the animal studies produced conclusive information about the break down of the DEHP *In-Vivo*. However, it did not shed any light on the human metabolism of DEHP.

Further studies have now shown that there are 15 main metabolites of DEHP found in urine produced by the breakdown of the chemical in humans (Silva et al, 2006; Barr et al, 2003). Many of these metabolites are similar in structure to those found in the rodent studies (Fredrikson et al, 2007).

The elimination characteristics of DEHP from the human species were investigated by Koch et al in 2004 and 2005. They exposed a 63 year old male to three doses of DEHP by oral administration. They showed that 67% of the initial dose was excreted via urine after the first 24hr. This excretion contained 5 of the major metabolites each in varying concentrations. This was followed by an excretion of a following 3.8% the following day. The above data show that the majority of an orally administered dose is excreted in urine after systemic absorption (Koch et al, 2006). The doses used in this study all fell below the current no observable adverse effects level (NOAEL) of DEHP which is set at 4.8mg/kg/day. Further studies compared the metabolic

distributions in two population groups who were exposed unknowingly to DEHP (Table 3.1). They showed similar metabolic profiles to the studies of Koch et al.

	German Population (n=19)		US Population(n=129)	
	Ng/ml (Mean)	%	Ng/ml Mean	%
MEHP Mono (2-ethylhexyl) phthalate	9.8	4.5	3.3	6.9
MEHHP Mono (2-ethyl-5-hydroxyhexyl) phthalate	47.5	21.7	15.1	31.7
MEOHP Mono (2-ethyl-5-oxy-hexyl) phthalate	39.7	18.1	7.8	16.4
MECPP Mono (2-ethyl)-5-carboxypentyl phthalate	85.5	39	16.2	34
MCMHP Mono (2-carboxymethyl)hexyl phthalate	36.6	16.7	5.2	10.9

Table 3.1: DEHP Metabolites extracted in Urine (Adapted from Frederikson et al, 2007), showing the levels of the most prevalent DEHP metabolites across both German and US populations. Data shown as the geometric Mean values for each metabolite. The % relates to the sum of the 5 listed metabolites being 100%.

Whilst the majority of the DEHP is excreted quickly from the body, there is a remaining amount which is unaccounted for in these studies. It is known that intestinal absorption of the DEHP and its metabolites occurs in both rodents and also at slower rate in humans. Once the DEHP is adsorbed in this way it is widely distributed throughout the body. It has a propensity to accumulate in the organs as well as the lungs and adipose tissue. It has also been found in the brain, heart, spleen, muscles, the reproductive organs as well as blood and plasma. The afore mentioned organs have also been shown to contain the enzymatic lipases that are known to break down DEHP (Albro and Thomas, 1973; Daniel and Bratt, 1974) indicating that the body is well equipped to dispose of the DEHP chemical no matter which tissue it appears in.

The ability of the human species to effectively break down DEHP and excrete it in a rapid manner, has supported the argument that it is safe to use DEHP in medical

applications. However, there is still a proportion that goes unaccounted and it is this proportion, be it DEHP or one of its metabolites, that continues to stoke the argument for its discontinuation in medical applications. Studies into the metabolism of DEHP in both humans and rodents are required to generate a fuller understanding of the behaviour of this chemical post exposure and if the adverse effects seen in the animals are in reality related to the DEHP and secondly translated to humans.

3.11. Toxicity of DEHP

3.11.1. Introduction

Substantial work has been performed into the possible toxic effects of DEHP due to the mounting concerns relating to both medical and environmental exposure to this chemical. Overview papers and risk assessments on DEHP toxicity have been published by individual researchers (Tickner et al, 2001; Latini, 1999), independent agencies (IARC, 2000) and also government institutions worldwide (Health Canada, 2002; FDA, 2002; European Commission, 2001, CSTEE 1998, 1999, 2002, SCENHIR 2008).

The general conclusions from these reviews state that in the case of the vast majority of adults there is no concern over the toxicity following exposure to normal levels of DEHP. This is not the case with regard to children. There have been studies reporting testicular toxicity and depressed fertility after oral exposures to DEHP.

However, the real concern lies with the treatment of seriously or chronically ill patients whether they are adults or children. Concern has arisen regarding the magnitude of the possible dose that these patients may be exposed to. This has been furthered by adding other at risk groups focusing on those who have very high short term exposures or repeated high exposures over a sustained period of time. These include interventions like ECMO, dialysis, extensive blood or blood product transfusions and parenteral nutrition. The following sections will briefly discuss relevant data from various studies from which the above conclusions have been drawn.

3.11.2. Acute Toxicity

DEHP has been shown to have a very low acute toxicity level with the LD_{50} value of >25g/kg in rodents by both oral and inhalation routes. However, the LD_{50} value increases to 200-250 mg/kg in rats when it is by the intravenous route and even more concerning is the LD_{50} value of MEHP, which is 5 time higher than DEHP (ECB 2002, 2006; NTP-CEHRHR 2005).

3.11.3. Genotoxicity and Mutagenicity

DEHPs possible role in gene mutation has been extensively studied using both *in vitro* and *in vivo* test systems ranging from human leukocyte cells, to rat and mouse liver cells as well as full rodent systems. The majority of the results for both DEHP and its metabolites have come back negative (ATSDR, 2002). However, it has been shown to produce some positive results in cell transformation and cell proliferation. This said, the susceptibility of these tests to other non-genotoxic substances has led the regulators to classify DEHP and its metabolites as non-genotoxic or mutagenic substances (ECB, 2001; CSTEE, 2002; SCENHIR, 2008).

3.11.4. Carcinogenicity

Reports began in the 1980s suggesting that many of the phthalates that were in commercial use were carcinogens in rodents. The phthalate DEHP was found to cause cancers of the liver of rodents and was deemed a carcinogen by the National Toxicology Program carcinogenesis bioassays in 1982 after it increased the incidence of hepatocellular neoplasms in F344 rats and B6C3F mice. This report produced a Lowest Observable Adverse Effects Level (LOAEL) value of 6000 mg/kg DEHP which was the level present in the animal feed (320 mg/kg/bodyweight for males) in rats. A NOAEL value was unobtainable in this study due to the increase in incidence of liver tumours at the lowest administered dose.

Ward et al in 1986 published work relating to the investigation into whether DEHP acts as a tumour initiator or a tumour promoter in rodents. The work was a long term study lasting 18months, administering high oral doses. It was reported that DEHP is a promoter of hepatocellular tumors initiated by an intraperitonial injection of N-

nitrosodiethylamine (DEN) in mice but no initiating activity was demonstrated in the mouse liver.

Studies by David et al in 2000, established a NOAEL level for tumour induction and adverse effects in the kidneys (minerlisation of the renal papilla, chronic progressive nephropathy and renal tubule pigmentation), testes (Interstitial cell tumours, bilateral aspermatogenesis) and liver (pigmentation in kupffer cells & spongiosis hepatis) of 500 mg/kg for rats which equates to 29 mg/kg body weight/day for male rats. DEHP is a known peroxisome proliferator. The carcinogenic effects of DEHP are thought to be related to it role as a peroxisome proliferator. The production of hydrogen peroxide by the peroxisome proliferators breaking down such compounds as fatty acids, along with the subsequent reactive oxygen species is thought to be the most conducive route of carcinogenicity by DEHP (Blass, 1992). The SCENHIR report of 2008, concluded that "DEHP induces liver tumours in rats and mice mainly by the Peroxisome Proliferator Activated Receptor αlpha (PPARα), which is currently believed to be non-relevant to humans".

The majority of the studies that relate to the hepatic effects of DEHP have been performed on rodents as they are the animals who have exhibited the most visible changes when exposed to DEHP and other plasticisers. The number of studies performed on other animals such as primates and guinea pigs have not shown the same results and it is for this reason that there is a debate as to whether the effects seen in rodents could be translated to humans.

Kevy and Jacobsen performed a study in 1982, designed to determine if there were any hepatic effects from plasticiser exposure following blood transfusions in the rhesus monkey. The rhesus monkey was chosen as the test subject because of the differences in the way it metabolises DEHP compared to rodents as well as the more direct correlation with the human species with its red blood survival, platelet preservation and serum protein pattern being similar to that of the human. It also performs in a similar manner to that of humans with regard to sulfobromophthalein (BSP) kinetics and also 99mTechnetium-labelled sulphur colloid scanning

techniques. The administered dose of plasma the monkey received over the year was equivalent to that expected in a human patient who undergoes chronic transfusion, which was to attempt to simulate the exposure routes seen in the clinical environment, something that had not been seen previously.

The results from the study showed that even after 26 months there was evidence of abnormalities in the hepatic scan and also in the BSP kinetics, with abnormal clearance curves. It was also shown that there was DEHP present in the liver after 14 months post procedure. One of the most interesting findings related to the comparison of the DEHP the monkey received during this study to that received by a patient undergoing maintenance dialysis. The dose that the monkeys received which was shown to produce some evidence of hepatotoxicity, was 10-20 times lower than what a patient who undergoes normal maintenance haemodialysis in a year (120-335mg/kg).

3.11.5. Reproductive Toxicity

There have been very few studies that highlight the reproductive anomalies in humans. The basis of the accusations levied at DEHP is all based on experimental animal work. It should be noted that the mechanisms of action between species especially the role of the peroxisome proliferator-activated receptor alpha (PPAR-α) are an integral part of determining the possible toxicity of DEHP in humans.

Testicular abnormalities have been shown to occur in both rodents and non-rodents exposed to DEHP. The No Observed Adverse Effects Level (NOAEL) has been shown to vary depending on the type of exposure. In a study by Schilling et al, 2001, involving two generations of rats subjected to an oral administration of DEHP, a NOAEL level for developmental toxicity was seen at 113 mg/kg/d and 340 mg/kg/d for reproductive performance and fertility. Similar NOAEL values from reproductive and testicular toxicity were produced by Wolfe et al in 2002 following an oral administration of DEHP. The lowest range of testicular toxicity is seen with a dose of DEHP in the range of 3.7-4.8 mg/kg/day. The findings by Sjoberg (1985, 1986) produced some critical data, showing that testicular toxicity is age dependent, with

developing males having a higher risk in developing testicular anomalies compared to their sexually mature counterparts.

In 1998, the scientific committee on toxicology, ecotoxicity & the environment (CSTEE) used the findings of a 13 week dietary study by Poon et al, 1997 which determined a critical end point of mild sertoli cell vacuolations as a sign of testicular toxicity in rats. They then set the NOAEL at 3.7 mg/kg/day which was the dose level that these abnormalities occurred.

However, the validity of these findings in relation to reproductive toxicity in humans had always been questioned because of the study design, which was not designed to evaluate reproductive performance and also because there was the appearance of minimal testicular tubular atrophies in some of the control animals (CSTEE, 1998).
The NOAEL level has since been modified by the European Chemical Board (ECB) in 2006, who in a risk assessment report, decided to increase the NOAEL value for testicular and developmental toxicity of DEHP exposure to 4.8 mg/kg/day based on the studies of Wolfe and Layton in 2003. This study demonstrated dose dependent effects on a selection of testicular related parameters and was deemed to have more robust endpoints than the previously used Poon study.
The debate on the effect of the species divide with regards to male reproductive toxicity is heightened due to the reported differences of effects between different species, with rodents being more susceptible than non-human primates (Rhodes et al, 1986). It was shown by Rhodes et al, that Marmosets receiving a dose of 2000 mg/kg each day for 14 days demonstrated no effect, where as male rats developed both liver enlargement and also testicular atrophy. Similar findings by Kurata et al, 1998 found that marmosets did not develop any signs of testicular toxicity at high doses of DEHP.
These findings are important with regard to the extrapolation of DEHP toxicity data between species. Marmosets have demonstrated similarities to humans, more so than rodents especially with regard to spermatogenesis. However, despite the issue surrounding the differences in species, DEHP has been classified as a toxic substance under the Council Directive 67/548/EEC, with effects on the following.

- Male and Female Fertility, Cat. 2, R60
- Developmental Toxicity, Cat. 2 , R61

DEHP is a Category 2 toxic substance which means that DEHP products must be labelled with a skull and cross bones and also adorn the appropriate safety and risk phrases. The risk phrases are the R60 and 61 which infer that DEHP may impair fertility and may cause harm to an unborn child respectively.

3.12. Mechanisms of Action

DEHP has been implicated in reproductive and developmental problems in rats and mice. (Hasegawa et al, 2004). The main organs that are seen to be targeted by DEHP and its metabolites are the testes, kidneys and liver. However, the actual mechanism that is responsible for the carcinogenetic effects of DEHP and other phthalate plasticisers is not fully understood at the present time. There have been two theories hypothesised to try and explain these effects particularly in regard to the liver effects. These are namely,

- Oxidative stress and generation of oxidative free radicals which cause damage to DNA, brought about by increased peroxisome proliferation due to activation of the PPARα receptor (Moody et al, 1978; Rusyn, 2001; Melnick, 2001).
- The formation of substantial levels of DNA damaged cells leading to the development of malignancies due to the suppression of apoptosis and increase in proliferation of cells within the liver (Melnick, 2001).

The peroxisome proliferator activated receptor alpha (PPARα) has recently been thought to play a major part in the control of the carcinogenic response of peroxisome proliferating compounds such as DEHP. It is this receptor that is thought to suppress the hepatocyte apoptosis of the damaged cells and also increase the activation of the regulated gene mRNA which results in increased hepatic DNA synthesis. (Melnick, 2001; Rusyn et al, 2006)

These two theories have been demonstrated in rodents, but the effect does not seem to manifest itself in primates or humans to the same degree (Cattley et al, 1998). Studies have used mice that have had the PPARα receptor knocked out of them to determine the effect on the induction of tumours within the liver. The studies failed to induce tumours even when administering very strong known peroxisome proliferators. (Peters et al, 1997; Ward et al, 1998) These findings lend weight to the argument that the PPAR α pathway is one of the main routes of initiation of tumours related to DEHP exposure, which in turn gives more weight to the argument that these adverse effects would not be as prevalent in humans due to the mechanism of action of the PPAR α being discounted in the human species (Cattley et al, 1998; IARC, 2000).

3.13. Medical Devices and a Toxic Chemical?

The debate on whether DEHP should be removed from medical devices is as intense as it was when the first indications of it causing problems emerged. Reports such as "The Koop Report" from the American Council on Science and Health in 1999 have stated that "DEHP used in medical devices is unlikely to pose a health risk even to highly exposed humans" They have highlighted evidence that in primates there is a decreased conversion of DEHP to its more toxic metabolite, MEHP, compared to rodents and also that most human medical exposure would be through intravenous exposure and not by oral exposure on which the animal studies are based. The exposure levels that are required for carcinogenesis, developmental and reproductive toxicity are not likely to be reached during medical interventions (Koop et al, 1999).

On the other hand a report by the Lowell centre for sustainable production at the University of Massachusetts, USA, interpreted the same data differently (Shea et al, 2003). They concluded that there is a problem with the exposure levels of DEHP significant enough to conclude that there was "a significant potential for serious adverse effects on human health" citing that the actual exposure levels of humans to

both DEHP and MEHP by intravenous method is not fully understood and documented at this time.

More recent reports by the Centre for the Evaluation of Reproductive Risks in Humans, (CERHR), concluded that for the general adult population the risks from exposure to DEHP are minimal and should be of no concern. Concern was raised regarding the exposure levels for infants and young toddlers and that they might have slightly higher exposure levels due to their dietary habits and mouthing behaviours. This exposure is also at a critical time when the male reproductive tract is developing and could be adversely affected. A higher level of concern was placed for young males who would undergo large amounts of intense medical or surgical treatment as they would be at most risk of receiving large doses of both DEHP and MEHP which again could cause some damage of the reproductive organs such as reduced testicular size and weight, mild Sertolli cell vacuolation which leads to germ cell depletion and reduced fertility (Poon et al, 1997; Wolfe et al, 2003).

The report concluded with remarks relating to the need for much more precise human exposure data to include multiple simultaneous exposure routes and better primate toxicity data to help improve the evaluation of the benefits and risks of medical exposure.

Other reports issued by the FDA in the USA and Health Canada, have reported similar results as above with concern over the exposure of young males to high levels of DEHP which may result in them being more susceptible to the risk of testicular toxicity. (Shea et al, 2003)

The “cloud of uncertainty” that surrounds the toxicity of DEHP and its use in medical products is there for all to see. There is currently no definitive answer or reasoning from the extrapolated results of the animal work that can say if exposure to DEHP through medical procedures could possibly cause toxic effects in the humans. The proposed mechanism of action of the activation of the PPARα receptor is one which has been discounted in humans. However, it cannot be said if this is indeed the mechanism that is the root cause of the adverse effects associated with DEHP and its

metabolites. More work is required to investigate not only the routes of exposure, but also the potential doses that the human race may be subjected to in an effort to determine if DEHP or its metabolites are actually responsible for the suggested adverse effects seen in animals.

3.14. Migration of DEHP

In 1970, Rubin and Jaeger reported the release of plasticisers from PVC medical devices into blood that had been stored in plastic blood bags. This was also reported by Marcel and Noel in the same year but as PVC had been used in medical devices and particularly in blood storage devices since the late 50s without any complications, there had been little concern expressed about the possible health effects on humans who were exposed to these leached agents (Autian, 1973).

It was the reporting of a side experiment in the same paper by Rubin and Jaeger that brought about substantial interest in the leaching of these agents. They discovered the presence of the plasticiser Di-2-ethylhexyl Phthalate, DEHP, in the tissues of two deceased patients who had received large quantities of blood that had been stored in PVC blood bags before being transfused into the patient. Since this discovery, the interest in this area has moved towards discovering not only more about the possible exposure of humans to the plasticiser DEHP but also the possible toxicological hazards of this compound and other phthalate plasticisers.

The focus in this thesis will be on the plasticiser DEHP, the most commonly used plasticiser in medical devices and medical disposables produced out of flexible PVC.

It is generally accepted that DEHP has low water solubility, < 0.04mg/ml @ 20^{o}C, (Jaeger and Rubin, 1973) and may migrate from the polymer when in contact with various media due to it not being covalently bound into the PVC matrix. As stated previously, it has been known that the DEHP is present in blood stored in PVC bags and also that it can be transferred into the recipient of this blood. This was corroborated by Marcel in 1973 and Hillman et al in 1975. Various plasticiser extraction rates have been published for different blood components producing such values as 5-7 mg/100ml/8hr for whole blood recirculated through a dialysis system and 0.25mg/100ml/ day for platelets stored at 4^{o}C (Jaeger and Rubin, 1970). Valeri

et al, 1973 produced results that showed the plasmas fraction of whole blood extracted at a rate of between 0.268-0.360 mg-% DEHP per day.

These results enforced the idea that the DEHP has a strong affinity to solutions that have a high lipoprotein concentration which was first described by Jaeger and Rubin in 1970. They showed that the higher the lipoprotein concentration the greater the amount of DEHP that was eluted into the solution, Figure 3.14. This figure indicates that the levels of DEHP eluted into each of the test fluids increased with the increased lipid content of the fluids, as both the 4% bovine serum albumin and perfusate (mixture of whole blood and albumin) show increased DEHP levels compared to the saline and solvent blank.

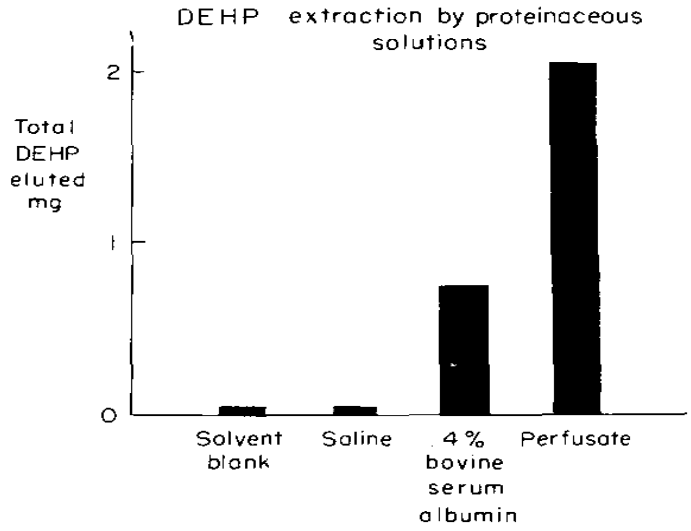


Figure 3.14: DEHP Extraction into proteinaceous solutions (Jaeger and Rubin, 1973) Reproduced with permission from Environmental Health Perspectives.

They furthered the work by determining which fractions in the blood contained the most of the DEHP, with results that indicated that the majority of the DEHP was found in the plasma section of the blood. The results showed that 60% of the total amount eluted into the whole blood was contained in the low-density lipoprotein portion (D<1.21 column) of the plasma, with the high-density plasma (D>1.21 column) containing another 20% and very little present in the triple washed Red Blood cells. This can be seen clearly in Figure 3.15.

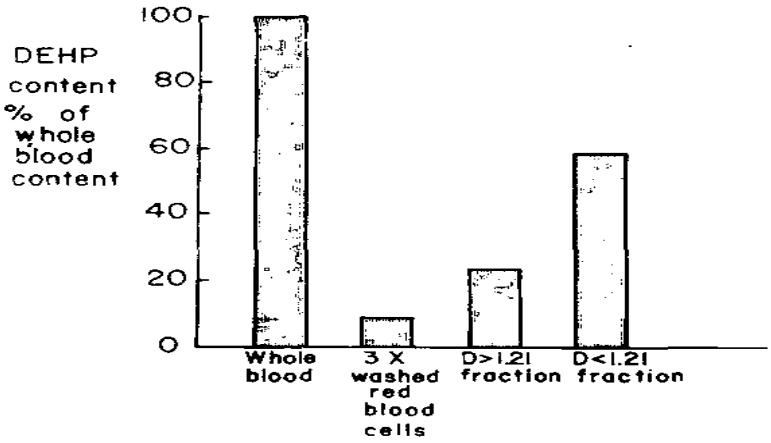


Figure 3.15: DEHP recovery from human blood and blood fractions after storage for 14days in PVC. (Jaeger and Rubin, 1973). Reproduced with permission from Environmental Health Perspectives.

The same authors also presented data that showed another important factor in the extraction of DEHP from the PVC material is temperature. They showed that by reducing the temperature of the blood from room temperature to around 4^{o}C it reduced the amount of DEHP that migrated out of the PVC.

These early indications that the lipid content of the fluid may have an influence on the migration of the plasticiser were confirmed by Faouzi et al (1999), who showed a direct linear correlation, (R=0.705), between plasma lipid concentration and the total amount of leached DEHP. This finding has a major implication on medical usage of PPVC, with PPVC coming into contact with many high lipid content fluids, be it biological or pharmaceutical on a regular basis throughout the hospital environment.

3.14.1. Extraction Media

Since the first reported migration of DEHP into blood, many studies have been undertaken to better understand the migration pattern of DEHP from the PPVC. The extraction of the chemical, DEHP, from the polymer PVC is one of the most studied migration profiles to date. This is due to it being the most commonly used plasticiser in medical device applications. These studies have used a variety of extraction media each with differing properties and hence results.

Extracting Fluid	Water	1% Soap	Mineral Oil	Hexane
Immersion Time (hr)	**24**	**24**	**24**	**1**
% weight loss by Extraction	**0.2**	**5.3**	**11.6**	**22.0**

Table 3.2: Migration of DEHP into various extraction media showing the increased removal of DEHP as an increase in % weight loss. (Adapted from Adams, 2001)

It can be seen from the above table (Table 3.2) that extraction of DEHP varies greatly depending on the type of media used in the extraction studies. Extraction is seen to be minimal in aqueous solutions, potentially due to the hydrophobic nature of DEHP. The extraction of DEHP increases with the additions of surfactants to the water. However, when oil based or organic solvents are used the extraction can be seen to increase at a much higher rate. This propensity for DEHP to migrate into lipid solutions is due to its highly lipophilic nature.

Recently, studies have focused on the use of organic solvents as the extraction media of choice because of their efficiency in removing the plasticiser from the PVC. The use of methanol in DEHP extraction studies has led it to be known as the un-official gold standard method in plasticiser extraction. (Lakshmi & Jayakrishnan, 1998; Zhao,1999; Gourlay, 2001a, 2002; French, 2001; Sharma, 2002).

It was the methanol studies by Kim et al (1976) and Zhao (1999) that have shown that the extraction of DEHP from PPVC follows a specific two phase process of dissolution and diffusion Figure 3.16. The dissolution phase of the extraction Vs time graph represents the removal of the plasticiser that is present on the surface of the PPVC by the solvent. This leads to what is described as a “clean” surface. From this point onwards, the extraction of the DEHP is classed as direct diffusion from the bulk of the material to the surface and then into the solvent. This is represented by the linear portion of the graph. The linear nature of the graph remains constant as long as there is sufficient plasticiser remaining in the bulk of the PPVC.

The understanding of the migration of the plasticiser from the PPVC is important on two levels. The leaching of the plasticiser into the environment leads to both a mechanical and biological concern which can in many cases be linked. The leaching of the plasticiser from the PPVC degrades the material by reducing the mechanical properties and hence making it less pliable and more likely to fail. Whilst this is an area of concern it is the introduction of the plasticiser into the biological environment and subsequently the patient which draws more of a concern.

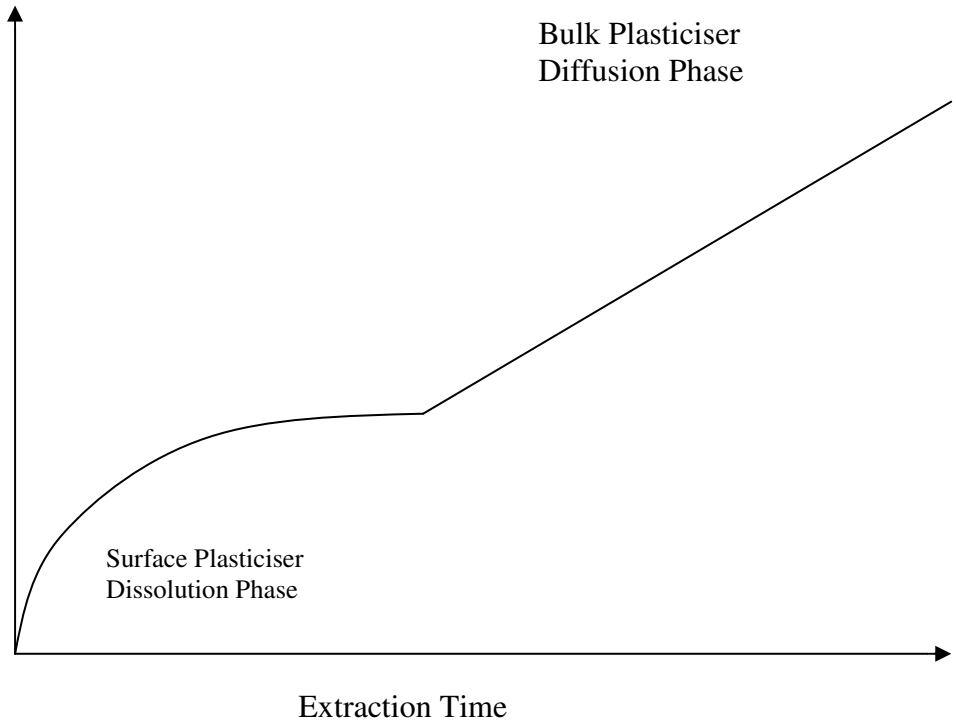


Figure 3.16: Extraction profile of DEHP from PPVC suggested by Kim et al, 1976. and Zhao 1999

3.14.2. Biological Migration Studies

The profiling of DEHP migration into organic solvents, especially methanol, is an excellent way of promoting fast accelerated studies removing large quantities of the plasticiser from the material. However, this does not give an accurate representation of the migration into blood and other biological fluids.

To counter this lack of knowledge, a number of studies have been performed using blood and other fluids such as plasma. The area of focus on these studies was initially blood and blood component storage bags. These devices were at the time, providing the longest continuous contact of these biological fluids with the PPVC and its plasticisers (Valeri et al, 1973, Vessman & Rietz, 1974, AuBuchon et al, 1988).

The results of these studies showed that the migrated DEHP actually has a positive effect on the life of the stored blood by lowering the osmotic fragility of the RBCs by binding with the membrane (Stern & Carman, 1980; Estep et al, 1984; Rock et al, 1984, AuBuchon et al, 1988).
These earlier studies opened a whole new dimension to the use of DEHP in blood contacting devices, by showing that the migrated DEHP conclusively has some benefit in the storage of blood and blood products compared to other alternative materials.

However, this theme of plasticiser migration from blood bags is still being investigated (Chawla & Hinberg, 1991; Inoue et al, 2005). A number of studies have focused more on the effects of DEHP migration in extracorporeal treatments such as dialysis, ECMO and CPB (Pollack et al, 1985; Kambia et al, 2001; Loff et al, 2004; Hildenbrand et al, 2005). These treatments expose patients to repeated large doses of migrated plasticiser because of the components involved in their complex systems. It is this type of repeated exposure which according to the various regulatory bodies around the world that places patients at a possible higher risk.

3.15. Everyday Exposure for the General Public

In modern society, the general population are exposed to a variety of environmental pollutants. Exposure to DEHP is generally found to be through either the ambient environment or in a medical setting.

DEHP's ability to leach from the host material into the environment means that it now a ubiquitous environmental contaminant and is being found more and more in water, soils and also food.

The largest area of exposure for the general population is though diet. Fatty foods such as dairy products and oils have been found to contain the largest amount of DEHP within the food chain (Moore et al, 2001; Wormuth et al, 2006).

The contamination of foodstuffs can be found during every aspect of food preparation through to consumption including significant contamination during the processing, handling, packaging and storage phases. The other significant forms of exposure to DEHP are from indoor air, household dust and consumer products like plastic toys and flooring. The Agency for Toxic Substances and Disease Registry (ATSDR) published a report in 1993 that estimated that the average American has a daily intake of around 0.28mg/day and of this 0.25mg/day comes from exposure in food stuffs.

The level of environmental exposure is hard to quantify. The methods of determination are broadly split into two categories, probabilistic calculations and urinary metabolite excretion. Both calculation methods produce varying results which are attributed to a wide range of factors that are introduced when trying to measure an exposure from a chemical that is ubiquitously found in the environment. The results from the probabilistic calculations produced varying results from 1µg-30µg depending on the author. (CERHR, 2005; Doull et al, 1999)

Measuring the excreted metabolites of DEHP is an avenue that has been explored by many authors for both environmental and medical exposure. These techniques are by no way fool proof, but they have managed to produce evidence that has shown that children are at an increased risk of exposure compared to adults.

3.16. Exposure through Medical Procedures

DEHP plasticised PVC has been used in the medical field since the early 1950s and is still the first choice polymeric material for storage containers for blood and or nutrients, catheters and tubing. Therefore, it is understood that a person undergoing certain medical procedures will probably be exposed to an amount of this DEHP plasticiser (FDA 2002, Health Canada 2002). Many studies have been performed to discover the quantities of DEHP that a patient may be exposed to during different interventions. The following list (adapted from SCENHIR, 2008) highlights a number of procedures that were deemed to have a high level of plasticiser exposure.

- Exchange transfusion in neonates
- ECMO in neonates and adults
- Total Parenteral Nutrition (TPN) in neonates
- Haemodialysis in prepubertal males
- Heart transplantation surgery
- Cardiac surgery using cardio pulmonary bypass (CPB)
- Large blood transfusions in trauma patients
- Transfusions in adults undergoing ECMO

The exposure type can be broken down into both acute and chronic exposure. Acute procedures are those that involve blood transfusion in trauma cases, those who undergo surgical procedures such as Cardio-Pulmonary Bypass (CPB) or extracorporeal membrane oxygenation (ECMO) as well as those patients who receive intravenous delivery of pharmaceuticals.(SCENIHR, 2008)

The chronic exposure comes from such interventions as haemodialysis, intubations of patients including feeding tubes, as well as repeated blood and blood product transfusions to patients who are suffering from haematological conditions such as sickle cell anaemia or haemophiliacs.

The level of exposure the patient receives depends on many factors. The exposure level has been shown to be strongly related to the following. (Gourlay et al. 2002;

Haishima et al, 2005; Hanawa et al, 2000,2005; Kambia et al, 2003; Loft et al, 2000, 2002, 2004)

.

- Lipid content of the fluid that comes into contact with the device
- The temperature & flow rate of the contacting fluid
- The surface area of the PVC exposed to the fluid
- The contact time

The following table (Table 3.3) gives a good representation of the exposure doses from the above mentioned procedures that an adult may experience for both acute and chronic interventions (Adapted from SCENIHR, 2008).

Medical Procedure	**Daily Dose (µg/kg/d)**	**Reference**
Long-Term Exposures		
Haemodialysis	**640 (150-2200)** **450 (270-1210) – delivered dose** **100 (20-360) – retained dose** **230 (50-850) – retained dose**	**Pollack et al (1985)** **Faouzi et al(1999)** **Dine (2000)**
Long term supply of blood and blood products	**6-90**	**Jacobson (1977)** **Doull (1999)** **Plonait (1993)** **Health Canada (2002)**
Long-term total parenteral nutrition	**130-280** **800-2000 (infants/children)**	**Loff (2000)** **Kambia (2003)**
Short Term Exposures		
Transfusions of Blood components: **Trauma** **ECMO**	 **8500 (63 units whole blood)** **1300-2600 (2.5l whole blood)** **3000-10000 (21-46 units combined blood products)**	 **Jaeger and Rubin(1972)** **Sjoberg (1985b)** **Butch (1996)**
Bypass op	**2400**	**Barry (1989)**
IV Drugs		
Lipophillic **Non-lipophillic**	**< 5** **Up to 1500**	**Health Canada (2002)** **Pearson (1993)**

Table 3.3: Possible DEHP dose levels from medical procedures for adults.

Recent calls by the NHS for more blood donors has led to an increase in the interest in exposure levels of volunteers who undergo such procedures as plasmapheresis or plateletpheresis to see if their repeated goodwill would perchance affect their own lives. A letter to the editor of Transfusion in 2006, by Koch and Weisbach, highlighted that these volunteers would be exposed to a varying amount of DEHP during these procedures. The amount received varied on the method of collection used by the health professional. It was shown that the plateletpheresis procedure produced a significantly higher level of DEHP exposure than the plasmapheresis. The levels of DEHP exposure varied considerably depending on the type of connection the donor received. The continuous connection method produced an exposure level which was nearly six times greater than in the control subjects and the non-continuous connection method producing a DEHP exposure level that was three times greater than the control subjects.

Table 3.4 highlights the previous work by Koch et al that led up to this letter showing the values of DEHP exposure calculated from this type of procedure. (Adapted from Koch et al, 2005)

Donation Procedure	**N**	**Exposure range (µg/kg/d)**
Controls	**5**	**3-11.6**
Plasma	**6**	**3.1-9.6**
Platelet (Continuous.)	**6**	**14.3-23.8**
Platelet (Non-continuous.)	**6**	**28.2-38.1**
Platelet (Continuous.)	**1**	**31.6**

Table 3.4: DEHP exposure from Blood Transfusion Procedures. Data reproduced with copyright permission from the publisher, Elsevier.

Even though this work showed that the volunteers would receive a considerable dose when donating platelets, the levels are well within the NOAEL effects for DEHP. As these volunteers are all healthy adults then the effects of the DEHP dose are deemed

to be insignificant. The same rational applies to the adults who undergo the procedures in Table 3.3.

Whilst there has been a considerable amount of concern shown for the possible exposure of adults to DEHP through medical devices, the levels of exposure are generally far off the current set limits that possible damage may occur at.

This is not the same case for younger patients who are not fully grown. Studies by Health Canada and the FDA in 2002, have shown that developing foetus and neonates are at considerable risk from DEHP exposure due to many factors. The main concern is that the adverse effects of DEHP are more pronounced in younger patients and are suspected of developmental and reproductive toxicity. Further concern is that many premature babies do not just undergo one single exposure; they are more than likely to be subjected to a multiple exposure from many different DEHP medical device interventions such as feeding tubes, transfusion and infusion tubing systems, PVC blood bags, and CPB.

The multiple exposures and small size of the patient means that they would be exposed to greater doses in terms of body mass than the adult population (Calafat et al., 2004). In fact the neonate can possibly be subjected to around 20 times the tolerable intake according to a study by Jaeger et al in 2005 and in some cases in a similar range to the reproductive toxicity inducing doses that were used in some animal studies (SCENIHR, 2008).

The following table (Table 3.5) adapted from SCHNEIR 2008 shows some of the doses that premature neonates may be exposed to during medical procedures.

Medical Procedure	DEHP Daily dose to 4kg Neonate (µg/kg/day)	Reference
Blood Transfusion Single dose Packard RBCs (20ml) Single dose Platelet rich plasma Single dose Fresh frozen plasma	36-152 3.16.1. 232 138-2020	Loff et al. 2000
IV Drugs Midazolam (24ml) Fentanyl (29ml) Propofol (1%, 10ml 24hr)	7 33 1640	Loff et al., 2000 Loff et al., 2000 Loff et al., 2000
ECMO Sub acute	Up to 14000 (14000µg/kg/10 days) 0 (heparin coated tubing) Up to 3490 (34900 µg/kg/10 days)	Schneider 1989 Karle 1997
Respiratory Aid Oxygen therapy Endotracheal tube	≤ 130 ≤700	Health Canada 2002 Health Canada 2002 FDA 2002
Total Parenteral Nutrition (TPN) Lipid Free Lipid emulsion 20% @ 27°C Fat infusion @ 33°C	30 2500 3250	Loff et al., 2000 and 2002

Table 3.5: DEHP exposure levels for neonates undergoing medical procedures

3.17. PVC Biomaterial influence on inflammatory response

3.17.1. Introduction

Much of the focus on the adverse effects of DEHP in recent years has centred on the carcinogenic and toxic effects of the compound. The fear that a compound used so extensively in medical products could cause more harm than good has led researchers to focus on proving the mechanisms of action of the compound in causing such diseases as cancer.

Whilst the general focus has been on the carcinogenic and toxicological effects, there is one effect which has been proven to have a clear clinical effect in humans but has currently gone unnoticed by the regulatory bodies, that DEHP is an inflammatory mediator. The first report of inflammation associated with DEHP exposure was by Jaeger in 1971. In his thesis, he reported that white lesions were seen on a number of organs harvested from rats which had undergone a single IP injection containing an industrial dose of DEHP. However, it was not until 2003 when Gourlay et al showed that DEHP evokes an inflammatory response in the blood of both rodents and more importantly humans. This common effect in rodents and humans is of key significance and has sparked a considerable research effort.

3.17.2. P-PVC and the Inflammatory response

All biomaterials, when exposed to biological fluids and tissues, invoke some sort of inflammatory response (Gourlay, 2001a). The magnitude of this response depends on a number of different factors such as type of material, length and type of contact and so on. One of the features of PPVC that has encouraged its use as a Biomaterial is its apparently good biocompatibility. Hence, it has been used for many years as part of the circuitry in extracorporeal procedures such as Dialysis and CPB.

As mentioned previously it has been shown that it is the DEHP plasticiser on the surface of the PPVC that influences the response of the material and not the PVC resin (Zhao 1999, 2003; Gourlay et al, 2003). The knowledge that the DEHP makes up 70% of the PPVC surface, its propensity to migrate from PPVC when in contact with biological fluids such as blood, makes DEHP PPVC a potentially significant

promoter of unwanted inflammatory responses in patients undergoing extracorporeal procedures.

3.17.3. Inflammatory response in Extracorporeal Procedures

For many years patients undergoing extracorporeal procedures such as CPB, have been shown to develop an unwanted inflammatory response during and post surgery. If this response goes unchecked, a clinical syndrome known as a systemic inflammatory response syndrome (SIRS) may occur. The severity of this syndrome can vary greatly between patients, with the worst case scenario resulting in multiple organ dysfunction (MODS), which is defined as "the presence of altered organ function in an acutely ill patient such as homeostasis cannot be maintained without intervention" (Bone et al, 1992.)

This systemic response is also associated with many clinical injuries such as major trauma, infection and burns (Asimakopoulos and Gourlay, 2003). However, it is more commonly associated with cardiac surgery and is the primary cause of mortality and morbidity associated with cardiac surgery.

The development of this SIRS is thought to have four main contributing factors (Asimakopoulos, 1999).

- Bio-material contact of blood and cellular components
- Leukocyte and Endothelial activation due to ischemia and reperfusion of tissues
- Leukocyte and Endothelial activation due to endotoxins
- Operative trauma (Butler et al, 1993; Ohri, 1996)

There are many different mechanisms involved in the generation of the inflammatory response and their interactions are complex. Many studies have been performed into the different molecules associated with the inflammatory response. One author, Franke et al, 2002, has since shown that the immune response associated with CPB is a two part process.

The initial phase occurs on first post operative day and represents the pro-inflammatory and anti-inflammatory reaction of the immune system. It is associated with the release of pro-inflammatory factors, complement, cytokines, adhesion molecules, endothelins, platelet activating factors and inflammatory factors such as

Interleukin-10 (IL-10) (Elahi et al, 2008). This phase starts to decline over the three days post-operation with base levels reached after 3 days. The second phase results in an anti-inflammatory reaction and a period of increased incidence of infection (Franke et al, 2002).

Recently, studies have focused on the activation of leukocytes and in particular the up regulation of adhesion molecules, L-selectins, P-selectins, β 2-integrins such as CD11b (mac-1) or CD11c , on the cell surface as a marker of inflammatory response (Galinanes et al, 1996; Moen et al, 1997; Asimakopoulos and Taylor, 2000).

As mentioned previously, the role of the material contact with the blood and cellular components is an important factor in SIRS. This has led to the study of many of the materials involved in extracorporeal re-circulation systems. One material that is found in abundance in these systems is DEHP PVC. It has been studied extensively to determine if it has a possible role in this adverse inflammatory response.

Gourlay et al, have published an important series of studies that have investigated the effect of the DEHP plasticiser in the possible inflammation response during a CPB procedure.

The studies involved measuring the CD11b (mac-1) integrin that had been studied extensively in the clinical environment and is deemed to be a good indicator of leukocyte activation (Asimakopoulos et al, 2000).

Gourlay et al, first demonstrated that the larger the exposed surface area of DEHP PVC the greater the CD11b expression and hence a higher level of neutrophil activation (Figure 3.17), with the expression of the CD11b molecule increasing not only at each sampling time of 30 and 60minutes but also with the level of exposed PPVC surface. It can be clearly seen from the figure below that the levels of CD11b expression at 60 minutes of exposure increase in a linear manner each time the material surface area doubles. They also reported that removal of the surface DEHP by pre-washing of the material with methanol also leads to a reduction in the CD11b expression (Figure 3.18) with a marked reduction in the CD11b expression at both 30 and 60 minutes of exposure when compared with the untreated 48cm of PPVC. It should be noted that the surface area of PPVC that was exposed to the methanol wash was also 48cm.

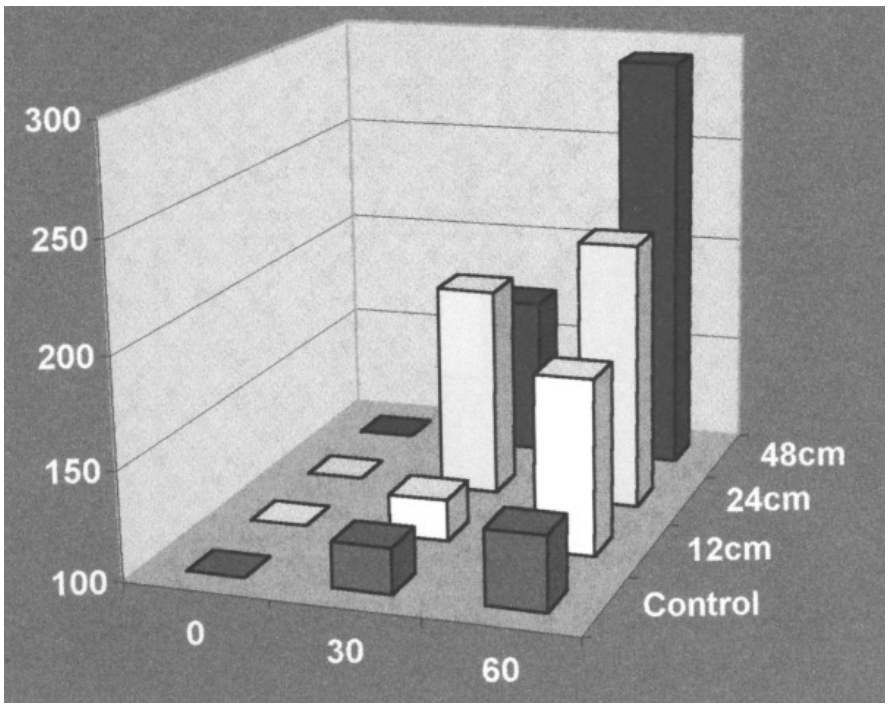


Figure 3.17: Effect of increasing the exposed surface area of PPVC showing an increase on the levels of expression of CD11b on isolated neutrophils over the sampling periods of the experiment (Gourlay, 2001a). X-axis is the sample times (min), Y-axis is the CD11b expression and Z-axis is the exposed surface area. Reproduced with copyright permission from the publisher SAGE as part of UMIs "Books on Demand" program

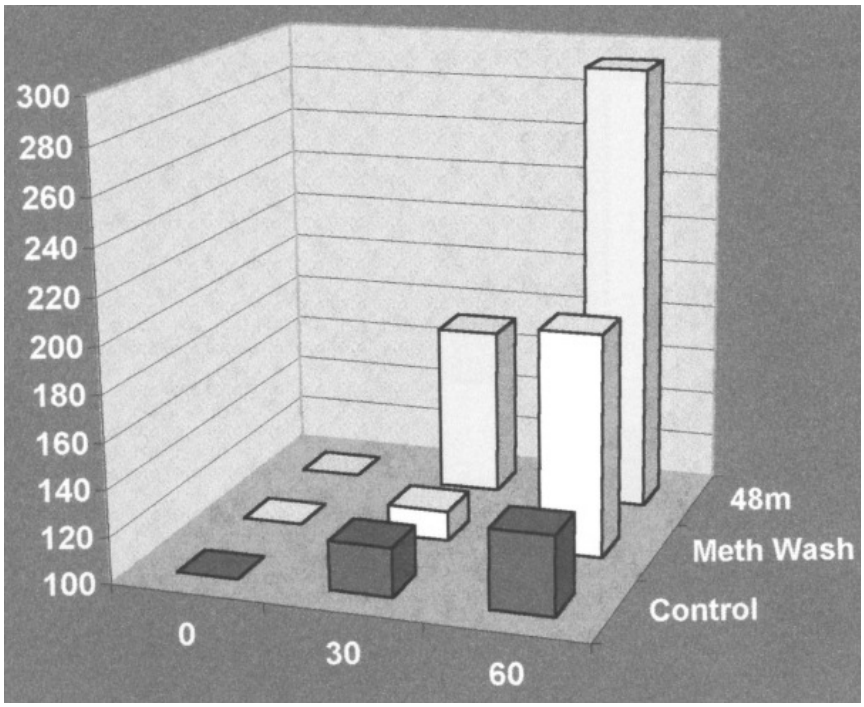


Figure 3.18: The effect of Methanol washing on the removal of DEHP from the surface of the PPVC which showed marked reduction of CD11b expression on neutrophils over the experimental sampling period (Gourlay, 2001a). X-axis is the sampling times (min). Y-axis is the CD11b expression values. Z-axis is the material exposed to the test fluid. Reproduced with copyright permission from the publisher SAGE as part of UMIs "Books on Demand" program

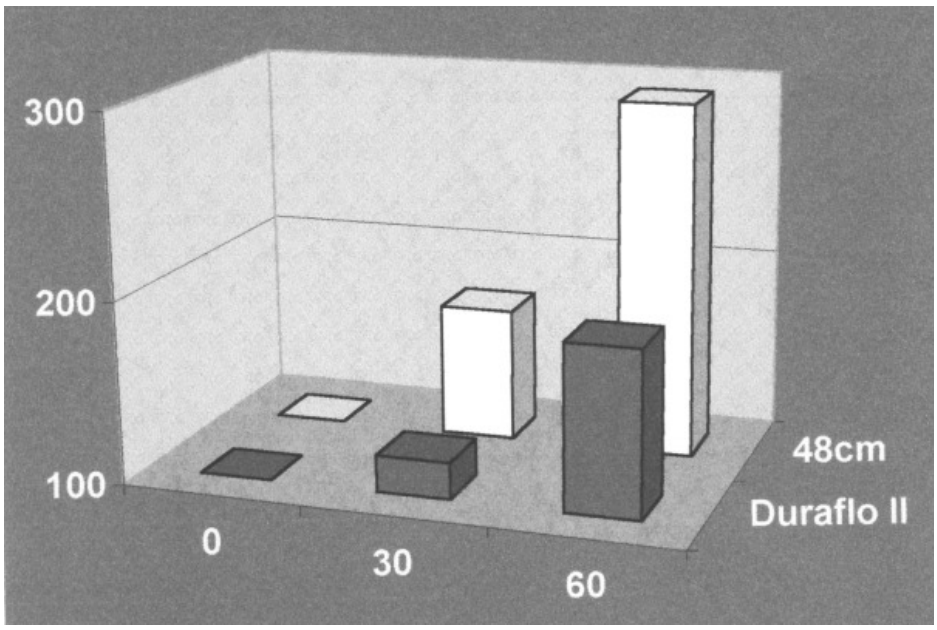


Figure 3.19: The effect of heparin bonding on the PPVC surface (Duraflo II) has a marked reduction on the CD11b expression on neutrophils compared to the untreated material (Gourlay, 2001a). X-axis is the blood sampling times (min). Y-axis is the CD11b expression. Shaded bars represent Duraflow II and the open bars are 48cm of PPVC. Reproduced with copyright permission from the publisher SAGE as part of UMIs "Books on Demand" program

Finally, they reported that the heparin coated PVC also had a reduced level of CD11b expression at both the 30 and 60 minute sampling times when compared with the same area of untreated PPVC material which was inline with many other clinical findings and confirmed that the addition of heparin coatings leads to improved biocompatibility in terms of neutrophil activation. (Figure 3.19).

Whilst the main concern lies with the inflammatory nature of DEHP in extracorporeal re-circulation procedures, there are other areas in which DEHP's inflammatory nature has been a cause of concern. The inflammatory properties of phthalates and especially DEHP have also been thought to be a factor in airway problems such as asthma and rhinitis (Bornehag et al, 2004; Jaakkola et al, 1999, 2004, 2006). The prevalence of these conditions in the western population is steadily increasing and many believe this is attributed to the abundance of chemicals such as phthalate plasticisers especially DEHP in the environment (Wormuth et al, 2006).
Furthermore, it has been shown in animal studies that DEHP and its metabolite MEHP, is immuno-modulatory by increasing the immunogenicity of a co-

administered antigen, immunogen ovalbumin (OVA) (Larsen et al. 2001, 2002. They looked at DEHP and other phthalates such as DOP and DINP and showed that DEHP was the most potent of the phthalates investigated, in that it produced elevated levels of IgG1 compared to the control OVA after only one administered dose compared to the other tested phthalates which only demonstrated elevated levels after two doses. (Larsen et al. 2002).

Whilst DEHP has been shown to promote an increase in lung inflammatory and IgG1 levels at high doses in a mouse model (300mg/m^3 DEHP), its effect at more realistic concentration levels (3.7, 18.4, 31.6mg/ m^3 DEHP) did not induce the same reaction (Larsen et al. 2007).

This response it not unexpected as similar findings have been seen in carcinogenic studies (David et al, 2000; Sjoberg et al, 1985) and other toxicological profiles, showing that DEHP can be challenging in high or sustained doses but small, one off exposures are generally not seen to be as much of a problem compared to the long term sustained doses seen during extracorporeal procedures.

DEHP has also been shown to act on the very first line of inflammatory response by producing an increased release in histamine from isolated basophiles (Glue et al. 2005). The importance of histamine release in biomaterial blood interactions is significant in the initiation of many of the bodies defence mechanisms and should not be ignored.

These studies have shown that DEHP may be a significant cause of the unwanted inflammatory response during the aforementioned procedures as well as being a cause of the increasing levels of respiratory problems in the general population. Therefore, the accurate determination of amounts of DEHP released during these procedures and the overall reduction of these levels associated with some interventional techniques is of clinical importance.

3.18. Conclusion

The preceding chapters have provided a background into PPVC, its constituent components such as the plasticiser and how it is used in the modern medical devices. An introduction to PPVC as a Biomaterial and the interactions of artificial materials with the bodies natural homeostasis was also described along with and the potential adverse effects of these materials on the human body with a particular emphasis on DEHP and the potential impact this chemical could have on the health of the general population.

Modern society's reliance on artificial materials, especially plastics, in everyday life has led to the contamination of the general environment. Whilst this contamination is small, it subjects the population to potentially harmful chemicals on a consistent basis.

This environmental exposure is not seen as an issue regarding public health; however, the exposure of both adults and children to these chemicals, especially DEHP, during medical procedures is a major issue. The possible side effects related to DEHP have the ability to cause major complications for sick people. The potential doses that a sick neonate or young child could receive is substantially higher per kg/body weight than that of an adult and can on occasions be very close to or exceed the current recommended safe dose. It is no wonder that this patient group are seen as one of the highest risk groups. It can therefore be said that continued study in determining not only accurate levels of the DEHP chemical that migrate during medical interventions but also the effects of these migrated doses is of critical importance to ensure that the patients are not exposed to a needless danger.

3.18.1. Project Aims

The investigation of DEHP migration has been studied extensively, but is in no way complete. Most of the work has centred on static, *In vitro* experimentation. Many of these experiments have involved the incubation of the test material in various serums and solvents and then determining the DEHP content in these solutions. This,

however, does not portray the type of conditions that the material would be exposed to in the clinical environment. The explorations of these conditions are an important part of the development into a more complete understanding of the migration of DEHP from medical plastics.

The aims of this research project are as follows:

- To investigate if plasticiser migration is an actual clinical problem
- To investigate whether the use of a radiolabel could be a possible standard method of determining plasticiser migration from PPVC
- To further the understanding of DEHP plasticiser migration from medical grade PPVC using *In vitro* experiments and a novel *Ex vivo* model.
- To determine the fate of migrated DEHP after exposure in a novel rat perfusion model and after gavages

4. The Clinical Problem

4.1. Introduction

The information gathered from the literature search showed that there is a major controversy surrounding DEHP migration from P-PVC, but the significance of these possible problems in the human species remains controversial. Therefore, it was decided to investigate if DEHP plasticiser migration was an actual concern in the clinical setting.

Discussions with various clinical staff showed that they are concerned about the possible side-effects of DEHP migration during acute and chronic interventions, such as ECMO or Dialysis, in patients especially neonates and young children.

The main area of concern did not lie with the possible carcinogenic effects of DEHP, but with DEHP being an inflammatory mediator. It was felt that this trait was the most damaging of all the possible side-effects of DEHP exposure during clinical interventions as it is at present the only one with a definitive and proven link to the human species.

4.2. ECMO Tubing Study

In response to the need for a more comprehensive understanding of the possible exposure of patients to plasticisers that are used regularly in ECMO, this study has been designed to investigated one particular issue associated with plasticiser migration; the effect of wet storage of ECMO circuits, a fairly common clinical practice, on the migration of plasticisers. In addition we investigated the effect of the makeup of the priming solutions and different plasticiser types employed on plasticiser migration under long term storage conditions.

Whether it is appropriate to store primed ECMO circuits for future use has been a matter of debate for some time, and the focus of this clinical debate has tended to revolve around possible infection issues, and effects on oxygenator performance. The potential clinical impact of DEHP exposure remains controversial, however, there is a body of literature that suggests such exposure is undesirable in the clinical setting. Our study, carried out under laboratory conditions, aims to determine whether wet storage is a safe technique in terms of plasticiser migration, which ultimately may have a negative clinical impact once the circuitry is deployed.

4.2.1. Materials and Methods

The study involved the testing of the four most commonly used tubing types that are found in ECMO circuits within in National Health Service Scotland and exposing them to three common priming solutions. The investigators were blinded to the tubing type and priming fluids employed, both of which were supplied unmarked by the clinical partners. The tubing was labelled A, B, C and D and the priming solutions numbered 1, 2 and 3. Only at the end of the experiment were the names and manufacturers of the tubing and fluids revealed to the investigators. These designations relate to the following materials:

Fluid 1: 0.9% (w/v) Sodium Chloride Solution
Fluid 2: 2/3 0.9% (w/v) Sodium Chloride Solution and 1/3 20% (w/v) Human Albumin
Fluid 3: 2/3 Gelofusine and 1/3 Hartmann's solution
The components of Fluid 3 are common clinical fluids have the following properties:

Gelofusine: It is a 4% (w/v) solution of succinylated gelatine which is used as a plasma substitute to expand and maintain blood volume after trauma. Its contains the following per 1000ml infusion.

- 40g Succinylated Gelatine
- 7g Sodium Chloride
- 1.36g Sodium hydroxide

Hartmanns Solution: This is also known as compound sodium lactate and is used to replace body fluids and mineral salts lost through medical reasons. Its make up can vary but is generally as follows.

- 131 mmol/L Sodium ions
- 111 mmol/L Chloride ions
- 29 mmol/L lactate
- 5 mmol/L Potassium ions
- 4 mmol/L Calcium ions

Tube A: EEC Blood line, Raumedic, Munchberg, Germany.
Tube B: Dideco XS, Sorin Group, Arvada, Colorado, USA.
Tube C: Action, Tekni-plex, Sommerville, New Jersey, USA.
Tube D: Tygon S-95E, Saint-Gobain, Akron, Ohio, USA.

4.2.2. Experimental Setup

Three 8cm sections of each tube type was cut and placed on a test rig (Figure 4.1). 5cm of this tube was then exposed to each of the test fluids and a non plasticised polyurethane stopper was then applied to the open end of the tube. The test rig was then placed on an orbital shaker plate at 150rev/min and kept at room temperature to simulate gentle recirculation a common clinical practice.

Samples of the test solutions were taken at were taken at 0hr, 1hr, 4hrs, 8hr, 24hr, 48hr, 7days, 14 days and 28 days. The plasticiser content of each sample was measured using a spectrophotometer (Ultrospec 4300pro, GE Healthcare Lifesciences, USA) over a wavelength range of 200-900nm, running a Wavescan analysis program. The reference sample used for all sample readings was a 100% Sodium chloride 0.9% solution.

A concentration curve for all plasticisers was also plotted using known concentrations, DOA and TOTM, suspended in methanol, and this was employed for calculating plasticiser concentrations.

4.2.3. Concentration Analysis

The concentration of plasticiser present in the test samples, which represents the plasticiser migration level, was determined by measuring the appropriate peak absorbance height. This value was then translated into a concentration by referring to the concentration curves plotted previously. This technique had been used successfully in previous published work by Gourlay et al., 2003 and also by Zhao and Courtney, 2003.

Statistical Methods

The statistical analysis in this study was performed using the MiniTab V15 statistical software tool (Minitab Ltd. Coventry, United Kingdom). Using this software, descriptive statistics were used to calculate the mean and standard deviations of the samples. The probability values quoted in this paper manuscript were also determined by performing a *t*-Test using the same software package.

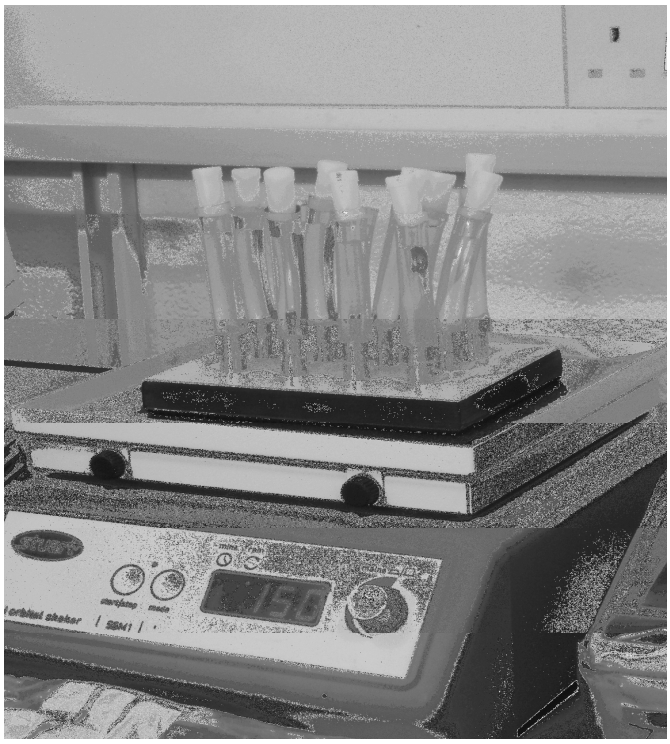


Figure 4.1: Experiment Test Rig Set up showing the constrained and sealed tubes on the tubing block positioned on an orbital shaker.

4.3. Results

The results from the recirculation migration experiments for Tubes A, B, C and D are shown in figures 4.2 to 4.5.

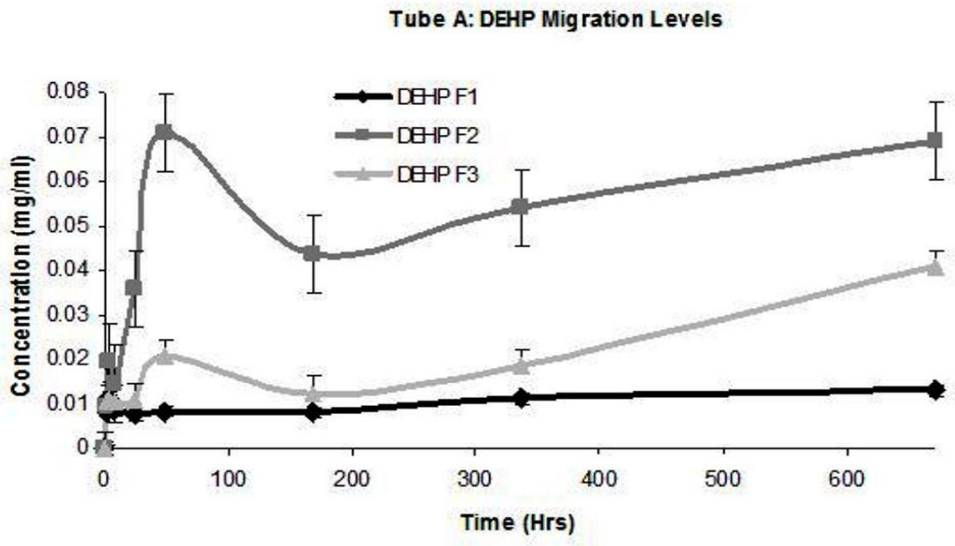


Figure 4.2: Graph of DEHP Concentration levels migrated from Tube A in mg/ml. (n=4) Data shown as Mean±SD

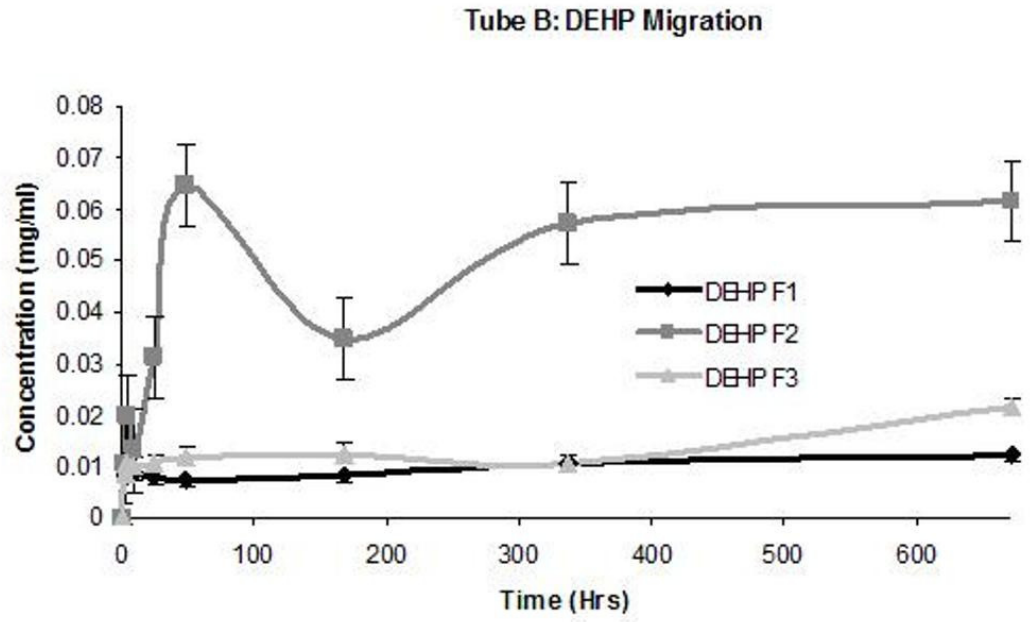


Figure 4.3: Graph of DEHP Concentration levels migrated from Tube B in mg/ml. (n=4); Data shown as Mean±SD

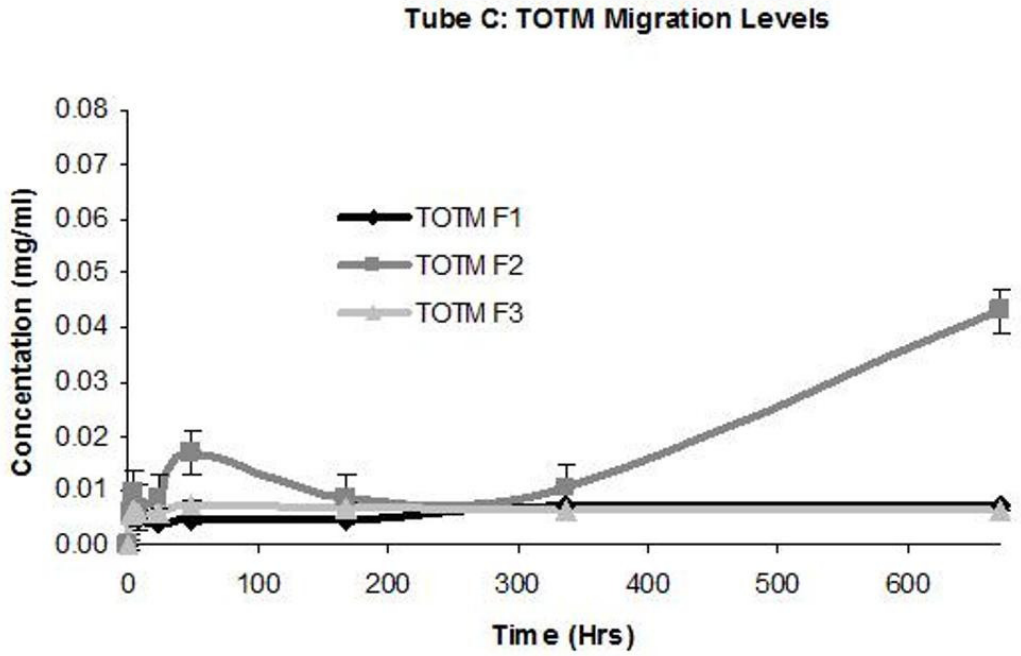


Figure 4.4: Graph of the migration concentration levels over the entire test period for Tube C in mg/ml. (n=4); Data shown as Mean±SD

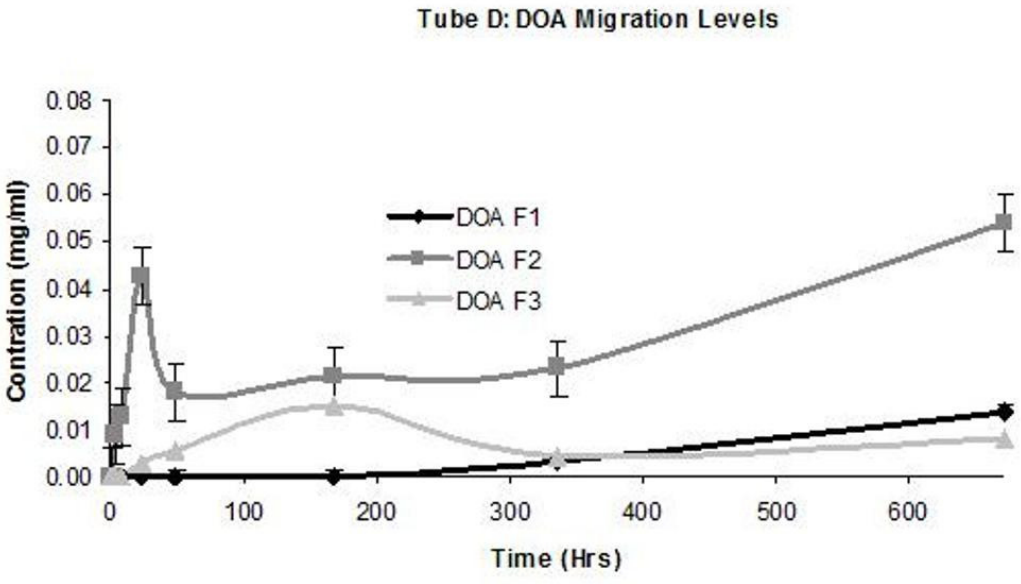


Figure 4.5: Graph showing the migration concentration levels over the entire test period for Tube D in mg/ml. (n=4); Data shown as Mean±SD

The results clearly demonstrate that in all tubing types, in the presence of all fluids, there was evidence of plasticiser migration. The level of migration, however varied

significantly over time and in response to fluid type. The DEHP migration profiles for both DEHP plasticised tubing types were fairly similar. Detectable levels of plasticiser migration were seen after as little as 1hr of exposure to the test fluids in both cases. Fluid 1 (sodium chloride 0.9% solution) was associated with the lowest level DEHP migration from the tubing with levels that varied between 0.0079±0.00031mg/mL and 0.008± 0.00031 mg/mL out to 7 days of exposure. The concentration levels tended to increase consistently from 14 to 28days of exposure with a final value of 0.0132± 0.0022 mg/ml and 0.0128±0.0043 mg/ml for tubes A and B respectively, although this difference was not statistically significant ($p>0.05$). In terms of DEHP migration, fluid 3 was associated with a similar migration profile as seen with the sodium chloride 0.9% solution. However, the overall DEHP concentrations were generally higher at around 0.0128±0.00082 mg/ml to 0.0125±0.000793 mg/ml at 7 days of exposure for tubes A and B. This difference in concentration was statistically significant in both cases, $p<0.01$ and $p<0.01$ respectively when comparing tubing types with respective fluid compositions. After 7 days, Tube A seemed to leech greater amounts of plasticiser than Tube B, with concentration levels of 0.019±0.0072 mg/ml and 0.0451± 0.019 mg/ml after 14 and 28days respectively compared to 0.011±0.0024 mg/ml and 0.0252±0.0156 mg/ml for Tube B. These differences were statistically significant at both 14 and 28 days, $p<0.03$ and $p<0.04$ respectively. In addition the differences in leaching rate at the 14 and 28 day exposure levels differed significantly between common tubing types, but with different priming solutions. Once again the plasticizer levels associated with Fluid 3 (Gelofusine and 1/3 Hartmann's solution) was significantly higher than that of sodium chloride 0.9% solution alone ($p<0.045$ for Tube A and $p<0.049$ for Tube B).

Fluid 2 (0.9% Sodium Chloride Solution and 1/3 20% Human Albumin) was associated with the highest level of DEHP migration into the priming fluid when compared to the other solutions. The migration profile over the first few hours was seen to follow a parabolic shape but after 8hrs (Figures 4.2 and 4.3) and out to 28days followed more of a linear pattern. The concentration levels at 24hrs reached 0.0464±0.027 mg/ml and this continued to increase linearly to 0.079±0.025mg/ml and 0.0624±0.0134mg/ml after 28days for Tubes A and B respectively. This was

statistically significantly higher than the other priming solutions employed ($p<0.039$, and $p<0.007$ when comparing Tube A and B with fluids 2 and 3 respectively).

The TOTM plasticised tube, Tube C was associated with a much lower migration rate than the DEHP plasticised tubes. Of particular interest was the finding that this slow migration rate was consistent with all fluid types. Fluids 1 and 3 exhibited a low level of extraction over the entire 28 day test period. Indeed the concentration levels were 0.0086±0.00057 mg/ml and 0.0067±0.0023 mg/ml for fluids 1 and 3 respectively at 28 days, around 10% of the levels observed at this time-point with the DEHP tubes. Only Fluid 2 (0.9% Sodium Chloride Solution and 1/3 20% Human Albumin) showed any significant increase in migration of the plasticiser with the TOTM plasticised tube, but this occurred only after 14days of exposure and this migration rate remained fairly constant out to 28 days at which a concentration of 0.047±0.024 mg/mL was recorded which was indeed statistically different to the levels seen in Fluids 1 ($p<0.018$ and 3 ($p<0.016$).

The DOA plasticised tubing, Tube D, exhibited no measurable migration over the first 4hr in any of the test fluids. This trend continues for both Fluids 1 and 3 out to 8hr and in the case of Fluid 1 it continues right out to 7days. For Fluid 1, concentration levels at 14days were seen to be around 0.0143±0.012mg/ml and this steadily increased to around 0.0252±0.02mg/ml at 28days. Fluid 3 followed a similar pattern to Fluid 1 with no discernable plasticiser migration being detected in the first 8hr. The concentration levels were then seen to increase after 24hr of exposure and further increased at a steady rate out to 0.019±0.016mg/ml after 28 days.
In common with the DEHP experiments, Fluid 2 (0.9% Sodium Chloride Solution and 1/3 20% Human Albumin) was associated with the highest level of DOA migration with detectable levels after only 8hrs. This level then steadily increased in a near-linear fashion from 48hrs out to 28days where a final concentration in the sample was determined as 0.054±0.047mg/ml, the highest level detected in this study.

4.4. Discussion

This study has shown quite clearly that plasticisers, utilised in the tubing element of perfusion circuits, migrate into the priming fluid. This is the case for all plasticisers investigated in the present study. However, there are clear differences in terms of the migration rate and the timeframe of migration between the tubing types. The study suggests that the concentration of plasticiser observed in the priming solution depends upon three factors:

1. The plasticiser type
2. The priming fluid
3. Storage time

In general DEHP was seen to readily migrate into all of the priming solutions. The two alternative plasticisers both exhibited a marked reduction in migration compared to the DEHP over the entire time period, and performed much better over the shorter time period out to 48hr and the concentration levels only started to increase after 14-28days (Figures 4.2 and 4.3). The extremes of the time-scale studied in the present study are probably not reflective of clinical practice, but the shorter term exposure levels, out to the 7 day level are within the margins of clinical practice. Critically, the early phase of this study is similar to that seen in previous studies, where DEHP plasticised tubing was exposed to migration media. Gotardo and Monteiro reported a very similar profile, with the exception that they recorded little migration in the first 3 hours. We on the other hand did see migration of DEHP into all media from the very outset. One possible explanation for this is the fact that we did not pre-wash our circuits and under these circumstances (which are similar to the clinical setting) one might anticipate a surge of DEHP from the surface of the tubing, followed by a slower emission. This precisely describes the profile we observed. The findings of this study are corroborated by the findings of studies by both Karle et al., 1997 and Burkart et al., 2007 as they also reported the detection of significant levels of migrated DEHP into whole blood and a high protein content priming fluid, Plasmalyte. In the case of Karle et al, they reported that exposure to migrated DEHP

can be as much as 20 to 70 times higher in ECMO than other procedures such as transfusions and dialysis. Furthermore, it is clear that the level of DEHP migration is effected by the priming fluid into which it comes into contact. The albumin and Gelofusin solutions, which contain both lipid and protein components were associated with greater migration rates. Whilst these studies show that DEHP migrates from the PVC used in clinical procedures, there are a number of studies that reported no accumulation of DEHP during storage experiments. The migration of DEHP from PVC is known to happen constantly from the bulk of the material to the material surface. Therefore, the findings of these studies by Han et al, 2005 and Riley et al., 1997 had to be questioned to why they found no DEHP. A simple explanation is that the samples of the priming fluid were taken from areas of the ECMO Circuit that were not exposed to DEHP PVC or the most likely scenario is that the PVC tubing used in the ECMO test circuits were of the coated type thus nullifying the DEHP migration into the test medium.

Our study confirms that there is a much lower migration rate from the alternative plasticisers TOTM and DOA. This was particularly clear during the early phase of the study where DOA was associated with virtually no migration for up to 7 days of exposure to sodium chloride 0.9% solution.

That DOA and TOTM moderate plasticiser migration confirms the information from the manufacturers of these materials, who market the tubes as low or non migration PVC tubing.

Resistance to migration is not the only factor in deciding if these tube types are a better proposition than DEHP Plasticised PVC in the clinical setting. Cost and toxicity are also factors. Currently, both alternatives are more expensive than the DEHP option and their toxicological profiles are not as yet fully described. Further studies therefore are required to establish if using these materials is subjecting patients to a low dose of a more toxic substance than DEHP. However, assuming that the toxicity profile of these molecules is similar to DEHP, and that our previous studies have shown that the inflammatory response to DEHP is dose dependent, the use of these low leaching plasticisers may offer some form of inflammatory benefit.

The second major finding, and not entirely unexpected, was that the makeup of the priming solution has an impact on the level of plasticiser migration. This is not

surprising as we know for example that DEHP and indeed the other plasticisers involved in this study are to some extent lipophilic. These are complex molecules with complex chemical properties which might affect migration tendency. In this study, it was clear that the simple sodium chloride 0.9% solution priming solution was associated with the lowest level of migration from all tubing types at over the entire timecourse of the study. The more complex priming solutions were associated with the highest migration rates. The high lipid solutions, those containing albumin and the gelofusine had the highest migration rates, possibly highlighting some of the complex affinities of the plasticiser molecules, which have not been investigated in this study. The presence of the Albumin in solution was associated with the highest migration rates, and its use in wet prep circuits should be applied with caution, as it seems to promote excessive migration of DEHP over short time periods (<48 hours). It also promoted a moderate migration response in the other plasticisers, but only at long term storage times of 14 days plus.

4.5. Limitations

The main limitation of this study is the fact that we utilised fairly small sections of tubing material; this resulted in small but significant levels of leached plasticiser. A larger surface area with a smaller priming volume may well have amplified the results. However, we were keen to use only clinically relevant materials and designed the test system and protocols to use these. The detection limitation of the spectrophotometer system was initially considered to be a concern however, previous studies and this current study conform that the levels detected are well within the limitations of the technology.

4.6. Study Summary

It was clear from this study that plasticisers migrate from PVC tubes at different rates depending on the time-course and the makeup of the priming solutions. The migration of DEHP was seen to be significant and the results of this study have clear clinical implications.

This study has clearly shown that plasticiser migration from P-PVC tubes used in extracorporeal procedures such as ECMO and CPB is a very real clinical problem

that could have serious effects on the patients who are exposed to significant amounts of DEHP by undergoing these extracorporeal procedures.

The knowledge gained from performing this small study has shown the importance of being able to accurately determine the amount of plasticiser migration from P-PVC that is used in the medical environment and has shown that the following work undertaken as part of this Doctoral thesis is not only useful to the PVC industry, who produce medical products, but also to help educate the clinicians who use these P-PVC products in the treatment of individuals on a regular basis.

These findings clearly demonstrate that plasticiser migration is a definite clinical problem and provides sufficient evidence to investigate the main aims of this thesis which are stated in section 3.18.1 with the intention of advancing the knowledge of plasticiser migration in scenarios that have greater relevance to medical interventions than is currently available.

5. Bio-material Test Cell Development and Evaluation

5.1. Introduction

This project was based on the concept of radio-labelling a fraction of the DEHP plasticiser with ^{14}C and incorporating labelled and unlabelled DEHP into the PVC sheets during the compounding process. The plasticiser would then be extracted from the PVC sheet by exposure to different liquid media. At various time periods during the experiment the radioactivity of samples of the media are measured in a scintillation counter and the amount of DEHP released may be determined. The purpose of these *In vitro* studies was to develop a direct and accurate method of determining how much DEHP plasticiser would be leached from the plastic during a set period of time. The results of the *In vitro* studies could lead to prediction of the DEHP release from PVC tubing during clinical procedures such as ECMO, CPB or dialysis. To compliment the *In vitro* studies radiotracer experiments would be conducted in a novel rat perfusion model that mimics a clinical therapeutic procedure. These data would also help to determine more accurate safe exposure levels for both adult and neonate patients.

To facilitate the DEHP migration experiments, a biomaterial test cell employed in previous studies in the Bioengineering Unit was evaluated for suitability of use in the planned experimentation to determine if a new test cell would have to be designed.

5.2. Evaluation of Current Test Cells

The Bioengineering Unit at the University of Strathclyde, Glasgow has been involved at the forefront of biomaterial research for many years with a primary emphasis on blood-biomaterial interactions. The research of Bowry (1981), Jones (1989), Yu (1993), Lamba (1994), Yin (1996) and Zhao (1999) have all yielded doctorates in biocompatibility related fields and these studies required the design of many test devices. Much of the design work was intuitive in nature due to the absence of computational analysis tools that are readily available today. As part of

this project, it was decided to ascertain if any of the existing material test cells would be suitable for the planned work. If none were found to be suitable the development of a new material test cell would have to be undertaken.

5.3. Investigation of Previous Test Cells

The project requires the utilisation of a test cell in which the test material is exposed to a well-defined flow of the extraction media. Robertson (1988) and Jones (1989) used test cells for *Ex vivo* perfusion studies on rats. However, the material samples were in tubular form and hence would not accommodate our flat sheet material. Of the three flat sheet material test cells used previously, only two allowed for controlled flow. Both were designed by Dr John Gaylor and were used by Robertson (1988) and Lamba (1994). The test cell described by Robertson (1998) was discounted as it could not be used on as small a scale as we were intending. The device used by Lamba in 1994 was more promising but was one of those designed purely on an intuitive basis. It was therefore decided to carry out a Computational Fluid Dynamics study of this device to characterise the velocity and shear stress distribution within the test cell and identify any regions of recirculation or stagnation.

5.3.1. Experimental Methods

5.3.1.1.Geometry

The study was undertaken using the computational fluid dynamics software package FLUENT (Ansys Inc). The models were built in the modelling package GAMBIT (Ansys Inc) from the dimensions for the test cells A and B given in the journal article by Lamba et al in 1998. A third model was created using the basic dimensions for Test Cell A but by reducing the channel height from 0.03cm to 0.02 cm. A fourth model was also produced using the basic dimensions of Test Cell A but having no tapered inlet or outlet flow ports. The dimensions of the test cell models are shown in Table 5.1. The geometry of the non -tapered and tapered test cells are shown in Figures 5.1, 5.2 and 5.3, 5.4 respectively.

	Cell A	Cell B	Cell C	Cell D
Ports	**Tapered**	**Tapered**	**Tapered**	**Non-Tapered**
Length, cm	**9.4**	**14**	**9.4**	**9.4**
Width, cm	**2**	**3**	**2**	**2**
Height, cm	**0.03**	**0.03**	**0.02**	**0.03**
Contact Area, cm^2	**37.6**	**84**	**37.6**	**37.6**

Table 5.1: Dimensions of original Test Cell models used in Computational Fluid Dynamics (CFD) Analysis of the tapered v non-tapered design.

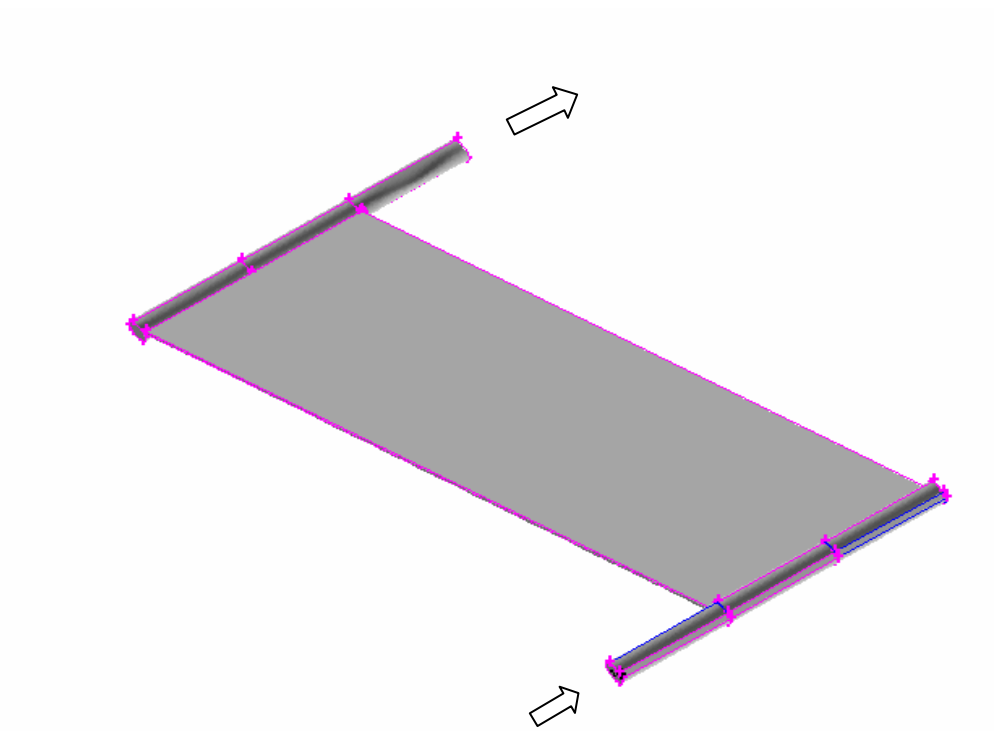


Figure 5.1: Geometry of entire non-tapered test cell.

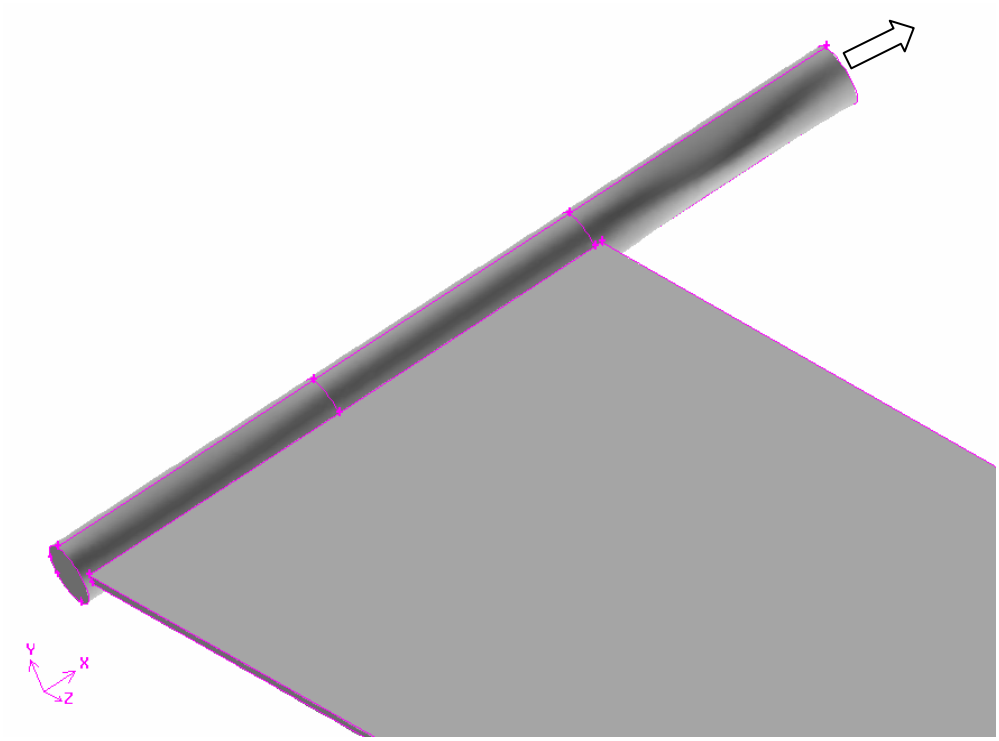


Figure 5.2: Close up of the geometry of the non-tapered exit port and channel

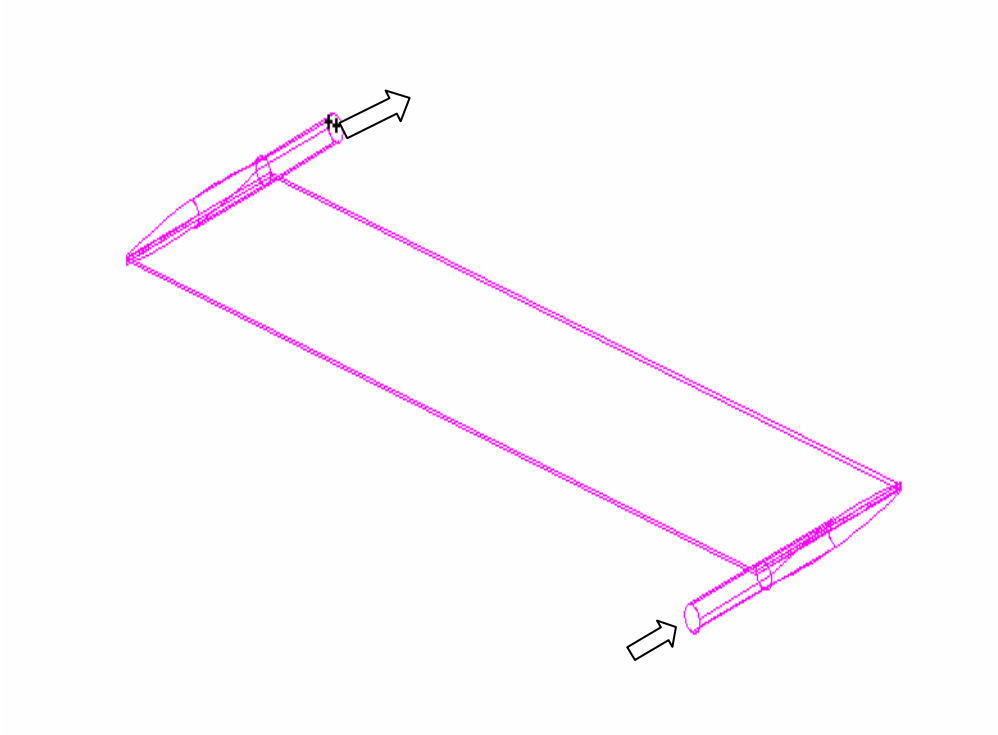


Figure 5.3: Geometry of the entire tapered test cell.

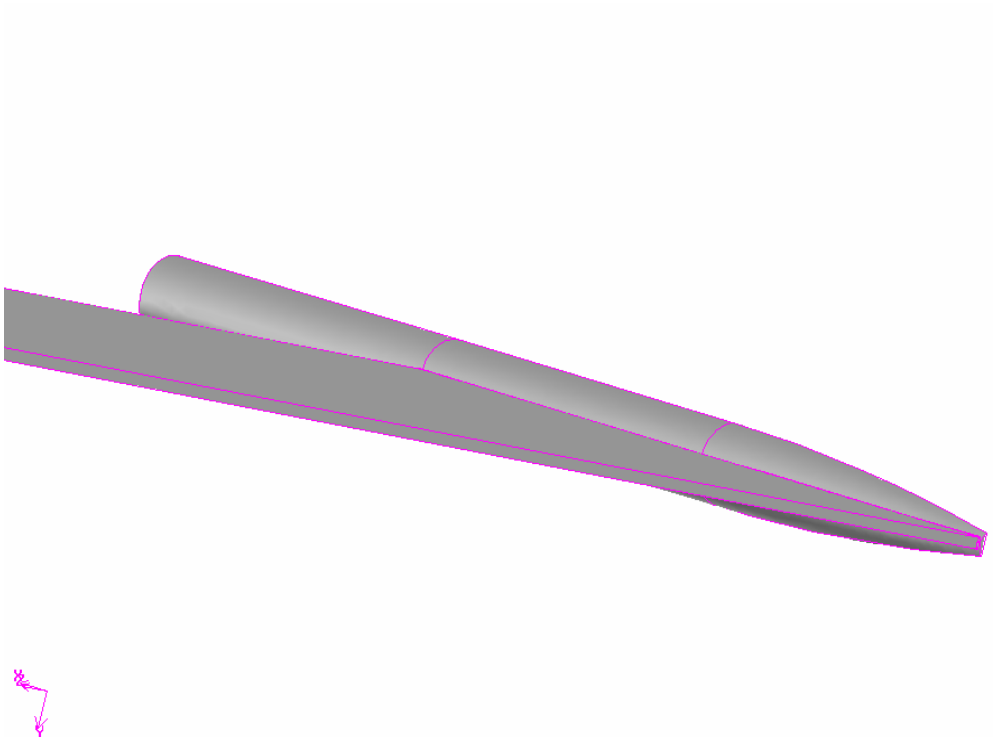


Figure 5.4: Close up of the tapered inlet port and channel

From the geometry illustrations it can be seen that the model is basically a channel terminating in either tapered or non-tapered cylinders. Each component was built separately and then assembled in GAMBIT. The components, namely the channel, one inlet port and one outlet port were joined using the "interface" command which is the standard procedure for models of this nature. This command allows for each shape to be individually meshed in the form that will provide the best quality of result for that component. It then interpolates the values at the exit of the first component and converts them so they can be used at the entry of the next component.

5.3.1.2. Mesh Production

The model was meshed in three separate sections. The first being the main channel section. This was meshed using a tetrahedral element. The mesh was graded along the longer sides of the channel so as to have more elements towards the ends of the channel as this is where most of the flow changes are expected to happen. The mesh was not graded across the width or the height of the channel, instead a larger amount

of elements were used as this helped to produce a better quality mesh which can be seen in Figures 5.5 and 5.6.

Both the inlet and outlet ports were meshed separately using triangular elements. This was because the more complex shape of the port would not mesh with a tetrahedral element. Again the elements were graded towards the narrower end of the port as this is where the flow conditions are expected to change by the greatest magnitude.

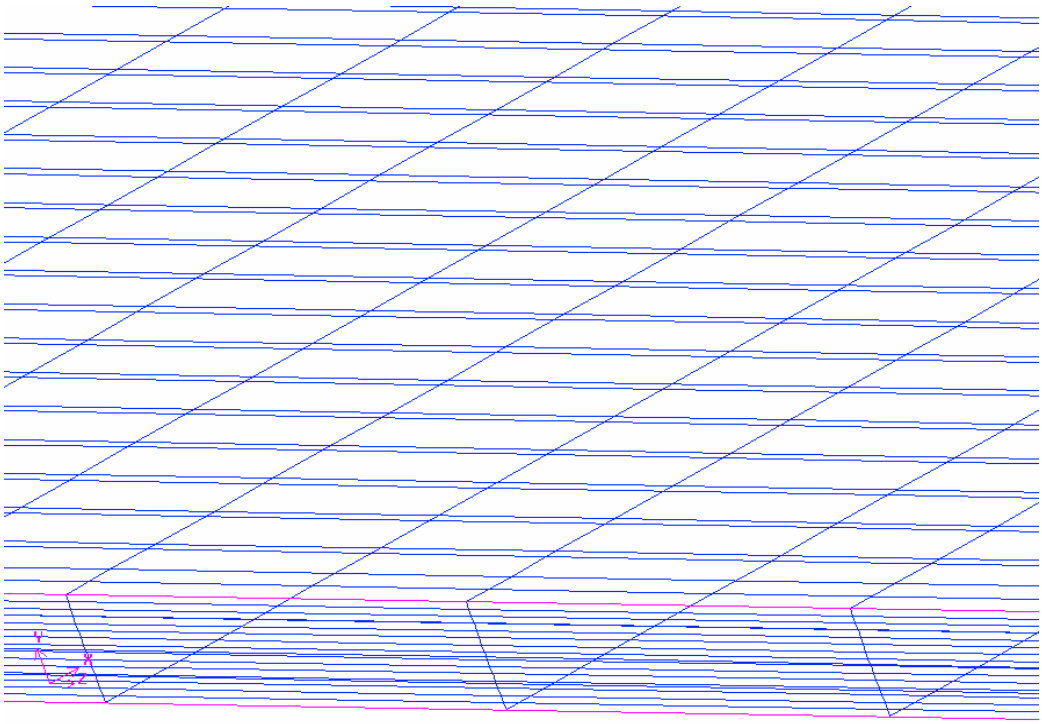


Figure 5.5: Cross-section of the middle of the channel showing the mesh.

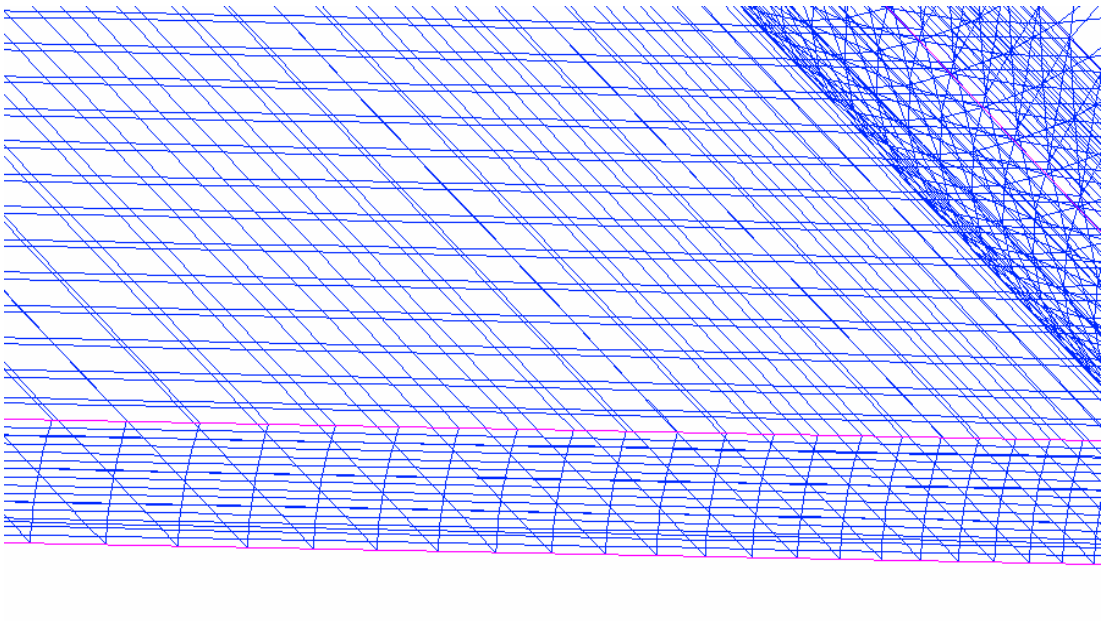


Figure 5.6: Cross-section of the mesh at the channel ends showing the increasing numbers of elements.

Each mesh was checked, using the element checker in the Gambit program, to ensure that there were no skewed or uneven elements which could lead to flawed results.
The meshes of the various models were then exported into the FLUENT program to enable the Computational Fluid Dynamics (CFD) analysis to be performed.

5.3.2. Computational Analysis

5.3.2.1.Background

Once the models had been meshed successfully, they were exported into FLUENT. Velocity profiles were predicted for the four test cells for the operating conditions specified in Table 5.2. The liquid media was blood which was modelled as a Newtonian fluid with a viscosity, μ of 3.5 mPa s and as a non-Newtonian fluid based on a power law relationship with parameters B and K. The relationship between shear stress, τ and shear rate, $\dot{\gamma}$ for these models are as follows:

Newtonian fluid	$\tau = \mu\dot{\gamma}$
Power law fluid	$\tau = B\dot{\gamma}^n$

The Newtonian viscosity and the power law constants are representative of human blood at 37°C and a haematocrit of 45%.

	Newtonian	Non-Newtonian
Vol. flow rate 1 (ml/min)	1.2	1.2
Vol. flow rate 2 (ml/min^1)	0.5	0.5
Density, ρ, (kg/m^3)	1050	1050
Viscosity (mPa s)	3.5	N/A
Temperature exponent, n	N/A	0.0235
Constant, B	N/A	0.7619
Cp	3.558	3.558
K	0.21	0.21

Table 5.2: Input conditions for CFD analysis

The convergence criteria for both the Newtonian fluid and the non-Newtonian fluids were set at 0.001. This convergence criterion is the basis of the validity of the test cells as each cell must converge for the results to be reliable.

5.3.2.2. Analysis

The processor FLUENT produces large amounts of data in each simulation, with different methods for presentation ranging from raw numbers to contour and vector plots. It was decided that the best form of displaying the results would be the filled velocity contour plot as this produces a picture of the flow conditions in the cell that are easy to understand and gave an instant overview of the performance of the entire cell.

The contour views that were taken were:

- Velocity Magnitude
- Velocities in the X, Y and Z directions

The majority of views that were taken were of a top-down view looking at the full length of the test cell at the mid-plane. Other views were also recorded, with section views through the depth of the channel over both the length and width of the test cell.

This helped to build up a 3-dimensional picture of the flow and also to identify any areas of poor flow or stagnation in the cell.

5.3.3. Computational Fluid Dynamics Results

Each test cell was subjected to different flow scenarios, outlined in Table 5.2, of which the results for the following are presented in this thesis.

- Test Cell A, Tapered Ports, Flow rate 1, Newtonian Fluid
- Test Cell A, Tapered Ports, Flow rate 1, Power Law Fluid
- Test Cell A, Untapered Ports, Flow rate 1 , Newtonian fluid

5.3.3.1.Scenario 1: Test Cell A, Flow rate 1 Newtonian model Contour Plots

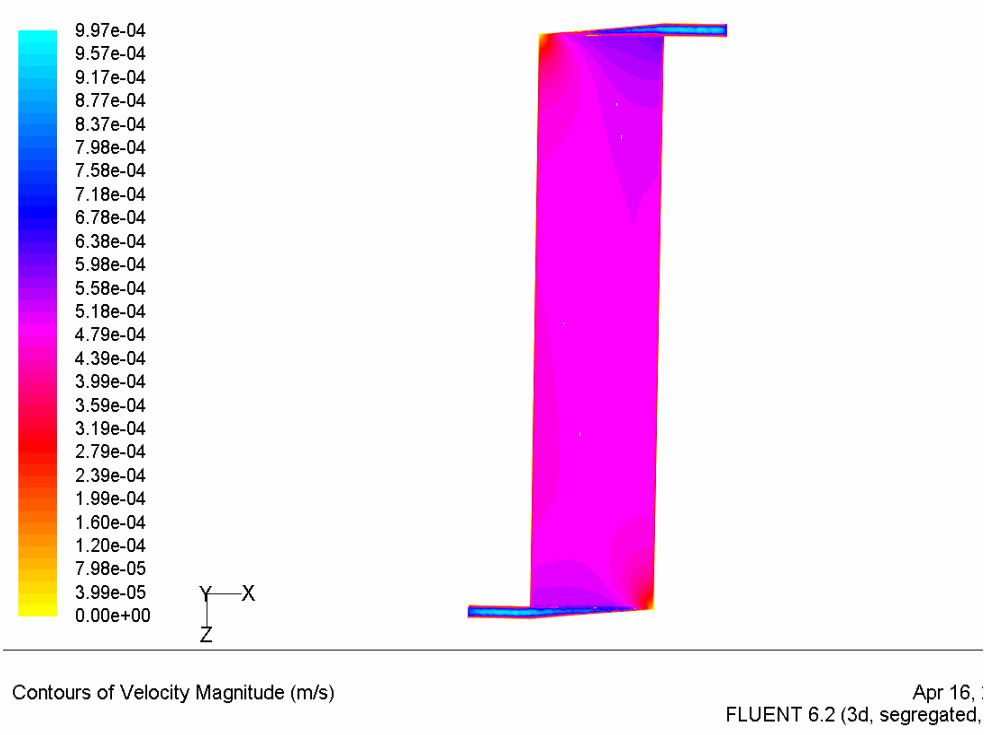


Figure 5.7:Full length contour plot of velocity magnitude, with inlet at top right

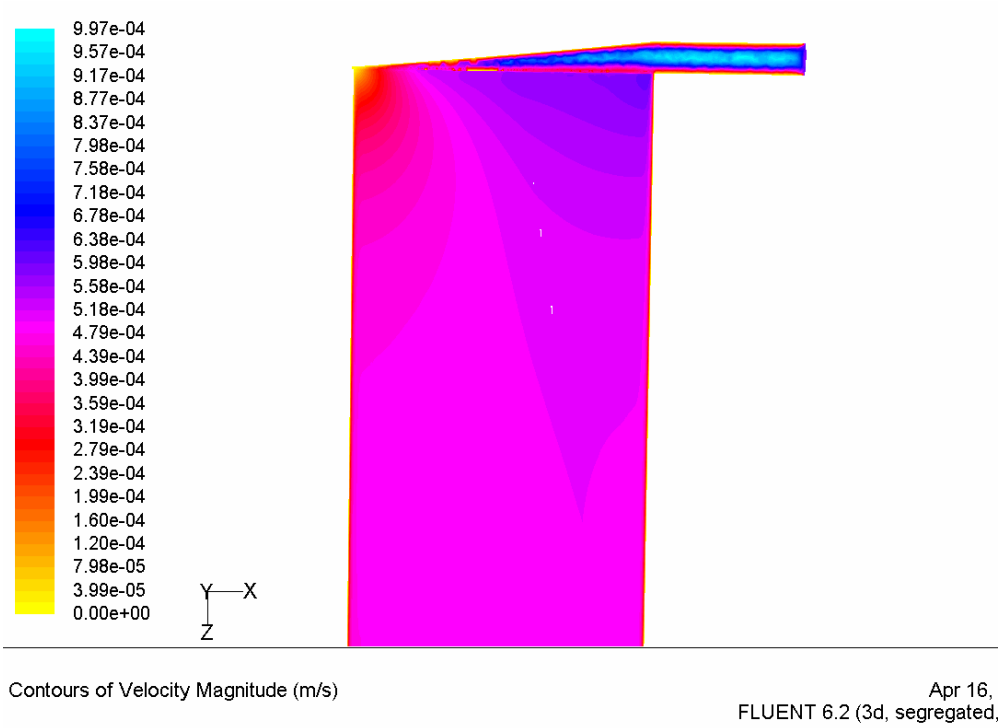


Figure 5.8:Close up of inlet port Velocity Magnitude Contour Plot

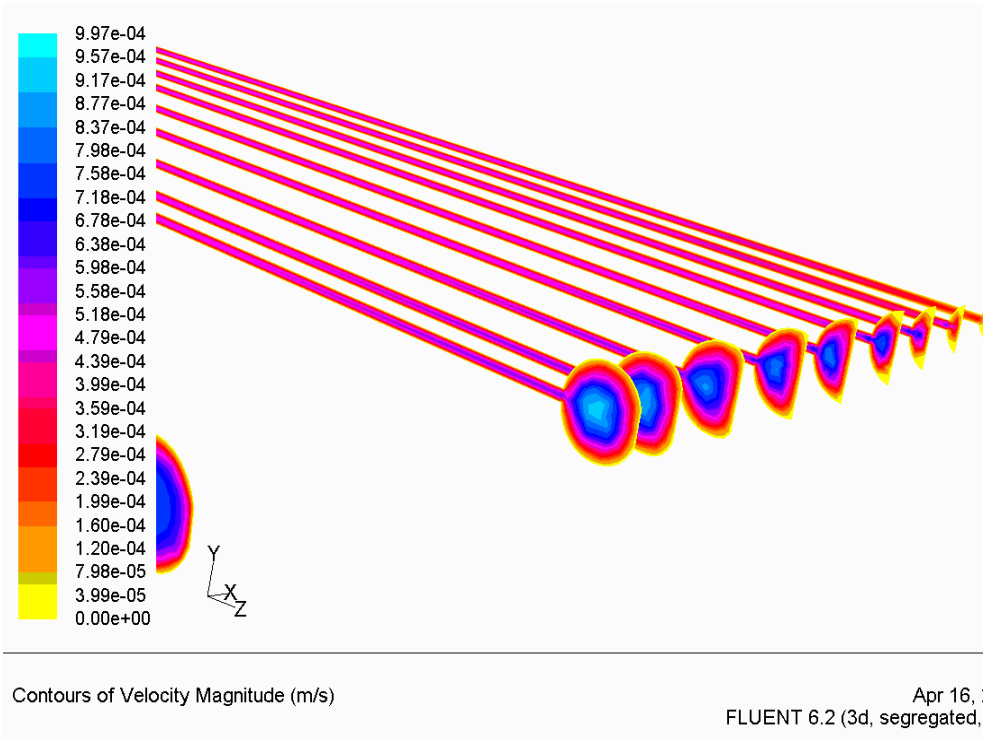


Figure 5.9: Velocity Magnitude Contour plot showing flow across width and depth of test cell.

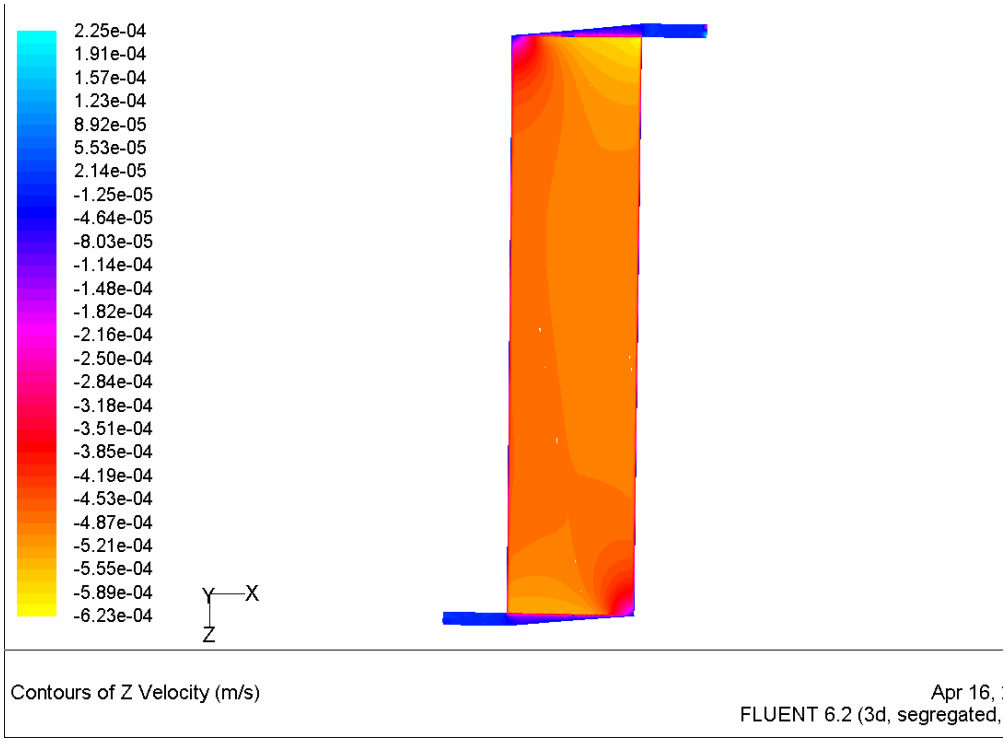


Figure 5.10: Full length Contour plot of the Z-direction flow

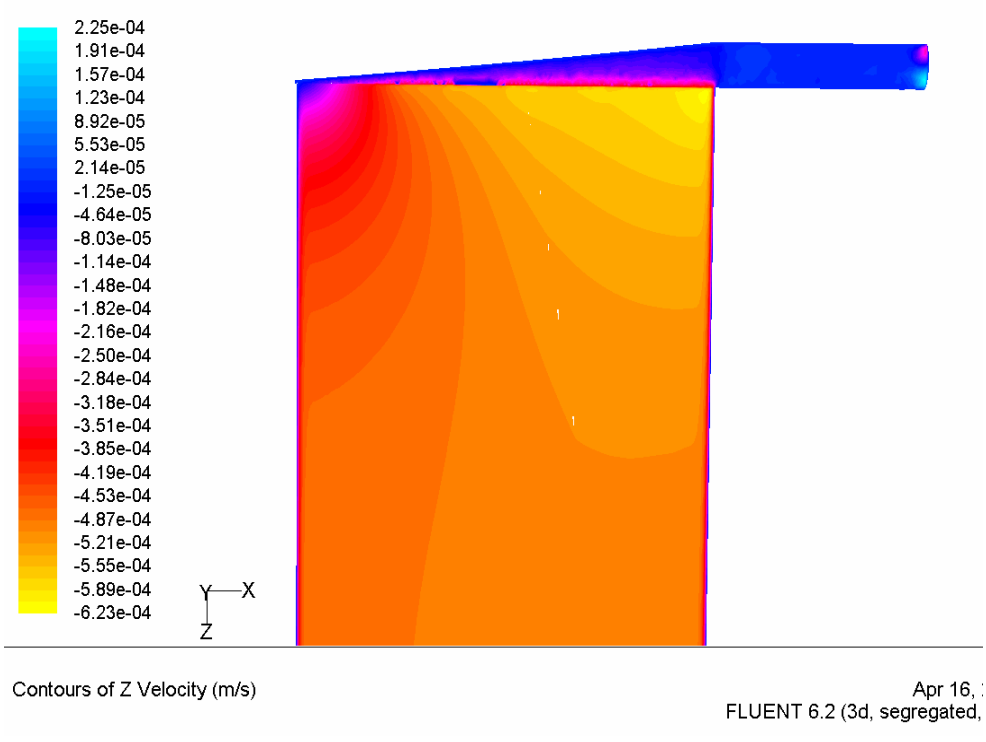


Figure 5.11: Close up of the Contour plot of the z-direction at the inlet port

5.3.3.2. Scenario 2: Test Cell A, Flow rate 1 with Power law model

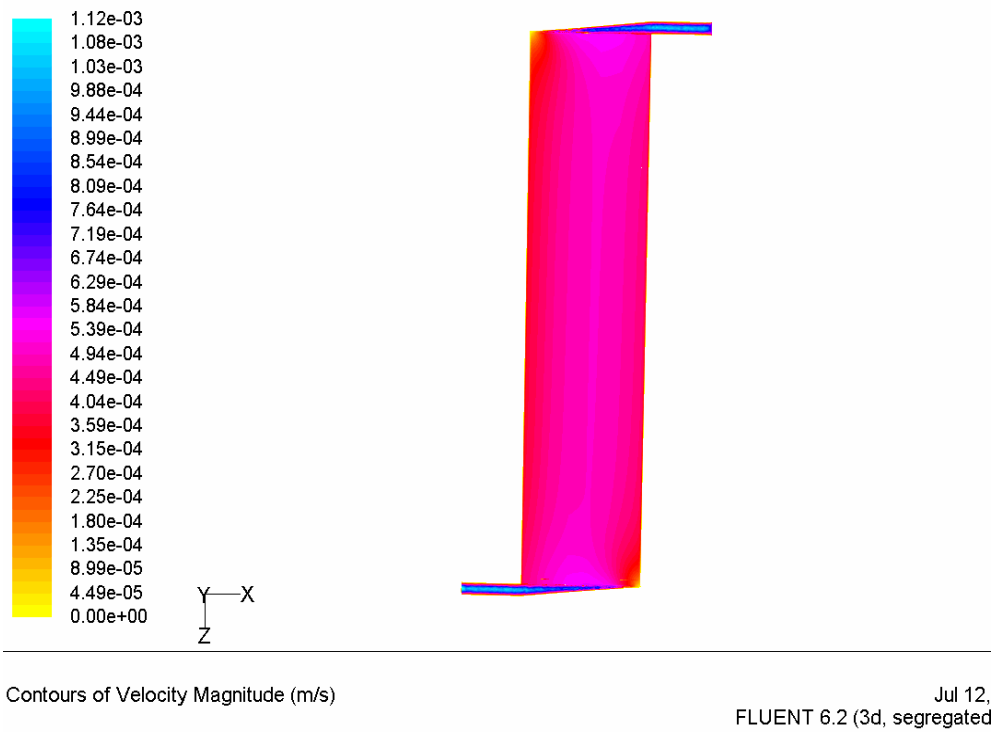


Figure 5.12: Full Cell Contour Plot of Velocity Magnitude, with inlet port at top right

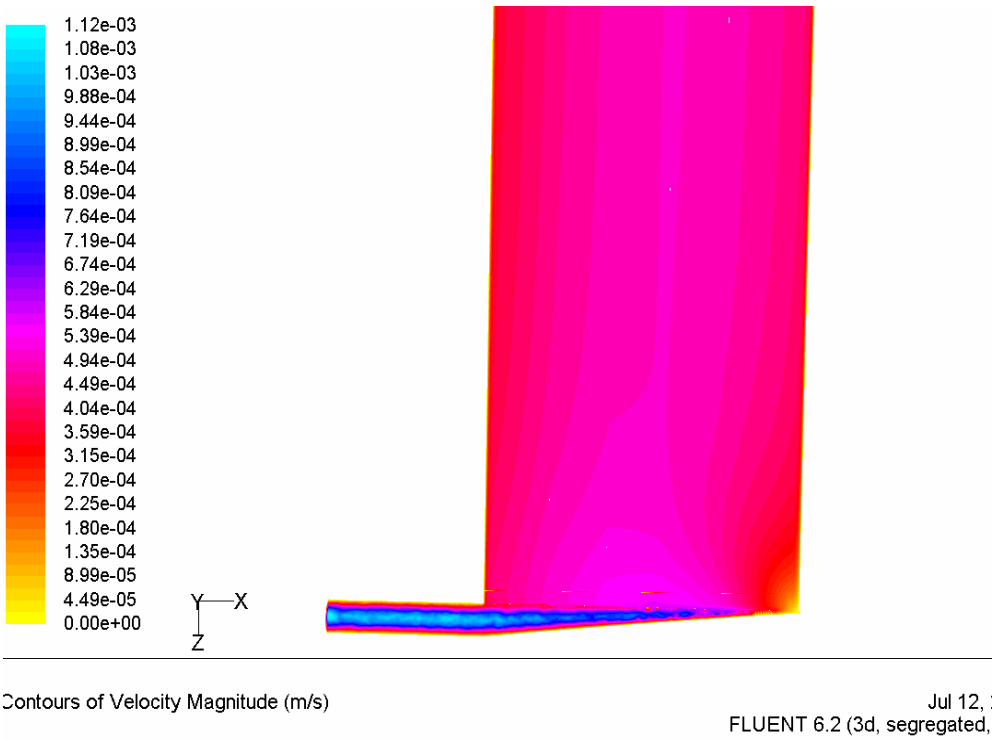


Figure 5.13: Close up of the Velocity Magnitude Contour Plot at the outlet port

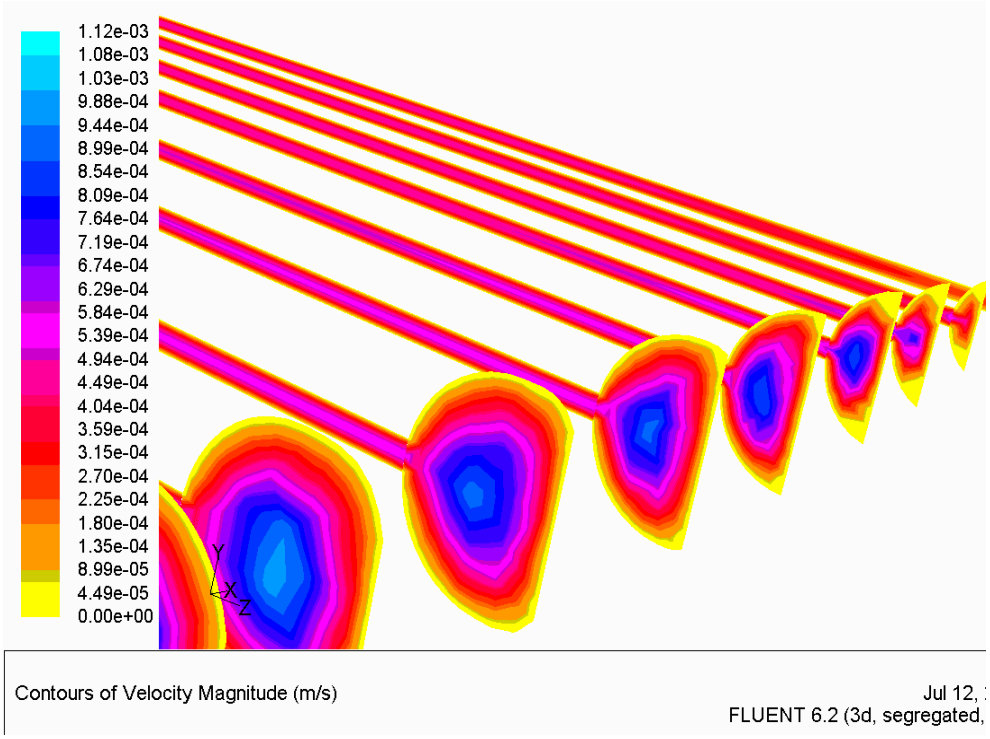


Figure 5.14: Close up of the Velocity magnitude flow across the tapered section of the inlet

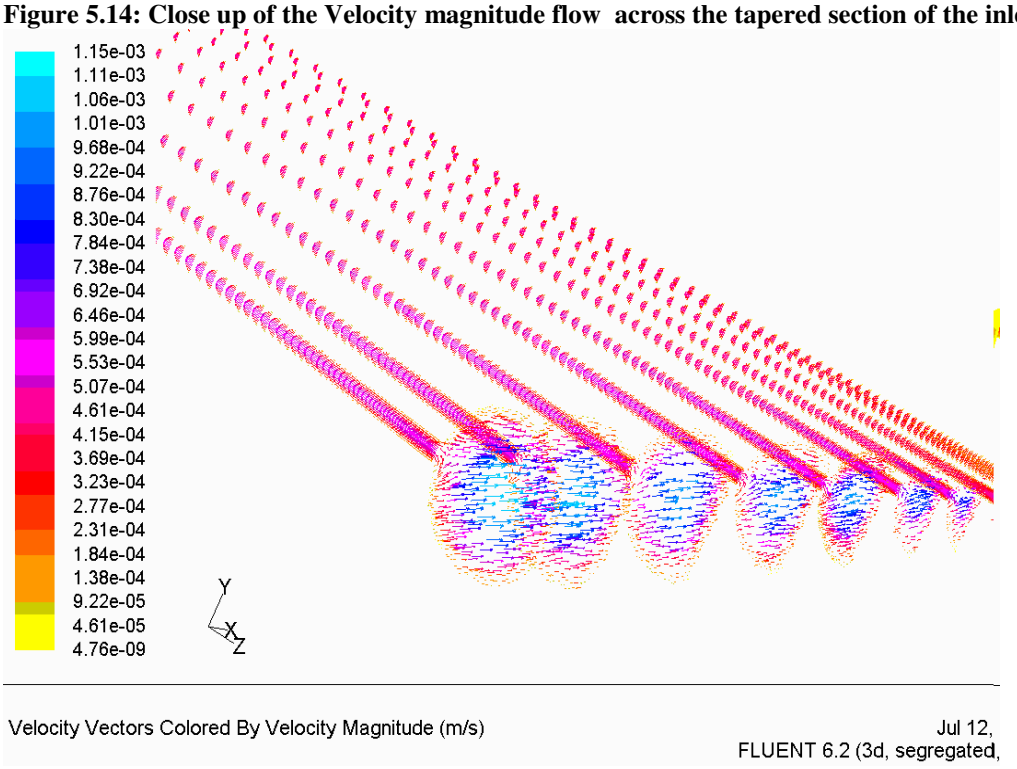


Figure 5.15: Vector plot of the flow through the taper and up the test channel.

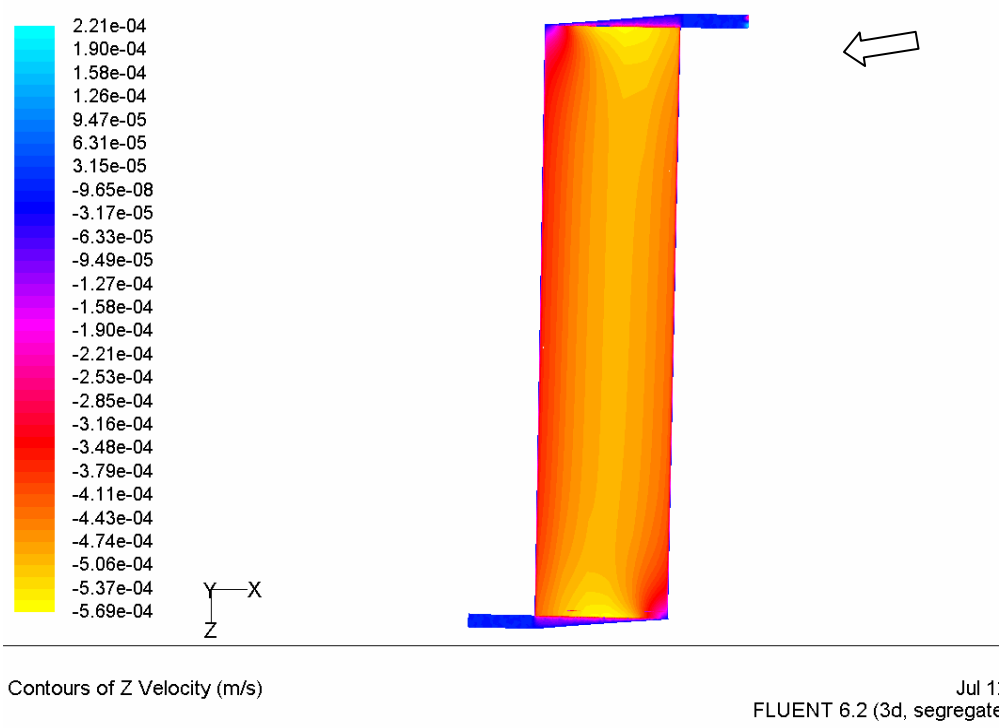


Figure 5.16: Full Cell Contour Plot of Velocity in the Z direction, with inlet port at top right

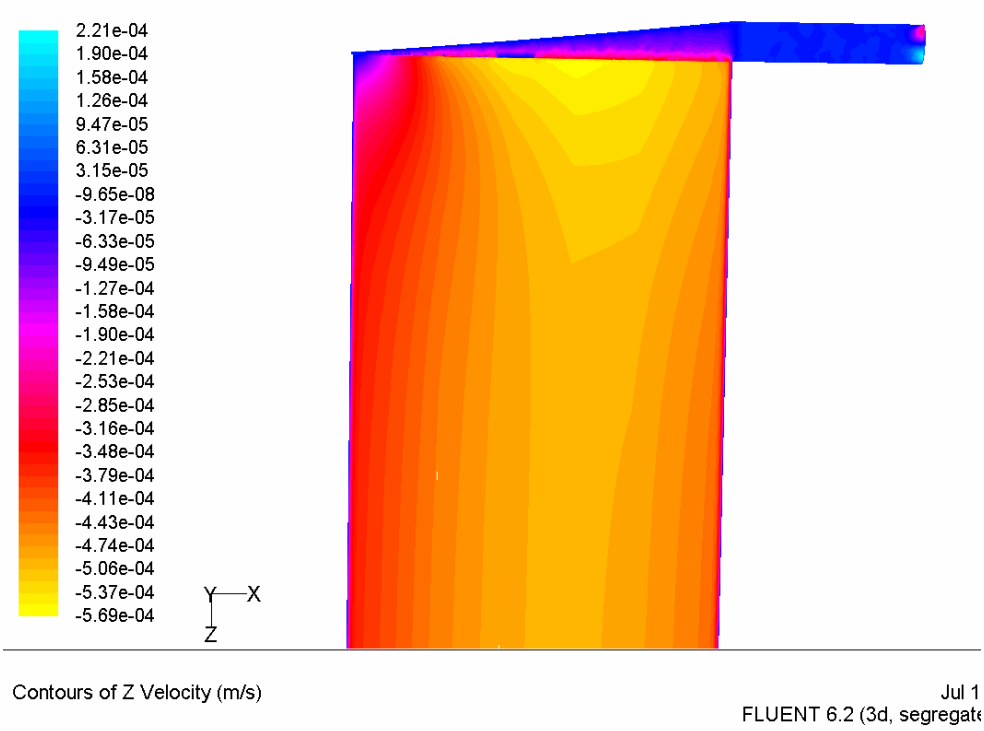


Figure 5.17: Close-up of the Inlet port showing the contours in the z-direction

5.3.3.3. Scenario 3: Test Cell A with non-tapered ports, Flow rate 1 - Newtonian model

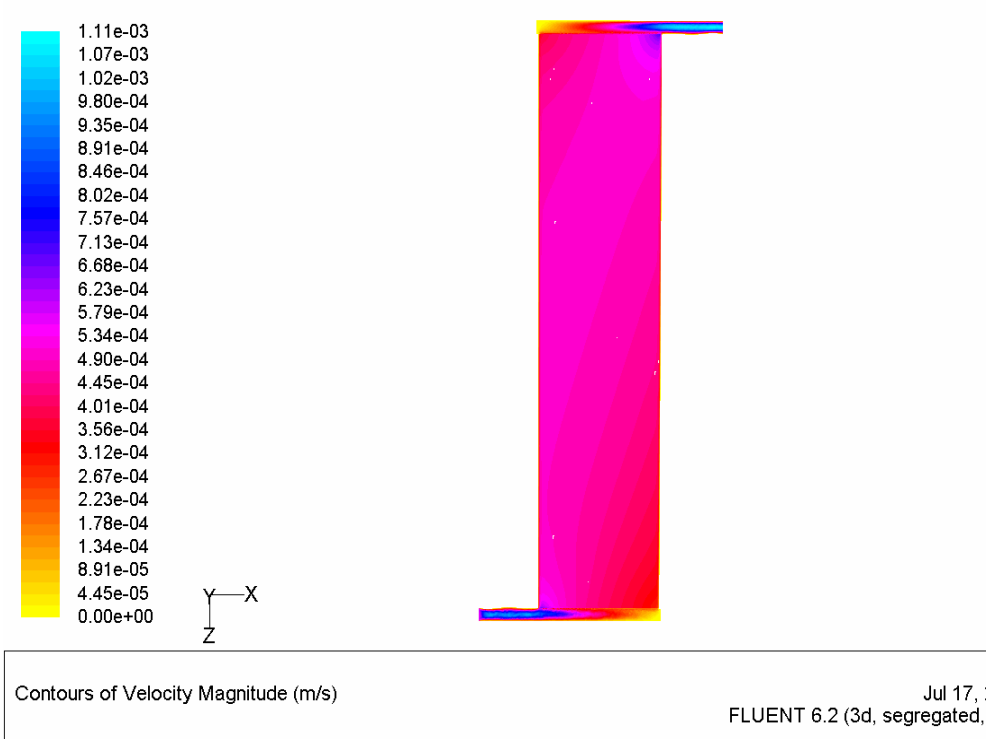


Figure 5.18: Full Cell Contour Plot of Velocity Magnitude

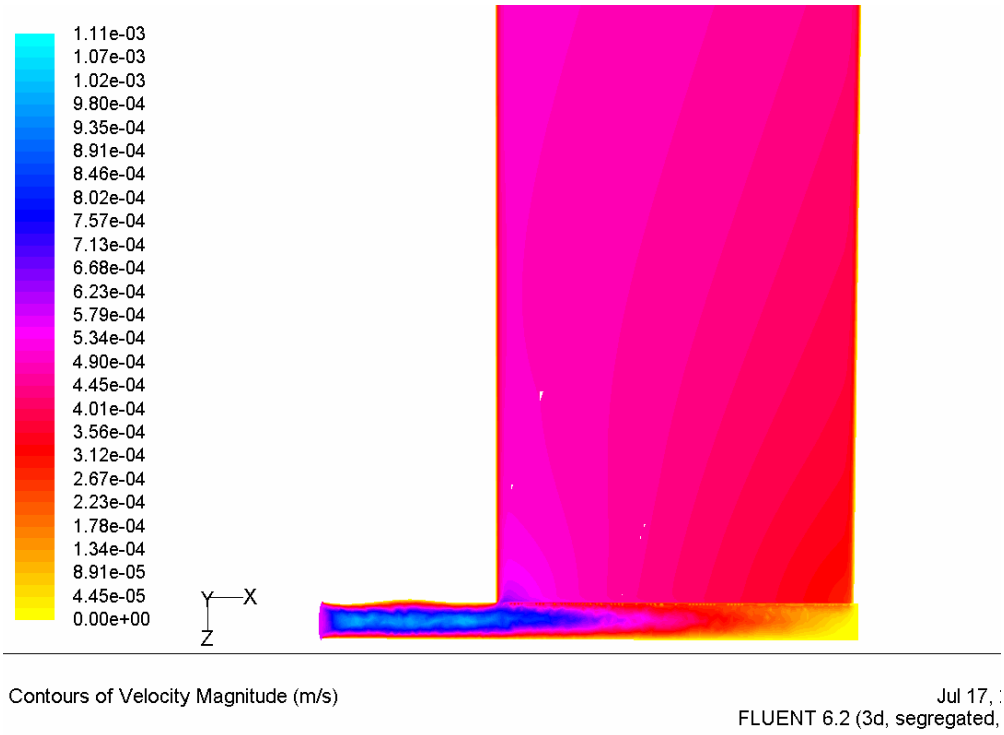


Figure 5.19: Close-up of the outlet port Velocity Magnitude Contours

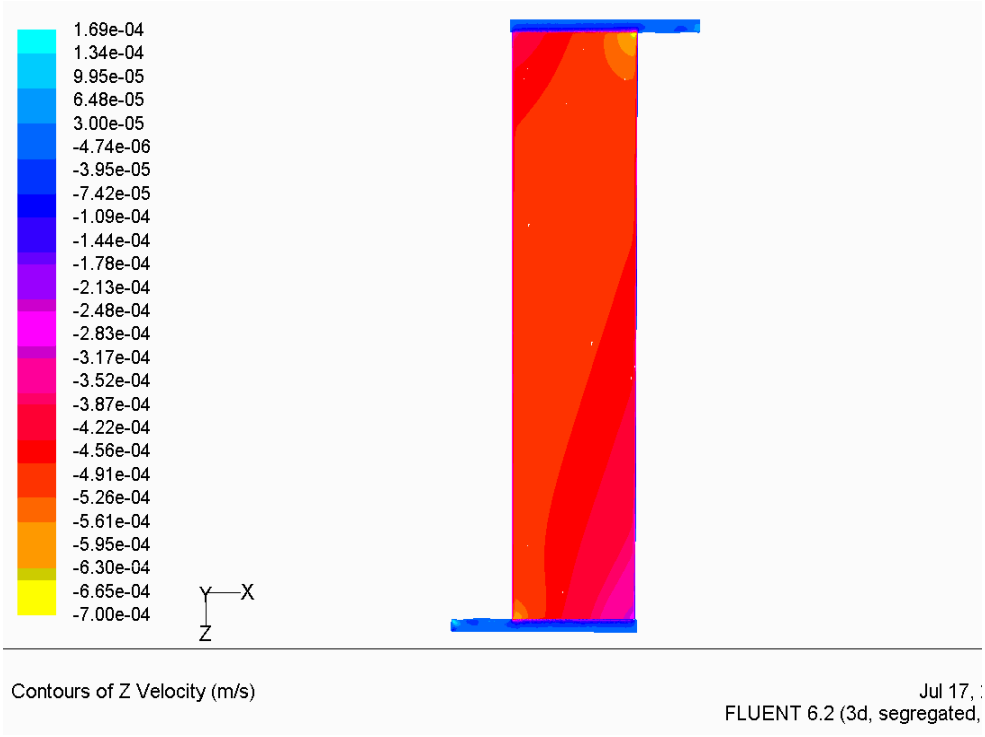


Figure 5.20: Full Cell Contour Plot of velocities in the Z-direction

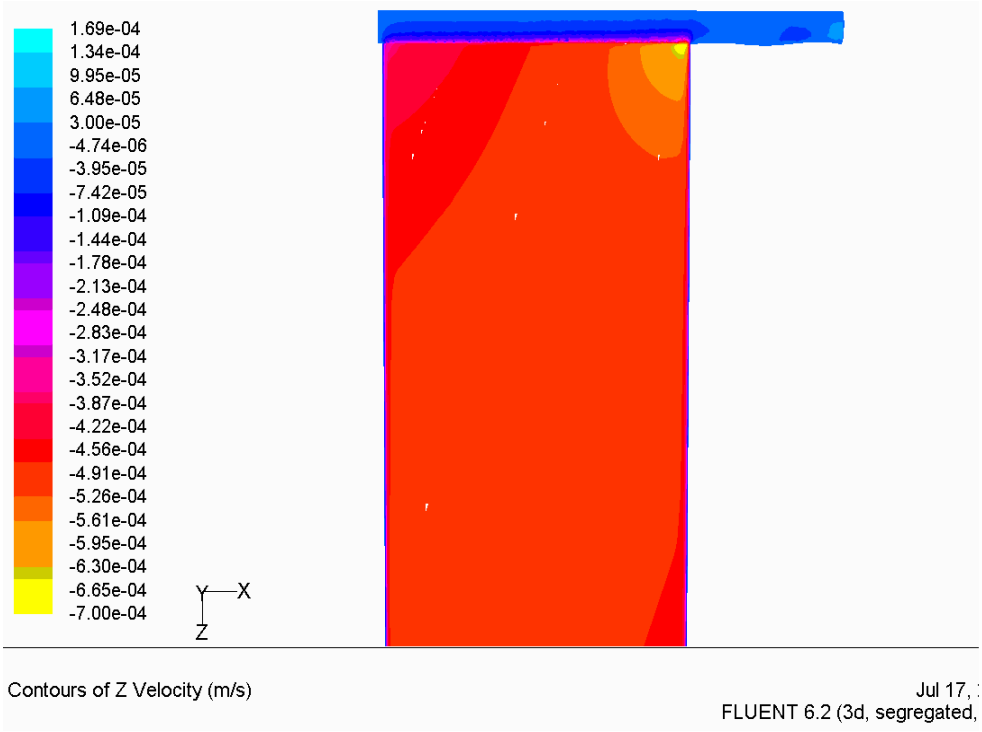


Figure 5.21: Close-Up of the Inlet Port Contours in the Z-direction

5.3.4. Discussion of CFD Results

The main aim of the study was to analyse the fluid dynamics of the generic biomaterial test cell that was developed by Gaylor and co-workers in the Bioengineering Unit (Lamba et al 1998). The results show that the tapering of the inlet produces an improved flow distribution along the parallel plate channel. There is also a slight difference between the Newtonian and power-law fluids when they are run through the tapered test cell.

When the Newtonian fluid is analysed it shows that for the central 7-8 cm length of the channel the flow is uniform across the entire width with velocities of around 4.39-4.79e^{-4}m/s. This is visible in Figures 5.7, 5.8 and 5.9. The main areas which do not show this uniform flow are found in the first and last cm length of the channel. At the inlet, there are some small velocity steps that show a decreasing of the fluid velocity as it enters the channel. These spread out in a fan like shape from the corner of the inlet, with the higher velocities being around 6.78e^{-4}m/s. This then slowly progresses down to around 5.98-5.58e^{-4}m/s before merging to produce the uniform flow that was mentioned before. This is illustrated in Figures 5.8 and also 5.13. Towards the outlet of the test channel, we see a similar pattern to that of the inlet region. This irregular flow velocity at the inlet may be caused by flow separation on the exit of the inlet needle just at the entrance into the channel. The same increase in velocity at the outlet could be attributed to the flow speeding up as it is forced from the larger channel into the narrower outlet needle. One might be lead to conclude that the inconsistencies of the flow in the first and last few centimetres would render only the middle section of the test channel useful for a material test cell. This would be justified if the velocities were greatly different. However, the values are only changing by 0.1e^{-4} m/s and therefore the test material will experience pretty much the same flow conditions along its entire length and width. Figure 5.10, 5.11 and 5.16, 5.17 show the flow velocities in the Z-direction down the cell. The same findings for the velocity magnitude are again applicable in this view. The main point of interest in these figures is the demonstration of the slight difference in flow velocity across the width of the cell. Figure 5.7, shows the majority of the flow across the cell is of the velocity 4.87e^{-4}m/s but there is a small area at the end furthest away from the inlet that has a velocity that is slightly slower at around 4.53-4.19e^{-4}m/s. This can be

attributed to the lack of velocity at the extreme end of the inlet taper which in turn produces a flow through the test channel that is slightly slower than the rest of the channel.

Overall, the lack of eddies or areas of stagnation in the flow were pleasing as it shows that the test cell design is sound, and only areas of slightly increasing or decreasing velocity in the initial and final extremities of the cell as well as small section at the far wall which has a slightly slower velocity are visible for the Newtonian fluid. Therefore, the current test cell design can be used successfully for extracting liquids of differing viscosities.

5.4. Development of New Test Cell

5.4.1. Introduction

The CFD analysis of the test cell indicates that the intuitive design approach used by Lamba and Gaylor was valid and that the incorporation of the taper was an important part of the design. There were however practical problems with the test cell namely leakage from the areas around the inlet and outlet ports which made the use of this test cell difficult.

The nature of the work that was performed in this thesis required a test cell that has no leakage for both safety and contamination reasons. Therefore it was decided that a new test cell should be designed incorporating the same features that were contained in the existing test cell of Lamba and Gaylor. The design process involved the use of a 3-D modelling package, Solid Edge, and then tested using the CFD package Fluent and Gambit.

5.4.2. Design and Testing Introduction

The design of the new test cell was initially undertaken as an open theme with no specific design characteristics being required other than the following:

- Creation of a uniform laminar flow over the material surfaces
- Minimal material contacts for the fluid other than the test material
- Ease of fabrication
- No leakage

As the test criteria had been left open, many of the initial ideas tried to incorporate the testing of the material in sheet or tubular forms. As the design process moved forward it was decided that the test cell should be designed for sheet material. This was based on many reasons, the main one being that sheet material is much easier to produce and also easier to perform surface characterisation tests on. The test cell was also to be of a total thickness of at least 4cm so as to "self shield" the operator from the β-radiation emitted from the labelled PVC situated within the cell. This was achieved by using two 2cm thick sheets of Perspex®; a material which was easy to machine and was compatible with the extraction media used in the experiments.

It was decided that the important (and desirable) features of the original parallel plate cell should be incorporated in the new cell design. These features were:

1. Tapered side entry
2. Upper and lower faces of parallel plate channel lined with the test material
3. Needle entry and exit systems

Feature 1: The tapered side entry had been proven to deliver a uniform flow through the test cell channel.

Feature 2: This reduces the number of different materials that the test fluid will be in contact with and also maximises the test material surface area to fluid volume ratio. In their study on the inflammatory response of rat and human neutrophils exposed to DEHP plasticised PVC, Gourlay et al (2001) have shown that the larger the surface area exposed to the blood the greater the inflammatory response. Therefore, a high surface area to volume ratio will increase the sensitivity of the test procedure.

Feature 3: The incorporation of the hypodermic needle entry and exit systems was based on the need for no leakages and ease of connectivity with the tubing used in the flow circuit. Furthermore, as the entry and exit ports incorporate a rubber stopper which is part of the sealing gasket, the stoppers are readily punctured by hypodermic needles and allow fluid to be passed through the test cell without leakage.

The original test cell used modified 14G needles to produce tapered inlet and outlet ports. The tapered was produced by bevelling the needle along its length and filling in the lumen with silicone rubber. This was a time consuming and awkward process and was avoided in the new test cell design.

The integration of the above features into the next generation was carried out with the aim of producing a test rig that would be easy to assemble and to operate whilst fulfilling the required design criteria.

5.4.3. The Next Generation Test Cell

As with all design projects many different concepts are considered before the most suitable design was selected and developed into the final product. The various concepts considered are detailed in Appendix B. After much discussion between supervisor and student, many of the designs were vetoed due to different reasons such as production complexity and poor sealing ability. It was decided that the design should be based around the initial design of two flat plates, each incorporating a built in taper section at fluid inlet and outlet and a recess to house the PVC sheet material. The plates are separated by a sealing gasket.

5.4.4. 3-d Parametric Modelling of the New Test Cell

The concept design that was chosen was constructed in the 3-d parametric modelling package called Solid Edge. In this package the test cell design was built in parts and then assembled to produce the full cell. The assembly also allowed for an initial check to see if the components fitted together the way they were designed to before it was manufactured. One of the many advantages to using this software package was the ease with which technical drawings could be produced from the original model. Another advantage is the ability to manufacture the test cell on a computer numerical control (CNC) milling machine direct from the design file. Both of these advantages helped to speed up the production of the test cell models.

The built-in taper section is machined directly into the base plate thereby eliminating the production of the tapered entry and exit needles. This in turn reduces the complexity of design whilst retaining the fluid dynamic benefits provided by the tapered profile. To facilitate this, an inlet channel that was wide enough to accommodate the inlet needle was also machined into the base, this inlet channel then becomes part of tapered section of channel, which then forces the fluid up a small incline into the actual test channel. The taper itself is of the same angle used in the previous test cell. This can be seen in Figure 5.22

The sealing gasket was required to ensure that the test cell was water tight to prevent leakages. The gasket channel was also machined around the main material holding pod into the base plate. This channel meets the two holes that had been drilled to accommodate the inlet and outlet rubber bungs to create a watertight seal. The gasket of choice was a silicone rubber cord of 3mm diameter which had been previously used in the original test cell. The channel for the gasket can also be seen in Figure 5.22. Initially, it was hoped that the pressure of the plates compressing the rubber bungs would be sufficient to seal the test cell. This seemed to be the case during the initial un-pressurised testing but as soon as a small amount of pressure was applied to the circuit the bungs began to deform and leak at the gasket joints. The leakage was stopped by using a small amount of liquid silicon to firstly, secure the rubber bung into the base plate, and secondly to secure the ends of the gasket to the rubber bung hence creating a watertight seal. A thin layer of silicone was spread on the bung holes in the upper plate to ensure that no fluid could infiltrate behind the bung when the cell was in use.

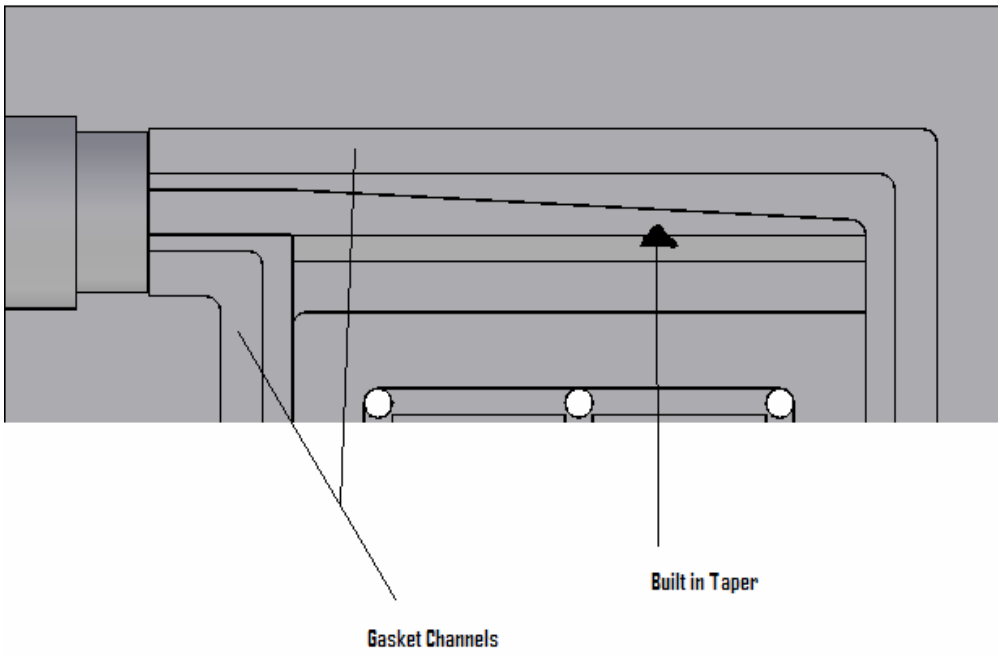


Figure 5.22: Diagram showing the Built in Taper and the Gasket channels of the Test Cell Base.

The base was created first by cutting out the main PVC positioning recess followed by other various channels and holes from a solid block. The upper part of the cell was created in the same way but without the inclusion of the gasket channel or the taper entry ports. Figures 5.23 show the base unit with the inbuilt taper and the gasket channel. Figure 5.24 shows the full assembly of the test cell.

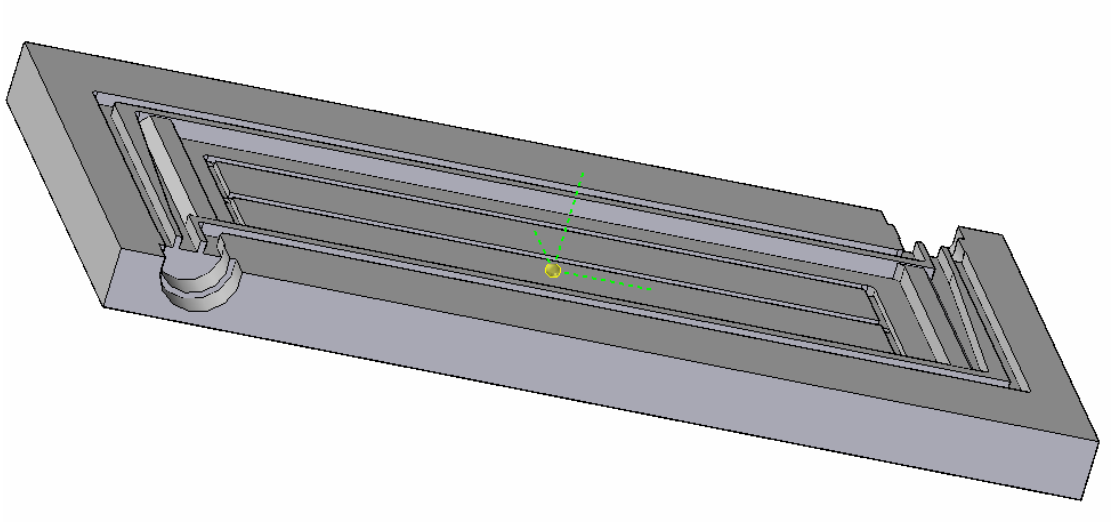


Figure 5.23: Base Unit of the Test Cell

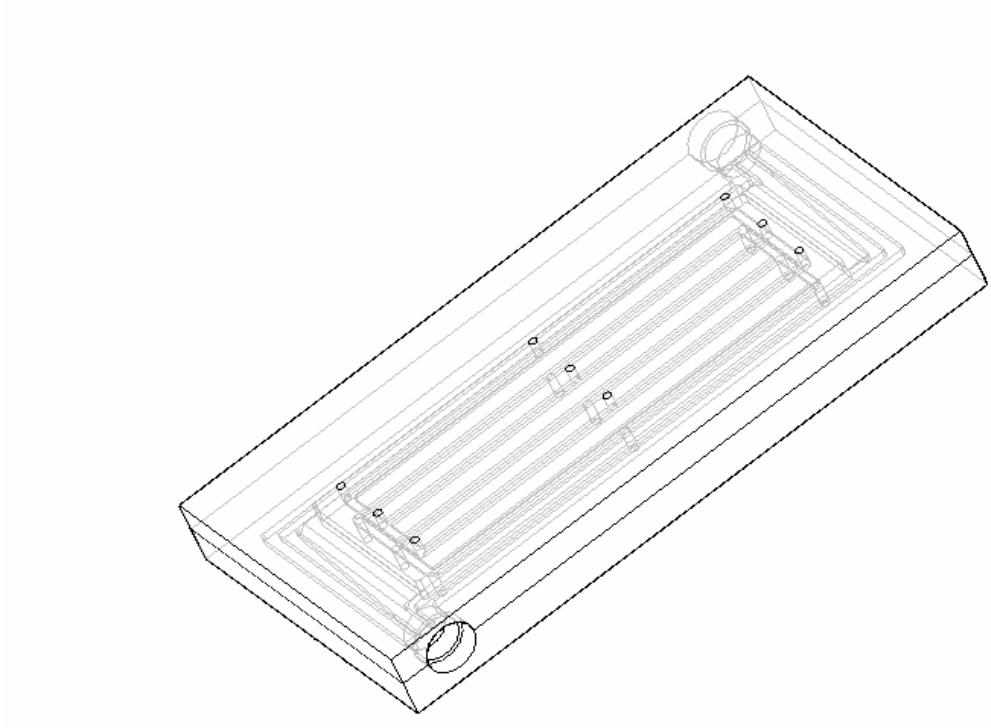


Figure 5.24: Full Assembly model of the Test Cell

5.4.5. Device Closure and Securing

The device is secured using a mechanical clamp system that consists of six clamps positioned in the configuration shown in Figure 5.25.

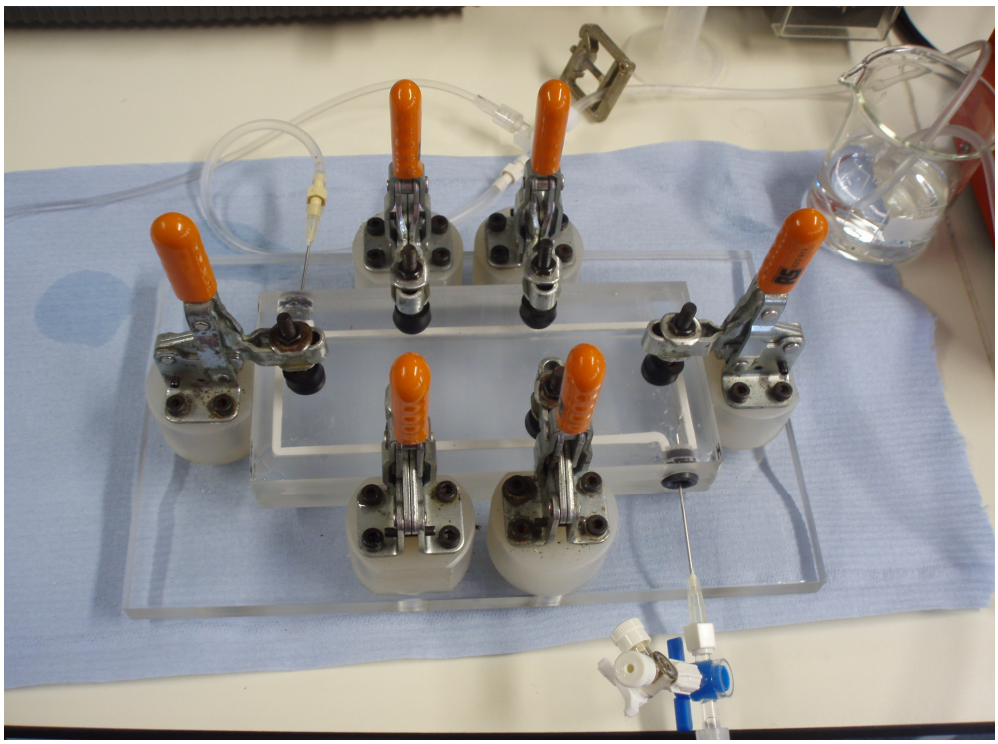


Figure 5.25: Configuration of the Clamping mechanism on the Test Cell

The positioning configuration ensures that the tip of each clamp positions itself on top of the gasket and seals it with enough force to make it water tight. When the cell is closed, the two end clamps were closed first to enable a better fit round the rubber bungs. The remaining clamps were then closed in a diagonal fashion but the starting point was not important. This closing sequence produced a good, watertight seal around the entire perimeter of the cell.

5.4.6. CFD Analysis of the Final Design

Once the design had been finalised, the flow characteristics were analysed using FLUENT computational fluid dynamics package Before this could be done the model was required to be built in modelling package Gambit so it could be uploaded into the post processor FLUENT. The new model dimensions were based around the original Test Cell A (Lamba and Gaylor, 1999) with the width and the channel height being the same as this model. The length of the channel was slightly longer due to

the make up of the new design. The geometry of the cell can be seen in the figures (Figures 5.26 and 5.27) below and the dimensions of the new cell are given in Appendix C.

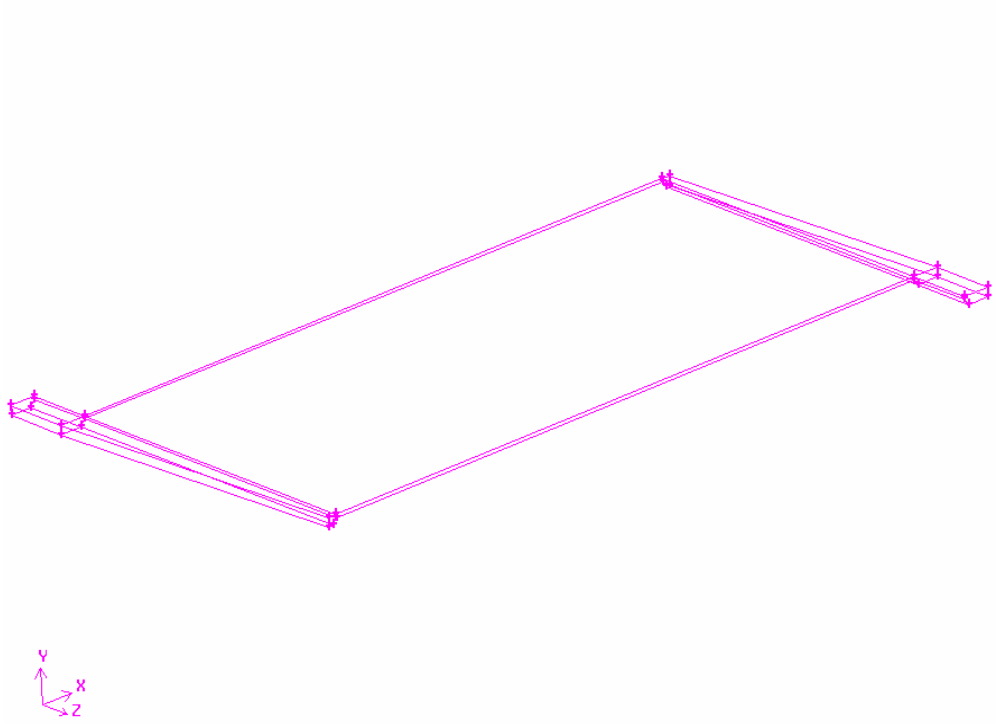


Figure 5.26: Geometry of the full cell

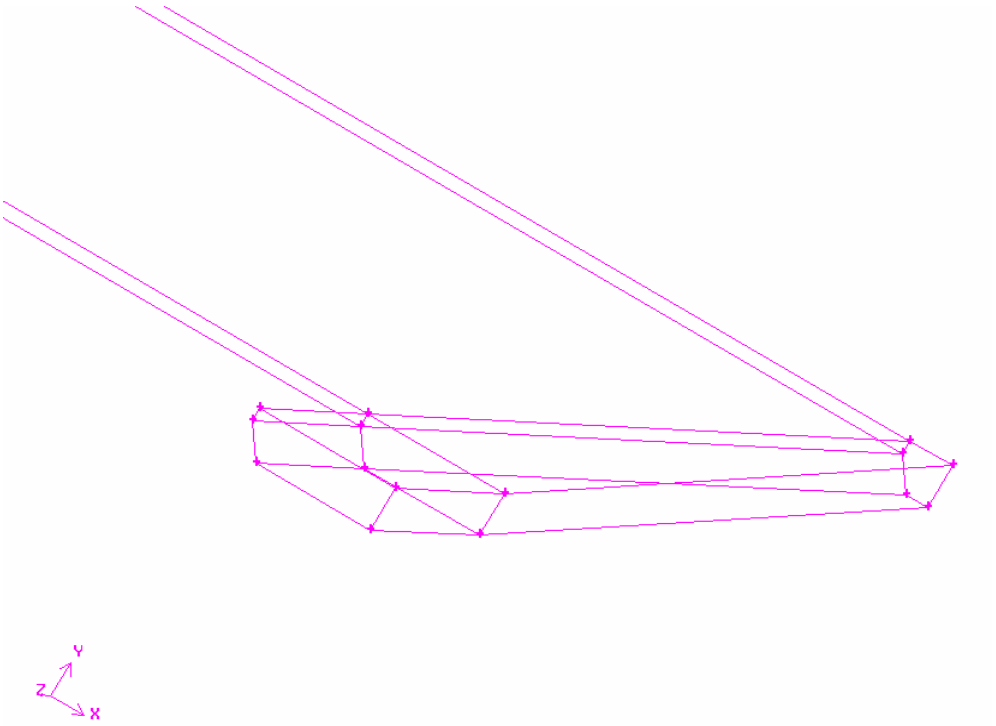


Figure 5.27: Close up of the inlet port geometry

5.4.6.1. Mesh Production

The model was produced in the same fashion as the previous CFD study with each section of the model being meshed separately and then connected using the "interface" method. The channel section was meshed using a tetrahedral element and was also graded in the same fashion as the previous model. The inlet and outlet ports were meshed using the triangular element as well as being graded in the same fashion for the reasons stipulated in the previous study.

5.4.6.2. Applied Conditions

The flow conditions that were applied to the new model were the same as reported in the earlier chapter, see Table 5.2. The flow rates were 0.021ml/s, 0.5ml/s and 1.2ml/s. Only the results from the 0.021m/s flow rate for both the Newtonian and Non-Newtonian fluids will be reported here due to the increased magnification of the areas of interest with the slower fluid velocity.

5.4.7. Results

The results for the Newtonian and Non-Newtonian fluids can be seen in the Figures on the following pages

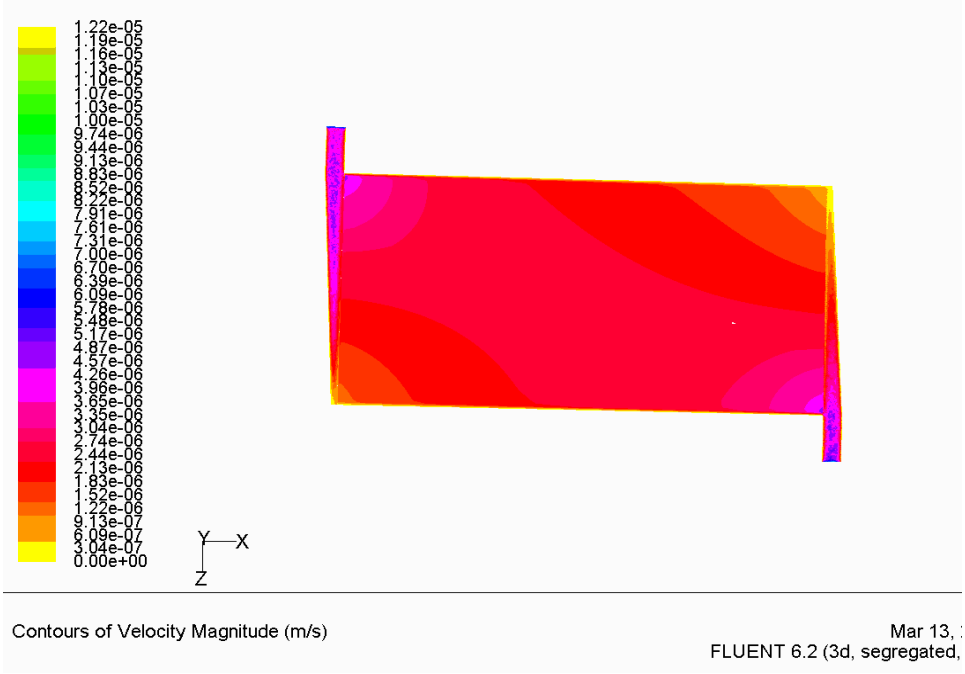


Figure 5.28: Contour plot of velocity magnitude of entire cell for Newtonian fluid

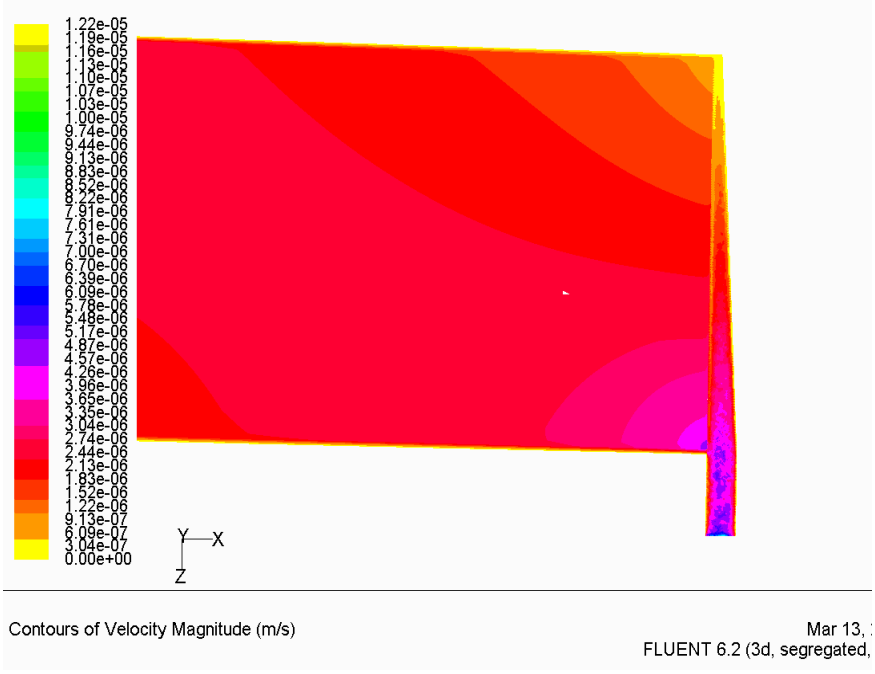


Figure 5.29: Velocity magnitude contour plot at outlet of cell for Newtonian fluid

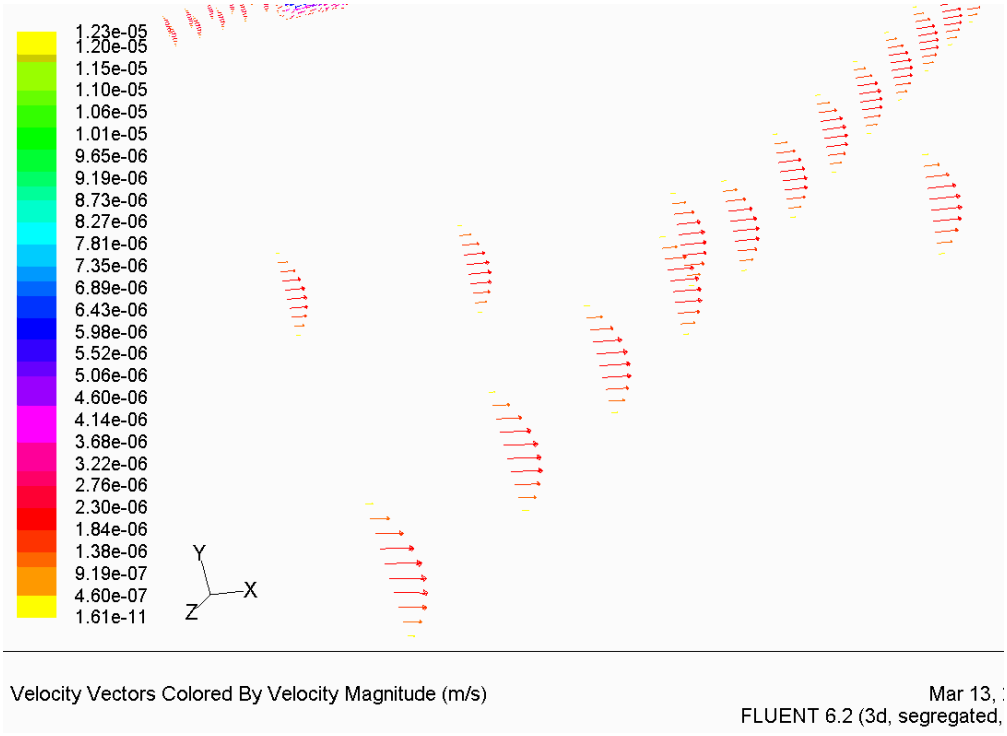


Figure 5.30: Vector plot showing developed flow across test cell for Newtonian fluid

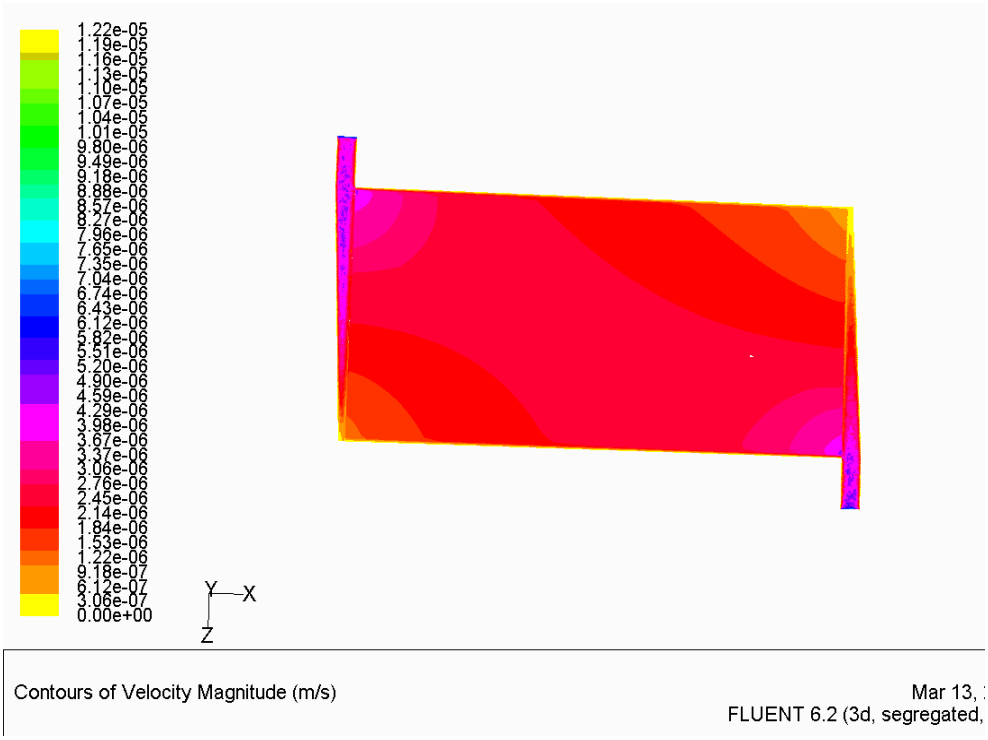


Figure 5.31: Velocity magnitude contour plot of entire cell for power law fluid

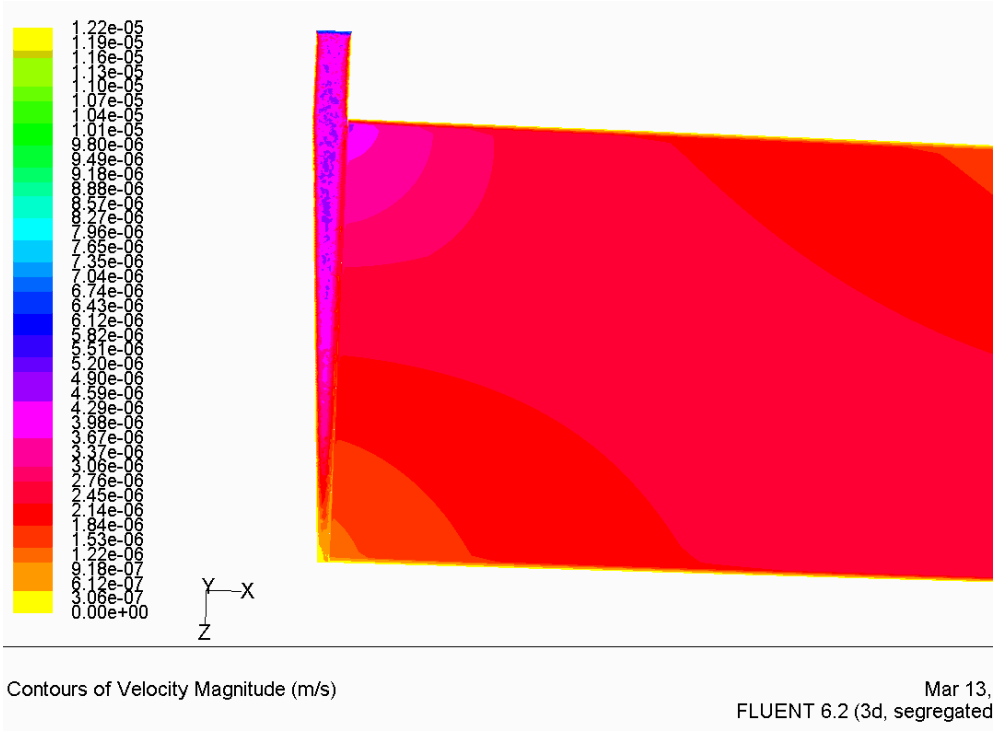


Figure 5.32: Velocity magnitude contour plot at inlet of cell for power law fluid.

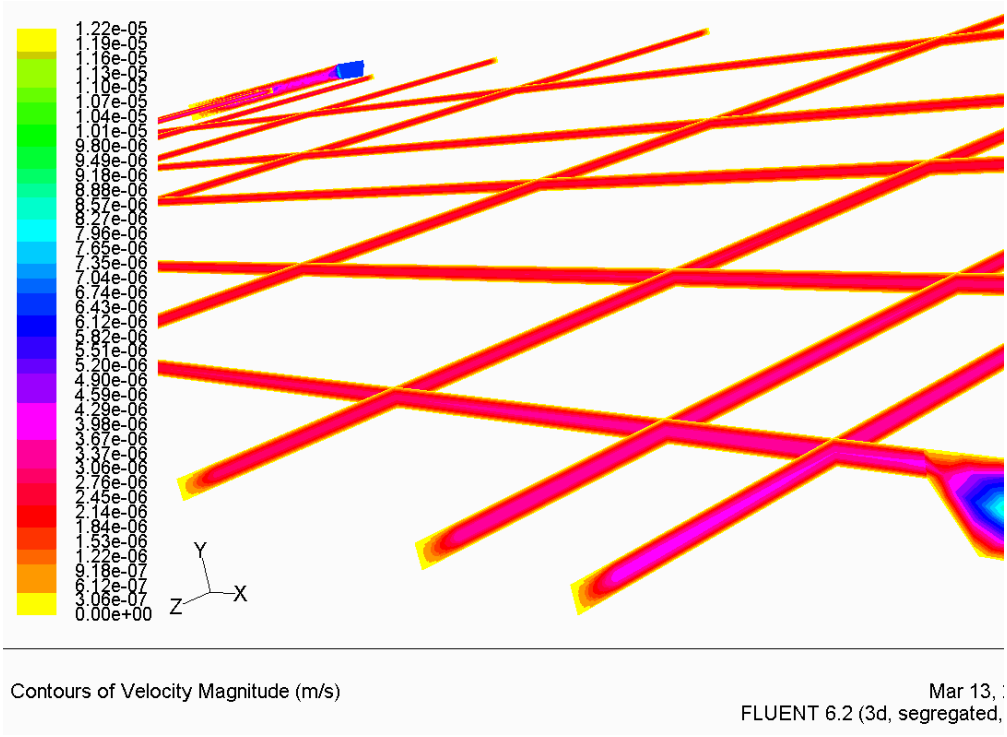


Figure 5.33: Velocity magnitude contour plot showing flow distribution across width and depth of cell for a power law fluid.

5.4.8. Discussion

The results show that the new design produces a similar set of results to the original test cell. There is however a larger area of velocity stepping at the end of the inlet port and also at the outlet. This leads to a proportion of the channel having a changing velocity along these sections which is the situation that we wanted to avoid for the main section of the channel. This small problem was thought to be caused by a combination of the substantially larger inlet channel and the reduced exit velocity. Various ideas were thought of to try and cure this problem, the first being to increase the velocity of the fluid through the channel. This had the opposite effect and actually made the problem worse by extending the velocity stepping further into the channel. The problem was solved by extending the length of the channel to allow the flow to fully develop before it comes in contact with the test material. This ensured that the flow across the test material was of a constant or nearly constant velocity. Whilst this solved the flow development problem it did slightly increase the non-test material surface that was in contact with the blood compared to the previous device. However, the selected material is one that has a good level of biocompatibility so exposure to this increase in non test material surface area was not deemed to have a major effect on the test fluid. However, the modifications of the test cell did increase the exposure of the test fluid to 60% more of the non-test material compared to the original cell by Gaylor and Lamba. The differences between the Newtonian and non-Newtonian flow are minimal with both fluids producing near identical flow plots. The flow velocities in the main channel range from 3.95-2.44e^{-5}m/s and have a main channel flow velocity of 3.35e^{-5}m/s for the Newtonian fluid and 3.98-2.45e^{-5}m/s and a main channel velocity of 3.37e^{-5}m/s for the power-law fluid. This can be seen in Figures 5.28 to 5.33. The differences in flow velocity are extremely small between the two fluid types, which suggest that the Test cell design would produce a similar flow profile for the lower viscous extraction fluids that were to be used in the experiments.

The taper unit performed well by maintaining the velocity across the width of the test cell. The main difference is that the taper does not reach the entry of the test channel; it has a small gap of 1mm as this was required to aid the manufacturing of the cell. This could also be contributing the increased stepping velocity lines in the initial

sections of the test channel. The gap in the model is slightly bigger than the one in the actual prototype because of issues with the meshing, so this may be why the initial flow plots are not as fluid as the original test cells. The differences in velocity between the different steps are also very small and should have a negligible effect on the plasticiser release characteristics from the test material.

Overall, the new test cell design produced satisfactory flow results for both Newtonian and the power law fluids, providing a good platform for the study of various liquid extractants with differing viscosities. Although there were velocity steps that were present in the test channel, the velocity differences are so small that they would not affect the efficiency of the test cell during use.

5.4.9. Construction and Testing of the Final Design

Once the cell design had produced favourable CFD results, it was decided to produce a couple of working prototypes. These were constructed out of 10mm thick PERSPEX sheet and were machined in the mechanical workshop in the Bioengineering Unit. The rubber bungs for the inlet and outlet ports were the lid component of Vacutainer (Becton Dickenson Ltd) blood collection tubes. The peripheral gasket was cut from 3mm diameter silicone rubber cord.

It was hoped that the mechanical clamp pressure would be enough to seal the two halves of the test cell and this was the case initially. However as soon as a small amount of pressure was applied via a hand pump, the cell leaked slightly at the areas where the gasket joined the rubber bungs. Leakage at the same area was also noticed when the cell was opened and then re-closed. It was concluded that if the bungs were not properly in-place and in contact with the gasket then leakage was possible.

To overcome this problem, the bungs were attached in the correct position using room temperature vulcanising (RTV) silicone elastomer to counter the leakage. This was to ensure that none of the liquid could make its way under the bung and hence out of the cell. At this point it was also decided to secure the ends of the gasket to the bung as well using RTV silicone elastomer. The elastomer was allowed to cure for a period of 24 hours.

Once the silicone had dried, the cells were again separately placed in the test rig and subjected to the following test sequence.

There were two prototypes that were initially built: Prototype A – cell without recesses for test material and Prototype B – cell with recesses for test material. Prototype A was used initially to test for leakages and to make sure that the cell could be pressurised. The test procedure was as follows:

- Test cell was clamped.
- 22 gauge hypodermic needles fitted with 3-way Luer stopcocks were inserted through the inflow and outflow bungs.
- With both valves opened and the cell in vertical position, the cell was filled with coloured water via a syringe attached to the lower inflow needle.
- After all air bubbles were removed the cell was then placed in its usual horizontal position and the valves closed.
- The syringe was removed and replaced with a sphygomanometer hand pump.
- . The inlet valve was opened and the cell slowly pressurised in increments of 20 mmHg up until 200mmHg.

At around 80mmHg applied pressure it was noticed that water leakage was occurring behind the gasket in the vicinity of the outflow port. Rebuilding the cell did not eliminate this problem and further leakage at higher pressures was observed near the inflow port. Further examination of the test cell revealed manufacturing variations in gasket channel depth preventing incomplete sealing under compression. A small amount of liquid silicone rubber was applied in the gasket channel to raise the gasket up slightly. This allowed for a better fit between the gaskets during compression which stopped the leakage completely.

Prototype B was also subjected to this form of testing with normal PVC test blanks being fitted into the material holding recesses. This also allowed investigation of the behaviour of the PVC fixing method under pressurised conditions. The lack of leakage from the material fixing troughs indicated that the chosen method was suitable for the task in hand.

The flow tests were undertaken on both prototypes, to ascertain if the CFD results were accurate. The following test procedure was used.

- The test cell was clamped and sealed.
- 22 gauge hypodermic needles fitted with 3-way Luer stopcocks were inserted through the inflow and outflow bungs. Both valves were opened.
- A collecting beaker was positioned under the outlet valve and a 50ml syringe was filled with blue dyed water and attached to the inlet needle.
- The cell was tilted vertically to reduce the chance of air bubble forming in the inlet. The inlet channel of the cell was primed with the liquid.
- The cell was returned to the horizontal position and the test liquid was slowly entered into the cell.
- The fluid flow was maintained through the cell until the syringe was emptied.

The flow through prototype A was encouraging, more so than what the CFD suggested. There were no areas stagnation or backflow seen during the flow tests, and the flow moved evenly up the length of the test cell with a good flow shape. The flow through prototype B, was as good as the uncut channel in prototype A in the first instance. However, further tests produced more varied results. The variation in flow distribution was dependent on the correct positioning of the PVC test material. If the fit was not entirely flush with the edges of the test cell, then the flow could be impaired.

A second set of combined pressurisation and flow tests were performed on the test cell once the initial pressurisation and flow tests were adjudged satisfactory. This second set of pressurisation and flow tests was to mimic the use of the cell in the proposed animal bypass study. The same set up procedure for the initial testing was followed again with the following modifications being added.

- Water was re-circulated through the test cell by small peristaltic roller pump similar to that to be used in the animal bypass study.
- An adjustable clamp was placed on the outlet tubing to simulate the resistance to flow produced by the animal (approx 250 mmHg).
- The pump was set to produce a flow rate of 1.5ml/min.

The outlet pressure was monitored by sensor (similar to that used in the bypass experiment) via a saline-filled line connected to the outflow tube via a T-junction.

The circuit set up can be seen in Figure 5.34. This new set of testing was important as the test cell should be capable of remaining leak free whilst subjected to a pulsatile flow at outflow pressures of between 250 mmHg and 300 mmHg. No leaks were seen during this testing sequence; confirmation that the sealing modifications were effective. It was observed that, with repeated testing, coring of the rubber bungs with needle insertion occurred with subsequent slight leakage. However, this leakage would not be present in the radiotracer studies as the bungs and gasket would be replaced after each experimental run.

The foregoing tests were conducted with normal saline solution, 40% glycerol and then methanol as the test fluids. Methanol has a surface tension and viscosity about one-third and one-half respectively of those of saline and would therefore present a greater challenge to the sealing integrity of the test cell. The compatibility of the test cell body, i.e. Perspex, with methanol was also examined by observing any structural changes in the cell body with extended exposure times.

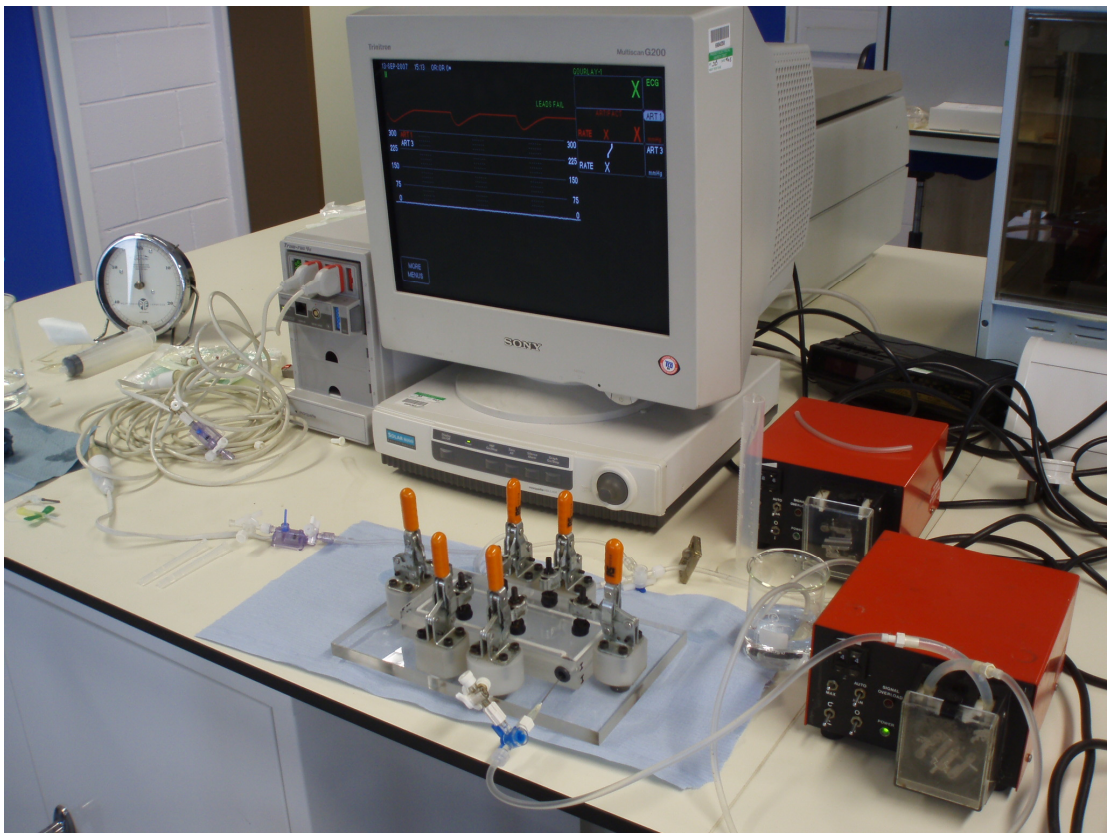


Figure 5.34: Set up for 2nd round of pressure and flow testing

5.4.10. Material Positioning Testing

Once the initial leakage and flow testing had been performed and each test cell had passed, the next stage was to test out the material securing method. The securing of the test material, in this case PVC sheets, is a very important aspect of the experiment. If the material is secured poorly then areas of the test sheet may become loose and block the flow channel, hence ruining the experiment. What was required was a securing method that would maintain the sheet in a tensile state enabling it to be perfectly flat without interfering with the sealing of the cell or the exposed surface area of the material under testing.

The PVC sheet was housed in a 3mm deep rectangular recess filled with a clear silicone elastomer, allowed the possibility for the testing of varying thicknesses of PPVC sheet using the same test cell. Two-part silicone elastomer was selected as this could cure even when overlaid with PVC sheet.

After much trial and error, the following sequence was adopted for the positioning of the test material.

- Prepared elastomer was mixed for 15 min until it started to become thicker.

- Elastomer was poured into the recess of lower plate and allowed to settle.
- PVC test sheet was placed onto elastomer and a spacer block pressed down on top of it.
- Block was held in place for 10 min to allow the elastomer to part cure so that elastomer would remain deformed when the block was removed.
- Assembly was left for 24 h to fully cure.

The same procedure was repeated for the upper plate of the test cell, but the spacer block was turned upside down so as to utilise the flat surface which helped to ensure that the lid PVC blank was flush with the rest of the cell.
Overall, this technique produced an ideal way of securing the PVC test material in place whilst also ensuring that the desired gap of 0.3mm was present.

5.4.11. Final Material Selection and Production

The methanol testing showed that the Perspex material would not be suitable for the final test cell material. During the methanol test, the Perspex showed signs of deterioration in its properties. This initially manifested as reduced opacity over the machined inlet and outlet channels after about 2hr of recirculation. Further degradation continued with small spider cracks appearing in the lid of the test cell over the same area, particularly near the two end clamps after around 3hr. It was not until about 4hr into the recirculation that major failure occurred. Large cracks, 4cm in length, suddenly appeared in the lid of the test cell. These cracks were the entire thickness of the lid and were severe enough to produce signs of leakage. The failure of the material only half way through the planned experimental time, required a suitable replacement to be found.
There were three requirements that the material had to fulfil:

- Extraction media compatibility.
- Transparency
- Machinability

Compatibility is arguably the most important of the three as the test cell material must be inert to the various extraction media, namely: blood, plasma and methanol.

Transparency of the material permits the direct visualisation of media flow through the test cell and the identification of flow abnormalities e.g. blockage.
Machinability is important in order to obtain a good surface finish and to enable a large number of the test cells to be produced easily using the general purpose machining facilities in the department workshop.
It was decided that the production of the test cells should be made using the computer numeric control (CNC) machining facilities available in the university as this would allow for a large number of completely identical cells to be produced. The material that met all of the desired criteria was a specially coated polycarbonate sheet with high abrasion rating, Lexan® Margard® MR5E (General Electric Plastics, The Netherlands)

5.4.12. Test Cell Choice

During the development and final testing of the modified test cell, certain issues arose which led to the viability of the test cells used in the project. The test cell was of sound engineering but was let down by the manufacturing quality of the machined parts. These machining problems led to each test cell having dimensional variations which led to leakage and required considerable time and effort to ensure that the cells could be used effectively. This greatly increased the time it took for a test cell to be prepared for an experiment and it was anticipated that this would be impractical especially when it came to the animal perfusion experiments.

Therefore, it was decided to revert to a previous test cell that had been specifically designed for animal studies by Prof. T. Gourlay at Imperial College in London as this negated the problems related to the manufacturing issues seen with the modified cell.

The test cell that was chosen for use in the experiments was a modified version of the test cell used in the previous DEHP studies by Gourlay et al (2001, 2002 and 2003).
The test cell was a parallel plate based cell that utilised a non compressible stainless steel gasket that maintained the 0.3mm channel depth over the test material. The material was initially adhered to the base using the hydrostatic effect of water, but this was not feasible for our study due to the thicker material used in this study. It

was decided to use some adhesive spray to mount the material on the base. The upper material was fixed to the test cell lid by the inlet and outlet valves, which were screwed into place. This test cell required some modification to accommodate the smaller size of the test material blanks. The design of the metal gasket that controls the flow reduces the surface area of the PVC that was exposed to the test fluid compared to the previously design cell by around 22%. However, the exposed level of PVC is comparable to the largest level used in the previously published studies by Gourlay et al. A 3-d parametric model was drawn up of the new test cell and gasket and these were translated into engineering drawings and can be seen in Appendix D. Schematics of this design are seen in Figures 5.35 & 5.36. The drawings were then sent to medical device manufacturer (Brightwake Ltd, Kirkby-in-Ashfield, UK) and 24 of the new test cells and gaskets were manufactured by CNC machining.

The viability of the test cell was demonstrated by its successful use in previous animal perfusion studies by Gourlay and his team. The fluid dynamics of the cell had also been extensively studied and validated as part of an MD thesis by Stefanou in 2006. Therefore, the flow conditions produced by this cell were deemed to be suitable for the proposed studies.

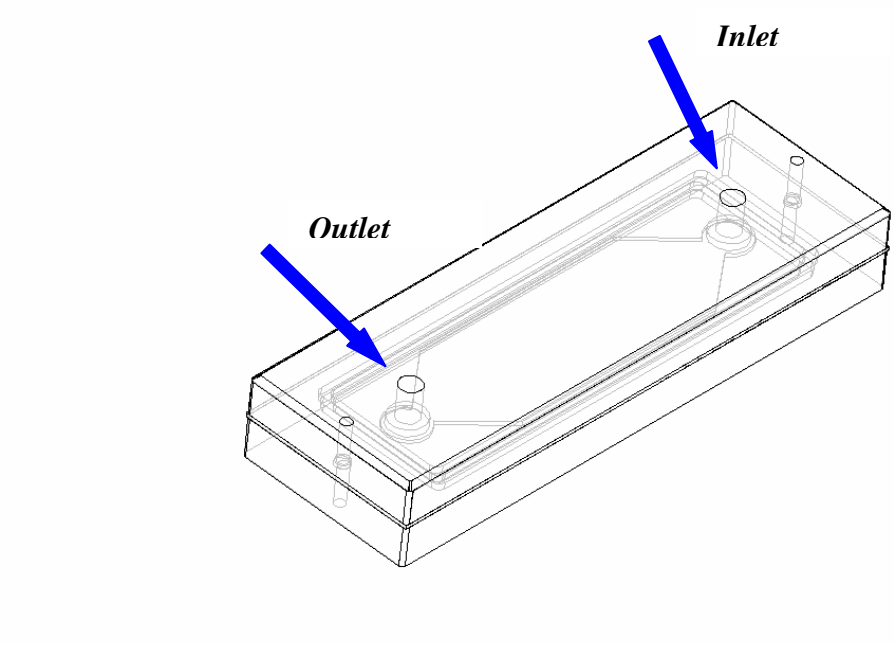


Figure 5.35: Schematic of the complete modified Gourlay Test Cell.

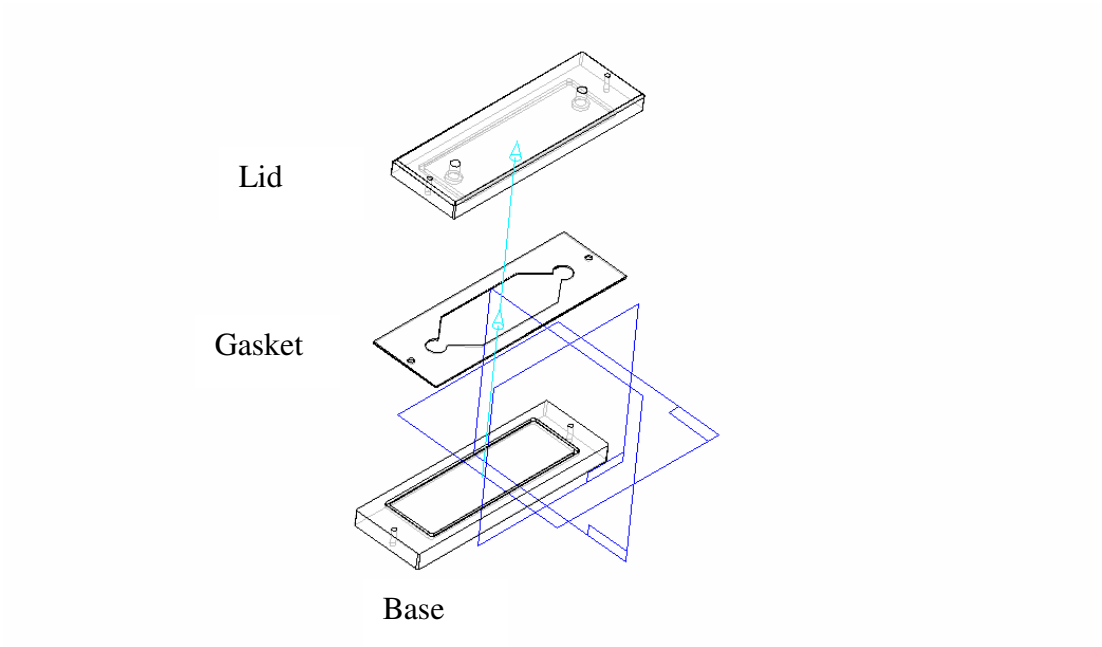


Figure 5.36: Exploded View of the modified Gourlay Test Cell, showing the make up of the Test Cell

6. Materials and Methods

6.1. Radiolabel Selection

At the beginning of the project the intention was to label a substantial amount of the DEHP plasticiser used in the fabrication of the test material as this would allow for direct correlations between the scintillation counts and the amount extracted. During the early investigation into the labelling process it was discovered that this would be impossible for a number of reasons, the two main ones being cost and also the holding limit for the radiation that was permitted in the Bioengineering Unit.

The two radiolabels that were considered for this study were Carbon-14 (^{14}C) and Tritium (^{3}H). These were selected as they could be substituted for either a normal carbon or hydrogen and could be placed anywhere on the DEHP molecule without altering its shape and properties as well as being the main types of radioisotopes that could be held in fairly substantial quantities in the Bioengineering Unit.

The carbon-14 radiolabel and the tritium were thoroughly investigated looking at various aspects such as safety, contamination, positioning and cost as listed in Table 6.1.

	^{14}C Label	**^{3}H Label**
Cost	Very Expensive Circa £13 000	Expensive Circa £3-5000
Contamination	Low risk	High risk- Easily contaminates surrounding surfaces
Positioning	Possible on both the carbon ring and branches	Possible only on branches
Shielding	Medium- 2 cm Perspex required to minimise exposure	Low- Exposure can be minimised by maintaining an air gap
Scintillation Counting	Easy due to high strength of emitter	Difficult due to weak emitter level
Type of Radiation	Strong Beta Emitter	Weak Beta Emitter

Table 6.1: Advantages and Disadvantages of ^{14}C and ^{3}H Radiolabels

The high cost of labelling the DEHP was unexpected but was due primarily to the positioning of the label and also because it would have to be custom synthesized as it is not readily available. Thankfully, generous financial donations by Dr Jason Leadbitter of Hydro Polymers (now INEOS ChlorVinyls, Newton Aycliffe, UK) allowed the project to continue.

After reviewing the literature relating to previous radiolabel work in this field we decided that it was of utmost importance to label the ring portion of the plasticiser. This would allow one to maintain a trace of the element through the animal even after it had been metabolised. Previous studies employed various label positions ^{14}C-tagged carbon atoms on the benzene ring (Albro et al, 1983), tagging the carbonyl carbon of the compound [7-^{14}C] (Albro et al 1986, Daniel and Bratt, 1974, Von Daniken et al, 1983) and ^{14}C-tagged carbon atoms in the branches, particularly C-1 of the alcohol chain (Von Daniken et al, 1983, Waddell et al, 1976, Tanaka et al, 1975). There were a number of studies that had been performed using tritium that had labelled the branches but these studies were not used for the same thing as we were intending.

The position of the label on the Benzene ring portion of the plasticizer was not so important. The options presented to us by the production company were either,

- Uniform substitution
- Single element substitution

Both of these would provide us with the required tracing ability, but we opted for the uniform substitution of a number of elements within the benzene ring. This choice was strongly linked to cost and time of synthesis of the product, with this being the most cost and time effective choice.

The contamination issue was one that initially was a major factor in deciding the type of radiolabel. This was due to the handling of the plasticiser during and after production of the PVC sheets. The initial plan for the production of the sheets was to compound them using a two roll mill and hydraulic press. These would then have to be decontaminated after use by running extra polymer batches through them until they were “clean”. Therefore, it would be ideal to have a label that would not easily contaminate any surfaces it came in contact with, to firstly ensure that majority of the

radiolabel was present in the finished PVC sheets and secondly to reduce the amount of decontamination that would be required once the work had been completed.

The next stage of the evaluation of the labels was again related to safety. Both labels are Beta emitters, with the Carbon-14 being a strong emitter and the Tritium and very weak emitter. The obvious choice for safety would be to use the weaker of the emitters, but this brought up other problems as the weaker emitter was not entirely suitable for scintillation counting, which was the chosen method of detection. Its counting efficiency for tritium is only around 20-30% where as for the stronger Carbon-14 this is nearer 90-95%. The stronger emitter combined with the higher counting efficiency of carbon-14 also suggested that suitable results could be achieved with substantially lower levels of carbon-14 present in the test material.

The shielding of the radiation source was another safety aspect that was required to be addressed. Tritium being a weak emitter does not penetrate the skin and therefore does not require extreme measures of shielding such as a containment box. Good laboratory practice requires the operator to wear a lab coat, protective gloves and also eye glasses and this combined with maintaining a distance of greater than 6mm from the radioactive source would be enough to shield the user from the tritium source.

The carbon-14 source on the other hand requires a medium level of shielding to protect the user. The source can be completely shielded by approximately 1-2cm of Perspex or polycarbonate. The use of a protective screen must be employed at all times as well as the usual safety equipment and as with the tritium maintaining a distance from the source also greatly reduces the exposure.

After all these points had been thoroughly looked at and discussed with the radiation safety officers, it was decided that the Carbon-14 label would be the best choice even though it is more expensive and also a stronger emitter. The reasons for this are listed below:

- Easier to Decontaminate PVC production machines and Test Cells
- Produce better results with the Scintillation Counting
- Better reliability on labelling of the Benzene Ring allowing for detection even after metabolism

- Carbon-14 has already been used in similar studies and is used in the clinical environment

6.2. Quantity of Radio-labelled Product

An Excel spreadsheet was drawn up to find the optimum label quantity that would work within the safety limits imposed by the University. Using previous data from other sources different combinations were tried until a suitable solution was reached. This worksheet can be seen in Appendix E. It was decided to use a label with the following characteristics.

- Specific Activity, (MBq), 74
- Total Activity, (MBq), 397.64
- % quantity of total DEHP, 0.007

The experiment now became a tracer study, as the levels of labelling were much lower than the 1% labelling that was previously thought of at the start of the project.

6.3. Raw Material Suppliers

The materials that were used in this project were substantial, with many being sourced internationally.

6.4. PVC Material

The raw materials for manufacturing the PVC sheets were supplied by INEOS Chloe Vinyl's (Newton Aycliffe, UK)
The company supplied the following raw materials that are used in their commercial medical grade PVC:

- B55089 K70 PVC Resin
- B62053 DEHP Med, Medical grade Di-2-ethylhexyl phthalate

- B66388 ESBO, Epoxidised Soybean oil
- B64334 calcium stearate
- B64032 zinc stearate

6.5. Radio-labelled DEHP

The sourcing of the radio-labelled DEHP proved to be more of a challenge than anyone involved in the project had previously thought. It was not a product that any of the major radiochemical suppliers stocked. It therefore had to be custom synthesised. Tenders were put out to numerous companies throughout the UK, Europe and worldwide. The majority of the responses received were far outside our project budget. The best quote came from American Radio-labelled Chemicals Inc. (ARC), St Louis, Missouri, USA. It was a partnership that proved to be very fruitful as the cooperation and help received from this company was outstanding especially considering the many problems we were to encounter.
An order was placed with ARC for the following product:

ARC 3163 Bis(2-ethylhexyl)phthalate [ring-^{14}C (U)]
Total Activity: 481MBq
Specific Activity 2-10mCi/mmol or 74MBq/mmol

6.6. Circuitry and Connectors

Connecting tubing and circuit components for *In vitro* and *Ex vivo* studies were sourced from Altec Products Ltd, Bude, UK. The following products were acquired:

AlteSil High Strength Silicon Tubing, Product code: 01-93-1414
Male Luer connectors, Product code: 05-44-5502
Female Luer Connectors, Product code: 05-44-5502
Barbed Straight Tubing Adapters Product code: 05-42-5354

The following connectors were also used as part of circuitry.
VYGON 3-way lockable Stopcocks
Model Number: 876.00

6.7. PVC Sheet Manufacture

Our first port of call involved contacting Dr Colin Hindle of Napier University as he had been involved in many of the previous PVC projects in the Bio-engineering Department. Dr Hindle believed that his institution would be able to offer a solution to our problem and set about investigating this further with the relevant authorities at Napier University. After much discussion about the finer points of the proposed work, a conditional agreement was made to perform the work at Napier.

Unfortunately, Napier discovered a discrepancy in their radiation license and had to withdraw their support at a critical stage of the project.

After this set back the search was broadened firstly to British Universities that had polymer engineering departments and latterly to those world wide. Many Universities were contacted during this search, such as Loughborough and Queens University, but the results were all the same. The universities in the UK all had their hands tied by “red tape” as their polymer processing areas were not designated radiation areas and therefore they could not help in the preparation of such a material. The case was the same regarding Universities in Europe and North America.

The Chinese universities, which were contacted by myself and also Professor Zhao, returned favourable results, in that they would be able to undertake the required work. However, due to the distances and logistics involved it was decided that this should remain as a back-up option if all other avenues of investigation failed.

Whilst the university search was being conducted, a search of possible industrial candidates was also undertaken. Polymer Laboratories were contacted throughout the world with varying success. One such laboratory in the USA, was again willing to perform the desired work for us, at a reasonable cost. This was an attractive option,

due to the radio-labelled DEHP also being synthesised in the USA, hence reducing the logistics of the work.

However, due to the industrial laboratory's inability to provide the correct documentation, this production route also failed. This then left the project in a precarious position, time was moving on and we still had no means of manufacturing the PVC material. The Chinese university routes were revisited. The initial contact made with Guangzhou University was no longer feasible due to logistical issues required to complete the work. Further enquiries were made by Professor Zhao to Langhzu University. Unfortunately, they could not accommodate the work in the required time scale and hence could not facilitate our request.

Another search was undertaken looking for polymer and radiation laboratories all over the world, in a last ditch effort to save the project. This search proved to be fruitful as a research institute in India was discovered and we entered into discussions with them over the project. They returned a proposal that was more than satisfactory on both product and cost, and hence The Shriram Institute, in Delhi, was commissioned to produce the PVC sheets.

With a manufacturer finally in place it was hoped that the project could progress at a quicker pace. However, this was just the start of the project problems. The University decided to invoke high levels of "red tape" relating to both the ordering and delivery of the ^{14}C DEHP product. This bureaucracy was so damaging that it came close to ending the project on a number of occasions. Only the timely interventions by a number of Prof. Gourlays many contacts, who had a genuine interest in the proposed work, allowed us to legally bypass the barriers that were put in place by Strathclyde University. By allowing us to perform the *Ex vivo* experiments in their respective laboratories.

Dr Thomas Modine of Service de Chirugie Cardiovasculaire, Hôpital Cardiologique, (CHRU) at Lille University Hospital arranged for the *Ex vivo* perfusion experiments to be conducted in his research laboratory in Lille. Whilst also allowing me to utilise the Industrial research partner for Lille University, Adrinord, which enabled us to order the full quantity of the ^{14}C DEHP and ship it to the required destinations.

Professor Serdar Gunaydin of Kirkali University in Ankara, had offered his laboratory services for the gavage study of the work, but again the red tape

surrounding the use of radiolabels put pay to this. However, he utilised further contacts and arranged for the work to be done in the laboratories of Professor Levant Kenar at the Gulhane Military Medical Academy in Ankara, Turkey.

This was the final piece of the jigsaw and we were now in a position to undertake every part of the planned experimentation. The project had grown from a small project with only three branches into a mammoth project which required the management of 11 different centres to try and obtain all of the desired project outcomes.

6.8. Experimental Methodology

6.8.1. Introduction

The experimental procedures were split into two distinct sections, *In vitro* and *Ex vivo*. These sections were designed to produce results that would demonstrate the effectiveness of the proposed assay test method in determining the fate the DEHP once it had been extracted from the PVC.

6.8.2. In vitro Studies

The first stage of the experimentation was the *In vitro* section. These experiments were conducted in the Departmental Radiation Laboratory, Level 5, Wolfson Centre. The *In vitro* studies involved recirculation of various extraction media through the material test cell described above.

The experiments were split into three groups based on the extracting fluid:

- Methanol
- Whole Blood (Bovine)
- Plasma (Bovine)

The extraction media were selected to allow comparison with experimental data obtained by previous investigators in this area. Methanol was used as a fast extraction rate test whilst the plasma is representative of extraction with lipid-containing media. Studies with whole blood are relevant to the clinical end use. The three test fluids allowed us to determine if the physical make-up of the contacting fluid had such a pronounced effect on the extraction levels as previously stated by Zhao et al (1999), Kim et al (1976) and French (2001).

For each of the three test fluids, five separate test runs were performed to provide statistically relevant data sets.

6.8.2.1.In vitro Experimental Set up

It was decided that the *In vitro* experimentation should compliment the *Ex vivo* studies, with the test circuitry being as similar as possible. This was to ensure that the conditions for both set of experiments were as close as possible. It should be noted that for the *In vitro* studies the entire test set up was contained within a specially built containment chamber. This was a stipulation made by the University Safety Services to protect the experimenter from any unnecessary radiation exposure and to limit contamination in the event of circuit leakage.

The layout of the re-circulation experiment circuit is shown in the schematic of Figure 6.1 and in the photographs of Figures 6.2 and 6.3.

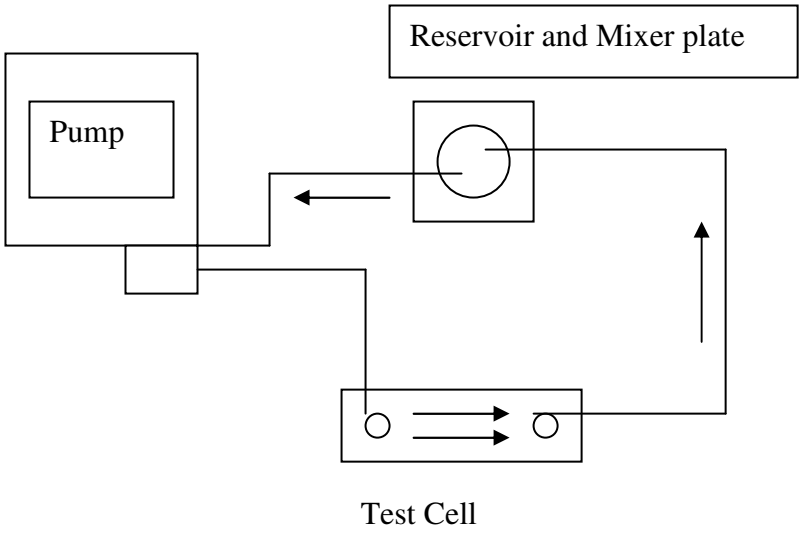


Figure 6.1: Schematic of the Test Circuit set up

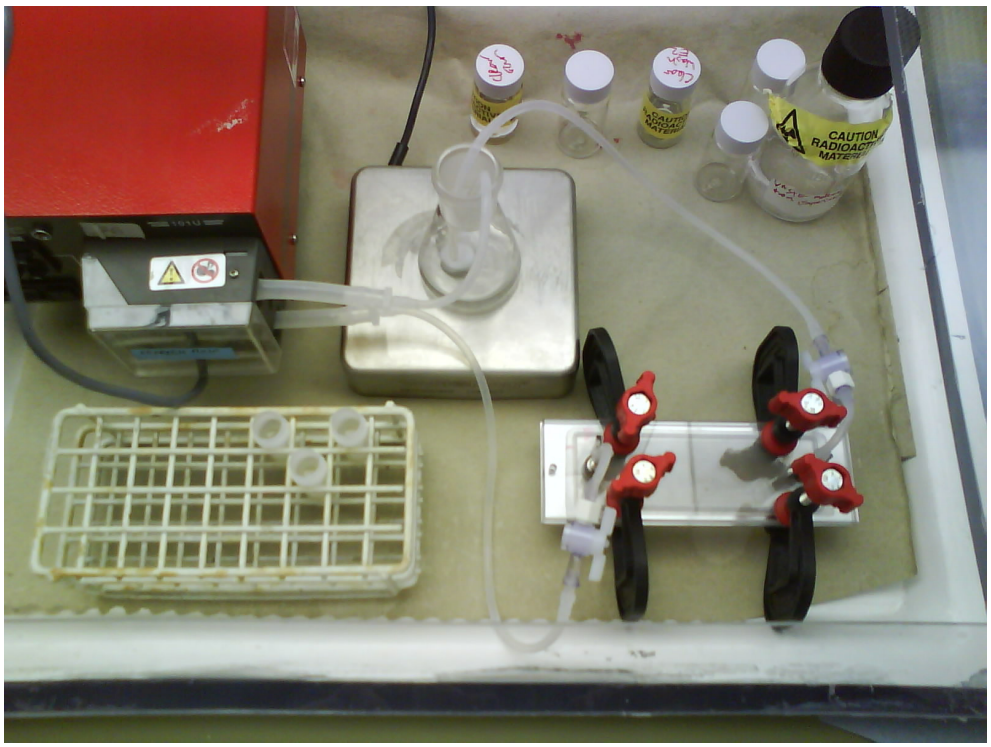


Figure 6.2: Picture of the re-circulation test set up during testing with methanol

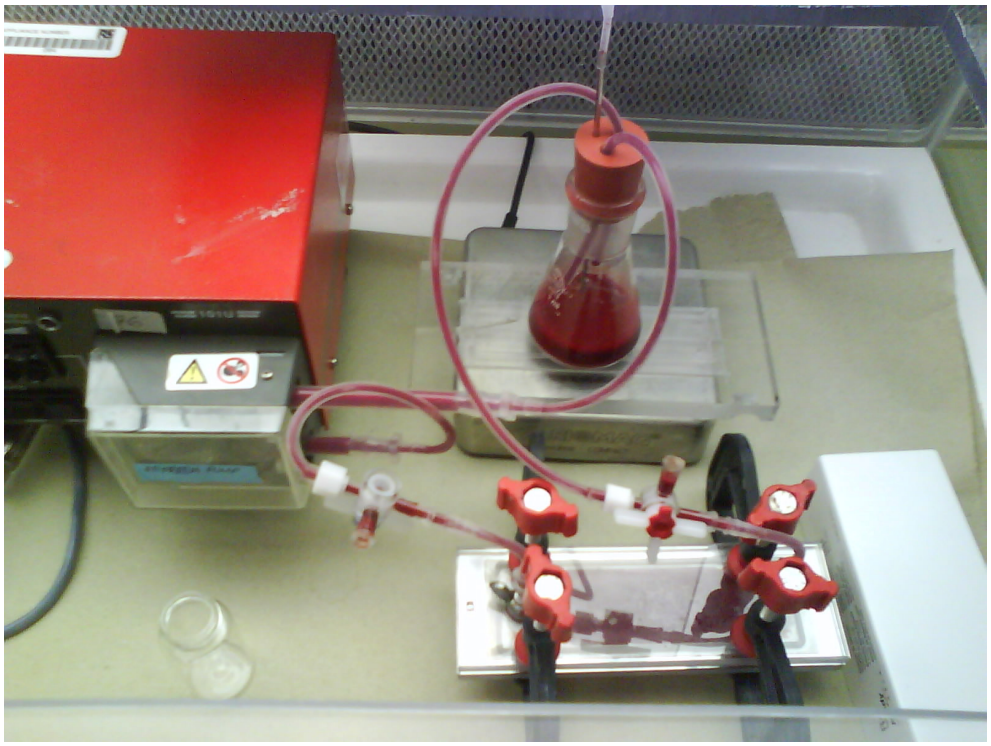


Figure 6.3: Picture of the test circuitry during an experimental run using bovine blood

6.8.2.2.In vitro Experimental Protocols

The experimental protocol for the *In vitro* work was split into three sections.

- Preparation of Test Material
- Preparation of Test Cell
- Experimental Procedure

The reason behind splitting of the experimental protocol is for safety, as by proceeding in this manner the possible exposure dose received by the experimenter is reduced by minimising the time spent with the radioactive material.

The protocols followed in the preparation of the test material and the subsequent preparation of the test cell can be found in Appendix F.

6.8.2.3.Experimental Procedure

The experimental procedure was as follows:

1. The test cell was removed from its storage box and connected to the tubing circuit.
2. The reservoir was then filled with the following volumes depending on the test fluids.
    a. Methanol: 42.5ml
    b. Plasma: 30ml
    c. Whole Blood: 30ml
3. The reservoir was then connected to the circuit and the pump started to fill the tubing before the inlet valve.
4. A measuring cylinder was placed at the outlet to siphon off 12.5ml of the methanol wash before the experiment was started properly. This was only performed for the methanol and not the other fluids.
5. The fluid was then pumped through the test cell to determine if the flow channel was suitable. The securing clamps were then adjusted to ensure that the channel was sufficiently open to promote the desired flow conditions.

6. Samples of 0.5ml were then taken using a pipette from the reservoir at intervals of 5, 10, 15, 30, 45, 60min, 2, 3, 4 and 5hr.
7. At the end of the run time, the test circuit was pumped empty and the fluid collected in the reservoir. The circuit tubing was then disconnected and disposed of in the solid waste bin. The test cell was removed from the containment chamber and dismantled in the radiation work tray with the used PVC material being disposed of directly into the solid waste bin. The remaining components were then placed into a decontamination box containing DECON and water and left to soak for 24hr.
8. In the case of the blood and plasma, the remaining liquid in the reservoir was treated with Chloros (sodium hypochlorite) until the biological organisms were killed and the resultant mixture disposed via the designated sink within the radiation laboratory. The methanol was collected and stored in a separate container for disposal by the University Safety Services.
9. The reservoir was then filled with DECON and left for 24hr to decontaminate.

6.8.2.4.Sample Preparation and Analysis

The radioactivity of all the extraction media samples was determined by Liquid Scintillation Counting (LSC). This was performed using a Packard Tri-Carb 1700T scintillation counter and the scintillation cocktail for all test fluids was Ultima Gold (Perkin-Elmer Inc.).

The LSC machine was calibrated each day before counting to ensure that it was working at its optimum levels by following the specific calibration program installed in the machine.

Prior to scintillation counting different sample preparation procedures were necessary due to the physical characteristics of the extraction media. The preparation of each medium type is discussed below.

6.8.2.4.1. Methanol Samples

The methanol sample required no specific treatment before counting because of its colourless nature. Samples of 0.5ml were placed directly into small plastic scintillation vials containing 4 ml of Ultima Gold scintillation cocktail. They were capped and inverted to mix the solution and then placed into the LSC machine. They were left for 1 hour to reach ambient temperature and light conditions before being counted.

6.8.2.4.2. Plasma Samples

Being a biological solution, plasma, required a certain amount of preparation work to help reduced the colour quench effect. The decolourising protocol that was followed was taken from application notes for LSC of biological samples. This can be found in Appendix G.

6.8.2.4.3. Whole Blood Samples

The counting of whole blood samples presented a problem for the experimenter due to the high colour intensity and particulate nature of the solution. The application protocol by Perkin Elmer was followed closely to try and minimise the colour quenching problem and produce reliable results. The protocol can also be found in Appendix G.

6.8.3. Ex Vivo Study 1: The Rat Perfusion Model

A series of *Ex vivo* experiments were undertaken in the Cardiac Surgery Research laboratory of CHRU at the University Hospital in Lille, France.

The experiments involved the following two groups;

- Control animals (n=5) - These animals underwent the 45 minute perfusion operation but had no contact with DEHP.
- Exposed animals (n=5) - These animals underwent the 45 minute perfusion operation but were exposed to the DEHP PVC in the Test Cell.

The aim of these experiments was to discover the amount of plasticiser that was removed from the PVC during a perfusion procedure under conditions which are representative of clinical perfusion procedures.

The perfusion experiments that were performed were based on the previous work by Gourlay et al, 2001. The normal accepted surgical procedure used for such perfusion experiments involves the use of the Femoral artery and vein, but as this was initially a survival study it was decided that this approach was too invasive for the animal to be allowed to survive post procedure. Therefore, it was decided that during the course of this project the caudal artery and vein should be used as an attachment point for the perfusion experiment. This approach proved initially successful as the surgery was much less invasive leading to improved recovery rates and low mortality.

However, due to licensing restrictions, it was not possible to perform these experiments as survival studies in the first instance and hence the femoral approach was adopted for the present study. The schematic (Figure 6.40) illustrates the adopted surgical approach.

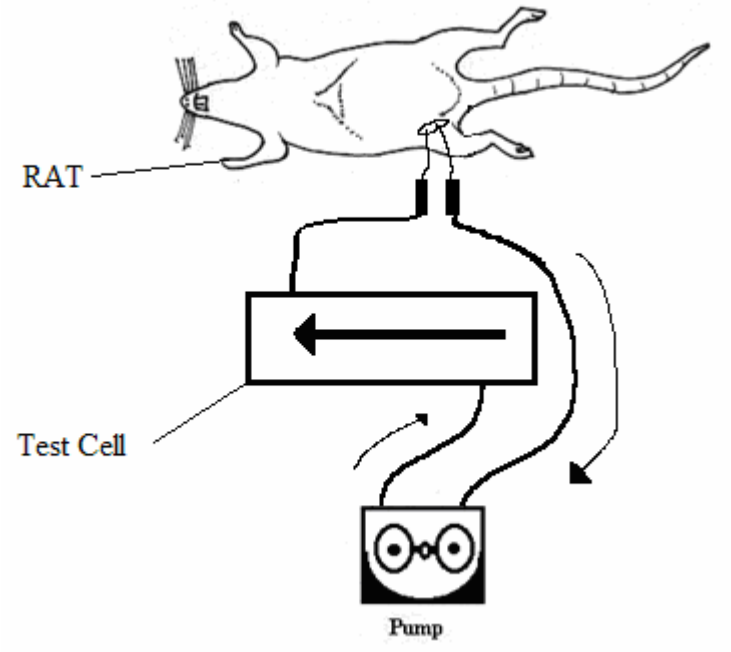


Figure 6.4: Schematic of the animal perfusion circuitry

The procedure itself was performed on 10 male Sprague Dawley rats with a weight range of between 350-480 grams. The animals were fully anaesthetised during the

experiments with anaesthesia induced by Enflurane inhalation and maintained with intraperitoneal injections of a Hypnorm (fentanyl citrate, fluanisone)/ Hypnovel (midazolam) cocktail. Top-up injections of anaesthesia and neuroleptanalgesia were administered every 30-40 minutes or as required. All procedures were carried out under strict adherence with the Home Office guidelines and in accordance with the Animals (Scientific Procedures) Act 1986.

The anaesthetised animals were placed on the operating bench in the supine position. The Femoral vein and artery were exposed by creating a small tear drop incision in the groin region of the animal. The vein and artery were then cannulated using two 3Fr portex cannulae that were positioned using an operating microscope. The cannulae were each held in place by a single suture and then taped to the operating table to ensure there was no risk of the cannulae slippage. This can be seen in Figure 6.5.

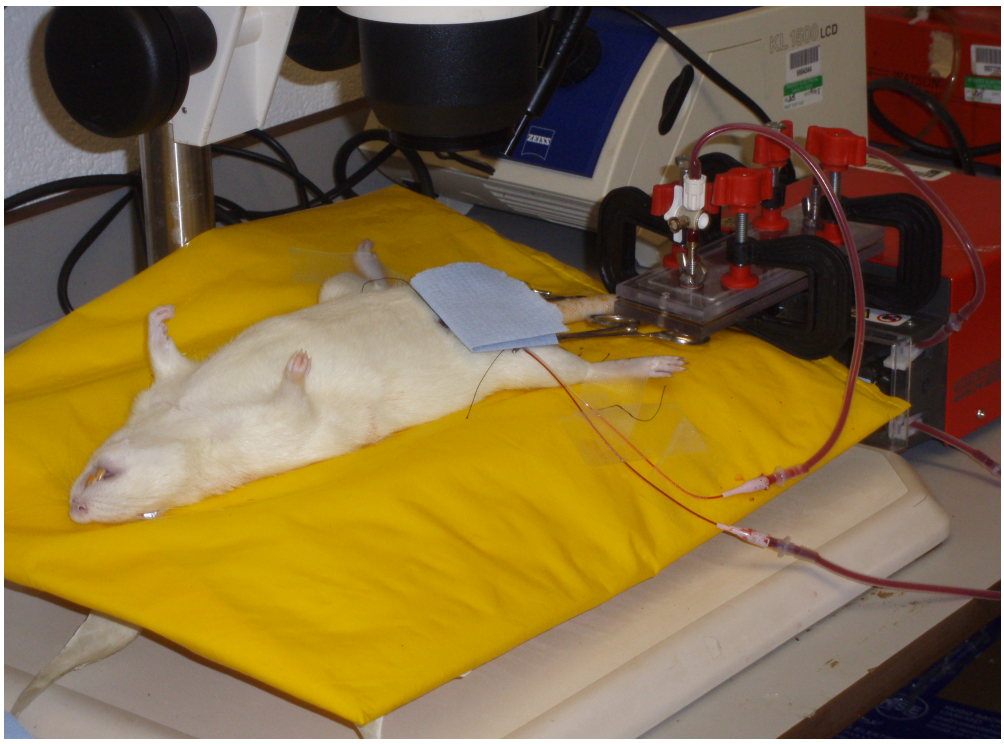


Figure 6.5: Picture of Test cell in place during Perfusion Experimentation

The animal's temperature was maintained by a heat blanket which was placed under the animal on the operating bench. Before the cannulae were attached to the test cell

apparatus, 0.7ml of 1000U Heparin was administered to the venous line. The test cell, which has been primed using a saline solution containing 0.3 ml of 1000U Heparin and left to re-circulate for a minimum of 15mins was then attached to the cannulae and the recirculation was then initiated with a flow rate of 1.4ml/min.

Recirculation was continued for 45 minutes. After which the procedure was terminated and the animals disconnected from the circuitry. The animals were then killed by administering a terminal dose of Potassium Chloride via the venous cannulae, until all signs of life had ceased. The cannulae were then re-attached and Ringers solution was then perfused through the animals and test circuitry for 10 minutes to haemodilute and exsanguinate. The cannulae were then removed and the animal prepared for organ harvesting. The test cells were then biologically decontaminated using Chloros and the radio-labelled PVC test strips were then disposed of in accordance with Home Office Guidelines. The test cells were then totally decontaminated to remove any trace of remaining radioactive contamination and prepared for re-use.

6.8.3.1.Sample Collection and preparation

As previously stated the rats were sacrificed post procedure and the following organs were harvested and frozen for transport to the Bio-engineering Unit at the University of Strathclyde for analysis.

- Brain
- Liver
- Kidney
- Lungs
- Muscle
- Skin
- Adipose
- Heart

6.8.3.2.Sample Analysis

The tissue samples were analysed using the Liquid Scintillation Counting technique. The accurate counting of biological samples can be a difficult process. Firstly, the tissues have to be solubilsed and then they must be decolourised to allow for accurate readings. A strict protocol issued by the manufacturer of the LSC machine was followed for both the solubilisation and decolourisation process for each sample. This can be seen in full in Appendix G. A brief outline of the processes is given below

6.8.3.2.1. Solubilisation

This procedure was a simple task, involving the following steps.

- 200mg of tissue (dry weight) from each organ was placed in a large glass scintillation vial.
- 1-2ml of the alkaline solubiliser, SOLVABLE™, was added and the lid securely fastened on the vial.
- The vials were then placed into a water bath at 55-60^{o}C until the tissue was fully dissolved. This took between 1-4hr depending on the tissue type.
- The vials were then removed from the water bath and allowed to cool to room temperature. The samples should be of a green/ brown or pale yellow colour at this point depending on the type of tissue sample.

6.8.3.2.2. Decolourisation

This procedure whilst simple in nature was a very time consuming process. Its basic steps are outlined below.

- 0.1ml of 0.1M EDTA-di-sodium salt solution was added to each vial to reduce the foaming of the subsequent reaction.
- 0.3-0.5ml of 30% Hydrogen peroxide solution was added in 0.1ml aliquots to each vial. Gentle agitation was performed between additions to allow the foaming to subside.

- The vials were left for 30minutes at room temperature to allow the reaction to complete.
- The vials were capped tightly and placed in a water bath at 55-60^{o}C for 1hr. Venting of the vials was performed every 15minutes to reduce the build up of gas.
- The vials were then left to cool to room temperature. The colour of the sample should now be pale yellow or translucent.

6.8.3.2.3. Final Sample Preparation

The final stage once the samples had been decolourised was to added the scintillate. 10-15ml of Ultima Gold scintillant was added to each vial. The vials were then inverted to mix the samples and then left to temperature and light adapt for 1hr before being counted.

6.8.3.2.4. Scintillation Counting

The sample vials were placed into the scintillation counting machine along with a back ground vial containing only scintillant. The correct program was selected to measure the Carbon-14 isotope and the machine was left to run until all samples had been fully counted. The counting protocol was repeated three times for each set of samples.

6.8.4. In Vivo Study: Rat Gavage Exposure

A large proportion of the previous work into DEHP migration, metabolism and toxic effects was conducted by gavages exposure in rodents. Therefore, as part of this study it was important to perform similar experiments to provide some data that could be compared with the perfusion exposure method to determine if there was a difference in distribution of the ^{14}C DEHP between the two methods.

The gavage experiments were performed at the Gulhane Military Medical Academy, Ankara, Turkey. This institute has a long experience of this procedure and have all of the necessary regulatory licenses in place.

6.8.4.1.Experimental Protocol

The protocol that was followed for these experiments was quite different to that of the perfusion study. The regulations in Turkey allowed for the animals to be kept for an extended time period post procedure. This allowed us to utilise along term time schedule in terms of illustrating DEHP distribution in the tissues.

The study was performed on 12 Sprague Dawley male rats of weights between 350 and 400g. Each animal was subjected to a dose of 0.375mCi\ ^{14}C DEHP, mixed with Corn oil at a ratio of 5ml/kg bw to ensure that all of the DEHP was deposited in the stomach by gavage. The procedure was performed after anaesthesia was induced by inhalation anaesthesia using Enflurane. The animals were then returned to metabolite cages and left to recover.

The animals were then sacrificed and the organs harvested at the following times:

- 4 at 2 days post procedure
- 4 at 14 days post procedure
- 4 at 28 days post procedure

The organs that were harvested were as follows:

- Brain
- Kidney
- Heart
- Liver
- Skin
- Testes

6.8.4.2.Sample Analysis

The samples were analysed in the Radiation Laboratory of the Bioengineering Unit at the University of Strathclyde. The samples were to be analysed by Liquid Scintillation counting. The sample preparation was performed using the same

protocols that were used for the perfusion study samples, described previously, and a full copy of the protocol can be found in Appendix G.

7. Results

7.1. In vitro Studies

7.1.1. Total DEHP Plasma Extraction

The extraction profile generated by plasma showed detectable levels of plasticiser migration after 5 minutes of recirculation, with a mean value of 0.0011±0.0003 mg/ml. The plasticiser efflux continued to increase but with a decreasing slope to a time of 15 minutes where the concentration was 0.0015±0.0003 mg/ml (Figure 7.1). Thereafter the concentration levels continued to increase in a linear fashion to a value of 0.0142±0.0017 mg/ml at 300minutes (Figure 7.2). On closer inspection of the early stages of the DEHP migration in Figure 7.2, the profile suggests that in the first 15 minutes the removal of the surface plasticiser is by dissolution, followed by steady state removal of the plasticiser from the bulk phase until termination of the test at 300 minutes. The steady state migration from 15 to 300 minutes is indicative of a diffusion rate limited process, which is only limited by the amount of plasticiser present in the bulk of the material. This profile is similar in nature to the migration profile originally proposed by Kim et al (1976) and confirmed by Zhao (1999) in his PhD thesis. However in their studies the extractions were performed in methanol and not biological fluids, but it is encouraging to see that the migration profiles in both fluid types are the similar in nature. The main differences being (a) the levels of extraction in the plasma are much lower than those obtained with methanol and (b) the point at which the dissolution phase ends and diffusion phase starts.

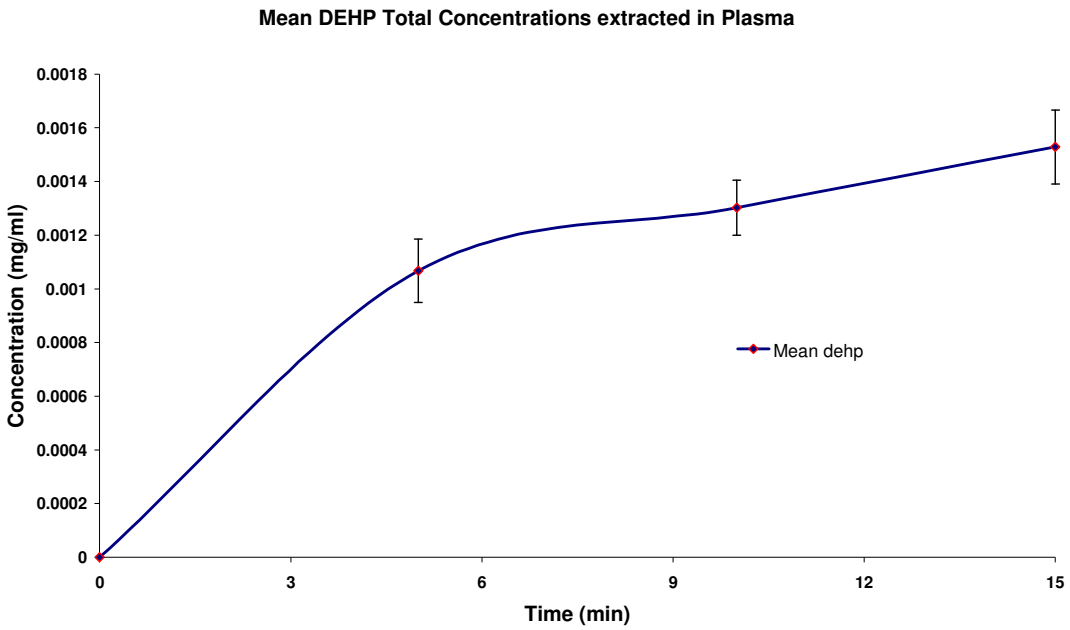


Figure 7.1: DEHP Concentration levels (mg/ml) in Bovine Plasma over initial 15 minutes of recirculation; number of runs (n=5); error (±SD)

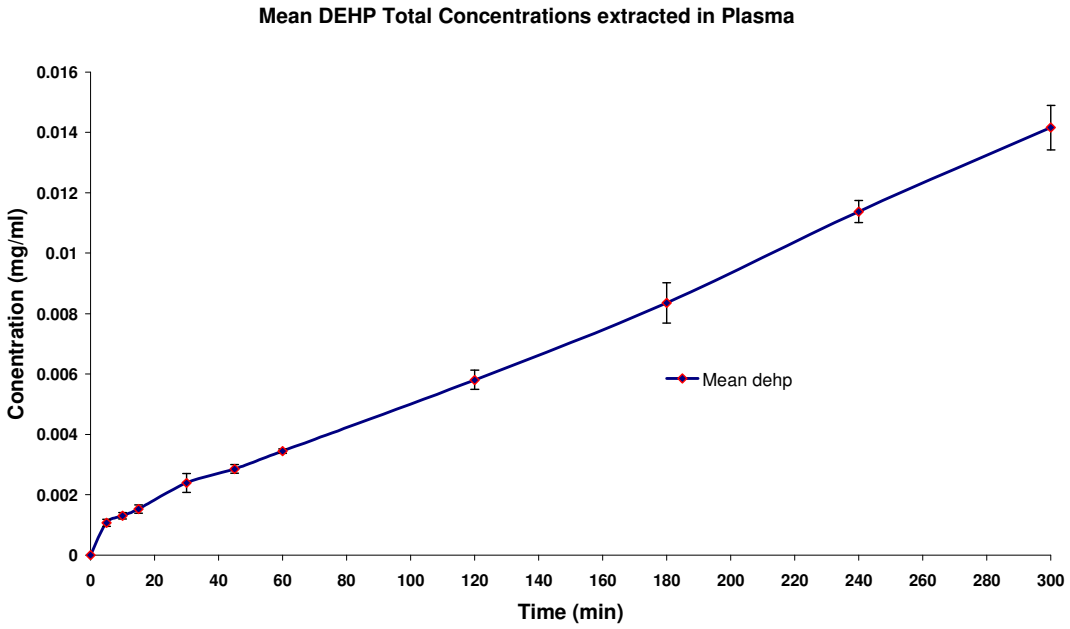


Figure 7.2: DEHP concentration levels (mg/ml) in Bovine Plasma over 5 hour recirculation; number of runs (n=5); error (±SD)

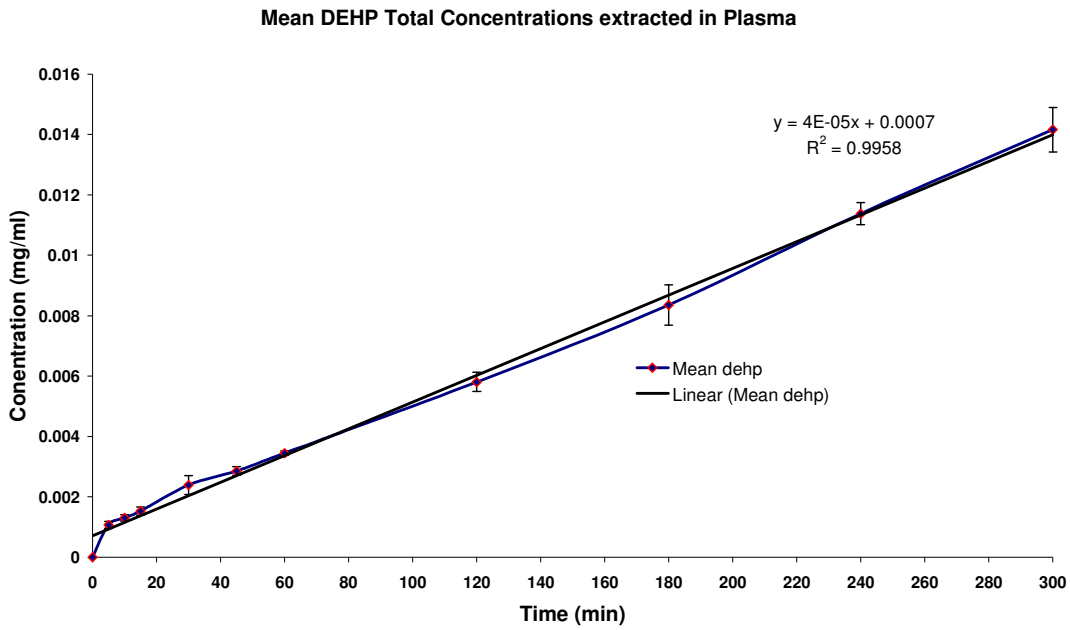


Figure 7.3: Trend line showing linearity of diffusion of DEHP from PPVC

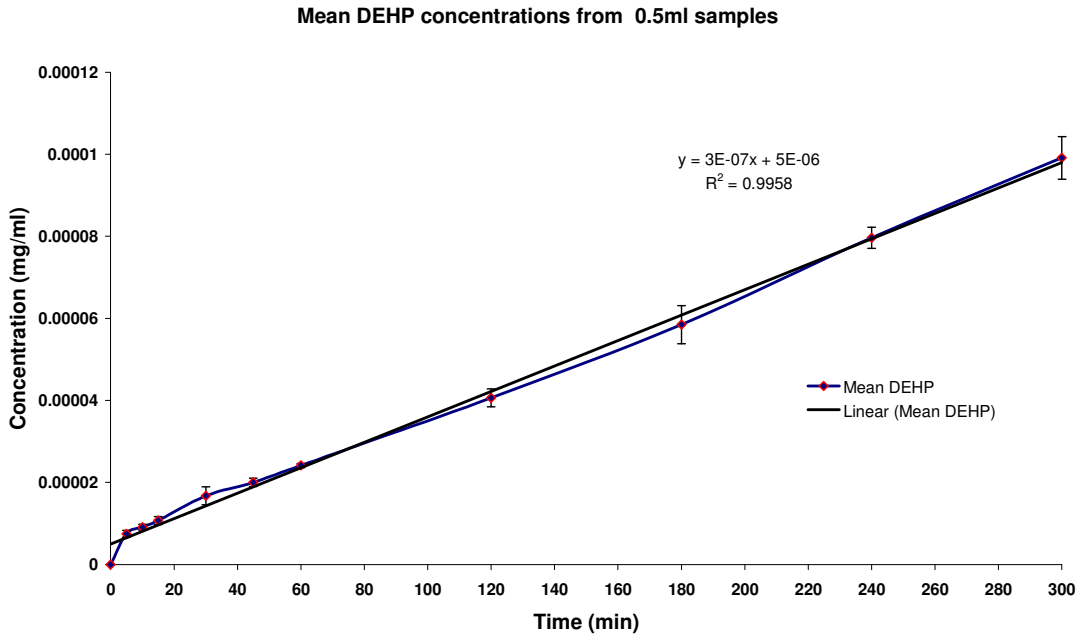


Figure 7.4: Extraction of 14C-labelled DEHP into plasma; number of runs (n=5); error (±SD)

7.1.2. ^{14}C- Labelled DEHP Extraction into Plasma

The concentration levels of the extracted ^{14}C-labelled DEHP show the same migration profile as the total DEHP migration over the entire experimental period. (Figure 7.4) The concentration levels at 5 minutes were found to be 7.47E-6± 1.84E-6 mg/ml. This was followed by a period of slower migration out to 15 minutes where the levels were in the region of 1.07E-5± 2.15E-6 mg/ml. At this point the migration showed signs of becoming diffusion limited. This produced a linear profile out to 300 minutes where the final concentration was found to be 9.91E-5± 1.16E-5 mg/ml. The gradient of the linear portion of the labelled DEHP curve shows a value that is two orders of magnitude smaller than that seen in the total DEHP curve. This indicates that the ratio of labelled to unlabelled material remains constant over each of the experimental runs, which were about 1:104 for labelled to unlabelled DEHP. This result indicates two important findings. Firstly, that the labelled DEHP was distributed at a fairly even rate in all of the test material which shows that the batch method of manufacture was indeed a suitable production method. Secondly, it indicates that the radio-labelling did not affect the migration properties of the DEHP molecule, from both the surface and also from the bulk to the surface of the test material.

7.1.3. Total Whole Blood DEHP Extraction

The results from the series of blood recirculation experiments produced a release profile that was similar in some respects to that obtained with plasma (Figures 7.5 & 7.6). Namely, there were detectable levels of plasticiser migration after 5 minutes of recirculation. However, the level reached after 5 minutes was substantially higher with a concentration of 0.0199±0.0118 mg/ml. The profile for blood increased slowly in a parabolic fashion out to 10 minutes and then a slight plateau occurred out to 15 minutes with the concentration reaching 0.024±0.0134 mg/ml. Even though this pattern was not seen in the plasma experiments, the levels of migration for the blood were an order of magnitude higher at this time period.

After 15 minutes, the DEHP release increases in a linear manner out to 300 minutes, finishing with a concentration of 0.1067±0.0636 mg/ml. As with the plasma experiments the detection of the point at which the diffusion phase occurs is harder

to determine than in the methanol studies (see Figure 7.6). This may be due to the levels of plasticiser that are seen to be extracted being lower than in the organic solvent.

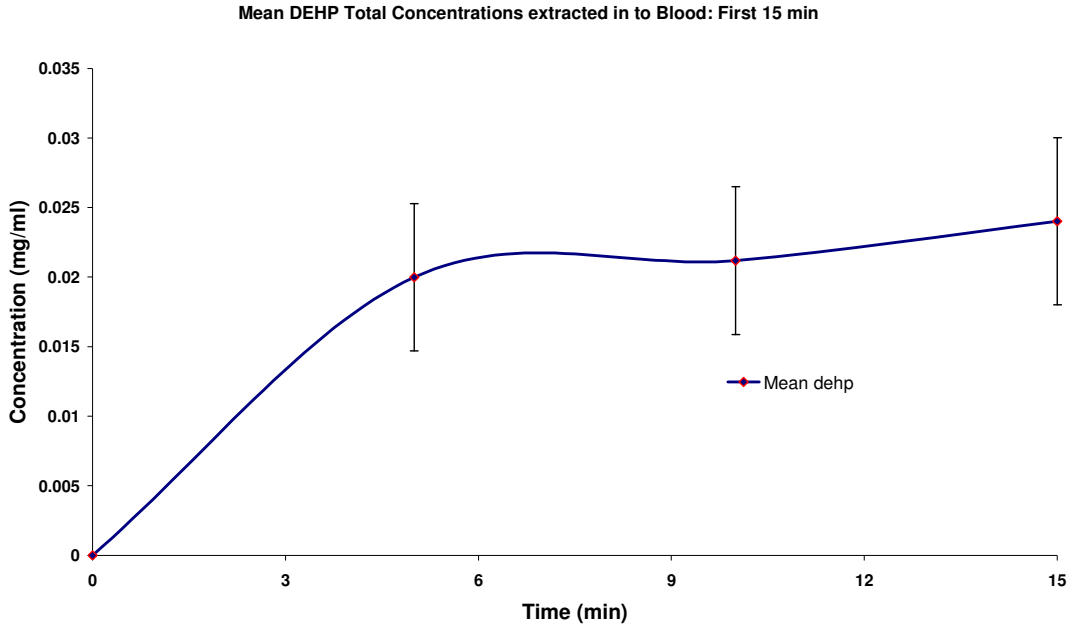


Figure 7.5: DEHP Concentration (mg/ml) in bovine blood after initial 15 minutes of recirculation; number of runs (n=5); error (±SD)

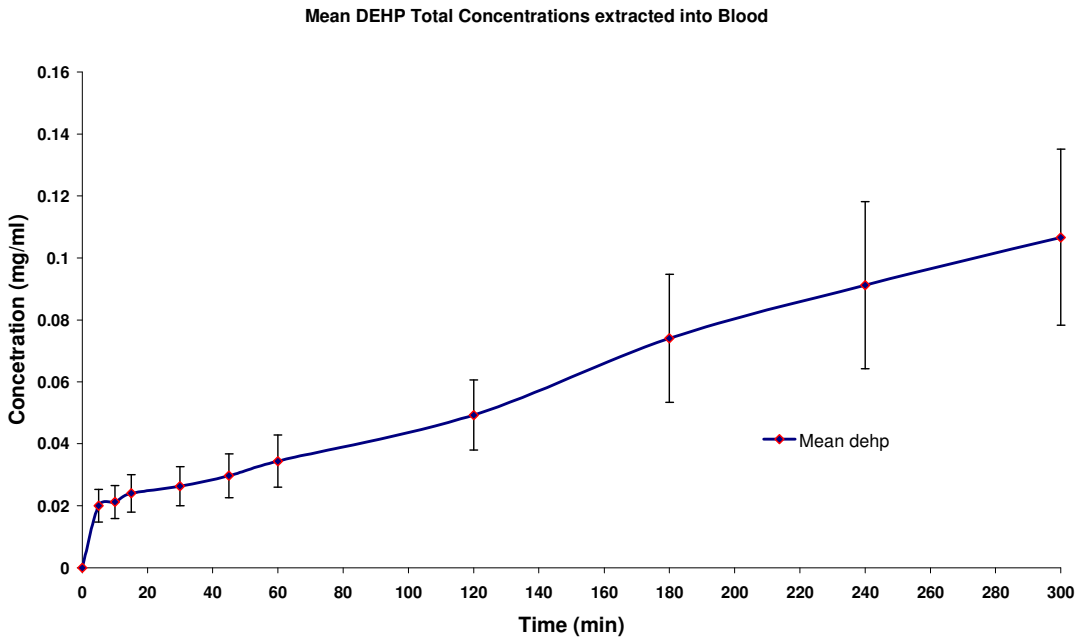


Figure 7.6: DEHP concentration (mg/ml) in bovine blood after 5 hours of recirculation; number of runs (n=5); error (±SD)

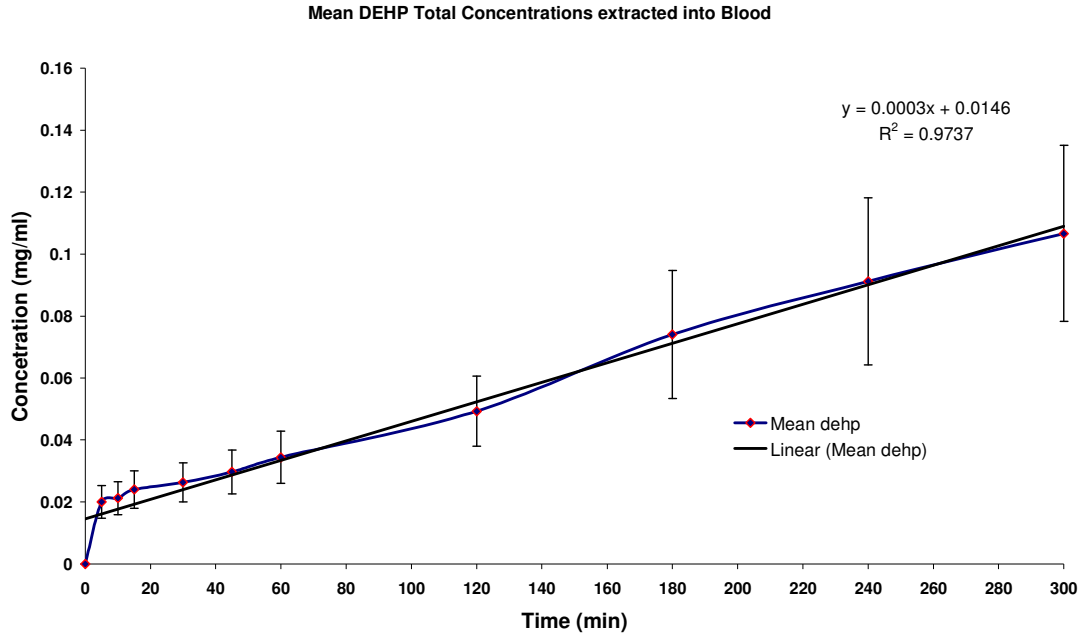


Figure 7.7: Trendline showing the diffusion gradient of DEHP into blood

7.1.4. ^{14}C-Labelled DEHP Extraction into Whole Blood

The extraction of the ^{14}C-labelled DEHP produced the same profile as the total migration of DEHP from the test material into whole blood (Figure 7.8). Again the levels of migration were two orders of magnitude smaller than those reported in the total DEHP migration, due to the non-inclusion of the unlabelled DEHP. At 5 minutes the concentration of ^{14}C DEHP was 2.36E-5±-7.19E-6 mg/ml. This then followed a similar plateau pattern out to 15 minutes were the concentration was 2.92E-5±7.5E-6 mg/ml. After this a linear migration out to 300 minutes was seen to produce a final concentration level of 0.000127± 3.72E-5 mg/ml. The consistency of the profile indicates again that the ratio of labelled to un-labelled DEHP remains constant throughout each of the experiments, further re-enforcing the results discussed previously.

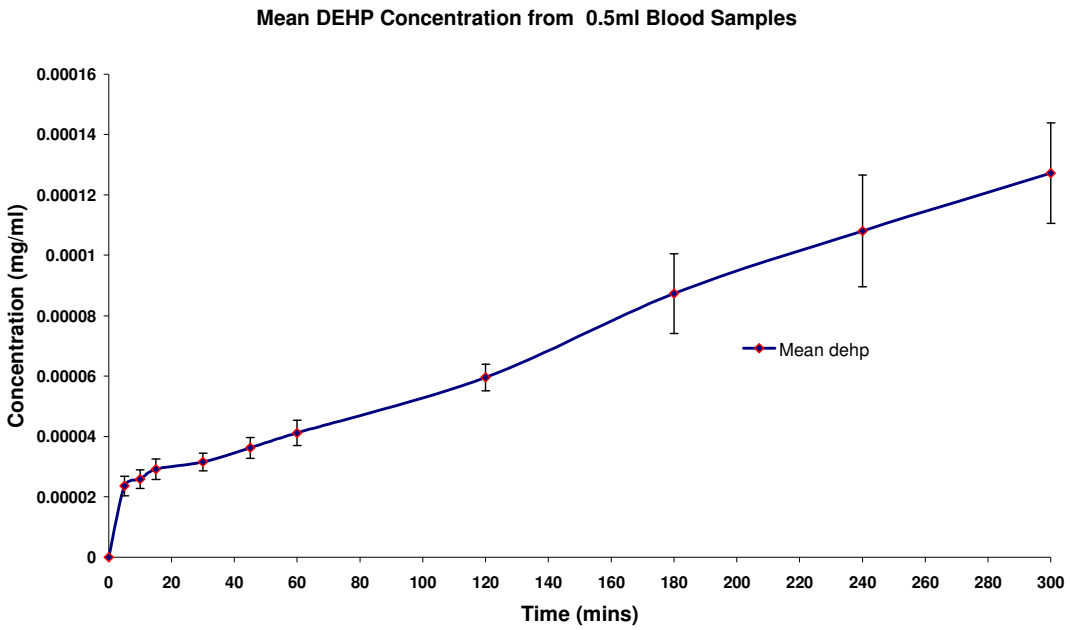


Figure 7.8: Concentration Profile of 14C-labelled DEHP released into whole blood; number of samples (n=5); error (±SD)

7.1.5. Total DEHP Extraction into Methanol

The organic solvent methanol when used in the recirculation experiment evoked a substantial level of DEHP migration from the test material. The levels were significantly higher than those seen with the biological fluids (see Figure 7.9).

Due to the known high extraction rate obtained with methanol, it was decided to wash the surface of the PVC test material with 5 volumes of methanol which equated to 12.5ml. This would then give a "clean" surface from which an accurate determination of the migration of the DEHP from the material could be found. After 5 minutes, there was substantial migration of the plasticiser, even after an initial methanol wash, with a concentration of 0.159±0.0423 mg/ml. The DEHP extraction at 5 minutes in methanol are an order of magnitude larger than the biological fluids . Between 5 and 10 minutes a slight slowing of migration with the concentration reaching 0.231±0.0514 mg/ml. This produces a small curve in the migration plot which can be seen in Figure 7.10. However this does not last long with the migration of the plasticiser following a fairly steep linear migration out to 60 minutes where the concentration was 0.741±0.154 mg/ml. From 60-300 minutes the plasticiser release

followed a linear pattern which had a slightly lower gradient than the period between 15 and 60 minutes (Figure 7.9). The final concentration was substantial at 2.21±2.16E-2 mg/ml.

The profile seen here is similar to the results presented by French (2001) in his MSc thesis, in which he produced a linear regression curve with an equation of $Y=0.0058X+0.2595$ mg ml^{-1} min^{-1}. The current study produced a correlation similar to that of French with $Y=0.007X+0.1909$ mg ml^{-1} min^{-1}.

Both these studies also have an overall similarity in the type of curve produced by Zhao in his PhD thesis in 1999. Zhao showed that the process of DEHP migration and extraction from PPVC is a two part process involving an initial dissolution period demonstrated by a parabolic curve. This was then followed by a diffusion section which was represented by a linear regression of the curve. Whilst both our and French's study follow a similar pattern to Zhao's work, the overall correlation of the diffusion section of Zhao's study was $Y=0.002237X+0.2564$ mg ml^{-1} min^{-1}, which was substantially lower than both of the other studies.

The initial 10 minutes of the graph suggest that the methanol washes performed did not entirely clean the surface of the test material indicated by the slight parabolic increase over this time period which indicates removal of the remaining surface based plasticiser from the PPVC. The short period taken to produce a linear migration pattern, indicates that the initial washes did strip off a substantial proportion of the surface plasticiser. This was also backed up by the substantial levels of ^{14}C DEHP activity present in the methanol washes as reported in Table 7.1.

7.1.6. Methanol Wash DEHP levels

The methanol wash was performed to produce a "clean surface" of test material before running the experiment. This was to ensure that there was uniformity of the different samples as methanol is known to extract substantial levels of DEHP plasticiser. Each of the test runs produced marked levels of migration into the wash fluid indicating that the PPVC test material had been stripped of the DEHP plasticiser that had accumulated on the surface.

Table 7.1 highlights that the distribution of ^{14}C DEHP in the PPVC test material varied across each of the eight manufactured sheets to some extent. This was to be expected due to the method of fabrication of the PPVC sheets which would not allow for an exact replication between test materials. However, the "washing" process undertaken had brought each of the test runs into line as the 5 minute samples taken are all of similar activity levels.

Wash number	Wash DPM	Wash Activity (MBq)
Experimental Run 1	**31945.8**	**0.000532**
Experimental Run 2	**38310.5**	**0.000639**
Experimental Run 3	**61428.0**	**0.001024**
Experimental Run 4	**75964.3**	**0.001266**
Experimental Run 5	**41052.7**	**0.000684**

Table 7.1: DPM and Activity levels of methanol washes taken from each of the 5 experimental runs.

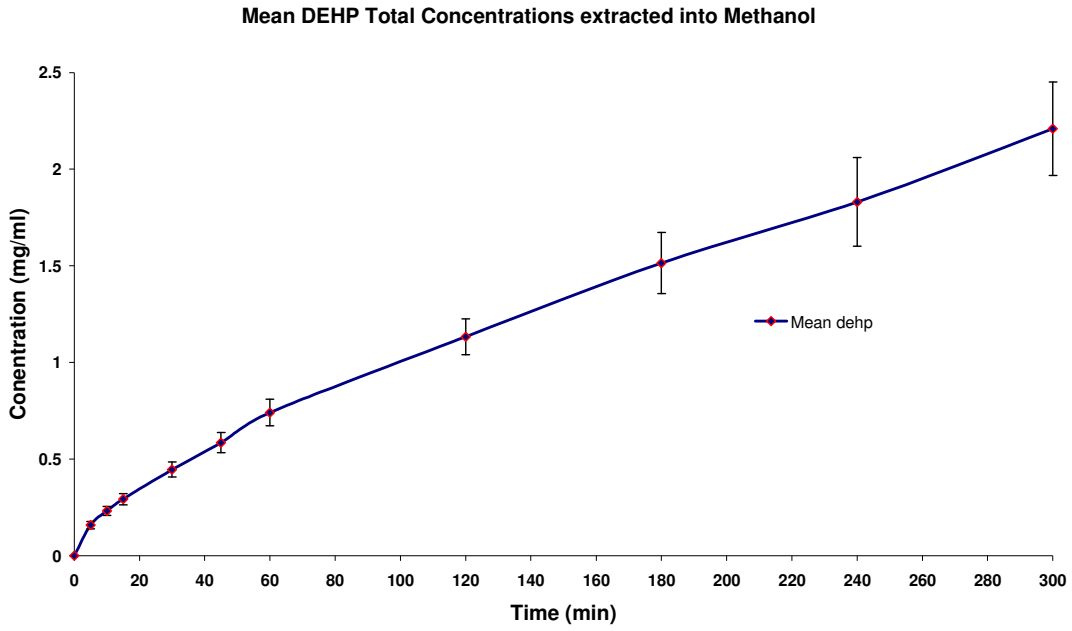


Figure 7.9: DEHP Concentration Levels (mg/ml) in Methanol after 5 hours of Recirculation; number of runs (n=5); error (±SD)

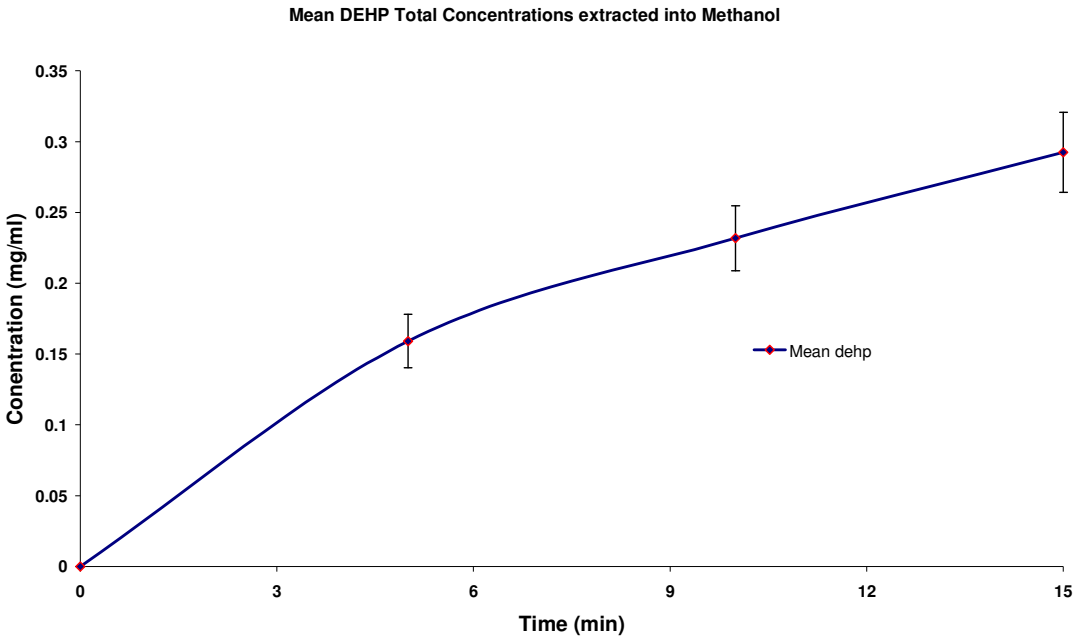


Figure 7.10: DEHP Concentrations (mg/ml) in Methanol after initial 15 minutes of Recirculation; number of runs (n=5); error (±SD)

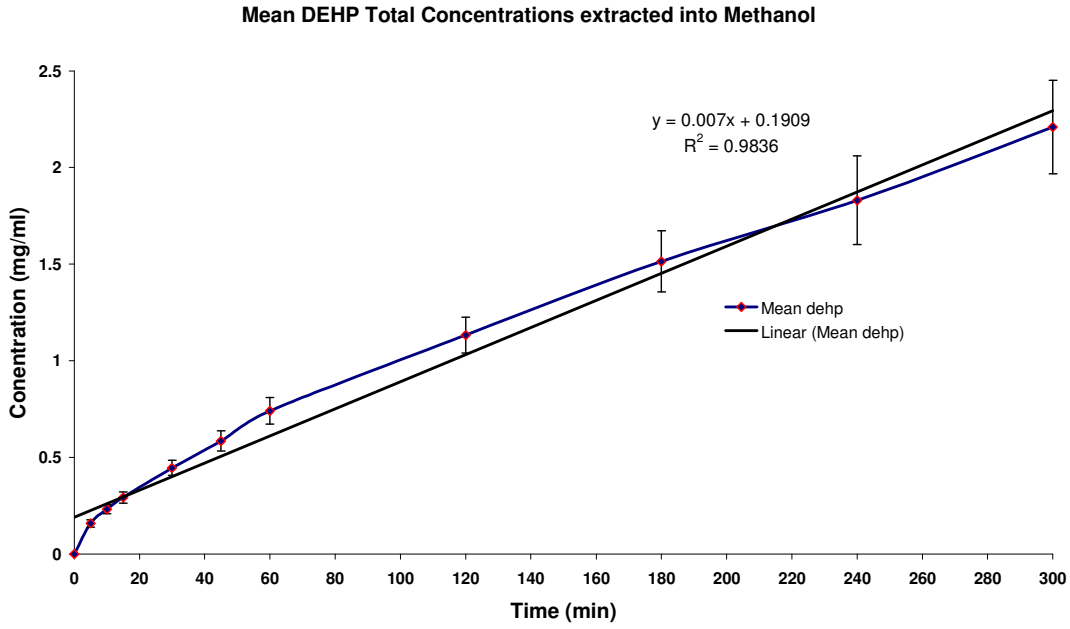


Figure 7.11: Trendline showing the diffusion gradient of DEHP into methanol

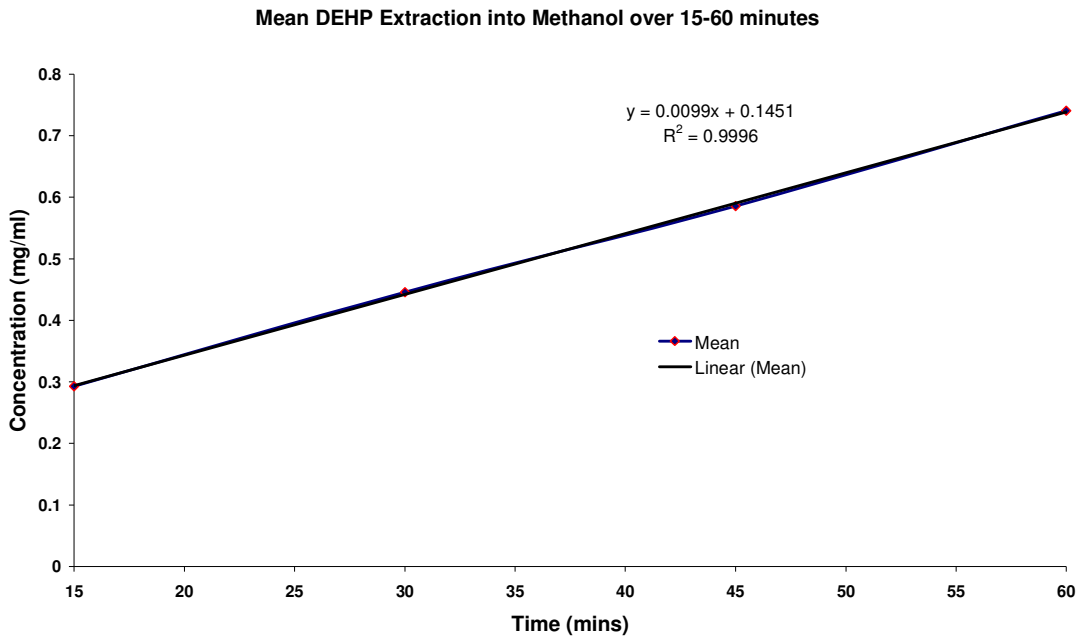


Figure 7.12: Trendline showing the linearity of migration of DEHP from 15-60 minutes of exposure

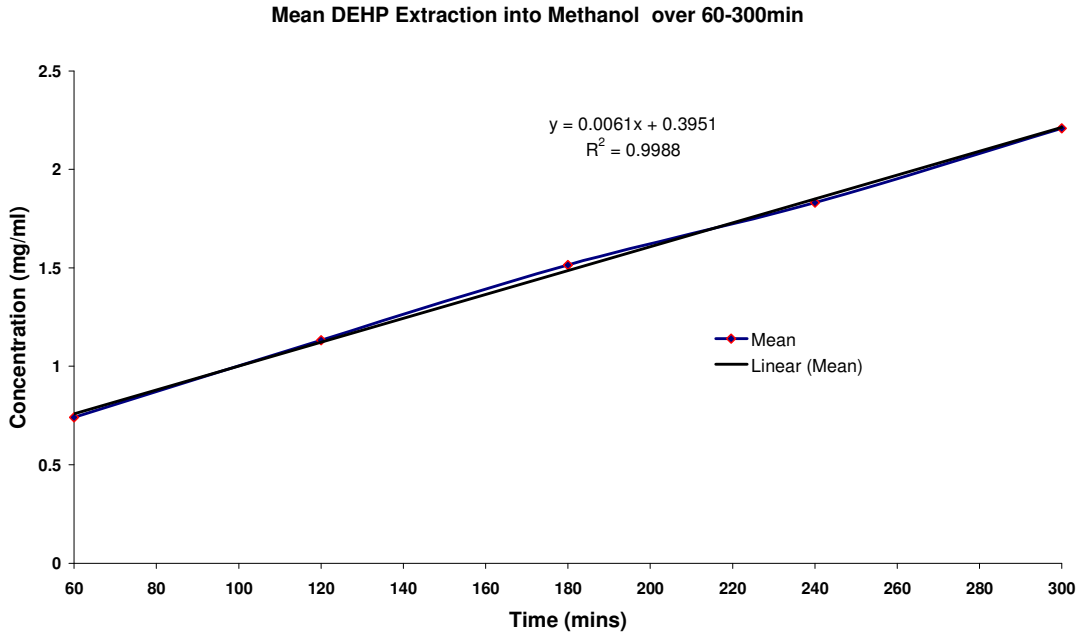


Figure 7.13: Trendline showing the linearity of migration of DEHP from 60-300 minutes of exposure

7.1.7. ^{14}C-Labelled DEHP Extraction into Methanol

Figure 7.14 shows the labelled DEHP concentrations found in the 0.5ml methanol samples over the experimental period. The profile was similar to that observed for the total DEHP release into the methanol as expected. The concentration at 5 minutes was 0.0011± 0.000295 mg/ml. The release then followed a linear profile which led to a concentration of 0.00205± 0.000441 mg/ml at 15 minutes. The linear profile continued out to 300 minutes, but at a slightly shallower gradient, where the final concentration value was 0.0155± 0.003768 mg/ml. Figure 7.14, highlights the linearity of the migration over the entire time course of the experiment, showing the continued pattern between the different fluid types. Whilst the linearity of this curve fit is good (R^2=0.9837) it is not the best fit. This is found from 60-300minutes (Figure 6.15) where the R^2=0.9988, which would suggest that the true diffusion occurs from 60minutes for our particular test material configuration.

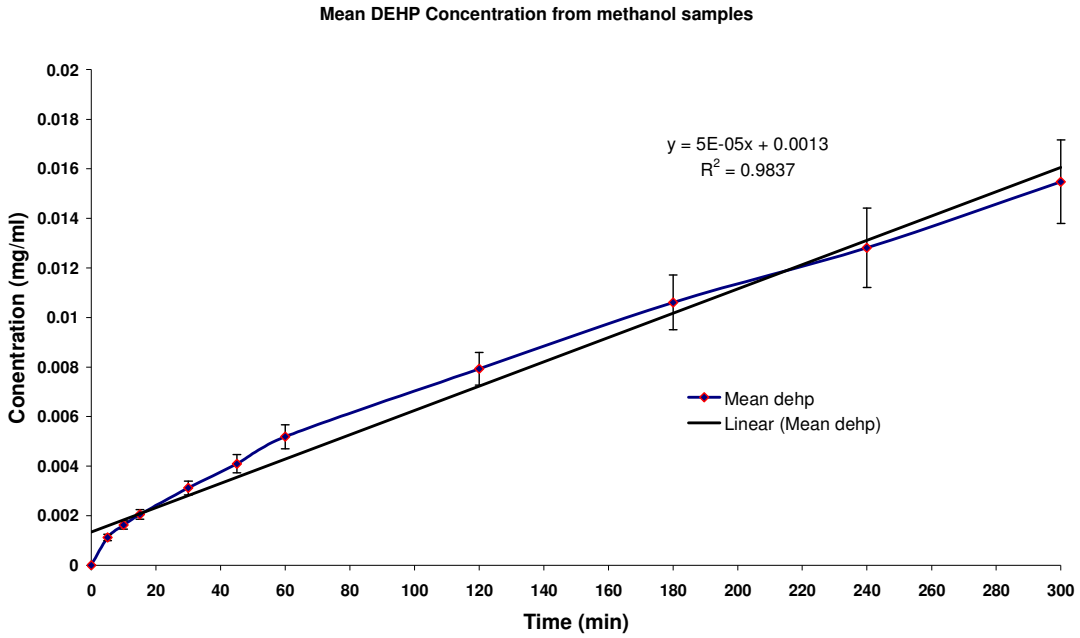


Figure 7.14: Concentration Levels of 14C labelled DEHP migrated into Methanol; number of runs (n=5); error (±SD)

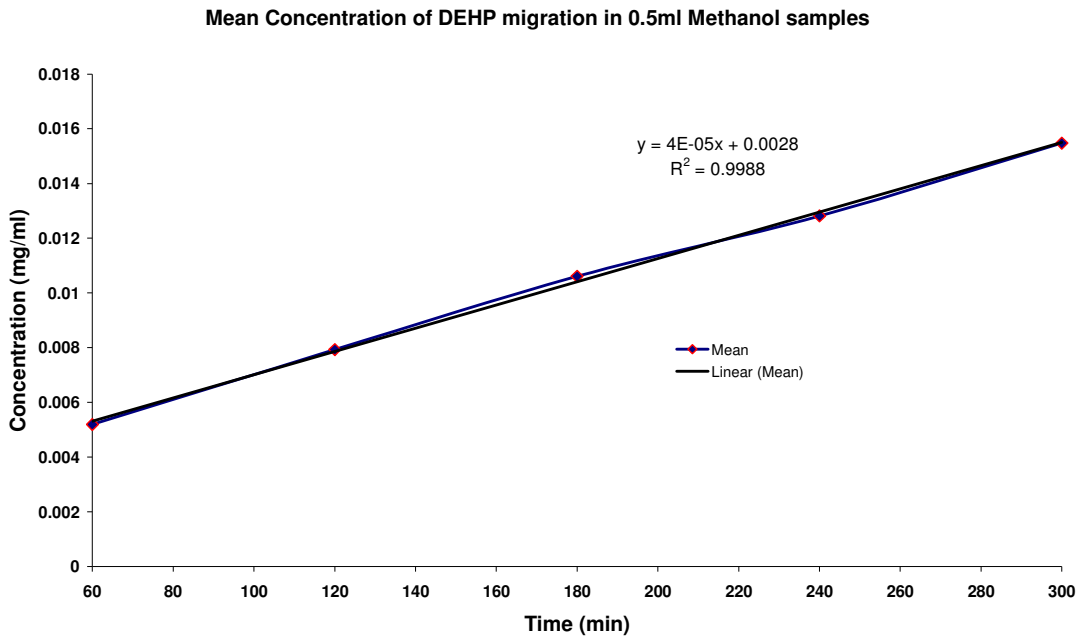


Figure 7.15: Trendline showing the linearity of migration of DEHP from 60-300 minutes of exposure in 0.5ml samples

7.2. Statistical Analysis of In vitro Experiments

The graphical data from the *In vitro* experimental runs, showed a marked difference in the amount of migration into each fluid. Statistical analysis was performed to determine if the differences in levels of migration between the tests fluids were significant or not ($p \leq 0.05$). The statistical analysis of a t-Test was performed in Microsoft Excel, using the data analysis add-in package to generate the p-values.

Figure 6.16 shows the mean values at selected intervals throughout the experiments with methanol and blood and clearly demonstrates the significant differences between the DEHP migration into these fluids. Table 7.2, shows the P-value relationship for methanol vs. blood and confirms the statistically significant differences in DEHP migration.

Figure 7.17, shows a plot of the concentrations for blood and plasma at selected time intervals. The P-values reported in Table 7.3 show that there is a statistically significant difference in the levels of migrated plasticiser between these biological fluids. This insinuates that the cellular components such as the RBCs that are present

in the whole blood, but are not found in the plasma, play some role in the extraction of the DEHP plasticiser from the test fluid.

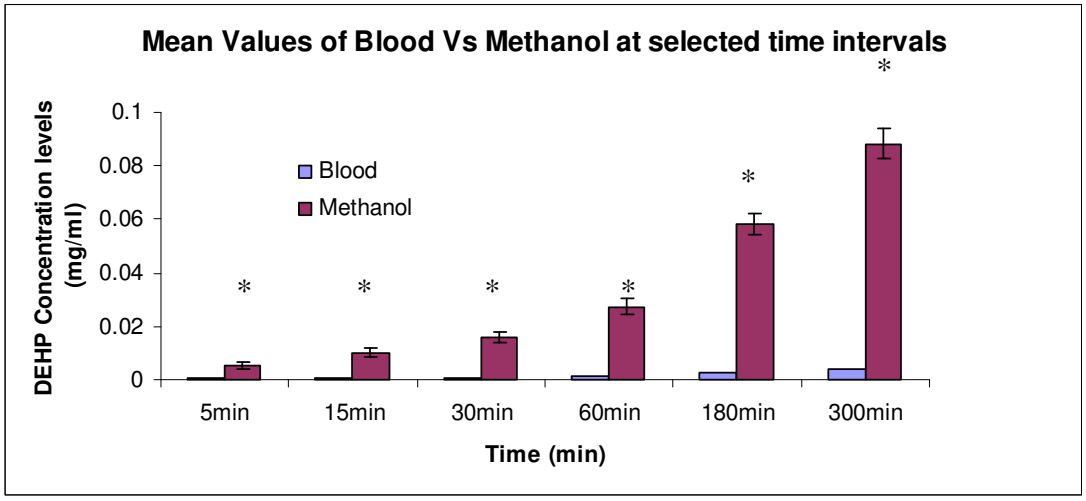


Figure 7.16: Graph of mean concentration values showing the statistical differences using a t-test (*) between methanol and blood (P≤0.05); n=5; error ±SD.

Methanol vs. Blood	P-Value
5 min	0.001461
15 min	0.000355
30 min	0.000181
60 min	0.000239
180 min	0.000358
300 min	0.000446

Table 7.2: P-values at selected intervals for methanol vs. plasma from the t-test.

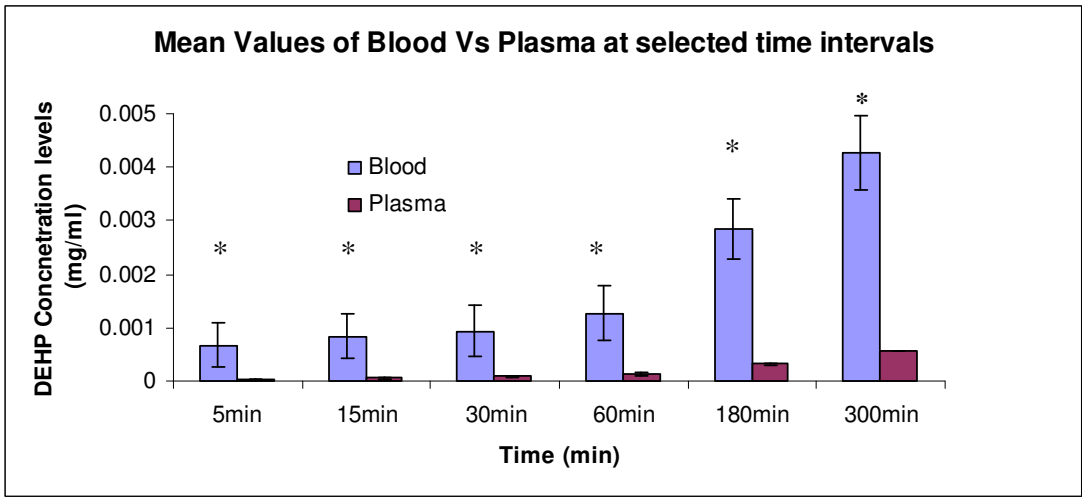


Figure 7.17: Plot showing the statistical differences (*) between blood and plasma (P≤0.05) from the t-test; n=5; error ±SD.

Blood vs. Plasma	P-Value
5 min	0.011822
15 min	0.010686
30 min	0.004419
60 min	0.010915
180 min	0.018338
300 min	0.016839

Table 7.3: P- values at selected intervals for blood vs. plasma from the t-test.

7.3. Discussion: In vitro migration studies

7.3.1. Introduction

The study into the extraction and migration of plasticisers from PPVC has been extensively investigated. This is partly due to the high volume usage of PPVC in many everyday products found in modern society. DEHP is now a ubiquitous environmental pollutant and hence the general population is exposed to it on a daily basis (Koch et al, 2005). However, the vast majority of migration tests have involved static (Yu, 1993; Bowry, 1983; Gotardo and Monteiro, 2005; Hildenbrand et al. 2005; Loff et al. 2004) or immersion (Lakshmi and Jayakrishnan, 1998; Bartlett, 1987; Zhao and Courtney, 2003) processes to remove the plasticiser from the PPVC. This work is relevant for many applications, but in the case of extracorporeal procedures such as CPB, ECMO and more importantly Dialysis, the means of exposure do not accurately represent the flow conditions in these procedures and the migration pattern of the DEHP may not be accurately profiled. It was hoped that the recirculation experiments performed by the author would help to fill in the current knowledge void as to whether the flow conditions have an effect on the plasticiser migration.

7.3.2. Discussion of Methanol Experiments

The results from the methanol studies corroborated the work by Kim et al (1973); Zhao, (1999) and French (2001) showing that there is a two part process to DEHP migration. The initial process is of dissolution, removing the original DEHP that has migrated to the surface of the PPVC. This level of DEHP can be fairly substantial. Courtney and Zhao (2003) reported that the surface of the PPVC can contain as much as 69% DEHP plasticiser. The levels in each of the methanol washes reported in our results give some indication that this is the case. The second part of the migration process is that of diffusion of the DEHP molecules from the bulk of the PPVC to the surface, where they are then removed by the circulating fluid. From the results generated in this set of experiments, it is quite clear that the migration in this process is diffusion limited. This is confirmed by the straight line trendline for this

portion of the graph (Figure 7.11) which has an R^2 of 0.9836. During the 60-300 minute section of the experiment, the R^2 value becomes near perfect with a value of 0.9988 (Figure 7.12). This diffusion-limited phase will continue as long as the DEHP reservoir in the PPVC material is maintained. It can be extrapolated that over time the gradient of the curve will become shallower and eventually plateau as the concentration of the extraction fluid becomes higher as more of the DEHP reservoir is depleted.

The linear migration pattern can be seen in each of the test fluids, with R^2 values of 0.9958 and 0.9737 for plasma and blood respectively.

The findings in this methanol study corroborate much of the previous work that was performed in this area by Kim et al (1973), Zhao (1999), French (2001) and Sharma (2002). This corroboration indicates that the ^{14}C radiolabel had no effect on the migration properties of the DEHP. It therefore concluded that this technique could be successfully used in determining DEHP migration in other recirculation experiments.

The studies by Zhao (1999), French (2001) and Sharma(2002) investigated the migration of DEHP plasticiser using a UV spectrometry method. However, their main aims were not to determine concentration levels of DEHP but to investigate the time profile of the migration process. Therefore, they were content to measure the absorbance of the DEHP in the test fluids but did not take it further. However, French (2001) and Sharma (2002) did present the means to determine DEHP concentrations and hence allow comparison with the findings of this study.

French (2001) and Sharma (2002) used the previously mentioned Bowry test cell. This was an incubation set up which produced conditions totally different from those pertaining in this study. French and Sharma both produced similar results with regard to the amount of DEHP that migrated in their studies. The concentration levels of migrated DEHP plasticiser derived from French's and Sharma's studies are given in Table 7.4 along with the results from this study.

Sample Time (min)	French (2001) Concentration (mg/ml)	Sharma (2002) Concentration (mg/ml)	This study Concentration (mg/ml)
5	0.0576	0.0301	0.1596
10	0.0845	0.0531	0.2322
15	0.0887	0.0731	0.2931
30	0.1099	0.1100	0.4462
45	0.1355	0.1213	0.5859
60	0.1502	0.1599	0.7419
120	0.2039	0.2607	1.1344

Table 7.4: Concentration levels of migrated DEHP into methanol

The DEHP levels obtained in the current study are substantially greater at each of the sample times. They are between 2 and 4 times greater than those produced by French and Sharma. There are a number of reasons why this would be the case. The differing test conditions would certainly be a major factor in explaining the differences seen between the three studies. In the Bowry incubation test cell the extracting fluid is static. The current study employed a non static flow test which subjected the PPVC test cell to re-circulated methanol at a steady rate. This may have had an effect on the levels of DEHP plasticiser that was extracted from the PPVC. However, whilst the flow across the test material may account for a small proportion of the difference it does not provide the full answer.

A more plausible explanation would be the difference in the exposed surface area between the two test methods. Our system employed a parallel plate channel with a PPVC contact surface area of 44 cm^2 compared to the Bowry test cell with $2cm^2$ of PPVC forming the base of each well of a 24-well plate. This substantial increase in exposed PPVC test material between the two experimental protocols provides a suitable explanation for the large differences in the concentrations of DEHP between the two different protocols. The volume to exposed surface area of PPVC, would explain a large portion of the difference between Sharma and Frenchs studies and our current study. Both French and Sharma had a surface to volume ratio of 1.25 ml/cm^2 compared to our study which had a ratio of 0.56 ml/cm^2 and when this ratio is taken

into account the respected concentration levels become a much closer match over the initial sample times.

Further weight is added to this hypothesis when the current test results are compared to the findings of Gourlay et al (2003). This group also performed methanol washing studies on PPVC. However, they used 0.5g of ground PPVC instead of sheets. The findings of that study produced values that were of the same order of magnitude with concentration levels of 0.11 mg/ml at 5 minutes compared to 0.15 mg/ml found in our study. The levels continue to be of a similar magnitude out to around 30 minutes, where the current findings approached 0.44 mg/ml compared to 0.22 mg/ml in the Gourlay study. However, the study by Gourlay et al, did not continue out for the time period so comparisons at the higher time periods cannot be made. Again the levels of PPVC and those of the DEHP used in both experiments are different, with our study having substantially greater amounts of PPVC present and hence there is the possibility of higher levels of DEHP being extracted into the methanol during the experiment.

Gourlay in 2001, utilising the test cell that was subsequently employed by the present author, performed other methanol washing experiments to study the effect on CD11b expression. He showed that there is a correlation between surface area exposure and the levels of CD11b expression. Although a direct comparison cannot be made this finding would suggest that the greater the surface area the higher the release rate of DEHP into methanol and hence higher concentrations of DEHP in the fluid.

A secondary consideration is that concerning the composition of the PPVC test material. The test material used in the current study was based on a standard Medical Grade PPVC produced by INEOS Chlorovinyls. It had a DEHP content of 50% of the polymer weight. Both Zhao and French do not enlighten the reader regarding the composition of the PVC sheet. Sharma reports at level of DEHP content of 48% of the polymer weight which would suggest to the author that the higher composition made little difference to the increased levels seen in the current study. It then adds further backing to the increased surface area being the main reason for the substantially higher levels of concentration seen in this work.

A further aim of the *In vitro* studies was to determine if there is any difference in the recorded levels of DEHP migration using the controlled flow test cell and the previously reported static immersion tests. The above discussion showed clearly that there is a substantial difference in DEHP migration between the two test methods but the main reason for this would seem to be the difference in the exposure areas and volumes of fluid used. However, the later samples tended to produce higher concentration levels that the surface area and volume ratio did not fully correct. Therefore, there must also be a further condition acting on the system to sustain the higher concentration levels seen in our study.

A plausible reason to explain this difference is the dynamic effect of the re-circulating methanol through the test cell. The forced flow of the fluid over the test material would appear to remove more of the DEHP from the surface compared to the static incubation test performed by French and Sharma. It could be suggested that the recirculation of the methanol removes much of the DEHP from the material/fluid interface and hence promotes greater migration of DEHP into the fluid passing over the test material, due to a lack of DEHP build up at this interface.

It should be stated that during the clinical study reported in chapter 4 of this thesis, recirculation and static migration were investigated as part of the study. This produced results that showed non statistically significant differences in concentration levels of plasticiser migration between the re-circulated and static fluids. However, this study did not re-circulate the fluid in the same manner as the current *in vitro* experiments, as the DEHP build up at the materials fluid interface would not be reduced over the experimental time course. This would tend to support the hypothesis that the recirculation does have a minor effect on the migration levels at later time intervals but is superseded by the surface area of the PPVC exposed to the material.

7.3.3. Discussion of Blood and Plasma Experiments

The findings from the methanol experiments were encouraging, and corresponded to a certain degree to previous studies in the same area, especially in regard to the migration profile. The migration profiles of both the biological fluids showed similar trends as that obtained with the methanol experiments and hence it was hoped the

concentration levels found in these fluids would be similar to previous studies in the same area.

The *in vitro* study by Jaeger and Rubin published in 1973, evaluated the migration of DEHP into blood from two common medical devices, a haemodialysis machine and a CPB unit. They reported concentration levels of 0.0924 and 0.0570 mg/ml after 8hr and 5hr in each of the respective devices. The concentration levels from this current study are slightly higher than those presented by Jaeger, with a 5 hour concentration level of 0.107 mg/ml. However, Jaeger's study involved full component systems which would have contained not just DEHP plasticised PPVC materials and hence would explain the slightly lower levels of DEHP found. They also used outdated blood that had been stored in PPVC blood bags which as the findings showed were already contaminated with DEHP. This could have led to a lower migration rate into the blood from the systems due to a saturation effect in the blood. This would also explain why the levels in Jaeger's study were slightly lower than the ones in this study. Further to this, the surface area to blood volume would also explain the lower concentration levels seen in this study. Jaeger used full clinical circuitry, which would lead to a substantial increase in both volume of blood and also surface area of PPVC and hence a reduced total concentration level in the test fluid.

Hildenbrand et al., 2005 investigated the migration DEHP in various PPVC tubing types. They reported a mean DEHP concentration of 0.0426 mg/ml in the blood sample after 90 minutes of exposure to a normal uncoated DEHP plasticised PPVC pipe. This finding compares very favourably with the results from our study, namely a concentration of 0.0344 mg/ml after 60 minutes and 0.0493mg/ml after 120 minutes. This indicates a similar level of migration rate of DEHP into blood between the two studies even though the two methodologies are different in nature.

A further study by Inoue et al, in 2005, measured the levels of DEHP migrated from different types of PPVC blood bags into various blood products. The concentration levels reported in whole blood were in the range of 0.015-0.083mg/ml depending on the type of blood bag used. It can be seen again that the levels are slightly lower as the findings of the current study. A more detailed comparison is difficult, as the composition of the blood bags employed by Inoue was not given and hence may not be purely DEHP PPVC. Incorporation of another plasticiser or a coating if present

would reduce the total level of DEHP that could migrate. As mentioned previously, the exposed surface area to volume ratio would also be plausible explanation to the lower concentration levels that reported by Inoue. Further to this, the method of detection used by Inoue, may be less sensitive than the one employed in the current study and hence the entire amount of migrated DEHP may not have been accounted for. This would again suggest that the reported levels in this study are robust in nature.

The results of the migration experiments into the whole blood have been shown to be fairly robust when compared to other studies in the same field. However, to fully evaluate the robustness of the methodology used in this work, it was important to also test the migration into another commonly used test fluid, plasma.

A study by Loff et al. in 2004 measured the migration of DEHP from different tubing lengths into a 20% lipid emulsion fluid. More importantly here they perfused the fluid through the tube at a slow flow rate. This was an important aspect as it allowed for direct comparison to our flow test methodology. Again the sample times were different with these samples being collected at 24hr compared to our 5hr sample time. The reported values varied depending on the type and length of tubing as would be expected. The table (Table 7.5) below shows the range of concentration levels that were seen in a selection of tubing types in the study by Loff et al, 2004.

Tube	Length (cm)	DEHP Concentration (mg/ml)
OPVC	**150**	**0.074±0.0036**
BPVC	**150**	**0.11±0.0071**
CPV	**75**	**0.039±0.0031**
OPVC	**10**	**0.0134±0.0012**

Table 7.5: DEHP concentration levels from different tube lengths that were extracted into 20% lipid solution, Mean ±SD (adapted from Loff et al. 2004). Data reproduced with permission of Lippencott Williams & Wilkins Inc.

Due to the time difference in the sample collection the comparison with our results would be best with the final 5hr figure. It is also pertinent to mention the difference

between the areas of PPVC used in the two studies. Our surface area was 44cm^2,which is similar to other studies with 20% lipid solution in the 10 and 75cm in the Loff study as these two tubing sections represent internal surface areas that are the closest to the surface area used in our study. We reported concentration levels of 0.01416 mg/ml of DEHP after 5hr of exposure to the plasma. This is comparable to the levels seen in the 10 and 75cm of tubing which showed a migration level of 0.0134 and 0.039 mg/ml respectively. Despite the differences in exposed area and sample time, the reported levels are of the same order of magnitude, which indicates a level of comparability between the two studies. This trend continues even at the vastly different tube length of 150. However, it must be said that the make up of this 20% lipid solution is not the same as the plasma used in our study which may have had an influence on the migration levels. This said they are both lipid solutions which would suggest that the lipid content would have some sort of influence on the migration of the plasticiser. But overall the correlations between the two studies are encouraging showing that the methodology used in the current work produced results that are similar to other studies.

The study by Inoue at al in 2005 also measured the migration of DEHP from PPVC into plasma. They reported a DEHP concentration level range of 0.0116-0.0185 mg/ml in the plasma samples. These values again correspond favourably with the results of this study (0.01416 mg/ml at 300min). This again shows that the results in this study correspond well to previously published work and highlights the validity of 14-C radiolabel compared with other analytical techniques such as High Performance Liquid Chromatography (HPLC) as used by Inoue and gas chromatography as used by Loff.

In an unpublished study for Hydro Polymers, Bartlett (1987), reported DEHP concentrations in human plasma of 0.047 mg/ml for after 48hr and 0.0622 mg/ml after 96hr of exposure. Despite the samples being taken at much longer time periods than ours, it does compare favourably as both results are of the same order of magnitude. It is of interest to note that Barlett (1987) used a ^{14}C radiolabel as the marker method and also employed a similar surface area of PPVC in their experiments. Interestingly, they could not produce any short term results due to the

low specific activity of the radiolabel involved. When the results of the current study are extrapolated out to 48hr a value of 0.1159 mg/ml is produced. This is substantially higher than Bartlett's results, but this could be attributed to the surface area to fluid volume ratio which would explain the lower levels seen in Bartletts study, which used a much smaller (2ml) of plasma This may be due to the low levels of activity being used in the marker which would suggest that not all of the migrated DEHP would be detected using their method. This would account for some of the difference between both the results. Another suggestion as to the difference between the values would be the lack of circulation of fresh plasma over the PPVC test material, the concentration gradient between the plasma and the material would be allowed to build up due to the static nature of Bartlett's experiments and hence could result in lower levels of migration in the allotted time period. This would also suggest that implementing a controlled flow test set up would provide more reliable results than just static incubation experiments.

Sjoberg et al, in 1985, investigated the concentration levels of DEHP in plasma intended for exchange transfusion in newborns. The time for a transfusion can range from 1-4hr and this provides a direct comparable time period with our results. Sjoberg reported DEHP concentrations in the range of 0.0034-0.011 mg/ml. This compares favourably with our results with concentration levels at 0.0034 after 60 minutes of exposure as well as 0.0114 mg/ml after 4 hr. This again adds substantial weight to the validity of the results for the migration levels of the DEHP into plasma.

In response to the findings of the clinical study from Chapter 4. The results from the biological fluid tests showed very simply that the more complex the fluid make up the higher the migration of DEHP. The concentration levels of DEHP found in plasma serum were significantly less than those seen in the whole blood studies. This suggests that the inclusion of various blood components and other molecules has a significant impact on the extraction of DEHP from the PPVC test material. This is in league with the findings of the original clinical study that was performed. However, the identification of whether it is an individual protein or group of certain proteins that has influenced the extra extraction of plasticiser is something that could not be

identified in these experiments due to the radioactivity that was present in the samples. Previous work by Rock et al, 1984 suggested that one of the main factors is the presence of red blood cells (RBC) which make up 40% of the cellular components of whole blood. DEHP is known to bind to the RBC membrane and this could possibly explain the significantly increased migration into whole blood compared to plasma. However, Jaeger and Rubin showed that the low density lipoprotein fraction contained the vast majority of the DEHP present in the whole blood sample. They also showed that the platelets tended to attract a higher % of the DEHP, than the plasma but were superseded due the volume of plasma. Stern et al, 1977, also showed that DEHP extraction was not affected by the lipid content of the fluid but was limited by the protein content. This would suggest that the difference in the concentration levels seen in the plasma and the blood would probably be due to the blood proteins and cellular matrix in the whole blood.

The idea that flow per se of the fluids may have caused increased migration is not a viable answer to the increased levels seen in the blood, mainly due to all of the experimental runs being performed at the same flow rate. Bearing this in mind, it adds further evidence that it is the presence of the blood proteins especially the RBCs and platelets have a major influence on the DEHPs migration into biological fluids.
Previous studies have indicated that the serum lipid levels in biological fluids have an effect on the DEHP migration (Jaeger & Rubin, 1973; Kambia et al, 2001; Pollack et al, 1985). However, other studies have indicated that this has no correlation on the migration of the DEHP into the particular test fluids (Stern et al, 1977; Friocout et.al, 1979). It has also been shown that whole blood tends to evoke higher migration of DEHP than other blood products (Inoue et al, 2005). The results in our *In vitro* studies between the blood and plasma would suggest that it is a combination of the cellular matrix and the lipid levels present in the biological fluids that determines the levels of migration of DEHP into the test fluid.

7.3.4. Overall Test Methodology Discussion

The biological fluid recirculation runs were not subjected to a pre-run wash. This was due to there being no need for a pre-prepared surface on the PPVC. The

manufactured PPVC was left in-situ for a number of months before the experiments were performed so the surface of the test material would have been saturated with DEHP, thus producing an already prepared surface. The second reason was to ensure that there was the maximum possible level of DEHP present on the surface of the PPVC. This would hopefully allow for the maximum levels of ^{14}C DEHP to be present, which was a desirable situation due to the extremely low levels of ^{14}C DEHP that were present in the PPVC sheets. Thus avoiding a similar situation that Bartlett found when they were unable to detect the ^{14}C labelled DEHP in short term studies due the low levels of radioactivity present in the PPVC. It was important to ensure that both the plasma and blood runs were treated the same to allow for accurate comparison of the migration into each of the fluids. The lack of a "pre-wash" for these tests was seen to enhance the level of the experimentation by mimicking the clinical setting, by having levels of surface plasticiser exposed to the passing fluid, which would not be the case if they were pre-washed.

It was quite clear that the migration profile that had been so successfully modelled in methanol by Kim et al, 1973 and then corroborated by Zhao (1999) and French (2001) also occurred in biological fluids as well. However, the levels of migration are substantially less than those seen in the methanol. This suggests that the migration profile of DEHP from PPVC would be the same no matter the type of fluid involved, be it a solvent or a biological fluid. It must also be said that the ability to determine the exact point of the beginning of the diffusion phase is also much more difficult to achieve in the biological fluids than in the methanol, this may be due to the lack of labelled plasticiser that is used in the experimentation or due to the lower levels of plasticiser removed by biological fluids. Another plausible reason would be that the definition of the results would be improved with a greater number of samples being taken as was the case with Zhao (1999).

The migration of DEHP into all of the test fluids has shown how the study compares with other previously published work. This would suggest that the methodology implemented in this section of the study was robust in nature and the results produced can be seen to be reliable.

7.4. Ex Vivo Results

7.4.1. Perfusion Experiments

7.4.1.1. Blood Samples

The main aim of the perfusion experiments was to determine the fate of the leached plasticiser after the *in vitro* experiments had clearly showed that DEHP migration from the test material occurs in significant levels into Blood. An important aspect of this was to discover if the plasticiser remained in the blood system re-circulating through the animal, or whether it was retained in the animal's tissues.

To address this issue blood samples were taken at specific time intervals from the venous outlet and arterial inlet of the test cell to measure any changes in DEHP levels across the vasculature. The results are shown in Tables 7.6 and 7.7.

Time (min)	Arterial (DPM)	Venous(DPM)	Net DPM Increase (V-A)
0	**9.79±1.75**	**6.4±1.55**	**~0**
5	**7.84±3.2**	**14.13±1.59**	**6.29**
45	**15.93±1.1**	**36.25±1.93**	**20.32**

Table 7.6: Mean DPM Counts at Arterial and Venous outlets of Test Cell during re-circulation experiments. (n=5); Error±SEM

	Mean Time period Increase (DPM)	Mean DPM Increase per Min
Arterial		
0-5 min	**~0**	**~0**
5-45 min	**8.09±2.8**	**0.202**
Venous		
0-5 min	**7.72±0.87**	**1.544**
5-45 min	**22.12±1.37**	**0.553**

Table 7.7: Mean DPM increases over full re-circulation period and also per minute of re-circulation. (n=5); data shown as Mean±SEM

The results in Table 7.6 illustrate that over the experimental time, there is a continual increase in disintegrations per minute (DPM) levels at the venous outlet side of the test cell. This indicated that there was indeed plasticiser being extracted from the test material. At the same time there was a negligible increase in DPM levels at the

arterial inlet side of the test cell over the first 5 minutes of exposure and only a small increase in the later part of the experiment. This implies that the majority of the ^{14}C DEHP plasticiser was being retained within the animal during the procedure and not returned to the test cell. This was an important finding as it suggests that it might be possible to investigate the destination of the DEHP by investigating the tissues.

This study also yielded data on the migration dynamics of the plasticiser. It showed that there is an initial large migration at 0-5 minutes, which agrees with the findings of other authors, Zhao, 1999 and Gourlay et al, 2001, who showed an initial large surface washing effect. After this the migration rate is slower as the plasticiser has to work its way through the bulk of the material to the surface where it is the removed. The initial rate of DPM increase per minute is substantially higher over the 0-5minute period compared to the 5-45 minute period (Table 7.7).

7.4.1.2. Investigation of DEHP Distribution within the Tissues.

Calculations were performed to determine if the levels of ^{14}C present in the tissue samples would present a case for uniform distribution throughout the organs. The calculations produced a theoretical level of ^{14}C migration into the animal over the 45 minute period based on the previously reported blood DPM levels. The next step was to determine a theoretical level of ^{14}C DEHP found in each organ. The calculations involved, scaled up the DPM levels from the organ samples to a weight of 1 gram of tissue and subsequently scaling this value up to the average organ weights taken from the literature (Suzuki et al., 2009; Tang et al., 2002). The results can be seen in Table 7.8.

	Activity (MBq)
Theoretical ^{14}C DEHP Deposit Level	**0.00017264**
Total Activity per gram of sampled tissue (All tissues are included in this value)	**0.0000838**
Total Activity per Organ of sampled Tissue (Not including the Muscle & Skin)	**0.00093**
Total Activity per Organ of sampled Tissue (Including Muscle & Skin)	**0.00819**
Difference	**-0.00801736**

Table 7.8: Theoretical levels of ^{14}C DEHP deposited in animal during the 45 minute perfusion procedure and total activity (MBq) per gram of tissue and per organ during 45 minute procedure.

The tabulated results show that the theoretical levels of ^{14}C DEHP that were calculated to have entered the animal (1.7E-4MBq) are smaller than the levels seen on a theoretical uniform distribution (0.00819 MBq) in each of the organs. This indicates that the distribution throughout the organs is not uniform in its nature, certainly at the levels taken from the sampled sections. This said, our samples were not guaranteed to be taken from the same site in each of the organs and hence would indicate there is a varied distribution pattern of ^{14}C DEHP in each of the organs.

The results that do not include the muscle or the skin produced an activity figure (0.00093 MBq) that was of the same order of magnitude as the theoretical level (0.000173 MBq) of ^{14}C DEHP that was administered during the 45 minute perfusion procedure and whilst the difference between the values is not small it does give some indication that the nature of the DEHP deposition in the organs is independent of the mass of tissue in the animal and is related to another aspect.

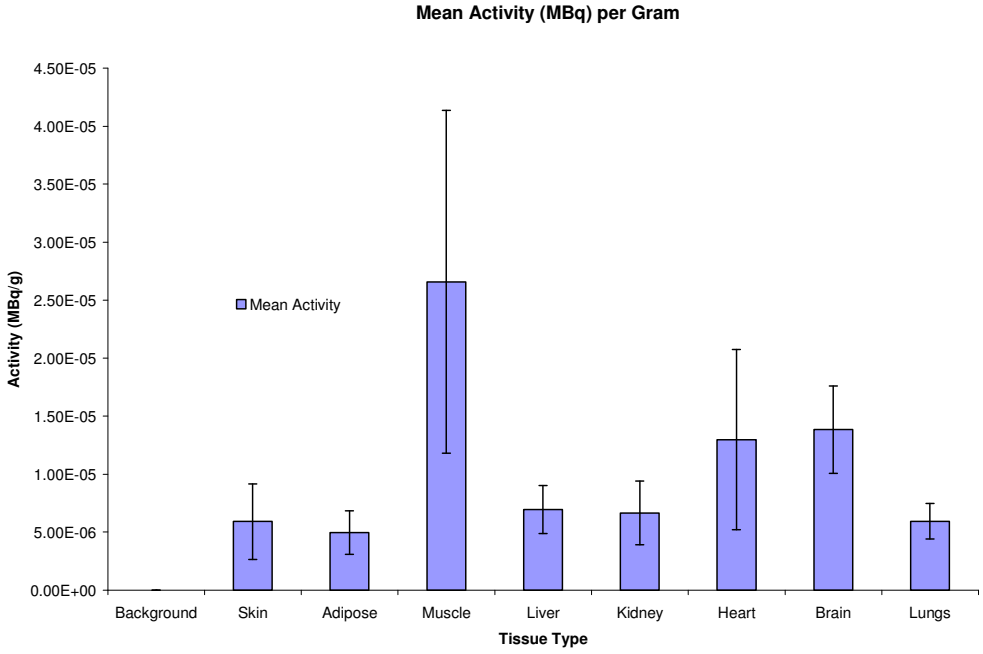


Figure 7.18: Deposited ^{14}C Dehp Activity per gram of tissue; n=5; Data shown as Mean±SD.

The low levels of radioactive labelling within the PVC material produced concerns about the possible results from the animal perfusion experiments, with regard to detection of the DEHP in the tissues.

However, these concerns were unfounded, it can be clearly seen in the above Figure 7.18 that there is distinct difference in distribution of Activity in each of the tissues sampled post procedure and hence DEHP. The main targets, such as adipose tissue, the brain and the liver that had been identified by previous authors contain substantial levels of ^{14}C DEHP. The values for the presented organs can be seen in Table 7.9. A major finding was the observed levels of DEHP present in the brain, which was substantially higher than expected. This was followed by the observed prominent levels of activity in the muscle, heart and skin. When the rate of ^{14}C DEHP deposit during the experimental period was looked at, a similar pattern to the overall depositing was seen. (Figure 7.19)

The results in the figure above have clearly shown that there is a distinct difference in deposition between the organs, but the question remained over the main actuator that is causing the retention of the ^{14}C DEHP in the organs.

To investigate this we looked at two governing factors, Bulk blood flow and organ lipid content, to ascertain if these two factors had any effect on the distribution of the DEHP.

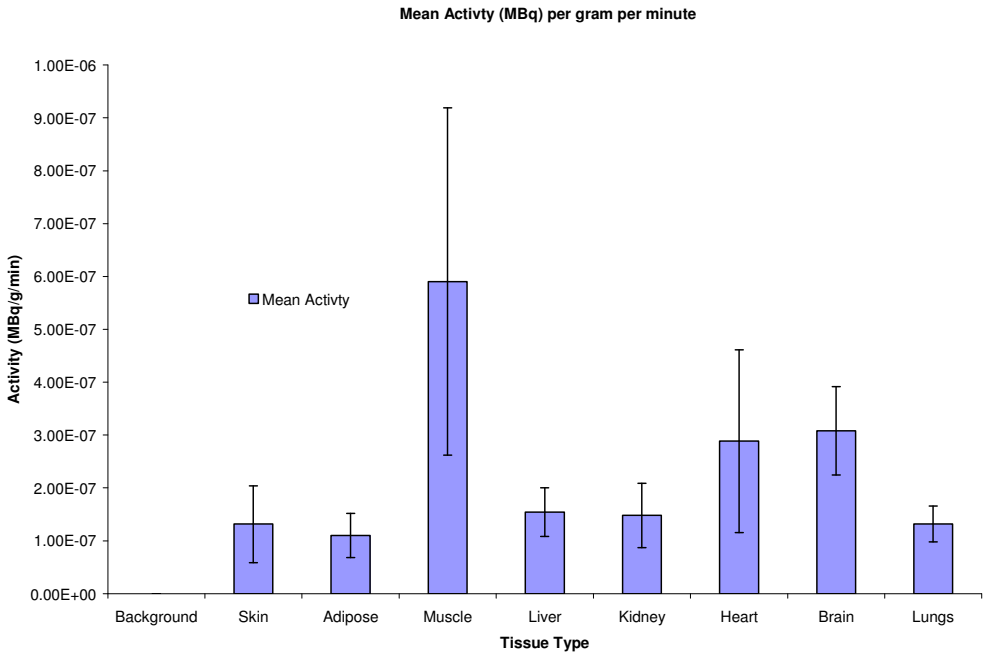


Figure 7.19: Deposited ^{14}C DEHP Activity levels per gram of tissue per minute of exposure; n=5; Data shown as Mean±SD.

7.4.1.3.Investigation of Bulk Blood Flow Effects

The first hypothesis to be investigated was the Bulk Blood Flow. To facilitate this we performed a set of calculations to determine how the rate of blood flow through the organ would affect the levels of ^{14}C DEHP being deposited in the tissue. Data from work by Kawahira et al (1993) on bulk blood flow through the various rat organs was used to calculate the bulk blood flow effects per gram/min/ml in each tissue. The results from this investigation had a dramatic effect on the levels of the ^{14}C DEHP in the tissues, these values can be seen in Table 7.11. It was shown to invoke a major reduction in all of the sampled tissues, with the kidney, heart and lungs being reduced to almost zero (Figures 7.20). It can therefore be said that in the case of these three organs that bulk blood flow may well be the governing factor that relates to DEHP deposition in these organs. A major decrease was also observed in the liver and muscle tissues however, the reduction was not as substantial and hence it was deemed not to be the main governing factor with these organs. This would therefore suggest that there is an alternative mechanism at work for these organs.

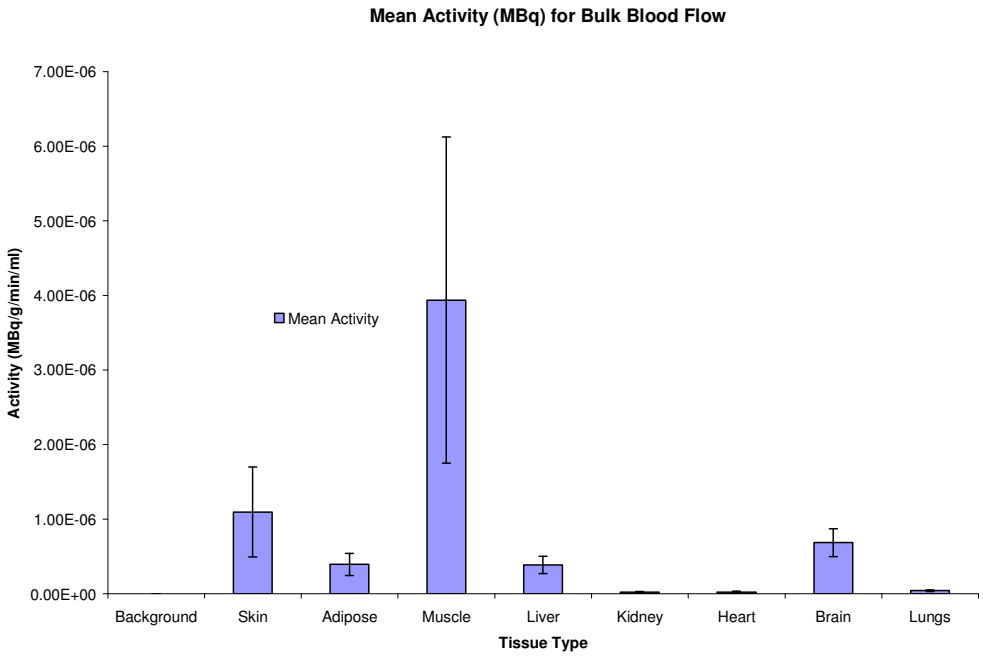


Figure 7.20: Bulk blood flow effects on ^{14}C DEHP Activity in sample organs; n=5; Data shown as Mean ±SD.

7.4.1.4.Investigation of Lipid Level Effects

The results from the bulk blood flow study showed that there was another factor at work that was governing the distribution of the DEHP in the organs and it was not linked to one individual effect. The second hypothesis was that the lipid content of the tissue would have a bearing on the deposition of DEHP in the tissues due to the lipo-phillic nature of DEHP. Again the initial activity levels of ^{14}C DEHP per gram of tissue were calculated to take into account the total lipid content per mg of tissue using values for organ lipid content taken from work by Gandemer et al 1983 and also from Masoro in 1967. The total lipid content values for each rat organ can be seen in Table 6.10.The results are shown in Figures 7.22 and tabulated in Table 7.11. The results shown in these two figures indicate that the average lipid content of the tissues has a more substantial effect on the deposition of DEHP in the organs, than the blood flow. Each of the test organs returned a near zero level, indicating that the lipid level of each organ has a major influence on the deposition of DEHP in each of the tissues. However, when it is scaled up, it can be seen that the muscle and the heart still have some presence on the graph (Figure 7.23). This suggests that the

overall deposition is not governed by a single factor and is potentially governed by a combination of both the blood flow and the lipid content.

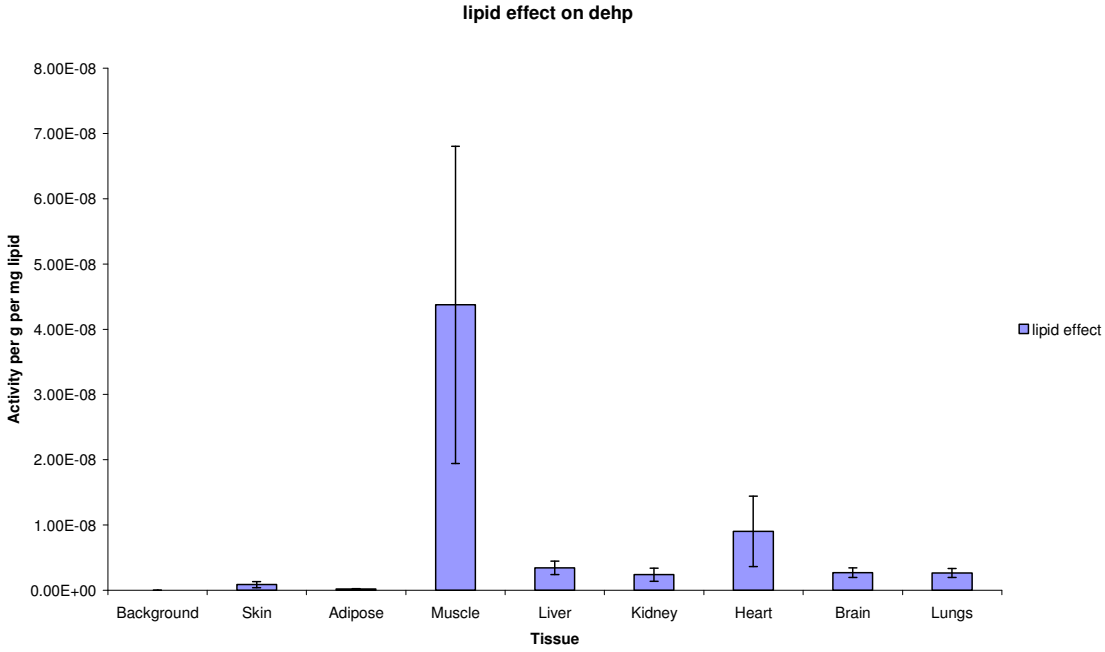


Figure 7.21: Effect of lipid content on ^{14}C DEHP Activity levels per gram of tissue per mg of lipid ;n=5; data shown as Mean±SD.

Tissue	Total Lipid Content (mg/g)
Skin	**151±41**
Adipose	**665±43**
Muscle*	**13.5±0.28**
Liver	**45±7**
Kidney	**62±0**
Heart	**32±0**
Brain	**115±0**
Lungs	**50±0**

Table 7.9: Total Lipid content per gram of tissue used in calculating the lipid level effect on DEHP deposition in the various tissues. n>5 All data reproduced from Gandemer et at 1983 except * Masoro, 1967 with permission from the publisher, ASBMB.

7.5. Statistical Analysis of Ex Vivo Tissue Samples.

Descriptive Statistics of the Re-circulation experiments reported above can be seen in the following tables. The descriptive statistics were performed using the Data Analysis function in Microsoft Excel.

	Mean	±SD	Error	Max	Min
Background	**0**	**0**	**0**	**0**	**0**
Skin	**5.92E-06**	**6.51E-06**	**3.26E-06**	**1.46E-05**	**8.25E-07**
Adipose	**4.96E-06**	**3.75E-06**	**1.88E-06**	**8.85E-06**	**8.01E-07**
Muscle	**2.66E-05**	**2.95E-05**	**1.48E-05**	**6.98E-05**	**3.6E-06**
Liver	**6.95E-06**	**4.14E-06**	**2.07E-06**	**1.1E-05**	**2.44E-06**
Kidney	**6.66E-06**	**5.48E-06**	**2.74E-06**	**1.37E-05**	**1.46E-06**
Heart	**1.3E-05**	**1.55E-05**	**7.77E-06**	**3.6E-05**	**3.38E-06**
Brain	**1.39E-05**	**7.53E-06**	**3.76E-06**	**2.49E-05**	**7.86E-06**
Lungs	**5.94E-06**	**3.07E-06**	**1.53E-06**	**9E-06**	**2.2E-06**

Table 7.10: Descriptive Statistics of Activity per gram of tissue

	Mean	±SD	Error	Max	Min
Background	**0**	**0**	**0**	**0**	**0**
Skin	**1.31E-07**	**1.45E-07**	**7.24E-08**	**3.24E-07**	**1.83E-08**
Adipose	**1.1E-07**	**8.34E-08**	**4.17E-08**	**1.97E-07**	**1.78E-08**
Muscle	**5.91E-07**	**6.57E-07**	**3.28E-07**	**1.55E-06**	**7.99E-08**
Liver	**1.54E-07**	**9.21E-08**	**4.61E-08**	**2.45E-07**	**5.41E-08**
Kidney	**1.48E-07**	**1.22E-07**	**6.09E-08**	**3.03E-07**	**3.25E-08**
Heart	**2.88E-07**	**3.46E-07**	**1.73E-07**	**7.99E-07**	**7.51E-08**
Brain	**3.08E-07**	**1.67E-07**	**8.37E-08**	**5.53E-07**	**1.75E-07**
Lungs	**1.32E-07**	**6.82E-08**	**3.41E-08**	**2E-07**	**4.89E-08**

Table 7.11: Descriptive Statistics of Activity per gram per min

	Mean	±SD	Error	Max	Min
Background	**0**	**0**	**0**	**0**	**0**
Skin	**1.1E-06**	**1.20624E-06**	**6.03E-07**	**2.69791E-06**	**1.53E-07**
Adipose	**3.93E-07**	**2.9771E-07**	**1.49E-07**	**7.02064E-07**	**6.36E-08**
Muscle	**3.94E-06**	**4.37728E-06**	**2.19E-06**	**1.03457E-05**	**5.33E-07**
Liver	**3.86E-07**	**2.30274E-07**	**1.15E-07**	**6.13511E-07**	**1.35E-07**
Kidney	**2.18E-08**	**1.79527E-08**	**8.98E-09**	**4.47594E-08**	**4.8E-09**
Heart	**2.17E-08**	**2.59405E-08**	**1.3E-08**	**6.00044E-08**	**5.64E-09**
Brain	**6.84E-07**	**3.71798E-07**	**1.86E-07**	**1.22789E-06**	**3.88E-07**
Lungs	**4.11E-08**	**2.12383E-08**	**1.06E-08**	**6.22892E-08**	**1.52E-08**

Table 7.12: Descriptive Statistics of Activity per gram per min per ml

	Mean	±SD	Error	Maximum	Minimum
Background	**0**	**0**	**0**	**0**	**0**
Skin	**8.71E-10**	**9.58604E-10**	**4.79302E-10**	**2.14E-09**	**1.21E-10**
Adipose	**1.66E-10**	**1.25352E-10**	**6.26759E-11**	**2.96E-10**	**2.68E-11**
Muscle	**4.37E-08**	**4.86364E-08**	**2.43182E-08**	**1.15E-07**	**5.92E-09**
Liver	**3.43E-09**	**2.04688E-09**	**1.02344E-09**	**5.45E-09**	**1.2E-09**
Kidney	**2.39E-09**	**1.96321E-09**	**9.81606E-10**	**4.89E-09**	**5.24E-10**
Heart	**9.01E-09**	**1.07977E-08**	**5.39887E-09**	**2.5E-08**	**2.35E-09**
Brain	**2.68E-09**	**1.45486E-09**	**7.27431E-10**	**4.8E-09**	**1.52E-09**
Lungs	**2.64E-09**	**1.3635E-09**	**6.8175E-10**	**4E-09**	**9.78E-10**

Table 7.13:Descriptive Statistics of Activity per gram per min per mg lipid

7.6. Ex Vivo Works Discussion

7.6.1. Blood Samples

The perfusion experiments undertaken using the radio-labelled PPVC were novel in nature. It is understood that no one has investigated the migration of the DEHP in this manner previously and hence there is little to directly compare the results to other than previous ^{14}C DEHP intravenous studies. The first findings from this group of experiments showed that the extracted DEHP from the test cell was retained within the animal during the procedure and not returned to the test cell. This finding was extremely important because of its relevance to extracorporeal procedures such as CPB, ECMO and Dialysis. It confirms that DEHP plasticiser that has migrated into blood or other fluids will enter the body of a patient undergoing one of the afore mentioned extracorporeal procedures. This thinking was re-enforced by the data from the activity levels seen in the tissues post procedure showing the retention of the ^{14}C DEHP or possibly one of its metabolites showing that there is definitive level of deposition in the animal.

The blood tests also confirmed a theory by Gourlay et al, 2001. It was suggested by the authors that the rate of migration would act like a "wave" in the initial instance being substantially higher than later in the procedure. The findings from these blood samples showed conclusively that the initial rate of migration was substantially higher for the initial 5minutes with a DPM increase of 1.54 per minute compared to 0.533 DPM per minute from 5-45 minutes of the procedure. This knowledge provides some very relevant data for clinicians using extracorporeal procedures. The potential dose of DEHP that a patient could be subjected to could be dramatically reduced if the process of filtration was employed on the venous return of the blood circuit before returning it to the patient even if it was only for the initial 10 minutes of the procedure.

7.6.2. Distribution of ^{14}C DEHP in Organs

The distribution of the DEHP within each of the animals tissues was something that was in-directly investigated. It has been assumed in other studies (Stern et al, 1977; Ikeda et al., 1980) that the distribution of the DEHP in each organ was uniform. This assumption is based on the tissues being macerated and a sample of this being used in the counting procedure. However, maceration of the organs was not possible in this series of experiments, due to the lack of a designated macerator for radioactive tissue work, so small samples of each organ were taken for LSC. It was attempted to take the samples from the same area of each organ but it was not possible to guarantee that each sample was from the exact same area. The DPMs generated in each tissue sample were then scaled up to a gram of tissue for comparison.

When this was combined with the information from the blood samples it provided an in-direct method of determining if the DEHP was uniformly distributed throughout the organs. It was shown that the two data sets do not match up with the theoretical levels of Activity that would have entered the animal during the 45 minute procedure being smaller than the levels calculated from the Activity of the tissues. The theoretical level that would be administered to the animal during the procedure was 1.7E-4 MBq and the total amount found in the animal based on a uniform distribution in the organs would be 8.1E-3 MBq. It is not uncommon to find that these calculations do not match the experimental data, due to the many different variables that are present. The difference between the two data sets is an order of magnitude which in many cases is seen as reasonable to prove the point.

However, it would be pertinent to try and account for this difference. The DPM levels found in the muscle samples per gram are substantially higher than the other organs that were tested. Using this abnormally high DPM level to calculate a uniform theoretical level of ^{14}C DEHP deposited in the entire muscle mass of the animal could be seen as the reason for the difference between the theoretical administered dose and the ^{14}C DEHP levels for uniform organ distribution. This would pertain to say that for those organs of substantial mass in the animal i.e. the skin or muscle, there is not a uniform distribution of the ^{14}C DEHP. However, for the remaining organs it would be possible to commit to a certain degree that the DEHP deposition

would be uniform in nature. This is an area which will require further work to determine if this is indeed the case.

7.6.3. Perfusion Experiments-Tissue Samples

The data from the blood samples ensured that there would be some levels of DEHP deposition within the tissues of the animals. The harvested tissues produced a specific deposition profile. The literature (Daniel & Bratt, 1974; Tanaka et al., 1975; Waddel et al., 1976;) had shown that after an IV dose certain organs were known to be exposed to substantial levels of the original dose. This was confirmed by the perfusion results, with the lipid rich organs such as the Brain and Adipose as well as the filtration organs having significant levels of DEHP present.

Moreover, the results highlighted significant levels of DEHP in the heart, muscle and skin. The result in the muscle tissue was exponentially high. The reason for this is unknown but it is believed that this is due to contamination of the sample by radioactive blood post procedure, as the samples were sourced from the same site as the cannulation. The surprising level in the heart can be deduced from the fact that this organ is seeing a substantially higher level of the ^{14}C DEHP as it pumps the blood round the cardiovascular system. However, the most interesting result was the levels seen in the Brain tissue, or more to the point the brain having the highest level of DEHP outside of the muscle. This finding has some interesting connotations regarding DEHP exposure through extracorporeal procedures. It would suggest that those undergoing these procedures are liable to subject their brain to substantial levels of the DEHP plasticiser, especially if they are undergoing repeated procedures as in the case of dialysis patients. It has been shown by numerous authors that people undergoing dialysis suffer from cognitive impairments the longer they are on these treatments, and it could be hypothesised that these impairments may be related in some way to the levels of DEHP or its more dangerous metabolite MEHP exposed to the tissue.

Jaeger and Rubin reported in 1973 DEHP distribution in various tissues after a single intravenous dose of DEHP given via the femoral vein. The lungs, spleen and liver were the top three reporting DEHP concentration levels of 8.18 mg/g, 1.89 mg/g and

1.6 mg/g respectively. They also reported low levels in the kidney (0.08 mg/g) and heart (0.0.8 mg) and negligible levels in the brain.

Stern and colleagues looked at a number of issues surrounding DEHP migration. Like our study they produced PPVC sheets to blood bag specifications whilst incorporating a ^{14}C radiolabel to allow the determination of the migration of the DEHP. They administered a single I.V. dose of 1mg/ml of DEHP to a level of 2.5 mg/kg weight. They reported levels in the liver, lungs and spleen with the highest value being the liver. However, they only sampled these organs due to the nature of their study.

This trend of only sampling a few organs is repeated by other authors. Daniel and Bratt published a study in 1974, stating that after a single I.V. dose of ^{14}C DEHP there was rapid accumulation in the lungs and liver, and this was excreted after 1-2 days. Again, this study does not paint the full picture due to only the lungs and liver being harvested post I.V. dose.

These examples show the knowledge in DEHP distribution following an I.V. administration is by no means complete. Most of the early authors reported levels of DEHP in a select group of organs such as the liver and the lungs, this would produce answers to questions relating to these organ groups but does not produce the full distribution picture within the animal.

In an attempt to quantify the distribution in the whole animal, Waddel et al, in 1976 performed a full body auto radiography study in mice to try and ascertain the full picture of DEHP distribution after an I.V. dose. They reported rapid accumulation in the Kidney and Liver which was followed by a rapid excretion. They also noted no accumulation in any other tissues. The findings from Waddell's study are strange, as other authors have reported levels of DEHP in other organs. The lack of DEHP in the other organs may be due to the change in animal or maybe too low an activity of the radio label hence there is none picked up by the detection method.

The most comprehensive I.V. study in the distribution of DEHP to date was performed by Tanaka et al., in 1975. They reported DEHP levels in twelve organs over a period of seven days post procedure. They subjected the animals to a single I.V. dose of ^{14}C DEHP. In concurrence with the other previous studies they showed the liver as one of the top organs for DEHP concentrations. This was the same for

both the brain and also the Testes who were shown to have a low level of DEHP 1hr post procedure. However, unlike the previous studies they showed the muscle and Adipose tissue were also fairly high. The lungs and kidneys were further down the list which was followed by the testes and the brain. Interestingly, the levels in the brain, whilst being small in comparison to the other organs were seen to have a much lower rate of excretion than the other organs. The levels in the brain were seen to remain at 0.02% from 1hr to 6hr, then increased slightly to 0.03% at 12hr before falling away to 0.01% after 24hr and then 7 days.

Whilst there are a number of studies that have used an I.V. route of exposure, none have used the constant perfusion technique that was used in our studies. The results from our I.V. perfusion studies have produced slightly different results than the other previously mentioned studies. We reported that the filtering organs, namely the liver, lungs and kidneys did not return the highest levels of ^{14}C DEHP. This does not correspond to both the findings of Stern et al (1980) and also Daniel & Bratt (1974), however as mentioned previously, they only harvested a few organs and hence could have missed substantial levels in other organs. There is substantial evidence for this when comparison is made to the more comprehensive studies by Jaeger & Rubin (1973) and also Daniel & Bratt (1974). Both these authors showed levels of DEHP in many different organs, and hence indicated that a broad range of tissue samples must be performed to understand the true distribution of DEHP within the body. This said the other studies may have been based on the idea of looking into the supposed target organs of DEHP, which at the time was the liver and other filtering organs.

Our findings correspond well in the main with both the findings of Jaeger & Rubin (1973) and Tanaka et al (1975). In that there is significant distribution in the liver, lungs and kidneys. They also correspond with Tanaka (1975) findings with regard to levels being found in the adipose tissue as well as suggesting a substantial level would be found within the muscle of the animal as well. This was also a finding of our work, however, as stated before the levels seen in our study do not reflect the true levels for this organ for the previously stated reasons. The main differences between the findings lie with our results producing significant levels in the brain and also the heart. This is something that contradicts the findings of both Jaeger & Rubin

(1973) and also Tanaka (1975), who reported that these organs had lower levels of DEHP. In response to this major difference, it can be said that the previous studies were based on a single injected dose of DEHP where as our study was based on a continuous feed of DEHP into the animal for a prolonged period of time. This would certainly explain why our profile was slightly different to the previous studies in this area. The continuous pumping of DEHP laden blood to this organ would allow more time for the DEHP molecule to pass over the blood brain barrier compared to a single I.V. dose. This could be a possible reason behind the higher levels seen in the brain. With regard to the levels seen in the heart, the continuous pumping of the blood during the procedure would subject the heart to a constant stream of DEHP laden blood and would therefore explain why this organ was seen to have a significant level of DEHP in it. Another potential reason for the higher levels present in the heart would be that there was some possibility of DEHP contaminated blood remaining in the heart chambers despite the animal undergoing a flushing post procedure in which Ringers solution was perfused round the test circuit and animal to help in the exsanguination. Whilst this procedure helps to dilute and remove a large amount of the blood it never runs clear indicating that there is still a quantity of blood remaining and hence the levels would be slightly higher than the other studies.

7.6.4. Lipid Level Discussion

The results from the perfusion studies clearly showed a differing level of deposition of ^{14}C DEHP in each of the tissues. However, the reason behind the deposition pattern was unknown. The initial hypothesis that bulk blood flow was the governing factor behind the DEHP deposition in the organs has been shown to be only partly true. The knowledge that there is possibly a secondary mechanism at work required a second hypothesis to be generated. It is common knowledge that DEHP is lipophilic in nature and the findings of the study that have suggested substantial levels of DEHP are present in the brain post perfusion led to the decision to investigate if organ lipid level is possibly the governing factor in DEHP deposition.

The initial findings (Figure 7.22) clearly showed that the level of lipid present in the tissues had a major influence on the deposition of the DEHP in each organ. This result corresponds to previous findings by Marcel, 1973; Kevy et al., 1975 who have

shown that the lipid levels have an influence. However, it was the magnitude of this influence in our study which was most interesting. Other authors have shown that the lipid level had no bearing on the level of plasticiser migration (Stern et al., 1977) but it has been clearly shown that in the case of intravenous reperfusion the migrated plasticiser will deposit itself in accordance with the lipid concentration in each organ. Closer inspection of the original lipid level results (Figure 6.23 and Table 7.13) showed that in the case of the muscle (4.37E-8 MBq/g/min/mg) and heart (9.01E-9 MBq/g/min/mg) samples there was still a small level of activity present. This promotes the thought that in the case of these particular organs, the lipid concentration of the organ is the secondary mechanism of action behind the Bulk blood flow.

It can be said that the tissue lipid concentration level is probably the main actuator behind DEHP deposition in the majority of the tissues sampled. However, there is evidence that the Bulk Blood flow does play a substantial role in conjunction with the lipid levels to determine the final fate of intravenously exposed DEHP in the animal.

7.6.5. Bulk Blood Flow Discussion

The reasons behind the deposition distribution in the organs were unknown. The initial perfusion results showed a propensity for DEHP deposition in many of the filtering organs such as the liver, kidney and lungs. This confirmed work by Tanaka et al., (1975) who had previously demonstrated the distribution of DEHP in rats after IV doses. Therefore the initial hypothesis for the governing factor behind the DEHP deposition was bulk blood flow through the organs.

It was clearly shown (Figure 7.20) that the Bulk blood flow through the tissue has a major bearing on the deposition of the DEHP in all of the organs that were tested. The main filtering organs saw substantial reduction, with the lungs and the kidneys both returning zero levels. On closer inspection (Figure 7.21) there is still a very small amount of activity present which indicates that whilst the bulk blood flow is certainly a main factor in the DEHP deposition in these organs. There is evidence that there is a another co-factor working in tandem with the bulk blood flow in these organs as well as the heart and also the liver. Closer inspection shows that the

peripheral tissues and the high lipid tissues have less of a reduction than the filtering organs which suggests that with these tissues the blood flow is not the main factor but still plays a substantial role.

The results have shown that the hypothesis on bulk blood flow is in part true for certain organs. However, it is also clear that there is another co-factor that is acting simultaneously to determine DEHP deposition in the organs after perfusion exposure.

*7.6.6. **In Vivo Studies 2: Oral Dosing by Gavage***

The data presented in this section, provide the results from the oral dosing of the 12 SD rats with ^{14}C DEHP using a gavage technique. The procedures were undertaken in the laboratory of Prof. L. Kenar at the Gulhane Military Medical Academy, Ankara, Turkey. The organs were harvested, dried then exported to the University of Strathclyde where they were analysed using the LSC technique to determine the levels of ^{14}C DEHP present in the samples. The aim of these experiments was to see if administration of ^{14}C DEHP by gavage at industrial levels, the currently preferred method of determining DEHP exposure, produced similar results to those of the extracorporeal exposure.

Tables 7.14, 7.15 and 7.16 show the descriptive statistics of Activity levels seen in each of the sets of animals sacrificed at the predetermined time intervals. Whilst Table 7.17 lists the P-values relationship from the t-test for the activity levels seen at 48hr and those at both 14 and 28 days. It can be clearly seen that the levels of Activity in the tissues are substantially higher than those seen after the extracorporeal exposure experiments; this was not a surprise as the initial dose by gavage, of 0.325mCi, was around 10,000 times greater. However, the levels seen at 14 days and 28 days post exposure, are comparable to the levels seen in the extracorporeal experimentation.

	Mean	±SD	Error	Range	Min	Max
Kidney	**0.000765**	**0.000386**	**0.000158**	**0.000715**	**0.000405**	**0.00112**
Liver	**0.001431**	**0.000352**	**0.000144**	**0.000654**	**0.001101**	**0.001755**
Brain	**2.13E-05**	**6.06E-06**	**2.47E-06**	**1.61E-05**	**1.68E-05**	**3.29E-05**
Heart	**8.73E-05**	**6.35E-05**	**2.59E-05**	**0.000122**	**2.44E-05**	**0.000146**
Skin	**0.000323**	**2.06E-05**	**8.43E-06**	**4.59E-05**	**0.000302**	**0.000348**
Testes	**0.000189**	**4.66E-05**	**1.9E-05**	**9.09E-05**	**0.000145**	**0.000236**

Table 7.14: Descriptive statistics of levels of ^{14}C DEHP present 48Hrs post Exposure

	Mean	±SD	Error	Range	Min	Max
Kidney	**1.59E-05**	**1.14E-05**	**4.64E-06**	**2.26E-05**	**5.23E-06**	**2.78E-05**
Liver	**1.03E-05**	**6.13E-06**	**2.5E-06**	**1.36E-05**	**4.74E-06**	**1.83E-05**
Brain	**5.11E-06**	**1.06E-06**	**4.31E-07**	**2.78E-06**	**4.38E-06**	**7.17E-06**
Heart	**3.92E-06**	**1.01E-06**	**4.1E-07**	**2.82E-06**	**2.82E-06**	**5.65E-06**
Skin	**8.41E-05**	**6.73E-05**	**2.75E-05**	**0.000127**	**2.13E-05**	**0.000148**
Testes	**6.51E-06**	**3.72E-06**	**1.52E-06**	**8.65E-06**	**2.93E-06**	**1.16E-05**

Table 7.15: Descriptive Statistics of Levels of ^{14}C DEHP present 14 Days Post Exposure

	Mean	±SD	Error	Range	Min	Max
Kidney	**2.18E-05**	**1.44E-05**	**5.89E-06**	**2.94E-05**	**7.43E-06**	**3.69E-05**
Liver	**1.15E-05**	**5.09E-06**	**2.08E-06**	**1.21E-05**	**4.56E-06**	**1.67E-05**
Brain	**4.27E-06**	**1.23E-06**	**5.02E-07**	**3.13E-06**	**2.34E-06**	**5.47E-06**
Heart	**4.15E-06**	**1.04E-06**	**4.26E-07**	**2.95E-06**	**2.43E-06**	**5.38E-06**
Skin	**3.23E-05**	**1.16E-05**	**4.72E-06**	**2.43E-05**	**2.02E-05**	**4.45E-05**
Testes	**4.63E-06**	**2.19E-06**	**8.95E-07**	**5.21E-06**	**1.79E-06**	**7.01E-06**

Table 7.16: Descriptive Statistics of levels of ^{14}C DEHP present 28 Days Post Exposure

48Hr Vs 14 Days	P-Value	48Hr Vs 28 Days	P-Value
Kidney	**0.0007**	Kidney	**0.00082**
Liver	**1.78E-06**	Liver	**1.76E-06**
Brain	**7.3E-6**	Brain	**5.02E-5**
Heart	**0.009**	Heart	**0.0093**
Skin	**8.4E-06**	Skin	**3E-11**
Testes	**2.3E-06**	Testes	**2.12E-6**

Table 7.17: P-Values from One-way ANOVA comparing the means of the Activity levels seen in each organ at 48hr with those levels recorded at 14 and 28 days.

Figure 7.22 shows a plot of the overall mean activity levels found per gram of tissue for each of the three time periods. Figure 7.23 shows the mean activity levels per

gram of tissue for 14 and 28 days post exposure. This was to provide a clearer picture of the levels for these data points originally presented in Figure 7.22.

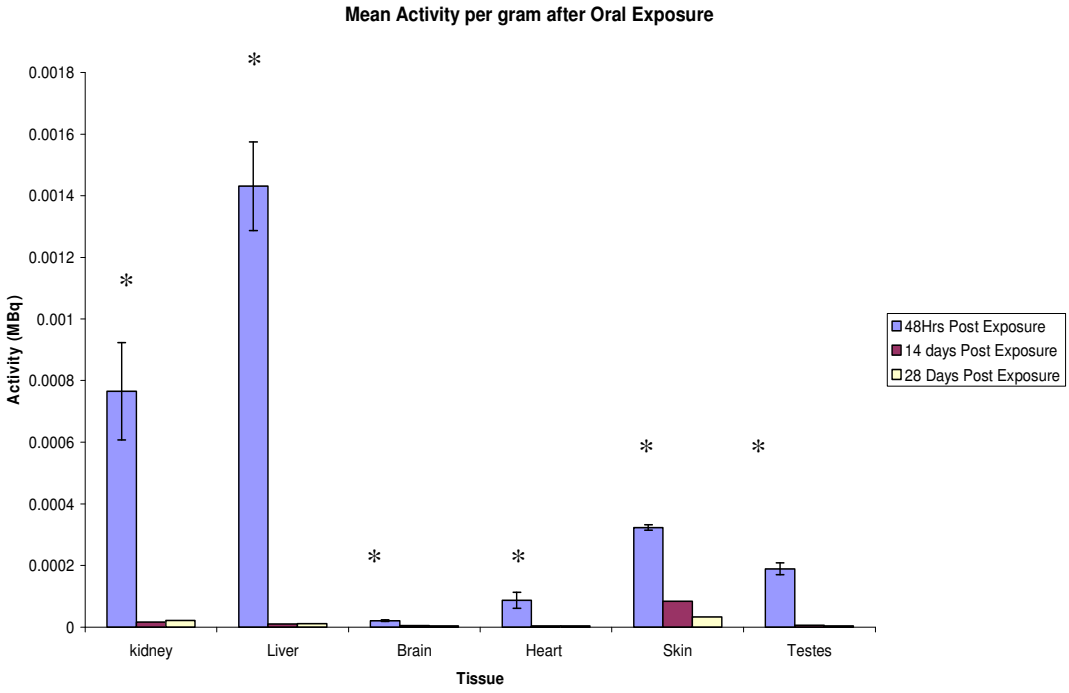


Figure 7.22: Overall Mean Activity per gram of Tissue after oral exposure, highlighting the statistical significant values (*) of each organ (P≤0.05) at 48hr compared to both 14 and 28 days using a one way ANOVA. (n=4); data shown as Mean±SEM

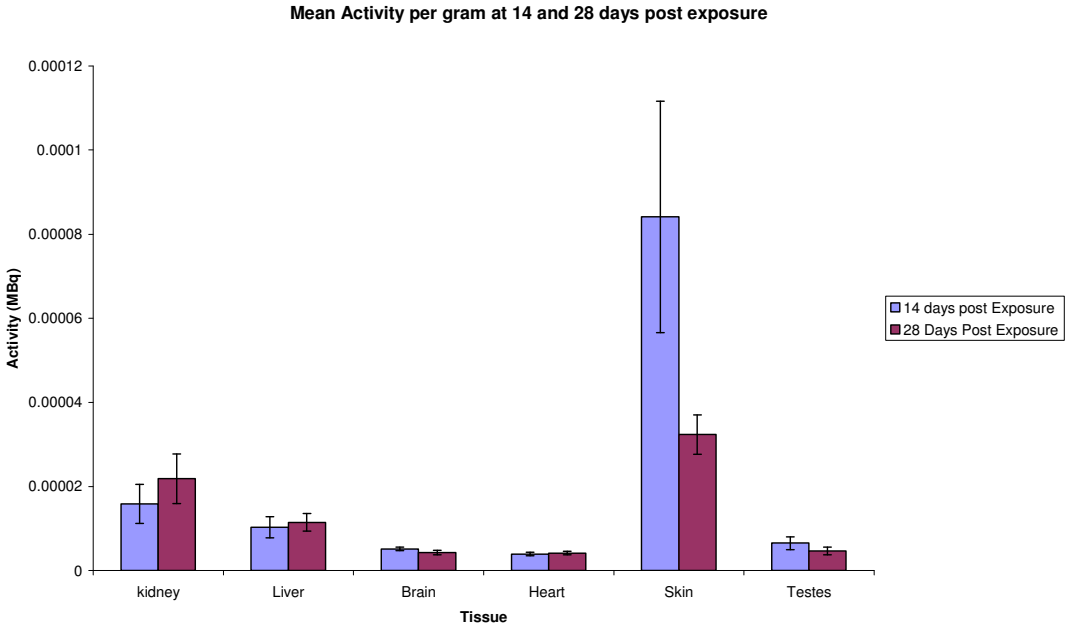


Figure 7.23: Mean Activity per gram of tissue at 14 and 28 post exposure. (n=4); data shown as Mean±SEM

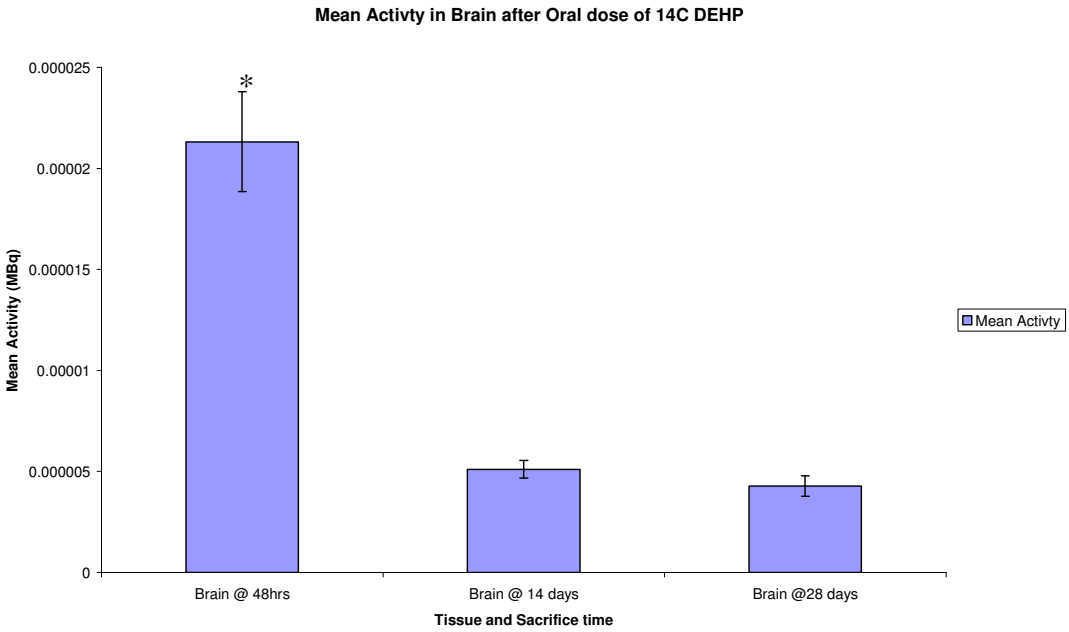


Figure 7.24: Mean Activity per gram of Brain Tissue over the test period, where (*) indicates the statistically significant (P≤0.05) values seen in the brain at 48hr compared to both 14 and 28days using a One way ANOVA. (n=4); data shown as Mean±SEM

The data produced from these sets of experiments led to a number of interesting results. The first point of interest lies in the levels of ^{14}C DEHP seen in the tissues. The levels reported in the tissues are minute in comparison with the initial dose. This suggests that the vast majority, greater than 95% of the initial dose is excreted in the first 48hr post exposure.

Secondly, the pattern of ^{14}C DEHP distribution after the gavage study was different to that produced by the extracorporeal mean of exposure (Figure 7.22). The gavage study was seen to favour the "processing organs" such as the liver and kidneys over the more lipid organs such as the brain.

It was also seen that the initially substantial levels seen at 48hr post exposure decreased as time progressed out to 14 days and less rapidly out to 28 days (Figure 7.22 & 7.23). This indicated continued excretion of the DEHP from the animal over time.

Surprisingly, the levels seen in the brain tissue were the lowest of all of the organs tested, with levels of 2.13E-5±6.06E-6 MBq per gram at 48hr post procedure. This was substantially lower than the other organs such as the kidney which had levels of 0.000765±0.000386 MBq per gram and the liver which reported 0.001431±0.000352 MBq per gram.

Figure 7.24 also shows that the levels of clearance from the brain of the DEHP from 48hr to 14 days were substantial and significantly different ($P \leq 0.0006$) as it dropped the DEHP levels to 5.11E-6±1.06E-6 MBq per gram. However, from 14 days to 28 days the level of clearance was not so marked, with the activity only dropping to 4.27E-6±1.23E-6 MBq per gram of tissue. This suggests that once the DEHP has made its way to the brain, it tends to remain in-situ even over extended periods of time.

The final finding from this study relates to the distribution of the DEHP within the organs themselves. Figure 7.22 showed that the levels of DEHP in each organ had some dependence on the area of tissue that was sampled. The graph shows that there is some occasional increases in activity per gram of tissue at 28 days post exposure compared to the samples at 14 days post exposure. This could be attributed to a non-uniform distribution of DEHP within the organs which is evident when different sections of tissue are taken as samples for LSC. This is something that was also discovered during the perfusion experimentation as well, which would indicate that there is a non-uniform distribution of the DEHP in each organ.

7.6.7. Gavage Studies Discussion

The use of gavage studies in determining the toxicological profiles of DEHP is common place (Daniel and Bratt, 1975; Albro, 1986; Tanaka et al., 1975). However, the viability of the results in relation to extracorporeal procedure exposure is questionable. A set of gavage studies using an industrial dose (0.325mCi) of ^{14}C DEHP was performed as part of this thesis to determine if there was any difference in the distribution profile produced by the perfusion study.

The gavage studies gave some suggestion that the majority of the gavage dose was excreted from the animal within 48hr when the values of ^{14}C DEHP present in the samples was compared to the initial dose. This concurs with previous work by Ikeda et al, 1980 and Tanaka et al, 1975 who also reported the same conclusions. The results also produced similar profiles to the work by Tanaka et al, 1975 in relation to the continued excretion of the ^{14}C DEHP over time. (Figure 7.22) This excretion pattern was seen to be substantial from 2 days to 14 days post procedure, but slowed between 14 days and 28 days post procedure. This suggests that some of the DEHP or possibly one of its metabolites are distributed deeply into tissues and hence cannot be shifted once lodged. This would explain why a number of authors (Jaeger and Rubin, 1973; Ikeda et al, 1980) were unable to account for the full initial dose given to the animals.

The findings from our study indicated a lower level of ^{14}C DEHP manifesting itself within the brain compared to the perfusion studies. Whilst the levels were small, they did have a propensity to linger within the tissue. The ^{14}C DEHP that was present in the brain did not clear the tissue at a quick rate over time. This finding was corroborated by the findings of Tanaka in 1975 and also Ikeda et al, in 1980. Both these authors showed that the DEHP was present in this tissue but continued to linger over time.

Both the above authors also reported a majority of ^{14}C DEHP manifestation in the liver and kidneys immediately after the dose. This initial level was quickly reduced by excretion. This was confirmed by our findings which reported the same pattern, all be it over a longer time period than the other studies.

Another similar finding between the three studies was the lower level of ^{14}C DEHP seen in the heart tissue compared to the I.V. route. Again this level was seen to reduce over time as with the other organs. This would add strength to the argument that the higher levels seen after the perfusion dosing would be due to the continuous exposure of the heart to the DEHP which was contained within the blood over the prolonged period of time.

Tanakas findings regarding the levels of DEHP seen in the testicles also correspond to our findings, with an initial reasonable exposure of the DEHP to these organs and then a sudden drop off. However, Tanaka's study had a shorter time span than ours

and hence showed that the testicles did not receive their highest dose until around 3-6hr post procedure. This would suggest the levels reported in our study at 48hr are only a portion of the dose they received.

The results of this particular study show conclusively that the oral dosing of DEHP to animals produces a significantly different distribution profile to that seen from an intravenous perfusion method. The filtering organs of the liver and kidney have substantially more levels of ^{14}C DEHP present than the other organs tested at 48hr post procedure. The skin saw a medium proportion of DEHP but this was found to be excreted at a much lower rate than in other organs. The reason for this is unsure, one suggestion is that the blood flow to the area from which the skin was taken is fairly low, hence the lower rate of removal from the area over time. The level seen in the heart was lower in comparison to the other organs suggesting that the levels that eventually migrate into the blood must be lower than was seen in the intravenous study. As reported by other authors the testes contain a level of DEHP that would suggest that it is one of the main target organs, hence the interest in the toxicological effects in this region.

The levels of ^{14}C DEHP reported in the brain were much lower than expected. It reported the lowest level of all the tested organs. The lipophilic nature of DEHP would suggest that the high lipid content in the brain matter would have returned totally different results from the one obtained. Possible reasons for this finding could be that the vast majority of the DEHP is processed by the gut, hence very little passes into the blood stream and makes its way to the brain. This would also explain why the levels in the heart were not as high as expected. However, the removal from the brain (Figure 7.24) is shown to be fairly slow, with relatively substantial levels still remaining in the tissue sample at both 14 days and 28 days post procedure.

7.7. Animal Study Overall Discussion

The use of ^{14}C labelled DEHP as a means of tracking migrated DEHP from PPVC has yielded a number of important results in both methods of exposure. It has successfully profiled the distribution in tissues after both gavage and perfusion exposures. The findings of this study may have far reaching consequences with

regard to previous thinking on DEHP toxicology studies and methods used in the determination of NOAELs for DEHP and its metabolites.

It was discovered at the outset of this project that the knowledge of DEHP migration and distribution in tissues in relation to extracorporeal procedures was extremely lacking in depth. The vast majority of toxicology studies focused on oral dosing of animals with large "industrial" levels of DEHP that were not representative of any extracorporeal procedures. Issues surrounding the safe use of DEHP in PPVC in such procedure have been based on experiments that have assumed that the exposure methods have no bearing on the fate of DEHP in the body.

However, it has been clearly shown by these sets of experiments that the route of exposure produces different distribution profiles within the tissues of the animal (Figure 7.25).

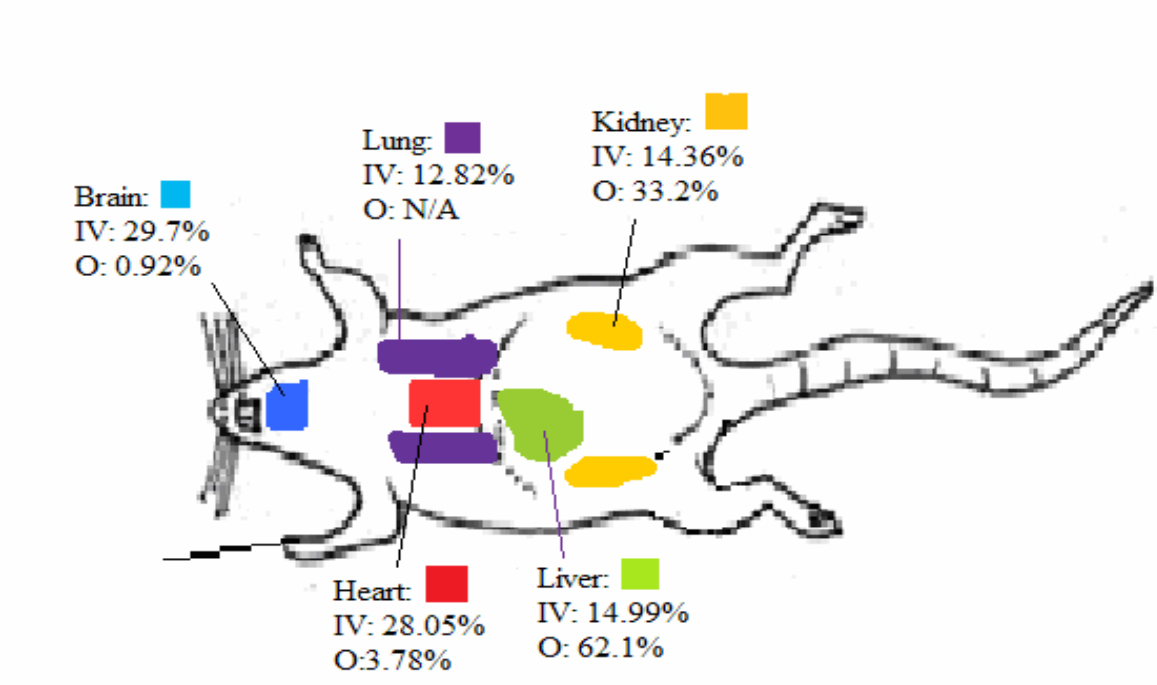


Figure 7.25: Comparison of the different distribution profiles between the Oral (O) and Intravenous (IV) methods of administration of DEHP. Differences were highlighted as the total ^{14}C DEHP activity per gram in each of the sampled organs. The % are based on the figures from Tables 6.9 and 6.13.

In both types of exposure, the same organs are targeted by the DEHP but the levels of activity present vary depending on the route of exposure. Oral dosing has a greater distribution proportion that favours the filtering organs whilst the intravenous favours a greater proportion in those organs with a higher lipid content. The organ

that has the greatest variation in DEHP levels between exposure routes is the brain. This organ contained one of the highest levels post perfusion exposure whilst it had the lowest by far post oral exposure. Interestingly, the levels seen directly after the 45 minute perfusion procedure had been ended were of the same order of magnitude as those found 48hr after the gavage dose. This may not seem too important but when it is considered that the magnitude of the oral dose was around 10,000 times greater than the dose from the perfusion experiments then it becomes quite clear that the route of exposure is important in determining the adverse effects associated with DEHP and other plasticisers. A final point relates to the possible differences not just between oral and I.V. exposure routes but between different I.V. exposure routes. The differences seen in the deposition profiles between our perfusion studies and the previously reported I.V. injection indicates that there may indeed be an effect on the target organs in continuously exposing the patient to DEHP contaminated blood or blood products compared to the one off dose.

Once it had been shown that the different routes of exposure produce different distribution profiles, it was important to try to define the overall cause of the different distribution found after the intravenous perfusion experiment. Two hypothesis, Bulk Blood Flow and overall lipid levels, were suggested by the author, to be possible reasons behind the specific distribution of the DEHP.

Both hypotheses were shown to have a profound effect on the levels of DEHP deposited in each of the organs, however, it was the overall lipid concentration in each organ that was the main factor with the bulk blood flow being a minor but nevertheless an important simultaneously acting co-factor in deciding the DEHP distribution with the different organs post intravenous exposure.

In conclusion, the findings from the various animal studies have shown, quite clearly, that judgements and conclusions relating to DEHP migration from PPVC medical products used in extracorporeal treatments cannot be based entirely on findings drawn from oral exposure studies due to the differences in distribution profiles.

7.8. Clinical Implications of Results

The study of the migration and toxicology of the plasticiser DEHP is a long standing area of research. However, it has been shown that there are certain areas in which the knowledge remains shallow. In order to shed light on these uncertainties, the objective of the work undertaken in this thesis was to gain a better understanding of the migration of the DEHP plasticiser under conditions that were closer to those found in the clinical environment. This was one area where the actual implications of DEHP migration were not well defined.

Plasticised PPVC is widely used in medical devices, particularly in extracorporeal systems. Interventions such as Dialysis, CPB and ECMO are becoming more common as population profiles change due to better social and clinical care. Various health agencies such as the FDA and the EC have issued warnings about the use of DEHP within certain age and patient cohorts with regard to possible adverse toxicological effects which may manifest after substantial single dose or prolonged exposure to this compound under clinical conditions. The results of the work performed in this thesis suggest that there may be a real problem with plasticiser migration in the clinical setting. One such area is the pre-priming of ECMO systems for immediate use. The current trend in healthcare of pre-empting the need for such interventions as ECMO has led to the pre-priming of the circuitry followed by prolonged storage. We have shown that the priming fluids used in these circuits have to be carefully selected to limit the levels of plasticiser migration before the system is finally deployed. This is just a small example of small measures that could be taken to try and limit the exposure of already seriously ill patients to a chemical that could be the basis of so many complications, particularly inflammation related complications, found under clinical conditions (Takahashi, Y. et al, 2009; Gourlay et al, 2003; O-Yurvati et al., 2004).

The findings of the present research clearly show that there is a migration of DEHP into the blood under perfusion conditions and that this DEHP migrates to the tissues of the subject. Studies of the method of exposure to DEHP further confirms that there was a clear effects on the level of DEHP in the major organs. We have shown that

continuous perfusion exposure produces a different distribution profile to that of the oral administration. The I.V. route has a propensity to be preferentially located in the brain and heart tissues, with the filtering organs being associated with lower counts, whereas the oral route favours the filtering organs over the brain and heart. In the main, our findings comprehensively show that no organ studied escapes DEHP infiltration and or its metabolite, regardless of the route of exposure. These findings have clear implications in extracorporeal procedures and the treatment of patients who undergo multiple exposures to DEHP plasticised PVC.

It has been shown by various authors that those undergoing CPB have a propensity to develop an uncontrolled inflammatory response, leading to a Systemic Inflammatory Response Syndrome or SIRS (Asimokopolous, 1999, 2001; O-Yurvati et al., 2004). One such reported cause is the contact of the blood and humoral components with the artificial materials that make up the circuitry of CPB and ECMO equipment (Asimokopolus, 1999, 2003). Gourlay et al., 2003 showed that DEHP itself is an inflammatory mediator in human blood. Further studies suggest that some of the complications seen in those presenting with symptoms of SIRS post surgery may be linked to the exposure of the DEHP and or its main metabolite, MEHP, which is known to be the more toxic.

The generation of an inflammatory response is not reserved for the CPB procedure. It is also prevalent in other extracorporeal procedures. One intervention in which an inflammation response is known to occur is dialysis. The morbidity and mortality of end stage renal patients is known to be unacceptably high and compares to that of many cancer patients with metastases (Yao et al, 2004). The main cause of the high mortality rate is cardiovascular disease. It is seen to be both twice as common and advances at twice the rate in patients who undergo dialysis (Yao et al., 2004). Recently, there has been accumulation of evidence that suggests that inflammation is a major contributor to the morbidity and mortality associated with dialysis (Chawla & Krishnan, 2009). It is thought to be associated with malnutrition and progressive atherosclerotic cardio vascular disease via several pathogenic mechanisms in which the pro-inflammatory cytokines are seen to play a pivotal role (Kaysen, 2004;Yao, et al., 2004). The activation of the inflammatory response is thought to be multi-factorial, in so far as it is caused by both dialysis and non-dialysis related agents.

However, the similarities to the inflammatory response seen during CPB procedure hints that the pro-inflammatory nature of DEHP may play a substantial role in initiating this response. It is this inflammatory nature of DEHP which the author believes presents the biggest issue of DEHP in the clinical setting especially with regard to extracorporeal procedures.

Patients undergoing CPB have been known to develop neurological problems that present as symptoms of confusional states, depressions and anxiety (Rimon et al, 1970). This discovery of neurological complications after extracorporeal interventions is not exclusive to CPB procedures. It has been known for many years that patients undergoing dialysis treatment for chronic kidney disease are known to suffer from cognitive impairment. Around 70% of all dialysis patients aged over 55 suffer some form of medium to severe levels of cognitive impairment (Murray, 2008). In a substantial number of these patients they present with vascular dementia (Krishnan and Kiernan, 2009). However, this condition is seen to improve on transplantation which would indicate that it is the dialysis procedure that is responsible for the onset of the condition. It has been suggested that the adverse cognitive effects seen in dialysis patients is caused by repeat occurrences of acute cerebral ischemia brought about by the dialysis treatment (Murray, 2008). However, further work suggested that this may not be the main reason behind the reduction in cognitive ability. One additional theory is that the reduction in cognitive function is due to brain degeneration brought about by toxic metabolic etiology (Fazekas et al., 1995).

The findings of our perfusion studies suggest that the brain is subjected to a more substantial dose of DEHP during an extracorporeal treatment than other organs, which when combined with the findings of Fazekas would indicate that the toxic nature of DEHP or more likely its more toxic metabolite, MEHP, may play a pivotal part in the degeneration of brain function. This contraindication would not have been discovered by studies favouring the oral route of exposure, which as we have shown does not produce the same levels of DEHP in the brain. Further weight to this argument is supported by clinical studies that show that the cognitive degeneration is reversed when the DEHP exposure is removed (Krishnan and Kiernan, 2009).

Studies have also shown that the clearance of DEHP from the brain or other highly lipid tissues such as adipose were much slower than from other tissues (Daniel and Bratt, 1974). Our findings showed substantial clearance after 48hr but little movement between 7 days and 28 days after oral administration. This would suggest that even though the levels of DEHP in the brain are small they linger and may accumulate over time with multiple exposures. The regime of dialysis treatment suggests that these patients would receive a dose of DEHP in the brain during their treatment, of which a substantial portion would be excreted before their next session. However, there would be consistent residual levels of DEHP that were not cleared between sessions and hence may continually accumulate in the brain during their treatment. This can be more easily described as a wave like exposure pattern continually throughout treatment. One study by Calabrese et al., in 2010 indicated that increases in oxidative stress can induce neuronal damage as well as modulating neuronal signalling and can ultimately lead to neuronal death by apoptosis or necrosis. DEHP is known to increase oxidative stress (Huber et al., 1996; Rusyn et al., 2006) and hence would provide a possible reason behind the adverse neuronal effects seen in patients who are exposed to substantial levels of DEHP via medical interventions such as dialysis.

The findings of our study would also lend weight to the argument that patients undergoing multiple procedures are indeed at more risk of adverse effects associated with this plasticiser, especially inflammation related problems.

Recently, inappropriate inflammation responses have been the subject of much work. It has been shown that these inappropriate responses are linked to most chronic diseases such as atherosclerosis, heart disease, diabetes and some cancers (Aggerwal & Geholt, 2009; Rauch et al., 2009; Erdmand & Poutahidis, 2009; Piperi et al., 2009). The identification of both transcription factors and their gene products have provided a molecular basis for the role of inflammation in cancer and the activation of these pathways by factors like stress, environmental stimuli and infectious agents are responsible for around 95% of all cancers (Aggarwel & Geholt, 2009). Therefore, it is clear that DEHP, as an inflammatory mediator could have some role in the initiation of certain cancers, especially in those undergoing chronic treatments like dialysis.

It has been well documented that DEHP causes reproductive issues in rats (Foster, 2006;). However, these effects as of yet have not been shown to affect the human species. However, it has been shown that DEHP does affect hormone levels in both the pituitary and thyroid glands. It has been shown to exert an age-dependent influence on the pituitary-adrenocortical axis in vivo and adrenocortical steroidogenesis ex vivo. This may have some significance in connection with disorders of the hormonal stress response, especially in very young people (Supornsilchai et al., 2007). The effects on the pituitary have also been shown to be reversible on the ceasing of DEHP exposure (Gayathri et al., 2004**).** This would indicate that one off treatments would not have such a major effect on the hormone levels, but those undergoing repeat and numerous interventions would be at a substantial risk especially if they are young. However, these findings are again based on animal studies and have yet to be proven in humans. But they would explain recent findings which have shown that DEHP exposure can make boys more feminine in nature. (Swan et al., 2009)

For many years the concern around DEHP related to its carcinogenic and detrimental reproductive properties. However, of equal concern may be its ability to invoke an unwanted inflammatory response. The findings that the route of exposure produces a different distribution pattern for DEHP suggests that those patients undergoing CPB, ECMO or more importantly dialysis are at a greater risk of developing complications that could be initiated by the exposure to DEHP. It would also suggest that the current thinking that the extrapolation of the possible effects of extracorporeal procedures can be successfully made from the effects seen in oral dosing studies is not true and effects from these procedures can only be successfully obtained by using perfusion models.

8. Conclusions, Limitations and Further Work

8.1. Conclusions

In summary, the work presented in this thesis attempted to meet the project aims that were previously outlined in the opening chapters. The results presented in this thesis, not only met the original hypotheses, but also generated extra findings that produced the following conclusions.

The work presented in this thesis:

- Firstly covered and outlined the various issues surrounding the use of the DEHP plasticiser in both non-clinical and clinical settings especially with regard to its use in multi-exposure procedures such as ECMO and dialysis.
- Demonstrated that plasticiser migration is something that can be seen as a clinical issue with regard to the pre-priming of ECMO circuits for immediate use. The study produced the following findings:
    - DEHP readily migrates from PVC tubing into different fluids, with a propensity to migrate at a higher rate into lipid rich solutions
    - The migration rate is dependent on fluid composition and also duration of fluid contact
    - Migration of the alternative "migration resistant" plasticisers was at much lower levels than the DEHP plasticiser
- Evaluated previous biomaterial test cells for suitability for use in this project and designed, developed and evaluated a new parallel plate test cell that produced specific controlled flow conditions over the test material using both Newtonian and non-Newtonian fluids for use in the perfusion experiments
- Sourced and manufactured be-spoke ^{14}C labelled DEHP and had this incorporated into plasticised PVC sheets that were to be used in the novel perfusion based plasticiser extraction experiments

- Developed and performed *In vitro* recirculation perfusion extraction studies using whole blood, plasma and methanol extraction fluids with the following results:
    - Methanol fluids were comparable to the previous studies in the literature
    - DEHP plasticiser migration was higher into blood than into plasma showing that the fluid composition has an influence on the plasticiser migration
    - The experimental set up was robust in nature as the results showed good repeatability

The use of a 14C radiolabel marker is indeed an excellent way of determining the migration of plasticiser into various organic and biological fluids.

- Performed migration experiments using a novel rat perfusion model which produced the following results:
    - Higher levels of activity were seen in the blood at the outlet of the test cell than at the inlet of the test cell, indicating that DEHP was being removed from the test material
    - The lower levels of activity in the blood exiting from the animal indicated that there was DEHP being deposited in the tissues of the animal
    - Activity was seen in each of the tested organs, confirming the presence of DEHP in the tissues
    - The distribution of DEHP in the organs was seen to be higher in those of high lipid contents such as the brain and not the filtering organs
    - The mechanism behind the deposition of DEHP in the tissues is strongly related to tissue lipid content and also in a minor way to bulk blood flow in the organ after perfusion exposure

- Performed gavage experiments to compare with the perfusion studies which showed the following:
    - Activity levels were seen in all organs that were tested, showing presence of DEHP at 48hr, 14 and 28 days after administration
    - Activity levels decreased over the time period indicating removal of DEHP from the animal, with a fast initial removal out to 14 days and a much slower rate from 14 to 28 days
    - After 28 days residual levels of DEHP were seen in all tissues
    - ^{14}C DEHP distribution profile was different to that of the perfusion experiments, favouring the filtering organs such as kidneys, liver and lungs

The *Ex vivo* results demonstrated that the method of administration of the DEHP leads to differing distribution profile in the tissues, which suggests that conclusions on the adverse effects associated with extracorporeal exposure cannot be entirely based on oral gavage studies

The most pertinent of the above findings is the varying profiles of DEHP deposition found during the different exposure experiments. It had been assumed for a number of years that the method of exposure had no bearing on the adverse effects or deposition of the DEHP seen in the tissues within the animal. A number of studies utilised a single intravenous exposure test, but this did not yield any significant differences to the gavage studies. Hence, this led to the oral administration of DEHP by gavage being used as the standard test method for many of the toxicological tests that have been used in determining the safety of DEHP within the medical environment.

Our studies clearly indicated that the recirculation perfusion method of exposure produces a different DEHP deposition profile to that seen by the gavage method. The perfusion exposure method produced a profile that favoured the deposition of DEHP in those tissues that were of high lipid concentration, especially the brain. To the best

of our knowledge this is the first time a recirculation perfusion system has been used in studies with the aim of determining the fate of the DEHP and its metabolites within a rat model. This leads to the conclusion that where extracorporeal exposure is the mechanism of concern, the results from gavage testing cannot be used. Therefore, to fully understand the adverse effects associated with extracorporeal exposure to DEHP and its metabolites, further toxicological studies should be performed using the perfusion exposure method.

The study reported in Chapter 4, clearly demonstrated that plasticiser migration remains very much a clinical problem with regard to the safe storage of pre-primed ECMO systems. Plasticisers were detected in each of the priming fluids and from each of the different tubing types tested. This showed that even the proposed alternative plasticisers do also migrate, albeit at much slower rate. The findings of this study clearly indicate that the first hypothesis of this dissertation, that plasticiser migration, especially DEHP, from PPVC is definitely a clinical problem that needs to be addressed to ensure that patients are not unnecessarily exposed to a potentially harmful chemical.

The second hypothesis of this thesis was to determine the feasibility of Carbon 14 labelled DEHP as a solution to current challenges associated with DEHP detection in complex tissues. This method had been used previously by other authors with varying degrees of success. A notable problem with the early work was ensuring that there was a high enough Specific Activity present in the radiolabel to ensure detection even at extremely low levels. The levels used in the present study clearly show that we could detect the low levels of DEHP migration even after just 5 minutes of recirculation, hence highlighting that the Specific Activity used in our study was high enough. The results of our study, both *In vitro* and *Ex vivo*, provided sufficient evidence that the use of Carbon 14 as a means of detection is a robust method of determining plasticiser migration from PPVC.
However, it does not represent a real option as a routine assay technique for the detection of plasticisers. There are a number of factors associated with radio-labelled testing which support these assertions, in particular:

- Cost
- Regulations
- Facilities

The cost category covers a number of areas. The first relates to the cost of producing the radio-labelled compound. ^{14}C DEHP was not readily available on the commercial market when this project started and was required to be custom synthesised to our specifications. This was achieved at a very high cost which provided only a very small amount that could be used in the PPVC sheets. The high cost of the custom synthesis, restricts the use of ^{14}C to tracer studies in this particular application. This does not lend itself to use as the standard test for plasticiser migration. Due to our work, it is now a listed commodity on American Radiochemicals supply sheet, but is again based on custom synthesis.
Another major cost relates to the implementation of dedicated facilities for radiation use. This can not be achieved without substantial investment, the gaining of a radiation license is no simple matter, and the continual support and maintenance of these facilities is also expensive. The second factor is that to produce radioactive PPVC sheets dedicated machinery would be required due to the high risk of contamination, again ruling out anything but research environments for such work as contamination of commercial plant may have considerable economic impact upon commercial concerns.

Currently, there is only one facility that is able to produce the ^{14}C PPVC. This is an industrial research unit in India, where the test material used in our work was produced. However, whether they could produce the ^{14}C PPVC in forms other than sheets is unknown. The production of other testable forms of PPVC would be the logical next step for this technology if it was to be used as the gold standard. The lack of possible producers of the test material is also not conducive for a gold standard test and hence this is substantial reason for this test assay being limited to small academic studies.

The regulations governing the use of ^{14}Cseverely limit its use as a possible standard assay method. This leads to only a few facilities being able to store and use these

products, which is not conducive to a standard industrial assay. The regulations govern every aspect of the radiochemical, from production, to shipping, use and disposal. These rules hamper the range of experiments that can be conducted using the technology. Therefore the answer to the second hypothesis of this dissertation is no, it is not feasible for this technology to become the gold standard assay test for plasticiser migration due to a number of different factors.

The remaining aspects of the original hypothesis were conclusively answered in this dissertation. ^{14}C DEHP provides an excellent method of determining the levels and migration profile of the plasticiser from the PPVC sheet both in *in-vitro* and *ex-vivo* studies. It was possible to

- Determine the concentration levels in both organic solvents and biological fluids used in the in-vitro studies.
- Detect and determine the plasticiser levels in the various tissues after the ex-vivo studies

These results were especially pleasing considering the very small amounts of the labelled DEHP that was used during these studies. These results combined with those obtained from the oral dosing clearly show that using a radiolabel as a means of tracing the plasticiser is an effective way of accurately determining plasticiser migration in both organic and biological media.

8.2. Limitations

The results from the investigations were encouraging especially after the number of problems that were encountered in trying to perform the studies. As with all experimental work, there were some limitations to the work.

The ECMO tubing study had a few limitations. The main being the use of small sections of tubing in the experiments; this resulted in small but significant levels of leached plasticiser. A larger surface area and a smaller priming volume may well

have amplified the results. However, we were keen to use only clinically relevant materials and designed the test system and protocols to use these.
The detection limitation of the spectrophotomer system was initially considered to be a concern, however, previous studies and this current study confirm that the levels detected are well within the limitations of the technology.

The *In vitro* and the *Ex vivo* series of recirculation experiments also had a number of limitations. The first related to the test material. Great care was taken to try and evenly distribute the radio-labelled plasticiser throughout the test material, but it was not guaranteed that each sheet contained the same amount of labelled plasticiser. The possibility of an uneven distribution of labelled product could be a factor for the slight differences found in the results of the experimental runs. A guaranteed distribution may have resulted in higher counts and therefore higher DEHP concentration levels.

The labelling of such a small proportion of the DEHP present in the test material was also a limitation. This involved a number of assumptions being used in the calculations of the concentration levels of DEHP that had migrated from the material. A higher labelling percentage may have increased the overall concentration levels of DEHP that had been seen to migrate from the test material, thus reducing some potential elements of experimental error.

Another limitation was the inability to determine the levels of 14C DEHP that were excreted by the animals during the gavage studies. This would have led to a certain amount of data that could have provided more information on the recovery of the initial dose of the ^{14}C DEHP administered to the animal as well as the rate of excretion of the compound over the experimental time period. However, this potential data was a casualty of international nature of this project, as it was not possible to safely store and transport the urine and faeces of the animals back to the University of Strathclyde for processing.

The biological samples used for the blood runs were freshly acquired before each test run. Hence, their make up would have been slightly different and when this

combined with the experiments being performed at room temperature and not at 37^{o}C, this may have had an effect on the extraction rate of the plasticiser from the surface of the material.

The final issue relates to the counting of the samples. Both the biological samples required a decolourisation treatment before they could be successfully counted using the Liquid scintillation technique. Therefore, each sample could not be guaranteed as having the same appearance as the run before, hence there could be some possible variations in the resulting counts between samples and also test runs. This colour issue should be corrected by the colour quench curve but may still have had a small effect on the resulting counts.

The total concentration levels that have been reported in this work are theoretical levels based on calculations that have scaled up the results of the tracer study. This method of deduction is open to a certain level of error due to the calculation process which assumes a uniform mixing of DEHP in the *In-vitro* reservoir and a certain amount of uniformity in the distribution of in the tissues of each organ. Therefore, the actual levels may well be higher or lower than those reported in this work.

Despite these limitations, the results produced in this dissertation highlight that the migration of DEHP plasticiser from medical PPVC is very much a clinical problem. The migration into blood and plasma raise serious questions for those patients who undergo extracorporeal procedures such as ECMO and Dialysis. These concerns are heightened by the significant findings of the animal studies. There were levels of either DEHP or its more toxic metabolite, MEHP, detected in organs that were previously not considered as a target after the ex-vivo perfusion experiment. This indicated that persons receiving chronic exposures via extracorporeal exposures may be at much greater risk of adverse effects than previously thought.

8.3. Further Work

The story of DEHP migration from PPVC is a never ending one. The work presented here has shown that, whilst the use of ^{14}C labelled DEHP is an excellent way of tracing the migration of the DEHP from the material to its end point, it is however not the ideal answer to an easy and reproducible assay due to the vast quantity of "red tape" that limits its use.

Further work is required to develop a new technique that is not subjected to vast amounts of rules and regulations that govern its use, but would also allow for similar types of experimentation to be undertaken.

In an attempt to find this new technique, we investigated the use of NMR, Nuclear Magnetic Resonance, to identify and quantify the levels of DEHP in both methanol and plasma. The results were generally positive, with excellent sensitivity possible in both fluids, but the most pleasing result is the level of sensitivity of 5μM DEHP in plasma which was a much lower concentration than we expected to see with an un marked molecule. However, the ability to detect in liquidised tissue samples is an area that must be investigated further.

The unearthing of a technique that can track DEHP unhindered in both biological and tissue samples is paramount to allowing for further work into plasticiser migration being completed in this country.

This would then allow for more accurate levels of DEHP to be detected in both *In-vitro* but more importantly *Ex-vivo* studies. Whilst continuing to study DEHP migration, it would be prudent to start to include further studies on the migration of the current alternative plasticisers that are available in commercial medical products. This would allow for a better understanding of the potential levels of exposure to these chemicals. Further work must also be performed into the toxicology profiles of these alternative plasticisers to determine if they pose any similar toxicological threat that is currently associated with DEHP.

8.4. Final Conclusions

DEHP PPVC will continue to be utilised within many medical products until a suitably priced alternative can be found to replace it. There is no doubting the issues surrounding the migration of the DEHP from PPVC, but it is clear that the utilisation of the perfusion method of exposure is required to fully define and understand the possible toxicological effects of the chemical with regard to extracorporeal procedures.

The Carbon 14 radiolabel, whilst being an excellent method of determining the plasticiser migration from the PPVC, is not the answer to the current detection problem. It is too tightly regulated to fully capitalise on its potential as an assay.

Further investigation into other alternative methods of detection such as the NMR approach, will hopefully provide an assay method that can be successfully applied throughout academia and also industry to finally answer the riddle of DEHP and its side affects once and for all.

Bibliography

Adams RC. (2001) A Comparison of Plasticisers for Use in Flexible Medical Products. Medical Device and Diagnostics Industry. 23:4: p54-59

Aggarwal B.B, Geholt P. (2009) Inflammation and Cancer: How Friendly is the Relationship for Cancer Patients? Current Opinion in Pharmacology. 9(4):p351-369

Albro PW., Corbett JT., Schroeder JL., Jordan ST. (1983) Incorporation of Radioactivity from Labelled Di-(2-ethylhexyl) Phthalate into DNA of Rat Liver *InVivo*. Chemico.-Biological. Interactions. 44:p1-16.

Albro PW. (1986) Absorption, Metabolism, and excretion of di(2-ethylhexyl) phthalate by rats and mice. Environmental Health Perspectives. 45:293-298.

Albro PW., Corbett JT., Schroeder JL., Jordan S., Matthews HB. (1982) Pharmacokinetics, Interactions with macromolecules and species differences in metabolism of dehp. 45:19.25.

Albro PW, Thomas RO. (1973) Enzymatic hydrolysis of di-(2-ethylhexyl) phthalate by lipases. Biochimica et Biophysica Acta. 306:380-90.

Asberg AE, Videm V. (2005) Activation of Neutrophil Granulocytes in an In Vitro Model of a Cardiopulmonary Bypass. Artificial Organs. 29(12);927-936.

Asimakopoulos G., Taylor KM. (2000) Effects of cardiopulmonary bypass on leukocyte and endothelial adhesion molecules. The Annals of Thoracic Surgery. 69:1192-7.

Asimakopoulos G. (1999) Mechanisms of the systemic inflammatory response. Perfusion. 14:269.

Asimakopoulos G. (2001) Systemic inflammation and cardiac surgery: An update. Perfusion. 16:p353-360.

Asimakopoulos G, Gourlay T. (2003) A review of anti-inflammatory strategies in cardiac surgery. Perfusion. 18:p7-12.

Agency for Toxic Substances and Disease Registry (ATSDR). (1993) Toxicological Profile for Di(2-ethylhexyl)phthalate. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

AuBuchon JP, Estep TN and Davey RJ. (1988) The effect of the plasticizer di-2-ethylhexyl phthalate on the survival of stored RBCs. Blood. 71: 448-452

Autain J. Toxicity and health threats of phthalate esters: A review of the literature. Environmental Health Perspectives. 1973;4: p3-26.

Baker RWR, (1978) Diethylhexyl Phthalate as a factor in blood transfusion and haemodialysis. Toxicology. 9: p 319-329.

Barr DB., Silva MJ., Kato K., Reidy JA., Malek NA., Hurtz D., Sadowski M., Needham LL., Calafat AM. (2003) Assessing human exposure to phthalates using monoesters and their oxidized metabolites as biomarkers. Environmental Health Perspectives. 111: p1148-1151.

Bartlett K,. (1987) A Comparison of the elution of Di-ethylhexyl phthalate, Trimellitiate and Polymericadipate from Polyvinyl chloride by biological fluids. Unpublished report for Norsk Hydro.

Baumgartner HR., Muggli R., Tschopp TB., Turitto VT. (1976) Platelet adhesion, release and aggregation in flowing blood: effects of surface properties and platelet function. Thrombosis Haemostasis. 35: p124-38.

Belboul A., Lofgren C., Storm C., Jungbeck M. (2000) Heparin coated circuits reduce myocardial damage during CPB: a randomised, single blind clinical trial. European Journal of Cardiothoracic Surgery. 17: p580-586.

Blass CR. The application of Plasticised poly (vinyl) chloride materials for the collection and delivery of blood and blood components. A critical review. 1992. MPhil Thesis, University of Strathclyde, Glasgow.

Bone RC., Balk RA., Cerra FB et al. (1992) American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: Definition for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Critical Care Medicine. 20: p864-68.

Bornehag, C.G., Sundell, J., Weschler, C.J., Sigsgaard, T., Lundgren, B., Hasselgren, M., Hagerhed-Engman, L. (2004) The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case–control study. Environmental Health Perspectives. 112: p1393–1397.

Bowry SK. Development of invitro blood compatibility assessment procedures and evaluation of selected biomaterials. 1981. PhD Thesis, University of Strathclyde.

Brash JL & Lyman DJ. (1969) Adsorption of plasma proteins in solution to uncharged, hydrophobic polymer surfaces. Journal of Biomedical Material Research. 3: p175-189.

Buck RH., Scarborough DE., Saba SR., Brinkhous KM., Ikenberry LD., Kearney JJ., Clark HG. (1969) Thrombogenicity of some biomedical materials: Platelet-interface reactions. Journal of Biomedical Materials Research. 3: p615-644.

Burkhart HM, Joyner N, Niles S, et al. (2007) Presence of plasticizer di-2(ethylhexyl)phthalate in primed extracorporeal circulation circuits. ASAIO American Journal of Artificial Internal Organs. 53(3): p365-7.

Butler J., Rocker GM., Westaby S. (1993) Inflammatory response to cardiopulmonary bypass. The Annals of Thoracic Surgery. 55: p552-59.

Calabrese V, Cornelius C, Mancuso C, Lentile R, Stella AM, Butterfield DA,. (2010) Redox Homeostasis and Cellular Stress Response in Aging and Neurodegeneration. Methods in Molecular Biology. 610: p285-308.

Calafat AM, Needham LL, Silva MJ, Lambert G. (2004a) Exposure to di-(2-ethylhexyl) phthalate among premature neonates in a neonatal intensive care unit. Pediatrics.113: p429-34.

Cattley, RC., DeLuca J., Elcombe, C., Fenner-Crisp P., Lake BG, Marsman, DS., Pastoor TA., Popp, JA., Robinson, DE., Schwetz, B., Tugwood, J., and Wahli, W. (1998) Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? Regulatory Toxicology and Pharmacology. 27; p47-60

CERHR (Center for the Evaluation of Risks to Human Reproduction) National Toxicology Program-CERHR Expert Panel Update on the Reproductive and Developmental Toxicity of Di(2-ethylhexyl) phthalate, 2005. Available at http://cerhr.niehs.nih.gov/news/index.html

Chawla AS, Hinberg, I. (1991) Leaching of Plasticisers from and Surface Characterisation of PVC Blood Platelet Bags. Biomaterial, Artificial Cells & Immobilization Biotechnology. 19(4): p761-783.

Chawla LS., Krishnan M. (2009) Causes and consequences of inflammation on anaemia management in haemodialysis patients. Haemodialysis International. 13(2):p222-234.

Chenoweth, DE. (1986) Complement Activation produced by Biomaterials, Transaction of American Society of Artificial Organs. 32: p226-232.

Colman RW., Hirsch J., Marder VJ., Salzman EW. Haemostasis and Thrombosis, 3rd Ed. 1994. Lippincott, New York, p1-660.

Courtney JM., Lamba NMK., Sundaram S., Forbes CD. (1994a) Biomaterials for blood contacting applications. Biomaterials. vol 15;10: p737

Courtney JM & Forbes CD. (1994b) Thrombosis on foreign surfaces. British Medical Bulletin. 50: vol 4:966-981.

Courtney JM, Sundaram S, Matata BM, Gaylor JDS, Forbes C. (1994c) Biomaterials in Cardiopulmonary Bypass. Perfusion. Vol 9: p3.

Courtney JM., Lamba NMK., Gaylor JDS., Ryan CJ., Lowe GDO. (1995) Blood-contacting biomaterials: bioengineer viewpoints. Artificial Organs. 19(8):852-856.

Courtney JM, Zhao XB, Qian H, Sharma A. (2003) Modification of polymer surfaces: optimization of approaches. Perfusion. vol 18, p33.

CSTEE (Scientific Committee on Toxicity, Ecotoxicity and the Environment). 26-27 November 1998, Phthalate migration from soft PVC toys and children-care articles.

CSTEE, (Scientific Committee on Toxicity, Ecotoxicity and the Environment). (1999) Opinion on 'The toxicological characteristics and risks of certain citrates and adipates used as a substitute for phthalates as plasticisers in certain soft PVC products, European Commission, Adopted on 28th Sept, 1999, Brussels, Belgium.

CSTEE (Scientific Committee on Toxicity, Ecotoxicity and the Environment). Opinion on The results of the Risk Assessment of bis (2-ethylhexyl) phthalate (DEHP). Report version Human Health, September 2001. Scientific Committee on Toxicity, Ecotoxicity and the Environment, European Commission,

Adopted on 9th Jan 2002, Brussels, Belgium.

Daniel JW., Bratt H., (1974) The absorption, metabolism and tissue distribution of Di-(2-ethylhexyl) Phthalate in rats. Toxicology. 2: p51-65.

David MR, Moore MR, Finney Dc, Guest D. (2000a) Chronic toxicity of di(2-ethylhexyl) phthalate in rats. Toxicological Sciences. 55: p433-443.

David MR, Moore MR, Finney Dc, Guest D. (2000b) Chronic toxicity of di(2-ethylhexyl) phthalate in mice. Toxicological Sciences. 58: p377-385.

Denes A, Thornton P, Rothwell NJ, Allan SM. (2009) Inflammation and brain injury: Acute cerebral ischemia, peripheral and central inflammation. Brain Behaviour and Immunity. In press.

Dine T, Luyckx M, Cazin M, Brunet C, Gondaliez F, Cazin JC. (1991) Rapid Determination by high-performance liquid chromatography of di-2-ethylhexyl phthalate in plasma stored in plastic bags. Biomedical Chromatography. 15: p94-97.

Doull J, Cattley R, Elcombe C, Lake BG, Swenberg J, Wilkinson C, et al. (1999) A cancer risk assessment of di(2-ethylhexyl)phthalate: application of the new U.S. EPA Risk Assessment Guidelines. Regulatory Toxicology and Pharmacology. 29: p327-57.

ECB (European Chemicals Bureau), (2001) Risk assessment bis(2-ethylhexyl) phthalate. Consolidated Final Report Institute for Health and Consumer Protection, Joint Research Centre, Ispra, Italy, (Doc R042 0109 env hh 0-3, and Doc R042 0109 env hh 4-6)

Elahi MM., Yii M., Matata BM. (2008) Significance of oxidants and inflammatory mediators in blood of patients undergoing cardiac surgery. Journal of Cardiothoracic and Vascular Anaesthesia. 22: p455-467.

Elwing H., Welin S., Askendal A., Nilson U., Lundstrom I. (1987) A wettability gradient method for studies of macromolecular interaction at the liquid/solid interface. Journal of Colloid and Interface Science. 119: p203-209.

Erdman SE, Poutahidis T., (2009) Roles for Inflammation and Regulatory T Cells in Colon Cancer. Toxicologic Pathology. Dec 17.

Estep TN., Pederson RA., Miller TJ., Stuar KR. (1984) Characterisation of erythrocyte quality during the refrigerated storage of whole blood containing di-(2ethylhexyl) phthalate. Blood. 64: p1270-1276.

Faouzi MA. et al. (1999) Exposure of hemodialysis patients to di-2ethylhexyl phthalate. International Journal of Pharmaceutics. 180: p113-121.

Fazekas G., Fazekas F., Schmidt R., Kapellar P., Offenbacher H., Krejs GJ. (1995) Brain findings and cognitive impairment in patients undergoing chronic haemodialysis treatment. Journal of the Neurological Sciences. 134(1-2): p83-88.

Favoreell, HW., Van der Walle, GR., Nauwynck, HJ. And Pensaert, MB. (2003) Virus complement evasion strategies. Journal of General Virology. 84: p1-15.

FDA Safety assessment of di(2-ethylhexyl) phthalate (DEHP) released from medical devices. (2000) Centre for devices and radiological health, US Food and Drug Administration, Rockville, MD, USA.

FDA Public Health Notification: PVC Devices Containing the plasticizer DEHP (2002). Centre for devices and radiological health, US Food and Drug Administration, Rockville, MD, USA.

French G. In-vitro assessment of plasticiser extraction rates. 2001. MSc Thesis, University of Strathclyde.

Forbes CD., Courtney JM. (1994) Thrombosis and artificial surfaces in “Haemostasis and Thrombosis”. Bloom AL., Thomas DP., Forbes CD., Tuddenham EGD. Edinburgh, Churchill Livingstone, pp1301-1324.

Foster PMD. (2006) Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. International Journal of Andrology. 24;140-147.

Franke A., Lante W., Fackaldey V. et al. (2002) Proinflammatory and anti-inflammatory cytokines after cardiac operation: Different cellular sources at different times. The Annals of Thoracic Surgery. 105: p1429-1435.

Frederiksen H., Skakkebaek NE., Andersson AM. (2007) Metabolism of phthalates in humans. Molecular Nutrition and Food Research. 51: p899-911.

Friocourt MP, Picart D, Saleun JP, Floch HH.(1979) Relargage du di(ethyl-2 hexyl) phthalate dans le sang conserve au contact du chlorure de polyvinyle. Revue Francaise de Transfusion et Immuno-hematologie. 22(4): p343-358.

Galinanes M., Watson C., Trivedi U., Chambers DJ., Young CP., Venn GE. (1996) Differential patterns of neutrophil adhesion molecules during cardiopulmonary bypass in humans. Circulation. 94: p364-9.

Gandemer G., Pascal G., Durand G. (1983) Lipogenic capacity and relative contribution of the different tissues and organs to lipid synthesis in male rat. Reproduction, Nutrition, Development. 23(3); p575-586.

Gayathri NS., Dhanya CR., Indu AR., Kurup PA.(2004) Changes in some hormones by low doses of di(2-ethylhexyl) phthalate (DEHP), a commonly used plasticiser in PVC blood storage bags & medical tubing. Indian Journal of Medical Research. 119(4): p139-44.

George, S. M., An in vitro evaluation of the biocompatibility of cyclodextrin modified PVC biomaterials 2007. PhD Thesis University of Strathclyde, Glasgow.

Glue, C., Platzer, M.H., Larsen, S.T., Nielsen, G.D., Skov, P.S., Poulsen, L.K. (2005) Phthalates potentiate the response of allergic effectors cells. Basic Clinical &. Pharmacology and Toxicology. 96, 140–142.

Gollamudi R., Prasanna HR., Rao RH., Lawrence WH., et al. (1983) Impaired metabolism of di(2-ethylhexyl) phthalate in the rat. Biochimica et Biophysica Acta. 760; p283-292.

Gorbet, MB., Sefton, MV. (2004) Bi-material associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials. 25: p5681-5703.

Gotardo MA, Monteiro M. (2005) Migration of diethylhexyl phthalate from PVC bags into intravenous cyclosporine solutions. Journal of Pharmaceutical and Biomedical Analysis. 38: p709-713.

Gott, VL., Whiffnen, JD., Olsson, P. (1963) Heparin Bonding on colloidal graphite surfaces. Science. 142: p1297-1298.

Gourlay T. (2001a) Biomaterial Development for Cardiopulmonary Bypass. Perfusion. 16: p381.

Gourlay T, Stefanou DC, Asimkopoulos G, Taylor KM. (2001b) The effect of circuit surface area on CD11b(mac-1) expression in a rat recirculation model. Artificial Organs. 25: 475-79.

Gourlay T, Stefanou D, Taylor KM. (2002) The effect of methanol washing of plasticized polyvinyl chloride on biomaterial-contact-mediated CD11b (mac-1) expression in a rat recirculation model. Artificial Organs. 26: p5-9.

Gourlay T, Samartzis I, Stefanou DC, Taylor KM. (2003) Inflammatory response of rat and human neutrophils exposed to di-(2-ethylhexyl)-phthalate and di-(2-ethyl-hexyl)-phthalate plasticized polyvinyl chloride. Artificial Organs. 27: p256-60

Gouzy MF, Sperling C, Salchert K, Pompe T, Streller U, Uhlmann P, Rauwolf C, Simon F, Bohme F, Voit B, Werner C. (2004) In vitro blood compatibility of polymeric biomaterials through covalent immobilization of amidine derivative. Biomaterials. 25: p3493-3501.

Gourlay T., Shedden L., Horne D., Stefanou DM. (2010) Simple surface sulfonation retards plasticiser migration and impacts upon blood/material contact activation processes. Perfusion. Jan;25(1):31-9.

Han J, Beeton A, Long P, Karimova A, Robertson A, et al. (2005) Plasticizer di(2-ethylhexyl)phthalate (DEHP) release in wet-primed extracorporeal membrane oxygenation (ECMO) circuits. International Journal of Pharmaceutics. 294(1-2): p157-9.

Hanawa T, Muramatsu E, Asakawa K, Suzuki M, Tanaka M, Kawano K, Seki T, Juni K, Nakajima S. (2000) Investigation of the release behaviour of diethylhexyl phthalate from polyvinyl-chloride tubing for intravenous administration. International Journal of Pharmaceutics . 210: p109-115.

Haishima Y, Matsuda R, Hayashi Y, Hasegawa C, Yagami T, Tsuchiya T. (2004) Risk assessment of di(2ethylhexyl) phthalate released from PVC blood circuits during haemodialysis and pump oxygenation therapy. International Journal of Pharmaceutics. 274: p119-129.

Hansen OG. (2006) PVC and Phthalates in Medical Devices: A never Ending Story. Medical Device Technology. 17(3): p16-18..

Hasegawa R., Koizumi H., Hirose A. (2004) Principles of risk assessment for determining the safety of chemicals: recent assessment of residual solvents in drugs and di)2-ethylhexyl) phthalate. Congenital Anomalies. Jun;44(2): p51-9.

Health Canada. (2002) An exposure and toxicity assessment. Medical Device Bureau, Therapeutic Products Directorate, Health Products and Food Branch, Ottawa, Canada.

Heindel JJ., Powell CJ. (1992) Phthalate ester effects on rat Sertoli cell function in vitro: Effects of phthalate side chain age of animal. Toxicology and Applied Pharmacology. 115: p116-123.

Hildenbrand SL, Lehmann HD, Wodarz R, Ziemer G, Wendel P. (2005) PVC-plasticiser DEHP in Medical Products: Do thin coatings really reduce DEHP leaching into blood? Perfusion. 20: p351-357.

Hillman LS., Goodwing SL., Sheman WR. (1975) Identification and measurement of plasticiser in neonatal tissues after umbilical catheters and blood products. New England Journal of Medicine. 292: p381-386.

Horne DC, Torrance I, Modine T, Gourlay T. (2009) The Effect of Priming Solutions and Storage Time on Plasticiser migration in different PVC tubing types-Implications for Wet Storage of ECMO Systems. Journal of Extracorporeal Technologies. 41(4): p199-205.

IARC (International Agency for Research on Cancer) (2000) Monograph on the evaluation of carcinogenic risk to humans, Some industrial chemicals, Di(2-ethylhexyl) phthalate.77, p41-148.

Inoue K, Kawaguchi M, Yamanaka R, Higichi T, Rie I, Saito K, Nakazawa H. (2005)Evaluation and analysis of exposure levels of di(2-ethylhexyl) phthalate from blood bags. Clinica Chemica Acta. 358:p159-166.

Ito R, Seshimo F, Haishima, Hasegwa C, Isama K et al. (2005) Reducing the migration of di-2-ethylhexyl phthalate from polyvinyl chloride medical devices. International Journal of Pharmaceutics. 303:p104-112.

ISO/TC194 Committee Draft 194 N50, Biological testing of material and dental materials and devices, Part 4: Tests for interactions of devices with blood. 1994.

Jaakkola, J.J., Oie, L., Nafstad, P., Botten, G., Samuelsen, S.O., Magnus,P., (1999) Interior surface materials in the home and the development of bronchial obstruction in young children in Oslo, Norway. American Journal of Public Health. 89, 188–192.

Jaakkola, J.J., Parise, H., Kislitsin, V., Lebedeva, N.I., Spengler, J.D. (2004) Asthma, wheezing, and allergies in Russian schoolchildren in relation to new surface materials in the home. American Journal of Public Health. 94, 560–562.

Jaakkola, J.J., Ieromnimon, A., Jaakkola, M.S. (2006) Interior surface materials and asthma in adults: a population-based incident casecontrol study. American Journal of Epidemiology. 164, 742–749.

Jaeger RJ. (1971) Studies on the extraction, accumulation and metabolism of phthalate ester plasticisers from polyvinyl chloride medical devices. PhD Thesis, The John Hopkins University.

Jaeger RJ, Rubin RJ. (1970) Plasticizers from plastic Devices: Extraction, Metabolism, and Accumulation by Biological Systems. Science. 170: 460-462.

Jaeger RJ, Rubin RJ (1972) Leakage of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its localization in human tissues. New England Journal of Medicine. 287:1114-1118.

Jaeger RJ., Rubin RJ. (1973) Extraction, localisation and metabolism of di-2ethylhexyl phthalate from PVC plastic medical devices. Environmental Health Perspectives. 95-101.

Jaeger RJ, Weiss AL, Brown K. (2005) Infusion of di-2-ethylhexylphthalate for neonates: a review of potential health risk. Journal of Infusion Nursing. 28:54-60.

Jones C. (1989) Blood response to plasticised poly(vinyl chloride). PhD Thesis, University of Strathclyde.

Kambia K, Dine T, Azar R, Gressier B, Luyckx M, Brunet C. (2001) Comparative Study of the leachability of di(2-ethylhexyl) phthalate and tri(2-ethylhexyl) trimellitate from haemodialysis tubing. International Journal of Pharmaceutics. 229: 139-146.

Kambia K, Dine T, Gressier B, Bah S, Germe AF, Luyckx M, et al. (2003) Evaluation of childhood exposure to di(2-ethylhexyl) phthalate from perfusion kits during long-term parenteral nutrition. International Journal of Pharmacceutics. 262:83-91.

Karle VA, Short BL, Martin GR, et al. (1997) Extracorporeal membrane oxygenation exposes infants to the plasticizer, di(2-ethylhexyl)phthalate. Critical Care Medicine. 25(4):696-703.

Kavlock R, Boeckelheide K, Chapin R, Cunningham M, Faustman E, Foster P et al. (2002) NTP Centre for the evaluation of risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reproductive Toxicology 16: 529-653.

Kaysen GA. (2004) Inflammation: Cause of vascular disease and malnutrition in dialysis patients. Seminars in Nephrology. 24(5):p431-436.

Kevy SV., Jacobson MS. (1982) Hepatic Effects of a Phthalte Ester Plasticiser Leached from Poly(Vinyl Chloride) Blood Bags Following Transfusion. Environmental Health Perspectives. 45;57-64.

Kicheva, YI., Kostov, VD., Chichovska, M. (1995) Invitro and in vivo studies of the effect of the concentration of plasticiser di (2-ethylhexyl) phthalate on the blood compatibility of plasticised poly(vinyl chloride) drain tubes. Biomaterials. 16;p575-579.

Kim SW, Petersen RV, Lee ES. (1976) Effect of Phthalate Plasticiser on Blood Compatibility of Poly vinyl Chloride. Journal of Pharmaceutical Sciences. 65(5):p670-673.

Koch HM, Angerer J, Drexler H, Eckstein R, Weisbach V. (2005) Di(2ethylhexyl) phthalate (DEHP) exposure of voluntary plasma and platelet donors. International Journal of. Hygiene and Environmental-Health. 208:p489-498.

Koch HM., Angerer J., Drexler H., Eckstein R., Weisbach V. (2006) Di(2-ethylhexyl) phthalate (dehp): human metabolism and internal exposure- an update and latest results. International Journal of Andrology. 29(1):155-65.

Koop E C, et al. (1999) Blue Ribbon Panel Report: *A Scientific Evaluation of Health Effects of Two Plasticizers Used in Medical Devices and Toys*. American Council on Science and Health. June 22, 1999.

Krishnan AV., Kiernan MC. (2005) Neurological complications of chronic kidney disease. Nature Reviews. Neurology. 5(10):p542-551.

Kurata Y, Kidachi F, Yokoyama M, Toyota N, Tsuchitani M, Katoh M, (1998) Subchronic toxicity of di(2-ethylhexyl)phthalate (DEHP) in common marmosets: Lack of hepatic peroxisomal proliferation, testicular atrophy, or pancreatic acinar cell hyperplasia. Toxicological Sciences. 42: 49-56.

Kuwahira I., Gonzalez NC., Heisler N., Piiper J. (1993) Regional blood flow in conscious resting rats determined by microsphere distribution. Journal of Applied Physiology. 74(1):p203-210.

Lakshmi S, Jayakrishnan A. (1998) Migration resistant, Blood Compatible Plasticized Polyvinyl Chloride for Medical and Related Applications. Artificial. Organs. 22(3):222-229.

Lamba NMK. (1994) Blood-Biomaterial interactions: application of a parallel plate flow system to study blood responses in-vitro. PhD Thesis. University of Strathclyde, Glasgow.

Lamba NMK., Gaylor JDs., Courtney JM., Lowe GD. (1998) Complement activation by cellulose: investigation of the effects of time, area, flow rate, shear rate and temperature on C3a generation in vitro using a parallel plate flow cell. Journal of Material Science. Materials in Medicine. 9(7):409-414.

Larsen, S.T., Lund, R.M., Nielsen, G.D., Thygesen, P., Poulsen, O.M. (2001) Di-(2-ethylhexyl) phthalate possesses an adjuvant effect in a subcutaneous injection model with BALB/c mice. Toxicology Letters. 125:11–18.

Larsen, S.T., Lund, R.M., Nielsen, G.D., Thygesen, P., Poulsen, O.M. (2002) Adjuvant effect of di-n-butyl-, di-n-octyl-, di-iso-nonyl and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c mice. Pharmacology and Toxicology. 91: 264–272.

Larsen ST., Hansen JS., Hansen EW., Clausen PA., Nielsen GD. (2007) Airway inflammation and adjuvant effect after repeated airborne exposures to di-(2-ethylhexyl) phthalate and ovalbumin in BALB/c mice. Toxicology. 235:119-129.

Lassen B., Malmsten M. (1997) Competitive Protein Adsorption at Plasma Polymer Surfaces. Journal of Colloid Interface Science. Feb 1;186(1):9-16.

Latini G, Avery GB. (1999) Materials degradation in endotracheal tubes: a potential contributor to bronchopulmonary dysplasia. Acta Paediatrica. 88:1174-5.

Lawson JH., Kalafatis M., Stram S., Mann KG. (1994) A model for the tissue factor pathway to thrombin. An empirical study. Journal of Biological Chemistry. 269:23357-66.

Lee Es., Kim SW. (1979) The role of adsorbed proteins in platelet adhesion onto polymer surfaces. Journal of Polymer Science. 66;p429-441.

Lee YK, Park JH, Moon HT, Lee DY, Yun JH, Byun Y. (2007) The short term effects on restenosis and thrombosis of echinomycin-eluting stents topcoated with a hydrophobic heparin-containing polymer. Biomaterials. 28:1523-1530.

Lhuguenot JC., Mitchell AM., Milner G., Lock EA., Elcombe CR. (1985) The metabolism of di(2-ethylhexyl) phthalate (dehp) and mono-(2-ethylhexyl) phthalate (mehp) in rats: In-vivo and in-vitro dose and time dependency of metabolism. Toxicology and applied pharmacology. 80:11-22.

Loff S, Kabs F, Witt K, Sartoris J, Mandl B, Niessen KH, Waag KL. (2000) Polyvinylchloride infusion lines expose infants to large amounts of toxic plasticizers. Journal of Pediatric Surgery. 35:1775-81.

Loff S, Kabs F, Subotic U, Schaible T, Reinecke F, Langbein M. (2002) Kinetics of diethylhexylphthalate extraction From polyvinylchloride-infusion lines. JPEN J Parenteral and Enteral Nutrition 26:305-9.

Loff S, Subotic U, Reinicke F, Wischmann H, Brade J. (2004) Extraction of Di(2-ethylhexyl) Phthalate from Perfusion lines of Various Material, Length and Brand by Lipid Emulsions. Journal of Paediatric Gastroenterology and Nutrition. 39:p341-345.

Lyman DJ., Klein KG., Brash JL. Fritzinger BK. (1979) The interaction of platelets with polymer surfaces. Thrombosis et Diathesis Haemorrhagica. 23:120-128.

Marcel YL., Noel SP. (1970) Contamination of Blood stored in plastic packs. Lancet 1:p35-36.

Marcel YL. (1973) Determination of di-2-ethylhexyl phthalate in human blood plasma cryoprecipitates. Environmental Health Perspectives. 3:119-121.

Marchant RE., Miller KM., Anderson JM. (1984) In-vivo biocompatibility studies, invivo leukocyte interaction with biomers. Journal of Biomedical Material Research. 18:1169-1190.

Martini FH. (2006a): Fundamentals of Anatomy & Physiology (7th Edition) Pearson Educational. Chapter 19: Blood. P660

Martini FH. (2006b): Fundamentals of Anatomy & Physiology (7th Edition) Pearson Educational. Chapter 19: Blood. P654-660.

Martini FH. (2006c): Fundamentals of Anatomy & Physiology (7th Edition) Pearson Educational. Chapter 22: The Lymphatic System: Complement. P779-780.

Masoro EJ. (1967) Skeletal Muscle Lipids- Analysis of the functioning of skeletal muscle lipids during fasting. The Journal of Biological Chemistry. 242(6);1111-1114.

Matthews G. (1996): PVC, Production, Properties and Uses. The Institute of Materials, Uk. Chapter 6: Plasticisation. P87,89.

Melnick, RL. (2001) Is Peroxisome Proliferation an Obligatory Precursor Step in the Carcinogenicity of Di(2-ethylhexyl)phthalate (DEHP)? Environmental Health Perspectives 109:437–442.

Missirlis YF. (1992) How to deal with the complexity of the blood polymer interactions. Clinical Materials. 11:p9-12.

Moen O., Hogasen K., Fosse E., Dregelid E. Brockmeier V., Venge P., Harboe M., Mollnes TE. (1997) Attenuation of changes in leukocyte surface markers and complement activation with heparin coated cardiopulmonary bypass. The Annals of Thoracic Surgery. 63:106-11.

Mohandas N., Hochmuth RM., Spaeth EE. (1974) Adhesion of red cells to foreign surfaces in the presence of flow. Journal of Biomedical Material Research. 8:p119-136.

Moody, DE. and Reddy, JK. (1978) Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. Toxicology and Applied Pharmacology. 45:497-504.

Moore RW, Rudy TA, Lin T-M, Ko K and Peterson RE. (2001) Abnormalities of sexual development in male rats with *in utero* and lactational exposure to the androgenic plasticizer di(2-ethylhexyl) phthalate. Environmental Health Perspectives. 109; p229-237.

Mosesson MW. (1990) Fibrin polymerisation and its regulatory role in haemostasis. Journal of Laboratory Clinical Medicine. 116:p8-17.

Murray AM. (2008) Cognitive impairment in the aging dialysis and chronic kidney disease populations: An occult burden. Advances in chronic kidney disease. 15(2):p123-132.

Nakamura, K. (1975) Dynamic Mechanical Properties of Plasticised Poly(vinyl) Chloride. Journal of Polymer Science. 13: p137-149.

Niino T, Ishibashi T, Itho T, Sakai S, Ishiwata H, Yamada T, et al. (2001) Monoester formation by hydrolysis of dialkyl phthalate migrating from polyvinyl chloride products in human saliva. Journal of health science. 47:318-322.

Norde W. (1996) Driving Forces for protein adsorption at solid surfaces. Macromolecules Symposim. 103:5-18.

Olivencia-Yurvati AH., Wallace N, Ford S., Mallet RT. (2004) Leukocyte filtration and aprotinin: synergistic anti-inflammatory protection. Perfusion. 19:13

Ohri SK. (1996) Systemic inflammatory response and the splanchnic bed in cardiopulmonary bypass. Perfusion. 11:200-12.

Packham MA., Evans G., Glynn MF., Mustard JF. (1969) The effect of plasma proteins on the interaction of platelet with glass surfaces. Journal of Laboratory Clinical Medicine. 73:p686-697.

Peters JM, Taubeneck MW and Keen CL. (1997) DEHP induces a functional zinc deficiency during pregnancy and teratogenesis that is independent of peroxisome proliferator-activated receptor-alpha. Teratology. 56, 311-316.

Piperi C, Themistocleous MS, Papavassiliou GA, Farmaki E, Levidou G, Korkolopoulou P, Adamopoulos C, Papavassilliou AG. Molecular Medicine. 2009;Oct 7

Piskin E. (1992) Biologically modified polymeric surfaces. Elsevier Applied Sciences. P1.

Pitt WG., park K., Cooper SL. (1986) Sequentila protein adsorption and thrombus deposition on polymeric biomaterials. Journal of Colloid Interface Science. 111:p343-362.

Pollack GM., et al. (1985) Circulating concentrations of di-(2-ethylhexyl) phthalate and its de-estrified phthalic acid products following plasticiser exposure in patients receiving haemodialysis. Toxicology and Applied Pharmocology. 79:257-67.

Poon R, Lecavalier P, Mueller R, Valli VE, Procter BG, Chu I. (1997) Subchronic Oral Toxicity of Di-n-octyl Phthalate and Di(2-Ethylhexyl) Phthalate in the Rat. Foot and Chemical Toxicology. 35:p225-239.

Rahman M, Brazel CS. (2004) The plasticiser market: an assessment of traditional plasticisers and research trends to meet new challenges. Progress in Polymer Science. 29:p1223-1248.

Ratner BD. (2007) The catastrophe revisited: blood compatibility in the 21st Century. Biomaterials. Dec;28(34):5144-7.

Rauch D, Gross S, Harding J, Bokhari S, Niewiesk S, Lairmore M, Piwnica-Worms D, Ratner L. (2009) T-Cell Activation Promotes Tumorigenesis in Inflammation-associated Cancer. Retrovirology. Dec 17;6(1): p116

Reading PC., Morey LS., Crouch EC., Anders EM. (1997) Collectin mediated antiviral host defence of the lung: evidence from influenza infection of mice. Journal of Virology. 71:8204-8212.

Rhodes C, Orton TC, Pratt IS, Batten PL, Bratt H, Jackson SJ, Elcombe CR. (1986) Comparative pharmacokinetics and sub acute toxicity of di(2-ethylhexyl) phthalate

(DEHP) in rats and marmosets: extrapolation of effects in rodents to man. Environmental Health Perspectives. 65:299-307.

Riley BJ, Sapatnekar S, Cornell DJ, Anderson J, Walsh-Sukys MC. (1997) Impact of prolonged saline solution prime exposure on integrity of extracorporeal membrane oxygenation circuits. Journal of Perinatology. 17:444-9.

Rimon R., Lehtonen J., Scheinin TM. (1970) Psychiatric disturbances after cardiovascular surgery. 31(5):326-328.

Robertson L. (1988) Blood compatibility of modified biomaterials: application of selected in-vitro and ex-vivo procedures. PhD Thesis. University of Strathclyde, Glasgow.

Rock G, Tocchi M, Ganz PR, Tackaberry ES (1984) Incorporation of plasticizer into red cells during storage. Transfusion. 24:493–498

Rock G, labow RD, Tocchi M. (1986) Distribution of di(2-ethylhexyl) Phthalate and products in blood and blood components. Environmental Health Perspectives. 65: 309-316.

Rusyn I, Peters JM, Cunningham ML. (2006) Mode of action and species-specific effects of di-(2ethylhexyl) phthalates in the liver. Critical Reviews in Toxicology 36:459-79.

Santin, M., Mikhalovska, L., Lloyd, AW., Mikhalovsky, S., Sigfrid, L., Denyer, SP., Field, S., Teer, D. (2004) Invitro host response assessment of biomaterials for cardiovascular stent manufacture. Journal of Materials Science: Materials in Medicine. 15:p473-477.

Sasaki K., Tsutsumi A., Wakamiya N., Ohtani K., Suzuki Y., Watanabe Y., Nakayama N., Kolke T. (2000) Monnose-binding lectin polymorphisms in patients

with hepatitis C virus infection. Scandinavian Journal of Gastroenterology.35:960-965.

Sathyanarayana S. (2008) Phthalates and Childrens Health. Current Problems in Paediatric and adolescent Healthcare. 38:p34-49.

Sawyer PN., Pater JW. (1953) Bioelectric phenomena as an etiologic factors in intravascular thrombosis. Surgery. 34:p491-500.

SCENIHR (Scientific Committee on Emerging and Newly- Identified Health Risks) Opinion on The Safety of Medical Devices containing DEHP- Plasticized PVC or Other Plasticizers on Neonates and other groups possibly at risk. 6th February 2008. Available at http://ec.europa.eu/health/ph risk/committees/04 scenihr o 014.pdf

Schettler T. (2006) Human Exposure to phthalates via consumer products. International Journal of Andrology. 29:p134-139.

Schmid P., Schlatter C. (1985) Excretion and metabolism of di(2-ethylhexyl) phthalate in man. Xenobiotica.15:251-256.

Sears JK., Darby JR. (1982a) The technology of plasticisers. John Wiely & sons Inc. Chapter 2: Mechanism of Plasticiser Action: Part 2: The Gel Theory, p39-42

Sears JK., Darby JR. (1982b) The technology of plasticisers. John Wiely & sons Inc. Chapter 2: Mechanism of Plasticiser Action: Part 4: The Free Volume Theory, p43 & 46.

Sharma CP. (1981) Surface Energy and interfacial parameters of synthetic polymers to blood compatibility. Biomaterials. 2:p57-59.

Sharma A. (2002) Plasticised poly (vinyl chloride): plasticiser selection, plasticiser migration and surface modification. MRes Thesis University of Strathclyde, Glasgow.

Shea KM. (2003) Paediatric Exposure and Potential Toxicity of Phthalate Plasticisers. Pediatrics.111: p1467-1474.

Shen, M., Horbett, TA. (2001) The effects of surface chemistry and adsorbed proteins on monocyte/macrophage adhesion to chemically modified polystyrene surfaces. Journal of Biomedical Materials Research. 57(3):p336-345.

Silva MJ, Samandar E, Preau Jr JL, Needham LL, Calafat AM. (2006) Urinary oxidative metabolites of di(2ethylhexyl) phthalate in humans. Toxicology 219:p22-32.

Sjoberg P, Bondesson U, Kjellen L, Lindquist N.G, Montin G. Ploen,L, (1985a) Kinetics of di-(2-ethylhexyl) phthalate in immature and mature rats and effect on testis. Acta Pharmacol.Toxicol.(Copenh). 56:30-7.

Sjoberg P, Lindqvist NG, Monti G, and Ploen L, (1985b) Effects of repeated intravenous infusions of the plasticizer di-(2-ethylhexyl) phthalate in young male rats. Archives of Toxicology. 58, 78-83,

Sreenivasan K. (1996) Effect of Blending β-cyclodextrin with poly (vinyl chloride) on the leaching of phthalate ester to hydrophilic medium. Journal of Applied Polymer Science. 56:2089-2093.

Stefanou DC. (2006) Investigation of the Inflammatory response to di(2-ethylhexyl) phthalate plasticised polyvinylchloride used in cardio pulmonary bypass. MD Thesis, Imperial College London.

Stern IJ., Miripol JE., Rodger S., Lueck JD. (1977) Physiochemical Aspects of the Extraction in Blood and the Disposition in Rats of Di-(2ethylhexyl) Phthalate Plastcizer. Toxicology and Applied Pharmacology. 43(3):507-522.

Stern IJ, Carmen RA: (1980) Haemolysis in stored blood: Stabilizing effect of phthalate plasticiser. Joint Meeting, 18th Congress International society of Haematology. p151.

Supornsilchai V., Soder O., Svechnikov K. (2007) Stimulation of the pituitary-adrenal axis and of adrenocortical steroidogenesis exvivo by administration of di-2ethylhexyl phthalate to prepubertal male rats. Journal of Endocrinology. 192(1):33-9.

Suzuki T., Kado S., Ando M., Nagata Y., Iwata S., Kobayashi T., Uchida K. (2009) Spontaneous Cardiac Hypertrophy in a Crl CD(SD) Rat. Journal of Toxicological Pathology. 22:83-87.

Svenmarker S., Sandstrom E., Karlsson T., Jansson E., Haggmark S., Lindholm R., Appelblad M., Aberg T. (1997) Clinical effects of heparin coated surface in cardiopulmonary bypass. European Journal of Cardiothoracic Surgery. 11:957-964.

Swan SH., Liu F., Hines M., Kruse RL., Wang C., Redmon JB., Sparks A., Weiss B. (2009)Prenatal phthalate exposure and reduced masculine play in boys. International Journal of Andrology. 32:p1-9.

Takahashi Y, Shibata T, Sasaki Y, Fujii H, Bito Y, Suehiro S. (2008) Di(2-ethylhexyl) Phthalate exposure during cardiopulmonary bypass. Asian Cardiovascular Thoracic Surgery Annals. 16:4-6.

Takatori S., Kitagawa Y., Kitagawa M., Nakazawa H., Hori S. (2004)Determination of di(2-ethylhexyl) phthalate and mono(2-ethylhexyl) phthalate in human serum using liquid chromatography-tandem mass spectrometry. Journal of

Chromatography B. Analytical technologies in the Biomedical Life Sciences. 804(2):397-401.

Takatori S., Okamoto Y., Kitagawa Y., Hori S., Izumi S., Makino T., Nakazawa H. (2008) Simulated neonatal exposure to dehp and mehp from pvc enteral nutrition products. International Journal of Pharmaceutics. 352(1-2):139-45.

Tanaka A., Adachi T., Takahashi T., Yamaha T. Biochemical studies on phthalic esters 1. (1975) elimination, distribution and metabolism of di-(2-ethylhexyl) phthalate in rats. Toxicology. 4:p253-264.

Tang, L., Eaton, J. (1993) Fibrin(ogen) mediates acute inflammatory responses to biomaterials. Journal of Experimental Medicine. 178:p2147-2156.

Tang H., Vassell JR., Wu EX., Boozer CN., Gallagher D. (2002) High resolution magnetic resonance imaging tracks changes in organ and tissue mass in obese and aging rats. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 282:890-899.

Tickner JA, Schettler T, Guidotti T, McCally M, Rossi M. (2001) Health risks posed by use of di-2-ethylhexyl phthalate (DEHP) in PVC medical devices: A critical review. American Journal of Industrial Medicine 39: p100-111.

Treleano A, Wolz G, Brandsch R, Welle F. (2009) Investigation into the sorption of nitroglycerine and diazepam into PVC tubes and alternative tube materials during application. International Journal of Pharmaceutics. 369: 30-37.

Valeri CR, Contreras TJ, Feingold H, Sheibley RH, Jaeger RJ. (1973) Accumulation of Di-2-ethylhexyl Phthalate (DEHP) in Whole Blood, Platelet Concentrates, and Platelet-Poor Plasma. 1. Effect of DEHP on Platelet Survival and Function. Environmental Health Perspectives. p103-118.

Vessman J. & Rietz G. (1974) Determination of Di(2-ethylhexyl) Phthalate in human plasma and plasma proteins by electron gas chromatography. Journal of Chromatography. 100: p153

Von Daniken A., Lutz WK., Jack R., Schlatter C. (1984) Investigation of the potential for binding of di(2-ethylhexyl) Adipate to Liver DNA invivo. Toxicology and Applied Pharmacology.73:p373-387.

Vroman, L., Adams, AL. (1969) Identification of rapid changes at plasma solid interfaces. Journal of Biomedical research. 3:p43-67.

Waddell WJ, Marlowe C, Miripol JE, Garvin PJ. (1976) The distribution in Mice of Intravenously Administered Plasma Solution of (14C) Di-2-Ethylhexul Phthalate Determined by Whole-Body Auto Radiography. Toxicology and Applied Pharmocology. 39:p339-353.

Ward JM, Peters JM, Perella CM and Gonzalez FJ, (1998) Receptor and non receptor mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator-activated receptor alpha-null mice. Toxicological Pathology., 26, 240-245.

Walker JE., Flook V., Ogston D. (1983) An artificial circulation for the study of thrombolysis. The influence of flow rate and pressure on thrombolysis and a comparison between urokinase and tissue activator as thrombolytic agents. Acta haematol. 63(1):41-6.

Weber N, Wendel HP, Ziemer G. (2002) Hemocompatibility of heparin-coated surfaces and the role of selective plasma protein adsorption. Biomaterials 23:429-439.

Weiss CH. In “Human Physiology”, Schmidt RF, Thews G. 1983. Chapter 16, Springer-Verlag, Berlin.

Whelan A., Craft JL. Developments in PVC production and processing. 1977. Applied Science publishers, London.

Wolfe GW, Layton KA. Multigeneration reproduction toxicity study in rats (unaudited draft): Diethylhexylphthalate: Multigenerational reproductive assessment when administered to Sprague-Dawley rats in the diet. TherImmune Research Corporation (Gaithersburg,
Maryland), TRC Study n° 7244-200, 2003

Wormuth, M., Scheringer, M., Vollenweider, M., Hungerbuhler, K., (2006) What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? Risk Anal. 26, 803–824.

Yao Q., Axelsson J., Heimburger O., Stenvinkel P., Lindholm B. (2004) Systemic inflammation in dialysis patients with end stage renal disease: Causes and consequences. Minerva Urologica e Nefrologica. 56(3):p237-248.

Yao Q., Axelsson J., Heimburger O., Stenvinkel P., Lindholm B. (2004) Chronic systemic inflammation in dialysis patients: An update on causes and consequences. ASAIO American Society for Artificial Internal Organs. 50(6)

Yin HQ, Zhao XB, Courtney JM, Blass CR, West RH, Lowe GDO. (1999) Blood Interaction with plasticised poly(vinyl chloride): relevance of plasticiser selection. Journal of materials science: materials in medicine. 10: p527-531.

Yin, HQ. (2004) Blood compatibility of poly(vinyl chloride) tubing: In-vitro and Ex-vivo assessment. PhD Thesis, University of Strathclyde.

Yu, J. (1993) Modification of polymeric Biomaterials for improved blood compatibility: Investigation of protein adsorption and in-vitro blood response. PhD Thesis, University of Strathclyde.

Zdolsek J., Eaton JW., Tang L. (2007) Histamine release and fibrinogen adsorption mediate acute inflammatory responses to biomaterial implants in humans. Journal of Translational Medicine. 5:p31

Zhao XB. (1999) Plasticised poly(vinyl) chloride: significance of plasticiser selection and surface modification for protein adsorption. PhD Thesis, University of Strathclyde, Glasgow.

Zhao, XB, Courtney JM. (2003) Blood Response to plasticised poly(vinyl) chloride: Dependence of fibrinogen adsorption on plasticiser selection and surface plasticiser level. Journal of Material Science: Materials in Medicine. 14:905-912.

Zhao XB., Courtney JM. (2007) Surface modification of polymeric biomaterials: utilization of cyclodextrins for blood compatibility improvement. Journal of Biomedical Materials Research A. 80(3):539-53.

Zhao XB, Courtney JM. Update on Medical Plasticised PVC. 2009. iSmithers Rapra Publishing ISBN: 978-1-84735-208-8.

Zucker MB., Vroman L. (1979) Platelet adhesion induced by fibrinogen adsorbed onto glass. Proceedings of the Society for Experimental Biology and Medicine. 131:318-320.

Published Work from Thesis

JECT. 2009;41:199–205
The Journal of ExtraCorporeal Technology

Original Articles

The Effect of Priming Solutions and Storage Time on Plasticizer Migration in Different PVC Tubing Types—Implications for Wet Storage of ECMO Systems

David C. Horne, MEng;* Ida Torrance, CCP;† Thomas Modine, MD;‡ Terence Gourlay, PhD, BSc*

**Bioengineering Unit, University of Strathclyde, Glasgow, Scotland; †Golden Jubilee National Hospital, Glasgow, Scotland; and ‡Service de Chirugie CardioVasculaire, Hôpital Cardiologique, CHRU Lille, France*

Abstract: The wet priming of extracorporeal membrane oxygenation systems and storage of these systems for rapid deployment is common practice in many clinical centers. This storage policy is, however, seen by many to be controversial due to the potential adverse effects associated with the migration of the di(2-ethylhexyl) phthalate plasticizer (DEHP) from the polyvinyl chloride (PVC) circuit tubing and issues surrounding the maintenance of sterility. This study was performed to evaluate the effects of both short and long-term storage and priming fluid type on plasticizer migration from four commonly used PVC tubes in extracorporeal membrane oxygenation therapy circuits. The four tubes incorporating three plasticizers, two DEHP, one tri(2-ethylhexyl) trimellitate (TOTM), and one dioctyl adipate (DOA) were exposed to each of the three priming fluids for a period of 28 days. Samples were taken at time intervals of 1, 4, 8, 24, and 48 hours, followed by samples at 7, 14, and 28 days. Each sample was processed using a spectrophotomer and the concentration of plasticizer leaching into each solution at each time-point determined. There was a time dependent increase in plasticizer leached from each tube. The migration was greatly affected by both the priming fluid and tubing type. The migration of DEHP was higher than that of TOTM and DOA over both the short and long-term exposure levels. Plasticizer migration occurs from all of the tubes tested over the long term. The TOTM and DOA tubes performed better than the DEHP counterparts in the short term. Selection of priming fluid has a major bearing on plasticizer migration with significant lipid and protein containing fluids promoting higher migration than simple sodium chloride .9% solution prime. The results suggest that DOA tubing and sodium chloride. 9% solution priming fluid should be selected if wet primed perfusion circuits are to be used over short terms of storage.
Keywords: di(2-ethylhexyl) phthalate, extracorporeal membrane oxygenation therapy, migration, plasticizer, storage. *JECT. 2009;41:199–205*

di(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer for medical grade polyvinyl chloride (PVC). It is used primarily to impart flexibility into what would otherwise be a ridged polymer and can represent up to 40% of the total polymer weight. The excellent physical properties and versatility of plasticised poly vinyl chloride (P-PVC) have made it one of the most commonly utilized polymers in clinical practice. DEHP itself is not covalently bonded to PVC resin, it is therefore mobile within the blend and has the potential to migrate from the plastic when exposed to blood, serum, or some pharmaceutical products. Hence, patients undergoing a variety of procedures such as blood transfusions, nutritional support, intravenous therapy, cardiopulmonary bypass (CPB), and extracorporeal membrane oxygenation therapy (ECMO) are exposed to a varying amount of P-PVC and in turn the mobilized or leached DEHP.

Previous studies have shown that DEHP and its metabolites have been identified in blood products (1–4) and tissues (5,6) as well as intravenous solutions (7) and pharmaceuticals (8,9). As a result of these findings, potential toxic effects of DEHP have been the focus of considerable research effort for some time. Much of this work has been carried out using animal models of various clinical interventions. These studies have given rise to some concern, in particular, the confirmation that DEHP is both a potential carcinogen and a toxic agent (10), particularly with

Received for publication July 1, 2009; accepted August 27, 2009.
Address correspondence to: Terence Gourlay, PhD, BSc, Bioengineering Unit, Wolfson Building, University of Strathclyde, Glasgow, Scotland. E-mail: terence.gourlay@strath.ac.uk
The senior author has stated that authors have reported no material, financial, or other relationship with any healthcare-related business or other entity whose products or services are discussed in this paper.

regard to the reproductive systems in male rodent species. More recently, DEHP has been shown in both rodents and humans to be a significant inflammatory mediator (11–14), and this trait has prompted concern in the clinical CPB and ECMO arenas, especially regarding the potential scope for inflammatory mediated complications during and post procedures. Various regulatory bodies have responded to these issues, and have reported on DEHP and its use in medical devices. These bodies have concluded that critically ill neonates and those patients who undergo chronic procedures, such as hemodialysis and multiple blood transfusions, are the most at risk from the adverse effects from DEHP exposure (15–18).

Despite these well-described issues, DEHP continues to be the most widely used plasticizer in medical practice. There are a number of reasons for this, but low cost and the very good mechanical properties associated with DEHP use are certainly two of these. However, bearing in mind the potential for adverse effects associated with the deployment of DEHP, there are a number of clinical interventions in which there may be real cause for concern. Principal amongst these are the various extracorporeal applications, hemodialysis, CPB, and in particular ECMO where the PVC surface area to body mass relationship and duration of exposure is at its most extreme. The knowledge of the possible adverse effects of DEHP has led many manufacturers in this field to investigate possible alternative plasticizers and other means of reducing the migration of the DEHP plasticizer from PVC tubing. Several approaches to this have evolved over the years including the use of elaborate surface modification and coating technologies (19) and the incorporation of more biocompatible molecules like Heparin into the plastic structure (20). These techniques have been shown to reduce the levels of DEHP migration into test solutions, but they have been only sporadically adopted by the clinical setting, as they increase the cost of the tubing material and the evidence for DEHP mediated complications has not been truly clinically defined.

As health providers around the world express an interest in alternatives to DEHP plasticized PVC, the commercial sector has been investigating alternatives, for example tri(2-ethylhexyl)trimellitate (TOTM) and dioctyladipate (DOA), both of which are more expensive than DEHP, but exhibit much lower migration levels and impart similar mechanical properties of DEHP plasticized PVC. These alternatives are very promising, but the cost/benefit balance associated with their deployment needs to be fully elucidated before they will be widely used. However, there are niche sectors of clinical practice in which these materials are already being deployed, and these include the high-risk groups highlighted by the regulatory bodies, including neonatal ECMO patients. It must be stressed however, that although the migration rate of these new plasticizers is lower than that of DEHP, their toxicology profile, particularly in blood contacting environments, is not, at present, well described.

In response to the need for a more comprehensive understanding of the possible exposure of patients to plasticizers that are used regularly in ECMO, this study has been designed to investigate one particular issue associated with plasticizer migration; the effect of wet storage of ECMO circuits, a fairly common clinical practice, on the migration of plasticizers. In addition we investigated the effect of the makeup of the priming solutions and different plasticizer types used on plasticizer migration under long-term storage conditions.

Whether it is appropriate to store primed ECMO circuits for future use has been a matter of debate for some time, and the focus of this clinical debate has tended to revolve around possible infection issues, and effects on oxygenator performance. Our study, carried out under laboratory conditions, aims to determine the levels of plasticizer exposure presented by wet storage of various ECMO tubing types and priming solutions. The potential clinical impact of DEHP exposure remains controversial, however, there is a body of literature that suggests such exposure is undesirable in the clinical setting.

METHODS AND MATERIALS

The experiments were conducted in the laboratories in the bioengineering unit at the University of Strathclyde, and materials used in the experiments were kindly provided by the perfusion staff at the Yorkhill Children's Hospital in Glasgow. The study involved the testing of the four most commonly used tubing types that are found in ECMO circuits within the National Health Service Scotland and exposing them to three common priming solutions. The investigators were blinded to the tubing type and priming fluids used, both of which were supplied unmarked by the clinical partners. The tubing was labeled A, B, C, and D and the priming solutions numbered 1, 2, and 3. Only at the end of the experiment were the names and manufacturers of the tubing and fluids revealed to the investigators. These designations relate to the following materials:

Fluid 1: .9% Sodium Chloride Solution
Fluid 2: 2/3 .9% Sodium Chloride Solution and 1/3 20% Human Albumin
Fluid 3: 2/3 Gelofusine and 1/3 Hartmann's Solution
Tube A: EEC Blood Line. Raumedic, Munchberg, Germany.
Tube B: Dideco XS. Sorin Group, Arvada, CO.
Tube C: Action. Tekni-plex, Somerville, NJ.
Tube D: Tygon S95-E. Saint-Gobain, Akron, OH.

Experimental Setup

Three 8 cm sections of each tube type was cut and placed on a test rig (Figure 1). Five centimeters of this tube were

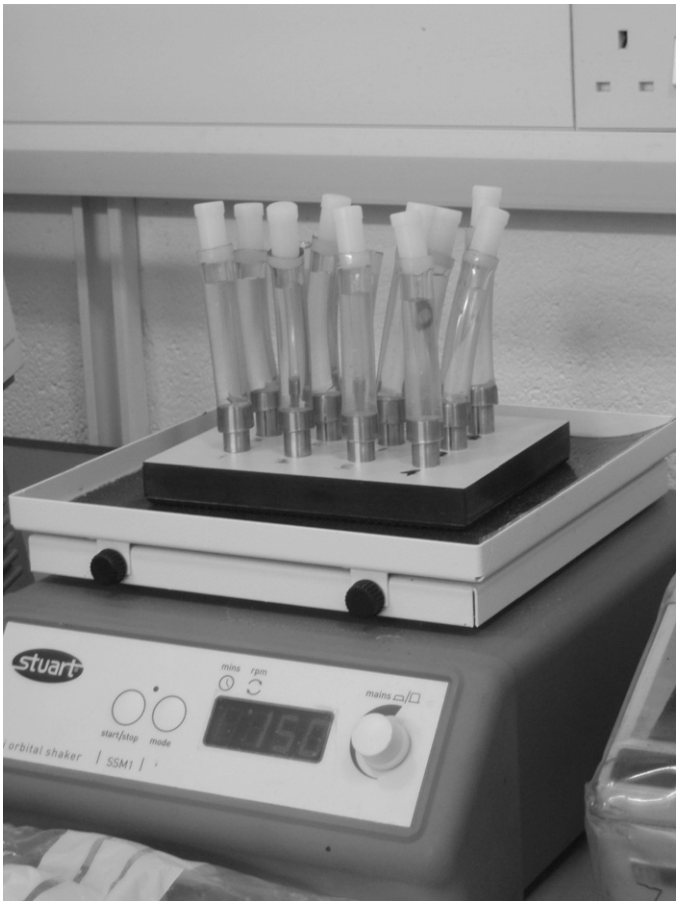


Figure 1. Experiment test rig set up showing the constrained and sealed tubes on the tubing block positioned on an orbital shaker.

then exposed to each of the test fluids and a nonplasticized polyurethane stopper was then applied to the open end of the tube. The test rig was then placed on an orbital shaker plate at 150 rev/min to simulate gentle recirculation, a common clinical practice.

Samples of the test solutions were taken at 0 hours, 1 hour, 4 hours, 8 hours, 24 hours, 48 hours, 7 days, 14 days, and 28 days. The plasticizer content of each sample was measured using a spectrophotometer (Ultrospec 4300pro, GE Healthcare Lifesciences, Piscataway, NJ) over a wavelength range of 200–900 nm, running a Wavescan analysis program. The samples were then returned to the tubes after the reading had been taken. The reference sample used for all sample readings was a 100% sodium chloride .9% solution. A concentration curve for all plasticizers was also plotted using known concentrations, DOA, and TOTM suspended in methanol, and this was used for calculating plasticizer concentrations.

Concentration Analysis

The concentration of plasticizer present in the test samples, which represents the plasticizer migration level, was determined by measuring the appropriate peak absorbance height. This value was then translated into a concentration by referring to the concentration curves plotted previously. This technique had been used successfully in previously published works by Gourlay et al. (14) and also by Zhao and Courtney (21).

Statistical Methods

The statistical analysis in this study was performed using the MiniTab V15 statistical software tool (Minitab Ltd. Coventry, United Kingdom). Using this software, descriptive statistics were used to calculate the mean and standard deviations of the samples. The probability values quoted in this paper manuscript were also determined by performing a *t*-Test using the same software package.

RESULTS

The results from the recirculation migration experiments for Tubes A, B, C, and D are shown in Figures 2–5. The results clearly demonstrate that in all tubing types, in the presence of all fluids, there was evidence of plasticizer migration. The level of migration, however varied significantly over time and in response to fluid type. The DEHP migration profiles for both DEHP plasticized tubing types were fairly similar. Detectable levels of plasticizer migration were seen after as little as 1 hour of exposure to the test fluids in both cases. Fluid 1 (sodium chloride .9% solution) was associated with the lowest level DEHP migration from the tubing with levels that varied between .0079 ± .00031 mg/mL, and .008 ± .00031 mg/mL out to 7 days of exposure. The concentration levels tended to increase consistently from 14–28 days of exposure with a final value of .0132 ± .0022 mg/mL and .0128 ± .0043 mg/mL for tubes A and B respectively, although this difference was not statistically significant ($p > .05$). In terms of DEHP migration, Fluid 3 was associated with a similar migration profile as seen with the sodium chloride .9% solution. However, the overall DEHP concentrations were generally higher at around .0128 ± .00082 mg/mL to .0125 ± .000793 mg/mL at 7 days of exposure for tubes A and B. This difference in concentration was statistically significant in both cases, $p < .01$ and $p < .01$ respectively when comparing tubing types with respective fluid compositions. After 7 days, Tube A seemed to leech greater amounts of plasticizer than Tube B, with concentration levels of .019 ± .0072 mg/mL and .0451 ± .019 mg/mL after 14 and 28 days respectively compared to .011 ± .0024 mg/mL and .0252 ± .0156 mg/mL for Tube B. These differences were statistically significant at both 14 and 28 days, $p < .03$ and $p < .04$ respectively. In addition the differences in leaching rate at the 14 and 28 day exposure levels differed significantly between common tubing types, but with different priming solutions. Once again the plasticizer levels associated with Fluid 3 (Gelofusine and 1/3 Hartmann's solution) were significantly higher than that

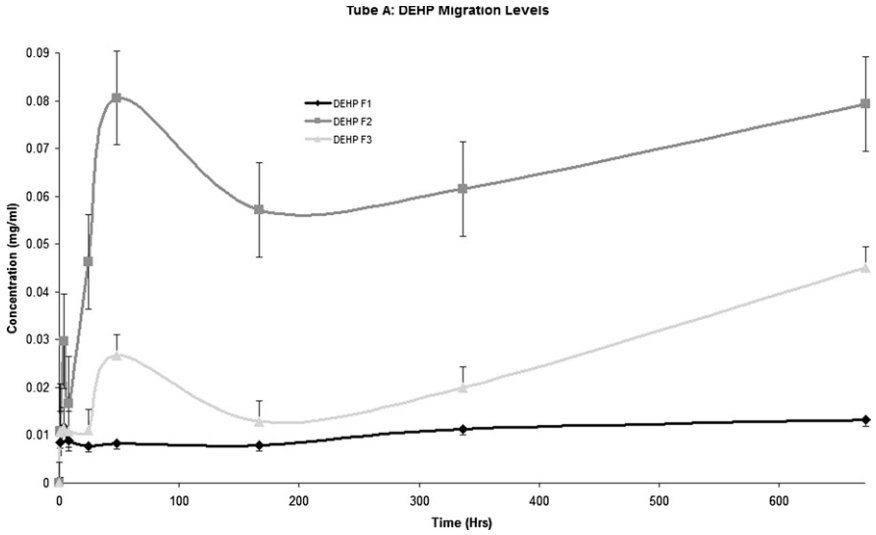


Figure 2. Graph of DEHP concentration levels migrated from Tube A in mg/mL.

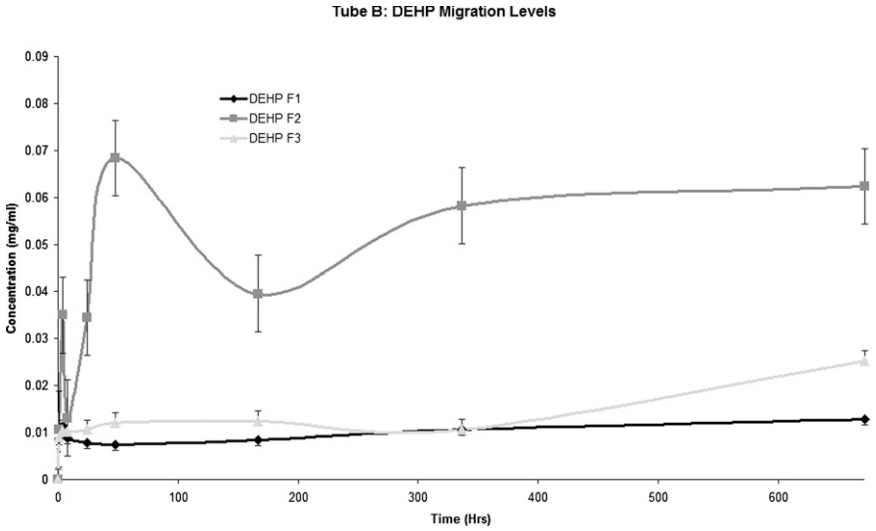


Figure 3. Graph of DEHP concentration levels migrated from Tube B in mg/mL.

of sodium chloride .9% solution alone ($p < .045$ for Tube A and $p < .049$ for Tube B).

Fluid 2 (.9% sodium chloride solution and 1/3 20% human albumin) was associated with the highest level of DEHP migration into the priming fluid when compared to the other solutions. The migration profile over the first few hours was seen to follow a parabolic shape but after 8 hours (Figures 1 and 2) and out to 28 days followed more of a linear pattern. The concentration levels at 24 hours reached .0464 ± .027 mg/mL and this continued to increase linearly to .079 ± .025 mg/mL and .0624 ± .0134 mg/mL after 28 days for Tubes A and B respectively. This was statistically significantly higher than the other priming solutions used ($p < .039$, and $p < .007$ when comparing Tube A and B with Fluids 2 and 3 respectively).

The TOTM plasticized tube, Tube C was associated with a much lower migration rate than the DEHP plasticized tubes. Of particular interest was the finding that this slow migration rate was consistent with all fluid types. Fluids 1 and 3 exhibited a low level of extraction over the entire 28-day test period. Indeed the concentration levels were .0086 ± .00057 mg/mL and .0067 ± .0023 mg/mL for Fluids 1 and 3 respectively at 28 days, around 10% of the levels observed at this time-point with the DEHP tubes. Only Fluid 2 (.9% sodium chloride solution and 1/3 20% human albumin) showed any significant increase in migration of the plasticizer with the TOTM plasticized tube, but this occurred only after 14 days of exposure and remained fairly constant out to 28 days, at a level only slightly, and not significantly, higher than that seen with Fluids 1 and 3.

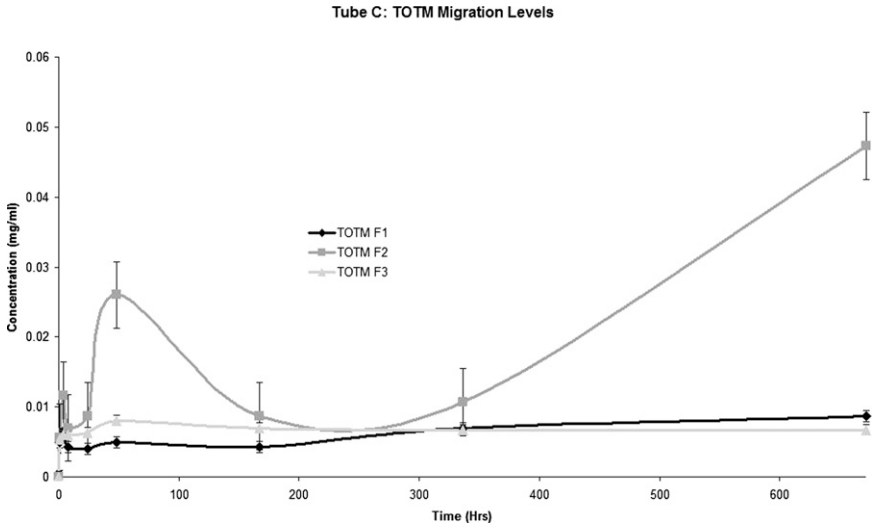


Figure 4. Graph of the migration concentration levels over the entire test period for Tube C in mg/mL.

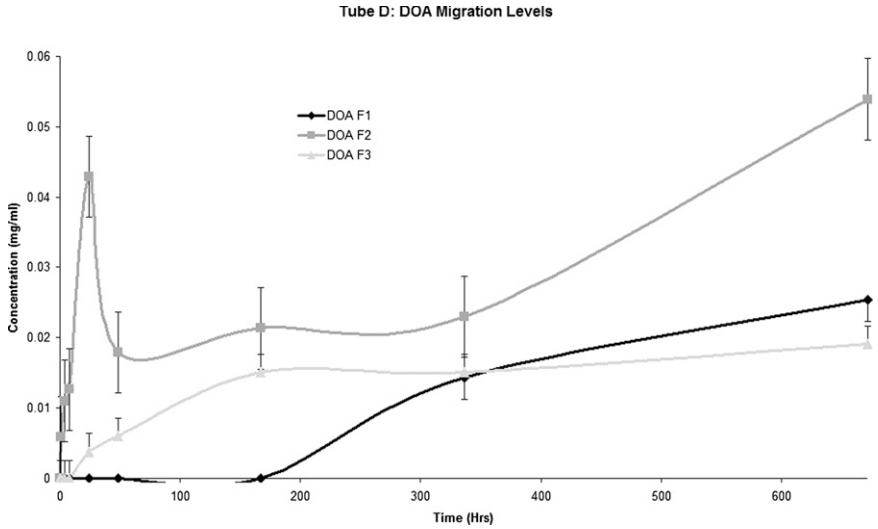


Figure 5. Graph showing the migration concentration levels over the entire test period for Tube D in mg/mL.

The DOA plasticized tubing, Tube D, exhibited no measurable migration over the first 4 hours in any of the test fluids. This trend continues for both Fluids 1 and 3 out to 8 hours and in the case of Fluid 1 it continues right out to 7 days. For Fluid 1, concentration levels at 14 days were seen to be around .0143 ± .012 mg/mL and this steadily increased to around .0252 ± .02 mg/mL at 28 days. Fluid 3 followed a similar pattern to Fluid 1 with no plasticizer migration being detected in the first 8 hours. The concentration levels were then seen to increase after 24 hours of exposure and further increased at a steady rate out to .019 ± .016 mg/mL after 28 days.

In common with the DEHP experiments, Fluid 2 (.9% sodium chloride solution and 1/3 20% human albumin) was associated with the highest level of DOA migration with detectable levels after only 8 hours. This level then steadily increased in a near-linear fashion from 48 hours out to 28 days where a final concentration in the sample was determined as .054 ± .047 mg/mL, the highest level detected in this study.

DISCUSSION

This study has shown quite clearly that plasticizers, utilized in the tubing element of perfusion circuits, migrate into the priming fluid. This is the case for all plasticizers investigated in the present study. However, there are clear differences in terms of the migration rate and the timeframe of migration between the tubing types. The study

suggests that the concentration of plasticizer observed in the priming solution depends upon three factors:

1. The plasticizer type
2. The priming fluid
3. Storage time

In general DEHP was seen to readily migrate into all of the priming solutions. The two alternative plasticizers both exhibited a marked reduction in migration compared to the DEHP over the entire time period, and performed much better over the shorter time period out to 48 hours and the concentration levels only started to increase after 14–28 days (Figures 4 and 5). The extremes of the timescale studied in the present study are probably not reflective of clinical practice, but the shorter term exposure levels, out to the 7 day level are within the margins of clinical practice. Critically, the early phase of this study is similar to that seen in previous studies, where DEHP plasticized tubing was exposed to migration media. Gotardo and Monteiro (8) reported a very similar profile, with the exception that they recorded little migration in the first 3 hours. We, on the other hand, did see migration of DEHP into all media from the very outset. One possible explanation for this is the fact that we did not prewash our circuits and under these circumstances (which are similar to the clinical setting) one might anticipate a surge of DEHP from the surface of the tubing, followed by a slower emission. This precisely describes the profile we observed. The findings of this study are corroborated by the findings of studies by both Karle et al. (22) and Burkhart et al. (23) as they also reported the detection of significant levels of migrated DEHP into whole blood and a high protein content priming fluid. In the case of Karle et al. (22) they reported that exposure to migrated DEHP can be as much as 20–70 times higher in ECMO than other procedures such as transfusions and dialysis. Furthermore, it is clear that the level of DEHP migration is effected by the priming fluid into which it comes into contact. The albumin and Gelofusine solutions, which contain both lipid and protein components, were associated with greater migration rates. Whilst these studies show that DEHP migrates from the PVC used in clinical procedures, there are a number of studies that reported no accumulation of DEHP during storage experiments. The migration of DEHP from PVC is known to happen constantly from the bulk of the material to the material surface. Therefore, the findings of these studies by Han et al. (24) and Riley et al. (25) have to be questioned as to why they found no DEHP. This may well be related to the sensitivity of the detection methodology, given that the levels being detected are fairly small.

Our study confirms that there is a much lower migration rate from the alternative plasticizers TOTM and DOA. This was particularly clear during the early phase of the study where DOA was associated with virtually no migration for up to 7 days of exposure to sodium chloride .9% solution. That DOA and TOTM moderate plasticizer migration confirms the information from the manufacturers of these materials, who market the tubes as low- or nonmigration PVC tubing.

Resistance to migration is not the only factor in deciding if these tube types are a better proposition than DEHP Plasticized PVC in the clinical setting. Cost and toxicity are also factors. Currently, both alternatives are more expensive than the DEHP option and their toxicological profiles are not as yet fully described. Further studies therefore are required to establish if using these materials is subjecting patients to a low dose of a more toxic substance than DEHP. However, assuming that the toxicity profile of these molecules is similar to DEHP, and that our previous studies have shown that the inflammatory response to DEHP is dose dependent, the use of these low leaching plasticizers may offer some form of inflammatory benefit.

The second major finding, not entirely unexpected, was that the makeup of the priming solution has an impact on the level of plasticizer migration. This is not surprising as we know for example that DEHP and, indeed, the other plasticizers involved in this study are to some extent lipophilic. These are complex molecules with complex chemical properties, which might affect migration tendency. In this study, it was clear that the simple sodium chloride .9% solution priming solution was associated with the lowest level of migration from all tubing types over the entire timecourse of the study. The more complex priming solutions were associated with the highest migration rates. The high lipid solutions, those containing albumin, and the gelfusine had the highest migration rates, possibly highlighting some of the complex affinities of the plasticizer molecules, which have not been investigated in this study. The presence of the albumin in solution was associated with the highest migration rates, and its use in wet prep circuits should be applied with caution, as it seems to promote excessive migration of DEHP over short time periods (<48 hours). It also promoted a moderate migration response in the other plasticizers, but only at long-term storage times of 14 days plus.

LIMITATIONS

The main limitation of this study is the fact that we utilized fairly small sections of tubing material; this resulted in small but significant levels of leached plasticizer. A larger surface area with smaller priming volume may well have amplified the results. However, we were keen to use only clinically relevant materials and designed the test system and protocols to use these. The detection limitation of the spectrophotometer system was initially considered to be a

concern, however, previous studies and this current study confirm that the levels detected are well within the limitations of the technology.

CONCLUSION

It was clear from this study that plasticizers migrate from PVC tubes at different rates depending on the time-course and the makeup of the priming solutions. This study would suggest that if wet-storage of the perfusion circuits is to be used, then DOA plasticized tubing combined with a simple sodium chloride .9% solution priming solution offers the best protection from the toxic effects of migratory plasticizers.

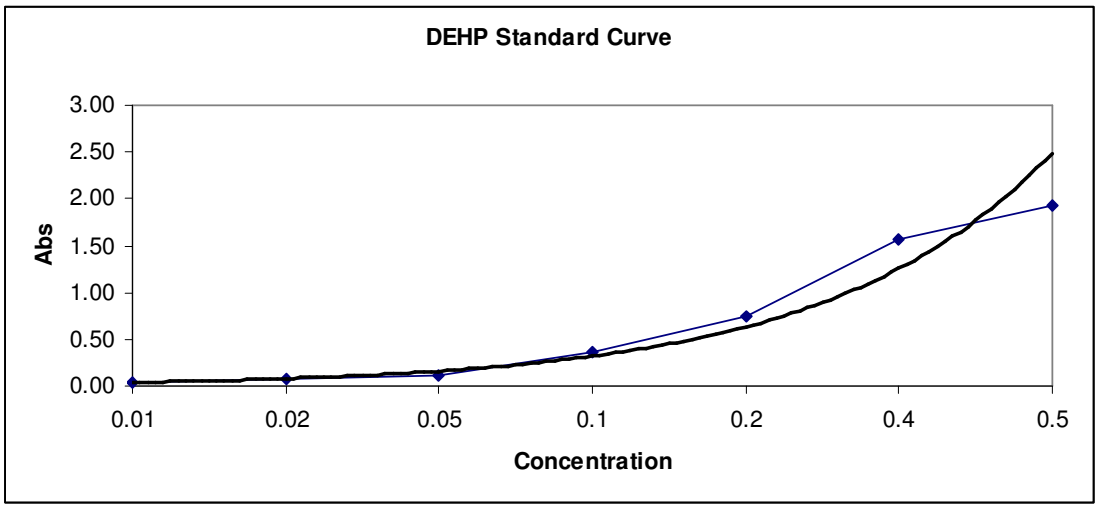
REFERENCES

1. Baker RWR. Diethylhexyl phthalate as a factor in blood transfusion and haemodialysis. Toxicology. 1978;9:319–29.
2. Dine T, Luyckx M, Cazin M, Brunet C, Gondaliez F, Cazin JC. Rapid determination by high-performance liquid chromatography of di-2-ethylhexyl phthalate in plasma stored in plastic bags. Biomed Chromatogr. 1991;5:94–7.
3. Jaeger RJ, Rubin RJ. Plasticizers from plastic devices: Extraction, metabolism, and accumulation by biological systems. Science. 1970;170:460–2.
4. Rock G, Labow RD, Tocchi M. Distribution of di(2-ethylhexyl) phthalate and products in blood and blood components. Environ Health Perspect. 1986;65:309–16.
5. Jaeger RJ, Rubin RJ. Leakage of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its localization in human tissues. N Engl J Med. 1972;287:1114–8.
6. Kambia K, Dine T, Azar R, Gressier B, Luyckx M, Brunet C. Comparative study of the leachability of di(2-ethylhexyl) phthalate and tri(2-ethylhexyl) trimellitate from haemodialysis tubing. Int J Pharm. 2001;229:139–46.
7. Hanawa T, Muramatsu E, Asakawa K, et al. Investigation of the release behaviour of diethylhexyl phthalate from polyvinyl-chloride tubing for intravenous administration. Int J Pharm. 2000;210: 109–15.
8. Gotardo MA, Monteiro M. Migration of diethylhexyl phthalate from PVC bags into intravenous cyclosporine solutions. J Pharm Biomed Anal. 2005;38:709–13.
9. Treleano A, Wolz G, Brandsch R, Welle F. Investigation into the sorption of nitroglycerine and diazepam into PVC tubes and alternative tube materials during application. Int J Pharm. 2009;369:30–7.
10. Kavlock R, Boeckelheide K, Chapin R, et al. NTP Centre for the evaluation of risks to human reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol. 2002;16:529–653.
11. Asberg AE, Videm V. Activation of neutrophil granulocytes in an in vitro model of a cardiopulmonary bypass. Artif Organs. 2005;29:927–36.
12. Gourlay T, Stefanou DC, Asimkopoulos G, Taylor KM. The effect of circuit surface area on CD11b(mac-1) expression in a rat recirculation model. Artif Organs. 2001;25:475–9.
13. Gourlay T, Stefanou DC, Taylor KM. The effect of methanol washing of plasticized polyvinyl chloride on biomaterial-contact-mediated CD11b (mac-1) expression in a rat recirculation model. Artif Organs. 2002;26:5–9.
14. Gourlay T, Samartzis I, Stefanou DC, Taylor KM. Inflammatory response of rat and human neutrophils exposed to di-(2-ethylhexyl)-phthalate and di-(2-ethyl-hexyl)-phthalate plasticized polyvinyl chloride. Artif Organs. 2003;27:256–60.
15. FDA. *Safety Assessment of di(2-ethylhexyl) Phthalate (DEHP) Released From Medical Devices.* Rockville, MD: Centre for Devices and Radiological Health, US Food and Drug Administration; 2000.
16. FDA. *Public Health Notification: PVC Devices Containing the Plasticizer DEHP*. Rockville, MD: Centre for Devices and Radiological Health, US Food and Drug Administration; 2002.
17. Health Canada. *An Exposure and Toxicity Assessment*. Ottawa, Canada: Medical Device Bureau, Therapeutic Products Directorate, Health Products and Food Branch; 2002.
18. SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks). *Opinion on The Safety of Medical Devices Containing DEHP- Plasticized PVC or Other Plasticizers on Neonates and Other Groups Possibly at Risk.* 6th February 2008.
19. Lakshmi S, Jayakrishnan A. Migration resistant, blood compatible plasticized polyvinyl chloride for medical and related applications. Artif Organs. 1998;22:222–9.
20. Weber N, Wendel HP, Ziemer G. Hemocompatibility of heparin-coated surfaces and the role of selective plasma protein adsorption. Biomaterials. 2002;23:429–39.
21. Zhao XB, Courtney JM. Blood response to plasticised poly(vinyl) chloride: Dependence of fibrinogen adsorption on plasticiser selection and surface plasticiser level. J Mater Sci Mater Med. 2003;14: 905–12.
22. Karle VA, Short BL, Martin GR, et al. Extracorporeal membrane oxygenation exposes infants to the plasticizer, di(2-ethylhexyl)phthalate. Crit Care Med. 1997;25:696–703.
23. Burkhart HM, Joyner N, Niles S, et al. Presence of plasticizer di-2(ethylhexyl)phthalate in primed extracorporeal circulation circuits. ASAIO J. 2007;53:365–7.
24. Han J, Beeton A, Long P, et al. Plasticizer di(2-ethylhexyl)phthalate (DEHP) release in wet-primed extracorporeal membrane oxygenation (ECMO) circuits. Int J Pharm. 2005;294:157–9.
25. Riley BJ, Sapatnekar S, Cornell DJ, Anderson J, Walsh-Sukys MC. Impact of prolonged saline solution prime exposure on integrity of extracorporeal membrane oxygenation circuits. J Perinatol. 1997;17: 444–9.

Appendix A

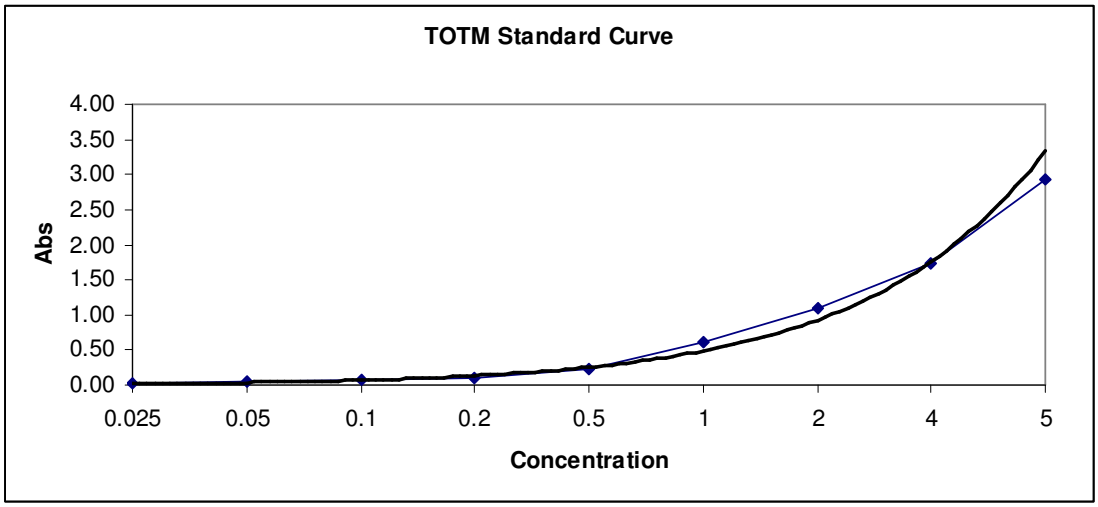
Standard Curves for Plasticiser Absorbance

DEHP Standard Curve Data



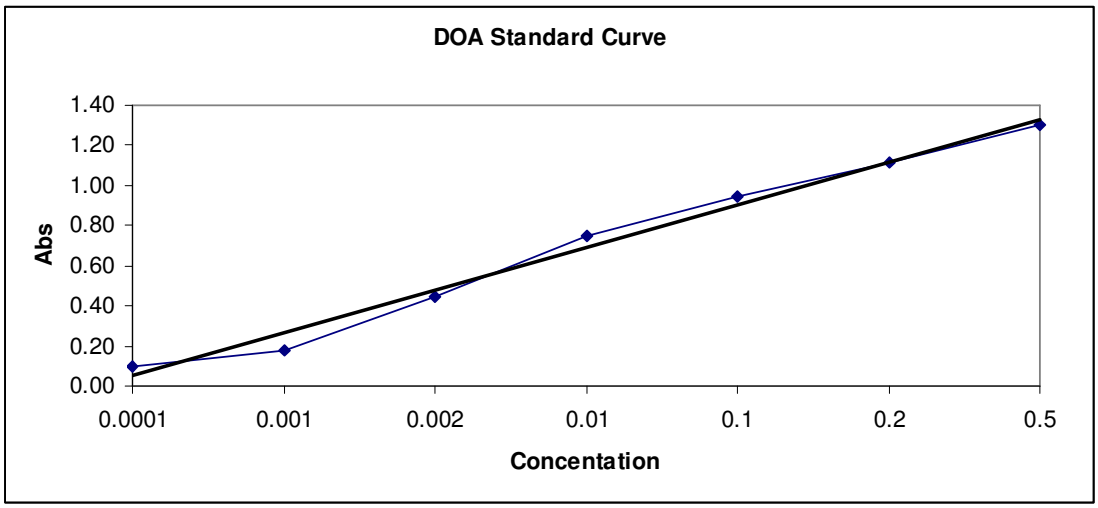
DEHP in Methanol				
Concentration	Absorbance			
	Run 1	Run 2	Run 3	Avg
0.01	**0.042**	**0.038**	**0.046**	**0.04**
0.02	**0.081**	**0.077**	**0.089**	**0.08**
0.05	**0.156**	**0.051**	**0.164**	**0.12**
0.1	**0.353**	**0.353**	**0.358**	**0.35**
0.2	**0.75**	**0.725**	**0.741**	**0.74**
0.4	**1.571**	**1.558**	**1.559**	**1.56**
0.5	**1.94**	**1.92**	**1.95**	**1.94**

TOTM Standard Curve Data



TOTM in Methanol				
Concentration	Absorbance			
	Run 1	Run 2	Run 3	Avg
0.025	**0.02**	**0.023**	**0.016**	**0.02**
0.05	**0.045**	**0.035**	**0.041**	**0.04**
0.1	**0.07**	**0.074**	**0.071**	**0.07**
0.2	**0.12**	**0.09**	**0.1**	**0.10**
0.5	**0.23**	**0.24**	**0.25**	**0.24**
1	**0.62**	**0.58**	**0.61**	**0.60**
2	**1.1**	**1.18**	**1.02**	**1.10**
4	**1.78**	**1.71**	**1.73**	**1.74**
5	**3**	**2.87**	**2.91**	**2.93**

DOA Standard Curve Data



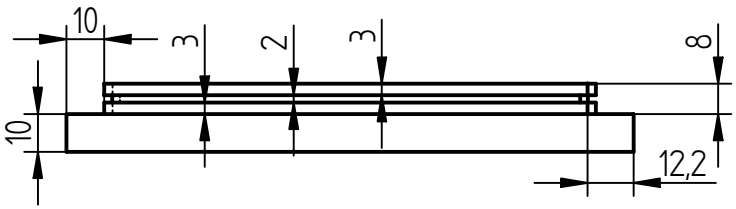
DOA in Methanol				
Concentration	Absorbance			
	Run 1	Run 2	Run 3	Avg
0.0001	**0.097**	**0.092**	**0.096**	**0.10**
0.001	**0.187**	**0.174**	**0.181**	**0.18**
0.002	**0.48**	**0.41**	**0.46**	**0.45**
0.01	**0.76**	**0.77**	**0.72**	**0.75**
0.1	**0.98**	**0.9**	**0.95**	**0.94**
0.2	**1.14**	**1.11**	**1.1**	**1.12**
0.5	**1.33**	**1.3**	**1.28**	**1.30**

Appendix B

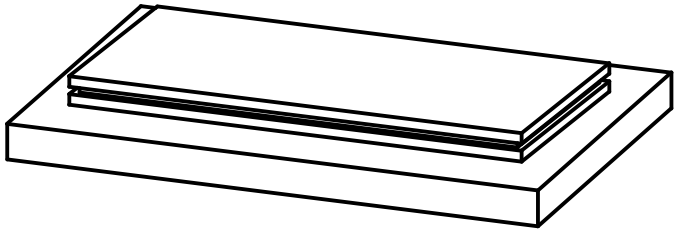
Test Cell Concept Drawings

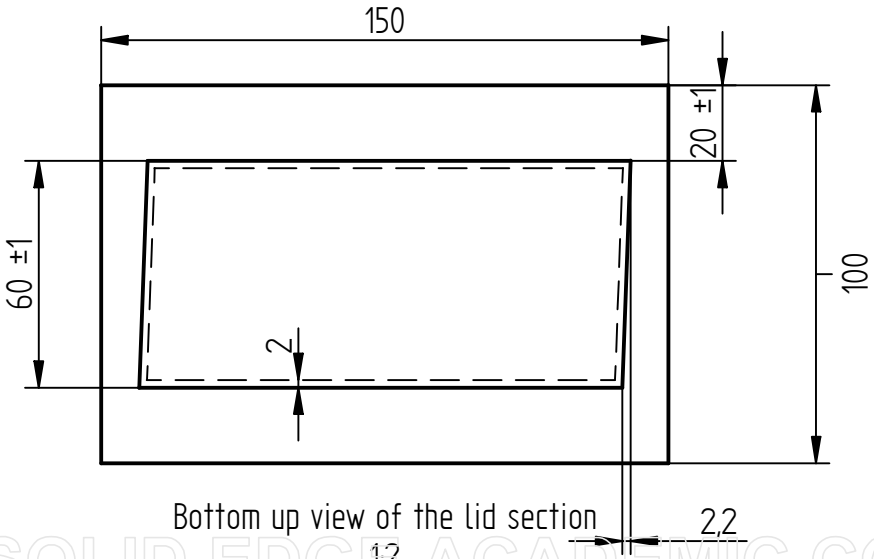
Concept 1 & Concept 2

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



Side view of the lid section
1:2





Bottom up view of the lid section
1:2

	NAME	DATE
DRAWN	Student	11/01/07
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

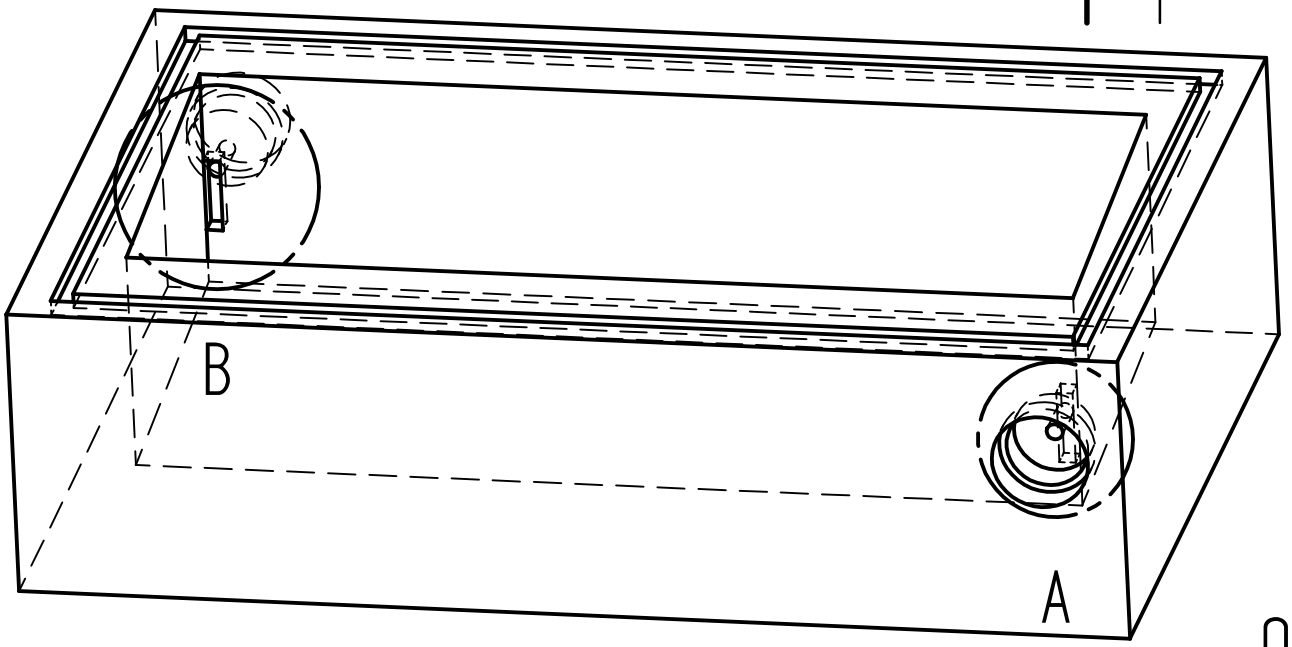
TITLE

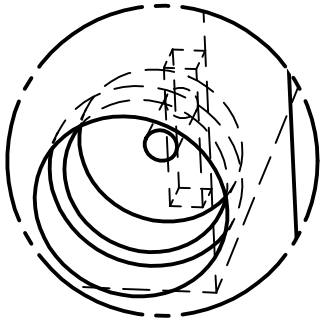
SIZE	DWG NO	REV
A4		

FILE NAME: Final_cell.dft

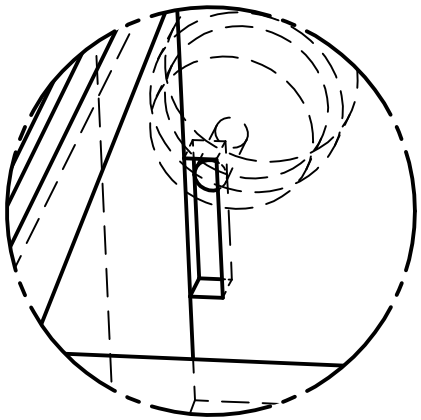
SCALE:	WEIGHT:	SHEET 1 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED





Outside of the entryport A



Inside of the entryport B

	NAME	DATE
DRAWN	Student	11/01/07
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

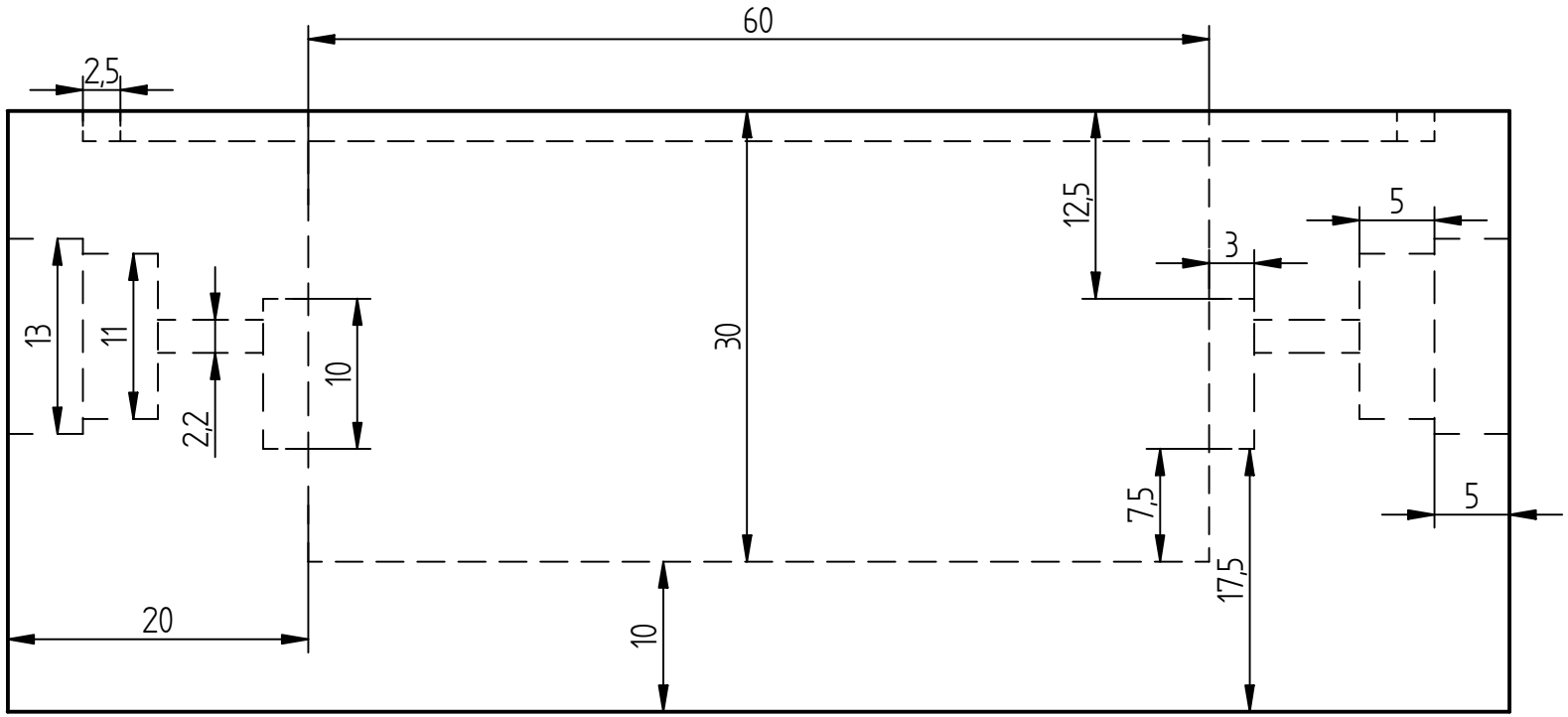
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: Final_cell.dft

SCALE:	WEIGHT:	SHEET 2 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



End View of Base Unit
2:1

	NAME	DATE
DRAWN	Student	11/01/07
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

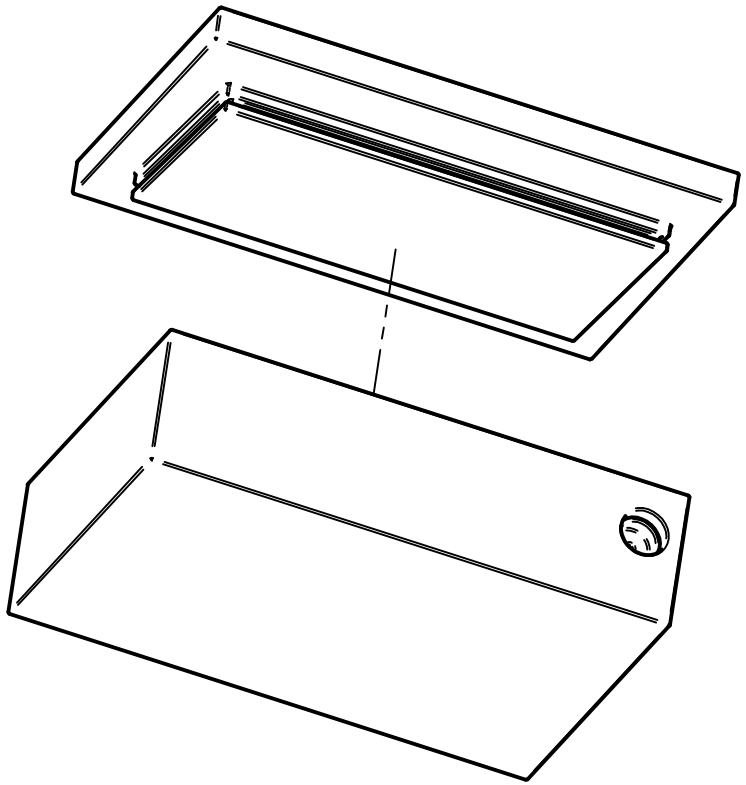
TITLE

SIZE	DWG NO	REV
A4		

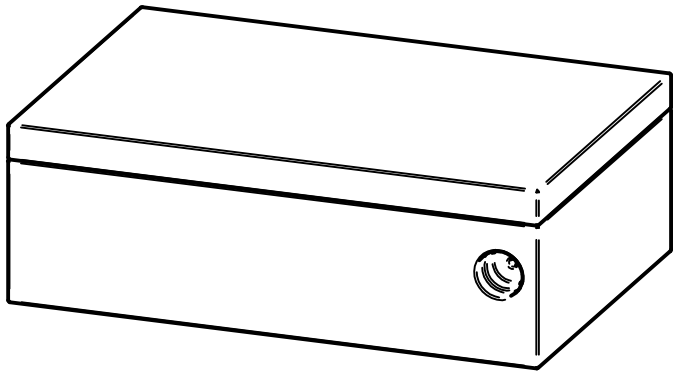
FILE NAME: Final_cell.dft

SCALE:	WEIGHT:	SHEET 5 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



exploded view of the complete cell
1:2



closed view of the complete cell
1:2

	NAME	DATE
DRAWN	Student	11/01/07
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

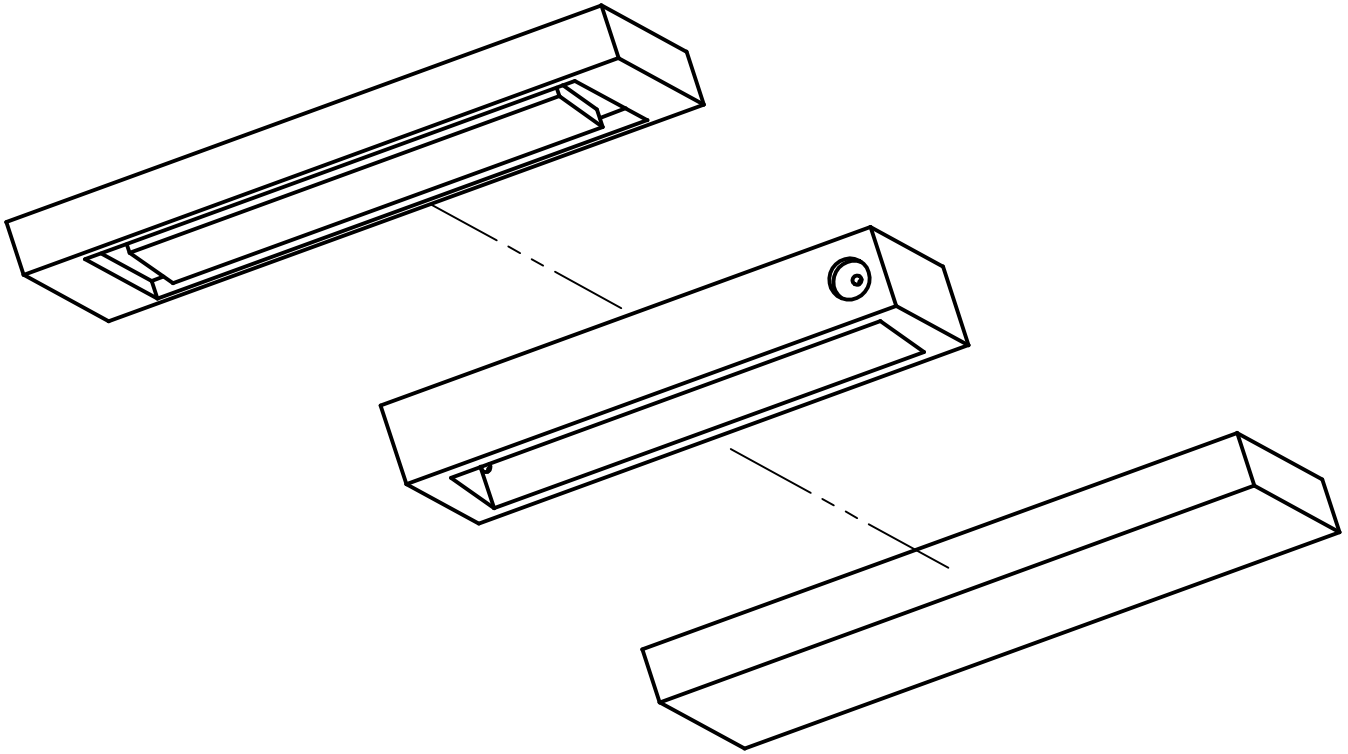
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: Final_cell.dft

SCALE:	WEIGHT:	SHEET 6 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



	NAME	DATE
DRAWN	Student	11/01/07
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

TITLE

SIZE	DWG NO	REV
A4		

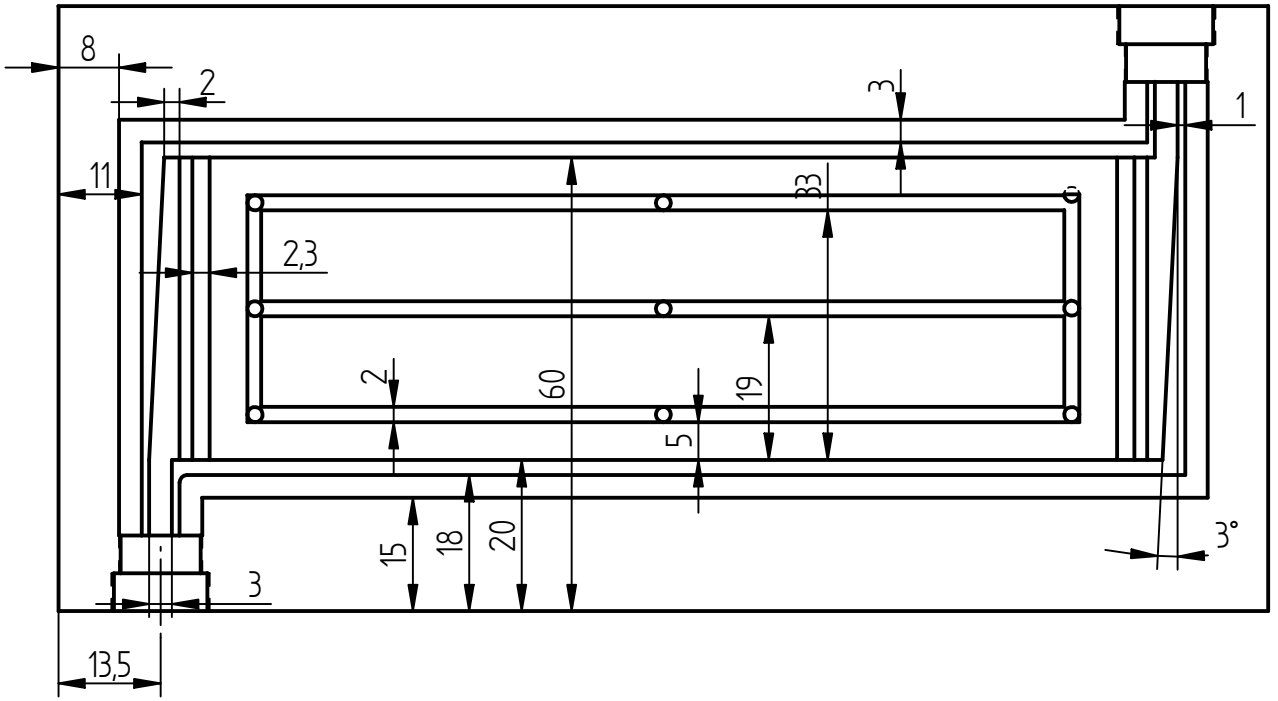
FILE NAME: concept_2_5mm.dft

SCALE:	WEIGHT:	SHEET 1 OF 1

Appendix C

New Test Cell Dimensions

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



Base Section Top View

	NAME	DATE
DRAWN	David	01/04/08
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

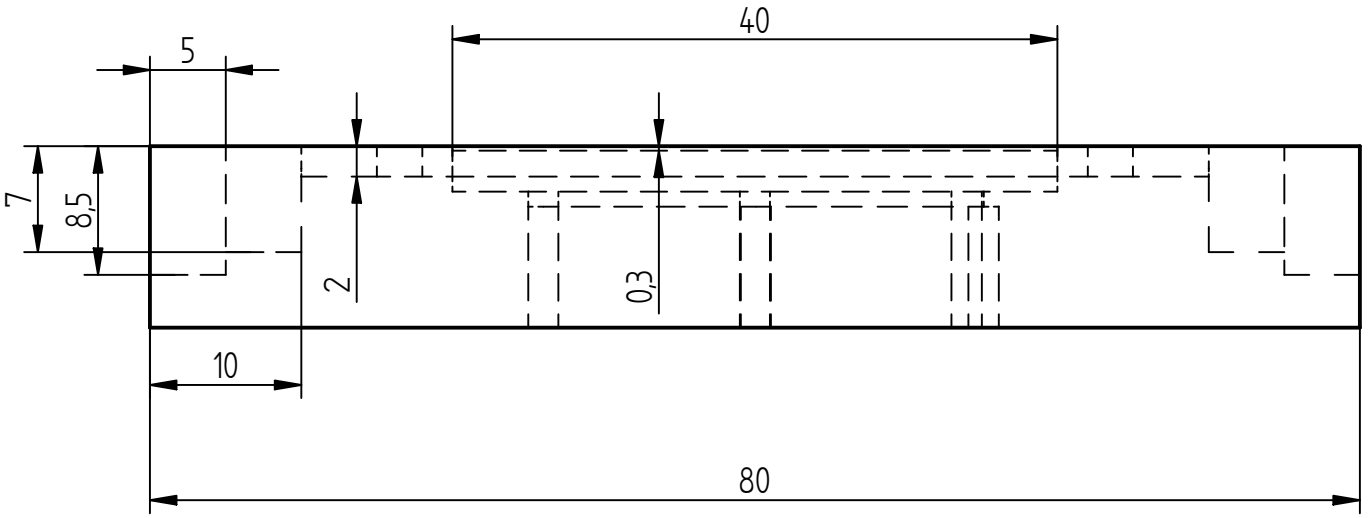
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: methanol_cellDMEM.dft

SCALE:	WEIGHT:	SHEET 1 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



Base Section Front View

	NAME	DATE
DRAWN	David	01/04/08
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

TITLE

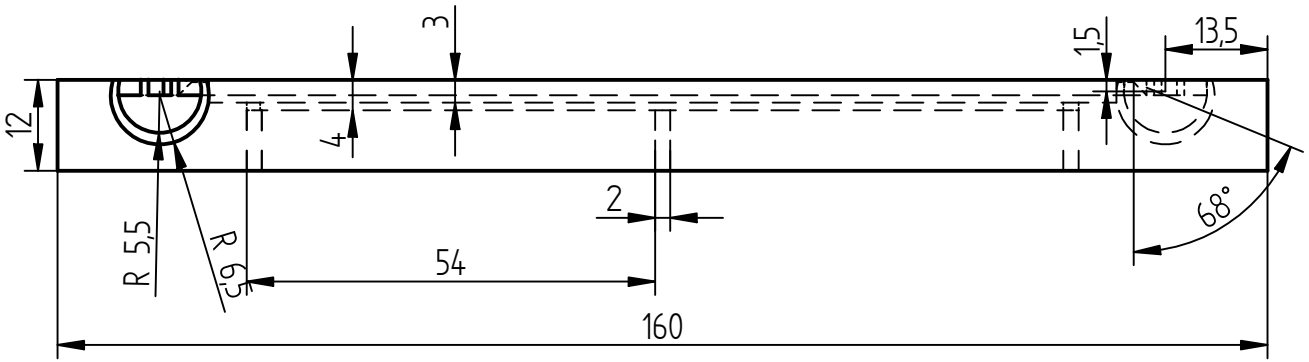
SIZE	DWG NO	REV
A4		

FILE NAME: methanol_cellDMEM.dft

SCALE:	WEIGHT:	SHEET 2 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED

Base Section Side View



	NAME	DATE
DRAWN	David	01/04/08
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

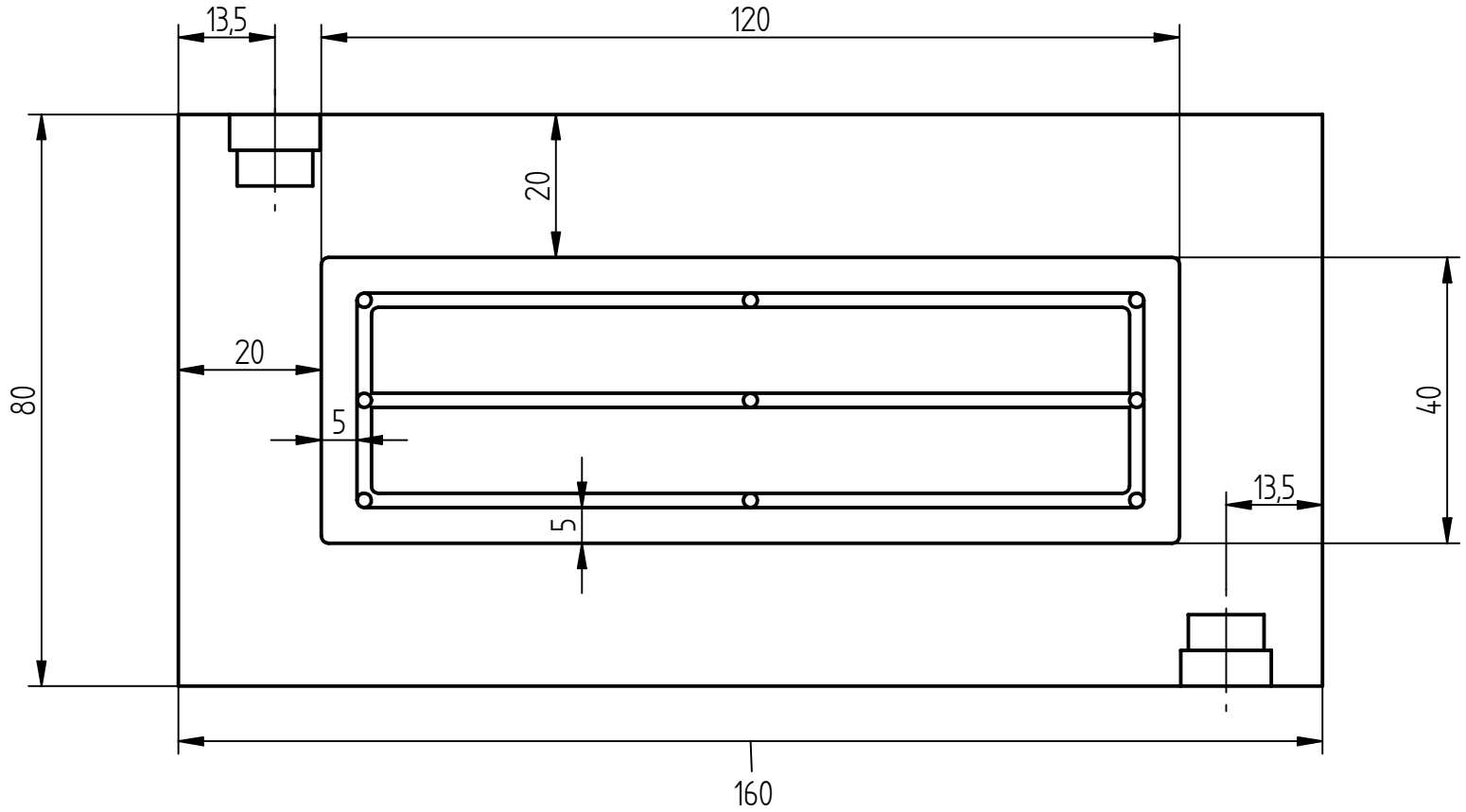
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: methanol_cellDMEM.dft

SCALE:	WEIGHT:	SHEET 3 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



Lid Section Top View

	NAME	DATE
DRAWN	David	01/04/08
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

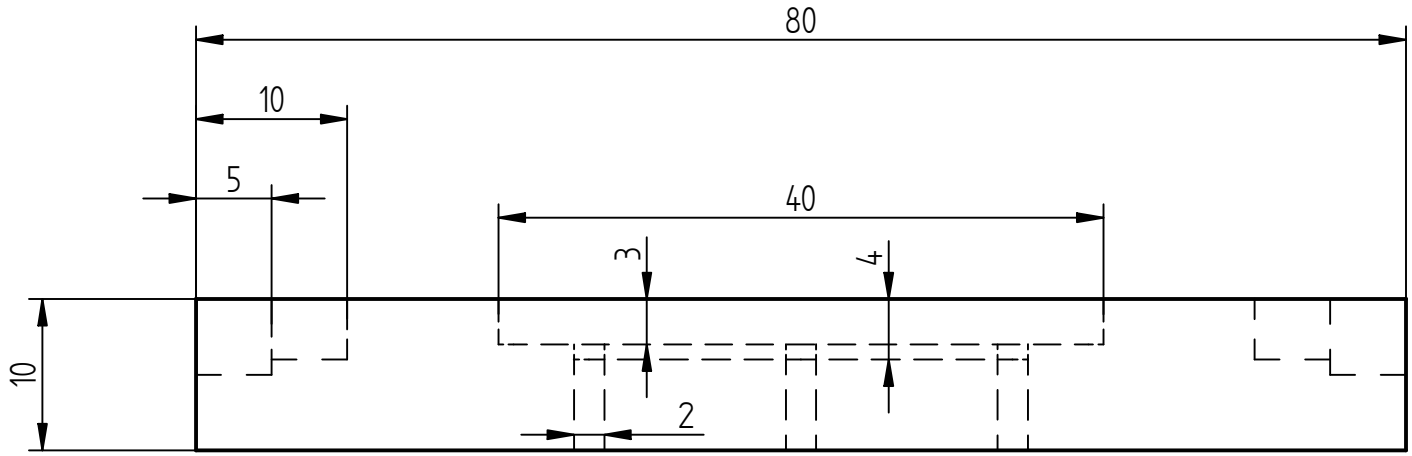
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: methanol_cellDMEM.dft

SCALE:	WEIGHT:	SHEET 4 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



Lid Section Front View

	NAME	DATE
DRAWN	David	01/04/08
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

TITLE

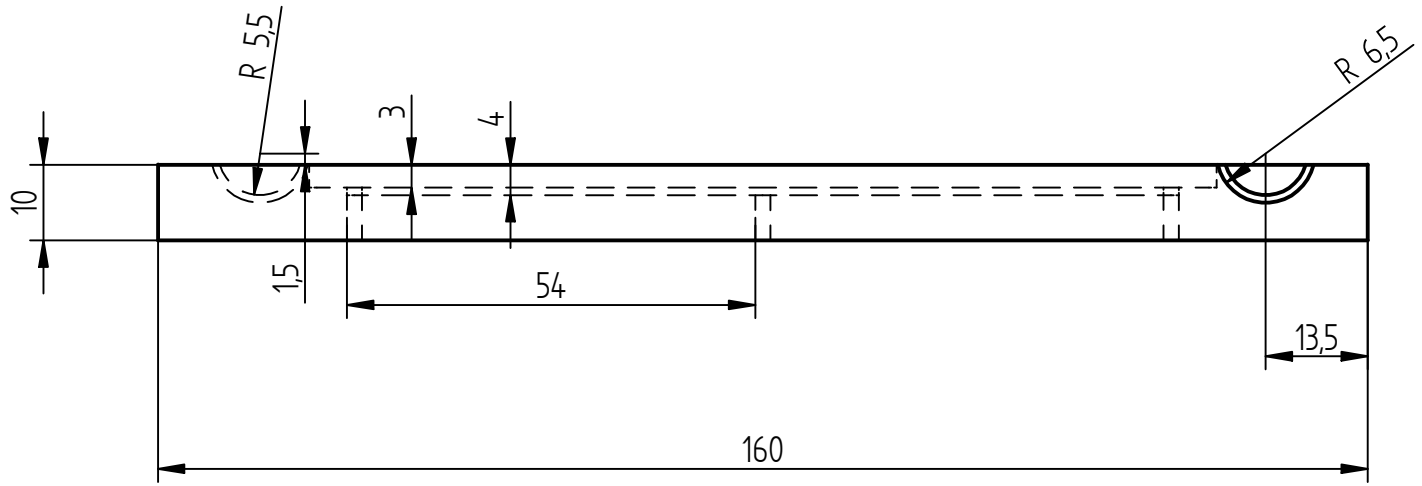
SIZE	DWG NO	REV
A4		

FILE NAME: methanol_cellDMEM.dft

SCALE:	WEIGHT:	SHEET 5 OF 6

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED

Lid Section Side View



	NAME	DATE
DRAWN	David	01/04/08
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

TITLE

SIZE	DWG NO	REV
A4		

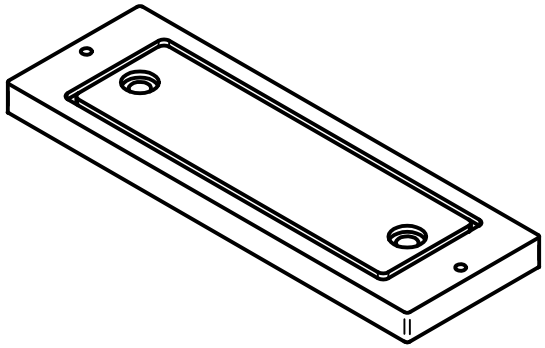
FILE NAME: methanol_cellDMEM.dft

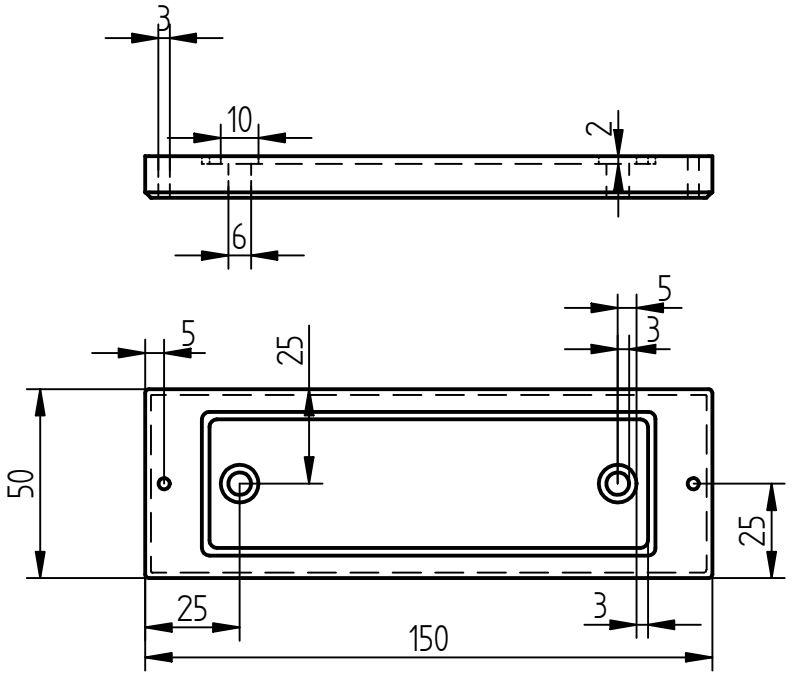
SCALE:	WEIGHT:	SHEET 6 OF 6

Appendix D

Drawings of Modified Gourlay Test Cell

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED





	NAME	DATE
DRAWN	David	02/26/09
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

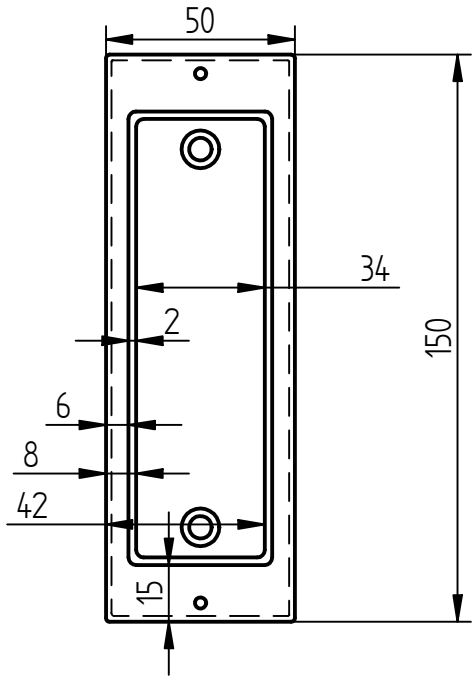
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: Narrow_TG_Cell_final_idea.dft

SCALE:	WEIGHT:	SHEET 1 OF 4

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



	NAME	DATE
DRAWN	David	02/26/09
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

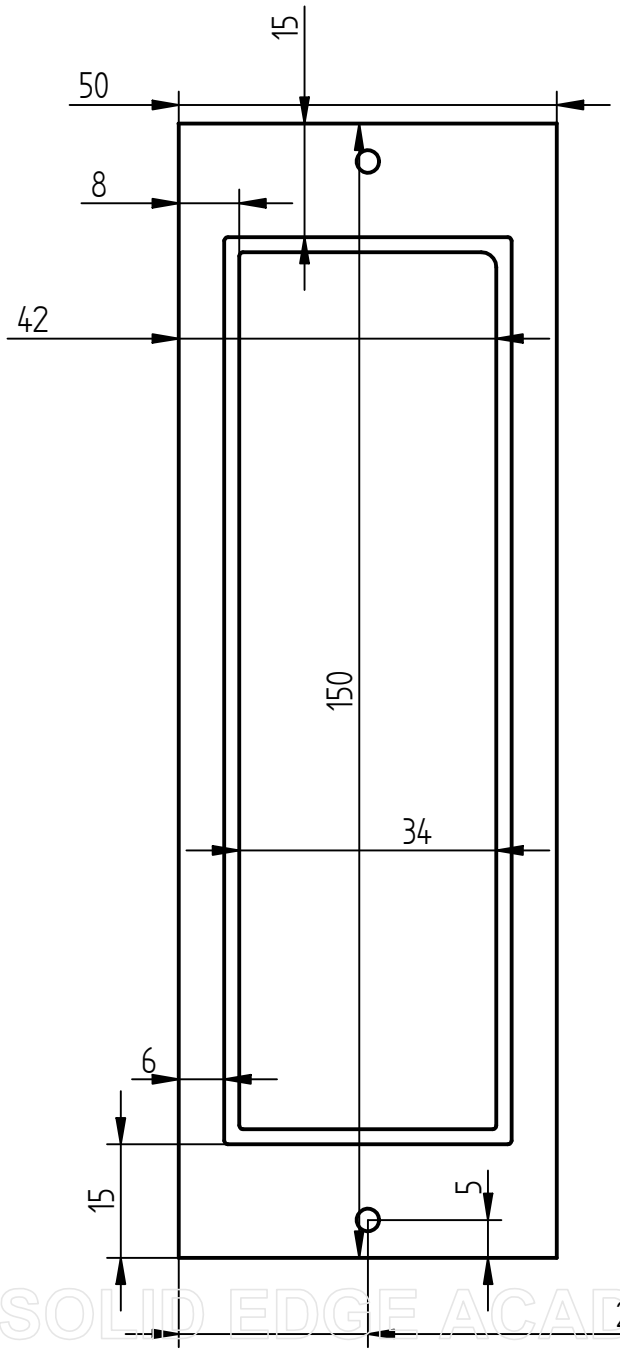
TITLE

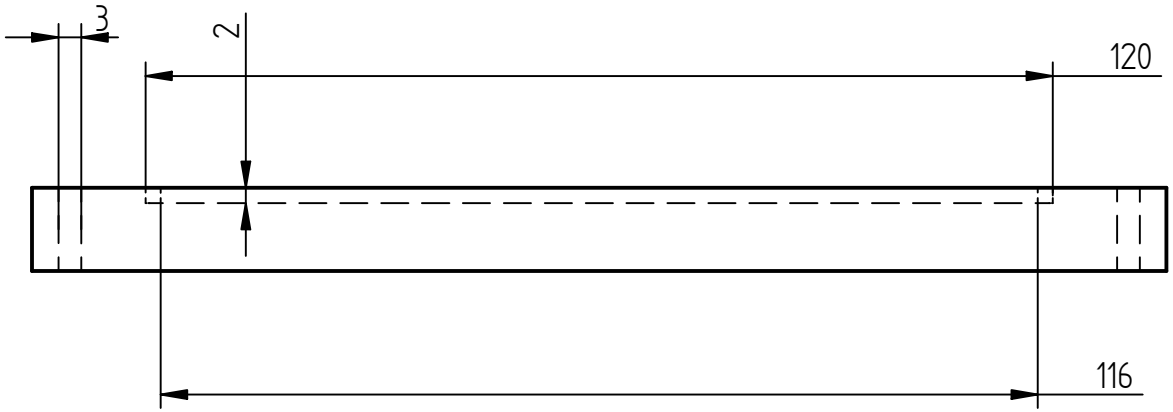
SIZE	DWG NO	REV
A4		

FILE NAME: Narrow_TG_Cell_final_idea.dft

SCALE:	WEIGHT:	SHEET 2 OF 4

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED





	NAME	DATE
DRAWN	David	02/26/09
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

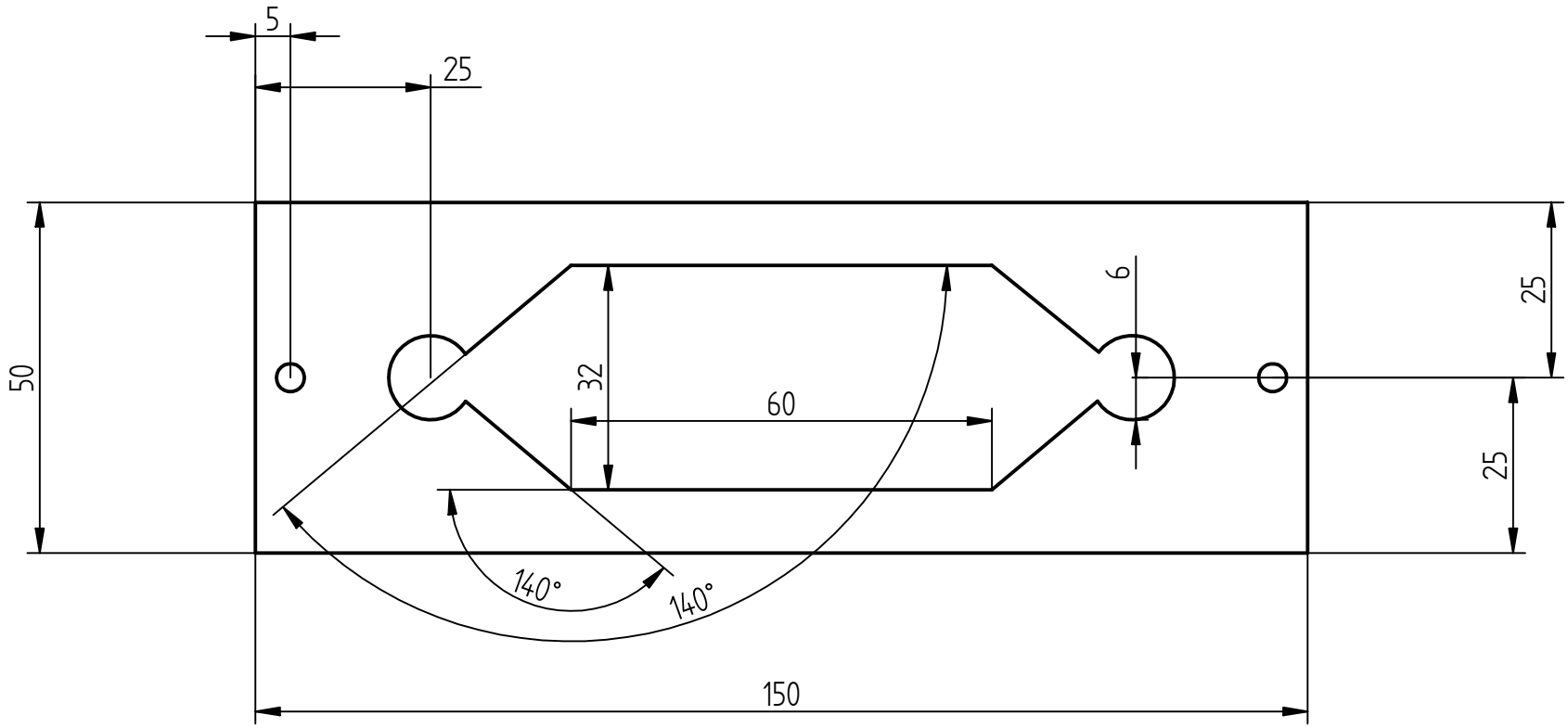
TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: Narrow_TG_Cell_final_idea.dft

SCALE:	WEIGHT:	SHEET 3 OF 4

REVISION HISTORY			
REV	DESCRIPTION	DATE	APPROVED



	NAME	DATE
DRAWN	David	02/26/09
CHECKED		
ENG APPR		
MGR APPR		

UNLESS OTHERWISE SPECIFIED
DIMENSIONS ARE IN MILLIMETERS
ANGLES ±X.X°
2 PL ±X.XX 3 PL ±X.XXX

SOLID EDGE
EDS-PLM SOLUTIONS

TITLE

SIZE	DWG NO	REV
A4		

FILE NAME: Narrow_TG_Cell_final_idea.dft

SCALE:	WEIGHT:	SHEET 4 OF 4

Appendix E

Radio-label Calculation Worksheet
&
Theoretical Extraction Worksheet

DEHP Calculation Sheet

Length (cm)	13											
B	25											
H	0.1											
Density of PVC	1.23					**mCi**	**MBq**	**Wastage (mCi) for methanol per run**	**mCi**	**MBq**	**% of rad**	
Density of DEHP	0.985		Amount of labelled DEHP (cm3)	0.142043147	**Total Activity (mCi)**	10.75	397.64					
Label quantity %	0.007							Activity of Unused PVC	0.08157	3.01800		
Molar mass of DEHP	390.56				**Total Activity (mCi)/sheet**	0.72	26.51	Activity of Fluid	0.01610	0.59586		
# of sheets	15		Amount of labelled DEHP (g)	0.1399125				Activity of Solid PVC	0.19553	7.23462	92.39051	
Specific Activity (mCi/mmol)		2	Amount of labelled DEHP (mg)	139.9125								
%of DEHP	0.5				**Total Activity/cm3**	0.02	0.82	**Total Solid waste (mCi)**	**0.27710**	**10.25262**		
# of test runs	45							**Total liquid waste (mCi)**	**0.01610**	**0.59586**	7.60949	
Vol of circuit (ml)	30				**Total Activity Unused section**	0.08	3.02					
used Section	28.8				**Total Activity Used Section**	0.63	23.49					
								Wastage (mCi) for Blood per run				
Volume of Sheet	32.5				**Total Activity/test run (mCi)**	0.21	7.830					
								Activity of Unused PVC	0.08157	3.01800		
			# of moles per large sheet	0.000358236				Activity of Fluid	0.00004	0.00138		
Mass of PVC sheet	39.975							Activity of Soild PVC	0.21160	7.82910	99.98236	
			# of moles for all sheets	0.005373534								
Mass of DEHP	19.9875		**# moles for all sheets (mmol)**	5.373534156				**Total Solid Waste (mCi)**	**0.29316**	**10.84709**		
Mass of DEHP(mg)	19987.5							**Total Liquid Waste (mCi)**	**0.00004**	**0.00138**	0.01764	
Vol of DEHP	20.29187817		**Total Hot DEHP(cm3)**	2.1306				**Total waste from experiment (mCi)**		**0.21163**	**10.76691**	
			Does not include DEHP for oral administration or invivo work									

Extraction Rates of DEHP

		Amount of DEHP Extracted																
Methanol mg/min	0.001497547	**60mins**	0.0899	**120mins**	0.2696	**180mins**	0.3594	**240mins**	0.4493	**300mins**	0.0225	**15mins**	0.0449	**30mins**	0.0674	**45mins**		
		% of labeled DEHP	0.0642		0.1927		0.2569		0.3211		0.0161		0.0321		0.0482			
		% of total DEHP	0.0004		0.0013		0.0018		0.0022		0.0001		0.0002		0.0003			
		Amount of Labelled DEHP (mg)	0.0006		0.0019		0.0025		0.0031		0.0002		0.0003		0.0005			
		Amount of Labelled DEHP (mmol)	0.0016		0.0048		0.0064		0.0081		0.0004		0.0008		0.0012			
		Activity of extracted DEHP (mCi)	0.0032		0.0097		0.0129		0.0161		0.0008		0.0016		0.0024			
Whole Blood mg/min	3.47E-06	**15mins**	0.0000521	**30mins**	0.0001562	**45mins**	0.0002083	**60mins**	0.0004167	**120mins**	0.0006250	**180mins**	0.0008333	**240mins**	0.0010417	**300mins**		
		% of labeled DEHP	0.0000372		0.0001117		0.0001489		0.0002978		0.0004467		0.0005956		0.0007445			
		% of total DEHP	0.0000003		0.0000008		0.0000010		0.0000021		0.0000031		0.0000042		0.0000052			
		Amount of Labelled DEHP (mg)	0.0000004		0.0000011		0.0000015		0.0000029		0.0000044		0.0000058		0.0000073			
		Amount of Labelled DEHP (mmol)	0.0000009		0.0000028		0.0000037		0.0000075		0.0000112		0.0000149		0.0000187			
		Activity of extracted DEHP (mCi)	0.0000019		0.0000056		0.0000075		0.0000149		0.0000224		0.0000299		0.0000373			

		Corrected	**microCi**							
Total Activity in 1 Test Run (mCi)	0.24	0.21	238.82							
			Activity for Methanol							
Total Activity per ml (mCi)	0.01	0.01	7.96	0.000107362	0.000322086	0.000429448	0.00053681	2.68405E-05	5.3681E-05	8.05215E-05
Size of scint. Sample (ml)	0.50	0.30								
Total Activity per sample size	0.00	0.00	3.98	5.3681E-05	0.000161043	0.000214724	0.000268405	1.34203E-05	2.68405E-05	4.02608E-05
			Activity for Whole Blood							
			TA per ml (mCi)	6.22322E-08	1.86697E-07	2.48929E-07	4.97857E-07	7.46786E-07	9.95715E-07	1.24464E-06
			TA per sample size	3.11161E-08	9.33483E-08	1.24464E-07	2.48929E-07	3.73393E-07	4.97857E-07	6.22322E-07

Bq to mCi 37000000
Efficiency of counter 0.6

			Methanol Extraction	**60mins**		**180mins**	**240mins**	**300mins**		**15mins**		**30mins**		**45mins**				
#of counts per sec	88364.7839	46982.87587	**Counts/s on extracted DEHP**		1191.72	3575.16	4766.87		5958.59		297.93		595.86		893.79			
#of counts per min	5301887.034	2818972.552	**Counts/min on extracted DEHP**		71503.11	214509.34	286012.46		357515.57		17875.78		35751.56		53627.34			
			time to reach 10K counts		8.39	2.80	2.10		1.68		33.56		16.78		11.19			
				10000														

			Whole Blood Extraction	**15mins**		**45mins**	**60mins**	**120mins**		**180mins**		**240mins**		**300mins**				
			Counts/s on extracted DEHP		0.69	2.07	2.76		5.53		8.29		11.05		13.82			
			Counts/min on extracted DEHP		41.45	124.34	165.79		331.57		497.36		663.15		828.93			
			Time to reach 10K counts (sec)		14476.45	4825.48	3619.11		1809.56		1206.37		904.78		723.82			
			Time to reach 10K counts (mins)		241.27	80.42	60.32		30.16		20.11		15.08		12.06			

Appendix F

Preparation of Test Material & Test Cell

Test Material Preparation

- Work Area prepared
- Storage box removed from cupboard
- One full tinfoil covered PPVC sheet removed from storage box and placed on work tray
- Storage box locked and returned to radiation store
- Tinfoil wrapper removed from PPVC sheet and moved off cutting board
- New tinfoil cover placed over PPVC sheet
- Cutting block placed on PPVC sheet
- Test blank cut out around cutting block
- Cutting block moved along large sheet and cutting procedure repeated until full sheet has been cut into 6 test blanks
- Cutting block and scalpel placed in decontamination box containing DECON and water and left to soak for 24hrs
- Three of the tinfoil wrapped blanks were replaced in original tinfoil wrapper
- Three remaining test blanks had the hole template placed on top and had the required holes stamped out.
- Waste material was then removed from hole punch into solid waste.
- Template was also disposed of in solid waste.
- Hole punch placed in decontamination box for 24hrs.
- Newly punched test blanks replaced into original tinfoil wrapper and this was resealed.
- Original tinfoil sheet, now containing 6 cut and punched test blanks was returned to storage box
- Storage box returned to radiation store
- Process repeated as required
- Work tray liners disposed in solid waste
- Work area cleaned and swabbed for records

Test Cell Preparation

- Work Tray prepared, lined with absorbent course and covered in tinfoil sheet
- Test cell laid out on second work tray
- Storage box removed from radiation store
- One full PPVC sheet removed from Storage box and 2 test blanks were removed and placed on work tray.
- Remainder of PPVC sheet rewrapped and replaced in Storage box
- Storage box then returned to radiation store
- Covered test blanks positioned on work tray

- Cover removed from base test blank and blank sprayed with thin layer of spray mount, Test cell base was then positioned correctly on top of sprayed PPVC and then recovered with tinfoil
- Process repeated for hole punched section
- Inlet and Outlet ports were then positioned through the punched test material and screwed into place
- Base of test cell was then uncovered and the metal gasket was positioned
- Test cell lid was then positioned on top of the gasket
- Test Cell was then secured using 4 C clamps
- Test cell was then placed into the containment box for use
- Work tray lining removed and disposed of in solid waste
- Work area cleaned and swabbed for records

Appendix G

LSC Sample Protocols

Blood and Plasma Decolourisation

The following decolourisation protocol is taken from the Perkin Elmer LSC Application Notes on "Sample Preparation and Counting of Biological Samples" by Jock Thomson. Available at www.perkinelmer.com/lifesciences

SOLVABLE™ Method (Alkaline solubiliser)
1. Add a maximum of 0.5 mL blood to a glass scintillation vial.
2. Add 1.0 mL SOLVABLE™.
3. Incubate the sample at 55 – 60°C for 1 hour. Sample at this stage will be brown/green in appearance.
4. Add 0.1 mL of 0.1 M EDTA-di-sodium salt solution which helps reduce foaming when the subsequent
hydrogen peroxide is added.
5. Add 0.3 mL to 0.5 mL of 30% hydrogen peroxide in 0.1 mL aliquots. Gently agitate between additions to
allow reaction foaming to subside. Hydrogen peroxide treatment helps reduce the amount of color present,
and thus reduces colour quench in the final mixture.
6. Allow to stand for 15 to 30 minutes at room temperature to complete the reaction.
7. Cap the vial tightly and place in an oven or water bath at 55 – 60°C for 1 hour. The colour will change from
brown/green to pale yellow.
8. Cool to room temperature and add 10 to 15 mL of either Ultima Gold, Opti-Fluor™, Hionic-Fluor or Pico-Fluor
40. If colour is present, use 15 mL cocktail, as this reduces colour quench by diluting the colour.
9. Temperature and light adapt for 1 hour before counting.

Tissue Preparation Protocol

The following protocols were used in the preparation of the various animal tissue samples that were taken during the Ex vivo and Gavage experiments. The protocols were taken from the following application notes supplied by Perkin Elmer. "LSC Sample Preparation by Solubilzation" By J. Thomson and J.D. Burns and are listed according to the type of tissue that has to be broken down.

1. Whole Tissue

The method of solubilizing whole tissue is relatively straightforward, and apart from colour formation with certain tissue types, no major problems should be encountered during sample preparation and LS counting.

(i) Muscle (50 - 200 mg)

Procedure

1. Place selected sample size in a 20 mL glass scintillation vial.
2. Add an appropriate volume of solubilizer (1-2 mL depending on sample size).
3. Heat in an oven or water bath at 50 - 60 °C for the specified time with occasional swirling.
4. Cool to room temperature and add 10 mL of a selected cocktail.
5. Temperature and light adapt for at least one hour before counting.

(ii) Liver (50 - 100 mg) Kidney / Heart / Sinew / Brains / Stomach Tissue

Procedure

1. Place selected sample size in a 20 mL glass scintillation vial.
2. Add 1-2 mL of solubilizer.
3. Heat in an oven or water bath at 50 - 60 °C for the specified time with occasional swirling.
4. Cool to room temperature.
5. Add 0.2 mL of 30% hydrogen peroxide in two aliquots of 0.1 mL, with swirling between additions. Allow any reaction to subside between additions of the hydrogen peroxide.
6. Heat again at 50 - 60 °C for 30 minutes to complete decolorization.
7. Add 10 mL of a selected cocktail and temperature and light adapt for at least one hour before counting.

Appendix H

Work sheet Examples for Invitro Experiments
&
Exvivo Experiments
&
Gavage Experiments

Plasma Averages

Run 1

Time Mins	CPM	DPM	MBq	MBq of res	Hot DEHP extracted mmoles	Total mmoles	Total moles	Extracted %	Concentration of Res Concentrations mg/ml	Concentrations mg/ml
0	0	0	0	0	0	0	0	0	0.00000000	0
5	50.29	54.57	9.1E-07	5.36616E-05	0.000725156	0.103607445	0.000103607	0.202445	0.00137169	4.6498E-05
10	58.14	63.2	1.05E-06	6.10946E-05	0.000825602	0.117958725	0.000117959	0.230486	0.00158862	5.478E-05
15	74	80.97	1.35E-06	7.6923E-05	0.001039501	0.148519676	0.00014852	0.290201	0.00203529	7.14138E-05
30	130.86	142.65	2.38E-06	0.000133143	0.001799225	0.257066096	0.000257066	0.502296	0.00358570	0.000128061
45	121.86	133.65	2.23E-06	0.000122515	0.001655607	0.236546568	0.000236547	0.462202	0.00335948	0.000122163
60	132.29	144.18	2.4E-06	0.000129765	0.001753576	0.250543869	0.000250544	0.489552	0.00362416	0.000134228
120	226.29	245.96	4.1E-06	0.000217269	0.002936068	0.419493613	0.000419494	0.819673	0.00618254	0.000233304
180	350.71	381.65	6.36E-06	0.00033077	0.004469864	0.638636313	0.000638636	1.247868	0.00959330	0.000368973
240	428.71	465.86	7.76E-06	0.000395989	0.005351202	0.764558282	0.000764558	1.493914	0.01171003	0.000459217
300	582.57	632.62	1.05E-05	0.000527194	0.007124242	1.01788314	0.001017883	1.9889	0.01590178	0.000636071
	0.074									

Run 2

Time Mins	CPM	DPM	MBq	MBq of res	Hot DEHP extracted mmoles	Total mmoles	Total moles	Extracted %	Concentrations mg/ml	Concentrations mg/ml
0	0	0	0	0	0	0	0	0	0.00000000	0
5	47.5	52.36	8.73E-07	5.14884E-05	0.000695789	0.099411505	9.94115E-05	0.194246	0.00131614	4.46149E-05
10	54.1	59.4	9.9E-07	5.74211E-05	0.000775961	0.11086627	0.000110866	0.216628	0.00149310	5.14862E-05
15	55.5	60.83	1.01E-06	5.77897E-05	0.000780941	0.111577768	0.000111578	0.218018	0.00152905	5.36507E-05
30	82.1	89.74	1.5E-06	8.3759E-05	0.001131878	0.161718272	0.000161718	0.315991	0.00225574	8.05621E-05
45	99.3	108.25	1.8E-06	9.92312E-05	0.001340962	0.191591216	0.000191591	0.374361	0.00272101	9.89459E-05
60	120.9	131.59	2.19E-06	0.000118433	0.001600451	0.228666027	0.000228666	0.446804	0.00330770	0.000122507
120	214.5	232.84	3.88E-06	0.000205679	0.002779452	0.397116982	0.000397117	0.77595	0.00585276	0.000220859
180	298.7	324.42	5.41E-06	0.00028117	0.00379959	0.54287015	0.00054287	1.060745	0.00815474	0.000313644
240	410.1	445.29	7.42E-06	0.000378504	0.00511492	0.73079929	0.000730799	1.427951	0.01119298	0.00043894
300	509.2	553.19	9.22E-06	0.000461001	0.006229742	0.890080576	0.000890081	1.73918	0.01390519	0.000556208

Whole Blood Averages

Run 1

Time Mins	CPM	DPM	MBq	MBq of res	Hot DEHP extracted mmoles	Total of mmoles	Total moles	Extracted %	Concentration of res Concentrations mg/ml	Concentrations mg/ml	MBq per ml
0	0	0	0	0	0	0	0	0	0.00000000	0.00000000	0
5	103.75	118.78	1.98E-06	0.0001168	0.001578414	0.225517543	0.00022552	0.440652	0.00293594	0.00009952	3.95941E-06
10	134.5	153.7	2.56E-06	0.0001486	0.002007833	0.28687114	0.00028687	0.560534	0.00373468	0.00012878	5.12344E-06
15	149.87	169.64	2.83E-06	0.0001612	0.002177854	0.311163121	0.00031116	0.608	0.00405093	0.00014214	5.65478E-06
30	150.87	171.45	2.86E-06	0.00016	0.002162476	0.308965875	0.00030897	0.603706	0.00402232	0.00014365	5.71511E-06
45	190.5	215.51	3.59E-06	0.0001976	0.002669659	0.381430235	0.00038143	0.745299	0.00496571	0.00018057	7.18381E-06
60	195.75	221.93	3.7E-06	0.0001997	0.002699203	0.385651275	0.00038565	0.753546	0.00502067	0.00018595	7.39781E-06
120	295.75	334.06	5.57E-06	0.0002951	0.003987733	0.569751327	0.00056975	1.11327	0.00741740	0.00027990	1.11356E-05
180	382.62	430.98	7.18E-06	0.0003735	0.005047614	0.721182964	0.00072118	1.409161	0.00938884	0.00036111	1.43663E-05
240	483.87	544.18	9.07E-06	0.0004626	0.006250841	0.893095192	0.0008931	1.74507	0.01162691	0.00045596	1.81397E-05
300	590.25	662.8	1.1E-05	0.0005523	0.007464113	1.066442644	0.00106644	2.083784	0.01388366	0.00055535	2.20938E-05

0.074

RUN 2

Time Mins	CPM	DPM	MBq	MBq of res	Hot DEHP extracted mmoles	Total of mmoles	Total moles	Extracted %	Concentrations mg/ml	Concentrations mg/ml	
0	0	0	0	0	0	0	0	0	0.00000000	0.00000000	0
5	163.13	183.07	3.05E-06	0.00018	0.002432736	0.34757953	0.00034758	0.679156	0.03220777	0.00109179	6.10246E-06
10	126.63	142.27	2.37E-06	0.0001375	0.001858519	0.265537782	0.00026554	0.51885	0.02502977	0.00086310	4.74243E-06
15	142.38	159.95	2.67E-06	0.000152	0.002053453	0.293389184	0.00029339	0.57327	0.02814023	0.00098738	5.33177E-06
30	167	186.74	3.11E-06	0.0001743	0.002355326	0.336519613	0.00033652	0.657545	0.03285344	0.00117334	6.22479E-06
45	173.67	194.48	3.24E-06	0.0001783	0.002409147	0.344209327	0.00034421	0.67257	0.03421515	0.00124419	6.4828E-06
60	225.5	252.12	4.2E-06	0.0002269	0.003066386	0.438112916	0.00043811	0.856054	0.04435584	0.00164281	8.40417E-06
120	313.13	349.18	5.82E-06	0.0003084	0.004168223	0.59553903	0.00059554	1.163658	0.06143174	0.00231818	1.16396E-05
180	485.13	539.59	8.99E-06	0.0004677	0.006319649	0.902926158	0.00090293	1.764279	0.09493085	0.00365119	1.79867E-05
240	543.5	604.13	1.01E-05	0.0005135	0.00693947	0.991483697	0.00099148	1.937317	0.10628546	0.00416806	2.01381E-05
300	657.63	713.28	1.19E-05	0.0005944	0.008032593	1.147664769	0.00114766	2.242488	0.12548838	0.00501954	2.37765E-05

Methanol Averages

Run 1

Time Mins	CPM	DPM	MBq	MBq of res	Hot DEHP extracted mmoles	Total mmoles	Total moles	Extracted %	Concentrations mg/res vol Concentrations mg/ml
0	0	0	0	0	0	0	0	0	0.00000000
5	8243.75	8857.4	0.000148	0.00870995	0.117702039	16.81679648	0.016816796	32.8593073	0.22264298
10	10511.3	11286.31	0.000188	0.01091032	0.147436728	21.06516994	0.02106517	41.1604489	0.28369699
15	12160.3	13061.41	0.000218	0.01240859	0.167683617	23.95796448	0.023957964	46.8128468	0.32831658
30	16526.8	17757.31	0.000296	0.01657382	0.223970551	32.0000165	0.032000017	62.5266754	0.44635452
45	21083.5	22635.51	0.000377	0.02074963	0.280400439	40.06249312	0.040062493	78.2804129	0.56897481
60	29665.5	31811.91	0.00053	0.02863129	0.386909346	55.28005974	0.05528006	108.014893	0.79963630
120	46400.9	49772.71	0.00083	0.04396677	0.594145583	84.88914433	0.084889144	165.869789	1.25110582
180	60046.4	64395.11	0.001073	0.05581021	0.754192048	107.755943	0.107755943	210.550544	1.61866004
240	68978	74033.51	0.001234	0.06292974	0.85040192	121.5020247	0.121502025	237.409805	1.86093454
300	83017	89138.21	0.001486	0.07428333	1.003828747	143.4230361	0.143423036	280.242531	2.24061204
	0.074		0.006379	0.33497366	1.00E-03				

Run 2

Time Mins	CPM	DPM	MBq	MBq of res	Hot DEHP extracted mmoles	Total mmoles	Total moles	Extracted %	Concentrations mg/ml
0	0	0	0	0	0	0	0	0	0.00000000
5	5966.25	6400.95	0.000107	0.00629439	0.08505937	12.15294256	0.012152943	23.7463345	0.16089672
10	9665.5	10369.15	0.000173	0.01002371	0.135455569	19.35334994	0.01935335	37.8156252	0.26064291
15	13204.3	14185.25	0.000236	0.01347626	0.182111581	26.0193743	0.026019374	50.8407541	0.35656585
30	20589	22086.25	0.000368	0.02061425	0.278570887	39.801094	0.039801094	77.7696501	0.55516840
45	27590.3	29613.65	0.000494	0.02714639	0.366843091	52.4130735	0.052413074	102.412923	0.74438000
60	33910.5	36391.85	0.000607	0.03275332	0.442612433	63.238694	0.063238694	123.565727	0.91475942
120	47330.2	50853.15	0.000848	0.04492118	0.607042985	86.73187356	0.086731874	169.470403	1.27826417
180	71510.1	76806.75	0.00128	0.06656718	0.899556504	128.5250351	0.128525035	251.132469	1.93064376
240	95137.3	101992.9	0.0017	0.0866957	1.171563498	167.3883064	0.167388306	327.069653	2.56373243
300	111971.5	120278.9	0.002005	0.10023442	1.354519207	193.5282862	0.193528286	378.146066	3.02337630
				0.4087268					

Animal 2 Tissue Samples

Tissue	Time	CPMA	CPMB	DPM1	DPM2	SIS	tSIE	Eff	Corrected DPM Levels	Corrected DPM2	Activity of Sample (MBq)	Sample Size (g)	Activity per g (MBq)	# of mmoles pr gram of tissue	total dehp mmoles
Background	7	40.71	31.71	42.97	33.81251	39.331	480	94.74052	0	0	0	0	0	0	0
Skin	7	255.14	172.14	275.36	185.962	13.944	331	92.65689	232.39	152.1495168	3.87324E-06	0.19	2.03855E-05	0.00027548	0.039359433
Adipose	7	162	119.71	174.15	128.9952	15.93	348	93.02326	131.18	95.18265266	2.18638E-06	0.21	1.04113E-05	0.000140694	0.020101725
Muscle	7	360.14	248.17	393.8	270.4334	11.161	277	91.45251	350.83	236.6208558	5.84728E-06	0.22	2.65786E-05	0.00035917	0.051316738
Liver	7	278.14	205	313.29	227.3965	10.521	185	88.78036	270.32	193.5839764	4.50542E-06	0.19	2.37128E-05	0.000320443	0.045783562
Kidney	7	260	192.86	288.4	212.4463	11.376	218	90.15257	245.43	178.6338195	4.09058E-06	0.2	2.04529E-05	0.000276391	0.039489592
Heart	7	580.29	409.71	633.75	446.2964	11.009	283	91.5645	590.78	412.4839122	9.84653E-06	0.22	4.4757E-05	0.000604824	0.086414795
Brain	7	1058.29	329.86	1140.25	355.8669	7.23	338	92.8121	1097.28	322.0544349	1.82884E-05	0.21	8.70875E-05	0.001176858	0.168144694
Lungs	7	399	285.71	451.33	317.3176	8.217	178	88.40538	408.36	283.5051205	6.80614E-06	0.19	3.58218E-05	0.000484078	0.069163123

Run 2

Tissue	Time	CPMA	CPMB	DPM1	DPM2	SIS	tSIE	Eff	Corrected DPM Levels	Corrected DPM2	Activity of Sample (MBq)	Sample Size (g)	Activity per g (MBq)	# of mmoles pr gram of tissue	total dehp mmoles
Background	7	44	37.57	46.94	40.06105	44.438	434	93.73669	0	0	0	0	0	0	0
Skin	7	197.71	149.43	213.67	161.4285	16.086	325	92.53054	166.73	121.3674678	2.77889E-06	0.19	1.46257E-05	0.000197645	0.028238729
Adipose	7	137.14	107.57	147.77	115.9135	19.985	338	92.80639	100.83	75.85248455	1.68053E-06	0.21	8.00254E-06	0.000108142	0.01545096
Muscle	7	287.57	221.43	313.76	241.2945	12.166	287	91.65286	266.82	201.233463	4.44709E-06	0.22	2.0214E-05	0.000273163	0.039028396
Liver	7	240.29	176.29	266.62	195.5499	13.648	216	90.12452	219.68	155.4888322	3.66141E-06	0.19	1.92706E-05	0.000260413	0.037206766
Kidney	7	262	187	288.55	205.9912	11.731	246	90.79882	241.61	165.9301506	4.02691E-06	0.2	2.01346E-05	0.000272089	0.038874956
Heart	7	534	400.86	581.67	436.6561	11.267	293	91.80463	534.73	396.5950784	8.91234E-06	0.22	4.05107E-05	0.000547441	0.078216228
Brain	7	534.57	215.71	577.03	232.7171	8.742	330	92.64163	530.09	192.6560343	8.83501E-06	0.21	4.20715E-05	0.000568533	0.081229787
Lungs	7	254.14	173.71	282.59	192.9273	10.917	210	89.93241	235.65	152.8661997	3.92758E-06	0.19	2.06715E-05	0.000279344	0.039911573

Run 3

Tissue	Time	CPMA	CPMB	DPM1	DPM2	SIS	tSIE	Eff	Corrected DPM Levels	Corrected DPM2	Activity of Sample (MBq)	Sample Size (g)	Activity per g (MBq)	# of mmoles pr gram of tissue	total dehp mmoles
Background	7	46	37.43	49.05	39.91177	39.512	436	93.78186	0	0	0	0	0	0	0
Skin	7	199.14	156.71	215.13	169.2931	16.096	326	92.56728	166.08	129.3813	2.76806E-06	0.19	1.45687E-05	0.000196874	0.02812864
Adipose	7	130.86	104.43	141.01	112.53	22.102	337	92.80193	91.96	72.6182168	1.5327E-06	0.21	7.29856E-06	9.86292E-05	0.014091741
Muscle	7	276	220.43	300.76	240.2048	14.225	292	91.76752	251.71	200.2930355	4.19525E-06	0.22	1.90693E-05	0.000257694	0.03681822
Liver	7	157.71	129.29	174.94	143.4151	17.122	218	90.15091	125.89	103.5033104	2.09821E-06	0.19	1.10432E-05	0.000149232	0.021321739
Kidney	7	193.29	159.71	212.92	175.9297	15.726	245	90.78057	163.87	136.017936	2.73122E-06	0.2	1.36561E-05	0.000184542	0.02636662
Heart	7	480.86	379.71	523.8	413.6175	12.246	293	91.80221	474.75	373.7057011	7.91266E-06	0.22	3.59666E-05	0.000486036	0.069442811
Brain	7	335.86	179	362.34	193.1128	11.698	332	92.69195	313.29	153.2010133	5.2216E-06	0.21	2.48648E-05	0.000336011	0.048007848
Lungs	7	124.29	102.43	138.04	113.7617	16.595	213	90.03912	88.99	73.84989211	1.4832E-06	0.19	7.8063E-06	0.00010549	0.01507206

Gavage Sample Study

Animal 1

				Run 1				Run 2				Run 3					Average Activity MBq	% of dose
Organ	**Weight taken**	**% of T. Dry weight**	**Wet Weight Equiv**	**CPM**	**DPM**	**Activity (MBq)**	**Activity MBq per g**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**			
Kidney 1	0.04g	45.30011325	0.1723665	10587	11574.5	0.0001926	0.001117386	10560.8	11554.2	0.000192288	0.001115574	10587	11596.8	0.000192971	0.001119541	0.0011175	0.009293141
Liver 1	0.05g	11.01321586	0.173404	16553.8	18282.7	0.000304403	0.001755458	16481.5	18225.6	0.000303478	0.001750119	16482.5	18248.5	0.000303833	0.00175217	0.001752582	0.014574488
Brain1	0.03g	67.41573034	0.1366872	188.25	201.69	3.05267E-06	2.23333E-05	173.75	186.16	2.82017E-06	2.06323E-05	151.25	162.27	2.3955E-06	1.75255E-05	2.01637E-05	0.000167681
Heart 1	0.02g	26.45502646	0.08369298	697.5	752.02	1.22249E-05	0.000146068	691.75	745.15	1.21367E-05	0.000145014	689.5	743.79	1.20875E-05	0.000144427	0.00014517	0.001207232
Skin 1	0.02g	9.00090009	0.048321	912.5	980.51	1.6033E-05	0.000331803	944	1014.88	1.66322E-05	0.000344202	955	1026.07	1.67922E-05	0.000347514	0.000341173	0.002837194
Testes 1	0.02g	27.54820937	0.0286416	395.75	423.84	6.75518E-06	0.000235852	385.75	413.23	6.60467E-06	0.000230597	383.5	410.68	6.53568E-06	0.000228188	0.000231546	0.001925537
			Background	17.5	18.53	0		16	16.95	0		17.5	18.54	0			

Animal 3

				Run 1				Run 2				Run 3						
Organ	**Weight taken**	**% of T. Dry weight**	**Wet Weight Equiv**	**CPM**	**DPM**	**Activity (MBq)**	**Activity MBq per g**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**	**Average Activity MBq**	**% of dose**	
Kidney 1	0.04g	25.95717067	0.135366645	215.75	234.5	3.66134E-06	2.70476E-05	225.25	244.73	3.76117E-06	2.77851E-05	193.5	210.66	3.19751E-06	2.36211E-05	2.61513E-05	0.000217474	
Liver 1	0.025g	46.38218924	0.075602968	90.5	97.95	1.3855E-06	1.8326E-05	77	83.32	1.071E-06	1.41661E-05	79.25	85.81	1.11667E-06	1.47702E-05	1.57541E-05	0.000131011	
Brain1	0.03g	100	0.1082	52.75	61.34	7.75335E-07	7.16576E-06	45	47.5065	4.74109E-07	4.38179E-06	44	47.38736	4.7629E-07	4.40194E-06	5.3165E-06	4.4212E-05	
Heart 1	0.04g	35.6824264	0.168064228	65.75	71.76	9.49002E-07	5.64666E-06	58.25	60.86	6.96668E-07	4.14525E-06	49.5	54.06	5.87501E-07	3.49569E-06	4.4292E-06	3.68333E-05	
Skin 1	0.04g	27.75850104	0.081582235	687.25	740.06	1.20874E-05	0.000148162	656.25	707.01	1.14659E-05	0.000140544	688.25	742.27	1.20577E-05	0.000147798	0.000145501	0.001209988	
Testes 1	0.02g	30.58103976	0.123547401	93.75	100.62	1.43E-06	1.15745E-05	80.5	86.47	1.1235E-06	9.09369E-06	77.25	83.03	1.07034E-06	8.66336E-06	9.77719E-06	8.13072E-05	
			Background	14	14.82	0		18	19.06	0		17.75	18.81	0				

Animal 5

				Run 1				Run 2				Run 3						
Organ	**Weight taken**	**% of T. Dry weight**	**Wet Weight Equiv**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**	**CPM**	**DPM**	**Activity**	**Activity MBq per g**	**Average Activity MBq**	**% of dose**	
Kidney 1	0.03g	19.54397394	0.151837134	90	97.58	1.33934E-06	8.82087E-06	83.75	90.89	1.51484E-06	9.97672E-06	82.25	89.4	1.12767E-06	7.42683E-06	8.74147E-06	7.26942E-05	
Liver 1	0.06g	23.83790226	0.191752086	64.75	69.73481	8.75249E-07	4.56448E-06	74.05	79.75077	1.32918E-06	6.93177E-06	122.75	134.6302	1.88151E-06	9.81218E-06	7.10281E-06	5.9067E-05	
Brain1	0.04g	22.77904328	0.15428246	36.08	38.85764	3.60628E-07	2.33745E-06	47	50.61832	8.4364E-07	5.46815E-06	48.5	52.23379	5.08231E-07	3.29416E-06	3.69992E-06	3.07686E-05	
Heart 1	0.04g	36.26473255	0.182194016	52.25	56.27249	6.50876E-07	3.57243E-06	47	51.85	8.64168E-07	4.74312E-06	43.75	48.29	4.42501E-07	2.42873E-06	3.58143E-06	2.97832E-05	
Skin 1	0.06g	10.67615658	0.093811388	136.5	146.29	2.15117E-06	2.29308E-05	117.75	126.34	2.10567E-06	2.24458E-05	126.25	135.37	1.89384E-06	2.01877E-05	2.18548E-05	0.000181744	
Testes 1	0.04g	32.05128205	0.225224359	38.5	41.46394	4.04066E-07	1.79406E-06	42.25	45.50264	7.58379E-07	3.36721E-06	58	62.34	6.76668E-07	3.00442E-06	2.7219E-06	2.26353E-05	