TITLE PAGE

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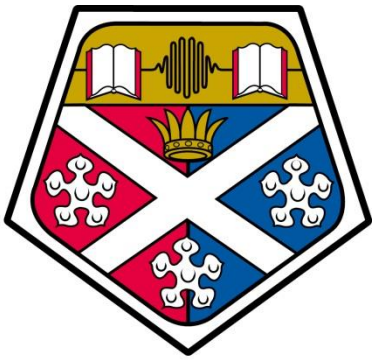

University of Strathclyde Glasgow

Department of Biomedical Engineering

2015 MSc Thesis (Literature review)

Project: Factors affecting the metabolism and toxicity of DEHP.

‘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: 13th of August 2015

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I would also like to thank my family, my cousins, fiancé and friends for supporting me during this critical time in life.

Abstract

Diethylhexyl phthalate (DEHP) is an organic compound belonging to the class of phthalates. Phthalates are chemicals that are added to Poly vinyl chloride (PVC) to make them soft and flexible. PVC is synthetic polymer, widely used in medical devices such as; blood bags, intravenous tubing and intragastric feeding equipment. During use, DEHP leaches out of the devices into the patients' bodies or into the fluids stored in the PVC bags.

This project concentrates on factors affecting the metabolism and toxicity of DEHP. Studies have reported that there is concern for DEHP effects in premature infants, which has led to withdrawal of the plasticiser from use in paediatrics in a number of countries. DEHP has been observed to cause reproductive defects in animals. The objective of my research was to find out if undeveloped neonatal metabolism could contribute to the adverse effects in premature infants exposed to DEHP.

Premature infants are exposed to DEHP when PVC tubing is used in nasogastric tubing. DEHP metabolism to water-soluble products, that can be excreted, involves Glucuronidation, which is catalysed by UDP- glucuronyltransferase (UDPGT) in the liver. This hepatic enzyme develops to its adult levels to a large extent after birth. Deficient UDPGT can lead to hyperbilirubinemia as it is responsible for metabolism of bilirubin thus leading to jaundice. Levels of UDPGT may be induced by Phenobarbitone both in animals and humans. We posed the question, 'Could Phenobarbitone be used to speed up metabolism and excretion of DEHP in premature infants, minimising risk of adverse effects on the reproductive tract?'

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1. Rationale for the project

1.2 Introduction

Plastics have been widely used since the mid-1940s. They were used to make toys, tools etc., and have also been used in medical applications since rubbers, glass and metals were not able to provide the service which were sought by the professionals. Constant sterilisation problems and cross infections were experienced using rubbers, glass and metals for medical devices. Also they were expensive, therefore could not be regularly discarded after a single use. Plastics were thought to be inert and unique, however after evaluation, it was determined that plastics can be modified according to their intended use by mixing other elements with the plastic. Plastics were low cost in comparison to other materials, therefore could be disposed after a single use, which would reduce the risk of infections and need for constant sterilisation. Using plastics for tubing such as intravenous tubes or catherisation provided optical clarity which would assist professionals to detect any contaminants or air bubbles within these tubes, thus preventing infections or obstructions (Guess and Haberman, 1968).

1.3 Polyvinyl chloride (PVC)

Polyvinyl chloride (PVC) is the most adaptable plastic in industry. It possesses thermoplastic properties; however it must be combined with other substances to achieve a variety of properties. PVC is an unstable polymer, thus requires a stabilizing agent. It mainly consists of resin, plasticiser, stabilising agents and other substances to acquire specific properties. This plasticised polymer is then used in medical applications; to store, collect or transport fluids such as blood, plasma, urine

etc. It can be inserted into the body to provide drug solutions to veins as an air-way in tracheotomy and many other purposes (Guess and Haberman, 1968).

At room temperature, PVC is a rigid and brittle material with a Young's modulus of 2.4 – 4.1 GPa. To soften and enhance its flexibility phthalates are added to the PVC as a plasticiser. Di-2-ethylhexyl phthalate (DEHP) is the only plasticiser used in medical devices. DEHP is not chemically bound to PVC and is shown to leach into solutions that it has contact with (Marcilla et al., 2004). DEHP is highly lipophilic therefore can dissolve in blood and plasma (Haishimaa et al., 2005). During *in vitro* tests, the major factors that may affect the leaching of DEHP from PVC medical devices are temperature, storage time, the concentration of DEHP in the PVC and the rate of PVC degradation. The migration process of DEHP may differ due to the polymeric properties of the PVC (molecular weight, tensile strength etc.), the process of plasticization, surrounding media, the media compatibility with the plasticiser and its effect on the polymer itself (Latini et al., 2010).

1.4 Background information on DEHP toxicity

A study was conducted by Jaeger & Rubin (1972), which demonstrated the leakage of DEHP from PVC plastic blood bags into the blood. The blood bags were stored at 4°C and administered at the rate of 0.25 ± 0.03 mg/100 ml/day. DEHP was found in lipid-containing and lip-free fractions of plasma. Detectable amounts of DEHP were present in the seven of twelve lung tissue samples taken from patients, at autopsy, who received transfusions from blood that had been stored. A patient who received open-heart surgery had DEHP metabolites excreted in the urine, the amount of metabolites was in excess of the calculated amount from the transfused blood, it was then suggested that the PVC tubing of the cardiopulmonary amount had also exposed

the patient to DEHP. The study did not consider the toxicological implications, but concluded the study with the idea of developing plastic medical equipment without any extractable materials to blood or its fractions (Jaeger and Rubin, 1972).

With regards to health effects, DEHP has exhibited its ability to interfere with the endocrine system in the body. It has been classified as an endocrine disruptor. Foetal exposure to these compounds produced reproductive and developmental toxicity. The effects observed in rodents have raised concerns regarding human health (Lyche et al., 2009). However, many reports have concluded their review with the thought that further investigation is required to obtain more information on the toxic interactions between the compounds, in humans.

Assessment of DEHP exposure in 6 premature new born babies was carried out. Levels of DEHP exposure were obtained by measuring levels of DEHP metabolites: mono-(2-ethylhexyl) phthalate (mEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), and mono-(2-ethyl-5-oxohexyl) phthalate (mEOHP), in urine samples. The study provided quantitative evidence verifying that neonates, who require therapeutic medical devices, are exposed to higher concentrations of DEHP than the general population. This could be due to their smaller size and under developed vital organs such as the liver (Calafat et al., 2004).

Reviews were published which evaluated human and environmental proof of the effects of DEHP on reproduction and development in humans. Rodents were used to observe the adverse effects of phthalates. It was determined that high levels of DEHP exposure had caused reproductive toxicity in rodent models. This brought about

concerns regarding the possible adverse effects of DEHP on humans (Lyche et al., 2009).

Hypospadias and DEHP have also been linked in previous studies. Hypospadias is a condition, in boys, where the urinary tract opening is not positioned at the tip of the penis, causing them to urinate in different directions. During sexual differentiation in mammals, exposure to anti-androgenic chemicals can cause abnormalities of the reproductive tract. DEHP has displayed anti- androgenic activity by suppression of fetal testicular testosterone production, which causes damage to the male rats' reproductive system (Ostby. et al., 2000). Another condition observed in rats was undescended testes (Moore et al., 2001).

1.5 Project Aim

This literature review;

•Investigates the factors that affect metabolism and toxicity caused by DEHP.

• Researches the metabolism of DEHP in Humans.

• Gives a detailed study of reproductive toxicity in animals and defects in male reproductive tract in the human population at birth.

• Investigates the amount of DEHP exposure and DEHP effects in neonates.

• Presents a novel idea of how to decrease the risk of toxicity effects in neonates by using the drug Phenobarbitone.

2. Di-2-ethylhexyl phthalate (DEHP)

2.1 Chemical structure

DEHP is composed of paired ester groups on a benzene ring. The chemical formula for DEHP is $C_6H_4(C_8H_{17}OO)_2$ and it has a molecular weight of 390 g/mol. Phthalates are diesters of phthalic acid, also known as 1,2-benzenedicarboxylic acids. The nature of the phthalates depends on the length of the alkyl or dialkyl side chains. The longer the side branches of the phthalates are, the more hydrophobic and lipophilic the compound becomes. It also creates a vast number of isoforms. Short branched phthalates with lower molecular weights, are commonly used in cosmetic products, i.e. dimethyl phthalate (DMP) and diethyl phthalate (DEP). Longer branched phthalates with higher molecular weights are used in many types of plastic products such as vinyl flooring, plastic bags, building materials etc., however, DEHP is the only plasticiser used in medical applications (Tickner et al., 2001a). Figure 1 shows the differences of chemical structures of phthalate esters.

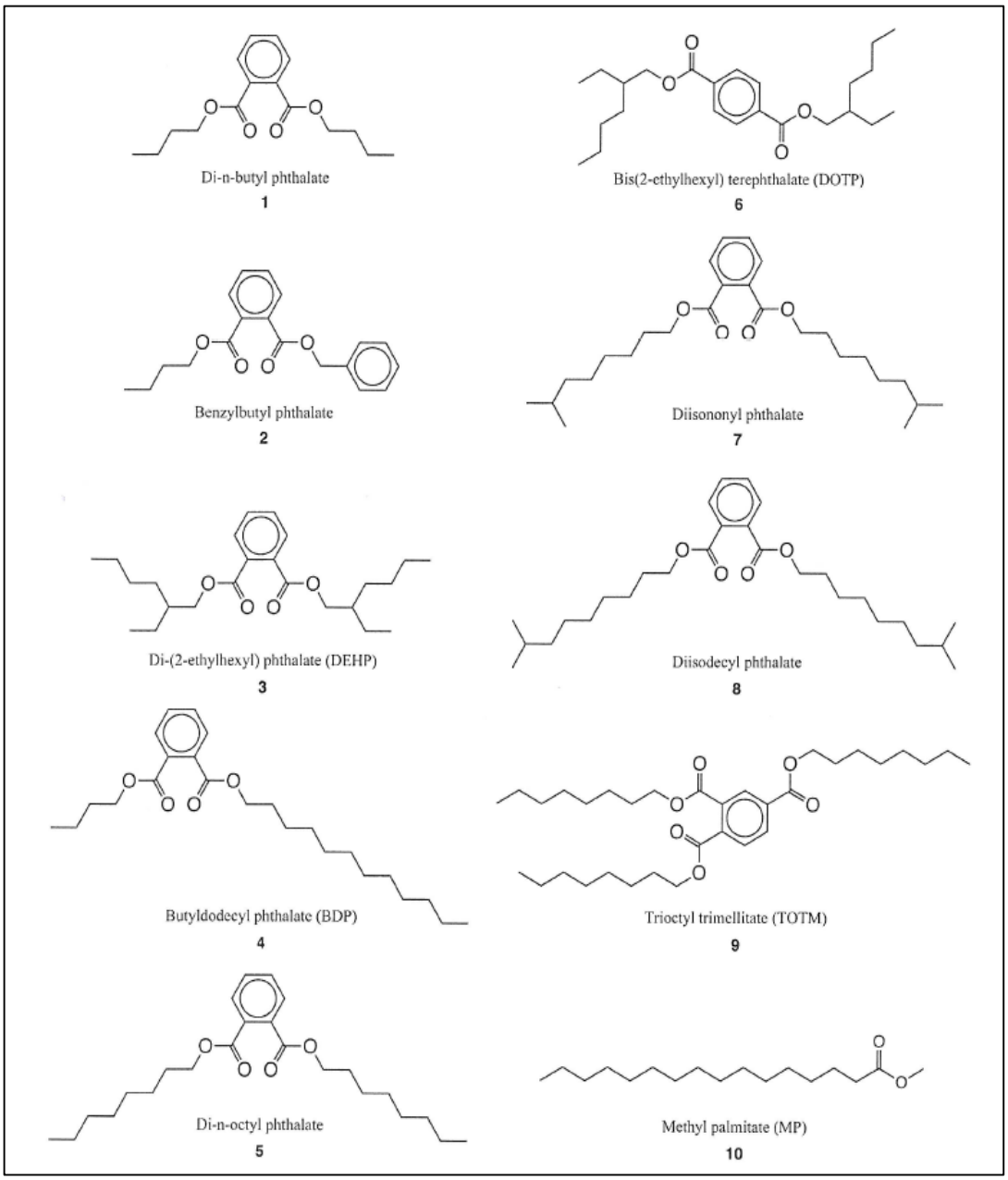

Figure 1: 2-D structures of phthalate esters. (Permission to use table was given by Søren T Larsen, from the website 'http: //www.biomedcentral.com/1471-2172/9/61'.)

DEHP in its pure form is a clear oily liquid, which is highly lipophilic. It is an aromatic diester and used to soften rigid polymer polyvinyl chloride (PVC). It contributes to the polymers flexibility, strength, durability, optical clarity and resistance to various ranges of temperature. PVC is used in medical devices, such as

intravenous tubing, ECMO, blood bags etc. DEHP acts as a semisolid in the PVC matrix; it is not bonded strongly with PVC and therefore can readily migrate into the blood or liquids that are stored in medical devices made of PVC. The rate of DEHP leaching into the blood systems or liquids stored in PVC bags, can lead to toxicity, if that liquid is administered to a patient.

2.2 Exposure

DEHP is present in the environment through sources such as food, air and water. The average total daily individual ambient exposure to DEHP, in the US has been observed to be of 0.27 mg/day. However, medical exposure of DEHP through PVC medical devices exceeds environmental exposure of up to three orders of magnitude (Tickner et al., 2001). Table 1 shows the total exposure of DEHP to humans during treatment.

Treatment	Total exposure (mg/patient)	Time period	Mg/kg body weight
Haemodialysis	**0.5-360**	**Dialysis session**	**0.01-7.2**
Blood transfusion in adults	**14-600**	**Treatment**	**0.2-8.0**
Extracorporeal oxygenation in infants	**-**	**Treatment period**	**42.0-140.0**
Cardiopulmonary bypass	**2.3-168**	**Treatment day**	**0.03-2.4**
Artificial ventilation in preterm infants	**0.001-4.2**	**Hour**	
Exchange transfusions in infants	**-**	**Treatment**	**0.8-4.2**

Table 1: Human exposure to DEHP during treatment with PVC medical devices. (Permission granted by Joel A. Tickner from critical review: 'Health Risks Posed by Use of DEHP in PVC Medical Devices.)

2.3 Absorption

Exposure to DEHP can be investigated by measuring the concentration of metabolites excreted by urine. Numerous studies have investigated DEHP absorption in rats, dogs, miniature pigs and marmosets. An example of measuring DEHP absorption was by giving rats a single dose of ^{14}C-carbonyl-DEHP(Daniel and Bratt, 1974). About 40-80% of that single dose was excreted in the urine of rats, within the first 24 hours. Repeated doses of ^{14}C-carbonyl-DEHP were also examined and over 15 days, 90-96% of the total dose was excreted in the urine (Williams and Blanchfield, 1974). In dogs, only 13% of the administered dose was found in its urine, implying that only small amounts were absorbed, however, there is a possibility that some amount of DEHP metabolites could be excreted in faeces or bile. This could lead to other suggestions such as a fault in binary excretion or just a lack of absorption (Wallin et al., 1974).

It was shown that majority of DEHP is absorbed in the rats and miniature pig, but this was not exhibited in dogs or marmosets. This implies that species plays an important role in metabolism of DEHP.

2.4 Metabolism of DEHP

Experiments in animals have been carried out suggesting that DEHP is hydrolysed to its monoester, following oxidation of the alkyl side-chain. In vitro experiments of the rats small intestine showed rapid hydrolysis of DEHP to mono (2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH), which is the obligatory first step in the metabolism of DEHP. Cells were obtained from the intestinal mucosa of the rat, which showed the ability to hydrolyse DEHP. Experiments further investigated the

enzyme responsible for the breakdown of DEHP to MEHP and 2-EH. It was shown that liver alkaline esterase is more efficient in catalysing DEHP, than the pancreatic esterase. Esterase enzyme exists mainly in the liver, kidney, pancreas and lung, but can be present in most tissues (Lake et al., 1977).

These experiments suggest that the origin of metabolism can affect the efficiency of DEHP hydrolysis, which in turn affects the amount of DEHP absorbed or released from the species.

DEHP metabolism in humans was investigated by Holger M. Koch et al., 2004. A single oral dose of 48.1 mg was administered to a healthy male volunteer. Background exposure of DEHP was avoided by adding a dose of D4-ring-labelled DEHP analogue. Three metabolites were excreted by urine and serum; MEHP, mono (2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) and mono (2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP). The urine was monitored for 44 hours and serum was monitored for 8 hours. The peak concentrations of all metabolites in serum were found after 2 hours. In urine, peak concentrations of MEHP were found after 2 hours and the peak concentrations of 5OH-MEHP and 5oxo-MEHP were found after 4 hours. The major metabolite observed in serum was MEHP and 5OH-MEHP in urine. After 44 hours, 47% of DEHP was excreted in urine; 7.3 % MEHP, 24.7 % 5OH-MEHP and 14.9 % 5oxo-MEHP.

DEHP can be excreted in several forms; parent form or its metabolites, which are the easily excreted. There are around seventeen metabolites that DEHP can be broken down into, as shown in figure 2. (Koch et al., 2006).

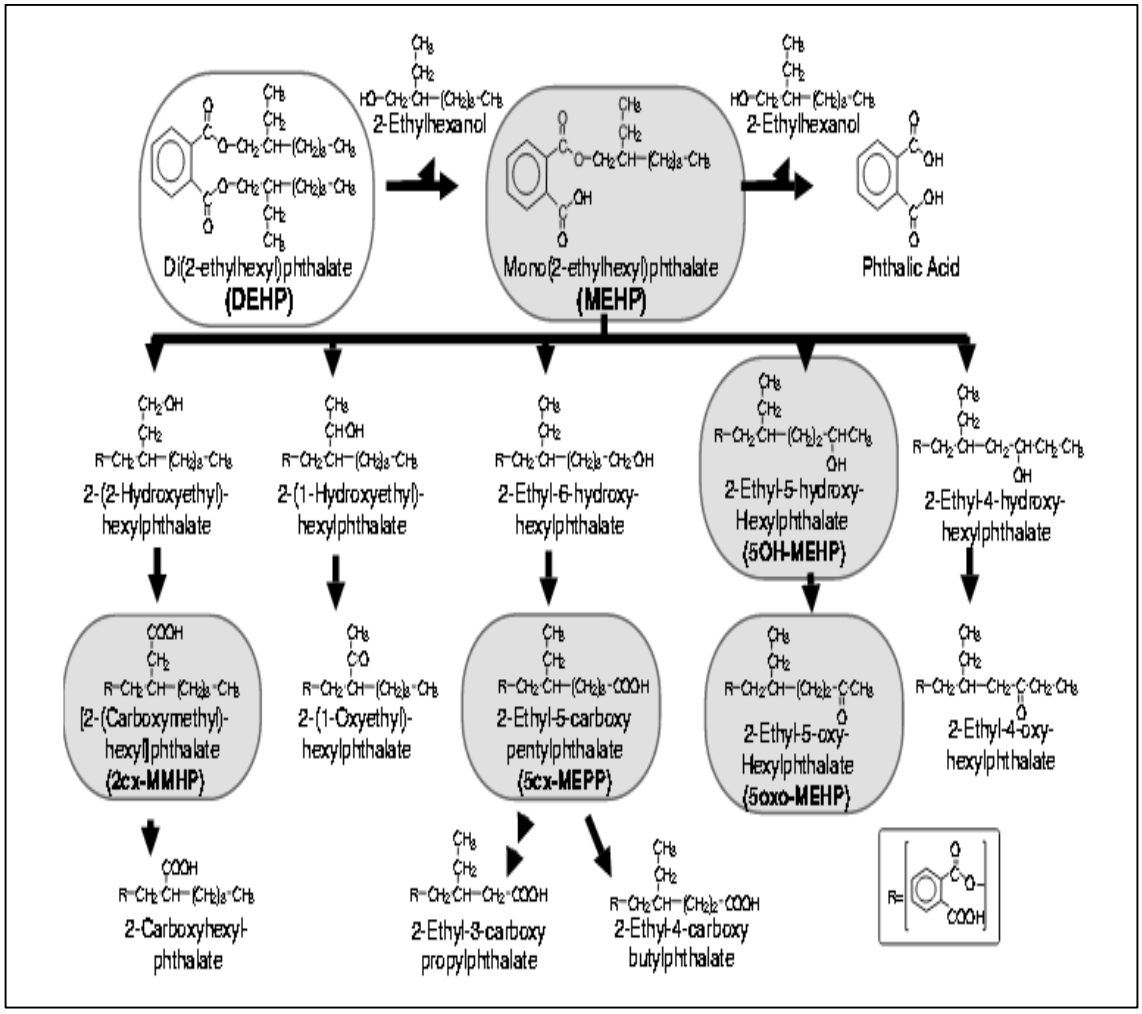

Figure 2: Metabolism of Di-2-ethylhexyl phthalate (DEHP). (Permission granted by publisher John Wiley & sons Inc.) (Koch et al., 2006).

2.4.1 Glucuronidation

Glucuronidation represents a major pathway which promotes the removal of lipophilic xenobiotics and endobiotics, by adding glucuronic acid to a compound, thus making it more water soluble (figure 3.) (King et al., 2000).

Glucuronidation is a pathway of phase 2 metabolism or conjugation reactions. These reactions involve different types of enzymes consisting of an ‘activated’ co-factor or substrate derivative which will encourage the elimination of compounds. The most widespread of the conjugation reaction is Glucuronidation. Enzymes that are vital for

the functionalization and conjugation reactions are Uridine Diphosphate - glucuronosyltransferase (UDP-GT or UGT) and Cytochromes P450 (CYP). Glucuronidation is also regulated by many cellular features, such as the access of substrates to UDP-GT and the mechanisms of transport or excretion of glucuronides from the cells (Burchell et al., 1990).

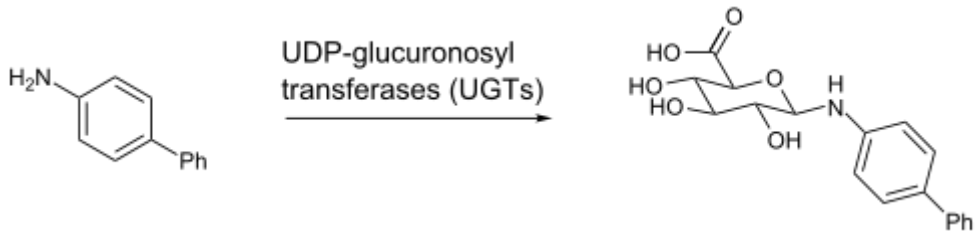

Figure 3: Glucuronidation reaction. (King et al., 2000).

UDP-GT represents a family of enzymes which plays a vital role in detoxification and homeostasis maintenance. It contributes to the biotransformation of endogenous compounds i.e. bilirubin, bile fats, fatty acids, steroid hormones, thyroid hormones. It also acts as a clearance system for drugs, such as analgesics, non-steroidal anti-inflammatory agents, anticonvulsants, and benzodiazepines etc. It can serve as a defence system by inactivating or increasing the water solubility of xenobiotics and drugs present in the liver, so that they can be secreted via the bile pathway or in the urine.

Studies of DEHP disposition and metabolism in man and the African green monkey were reported. It showed the compound is rapidly and extensively metabolised. In this study, it showed that over 90% of DEHP is largely excreted in the urine as conjugated glucuronides oxidation products of MEHP. Elimination of the 10% left can be excreted in faeces (Peck and Albro, 1982).

Albro in 1986 also investigated the absorption, metabolism and excretion of DEHP in rats and mice. It was evident that UDP-GT is involved with the elimination DEHP metabolites. The reaction only requires the need of UDP-GT and microsomes (Albro, 1986).

DEHP is hydrolysed to MEHP; MEHP is excreted as mono (2-ethylhexyl) phthalate O-glucuronides, after Glucuronidation. It is usually excreted by urine in humans (Peck and Albro, 1982). This knowledge can be used to remove DEHP and its metabolites in neonates since they are at a higher risk for DEHP toxicity, as reviewed in chapter 3.

2.4.2 Human cytochrome P450

Human Cytochrome P450 (CYP) is a family of enzymes that belong to a family of biotransformation enzymes, which are involved in the metabolism of xenobiotics, endogenous and exogenous compounds. CYP metabolism produces more hydrophilic compounds, which makes it easier for the body to remove, thus preventing toxicity. The metabolism of drugs plays a vital part in creating pharmaceutical and toxicological effects in humans. The enzymes are found to be anchored to the membrane of the endoplasmic reticulum (Berka et al., 2011).

An in vitro experiment was carried out, investigating the metabolism of DEHP by subcellular fractions of human brain, liver, kidney, liver, lung, testis, rat liver and CYP isoforms of human and rat, using Liquid chromatography–mass spectrometry (LC-MS). DEHP was hydrolysed to mono (2-ethylhexyl) phthalate (MEHP) in selected human organs and rat liver. It was not found to be metabolized in human brain or female skin. This suggests that the metabolism of DEHP may also be organ

dependent. MEHP was further metabolized to dealkylated and oxidative metabolites. CYP isoforms are the major enzymes involved in metabolism of DEHP. (Choi et al., 2011). Human CYP1C9*1 and CYP2C19 were observed to be the major human CYP isoforms that produces 5-OH MEHP and 5-oxo MEHP metabolites, only CYP2C9*1 and 2C9*2 produces 5–carboxy MEPP from MEHP (Choi et al., 2012).

2.5 Distribution

In vitro experiments have suggested that DEHP is associated with blood lipids. DEHP was shown to be linked with lipoproteins, present in the plasma, rather than with free lipids (Stern et al., 1977). In one experiment, DEHP was given to rats for 15 days, 59% of the radioactivity was present in the tissues of the liver, 27% in the kidney, 10% adipose tissue, approximately 1% in the heart and lungs and 1% in the testes (Williams and Blanchfield, 1974). In another study, female rats were shown to accumulate DEHP in the liver and fat, whereas in males rats, DEHP localised in the epididymal fat pad the heart (Stein et al., 1973).

Highest levels of the compound were present in the liver, after giving oral doses to rats, mice and other species. DEHP was shown to accumulate in fatty tissues, however, it would decrease in concentration after prolonged feeds. When DEHP was given intravenously, it was localised in the lungs of the species (Tanaka et al., 1978).

2.6 Excretion

Many experiments have demonstrated the rapid and extensive nature of DEHP excretion. In rats, after oral or intravenous administration of the chemical, up to 60% of it is present in the urine, within 24 hours. The rest is excreted over a period of days (Ikeda et al., 1980). The dog excretes 56-75% of the chemical in its faeces, with

some amounts excreted via urine. The DEHP metabolite, MEHP, is also found to be excreted in the urine of species. Substantial amounts are excreted in the bile, as some studies have noted. Dogs and marmosets eliminate the compound by faeces, whereas rats and miniature pigs appear to excrete DEHP or its metabolites via the urine. Many species also excrete DEHP and its metabolites as glucuronides (Schulz and Rubin, 1973).

2.7 Acute toxicity

A short-term toxicity study of DEHP in rats was carried out on groups of 15 male and 15 female rats. These groups were given diet containing different concentrations of DEHP; 0 % (control), 0.2 %, 1.0% or 2.0%, for 17 weeks. A reduced rate of body-weight gain and food intake was observed in rats with diet containing 1.0% and 2.0% DEHP. The food intake did not account fully for the reduced growth rate; this was determined by a paired-feeding study. There was a decreased packed blood cell volume in both sexes given the two higher treatments, however in male rats; the level of haemoglobin concentration was also reduced. No decrease in erythrocyte total count was observed. Female rats that received 2% DEHP showed reduction in renal concentrating and diluting activities (Gray et al., 1977).

After 17 weeks, the testis weight of male rats that were fed 0.2% DEHP did not show any reduction, but there was evidence to prove decreased spermatogenesis. Male rats fed 1% and 2% DEHP showed a major decrease in testis weight, histopathological examination declared severe seminiferous tubular atrophy and no spermatogenesis activity (Gray et al., 1977).

2.8 Sub-acute toxicity

Many investigations have been carried out on animals to assess sub-acute toxicity, by using oral, intravenous and intraperitoneal routes for DEHP administration. It was noted that oral administration of DEHP in rats, cause a decreases in rate of weight gain (Nikonorow et al., 1973). Large doses of DEHP given daily led to considerable amounts of death. Major effects of DEHP were observed in the testes and liver of species. Other effects noted were the reduced number of erythrocyte cells, haemoglobin and cell packed volume (Gray et al., 1977). When MEHP was administered to rats, the main effects of MEHP were found in the heart and liver. Necrosis and cholestasis was reported in the liver and chromatin precipitation was reported in the heart (Chu et al., 1981). The main findings in mice, after large daily intake of DEHP were cystic kidneys with tubular atrophy, splenic atrophy and thickening on uterine and intestinal mucosa. The intraperitoneal route of DEHP administration in rats, caused hepatotoxicity of carbon tetrachloride (Seth et al., 1979).

2.9 Effects on reproductive organs

Phthalate induced testicular damage has been of high importance, since it is the major effect of DEHP toxicity. Zinc is important for testicular function and is shown to be depleted in species after given a dose of DEHP. Zinc depletion leads to testicular atrophy. Enzymes such as dehydrogenase, which plays a part in the synthesis of testosterone are zinc dependent. Inhibition of dehydrogenase enzymes can also have an effect on NADP and NAD leading to low levels of hormones (Foster et al., 1982). Studies have indicated that rats are more at risk of DEHP toxicity on the testes than mice, hamsters and marmosets.

2.10 Chronic toxicity and carcinogenicity

Rats which were dosed with relatively high dietary levels of DEHP over a period of 1-2 years and produced testicular atrophy, kidney hypertrophy and hepatomegaly (HARRIS et al., 1956). The National Toxicology Program (NTP) rodent bioassays reported that DEHP acted as a hepato-carcinogen in rats and mice (Kluwe et al., 1982).

DEHP has also shown characteristics of a hepatic tumour promoter after administration of diethyl nitrosamine, which is an initiating agent. However, in other studies, DEHP did not promote the carcinogenic activity of dimethylbenz[a]anthracene. This suggests that DEHP does possess carcinogenic properties but it is limited and species specific. Evidence regarding carcinogenesis due to DEHP, in humans are very limited, and has only been exhibited in rodents (Reddy and Lalwai, 1983).

3 DEHP problems; neonates and reproductive tract

3.1 Neonate Human

DEHP toxicity has been intensively investigated in animals but humans have not been investigated as much as animals. Observations obtained from animal studies have caused concern about the risk associated with DEHP exposure in humans, especially the immature infants. Neonates require medical devices such as intravenous tubing's, enteral, nutritional support, dialysis and blood transfusions, to help them develop their immature organs outside the mothers' body. They are exposed to a large amount of DEHP at such an early stage and vulnerable time in their life. Their small bodies and underdeveloped organs may not be able to protect themselves from DEHP toxicity as well as adults. Due to their smaller size, neonates may receive a larger dose of DEHP than adults if the same medical devices are used for treatment such as ECMO etc. (Green et al., 2005).

Green et al (2005) made the association of the level of MEHP metabolites, in the urine, with the level of exposure of DEHP from medical devices. 54 neonates were studied in this experiment. These neonates were admitted to the neonatal intensive care unit for3 days to enrol in this study. Three groups were studied; low exposure of DEHP, medium exposure of DEHP and high exposure of DEHP. In the low exposure DEHP group, infants received bottle and/or gavage feedings. Infants in the medium DEHP exposure group received enteral feeding, intravenous hyperalimentation and nasal continuous positive airway pressure. The high exposure to DEHP group infants received umbilical vessel catheterization, endotracheal intubation, indwelling gavage tubing and intravenous hyperalimentation. The MEHP metabolites in the urine were

measured using automated solid-phase extraction, isotope dilution, high performance liquid chromatography and tandem mass spectrometry. After analysing the level of MEHP in the 81 urine samples collected from these infants, the result demonstrated a high correlation of MEHP metabolites with DEHP exposure. The medium exposure group excreted MEHP metabolites twice as much as the low DEHP exposure group had excreted. The high DEHP exposure group showed 5.1 time higher MEHP excretion in urine, than the low DEHP exposure group. This proves the association of DEHP exposure with MEHP metabolite excretion in urine (Green et al., 2005).

Sex	Number	Low DEHP exposure (n = 11)	Medium DEHP exposure (n = 23)	High DEHP exposure (n = 17)
Female	34	8	15	11
Male	18	4	8	6

Table 2: Number of neonates in each DEHP exposure group (Adapted from Green at al., 2005).

Factor	25th percentile	Median	75th percentile	P-Value
Sex				0.15
Female	3	20	64	
Male	19	39	75	
DEHP exposure group				0.001
Low	0.87	4	18	
Medium	3	28	61	
High	21	86	171	

Table 3: Mean concentrations of MEHP metabolites in urine by sex and DEHP exposure group. (Adapted from Green et al., 2005).

Assessment of the level of exposure of DEHP can be obtained by measuring DEHP metabolites in the urine. Six premature new born urine samples were assessed. These six neonates were to receive intravenous treatment for over 2 weeks and survive to be eligible for this experiment. Three DEHP metabolites were measured: MEHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). 41 urines samples were assessed, MEHHP and MEOHP were present in all the urine samples. MEHP was detected in only 33 urine samples. The median percentage of the free unconjugated DEHP metabolites in the neonate samples were higher than those found in urine samples collected from a demographically large diverse population group in 2001 (neonates: 22% MEHHP & 21% MEOHP, population: 5% MEHHP & 12% MEOHP). This experiment provides quantitative proof that neonates are exposed to higher concentrations of DEHP than

the general population (Calafat et al., 2004). The table below present the Urinary levels of MEHP-, MEHHP- and MEOHP concentrations in 6 critically ill neonates.

Phthalate Monoester	Mean (ng/ml)	Geometric mean (ng/ml)	Median (ng/ml)
MEHP	205	100	129
MEHHP	3419	2003	2221
MEOHP	2962	1617	1697

Table 4: Urinary levels of MEHP, MEHHP and MEOHP metabolites in 6 critically ill neonates. (Adapted from Calafat et al., 2004)

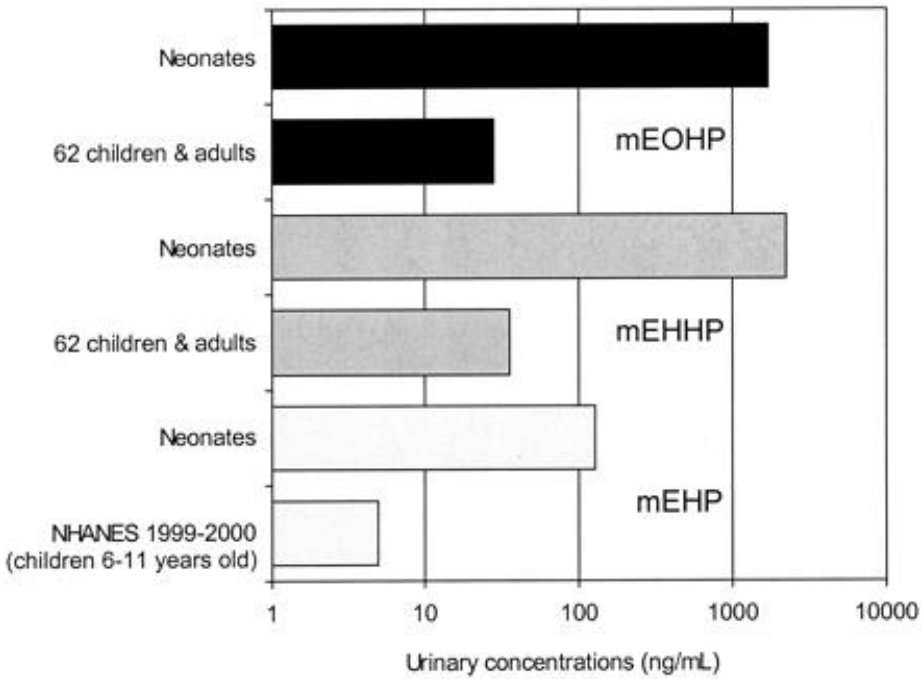

Figure 4: Comparison of median concentrations of DEHP metabolites in 6 critically ill neonates, in 328 children (6 – 11 years in age) and a diverse group of 62 children & adults. (Adpated from Calafat et al., 2004, with kind permission from publisher: American Academy of Paediatrics).

The median concentrations of DEHP metabolites; MEHHP and MEOHP, was compared amongst neonates and a diverse group of 62 children & adults. Median concentrations of MEHP were compared against 328 children. The children were of age 6 – 11 and data of MEHP median concentrations were obtained from the 1999-2000 National Health and Nutrition Examination Survey. The levels of MEHP were obtained for 2 published articles based on children's environmental exposure to phthalates. It can be seen that the levels of DEHP metabolites in neonates are much greater than the median concentration rate observed in children and adults.

In another study, longitudinal phthalate exposure and metabolism was evaluated in full-term and preterm infants. 58 full-term infants and 67 preterm infants (24.7 to 36.6 weeks, gestational age) were investigated for phthalate exposure, from birth to 14 months. 894 urine samples were assessed for metabolite concentrations of diethyl phthalate (DEP), dibutyl phthalate isomers (DiBP and DnBP), butylbenzyl phthalate (BBzP), DEHP and diisononyl phthalate (DiNP). In preterm infants, BBzP, DiNP and DEHP metabolites were shown to be 5-10 times higher at day 7 and month 1, than full term infants. Results also showed that 7 days after DEHP exposure, more than 80% of preterm infants and above 30% of full-term infants exceeded the antiandrogen threshold. This proves how hazardous DEHP exposure can be in infants. The European Food Safety Authority have recommended limits of daily exposure to infants since high levels of DEHP exposure at such an early stage of life, whilst the infants detoxification functions and organs are still very immature, can be very harmful and dangerous for infants (Frederiksen et al., 2014).

At a public hearing on PVC, organised by the European Commission in 2000, it was demonstrated that a feeding tube, inserted in a neonate's stomach, became rigid and

stiff after use. In 2000, the Stockholm County Council had reported that 50% of DEHP was leached out of a used PVC tube. During a feeding period of 10 weeks, feeding tubes are replaced after every three days; therefore 20-30 tubes are needed. The hospital reported that 30mg of DEHP is released per 24 hours (Karoline et al., 2204)

Another experiment was set out, in vivo simulated, to analyse the extraction of DEHP from PVC nasogastric tubes in gastric juices and feeding solution. 5cm of the PVC tube was incubated with gastric juice for 1 week and a feeding solution for 4 weeks. The rate of DEHP leakage was measured on a daily basis for the gastric juice group and a weekly basis for the feeding solution group. The results showed that in the feeding solution group, 200 – 542 µg of DEHP was extracted from the tube after 1 week and 660 – 1700 µg of DEHP was extracted after 4 weeks. The gastric juice group showed that 635 – 1043 µg of DEHP was leached from the tube. This shows that within 1 week, 1mg of DEHP can be extracted from a 5cm long VC tube, this represents an in vivo load of up to 4mg. Neonates can easily accumulate more than 4mg load in an intensive care unit, depending on the size and age of the baby, length of the PVC tube, the duration of the feeding tube whilst in contact with the neonate and the amount of DEHP present in the PVC tube (Subotic et al., 2007).

3.2 Male Animal Reproductive System

Shaffer et al (1945) reported the first phthalate induced testicular injury in animals. Testicular injury was determined by reduced testis weight, atrophy of seminiferous tubules, and degeneration of germ cells, spermatocytes and spermatids. From histological examinations of the reproductive system in animals, it seems that sertoli cells, germ cells and leydig cells are the target cells for DEHP toxicity.

Sertoli cells act as ‘nurse’ cells of the testicles by contributing to spermatogenesis – production of sperm (Rato, 2012). Dostal L. A. et al (1988) carried out an experiment in which histological changes of the testis in neonatal and adult rats (1, 2, 3, 6 and 12 weeks old) were examined. The rats were given five daily oral doses of DEHP (0, 10, 100, 1000, 2000 mg/kg). The testes were examined 24hr after the last dose. Testis weights were reduced at doses of 1000mg/kg in all rats apart from the 12 week old rats. DEHP doses of 2000mg/kg were fatal to the neonatal rats and showed a significant decrease in testis weight. It did not cause death in rats that were 6 weeks and 12 weeks old. One week old neonatal rats that were given a dose of 1000mg/kg DEHP showed a 35% decrease in sertoli cell numbers. Two and 3 week old rats did not show a decrease in sertoli cells number but did show a reduction in spermatocytes. At doses of 1000mg/kg and 2000 mg/kg, 6 week and 12 week old rats showed a decrease in spermatids and spermatocytes. These results obtained demonstrated that the sertoli cell is the primary cell target of DEHP toxicity, when observed that toxicity effects only showed when sertoli cells were present (Dostal et al., 1988).

Sperm production is dependent on sertoli cells, germ cells and hormonal factors. Sertoli and germ cells make up the seminiferous epithelium (Figure 5.). Leydig cells are present in the interstitium. These cells are vital for spermatogenesis – production of spermatogonial stem cells, which then mature into sperm cells.

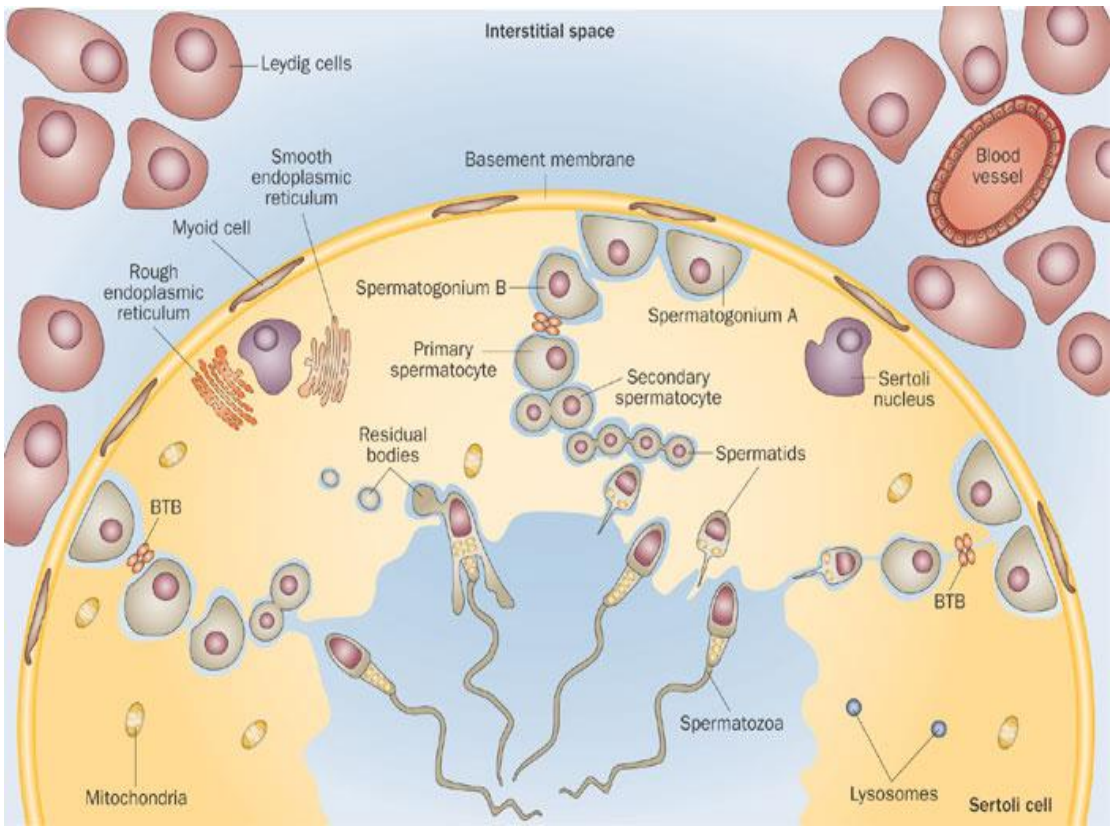

Figure 5: Seminiferous epithelium consisting of sertoli, leydig cells and germ cells at different stages. (Adapted from (Rato et al., 2012), kind permission from publisher: Nature Publishing Group.)

MEHP is the metabolite that has been linked to testicular atrophy in rats. Gray and Beamand (1984) conducted an experiment that showed the effect of MEHP in mixed cultures of sertoli and germ cells. The culture consisted on a monolayer of sertoli cells with clusters of spermatocytes and spermatogonia adhered to it. With time, the cells would mature into germ cells, which would then detach from the monolayer of sertoli cells into the medium. DEHP, MEHP and 2-EH were then added separately to these cultures, MEHP was the only phthalate ester to increase the rate of germ cell detachment over the range of 10^{-7} – 10^{-4} M, implying that the toxicity effect is concentration dependent. DEHP and 2-EH produce no effect on the cell cultures (Gray and Beamand, 1984). The separation of germ cells from sertoli cell monolayer

would prevent the transfer of nutrients from the sertoli cells to the germ cells, thus leading to death of germ cells.

There are experiments that also suggest that DEHP toxicity targets leydig cells (Jones et al., 1993). Leydig cells produce testosterone, which is required for the starting of spermatogenesis, differentiation of the male urogenital system and maintenance of the reproductive function (Benton et al., 1995). Testosterone is the primary steroid hormone that supports male fertility. Luteinizing hormone (LH) is secreted by the pituitary gland; it regulates the expression of 17 - β hydroxysteroid dehydrogenase, this enzyme is involved in the conversion of androstenedione to testosterone. Testosterone is the precursor cell for the development of androgen dihydrotestosterone (DHT): male sex hormone. An experiment was conducted to observe the effect of DEHP on leydig cell androgen biosynthesis (Akingbemi et al., 2001). It was concluded that DEHP toxicity on leydig cell steroidogenesis is dependent on the stage of development when exposed to DEHP.

Effects of MEHP on steroidogenesis by primary cultures of immature and adult rat leydig cells *in vitro* were investigated by Konstantin Svechnikov et al., (2008). The study showed that 250 μM MEHP suppressed gonadotropin-simulated steroidogenesis in immature and adult rat leydig cells. The up-regulation of steroidogenic acute regulatory protein (StAR), which was evoked by the human chorionic gonadotropin (hCG), was inhibited significantly. This resulted in a decrease of cholesterol transport into the mitochondria; however, it did not affect the downstream steroidogenic enzymes. Mitochondrial cholesterol is essential for functions such as the production of steroid hormones in specialized tissues or synthesis of bile acids; it fulfils vital physiological functions (Marí et al., 2009).

MEHP also suppressed 5α-reductase activity in immature rat leydig cells, but the activity remained unaffected in adult rat leydig cells. This study is the first to represent an age-dependent negative effect on the enzyme 5α-reductase. This enzyme is required for the transformation of testosterone into DHT (Labrie et al., 1992). Inhibition of DHT induces reproductive malformations in both humans and animals. Since MEHP inhibits the regulation of DHT, it can be classified as an anti-androgenic. Decreased levels of DHT can result in malformations such as hypospadias (ectopic opening of the urethra), testicular atrophy, epididymal agenesis and anogenital distance (Gray et al., 2001). 100μM – 250 μM of MEHP was used in this experiment. This is equivalent to 22μ – 55 μg/mg; 1780 μg/kg/day of DEHP is exposed to neonates in an intensive care unit (Koch et al., 2006). Exposure of 300mg DEHP can be achieved just by blood transfusions. This highlights the risk of neonates being exposed to such a large amount of DEHP.

Experiments regarding DEHP toxicity in male reproductive systems in animals can represent the risks associated in male neonates. Solutions that have been put forward by researchers were that DEHP free medical devices should be used. Alternative plasticisers should be used to make PVC medical devices softer, however it would still not be able to remove the risk of toxicity from these compounds since they are not chemically bound to PVC.

4. The UDP-glucuronosyltransferases

Humans are exposed to many lipophilic, non-polar drugs and xenobiotic compounds. The lipophilic characteristic makes it difficult for the elimination of these compounds by renal excretion. It is essential for these compounds to be converted into a hydrophilic form for excretion. Xenobiotic metabolism is classified into two types of reaction: functionalization reaction and conjugate reactions. Functionalization reaction involves adding a polar functional group such as a hydroxyl, amino or carboxyl with a molecule. A conjugation reaction involves linking a polar endogenous compound such as a glucuronic acid, glutathione, acetyl or sulphate (Rowland et al., 2013).

Uridine Diphosphate-glucurosyltransferase (UDPGT) and Cytochrome P450 are vital families of enzymes that mediate reactions involved in drug metabolism. Clearance of more than 90% of these drugs relies on these enzymes. These enzymes mainly act as a detoxification mechanism for the body in that they facilitate excretion (Rowland et al., 2013).

Neonates lack protective enzymatic mechanisms against xenobiotics and toxic compounds i.e. DEHP. This is due to under development of vital organs such as the liver, where most of the metabolism of xenobiotics occurs. This leads to a lack of neonatal glucuronidation, hence why neonates are at such high risk of toxicity caused by absorbing DEHP (Miyagi et al., 2012).

4.1 UDPGA synthesis

UDPGT enzymes are found to be attached to the membrane of the endoplasmic reticulum (ER). They are highly water soluble and stable. The cofactor - UDP-glucuronic acid (UDPGA) is formed inside the cell. The catalysing reaction of glucose-1-phosphate and UTP by UDPGT pyrophosphorylase forms UDPGA for the UDPGT enzymatic activity.

4.2 Induction of UDPGT

The most common conjugation reaction in humans is glucuronide formation. The reason is presumed to be due to the ready availability of glucuronic acid in tissues. G. J. Dutton has intensively studied the enzymes that are involved in glucuronide synthesis. The differential induction of UDPGTs is brought about by many xenobiotics, and was originally used to classify the different isoenzymes into families before the development of DNA based technologies. Two major types of inducing agents have been identified – Polycyclic aromatic hydrocarbon (PAH)-type and phenobarbitone type (Bock et al., 1973), and most of the work was carried out in animals.

However, in humans it is also established that both of these agents also induce UDPGT activity. The evidence for this comes from data obtained from patients who smoke and are on drug therapy, and from people taking the drug phenytoin to treat epilepsy (Treluyer et al., 1996).

In an experiment conducted by Bock et al. (1973), it was shown that in animals PAH - type inducers, encouraged UDPGT activity towards planar phenols. Examples include 1 - naphthol, 4 - nitrophenol, 4 - methylumbelliferone and 2 - aminophenol. PB - type inducers have induced 4 - hydroxybiphenyl UDPGT activities etc. Several compounds have also been reported to induce specific UDPGT isoenzymes. Treatment of patients with PB – type drugs (PB, phenytoin) was also carried out, in which it was reported that the liver microsomes displayed considerably high levels of UDPGT activity towards compounds such as bilirubin etc. (Bock et al., 1984). Major contrasts have also been established between humans and rat livers regarding PB-type inducers. Bock Ullrich and Bock-Hennig (1987) demonstrated that the conjugation of morphine and 4 – hydroxybiphenyl were unaffected in humans who were treated with PB/phenytoin, whereas in rat liver, conjugation of morphine and 4 – hydroxybiphenyl are the major UDPGT enzyme activities that are induced by PB/phenytoin. Smoking has shown to simulate certain UDPGT enzyme activities in humans also (Bock et al., 1987). These studies give evidence that differential induction of UDPGT enzyme activities are present in humans, but very different in comparison with rats.

4.3 Tissue distribution

UDPGT activity has been retained in most of the major extrahepatic organs, i.e. skin, intestine and lungs, although liver is the most important site of Glucuronidation due to the majority of the enzyme protein (Hartiala, 1973).

Majority of reports regarding UDPGT activity concerns the rat. The main UDPGT enzyme activity that is exhibited is the planar phenol UDPGT, which is considered the major xenobiotic-metabolizing form. However, this activity is shown to be

expressed in the rat lung only, the kidney shows a very limited expression of major UDPGT activities such as morphine and testosterone. Steroid- metabolizing UDPGT or bilirubin UDPGT activity has not yet been proven to be present in the rat intestine (Peters et al., 1987).

In man, bile acids are conjugated in the kidney, where UDPGT activity is present; however the kidney is unable to metabolize bilirubin. The human intestinal mucosa is capable of glucuronidation of bile acids, which is found to be similar in rats. These investigations were carried out at a molecular level by immunoblot analysis in rats, and using a monoclonal antibody, which is directed against a common epitope on UDPGT activity in human liver (Peters et al., 1987).

4.4 Development of UDPGT isoenzymes

Variation in UDPGT activities during development are the best studied when investigating the differential regulation of UDPGT gene expression. It was established that the development of rat UDPGT activities can be divided into two groups – Steroidal and non-steroidal groups (Greengard, 1971). The non-steroidal group stimulated activities towards planar phenols and developed late-foetally, the steroidal group consisted of activities leading towards steroids. There have been findings that suggest that hormones can also play a part in regulating the gene expression of the non-steroidal group in vivo. A suggestion was presented that the activities in the non-steroid group can be induced by the dispensation of glucocorticoids (Wishart, 1978). Four key periods have been acknowledged during the development of different UDPGT in rat liver. First period, known as the late-foetal cluster, consists of activities that stimulate the production of planar phenols. The second period involves activities that progress towards testosterone, bilirubin

and morphine; this is referred to as the neonatal cluster. The third period is called the post-weaning cluster, comprising of activities towards androsterone and pregnanediol. The last key period is known as the pubertal cluster, where activities towards pregnanediol develop (Wishart, 1978).

It has been validated that the variations that occur during the development of enzyme activity relate to the differences in the amount of enzyme proteins that represent their UDPGT isoenzyme. These changes were determined by immunoblot analysis (Coughtrie et al., 1988).

There is sufficient evidence that recognises the existence of UDP-GTs as a multigene family, which results in a range of isoenzymes, each possessing different physical and catalytic properties. The understanding of the multiple nature of UDP-GTs has indirectly progressed from the differential induction, ontogeny, tissue distribution and many inherited defects of UDP-GT activity.

There are sixteen different types of UDP-GT human enzymes. 9 of these enzymes are encoded by the UGT1 gene. The UGT1 gene can express 12 isoforms by splicing of a primary transcript. Each isoform possesses specificity for their substrates. UGT1A1 is responsible for the conjugation of bilirubin and mutations of the gene which results in hyperbilirubinemias. Studies have suggested that polymorphisms of UGTs can lead to individual variations of drug metabolism, inherited diseases and toxicity (Maruo and Sato, 2002).

4.5 Bilirubin and Jaundice

When red blood cells are broken down, they release haemoglobin. The destruction of haemoglobin results in the production of bilirubin. Red blood cells have a shortened lifespan in neonates. It has been investigated that from each gram of haemoglobin, 35 mg of bilirubin in its unconjugated form is produced. New born babies produce up to 10 mg/dl/day. In the first 24 – 48 hr of a baby's life, bilirubin production is raised. Unconjugated bilirubin is lipid soluble, therefore cannot be secreted in its initial form. Transportation to the liver is required, where enzymes will catalyse the metabolism of bilirubin and produce it in a water soluble form i.e. glucuronidation, where it can be excreted from the body in faeces or urine. Bilirubin that is not excreted will reabsorb back into circulation by a process called enterohepatic recirculation (Smith, 2004).

Bilirubin diglucuronide (BDG) has been reported to be main conjugate found in bile. It is formed by two enzyme-catalysed steps. Synthesis of bilirubin monoglucuronide (BMG) occurs, which is then converted to BDG. The hepatic formation of BMG is regulated by UDP-GT (Blanckaert et al., 1979).

Hyperbilirubinemia refers to high levels of bilirubin in the blood. Neonatal hyperbilirubinemia is the leading cause for hospital admission. Excessive levels of bilirubin can be controlled by treating neonates with ultraviolet light or exchange transfusion. It has been estimated that severe hyperbilirubinemia affects at least 481,000 new-born babies annually, worldwide. 114,000 of these affected infants die,

and more than 63,000 survive but with moderate or severe disabilities (Olusanya et al., 2015).

Jaundice is yellow-pigmentation caused by high levels of bilirubin, which causes the affected individual to appear yellow in colour. It results from deficient ligandin, the main hepatocyte intracellular bilirubin binding protein, and insufficient expression of hepatic UGT1A1. This prevents the clearance of bilirubin from the plasma and metabolism by the liver. This illustrates the importance of UGT1A1 gene regulation for the maintenance of normal levels of serum total bilirubin. Induced bilirubin toxicity can result in a variety of toxic responses in new born babies. It can lead to ocular muscle paralysis (ophthalmoplegia), mental retardation, long-term physical impairment, dystonia and death. Crigler-Najjar type 1 (CN1) disease is described by the complete inactivation of UGT1A1-dependent bilirubin Glucuronidation, which leads to the development of hyperbilirubinemia and resulting central nervous system toxicity. Severe hyperbilirubinemia can also be progressed by infection, ischaemia, breast feeding, biliary obstruction and a metabolic deficiency in glucose-6-phosphate dehydrogenase activity (Fujiwara et al., 2010).

4.6 Neonatal development of Jaundice

Bilirubin is an end-product created by the degradation of haemoglobin, it is toxic therefore elimination of this compound is essential. 75% of bilirubin is formed due to haemolysis and 25% if formed due to ineffective erythropoiesis. Haemoglobin first breaks down into biliverdin and then catalysed by biliverdin reductase to form unconjugated bilirubin (figure 6).

Unconjugated bilirubin is neurotoxic, lipid soluble and water insoluble, it has to undergo glucuronidation for excretion in the bile. Unconjugated bilirubin in its 'free' unbound form can easily enter the interstitial fluid, cerebrospinal fluid and the brain. Neurotoxicity is caused when unconjugated bilirubin, it its free form, is present in the brain. Unconjugated bilirubin can bind to a blood-transport protein called albumin. Albumin bound-bilirubin cannot pass the blood-brain barrier, therefore does not enter the brain. It is transported to the liver, where glucuronidation takes place (Steven M Shapiro, 2003).

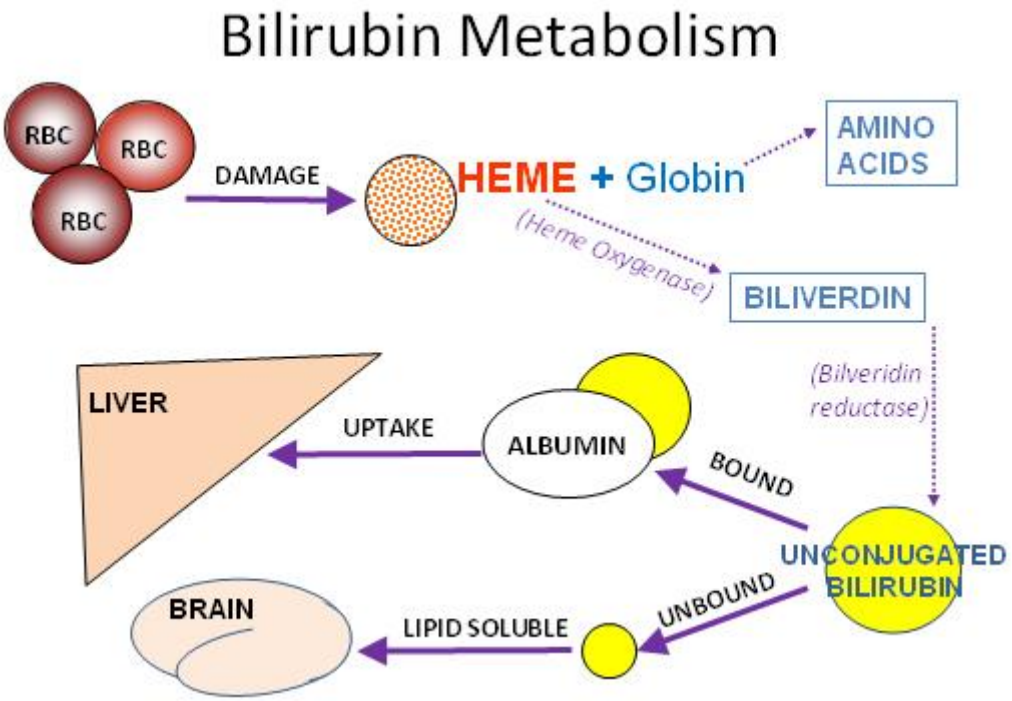

Figure 6: Bilirubin metabolism.

Uridine Diphosphate- Glucuronic Acid is a compound that donates the glucuronyl group necessary for the formation of bilirubin glucuronides. UDP-GT is essential for the transformation of bilirubin into a water soluble form (Barbara H, 1963). The figure below shows a possible mechanism for the glucuronidation of bilirubin.

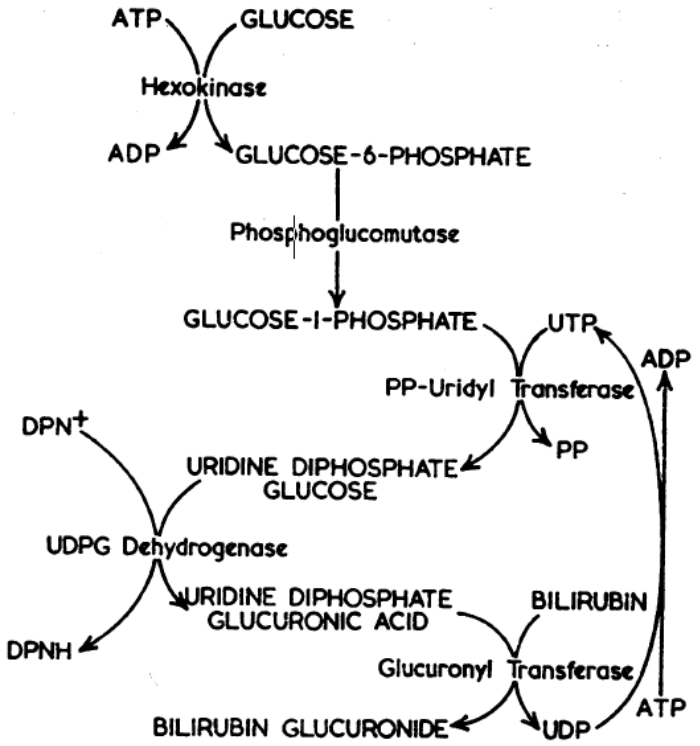

Figure 7: Synthesis of bilirubin glucuronide by UDPGT activity.

During gestation, the foetus is reliant on the mother's metabolic activities, whilst the foetus is developing. Liver organogenesis initiates during the 4th weeks of embryogenesis. It begins to develop from a liver bud, also referred to as the hepatic diverticulum. The cranial portion of the liver bud progresses into the liver and intrahepatic biliary tree, whilst the caudal portion develops into the extrahepatic biliary tree and the gall bladder. The primitive endodermal cells of this liver bud are bipotential cells and can differentiate into either hepatocytes or biliary epithelial cells. Slowly, other functions of the liver start to progress; plasma protein synthesis, glucose and fatty acid metabolism, bilirubin metabolism, bile synthesis etc. (Grijalva and Vakili, 2013).

In neonates, the expression of UGT1A1 and UGT1A9 are minimal. It has been reported that between weeks 30 and 40, expression of UDP-GT is approximately 1%

of adult levels. After birth, the expression levels do tend to increase during the first few weeks of life (J.F. Watchko, 2010). Due to reduced expression of this enzyme, unconjugated bilirubin is increased in the blood, which then undergoes enterohepatic absorption.

Neonatal liver rapidly matures during the first year of life. The human cytochrome P450 family catalyses the oxidative metabolism of the xenobiotic compounds i.e. DEHP that the neonate is exposed to. CYP450 concentrations remain stable throughout the gestation period, and at birth the levels are equivalent to 30% adult levels (Treluyer et al., 1996).

Since infants are at high risk of exposure to DEHP, which is proved by high levels of DEHP metabolites in the urine, the lack of enzymes present in the liver that could convert DEHP into a more water-soluble form for excretion can prove to be dangerous for the neonate.

5. Introduction of Phenobarbitone

Phenobarbitone (PB) is used as an antiepileptic drug (AED). It was first synthesised in 1911 by Emil Fischer. Epilepsy is a serious brain disorder and often occurs after brain injury, stroke, brain tumour and substance abuse. It is characterised by unpredictable interruptions of normal brain function that will keep reoccurring; it is referred to as epileptic seizures (Robert S. Fisher 2005). Patrick Kwan and Martin J Brodie (2004) published a review about the therapeutics and clinical pharmacology of PB. It was concluded that PB remains one the most important AEDs of the 21st century, due to its continuous widespread use in developed and developing countries. From an economical aspect, the greatest advantage of PB is its low net cost. For developing countries, the cost effectiveness of treatment was of top priority. Other advantages included were that it was easy to use, reliability of supply and the drugs various actions (Kwan and Brodie, 2004).

5.1 Pharmacokinetics

5-ethyl-5-phenylbarbituric acid (PB) is a substituted barbituric acid. Its bioavailability reaches over 95%, implying it is well absorbed. Experiments have shown that PB can reach its peak plasma concentration between 0.5-4 h. It has also shown a low clearance rate and long elimination half-life after one daily dosing in adults and children. The half-life of PB in adults is 3-5 days and in children it is 1.5 days. About 25% of a PB dose is eliminated from the body by urination. Most of the PB is absorbed in the liver – the main site for drug metabolism. PB undergoes aromatic hydroxylation and forms p-hydroxyphenobarbitone. It also undergoes Glucuronidation in the liver. The metabolites of PB are inactive. The cytochrome

P450 enzyme system conducts the hydroxylation of PB, particularly CYP2C9 (Nelson et al., 1982).

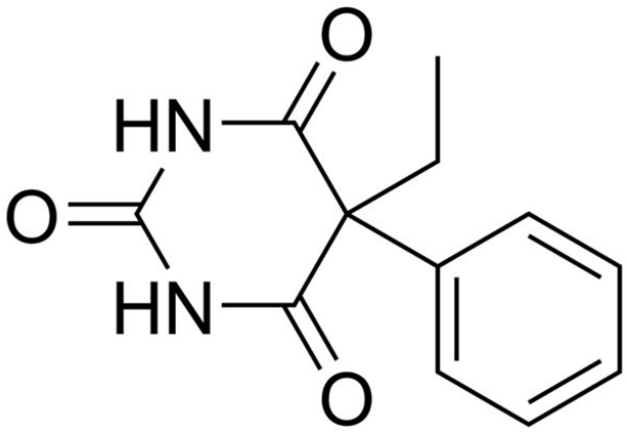

Figure 8: Structure of phenobarbital.

5.2 Drug interactions

PB has been classified as the archetypal enzyme inducer as it has been shown to increase phase II metabolizing enzymes and a variety of CYP enzymes. PB induction of these enzymes contributes towards the clearance and detoxification mechanisms of xenobiotic that go through hepatic biotransformation.

An experiment was conducted by Gunner Bengtsson et al., (1997) to investigate the effect of PB on the distribution of drug metabolising enzymes between the Periportal (PP) and Perivenous (PV) rat hepatocytes. The PV region of the liver is more vulnerable to toxicity caused by xenobiotics, which is believed to be due to the faster production of electrophilic metabolites in comparison to PP. Although this experiment focused on the different regions of the liver, and concluded that the PV region for the liver is the dominant region for drug metabolism than the PP region, it did successfully demonstrate the effect of PB on hepatocytes. For enzyme induction 100mg/kg of PB was suspended in water and was given to male rats of 7 to 8 weeks

of age, for 3 consecutive days. The control group of rats were fed a standard diet. The yield of microsomal protein (% of cell protein) was measured. In the PP region, the control group demonstrated a value of 2.58 ± 1.24 %, whereas the PB group demonstrated a value of 5.05 ± 3.01 %, almost twice the amount of microsomal proteins normally induced by PB-untreated rats. In the PV region, the control group yield of microsomal proteins were 3.35 ± 0.93, the PB-treated rats showed a value of 5.42 ± 1.55, again almost twice the amount of microsomal proteins induced by normal rats. PB induced enzymes such as glutathione transferase, cytochrome P450, NADPH-cytochrome c reductase and UDP-glucuronosyltransferase. The table below compares the activity of CYP 450 and UDPGT in normal rats and PB-treated rats, by

Enzyme	Control Group (nmol/mg cell protein/min)	Control Group (nmol/mg cell protein/min)	PB – treated Group (nmol/mg cell protein/min)	PB – treated Group (nmol/mg cell protein/min)
	PP region	PV region	PP region	PV region
P-450 (nmol/mg protein)	7.9	12.9	39.6	46.1
UDPGT	67	97	202	240

using enzyme assay techniques for measurement (Bengtsson et al., 1987).

Table 5: Comparison of inducing effect of PB in CYP 450 and UDPGT activities in PP and PV hepatocytes. (Adapted from Bengtsson et al., 1986)

Table 5 illustrates the effect of PB on cytochrome P450 and UDPGT, in rats. This could prove to be beneficial for rats that are not able to express these enzymes due to undeveloped organs which can affect enzyme expression in the body.

5.3 Effect of Phenobarbitone on hepatic microsomes

An experiment conducted by Nicole Tavoloni et al., (1983) observed the effects of PB in rats. The dose administered to rats was 0.5 – 7.5 mg/kg, similar to doses administered to humans. Rats were treated with PB doses ranging from 1 – 125 mg/kg/day for 6 days. UDPGT and CYP450 activities were measured from microsomal preparations and in liver homogenate. No significant changes were noted in liver weight and protein content of the homogenate, UDPGT and CYP450 were enhanced significantly.CYP450 was increased by 30% and UDPGT activity was increased with 15-24 and 45-66%. The data obtained from this confirmed the enhancement of Phase II metabolizing enzyme, UDPGT, ad CYP-450 activity (Tavoloni et al., 1983).

5.4 Phenobarbitone effect on bilirubin excretion in animal studies

Bilirubin requires to be conjugated into a glucuronide form for excretion from the body in urine. It is catalysed by UDPGT. In neonates UDPGT is absent due to underdeveloped liver systems. Due to the lack of UDPGT, bilirubin remains in its unconjugated form and build up which causes jaundice. A study was designed to determine whether UDPGT levels can be modified with Phenobarbitone and enhance bilirubin conjugation and excretion in animals. The experiment was carried out on

mice and rabbits. The mice were dosed with sodium barbitone and the rabbits were dosed with Phenobarbitone (Catz and Yaffe, 1968).

5.4.1 *In vitro* enzymatic activity in mice

Male and female mice of the Swiss-Webster strain were used in this experiment; 3 month old adult mice, 10-14 and 20 day old young mice and newborn mice which were less than 24 hrs in age. In the control group adult mice were given a normal laboratory diet, the young and newborn mice were fed by their mothers. The adult experiment mice were administered 300 mg/kg/day of sodium barbitone by intraperitoneal injection, for 3 days. Young mice were administered 150 mg/kg/day of sodium barbitone by the intraperitoneal route and newborn mice were administered a dose of 50 mg/kg/day by the subcutaneous injection. Pregnant mice were also administered sodium barbitone similar to the way adult mice were handled, however they were treated for 4 – 6 days. All animals were sacrificed, and levels of conjugated bilirubin were measured 18 – 24 hours after the last injection. Their livers were excised and gall bladders were removed for investigation.

The male adult mice in the control group specific activity was 103 ± 3.7 µg/g protein/20 min, the adult male mice that were treated with sodium barbitone showed a significant increase in conjugated bilirubin (181± µg/g protein/20 min). In control females, the bilirubin activity was much higher than the male control group. The specific activity was measured to be 177±11 µg/g protein/20 min, in this report there were no data recorded of the measurement of treated female group, but it was presented in the graph, and it showed to be between 200 – 250 µg/g protein/20 min. Figure 9 shows an approximate representation of the result obtained in this experiment.

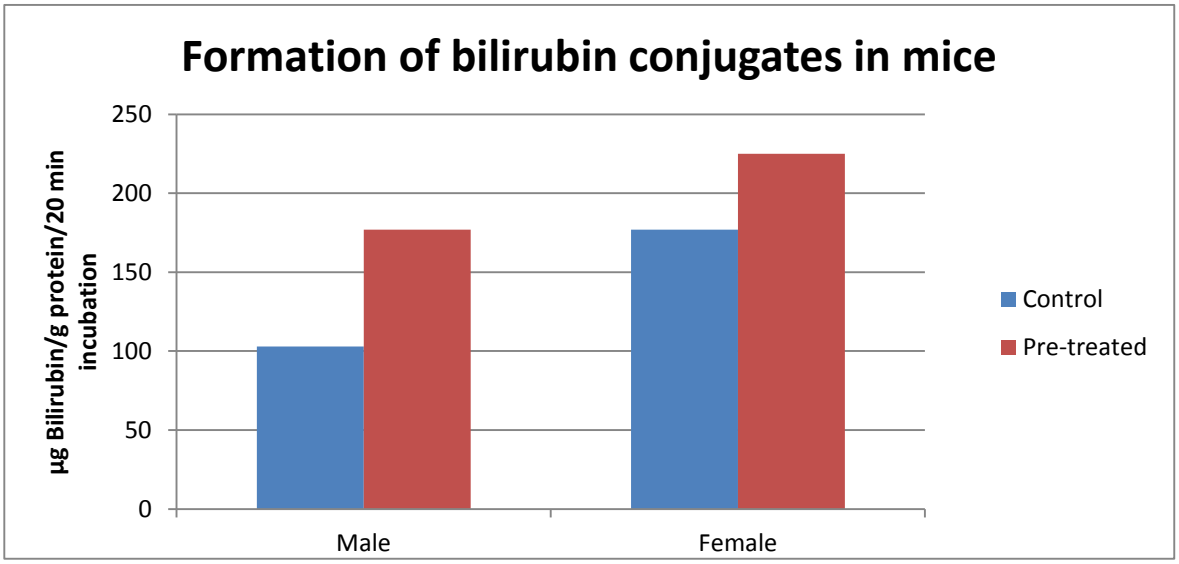

Figure 9: Effects of sodium barbital on conjugated bilirubin formation achieved by liver homogenates in male and female adult mice. (Adapted from (Catz and Yaffe, 1968)).

5.5 Phenobarbital and neonates

Phenobarbital is frequently administered to neonates for treating neonatal seizures. Achieving the right dose and maintaining the effectiveness of the treatment is essential for avoidance of drug toxicity especially in neonates. Fischer et al., (1981) has previously performed the pharmacokinetic studies in neonates and have demonstrated that 16-20mg/kg doses of phenobarbital and maintenance doses of 3.1 – 3.8 mg are required to obtain serum concentrations of 20-25 mg/ml (Fischer et al., 1981) 10 – 30 mg/l had been suggested by Buchthal et al., (1968) to be the effective serum concentration range of phenobarbital.

The effectiveness and safety of prenatal PB for the treatment of neonatal jaundice was investigated by Valaes et al., (1980). 100mg of PB was administered at bedtime during the last weeks of pregnancy. Mothers who took less than 10 tablets of 100 mg PB demonstrated no effects of the drug. The incidence of jaundice (bilirubin levels greater than 16 mg/dl) and requirement of an exchange transfusion in 1310 newborns, from mothers treated with PB $\geq$ 1.0 g, was reduced by a factor of 6 in

comparison to the incidence in 1153 control infants. 415 children were randomly selected for re-examination when they were 61 to 82 months of age. 182 children were from the control group and 233 children were from the PB-treated group. A detailed neurological assessment was carried out and there was no significant difference recorded between the groups. Experiments were carried out showing that the effect of PB in humans and animals are age-dependent. As time went on, the effect of PB started to decline. Therefore in this experiment, PB may have shown a significant difference when the neonate when born, in comparison to children of 61 to 82 months of age. Valaes did conclude the experiment with the following statement: "Prenatal PB is a practical, effective, and safe method for decreasing the incidence of neonatal hyperbilirubinemia." (Valaes et al., 1980).

5.6 Summary

The severity of DEHP toxicity in this thesis has been reviewed, and it has been noted that neonates are a high risk due to the amount of DEHP exposure, from PVC medical devices. DEHP is used to induce flexibility and soften PVC medical devices. PVC medical devices have proven to provide much more efficient and advantageous properties for the medical and pharmaceutical professions, in comparison to medical devices made from rubber, metal and glass. DEHP is not chemically bound to PVC and studies have confirmed the migration of DEHP from PVC devices, leaching into solutions where lipid soluble compounds are absorbed i.e. plasma and blood. DEHP has confirmed its toxic effects in animal studies, with numerous results demonstrating reproductive damage. DEHP can be broken down into toxic MEHP metabolites by the process of oxidation, mediated by the CYP 450 enzyme family. These metabolites are then excreted in the urine and faeces. Researchers are able to take the urine samples and investigate the level of DEHP that the subject has been exposed to.

The liver is the main site of metabolism of xenobiotics that the human and animals are exposed to. This is due to their multifunction systems; e.g. the bile pathway and the urea pathway. The main enzyme of interest is the UDPGT. Neonates lack this enzyme when they are born. Neonates have under developed liver systems and therefore are unable to express this enzyme. This enzyme is responsible for the process of Glucuronidation, which is the addition of a glucuronic acid, to transform a lipophilic compound into a water soluble compound. Xenobiotics are non-polar and water insoluble; it must be converted into a water soluble form for elimination via

urine or faeces. Therefore, Glucuronidation is a vital process for the clearance and detoxification of the body. The lack of this enzyme also reduces bilirubin excretion in the body. Bilirubin is produced in result to haemoglobin degradation. Unconjugated bilirubin accumulation in the body could cause serious brain damage, thus conjugation of bilirubin in to a water soluble form is necessary for excretion in the urine or faeces.

5.7 Proposal

Neonates are at high risk of DEHP toxicity. Studies have demonstrated high levels of DEHP exposure to neonates by the level of MEHP metabolites found in their urine. Elimination of DEHP is essential, as animals studies have shown adverse effects of DEHP on the reproductive system especially in male rats. Neonates also suffer from severe jaundice (high levels of bilirubin > 25 mg). PB is a drug that has proven its ability to induce CYP 450 and UDPGT activity in animals. It has also been administered to neonates that suffer from jaundice and has shown its ability to decrease levels of total bilirubin in the blood. Proposal of using PB in neonates to enhance UDPGT activity, which promotes Glucuronidation of DEHP and its metabolites, may decrease the risk of DEHP toxicity in neonates. Neonates have been administered PB by the intravenous route. 19.4mg/kg was the mean loading dose, which ranged from 16.4 to 20.5 m/kg. The mean maintenance dose was 4.0 mg/kg/day, ranging from 2.6 – 5.0 mg/kg/day. This experiment recorded no side effects to the neonate. Studies have confirmed the reliability, efficiency and safety of administering the drug to neonates. Dosage of the drug is highly dependent on the age and body weight of the neonate. PB administration could be a possible solution to increase the elimination of DEHP in neonates, preventing toxicity caused by this compound.

5.8 Future Investigations

The question posed by this project was whether the administration of Phenobarbitone (PB) to neonates can increase the rate of DEHP excretion, thus lowering the risk of DEHP toxicity. Experiments carried out in order to investigate the effects of PB on DEHP excretion must be planned and researched thoroughly. Ethical permission is of utmost importance in all clinical studies, particularly those involving neonates; therefore it is necessary to seek permission to administer PB to neonates suffering from jaundice. The PB will be dosed as if giving the drug to a neonate suffering from epilepsy. After PB administration, urine samples of the neonates can be collected at different times for assessment and detection of DEHP and its metabolites. Two groups can be studied; PB treated neonates and a control group. The urine samples of neonates in each group can be collected and compared to analyse the levels of DEHP and metabolites present in the urine. If the hypothesis of treating neonates with PB increases the levels of DEHP excretion is proven, then the levels of DEHP and its metabolites in the PB treated group would be higher than the levels observed in the control group.

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