ANALYSIS AND PHARMACOKINETICS OF NON_STEROIDAL ANTI-INFLAMMATORY DRUG COMBINATIONS IN MAN

A Thesis Submitted in Fulfillment of the Requirement for the Degree of Doctor of Philosophy of the University of the University of Strathclyde,Glasgow.

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DEDICATION

To my parents

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ABSTRACT

High Performance Liquid Chromatographic methods for quantifying 11 commonly used non-steroidal anti-inflammatory drugs in serum were developed.Rapid , specific and sensitive adaptations of the methods were achieved by extraction with chloroform : acetonitrile 3:2 or diethylether : n-hexane 1:1 , giving recoveries of 85-98 %.

The methods were used to study the in-vivo kinetic properties of aspirin in healthy volunteers when aspirin (652mg) was taken alone (I) , with paracetamol (1000mg) (II) or with indomethacin (100mg) (III). The salicylate absorption rate for I was 0.75 ± 0.03 hr^{-1} (mean ± S.E.M) but for II the absorption rate was 0.99 ± 0.03 hr^{-1} ; for III the absorption rate was 1.14 ± 0.05 hr^{-1} . These constants for II and III were different (p = 0.05) from that for I but not from each other. Statistically significant differences were not found between other pharmacokinetic parameters viz:

(mean ± S.E.M.)

	I	II	II
<u>Distribution</u>			
Volume (L)	8.60 ± 0.79	7.97 ± 0.57	7.27 ± 0.45
Rate (hr^{-1}) blood to tissue	0.07 ± 0.02	0.11 ± 0.02	0.23 ± 0.03
Tissue to blood	0.15 ± 0.01	0.20 ± 0.02	0.23 ± 0.03
<u>Elimination</u>			
rate (hr^{-1}) Pseudo-distribution (body)	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
Central compartment (plasma)	0.12 ± 0.01	0.13 ± 0.02	0.13 ± 0.01

Relating the findings to changes in electropotential differences across the gastric mucosa it is apparent that a reduced gastric mucosal distribution of aspirin with an increased intestinal mucosal transport of aspirin when combined with paracetamol or indomethacin confer protective effect on the gastric mucosa.

1 INTRODUCTION

Therapeutic approaches to the suppression of fever,pain and inflammation often include the combination of aspirin with another analgesic or non-steroidal anti-inflammatory drug (NSAID).In addition,the painful episodes associated with inflammatory diseases can force patients to take additional analgesics without the physician's knowledge [1].

Opinions may differ among clinicians and rheumatologists as to the benefit of such combinations but it is desirable that drug combinations reduce side effects without compromising their therapeutic efficacy.

Different synthetic derivatives,combinations,formulations and modes of administration have greatly minimised the side effects and acute toxicity of aspirin.After an accidental ingestion of large doses of timed-release tablets containing aspirin and paracetamol there is a possibility of under-estimating the risk of poisoning since absorption of one may be continuing at a relatively later time [2].The ingestion of a liquid mixture of aspirin and another analgesic or NSAID may influence the blood concentration of either or both drugs.

Various reports indicate a correlation of salicylate therapeutic effects and side effects including acute toxicity with serum salicylate concentrations.This has been demonstrated by in vivo salicylate kinetic studies [3-5].When aspirin is combined with another analgesic the pharmacokinetic outcome may be a result of interplay between their physicochemical properties and their disposition in physiological systems.This can occur during absorption,distribution,metabolism and excretion of either of the interacting drugs.A change in these processes would be reflected in the pharmacokinetic parameters derived from serum concentration determinations.However,inter- and intra- individual differences in salicylate absorption and disposition present problems in establishing parametric relationship between salicylate kinetics and effects [6,7].

In order to evaluate clinical pharmacokinetic parameters during salicylate therapy a critical examination of some factors which contribute to the differing salicylate kinetics is required.For the purposes of this thesis the main pharmacokinetic characteristics of aspirin are presented as well as data on the potential for some of the most commonly used anti-inflammatories to influence the analysis and monitoring of salicylate concentration-time profiles in serum.

1.1 PHYSICOCHEMICAL PROPERTIES OF ASPIRIN AND SALICYLIC ACID

1.1.1 STRUCTURES OF ASPIRIN AND SALICYLIC ACID

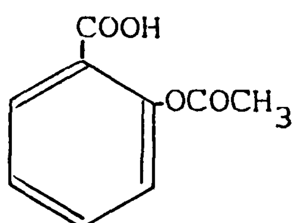


Figure 1 ACETYL SALICYLIC ACID

Aspirin,acetylsalicylic acid or 2-acetoxybenzoic acid, $C_9H_8O_4$,has molecular weight of 180.15 and an acid pKa of 3.5.

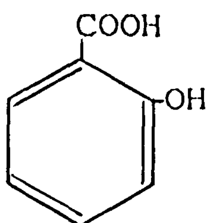


Figure 2 SALICYLIC ACID

Salicylic acid or orthohydroxybenzoic acid , $C_7H_8O_3$, has molecular weight of 138.12 and an acid pKa of 2.97.

Both compounds as drugs are relatively strong acids.

The ultraviolet absorption spectrum of aspirin in 0.1N sulphuric acid and in dilute trichloroacetic acid exhibits maxima at 229nm and 276nm respectively.In chloroform a maximum was found at 277nm [8].

The fluorescence excitation wavelength for aspirin is maximum at 280nm and the emission maximum is at 335nm. Salicylic acid[3] maxima are at 308 and 450nm respectively.[3]

1.1.2 SOLUBILITY OF ASPIRIN AND SALICYLIC ACID

At 25°C aspirin is soluble in carbon tetrachloride, chloroform,ether,ethanol and water in descending order.It is sparingly soluble in absolute ether and is insoluble in petroleum ether.The solubilty of salicylic acid differs a little from that of aspirin,the former being relatively more soluble in ether than in chloroform Elevated temperature improves the solubility of both compounds in water but aspirin is hydrolysed to salicylic acid under such conditions.

1.1.3 PARTITION COEFFICIENT (P) OF ASPIRIN

This ranges between P=17.7 at pH 1 buffer/ octyl alcohol to P=0.025 at pH 7 buffer/octyl alcohol. In chloroform/water the partition coefficient was 1.81 and in toluene/water it was 0.32 [8].

1.2 HYDROLYSIS OF ASPIRIN

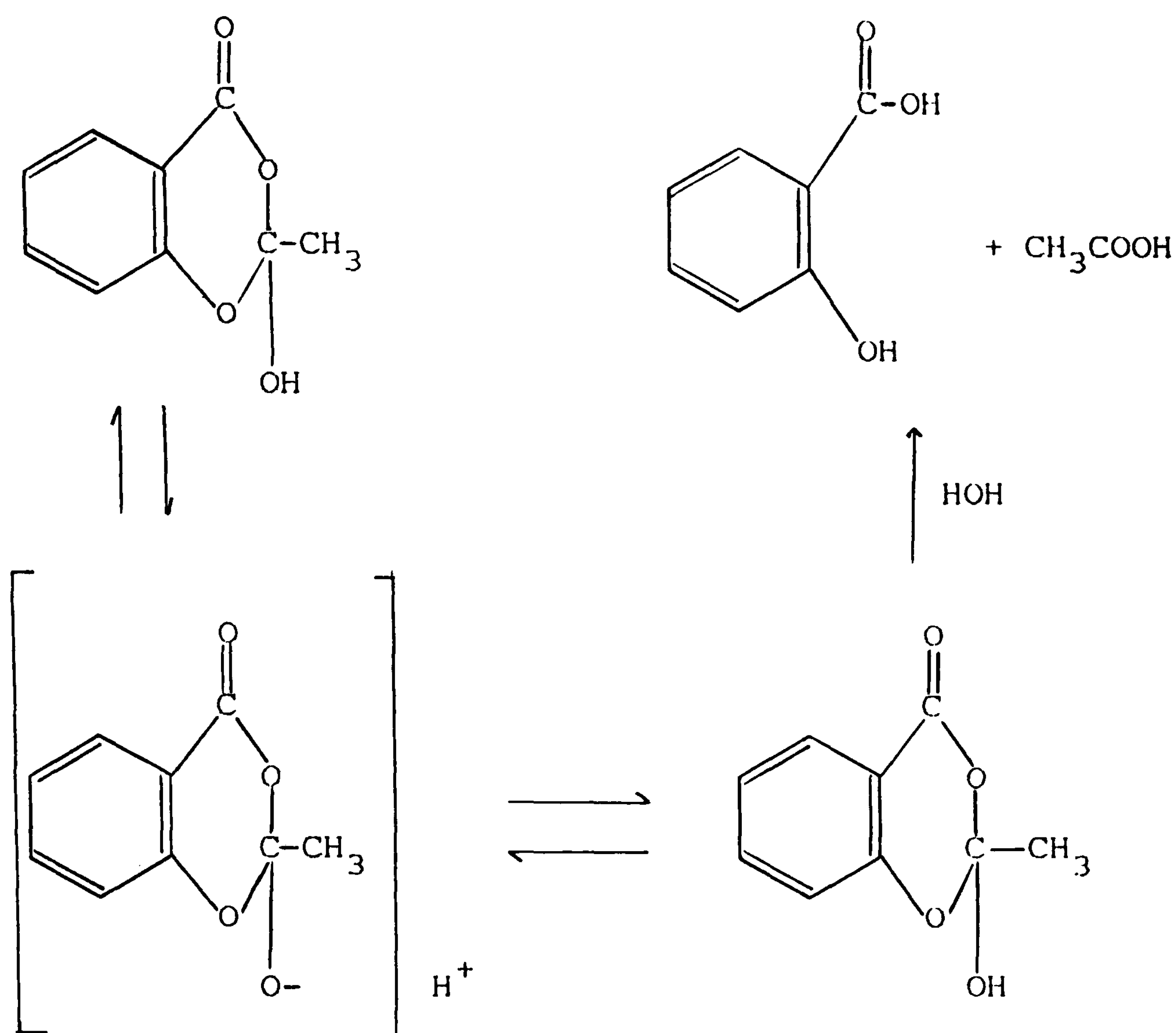
Aspirin is an ester which is readily hydrolysed by both non-enzymatic and enzymatic processes.The atmospheric hydrolysis of aspirin tablets in the home is commonly evident by the odour of acetic acid,a by-product of the

hydrolysis,perceived when improperly sealed aspirin tablet containers are opened.Non-enzymatic hydrolysis also occurs in solution in some solvent combinations.

1.2.1 SOLVOLYSIS

The solvolytic reactions involving uncharged aspirin , acetyl salicylate ion and water or solvent may or may not be dependent on bimolecular attack of the solvent on acetyl salicylic acid.This confers first order characteristics with respect to aspirin on the rate constant of the reaction [9] . However , alternative solvolytic routes result in a complex of kinetic dependencies which make aspirin possess maximum stability at about pH 2.5 against pH-dependent hydrolysis.

A significant pH-independent solvolysis of aspirin exists in the pH range 5-9 . This pH-independent hydrolysis of aspirin has been attributed to intramolecular catalysis as shown in scheme 1 (page 6)



Scheme 1: Intramolecular catalysis of aspirin.

Substituents,molecular structure and steric effects can therefore influence the hydrolysis of aspirin and similar salicylate esters.

1.2.2 ENZYMATIC HYDROLYSIS

It was reported that enzyme hydrolysis of aspirin *in vitro* obeys first order kinetics [10]. The mean half-lives of hydrolysis were 32mins in human whole blood and

66 mins in plasma at 13μg/ml concentration . At 6.5μg/ml the half-lives were 30 mins in whole blood and 69 mins in plasma.

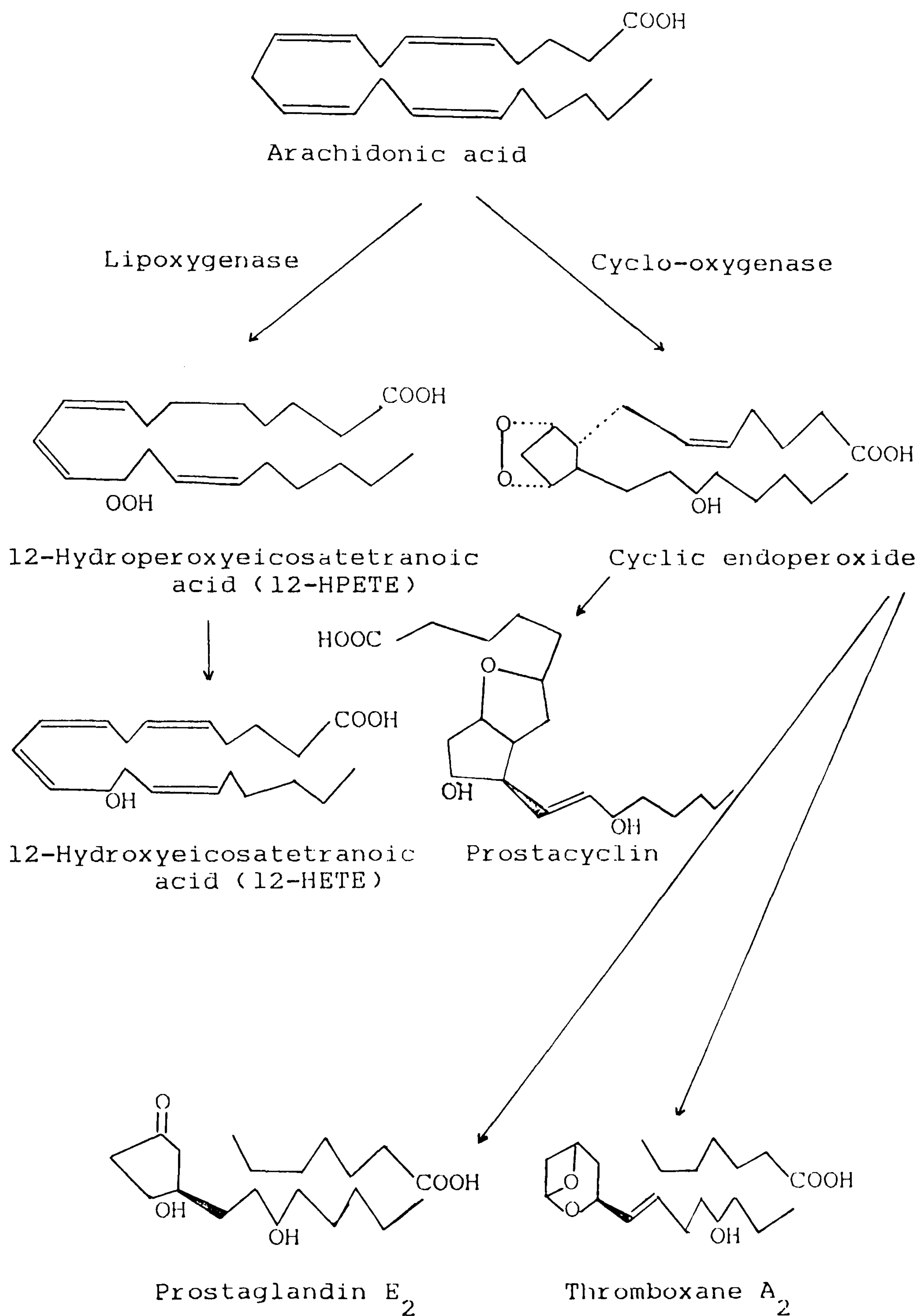
The mucosal cells and liver in man have been shown to play a role in the elimination of aspirin [11]. Enzymatic hydrolysis of aspirin has been demonstrated using the intestinal sac of rats , suggesting that some hydrolysis occurred during passage across the intestinal wall [12].

The half-life of aspirin in vitro is double the value (15 mins.) of that in vivo . Since only negligible amounts of aspirin is excreted through the kidney and aspirin is not hydrolysed appreciably in gastric and duodenal fluids,then hydrolysis of aspirin must proceed even more rapidly in certain tissues than in blood.

1.3 PHARMACOLOGICAL ACTIONS OF ASPIRIN

At therapeutic concentrations aspirin inhibits cyclo-oxygenase and lipoxygenase enzymes that are responsible for the conversion of arachidonic acid to various prostaglandins and related compounds. These endogenous compounds are involved in diseases and physiological homeostasis.

Simplified pathways of arachidonic acid metabolism involving the enzymes inhibited by aspirin are :



Scheme 2: <u>In vivo</u> Production of Prostanoids.

1.3.1 ASPIRIN IN THE CYCLO-OXYGENASE PATHWAY

Many pharmacological actions of aspirin have been ascribed to the inhibition of cyclo-oxygenase enzyme.This prevents the synthesis of prostaglandins E,F or D,as well as prostacyclin and thromboxane A_2.It has been strongly postulated that prostaglandin production is necessary to induce sensitisation of pain nerve-endings and this does not occur unless there is some degree of tissue damage [13].Prostaglandin E_2 (PGE_2) has been stated to be the predominant compound in situations where aspirin exerts its most clinically important pharmacological actions [14].

One of the signs of inflammation is pain due to the hyperalgesic effect of PGE_2 which is cummulative and long-lasting.Aspirin reduces pain and headache by removal of hyperalgesic cyclo-oxygenase products,the formation of which is stimulated by tissue damage.

Fever is another sign of inflammation associated with the presence of PGE_2 which is one of the most potent pyretic agents known.Elevated concentrations of PGE_2 have been found in cerebrospinal fluids taken from pyrexic patients suffering from bacterial or viral infections such as pyrexias of unknown origin,encaphalitis or pyrogenic

meningitis.Investigations of pyrogenic actions of cyclo-oxygenase products led to the conclusion that aspirin and related compounds exert their antipyretic action by inhibiting cyclo-oxygenase.

1.3.1.1 HAEMODYNAMIC ACTIONS

Other sequelae of cyclo-oxygenase inhibition are the prevention of prostacyclin production and thromboxane A_2 (TxA_2) formation.These have haemostatic implications . Cyclo-oxygenase catalyse prostacyclin production within the vascular endothelial cells. TxA_2 is separately contained in platelets.By selectively inhibiting TxA_2 formation while allowing prostacyclin production to continue,aspirin prolongs bleeding time [15,16].This occurs only with small doses of aspirin,large doses having no effect . High doses are known to block both prostacyclin and thromboxane formation. This could account for variations in reported haemodynamic and cardiovascular actions of aspirin [17-20].However,other endogenous compounds such as ADP and/or noradrenalin are also involved [21].

One approach in characterising the dose-effect relationship is the activity ratio - prostaglandin inhibition:thromboxane inhibition [22].

1.3.1.2 THE ROLE OF TARGET CYCLO-OXYGENASE PRODUCTS IN THE ABSORPTIVE AND EXCRETORY ORGANS

1.3.1.2.1 GASTROINTESTINAL TRACT

PGE_2 has been shown to be present in sustantial amounts in gastric juice .Oral administration of PGE_2 to normal human volunteers prevented the microbleeding induced by aspirin [23] . PGE_2 inhibits gastric acid secretion in a dose higher than that required to protect the mucosa . Parenteral administration of aspirin or NSAIDS induce gastric ulceration as a result of cyclo-oxygenase inhibition.PGI_2 and TxA_2 are also,present in gastric mucosa .Their role in the mucosa may serve haemodynamic purposes.

In the intestines prostaglandins E and F reverse the inhibitory effects of NSAIDS on the contractions of the longitudinal ileal muscle of guinea-pigs induced by acetylcholine,histamine and transmural stimulation [16,24].While the result may be different in man the findings suggest that prostaglandins play a role in maintaining the balance that ensures normal gastric emptying and intestinal motility.

1.3.1.2.2 RENAL FUNCTIONS

A complex physiological interplay exists between the prostaglandin-angiotensin , kinin-angiotensin and renin-angiotensin systems that modulate renal fuctions . In the normal kidney inhibition of cyclo-oxygenase would cause only minor blunting of renal functions mainly by decreasing renal blood flow [25-27] . However, in inflammatory conditions , as in patients with systemic lupus erythematosus , removal of PGE_2 significantly impairs renal functions .

1.3.1.2.3 THE LIVER

Little is known about the possible effects of prostaglandins and related compounds on the liver .

1.3.2 ASPIRIN IN THE LIPOXYGENASE PATHWAY

Arachidonic acid may also be metabolised by lipoxygenase enzyme to give rise to arachidonate hydroperoxides.In addition to the inhibition of cyclo-oxygenase aspirin blocks the peroxidation of 12-hydroperoxyeicosatetranoic acid (12-HPETE) to 12-hydroxyeicosatetranoic acid (12-HETE). Quite a few studies with aspirin and NSAIDS have included investigation of their effects on 12-HETE production . However,there seems to exist a strong

correlation between aspirin (as well as other NSAIDS) anti-rheumatic dosage and their inhibition of peroxidation [28].

1.3.3 ENZYME INHIBITION AT TOXIC ASPIRIN CONCENTRATIONS

Aspirin may affect the function of many enzymes , particularly when the drug reaches toxic concentrations [29-30].At those concentrations uncoupling of oxidative phosphorylation and inhibition of protein synthesis ensues.The range of toxic concentrations studied is about 83-691mg/litre.In a previous study [29] every plasma protein exposed to aspirin under physiological conditions was acetylated . Salicylate molecules were bound to human serum albumin but not to other proteins investigated . More over, human serum albumin could be acetylated *in vivo*. However, it has been emphasised [30] that it is the *in vivo* pharmacokinetic human plasma salicylate concentrations that serve clinical intentions.Against this background it was stated that salicylate at concentrations in the range 63.36 to 636mg/litre uncouples oxidative phosphorylation reactions in mitochondrial suspensions.This range is within that observed in the unbound form in plasma of patients receiving therapy for arthritis or with salicylate poisoning.

1.3.4 DIRECT ACTIONS OF ASPIRIN ON THE STOMACH

Orally administered aspirin causes aggregation and sloughing of the protective mucous layer in the stomach . This impact permits aspirin molecules to penetrate the mucosal barrier and induce direct denaturation of the underlying mucus-secreting cells [31]. The exposed mucosal parietal and capillary cells are then attacked by hydrochloric acid and pepsin. The distribution of focal points of damage is most probably determined by local concentrations of hydrochloric acid and the pH-partition characteristics of aspirin. Thus aspirin accumulates in large quantities inside acid-secreting parietal cells. The net action of aspirin in the gastric mucosa and submucosa is to increase the release of acid into the gastric lumen.

1.4 THERAPEUTIC USES OF ASPIRIN

The use of aspirin in clinical therapeutics dates back to 1899.The metabolite salicylic acid had earlier been used mixed with naturally-occuring prodrugs extracted from powdered bark of the Willow plant Salis alba vulgaris . Sustained clinical investigations and trials , buttressed with greatly improved understanding of the pharmacological actions of both compounds have established the uses of aspirin and commonly used

analgesic anti-inflammatory drugs in the clinic as follows:

1.4.1 RELIEF OF ACUTE PAIN

Headache and transient musculoskeletal pain are relieved with aspirin and this effect seems related to the suppression of acute inflammatory developments. In this circumstance the formulation of aspirin for rapid onset of action facilitates the delivery of aspirin molecules at the site of inflammation . Thus MIGRAINE headache requires the administration of dispersible or effervescent preparation to hasten peristalsis which is often reduced during migraine attacks [32] . For transient musculoskeletal pain the dosage of 600mg up to 3 times a day gives sufficient relief to the subject.The serum concentrations achieved possibly reduces PGE_2.

1.4.2 TREATMENT OF FEVER

This is another condition in which PGE_2 is implicated.It includes pyrexia due to viral,bacterial and protozoal infections.The specificity of PGE_2 in this situation is gradually being investigated and observations with other NSAIDS demonstrating antipyretic effects imposes some reservations on the role of PGE_2 per se in pyrexia [33].

1.4.3 PREVENTION OF THROMBOSIS

The inhibition of platelet aggregation is an important basis for the use of aspirin in the prevention of thrombosis.This has application in the treatment of cardiovascular conditions such as coronary artery disease,myocardial infarction,cardiac replacement surgery and thrombosis-ischaemic extremities syndrome.Clinical trials are in progress toward establishing optimum dosage and therapeutic requirements in these applications.

1.4.4 SUPPRESSION OF CHRONIC INFLAMMATION

In conditions of chronic inflammation such as rheumatoid arthritis (juvenile and adult) , rheumatoid fever, osteoarthritis and systemic lupus erythematosus not only are prostaglandins implicated but also 12-HETE production.At this point there occurs a significant departure from the occasional use of aspirin in terms of dosage.Interference with cell migration is thought to be of great importance in the remission of these inflammatory processes [34].

1.4.5 MANAGEMENT OF DYSMENORRHEA

Aspirin is recommended for use in the management of dysmenorrhea,a situation in which the role of aspirin is traditional.PGE_2,$PGF_{2\alpha}$ and the ratio $PGF_{2\alpha}$:PGE_2 are all increased in primary dysmenorrhea [35].Spasmodic actions

of the prostaglandins and the net vascular effects modulate pain intensity during dysmenorrhea.Suppression of these symptoms could be achieved with aspirin.

1.4.6 CLINICAL USES FOR THE REDUCTION OF PUTATIVE ENDOGENOUS AND EXOGENOUS COMPOUNDS

Aspirin and some commonly used analgesic anti-inflammatories are employed in various clinical circumstances based on the involvement of PGE_2 and specified prostaglandins.In these situations other NSAIDS are often reported to be more effective .This may be due to the inherent potency of the NSAID ,the pharmacokinetic characteristics of the drugs and the formulations or the patients clinical conditions.

1.4.6.1 CLOSURE OF PATENT DUCTUS ARTERIOSUS (PDA)

Various clinical trials [36-38] have placed indomethacin ahead of aspirin and other NSAIDS in the pharmacologic closure of PDA.This opening between the main pulmonary artery and aorta permits blood to be shunted away from the pulmonary vasculature *in utero* fails to close at birth in premature infants.PGE_2 is thought to relax ductal smooth muscle,maintaining the PDA.Inability of the PDA to close is associated with high concentrations of PGE_2. The success of aspirin in this use is rated far

below indomethacin and other NSAIDS.

1.4.6.2 BARTTER'S SYNDROME

The report of the syndrome of disturbed renal physiological interplay between renin,angiotensin II,kinin and aldosterone was associated with inreased output of PGE_2 and $PGF_{2\alpha}$ .Prostaglandin inhibitors,including aspirin provide effective treatment [39-40].

1.4.6.3 DIARRHOEA

Infusion of prostaglandins for the induction of labour or abortion causes diarrhoea.Studies in vitro provided evidence that bacteria which cause diarrhoea release prostaglandins.The release of prostaglandins by such bacteria as salmonella,shigella and cholera is blocked by aspirin or indomethacin [41].However,increased vulnerability of the gastrointestinal tract in such conditions limits the benefit of aspirin intake.The use of salicylate prodrugs as a way of avoiding this risk was suggested.

1.5 SIDE EFFECTS OF ASPIRIN

Chronic side effects of aspirin relate more to the duration of aspirin usage.Frequency of prescription and

administration are associated with various iatrogenic diseases.The acute side effects occur according to the therapeutic requirement of patients once a choice of salicylate therapy is made.Acute systemic side effects of aspirin may be monitored most appropriately in association with serum concentration measurement.

The major side effect of aspirin is upper gastrointestinal mucosal damage (see 1.3.4).This leads to erosive gastritis,gastric and duodenal ulcers.Secondary effects are occult blood loss and malaena.Haematemesis sometimes follows nausea,as in milder conditions,often due to gastric intolerance to aspirin.Anaemia is sometimes associated with chronic blood loss.

As serum concentration of salicylate inreases the limiting range of salicylate therapy is approached and occurs with anti-inflammatory dosage (1.4.4) Figure 3 shows the relationship of plasma salicylate concentrations to response and toxicity [42].When this relationship is viewed against the background of long-term monitoring [43] and spontaineously reported adverse reactions [44] the properties of aspirin that predispose the subject to acute gastrotoxicity and ototoxicity may be rationalised along the pharmacokinetic course.

PLASMA SALICYLATE (μg/ml)	EFFECTS	COMPLICATIONS
900 -		Renal and respiratory failure
800 -		Cardiovascular collapse
700 -	Intoxication	Fever,coma
600 -		Metabolic acidosis
500 -		Respiratory alkalosis
400 -		Central Hyperventilation
300 -	Rheumatic fever	Nausea and vomiting
200 -	Anti-inflammatory range	Deafness,headache, vertigo,tinnitus
100 -	Analgesic range Antipyresis Antiplatelet effect	Gastric intolerance and bleeding Hypersensitivity reactions

Figure 3:Relationship of plasma salicylate concentrations to response and toxicity [42]

1.6 THERAPEUTIC CONCENTRATIONS

In various clinical trials for analgesia and salicylate disposition [45-47] the concentrations in plasma or serum are in the range of 15-80μg/ml depending on the sampling times and study design.

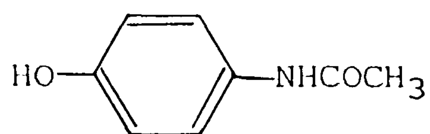
The therapeutic concentration of aspirin is often considered as being that required for the treatment of rheumatoid arthritis.The plasma salicylate concentration range is 120-350μg/ml [42,48,49].Figure 3 (1.5) shows the anticipated effects of salicylate concentrations.

1.7 COMMONLY USED ANALGESIC AND ANTI-INFLAMMATORY DRUGS

The clinical uses of aspirin may be fulfilled with drugs which possess analgesic, antipyretic,antithrombotic and anti-inflammatory actions.Further specifications based on structure-activity relationships result in groups of compounds from paracetamol with mainly analgesic and antipyretic action through aspirin with all the actions to indomethacin with powerful anti-inflammatory action. Figure 4 (a-d) shows the structures of the compounds.

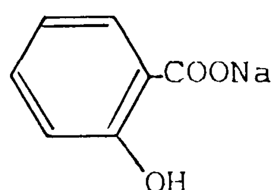
Figure 4: COMMONLY USED ANALGESIC AND ANTI-INFLAMMATORY DRUGS

(a) PARAMINOPHENOL DERIVATIVE

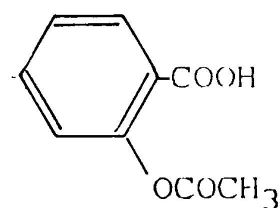


Paracetamol

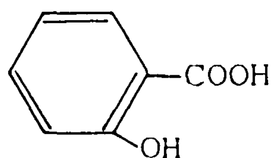
(b) SALICYLATES



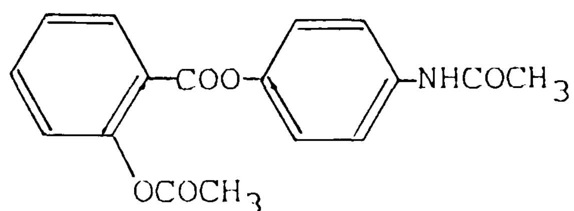
(i) Sodium salicylate



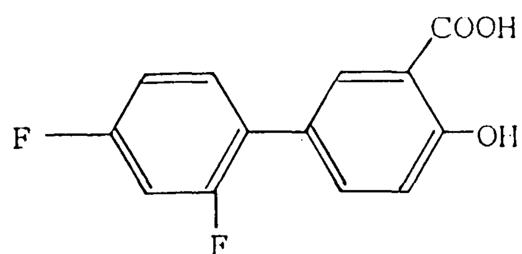
(ii) Acetylsalicylic acid



(iii) Salicylic acid

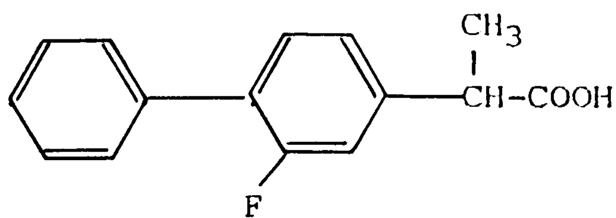


(iv)Benorylate

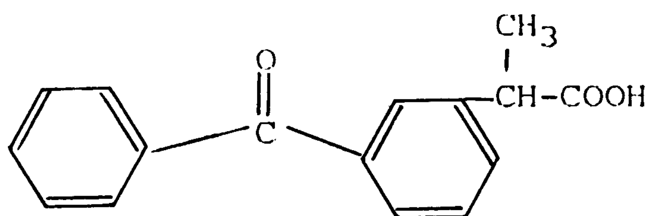


(v) Diflunisal

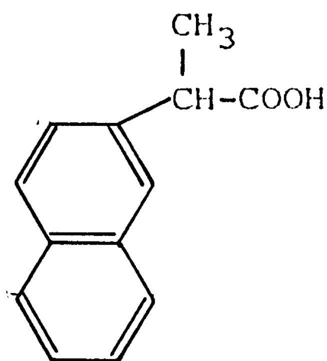
(c) PROPIONIC ACID DERIVATIVES

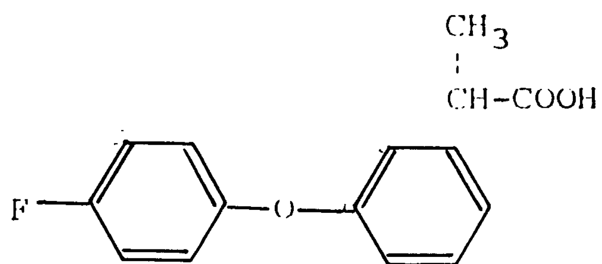


(i) Flurbiprofen



(ii) Ketoprofen

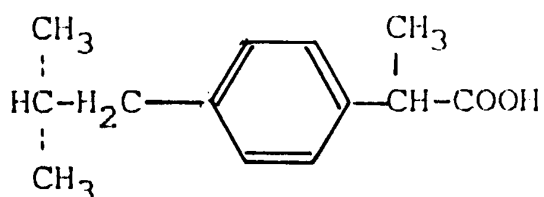




(iv) Fenoprofen

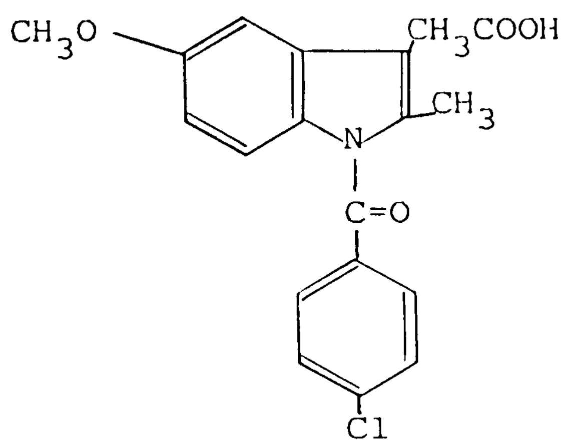
CH_3O

(iii) Naproxen



(v) Ibuprofen.

(d) INDOLE DERIVATIVE



Indomethacin

1.7.1 The PARAMINOPHENOL DERIVATIVE (Figure 4a).

Paracetamol (Acetaminophen) has analgesic and antipyretic effects similar to aspirin but it is very weekly anti-inflammatory.

Comparative literature reports on the modes of actions of paracetamol and other NSAIDS indicate that paracetamol, even in very high doses has only minor inhibitory effects on cyclo-oxygenase [28].In some reports paracetamol was shown to enhance PGE_2 production [26,31].In one report the differing properties of paracetamol and some analgesic anti-inflammatory drugs were related to their pharmacokinetic characteristics _in vivo_ as different from _in vitro_ experiments [26].

1.7.1.1 THE PHYSICOCHEMICAL PROPERTIES OF PARACETAMOL

Paracetamol is N-acetyl p-aminophenol,also called acetaminophen,p-acetylaminophenol,N-acetylparaminol or p-hydroxy acetanilide,$C_8H_9NO_2$. Its molecular weight is 151.16 and it is weakly acidic with a pKa of 9.5.

The ultraviolet spectrum of paracetamol shows absorption maxima ranging from 242.5nm in water and various solvents to 250nm in isopropanol.Addition of acid to aqueous and alcoholic solutions does not change the position of the maxima [50,51].

1.7.1.2 SOLUBILITY AND STABILITY

At $25^{\circ}C$ paracetamol is soluble in water , alcohol and chloroform.It is insoluble in ether, petroleum ether and pentane [52,53].Aqueous solutions of paracetamol remain stable at $90^{\circ}C$ and pH of 5 for saturated solutions of the compound up to 20 hrs.At $70^{\circ}C$ in hydrochloric acid of varying strengths hydrolysis of paracetamol has maximum stability in the acidic medium of pH 5-7,having a half-life of 9 months at pH of 2,20 years at pH of 5,22 years at pH of 6 and 2 years at pH of 9 .

1.7.1.3 PARTITION COEFFICIENT

At pH of 7 91% of paracetamol is extracted into the ether phase with ether:water volume ratio of 5.In the same volume ratio at pH of 4 the partition coefficient is 88%.In octyl alcohol/water the partition coefficient is $6 \pm 2\%$ at pH of 7.2. Chloroform-ethanol:water partition coefficient is about 0.44.In alkaline pH (0.1N NaOH) paracetamol fails to partition into the ether phase.

1.7.2 The THERAPEUTIC CONCENTRATION range of paracetamol [54,55] is 5-15µg/ml.

1.7.2 SALICYLATE DERIVATIVES (Figure 4b)

With the exception of sodium salicylate which is

contemporary in usage with aspirin other salicylate derivatives synthesised to provide sufficient serum salicylate whilst being devoid of gastrotoxic effects are benorylate , choline magnesium trisalicylate and diflunisal.These drugs invariably exert their pharmacological action through the salicylate anion liberated in vivo by metabolic degradation.

1.7.3 THE PROPIONIC ACID DERIVATIVES (Figure 2c) are mainly used for analgesic and anti-inflammatory purposes. Flurbiprofen,naproxen,ketoprofen,fenoprofen and ibuprofen are prescribed interchangeably for patients.

In their pharmacological actions flurbiprofen is reported to be more potent than indomethacin in the inhibition of PGE_2 [14].Naproxen inhibited PGE_2 production associated with coronary vasodilator and vasoconstrictor actions of arachidonic acid in the isolated perfused heart of the rat [17].It was without effect on renal PGE_2 ,PGI_2 and TXA_2 formation.This was similar to other aspirin-like drugs tested for effects on rabbit renal prostaglandins and sodium balance [27].Flurbiprofen and ibuprofen are placed intermediary between aspirin and indomethacin in the range of per cent inhibition of PGE_2 induced by selected NSAIDS at specified molar concentrations.

The PHYSICOCHEMICAL properties of the propionic acid derivatives are similar to those of indomethacin (see below) being acids with mean pKa of 4.50.

1.7.4 The methylated INDOLE DERIVATIVE (Figure 2d),indomethacin,has analgesic antipyretic and anti-inflammatory effects.It is one of the most potent inhibitors of the cyclo-oxygenase enzyme.Indomethacin also inhibits leucocyte migration.Comparative literature reports indicate that in equimolar concentrations indomethacin exerts greater inhibitory actions than aspirin and most NSAIDS [16-20].

1.7.4.1 PHYSICOCHEMICAL PROPERTIES OF INDOMETHACIN

Indomethacin is 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid,also named as (1-p-chloro-benzoyl-1-5-methoxy-2-methylindole-3-yl) acetic acid.It is strongly acidic with pKa 4.5 and has molecular weight of 357.80.

Indomethacin exhibits ultraviolet maxima at 318nm at a concentration of 14.29µg/ml in methanolic 0.1N hydrochloric acid [56].The compound in 0.1N NaOH decomposes to a product which fluoresces at 385nm after excitation at 312nm. This phenomenon does not permit discrimination of indomethacin from salicylates [56].

1.7.4.1.1 SOLUBILITY AND STABILITY OF INDOMETHACIN

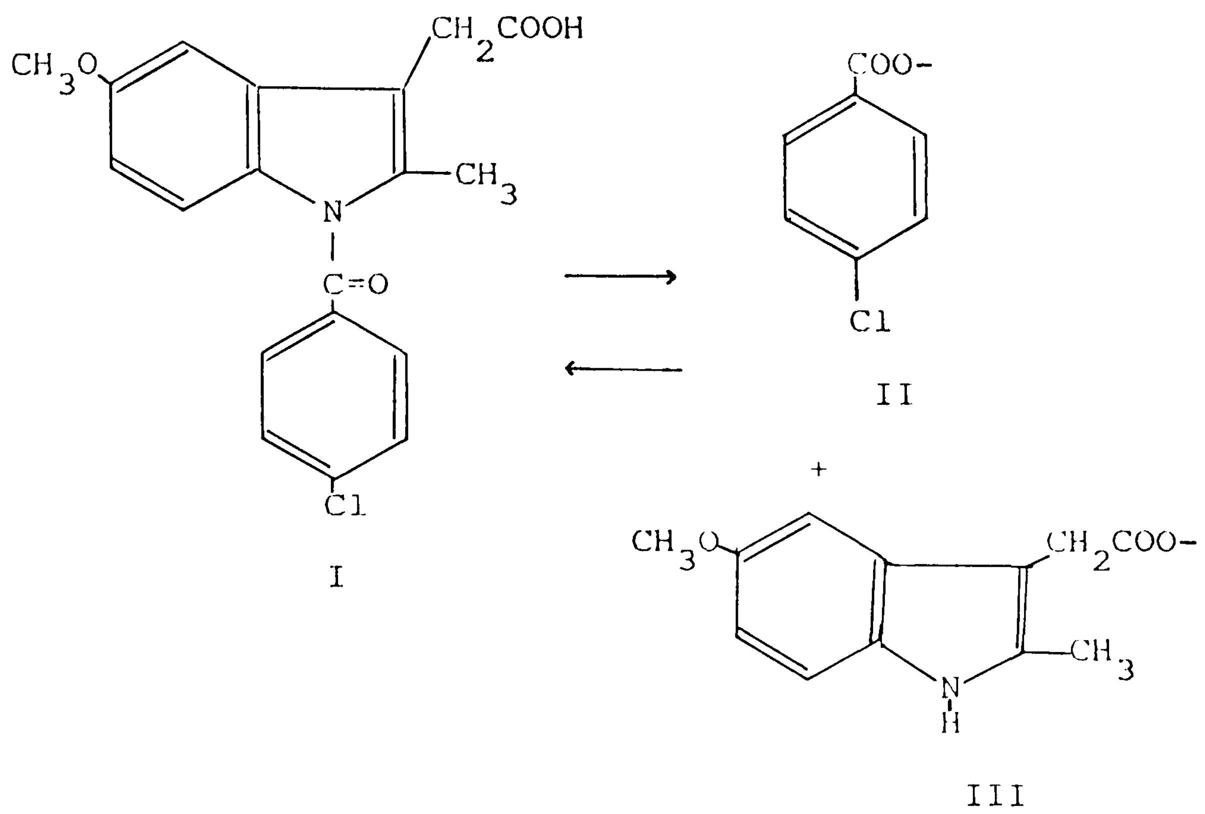
Indomethacin is soluble to appreciable degrees in chloroform,ether,ethanol,methanol and water at 25°C and room temperatures.The polymorphic varieties of the compound has different solubility properties in water and phosphate buffer.Solubility of indomethacin inreases with increase in buffer pH from 30μg/ml at buffer pH 5.6 to 800μg/ml at buffer pH 7.0 at 25°C.

HYDROLYSIS of indomethacin (I,scheme 3 p.31) occurs in alkaline conditions yielding p-chlorobenzoyl anion (II) and 2-methyl-5-methoxy-indole-3-acetate (III).

1.7.4.1.2 PARTITION COEFFICIENT (P) OF INDOMETHACIN is 16.4 for methylene chloride : phosphate buffer pH 7.1 but 8.2 in solvent pair of ether:phosphate buffer pH 7.1.

1.7.5 THERAPEUTIC CONCENTRATION

Serum therapeutic concentration of indomethacin is in the range 0.5-3μg/ml for analgesic and anti-inflammatory effects [57-59]



Scheme 3: Alkaline hydrolysis of indomethacin.

1.8 ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION

1.8.1 ASPIRIN

1.8.1.1 ABSORPTION of aspirin from the stomach and intestine is rapid and rate-limited by dissolution [60] The rate of dissolution of aspirin is strongly dependent upon the size of the particles. According to the pH-partition hypothesis ,the pH of the stomach and the pKa of aspirin favours greater absorption in the stomach. However,the unionised,lipophilic aspirin molecules and negligible ionised species in solution are more rapidly absorbed in the upper intestine because of a much larger surface area and greater blood flow.Consequently,gastric emptying affects the absorption of aspirin.

At an average pH of 6 in the upper intestine,a partition factor contributing to absorption rate constant would be:

e.g (i)

$$\frac{\text{Concentration in the intestine}}{\text{Concentration in the blood}} = \frac{1+10^{\text{pHintest-pKaASA}}}{1+10^{\text{pH blood-pKaASA}}}$$

$$= \frac{1+10^{6-3.5}}{1+10^{7.4-3.5}}$$

$$= 0.0399$$

During absorption some proportion of aspirin is hydrolysed to salicylic acid.Absorption of aspirin quantified from salicylic acid concentrations may not differ from computation with aspirin due to rapid rate of aspirin hydrolysis when total salicylate is the objective of therapy.

Individual variability in the rates of absorption of aspirin is one major source of intrasubject variability of serum salicylate concentration.A sufficiently large panel of subjects to represent the types of absorption variability after aspirin ingestion has been suggested [61].However,not more than 3 types of absorption have been identified.A plot of average concentrations against time could therefore result in a dramatic plateau curve [6,7].

1.8.1.2 DISTRIBUTION

Salicylate is distributed throughout the body fluids and tissues.The highest concentrations occur in plasma,kidney,liver,heart and lung [62].About 70% of salicylate in the blood is bound to plasma proteins.

In consideration of pH-partition hypothesis the distribution of salicylate from the blood into the tissues when the lowest pH is assumed to be 6.5 (as in

kidney/urine) would influence the distribution rate constant (in the direction of concentration/pH gradient) by a factor of:

$$\frac{1 + 10^{7.4-3.0}}{1 + 10^{6.5-3.0}}$$

$= 7.94$ (c.f. eg.i)

and a consideration of 30% free salicylate further modifies the distribution factor to 2.38.This could be modified by the equilibration process,especially at limiting concentration difference.

If urine pH of 6.0 is used when the relatively negligible salicylate concentration (see below) in urine is considered as a lower reference zone then the distribution rate would be influenced by a factor of:

$$\frac{1+10^{4.4}}{1+10^{3.0}}$$

$= 25$, corrected for free fraction the factor would be 7.50.

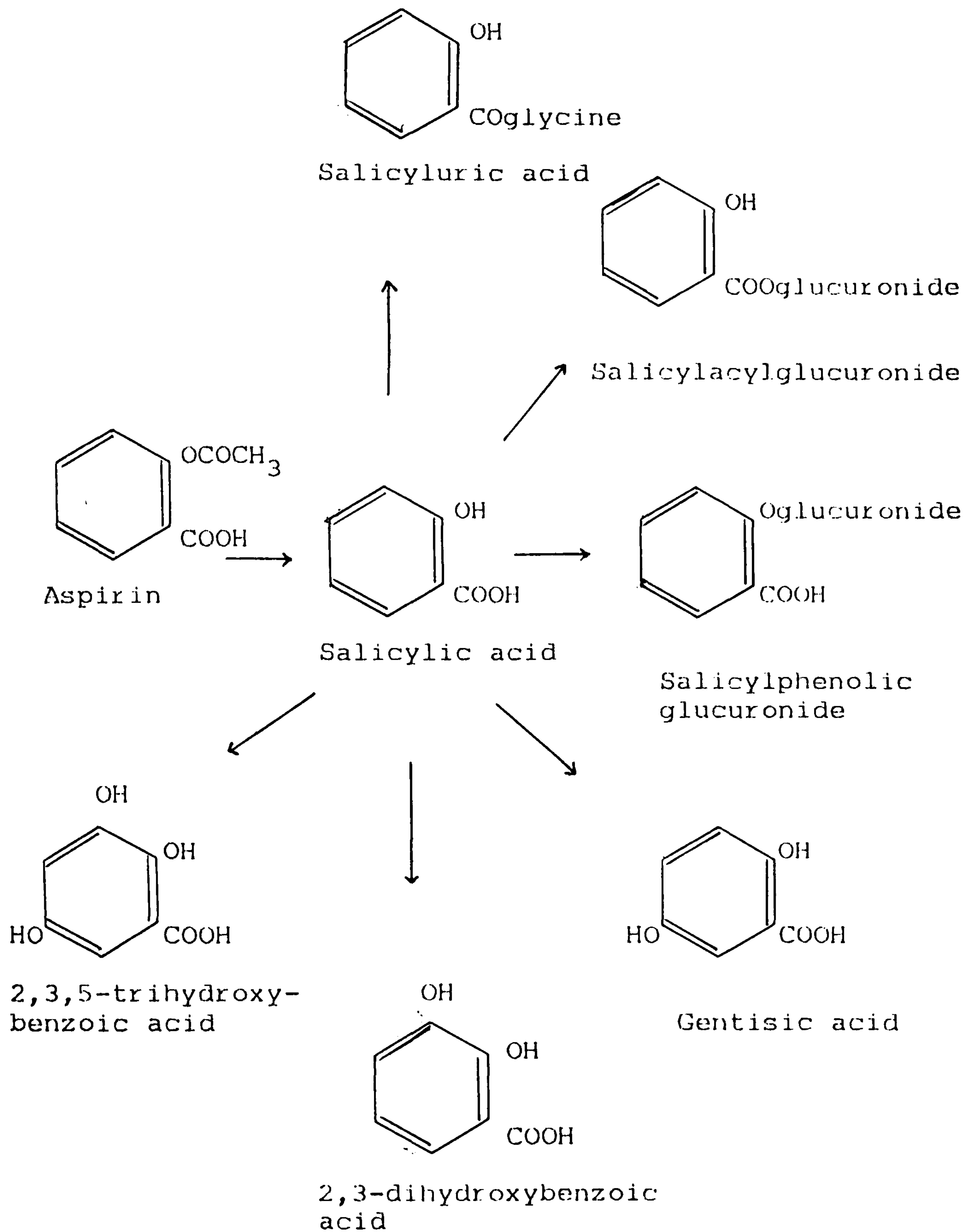
1.8.1.3 METABOLISM

Aspirin is rapidly hydrolysed in the plasma to salicylic which is also active agent [63].

Conjugation with glycine to form salicyluric acid

accounts for 50-80% of the administered dose and 10-30% is conjugated with glucuronic acid to form salicyl-o-glucuronide,5% as salicylacylglucuronide,5-10% as free free salicylic acid and small amounts of gentisic acid and gentisuric acid.Other metabolites are dihydroxy and trihydroxy derivatives of salicylic acid.Scheme 4 shows the main metabolites of aspirin and their relationship to each other.

1.8.1.4 EXCRETION of salicylate in the urine is low and is influenced greatly by urine pH. 5-10% of the normal therapeutic dose is present in urine as free salicylic acid.This low concentration renders reabsorption negligible.



Scheme 4 : Metabolites of aspirin

1.8.2 PARACETAMOL

1.8.2.1 ABSORPTION

Various reports show that the absorption of paracetamol from tablets or solution is rate-limited by gastric emptying [64-65].Absorption curves for paracetamol and gastric emptying were consistent with negligible absorption from the stomach.Paracetamol is rapidly absorbed from the upper intestine . Like aspirin ,factors which inhibit gastric emptying or intestinal motility delay the absorption of paracetamol,the effect being more pronounced in the case of paracetamol [65].Large doses,the presence of food and time of the day cause variations in the absorption of paracetamol.

The rapid absorption of paracetamol is consistent with pH-partition hypothesis since a high proportion of unionised ,lipophilic molecules would be present in the upper intestine,pH 5-7 with approximate surface area of $200m^2$ [66].Considered as a monobasic acid and in a mean intestinal pH of 6,unionised molecules of paracetamol diffuse through the membrane demonstrating an <u>in vivo</u> characteristics which contributes to the absorption rate constant by a factor of

$$\frac{1+10^{6-9.5}}{1+10^{7.4-9.5}} = 0.99$$

This,however,assumes that back difussion into the membrane and aqueous resistances are negligible [67-69].

1.8.2.2 DISTRIBUTION

Paracetamol is widely distributed throughout body fluids.Tissue-water:plasma concentration ratios for paracetamol were reported following studies with dogs [53].Phenacetin (2.7g) administered to a dog resulted in slightly varying ditribution characteristics of paracetamol in different tissues. With the exception of red cells (1.5) and cerebrospinal fluids (0.48) the main organs including the brain had ratios of about 1.0.The liver (1.32) and the kidney (1.29) had slightly higher ratios.The situation may be different in man but there seems to be minor differences between plasma and tissue paracetamol concentrations.However,with higher doses protein binding occurs up to a mean of 25% in serum.

On the pH-partition hypothesis the various tissues may be a reflection of varying pH zones . The distribution from blood (pH 7.4) to tissues of lowest pH, assumed 6.5 (kidney) would be influenced by the ratio:

$$\frac{1+10^{7.4-9.5}}{1+10^{6.5-9.5}}$$

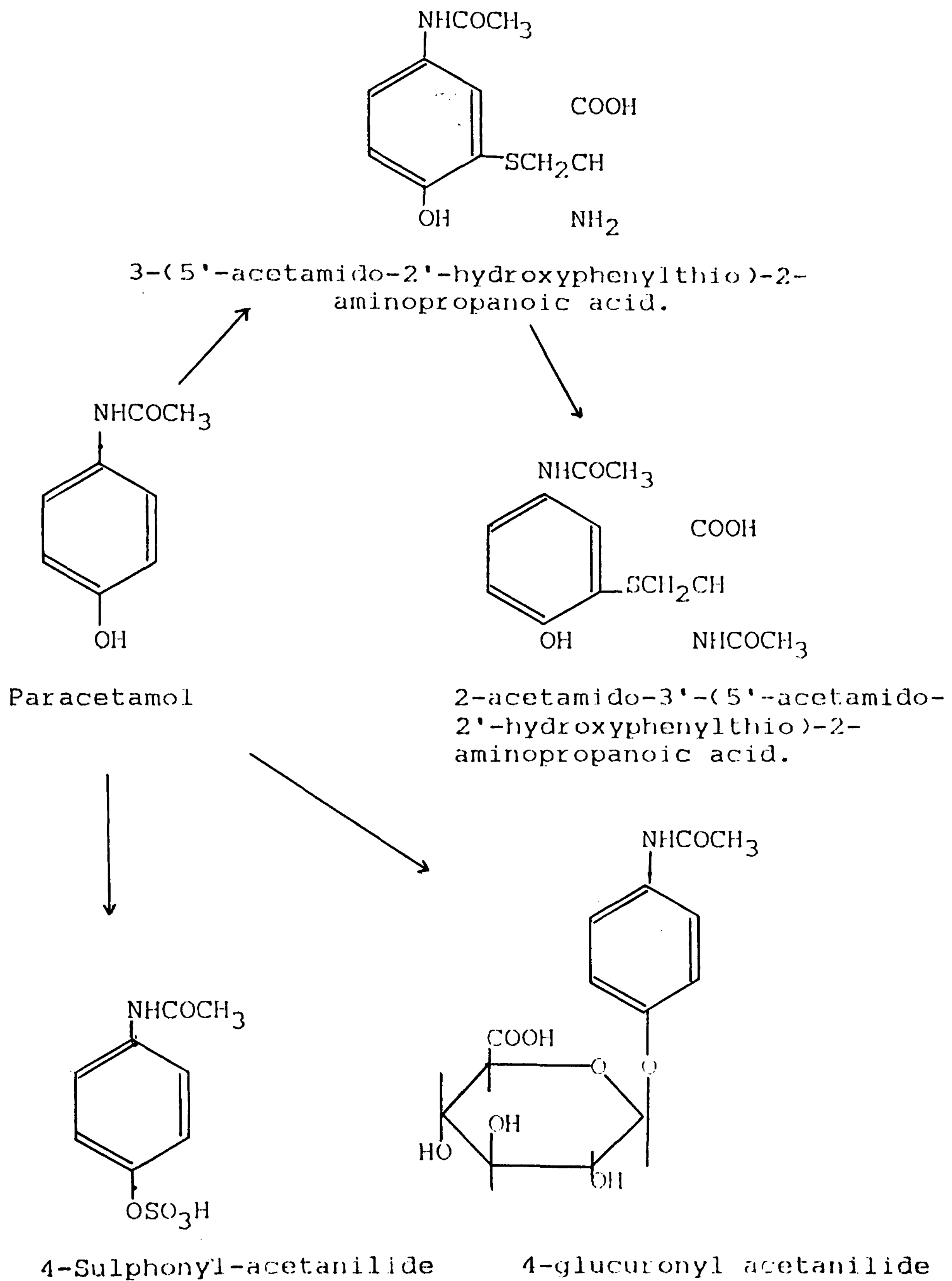
$$= 1.007$$

If urine pH of 6.0 is assumed as the lower reference point then the ratio would be 1.008.

Access to metabolising enzymes and glomerulus may be related to the distribution characteristics.

1.8.2.3 METABOLISM of paracetamol occurs mainly in the liver and partly in the gut wall.Following the administration of a therapeutic dose about 36-54% is conjugated with glucuronic acid . Conjugation with sulphate is 18-27%.The 3-hydroxy sulphate,the 3-methoxy glucuronide and the 3-methoxy-3-sulphate make up 4.5-9% About 4.5-9% consist of the mercapturic acid and cysteine conjugates.Scheme 5 shows the main metabolites of paracetamol and their relationship to each other [70]

1.8.2.4 EXCRETION of paracetamol through the kidney is minimal,being 0.9-3.4% of the therapeutic dose in 24 hours in the urine.



Scheme 5 : Main metabolites of paracetamol

1.8.3 INDOMETHACIN

1.8.3.1 ABSORPTION

Indomethacin is promptly absorbed after oral administration to man [71].The rate of absorption greatly depends on the dose and particle size of the drug.Thus 200mg in small particle size to 50mg in large-particle size of indomethacin resulted in 5μg/ml-0.2μg/ml serum concentration in fasting subjects in 30 mins - 1h.

On the pH-partition hypothesis,at a mean upper intestinal pH of 6 the absorption rate of indomethacin would be influenced by a factor of 0.041.

1.8.3.2 DISTRIBUTION

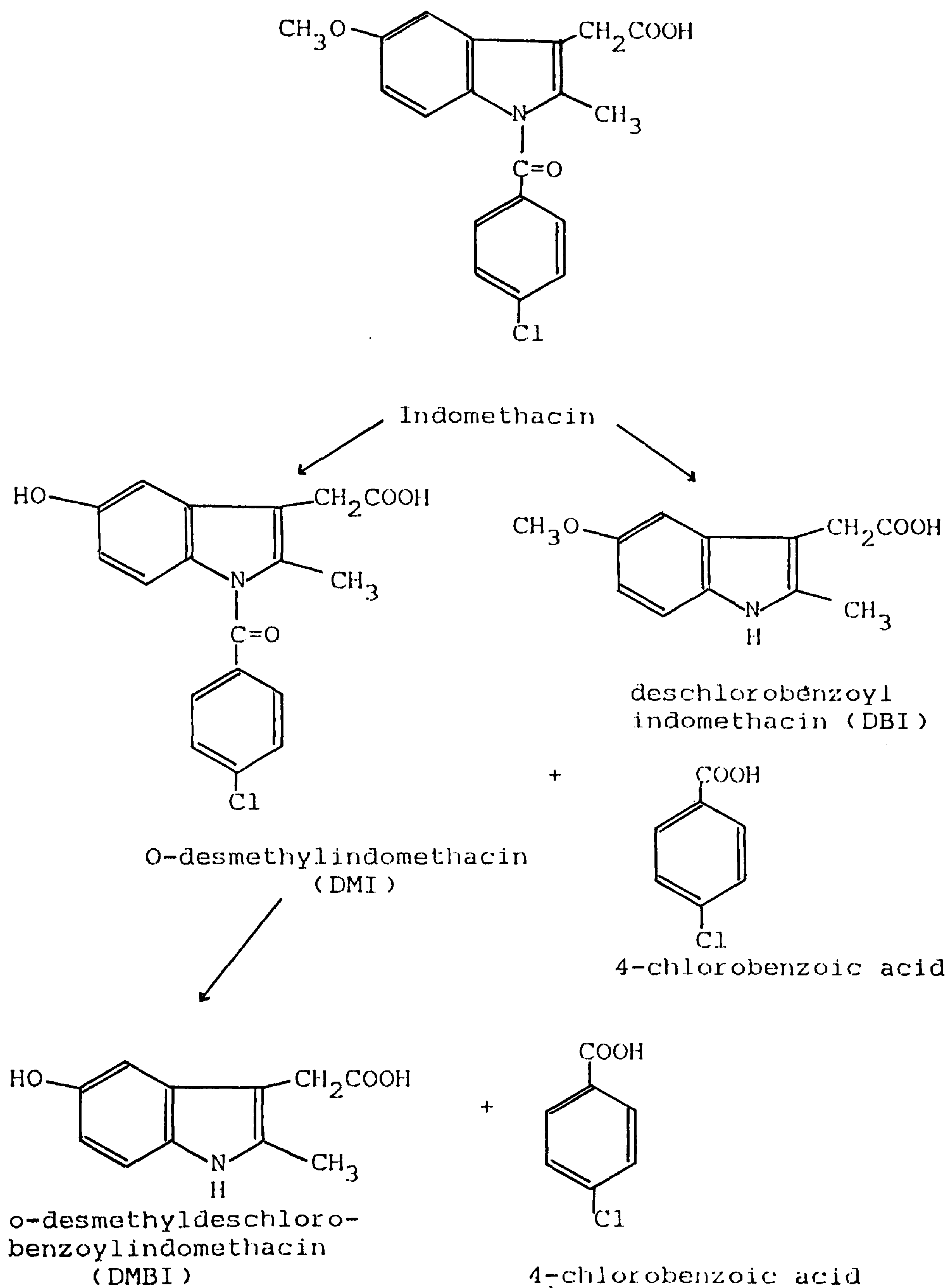
Indomethacin is slowly but widely distributed in the body fluids and tissues.The drug binds to plasma proteins to the extent of 95-98%.Tissue plasma concentration ratios were reported for rat and guinea-pig [71].At 120min the liver (0.50) and kidney (0.54) ratios are similar in the rat as is the case in the guinea-pig liver (0.50)and kidney (10.40).It was 0.36 for rat intestine but 25.1 for guinea-pig intestine. No accurate projection of indomethacin distribution characteristics in these species can be made to humans.

The pH-partition contribution of indomethacin would be of

the factor of 7.87 if the lowest pH of tissue is assumed as 6.50.If urine pH of 6.0 is the lowest limit then it is 24.4. Considering protein binding,the factors would be 0.8 and 2.4 respectively.

1.8.3.3 METABOLISM of indomethacin in man proceeds at rates suggestive of the sequence whereby indomethacin is o-demethylated to desmethylindomethacin (DMI) then n-deacylated to desmethyldeschlorobenzoylindomethacin(DMBI) as a major pathway [72].In the terminal stage direct deacylation of indomethacin to desbenzoylindomethacin (DBI) constitutes a competing pathway.In 48hr urine 8.14% is free DMI and 9.4% is the glucuronide;7.9% is free DBI and 6.24% is the glucuronide conjugate while 2.14% is the free DMBI and 0.26% the glucuronide of DMBI,after oral administration of 25 mg.Enterohepatic cycling of conjugates occurs.Scheme 6 shows the metabolites of indomethacin and their routes.

1.8.3.4 EXCRETION of indomethacin by the kidney is pH-dependent and 16.05% of a 25mg dose is recovered in urine as free drug in 48h,11.6% being the glucuronide.Indomethacin is secreted by renal tubles and has been shown to compete with acidic drugs that are similarly secreted.Negligible amount (1.4%) is present in 96h faeces as indomethacin.



Scheme 6: Metabolism of indomethacin

1.9 COMBINATION OF ASPIRIN WITH ANALGESIC OR ANTI-INFLAMMATORY DRUGS

1.9.1 CLINICAL RATIONALE

Aspirin is an established anti-inflammatory drug whilst paracetamol is not (1.4).Evidences in support of the different clinical uses of both drugs apparently do not point to a concensus of opinion based on the findings in pharmacological assessments in rats [73].These show paracetamol to possess greater antipyretic,analgesic and inflammatory actions than aspirin.However,in humans other factors could have more profound effects than is suggested by discrete biochemical pharmacological events.

With the exception of paracetamol and sodium salicylate all aspirin-like drugs have consistently been found in therapeutic concentrations to inhibit PGE_2 synthesis (1.6).Some findings [24,28] indicate that the actions of paracetamol at cyclo-oxygenase level of arachidonic acid cascade are opposed to those of aspirin.In addition, paracetamol has been shown to protect the gastric mucosa against the ulcerogenic effect of aspirin [74-76].Nevertheless it was reported that paracetamol has an additive action with aspirin in the inhibition of prostaglandin release from macrophages [77] . A potentiation effect was also shown to result from the

phenolic co-factor role of the compounds in combination [78].There seems to exist,therefore,an anatomical basis for the different effects of cyclo-oxygenase inhibition achieved with the combination of commonly used anti-inflammatory drugs.

Paracetamol in relatively high concentrations was shown to inhibit cyclo-oxygenase in the brain and this action was stated to be responsible for its antipyretic effect [79].Some other factors are involved in pyrogenesis.One notable factor is the stimulation of thermosensitive preoptic and anterior hypothalamic neurons [81] . This would require the compounds to cross the blood-brain barrier to an effective extent,the evidence of which is lacking,except perhaps in over-dose situations.

An additive effect of aspirin with an analgesic or non-steroidal anti-inflammatory drug would depend upon the ability of either compound to reach the sites where their pharmacological actions mediate therapeutic and side effects . The products and mediators of inflammation are often transported within fluid systems and across cell membranes and junctions.Also clinical manifestation of inflammation are of a localised nature such as the tophaceous growths in gauty arthritis.The presence of the drugs within inflammation sites would be of

antinociceptive advantage.Indomethacin may have an additive anti-inflammatory effect with aspirin but might also increase side effects.

1.9.2 PHARMACOKINETIC CONSIDERATIONS

It is thought that faster pain releaf and improved safety that might be obtained from the combination of aspirin and paracetamol or other NSAIDS may be reflected by increased serum concentration of either aspirin or both drugs.

When both compounds are present at their sites of action an increased serum concentration may be a result of reduced physiological distribution.This would support the concept of additivity of their actions.However,the synchronous and simultaneous absorption and distribution of the co-administered drugs would result in a concentration-time curve which requires resolution in terms of the processes of disposition.

1.10 AIMS AND OBJECTIVES

The current position of salicylate therapy against the background of specific pharmacological mediators of inflammation apparently does not comform to a single concept.A combined administration of aspirin with two compounds possessing varying degrees of similarity in

actions might point towards probable kinetic implications with regards to therapeutic and side effects of aspirin.

The present study was undertaken to:

1. Develop methods for the simultaneous determination of the concentrations of paracetamol and anti-inflammatory drugs in serum.

2. Analyse human sera for concentrations of salicylic acid and the co-administered drug when given orally.

3. Assess the in vivo kinetic consequences of administering aspirin with either of the counter-putative analgesics: paracetamol and indomethacin.

It was hoped that these procedures would provide a pharmacokinetic view that probably complements the systemic and gastric effects observed upon the consumption of aspirin.The alteration of electropotential difference across the gastric mucosa following the administration of aspirin is perhaps appropriate as a measure of gastric effects.This would be noted for possible relationship to independent pharmacokinetic assessments.

2. DETERMINATION OF ASPIRIN AND COMMONLY USED ANTI-INFLAMMATORY DRUGS

2.1 METHODOLOGY

Various methods for quantifying aspirin and other non-steroidal anti-inflamatory drugs have been developed for quality control of their pharmaceutical dosage forms.Some of these methods have been applied to the routine monitoring of the concentrations of these drugs in plasma or serum.These methods are required to take into account the need for specificity,sensitivity,reproduciblity and speed of analysis.

2.1.1 COLORIMETRY

One of the early methods of analysis of aspirin involved procedures in which salicylate anion was first converted to a coloured complex with ferric or ferrous salts and the absorbance in the visible electromagnetic wavelengths used to determine the concentrations in the samples [82-84].Subsequent modifications of these procedures involved the use of Folin Ciocalteau phenol reagent in place of ferric or ferrous salts.The applicability of each of these procedures to serum samples required not only the extraction process but also the hydrolysis of aspirin to salicylic acid under relatively vigorous conditions for

favourable chromogenic reaction with the phenolic group.

Paracetamol,possessing the phenolic group,was also analysed by colorimetry [85-86].The compound was first extracted from plasma saturated with sodium chloride using ether as the extraction solvent.Back extraction of paracetamol into dilute alkali provided an aqueous solution of the compound on which colorimetry was performed.

Quantitation of total salicylate or total paracetamol in plasma or serum after the ingestion of the drugs requires prior hydrolysis of the gluronide and sulphate conjugates before colorimetry.Consequently the method is slow,involves many potential sources of error and the specificity is difficult to maintain for large numbers of samples.

2.1.2 ULTRAVIOLET (U-V) SPECTROPHOTOMETRY

Absorption spectrophotometric methods have been used as alternatives to colorimetry.The procedure requires the extraction of aspirin from an aqueous acidic medium with chloroform [87-89] or with ether [92].The chloroform extract was quantified by direct u-v spectrophotometry.The acid ether extract was evaporated to dryness and the extract reconstituted in alcohol for

subsequent determination of the ultraviolet absorbance. This last step enabled the avoidance of ether interference in the u-v spectrum in the wavelength at which the compound was determined.An acceptable measure of reproducibility (c.v = 2%) was obtained and either procedures was suitable for application to pharmaceutical mixtures.However,the method was as time-consuming as the colorimetric procedure and did not possess the required specificity when applied to blood samples.

2.1.3 FLUORIMETRY

Various workers have explored the fluorescence of salicylic acid and some NSAIDS as a means of providing the necessary sensitivity for the analysis of these compounds [90-92].

A fluorometric procedure for the determination of aspirin and salicylic acid in blood and plasma [90] involved the prior separation of the compounds by paper chromatography.The eluted compounds were recovered by alkaline extraction before the determination of the fluorescence.In this procedure the susequent hydrolysis of aspirin was unnecessary,having occured under ammonia vapour spray.The fluorescence emmission of salicylic acid was in the range of 350-480nm.The maximum concentration determined for calibration was 10μg/ml of aspirin and

10mg/ml of salicylic acid added to whole citrated blood.However,the plasma concentration was derived by assuming 50% haematocrit.

A subsequent report [91] showed that aspirin per se could be excited at 280nm with the resultant emission maximum at 335nm.This method permitted simultaneous fluorometric determination of aspirin and salicylic acid dissolved in acetic acid-chloroform solvent.This approach was not applied to serum nor plasma samples.

INDOMETHACIN was analysed in plasma, bile and tissue homogenates by spectro-photofluorimetry [71].The drug was extracted at pH 5 into heptane and back-extracted into the aqueous phase by shaking with 0.1N sodium hydroxide.The aqueous extract was then measured by spectrophoto-fluorimetry.The excitation maxima had been at 295 and 385nm respectively.Huidberg et al [93] showed that the intensity of the fluorescence could be increased by adjusting the pH to 11.6 $\pm$ 0.1.

The metabolites of indomethacin (DBI and DMBI) fluoresce strongly at these wavelengths and so could interfere with the assay for indomethacin.However,the specificity of the assay for indomethacin was ensured by verifying the absence of the metabolites in the extracts using the

method of comparative distribution ratios [72].Also paper chromatography was used to check for such metabolites.

These procedures implied grave potentials for error when applied to several samples not only because of the time of analysis but also the potentials of other co-administered drugs ,especially aspirin to cause interference with the fluorescence.

Analgesic mixtures and drug metabolites require _in situ_ separation prior to detection and quantification.The manipulations involved in colorimetry, spectrophotometry and fluorimetry led some analysts to explore gas chromatography for the analysis of aspirin and other NSAIDS.This system incorporates electronically based detectors viz: flame ionisation (FID),Nitrogen-sensitive ionisation (N-FID) and electron-capture (E-C).Ocassionally,thermal sensitivity detectors have been used.

2.1.4 GAS CHROMATOGRAPHY

Early applications of gas chromatography for the determination of aspirin used helium at a flow rate of 100ml/min. under 25 p.s.i pressure [94].The chromatograph consisted of a thermal condutivity detector coupled to a 0.60m x 0.6cm o.d. column of copper tubing packed with

chromosorb W (80-100 mesh) coated with 30% carbowax 20M as stationary phase.The instrument oven was set at 175° C and the injection port at 275°C.

Aspirin in tablets was isolated by sohxlet extraction with hot anhydrous methanol and methylated in the presence of boron trifluoride reagent.5μl was injected directly into the chromatograph.

Further developments allowed the simultaneous analysis of aspirin and detection of the hydrolysis products,acetic acid and salicylic acid [95].The chromatograph consisted of a flame ionisation detector coupled to a 2m x 0.6cm o.d. aluminium tubing packed with 200μ-size glass beads coated with 0.25% carbowax 20M and 4% isophthalic acid as the stationary phase.The analysis was performed isothermally at 125°C oven temperature and injection port temperature of 250°C.The mobile phase was helium at a flow rate of 60ml/min.This considerably improved the resultant chromatogram,the peak shape being symmetrical.

In another system incorporating a flame ionization detector and column of 100-120 mesh Gas Chrom Q coated with 5% OV17 in 1.8m x 0.6cm o.d. glass tube aspirin and salicylic acid after extraction from serum were separated and quantified simultaneously [96-97].This, however, involved the silylation of the extract by placing 40μl of

"Regisil" with the extract in the test tube and keeping for 1h in a water bath at 50°C.

Other NSAIDS have been quntified by gas chromatography.

DIFLUNISAL [98] and an internal standard extracted from acidified plasma using heptane were converted to methyl esters and separated on a column of 1% QF-1 on Gas Chrom.Q.

FLURBIPROFEN [99] was extracted from acidified plasma , purified by thin layer chromatography and converted to pentafluorobenzyl ester for gas chromatography on a stationary phase of 3% OV17 using an electron capture detector.A similar procedure was adopted for KETOPROFEN [100] in which the derivatives were methyl esters.Separation was by a column of 5% OV17 coated on "Embacel". In another study NAPROXEN methyl ester was resolved on a column of 3.85% S.E. 30 as the stationary phase.

INDOMETHACIN. Submicrogram quantities of indomethacin were measured using an electron-capture detector after separation of material on a column of 2% OV-1 coated onto acid-washed , silanised Chromosorb W. Prior derivatisation was performed using diazoethane in hexane and excess solvent removed by evaporation [101].

The low volatility and high polarity of analytes necessitate derivatisation of many NSAIDS before gas chromatographic separation.Also,thermolabile analytes would decompose at unfavourable column temperatures.

2.1.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Analgesic anti-inflammatory drugs present in biological fluids have been determined by liquid chromatography using U-V and fluorometric detector systems.

Simultaneous determination of analgesic drug mixtures, including aspirin and paracetamol was performed with a stationary column made of controlled-pore glass support.Detection was by ultraviolet absorption at 254nm [102].The mobile phase was composed of 85% acetic acid in chloroform.

Aspirin and salicylic acid have also been analysed on systems employing fluorescence detection [103]. The fluorescence of aspirin in ethanol-water 3:1 at pH 3.4 was sufficient to to determine 5μg/ml of the compound in serum.However,this procedure could not be applied to samples in which analytes have poor fluorescence.This was therefore not applicable to paracetamol singly or simultaneously with aspirin.

Finer separations have been achieved by column packings of various sizes and coatings.The column packing which has most widely been used for the separation and detection of anti-inflammatory analgesic drugs is the "reverse phase" packing ODS (octadecyl silane).This consists of the inert support coated with a stationary phase which is less polar than the stationary phase. The particle size used by most workers fall in the range 5-10μm diameter.These columns were particularly suitable for polar non-ionic compounds.With adequate pH conditions many of the analgesic anti-inflammatory drugs are analysable on these columns using appropriate solvent combinations.Extraction of the compounds from serum is nevertheless necessary and ensured the long life of the columns.

The microparticulate reversed-phase column was used for the simultaneous determination of aspirin and its metabolites in plasma [104,106].Quantification of aspirin,salicylic acid,salicyluric acid and gentisic acid was carried out by monitoring at 313nm. Addition of phthalic acid or ortho-methoxy-benzoic acid to the extraction solvent served as an internal standard, subsequent extraction of analytes being subject to minimal variability.A similar column was used by Lo and

Bye for the determination of aspirin and employing 3,4-dimethylbenzoic acid 10μg/ml in acetonitrile as internal standard [107].

These procedures yielded good chromatograms with peaks well separated.However,subsequent observations showed that the volume of sample injected and the solvent polarity caused peak broadening,ultimately leading to shoulders and doublets [108].This effect was stated to be one of the sources of deviation from linearity of calibration curves. Other sources of peak distortion identified subsequently were ferric ion and edetic acid that are often present in blood samples [109].

A subsequent report in which a column packed with "Hypersil" ODS 5μm particle size was used instead of 10μm and the extraction of aspirin and salicylic acid was conducted with buffer at pH 2.2,showed sharper peaks.The use of an injection loop also obviated the requirement for an internal standard without loss of reproducibility when applied to many samples [110].

PARACETAMOL was determined simultaneously with aspirin in pharmaceutical formulations using reversed-phase HPLC [108,111].However,for the determination of paracetamol and aspirin in serum a procedure devoid of interfering metabolite peaks is required.

Determination of paracetamol and its metabolites in plasma using "Hypersil2 ODS for separation after protein precipitation and direct injection gave well separated peaks on the chromatogram [112,113].

Simultaneous determination of paracetamol and salicylic acid in plasma was achieved on an ODS -Sil-x-1 reversed phase column [114].The extraction solvent mixture of chloroform/isopropanol 1:1 v/v containing 8-chlorotheophylline as the internal standard yielded extracts with negligible peak interference.The effect of intact aspirin,if present in the sample,would have necessitated a different procedure.

INDOMETHACIN in plasma was analysed on a column packed with C_{18} 37-50μm particle size [115].The plasma was adjusted to pH 5 with citrate buffer and 1ml of aqueous solution of flufenamic acid added as the internal standard.Indomethacin was extracted into ether,the extract evaporated to dryness and the residue redissolved in the mobile phase .Excellent peak separation was achieved.

Simultaneous determination of salicylic acid and indomethacin in serum using a reversed phase ODS 5μm alkyl-modified column packing [117] was sensitive at

0.5µg/ml aspirin (signal:noise ratio > 2).The serum was deproteinated with 0.3M perchloric acid and the compounds extracted into dichloromethane.The organic phase was transfered to another centrifuge tube and evaporated Good recoveries of the compounds were reported .The linearity of the method was sustained over a concentration range of indomethacin 0.1-2.5µg/ml and salicylic acid 1-25µg/ml.The study was applied to the serum of subjects continuously taking both aspirin and indomethacin.Different dosage application, in addition to single-dose study would require the development of a procedure particularly applicable to the design of the present study.

2.1.6. METHOD OF CHOICE IN THIS STUDY.

Several samples of blood are expected to be analysed in order to study the in vivo kinetics of aspirin when combined with one or more analgesic/anti-inflammatory drug(s).

It was stated (1.1.2) that the hydrolysis of aspirin is enhanced under elevated temperature.In case some intact aspirin is present in the blood a work-up procedure such as derivatisation,for gas chromatography constitutes a source of error in the measurement of aspirin concentration (2.1.4). Furthermore , simultaneous

derivatisation would impose a great demand on sample processing time without a distinct advantage,particularly of sensitivity.HPLC has the potential to meet the requirements of the study protocol for the purposes of this investigation.When coupled to U-V detection the procedure to be developed obviates the necessity for derivatisation of the drugs under investigation.HPLC was therefore explored as the the analytical method of choice in this study.

3. EXPERIMENTAL

3.1 SIMULTANEOUS DETERMINATION OF PARACETAMOL, ASPIRIN AND SALICYLIC ACID IN SERUM

3.1.1 INSTRUMENTATION AND ANALYTICAL CONDITIONS

Solvent was delivered at 1ml min by an Altex Hitachi 100-10 pump.The column was a 25cm x 4.5mm i.d. stainless steel tube packed with spherisorb 5μm O.D.S. and fitted with a Rheodyne injection system incorporating a 20μl loop. The eluent was monitored at 234nm with a Hitachi 100-10 variable wavelength U-V detector. Detector sensitivity was 0.2 aufs.The chart speed was 2mm min^{-1}.

3.1.2 MATERIALS

Salicylic acid and acetylsalicylic acid (B.P. grades) were obtained from Thornton and Ross and paracetamol from Dista.All solvents were HPLC grade (Rathburn Chemicals).All other chemicals were analytical reagent grade. Blood samples were obtained from healthy volunteers after oral administration of aspirin and paracetamol.

3.1.3 DEVELOPMENT OF THE MOBILE PHASE

Various solvent combinations were tested for appropriate separation characteristics on reverse-phase O.D.S.

column.Table I shows the solvents investigated and references which were starting points for the development of a suitable eluent.When good peak resolution and sensitivity high enough to detect the compounds in serum (signal to noise ratio > 2) was achieved,the eluent was chosen.This was water:acetonitrile:methanol (4:2:1 v/v) adjusted to pH 3.0 with orthophosphoric acid.

3.1.4 PREPARATION OF STANDARD AND CALIBRATTION CURVE

Blood (50ml) was withdrawn from the antecubital vein and transfered into plastic tubes.It was allowed to clot. After centrifugation serum was decanted ,in portions into 10ml glass tubes , labelled and frozen at $-20^{o}C$.A portion was allowed to thaw before use.

Separate and combined stock solutions of paracetamol (100μg/ml) aspirin (100μg/ml) and salicylic acid (200μg/ml) in methanol were prepared and kept in a refrigerator.Aliquots of each stock were diluted with distilled water in washed volumetric flasks (25ml) to give solutions containing 4.0,8.0,12.0,15.0,20.0 and 30.0μg/ml of paracetamol,aspirin and salicylic acid standard solutions.

Stock solution of concentration 5mg/ml (2ml,4ml,6ml,8ml) diluted to 25ml to give 400,800μg/ml and 1.2,1.6mg/ml

standard solutions respectively were used to prepare serum containing aspirin and paracetamol separate and combined as follows:

50μl of standard (400μg/ml) in 5ml serum = 4μg/ml

50μl of standard (800μg/ml) in 5ml serum = 8μg/ml

50μl of standard (1200μg/ml) in 5ml serum = 12μg/ml

50μl of standard (1200μg/ml) in 4ml serum = 15μg/ml

50μl of standard (1600μg/ml) in 4ml serum = 20μg/ml

50μl of standard (1200μg/ml) in 2ml serum = 30μg/ml

For salicylic acid,stock solution of 10mg was used.These serum standards were frozen at -20°C and used for calibration procedures as well as between assay evaluations as necessary.

3.1.4.1 EXTRACTION

Diethylether and chloroform were evaluated for selective extraction of the compounds from serum (aqueous phase) into the organic phase.Chloroform : acetonitrile 3:2 was employed in the extraction , having been found to give the best extraction.

Serum (0.5ml) was acidified with 0.1M hydrochloric acid and extracted by shaking with 2ml aliquots of the extraction solvent in three successive operations and the

organic layer combined.The extract was evaporated to dryness under a stream of nitrogen.The residue was redissolved in 100µl of the eluent and 20µl samples injected onto the column.

3.1.4.2 CALIBRATION CURVES

Serum (0.5ml) containing paracetamol,aspirin and salicylic acid in concentrations of 4,8,12,15,20 and 30µg/ml as described in 3.1.3 were extracted and chromatographed under the conditions described above.The mean peak areas for 10 determinations at each concentration were calculated and plotted against the corresponding concentrations for the individual drugs present in the serum.

3.1.4.3.1 LIMIT OF DETECTION

A solution containing aspirin (0.2µg/ml) was diluted serially and each dilution monitored at 0.02 aufs until the signal to noise ratio was less than 2.The procedure was repeated for each compound singly.

3.1.4.3.2 PERCENTAGE RECOVERY was calculated by the equation :

$$\frac{\text{mean peak area of extracted drug}}{\text{mean peak area of standard solution}} \times 100\%$$

for each concentration used in the calibration curves.

3.2 SEPARATION AND DETECTION OF OTHER ANTI-INFLAMMATORY DRUGS

3.2.1 INSTRUMENT AND HPLC CONDITIONS

A Spectra Physics 8700 XR extended range LC pump was used to deliver solvent at 1ml min^{-1}.The column was 25cm x 4.5 mm i.d. packed with spherisorb 5μm O.D.S. fitted with a Negretti and Zambra injection system and a 20μl loop.The eluent was monitored with a Pye-Unicam P.U. 4020 variable wavelength U-V detector set at 248nm and sensitivity scale of 1.28 aufs .The chart speed was 3mm min^{-1}.

3.2.2 MATERIALS

Salicylic acid and acetyl salicylic acid ,as previously described were obtained from Thornton and Ross.Indomethacin and diflunisal were from Merck Sharp and Dohme,ibuprofen from Boots,ketoprofen from Synthex and Mefenamic acid from Parke Davis.Blood samples were obtained from healthy volunteers and hospitalised patients after oral administration of individual drugs.

3.2.3 THE MOBILE PHASE

Various modifications of the elution solvent for the paracetamol-aspirin study were examined for the

separation of some commonly used anti-inflammatory drugs (Table II).Separation was finally achieved with an eluent of water-orthophosphoric acid pH 3.0: acetonitrile : methanol in the ratio of 52:35:13.

3.2.4 EXTRACTION

Various solvent combinations were tested for selective extraction of aspirin,salicylic acid,ketoprofen,naproxen fenoprofen,flurbiprofen,diflunisal,ibuprofen,indomethacin, and mefenamic acid from serum into the organic phase.Two solvent systems adaptable for specific analyses were compared and minor modifications applied.

Serum (0.5ml) was acidified with molar hydrochloric acid (0.1ml) and extracted by shaking with 2ml aliquots of the extraction solvent consisting of either chloroform:acetonitrile 60:40 or hexane:ether 50:50. Aliquots (three) from each sample were combined in a conical glass tube and evaporated to dryness under a stream of nitrogen.The residue was redissolved in 100μl of methanol.20μl was injected onto the column.

3.3 SIMULTANEOUS DETERMINATION OF SALICYLIC ACID AND INDOMETHACIN

3.3.1 INSTRUMENTS AND HPLC CONDITIONS were the same as in 3.2.1 except for the use of gradient elution.

TABLE I: Elution Solvents Investigated For Separation of paracetamol,aspirin and salicylic acid (3.1.3)

Solvent	Ref.
2-propanol 30ml:970ml phosphoric acid 0.2%	114
2-propanol 50ml:950ml phosphoric acid 0.2%	
Orthophosphoric acid (0.15%,0.25%, 0.3%):Methanol 20:80,30:70	
Phosphoric acid 0.05%:acetonitrile 30:70,20:80 25:75.	105
Phosphoric acid 0.15%:acetonitrile: methanol 3:2:1,2:2:1,4:2:1	This work
Potassium dihydrogen phosphate 0.01M in water 20% methanol,phosphoric acid (85%) 1ml in 1L.	111

TABLE II: Development of Elution Solvent for Ten anti-inflammatory drugs (3.2)

Solvent	Ref.
Potassium dihydrogen phosphate in water (0.01M):methanol 80:20,70:30, 75:25,pH adjusted to 2.3 with phosphoric acid .	111
Potassium dihydrogen phosphate in water (0.05M);perchloric acid 0.08M: n-butanol:methanol 60:25:15,70:20:10, 65:20:15,50:30:20 each mixture adjusted to pH 2.5 with phosphoric acid.	116
Phosphoric acid (0.15%):acetonitrile: methanol 60:20:10,65:25:10,65:20:15, 50:35:15,52:35:13	This work.
Potassium dihydrogen phosphate (0.05M) in water : methanol; 60:40,70:30 each adjusted to pH 3.5 with phosphoric acid	117.

The LC pump was programmed to deliver solvent at a rate of 1ml/min with simultaneous change of the solvent composition from water-orthophosphoric acid (pH2.5): acetonitrile:methanol (52:35:13) to water-orthophosphoric acid (pH2.5):acetonitrile:methanol (35:52:13) in 15 minutes.Table III shows the solvent development sequence.

The u-v detector was set at a sensitivity scale of 1.28 aufs for salicylic acid peaks and 0.16 aufs for indomethacin peaks.

3.3.2 EXTRACTION

Aliquots (0.5ml) of serum were diluted with 1ml of distilled water and acidified with 0.15ml of molar hydrochloric acid.The samples were extracted three times with 2ml aliquots of ether:hexane mixture 1:1 v/v.The mixture was centrifuged at 2500 rpm for 5min.This procedure was performed three times for each sample and the organic layer aspirited and combined.The extract was evaporated to dryness under a stream of dry nitrogen and the resultant residue redissolved in 50μl of methanol. 20μl was injected unto the column.

TABLE III Simultaneous Determination of Salicylic Acid and Indomethacin (3.3): Development of the Mobile Phase

Solvent	Ref
Potassium dihydrogen phosphate (0.01M) in water : methanol 60:40, 75:25 each adjusted to pH 2.8 with phosphoric acid solution 15%	117
Phosphoric acid (0.15%):acetonitrile: methanol 52:35:13; 50:30:20	This work.
Phosphoric acid (0.15%):acetonitrile: methanol 52:35:13 change over 15 min to 35:52:13; hold 52:35:13 for 5 min then change to 35:52:13 in 10 min.	This work.

3.3.3 CALIBRATION CURVE

Methanol solutions of indomethacin (25μg/ml) and salicylic acid 500μg/ml were diluted serially with water to give decreasing concentrations of indomethacin and salicylic acid.Appropriate solutions were added to 0.5 aliquots of serum to give indomethacin concentrations : 0.25, 0.5, 2.0, 2.5 and 3.0μg/ml. Salicylic acid was 5, 10, 20, 40, 50 and 60μg/ml.

These samples were subsequently extracted and chromatographed by gradient elution as described above. Quantification of the concentrations was by peak area measurements. The peak areas were plotted against the corresponding concentrations for each compound singly and in admixture.

The procedure over the period of study was evaluated as for the paracetamol-aspirin study (3.1.3.3). The limit of detection,coefficient of variation and percentage recovery were calculated after final adjustments suitable for the actual subject samples.

4. RESULTS OF CHROMATOGRAPHY

4.1 SIMULTANEOUS DETERMINATION OF PARACETAMOL, ASPIRIN AND SALICYLIC ACID

4.1.1 MOBILE PHASE AND COLUMN PERFORMANCE

The solvent mixture containing water-acetonitrile-methanol (4:2:1 v/v) adjusted to pH 3.0 with orthophosphric acid gave reliable separation of paracetamol,aspirin and salicylic acid .The retention times were 3.5,6.5 and 10 mins respectively.The elution characteristics were sustained after several injections of the standard solutions.Using methanol as the reference standard the capacicity ratio for the elution system was 0.7,2.3 and 4.0 respectively.

4.1.2 EXTRACTION

Chloroform-acetonitrile (60:40) as the extraction solvent yielded clean extracts with between 97% and 100% recovery for each drug.No change in peak shape was observed up to 50 injections of extracts from serum.Figure 5 shows the chromatogram of (a) a blank serum extract, (b) the chromatogram of serum spiked with paracetamol, aspirin and salicylic acid ; (c) that of an individual taking aspirin and paracetamol.

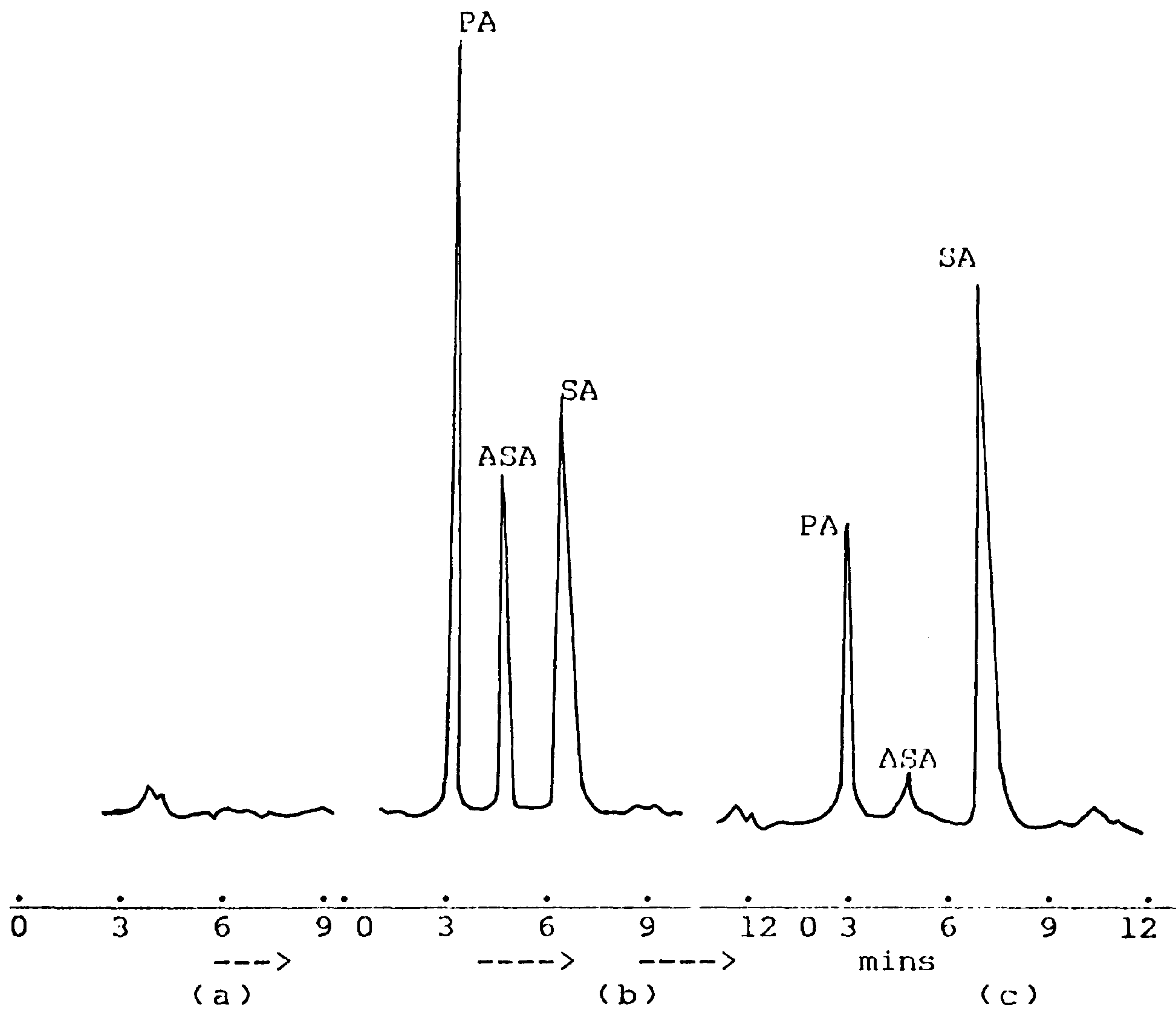


Figure 5: Chromatogram for simultaneous analysis of paracetamol (PA), aspirin (ASA) and salicylic acid (SA)
(a) blank serum (b) spiked serum (c) subject sample

4.1.3 CALIBRATION CURVES

Straight-line calibration graphs were obtained for paracetamol aspirin and salicylic acid when each was determined singly and when all the three compounds were determined simultaneously.Tables IV-IX show the concentrations,the variations in peak heights/peak areas and the regression lines with mean signals of 10 determinations for each concentration point.The correlation coefficients were 0.998-1.00.Figures 6-9 show the representative calibration curves for salicylic acid for the occasions stated on the legends.

The LIMITS OF DETECTION were 2ng on-column for salicylic acid and aspirin and lng for paracetamol.

Concentration (μgml) / Anal.No	Peak height (mm): Single Assay					
	4	8	12	15	20	30
1.	32.4	67.6	100.4	117.8	166.6	243.8
2.	31.6	67.8	100.6	117.4	165.8	242.4
3.	32.2	66.6	99.8	118.0	166.2	243.4
4.	31.8	67.4	100.2	118.2	165.2	242.6
5.	31.0	67.2	100.4	118.6	166.0	242.4
6.	32.8	66.8	99.8	117.8	166.6	243.6
7.	31.4	67.4	99.6	118.2	166.2	242.8
8	31.6	67.2	100.2	118.6	165.4	243.2
9	31.8	66.6	100.4	117.4	166.4	242.6
10	32.0	67.4	100.6	117.6	166.2	243.4
MEANS	31.8	67.2	100.2	117.8	166.2	243.0
S.E.M	0.17	0.14	0.12	0.17	0.17	0.17
REGRESSION LINE	Peak Height=8.11 x Concentration + 0.79					
Correlation	0.999					

TABLE IV (a) Variations in the peak heights for paracetamol single assay concentrations used for construction of calibration curves. S.E.M. = standard error of mean.

Concentration (μg/ml) / Anal No	Peak height (mm): Simultaneous Assay					
	4	8	12	15	20	30
1.	32.2	67.7	100.0	117.8	166.0	243.0
2.	32.0	67.8	99.4	118.2	165.2	242.2
3.	31.6	67.2	100.4	117.4	165.8	243.0
4.	31.0	67.2	100.0	117.6	165.0	242.0
5.	32.6	66.6	99.8	117.0	165.6	241.8
6.	33.2	67.0	99.2	118.0	166.4	243.4
7.	31.4	66.4	989.6	118.2	165.8	242.4
8.	32.0	67.8	99.0	117.0	165.4	242.6
9.	31.8	67.0	100.2	118.0	166.2	242.4
10	31.4	67.4	100.4	117.6	165.8	243.0
MEANS	31.9	67.2	99.8	117.7	165.7	242.6
S.E.M.	0.20	0.15	0.16	0.15	0.14	0.17
REGRESSION LINE	Peak Height=8.09xConcentration + 0.87					
Correlation	0.999					

TABLE IV (b) Variations in the peak heights for paracetamol upon simultaneous assay with aspirin and salicylic acid used for construction of calibration curves. S.E.M. = standard error of mean.

Concentration (μg/ml) / Anal No.	Peak Area (mm^2): Single assay					
	4	8	12	15	20	30
1.	15.0	25.4	40.2	48.6	69.0	99.2
2.	14.3	25.6	41.4	48.4	68.4	98.8
3.	13.4	25.2	39.6	49.2	68.4	97.6
4.	13.9	25.6	40.0	48.6	67.4	98.8
5.	13.6	25.2	40.4	49.6	67.8	99.4
6.	14.2	24.8	40.2	49.4	68.2	97.4
7.	14.4	24.6	41.0	48.8	68.8	98.6
8.	13.6	26.0	40.4	47.8	67.6	98.4
9.	14.8	25.2	39.8	49.2	67.8	97.6
10	14.2	25.0	40.2	48.8	68.6	99.0
MEANS	14.0	25.4	40.2	49.2	68.4	99.2
S.E.M	0.17	0.16	0.17	0.18	0.18	0.19
REGRESSION LINE	Peak area=3.3 x Concentration + 0.043					
Correlation	0.997					

TABLE V (a) Variations in peak areas for standard aspirin in elution solvent and concentrations determined singly and used for construction of calibration curves.

S.E.M = Standard error of mean.

Anal. No. \ Concentration (μg/ml)	Peak area (mm^2): Simultaneous assay					
	4	8	12	15	20	30
1.	14.2	25.4	40.6	49.4	68.6	98.8
2.	13.8	25.8	39.8	48.6	69.2	99.6
3.	14.2	25.4	40.2	50.2	69.0	99.6
4.	12.6	25.0	40.8	49.2	67.8	98.8
5.	13.4	25.4	40.6	49.0	68.4	100.0
6.	13.2	24.2	40.2	48.6	69.2	99.8
7.	14.0	25.8	40.8	49.4	68.8	99.0
8.	13.6	25.1	40.2	48.8	69.2	99.4
9.	14.4	25.6	40.4	49.6	68.6	98.0
10.	14.0	25.4	40.6	49.2	69.2	99.2
MEANS	14.2	25.3	40.4	49.4	68.8	99.6
S.E.M.	0.18	0.16	0.12	0.17	0.16	0.18
REGRESSION LINE	Peak area=3.34 x Concentration + 0.05					
Correlation	0.998					

TABLE V (b): Variations in the peak areas for standard aspirin in elution solvent and concentrations determined simultaneously with paracetamol and salicylic and acid used for construction of calibration curves.S.E.M. = Standard error of mean.

Anal.No \ Concentration (μg/ml)	Peak area (mm^2):Single assay					
	8	16	24	30	40	60
1.	17.5	37.5	55.8	68.4	90.4	138.4
2.	16.8	36.8	56.4	66.6	89.6	138.6
3.	17.6	37.4	55.6	67.2	91.2	138.2
4.	16.2	37.6	56.2	67.8	90.8	137.4
5.	16.5	36.5	55.8	67.6	90.6	137.8
6.	17.2	37.4	56.2	66.8	89.8	137.2
7.	15.8	36.8	56.4	67.0	89.6	138.6
8.	17.0	36.5	56.8	66.6	90.4	139.0
9.	16.6	37.2	57.2	67.4	91.0	138.2
10.	17.2	37.6	56.4	66.8	90.8	137.6
MEANS	16.8	37.1	56.3	67.2	90.4	138.1
S.E.M.	0.19	0.15	0.16	0.19	0.19	0.19
REGRESSION LINE	Peak area = 2.31 x Concentration - 0.76					
Correlation	1.00					

TABLE VI (a): Variations in the peak areas for standard salicylic acid in elution solvent and concetrations determined singly and used in the construction of calibration curve.

Concentration (µg/ml) \ Anal.no.	Peak area (mm^2): Simultaneous assay					
	8	16	24	30	40	60
1.	17.6	38.2	56.4	66.2	90.8	138.6
2.	17.5	37.8	56.8	67.8	91.2	137.8
3.	16.2	36.8	55.6	66.8	91.4	138.2
4.	15.8	37.6	57.2	67.4	90.6	137.4
5.	16.4	38.4	56.8	68.0	91.0	138.8
6.	16.8	37.6	57.0	67.8	92.0	138.4
7.	17.0	36.8	56.4	66.8	90.8	138.2
8.	16.8	37.2	57.4	67.2	91.4	139.0
9.	17.2	37.8	55.8	67.6	92.2	137.6
10.	16.6	36.8	55.8	66.8	91.6	138.6
MEANS	17.0	37.5	56.6	67.2	91.3	138.3
S.E.M	0.19	0.19	0.19	0.19	0.18	0.18
REGRESSION LINE	Peak area = 2.31 x Concentration - 0.49					
Correlation	1.00					

TABLE VI (b): Variations in the peak areas for standard salicylic acid in elution solvent and concentrations determined simultaneously with paracetamol and aspirin and used in the construction of calibration curve.

Concentration (µg/ml) / Anal.No	Peak heght (mm):Single Assay					
	4	8	12	15	20	30
1.	31.8	63.8	95.8	117.8	159.4	238.8
2.	32.6	63.6	95.6	117.6	159.8	239.2
3.	32.0	64.0	96.2	117.8	160.2	239.8
4.	31.8	63.8	95.6	118.2	159.8	239.0
5.	32.2	65.0	95.8	118.6	160.6	239.6
6.	33.4	64.6	96.0	118.0	160.2	239.8
7.	32.6	63.8	96.4	118.8	159.6	240.0
8.	31.8	64.0	95.8	118.4	159.8	240.2
9.	32.0	63.6	95.0	117.8	160.2	239.0
10.	31.8	63.8	96.0	119.0	159.6	239.4
MEANS	32.2	64.0	95.8	118.2	159.8	239.5
S.E.M.	0.17	0.17	0.12	0.16	0.13	0.18
REGRESSION LINE	Peak height=7.98 x Concentration - 0.53					
Correlation	1.00					

TABLE VII(a) : Variations in the paracetamol concentrations in spiked serum extracted and determined singly for construction of calibration curves.

Concentration (µg/ml) \ Anal.No.	Peak height (mm^2): Simultaneous assay					
	4	8	12	15	20	30
1.	32.8	63.6	95.6	118.4	159.2	238.8
2.	33.2	63.8	96.4	119.0	160.2	239.2
3.	31.8	64.6	95.8	118.6	159.8	239.8
4.	31.6	63.6	96.0	117.6	160.0	240.2
5.	32.6	64.0	96.4	117.8	159.6	239.4
6.	32.4	64.6	96.6	117.4	160.2	239.0
7.	31.8	65.0	95.4	118.2	159.8	238.8
8.	32.4	64.4	96.0	117.6	160.4	239.4
9.	32.2	63.8	95.6	118.2	159.6	239.8
10.	33.0	64.4	95.8	118.0	159.2	239.2
MEANS	32.4	64.0	96.0	118.0	159.8	239.4
S.E.M.	0.18	0.17	0.13	0.17	0.14	0.15
REGRESSION LINE	Peak height = 7.98 x Concentratio + 0.11					
Correlation	1.00					

TABLE VII (b) : Variations in paracetamol concentrations in spiked serum extracted and determined simultaneously with aspirin and salicylic acid for the construction of calibration curve.

Concentration ($\mu g/ml$) \ Anal.No.	Peak area (mm^2): Single assay					
	4	8	12	15	20	30
1.	14.6	25.2	39.8	50.2	67.6	98.2
2.	13.4	24.8	39.6	50.4	68.4	99.0
3.	13.6	25.0	40.0	49.8	68.2	99.6
4.	14.0	25.4	39.4	50.6	69.0	99.0
5.	14.2	24.6	40.6	49.8	68.8	98.6
6.	13.2	24.0	40.4	49.6	68.0	99.6
7.	14.0	24.4	39.6	50.0	68.4	99.2
8.	13.6	25.2	40.0	50.6	68.0	98.8
9.	14.4	24.8	39.8	50.0	68.2	99.4
10.	13.8	24.6	40.2	50.4	68.4	99.4
MEANS	13.8	24.6	40.2	50.4	68.4	99.4
S.E.M.	0.15	0.14	0.13	0.12	0.13	0.16
REGRESSION LINE	Peak area = 3.34 x Concentration - 11					
Correlation	0.999					

TABLE VIII (a): Variations in the peak areas for the concentrations of aspirin in spiked serum extracted and determined singly for the construction of calibration curve.

Concentration (μg/ml) / Anal.No.	Peak area(mm) Simultaneous assay					
	4	8	12	15	20	30
1.	14.8	25.6	40.2	49.8	69.4	99.6
2.	14.2	25.0	39.4	49.0	68.6	99.8
3.	14.0	24.6	38.8	48.8	69.0	100.4
4.	14.2	24.8	39.6	49.2	68.4	100.2
5.	13.2	25.2	40.0	50.0	69.6	100.0
6.	13.8	24.6	39.8	50.2	69.2	99.8
7.	13.2	25.0	40.2	49.4	68.6	99.6
8.	13.6	25.8	39.8	50.0	69.0	99.8
9.	14.6	25.4	39.0	49.0	69.4	99.4
10.	14.0	24.6	40.0	49.6	68.4	99.2
MEANS	14.0	24.9	39.7	49.5	69.0	99.8
S.E.M.	0.18	0.13	0.16	0.16	0.15	0.12
REGRESSION LINE	Peak area = 0.37 x Concentration - 0.52					
Correlation	0.999					

TABLE VIII (b) : Variations in the peak areas for the concentrations of aspirin in spiked serum extracted and determined simultaneously with paracetamol and salicylic acid for the construction of calibration curves.

Concentration (μg/ml) \ Anal.No.	Peak area (mm): Single assay					
	8	16	24	30	40	60
1.	16.4	38.9	55.8	66.4	89.2	137.2
2.	17.0	38.2	56.6	67.0	89.6	137.0
3.	16.0	38.8	56.2	66.8	88.4	137.0
4.	15.8	39.0	55.8	66.2	89.0	136.8
5.	16.6	38.2	56.8	66.6	89.4	137.0
6.	16.8	37.8	56.2	66.4	90.0	136.8
7.	15.6	38.6	55.6	66.2	89.4	136.6
8.	16.4	39.0	56.8	67.8	88.2	137.0
9.	17.2	39.0	56.6	67.6	89.0	137.4
10.	16.8	38.2	56.6	66.8	89.8	137.8
MEANS	16.5	38.6	56.4	66.8	89.2	137.0
S.E.M.	0.18	0.15	0.15	0.18	0.19	0.12
REGRESSION LINE	Peak area = 2.27 x Concentration + 0.05					
Correlation	0.999.					

TABLE IX(a): Variations in the peak areas for the concentrations of salicylic acid in spiked serum extracted, determined and used for the construction of calibration curve. S.E.M. = standard error of mean.

Concentration (µg/ml) \ Anal.No.	Peak Area (mm): Simultaneous assay					
	8	16	24	30	40	60
1.	16.8	38.4	56.6	66.6	89.4	137.4
2.	17.0	39.4	55.8	66.0	89.6	137.6
3.	16.0	38.8	56.2	66.8	88.6	137.2
4.	16.6	39.8	56.8	67.0	89.4	137.6
5.	16.2	39.4	56.0	66.0	89.2	137.8
6.	15.4	38.8	55.6	66.4	89.6	138.2
7.	17.2	39.2	56.6	67.4	90.0	136.6
8.	16.0	38.6	56.2	66.8	88.6	137.8
9.	16.8	38.2	55.8	66.6	90.0	137.0
10.	16.2	39.4	57.2	66.4	90.0	137.8
MEANS	16.4	39.0	56.3	66.6	89.4	137.2
S.E.M.	0.18	0.17	0.17	0.14	0.17	0.16
REGRESSION LINE	Peak area = 2.27 x Concentration + 0.00					
Correlation	0.999					

TABLE IX (b): Variations in the peak areas for the concentrations of salicylic acid in spiked serum extracted, determined and used for the construction of calibration curve. S.E.M. = standard error of mean.

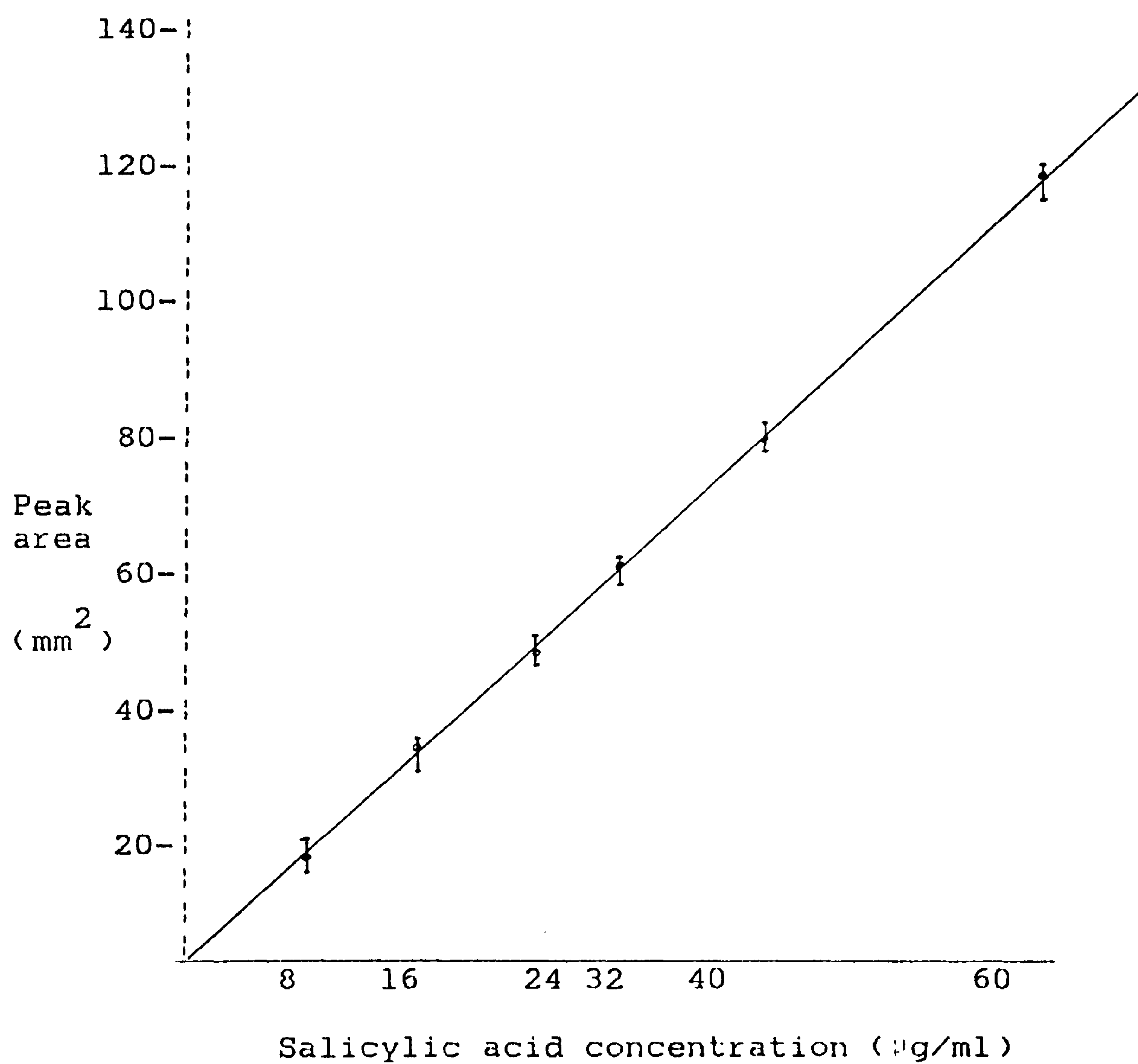


Figure 6 : Calibration curve of standard solution of salicylic acid in eluent determined singly

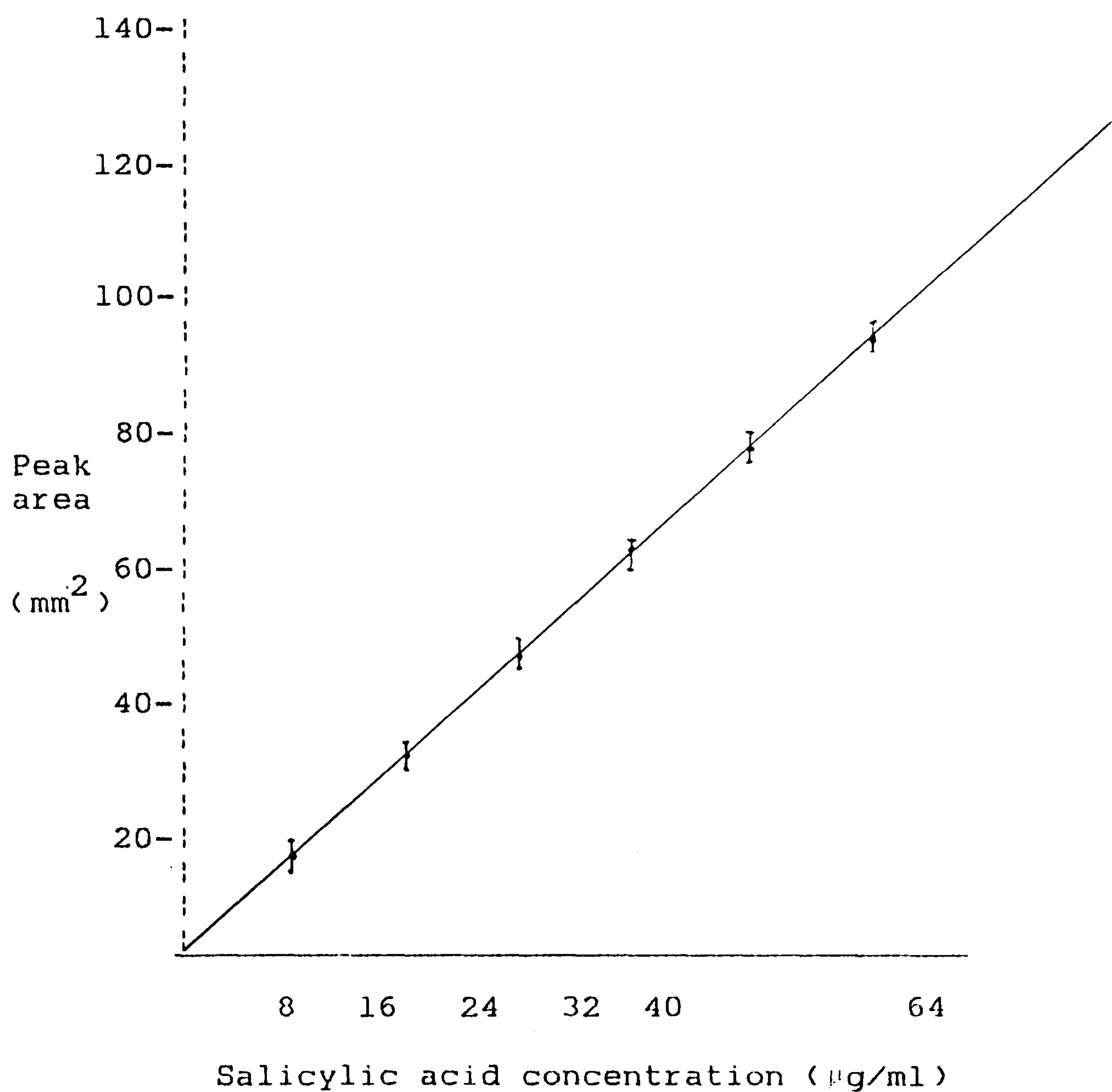


Figure 7 : Calibration curve of standard solution of salicylic acid in eluent determined simultaneously with paracetamol and aspirin.

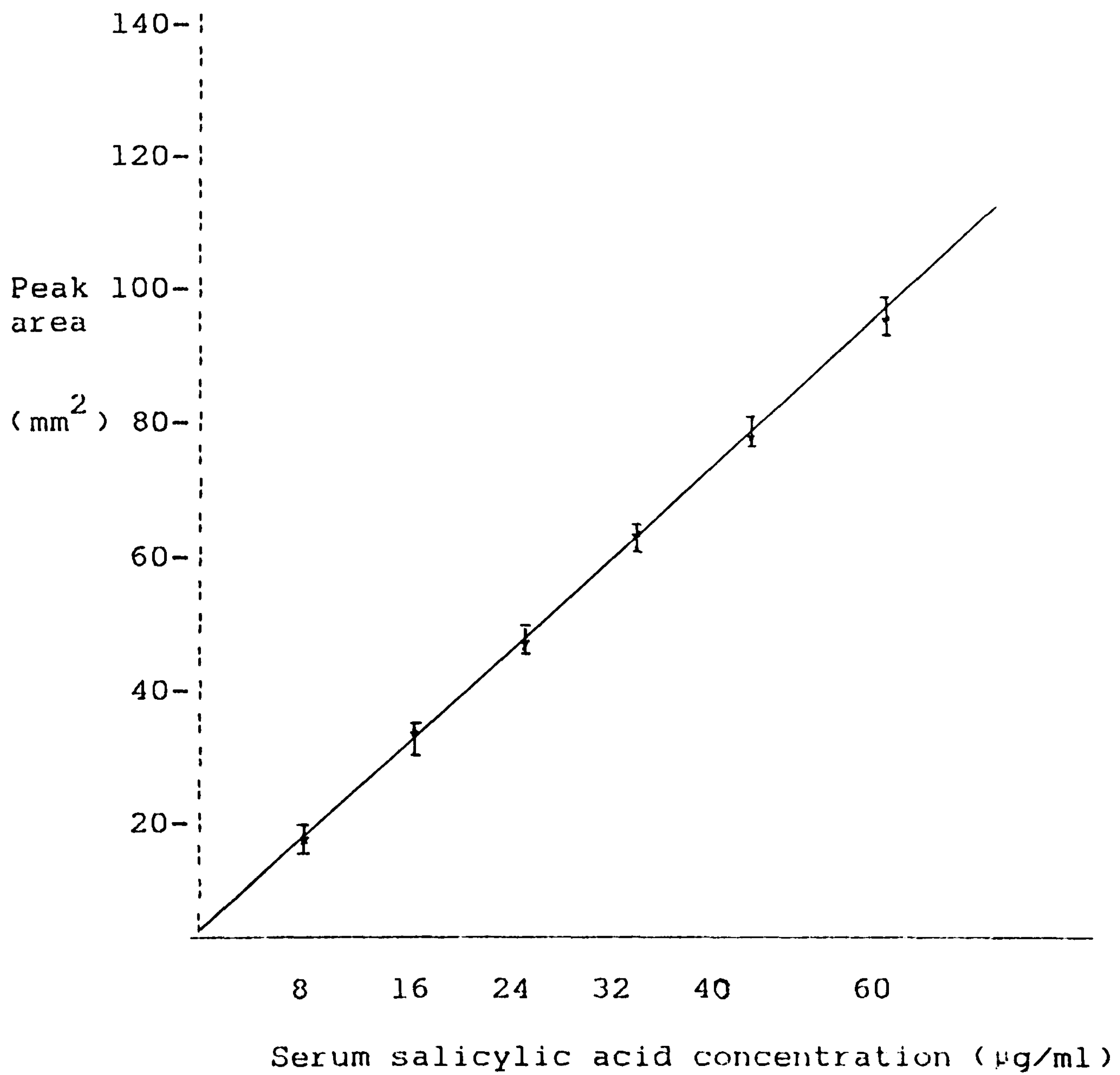


Figure 8 : Calibration curve for serum salicylic acid concentrations determined singly.

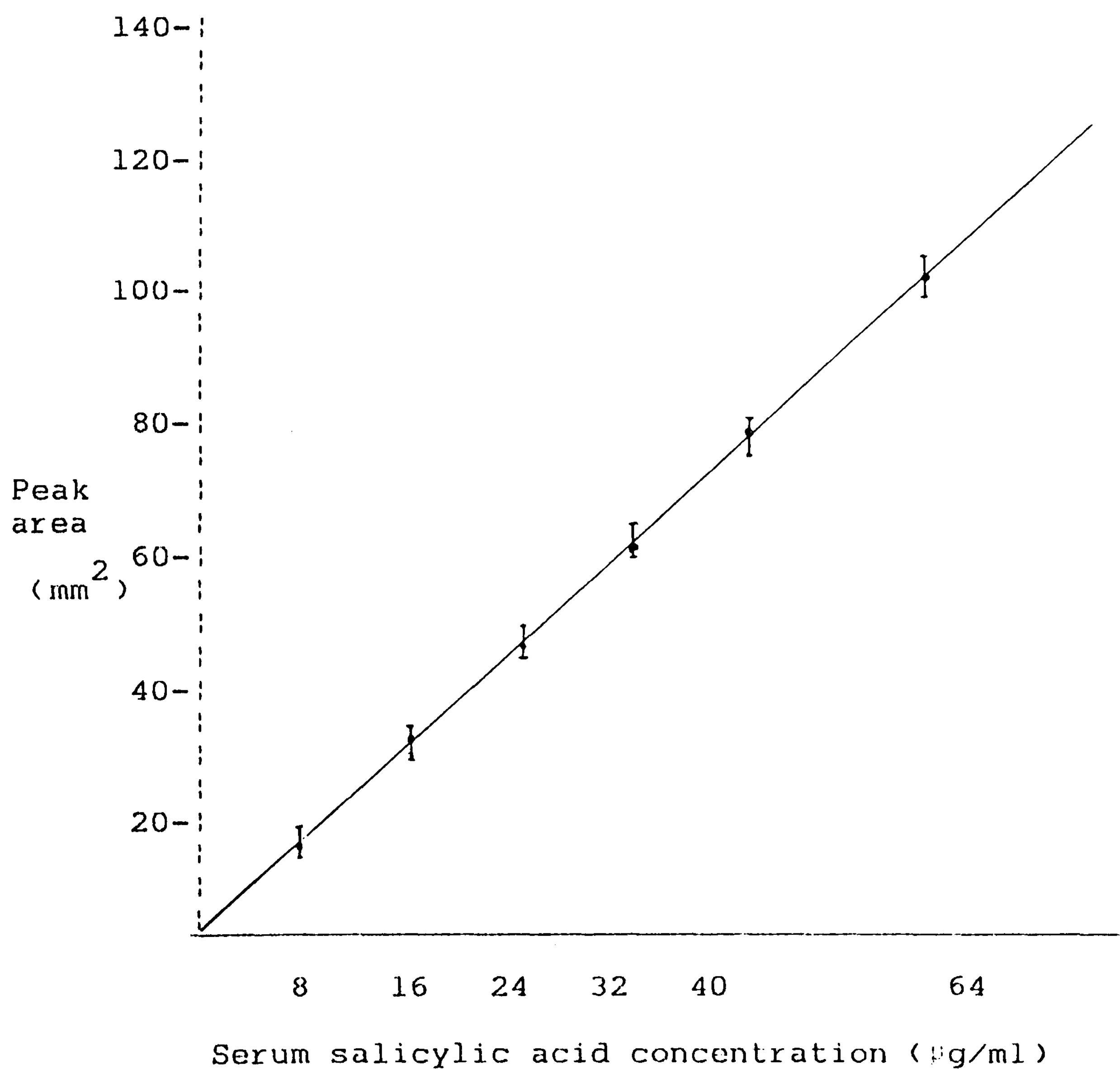


Figure 9 : Calibration curve for serum salicylic acid concentration determined simultaneously with aspirin and paracetamol

4.2 OTHER ANTI-INFLAMMATORY DRUGS

Separation of the ten most-commonly used anti-inflammatories was achieved with an eluent consisting of water-orthophosphoric acid : acetonitrile : methanol (52:35:13).

EXTRACTION with chloroform : acetonitrile 60:40 resulted in a chromatogram of drugs devoid of interfering endogenous compounds.Figure 10 shows a chromatogram of extract from serum spiked with a solution containing the drugs shown in table X. Also included are the minimum detectable concentrations of the compounds on column. It was observed that better sensitivity was obtained for indomethacin using hexane : ether 1:1 as the extraction solvent.

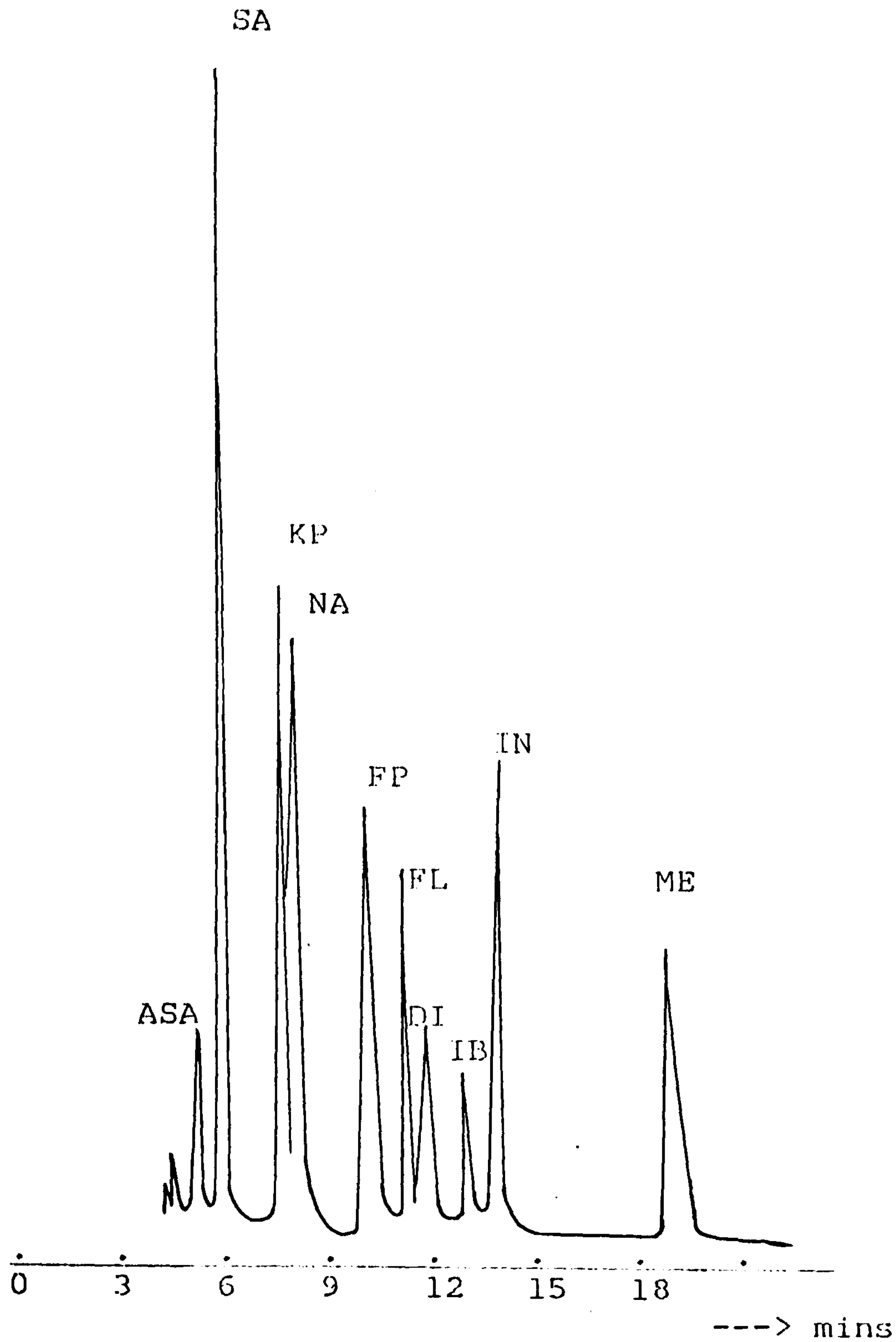


Figure 10 : Chromatogram of serum spiked with 10 anti-inflammatory drugs.

Symbols :

ASA : Aspirin
SA : Salicylic acid
KP : Ketoprofen
NA : Naproxen
FP : Fenoprofen
FL : Flurbiprofen
DI : Diflunisal
IB : Ibuprofen
IN : Indomethacin
ME : Mefenamic acid

Code	Drug	Minimum detectable level x 10 ng at 250nm	on column (ng)	Recovery (%) Chloroform / acetonitrile	Hexane / Ether
ASA	Aspirin		5	97	70
SA	Salicylic acid		4	97	70
Kp	Ketoprofen		4	100	70
NA	Naproxen		4	100	70
FL	Flurbiprofen		1	95	60
D1	Diflunisal		2	100	70
IB	Ibuprofen		50	95	60
IN	Indomethacin		2	70	80
ME	Mefenamic acid		20	70	40

TABLE X : Chromatogram of ten commonly used anti-inflammatory drugs extracted from serum and determined at 250nm wavelength and the estimate of their limits of detection.

4.3 SIMULTANEOUS DETERMINATION OF ASPIRIN AND INDOMETHACIN

4.3.1 MOBILE PHASE AND COLUMN PERFORMANCE

The gradient elution of salicylic acid and indomethacin with a solvent composition of aqueous phosphate buffer 52% : acetonitrile 35% : methanol 13% changed over 15mins as stated in 3.3.1 improved the sensitivity of both compounds whilst maintaining elution time of 15 mins for the chromatogram. The elution times for salicylic acid and indomethacin were 5 mins and 15 mins respectively.

4.3.2 EXTRACTION

The extraction of salicylic acid and indomethacin using ether : hexane 1:1 yielded extracts without interfering endogenous compounds or metabolites on the chromatogram. Approximately 85% recovery of salicylic acid and indomethacin was obtained within the observed concentration range in the subjects' samples.Figure 11 shows the chromatogram of (a) blank serum extract (b) extract of spiked serum and (c)extract of serum from a subject after taking aspirin and indomethacin.

4.3.3 CALIBRATION CURVE

Straight-line calibration graphs were obtained for salicylic acid and indomethacin.Tables XI - XII show the responses for concentrations of standard and extracted salicylic acid determined singly and simultaneously by gradient elution.Means of ten determinations were used for the regression analyses as shown by the equations at the bottom of the tables.Calibration curves for salicylic acid are shown in figures 12 - 13 . A similar procedure was adopted for the calibration process for standard indomethacin solutions and indomethacin extracted from serum.Tables XIII - XIV show the responses for corresponding concentrations of indomethacin and the regression lines. Figure 14 is a calibration curve shown for indomethacin for the occassion stated on the legend.

The LIMITS OF DETECTION for salicylic acid and indomethacin on column were 8ng and 4ng respectively.

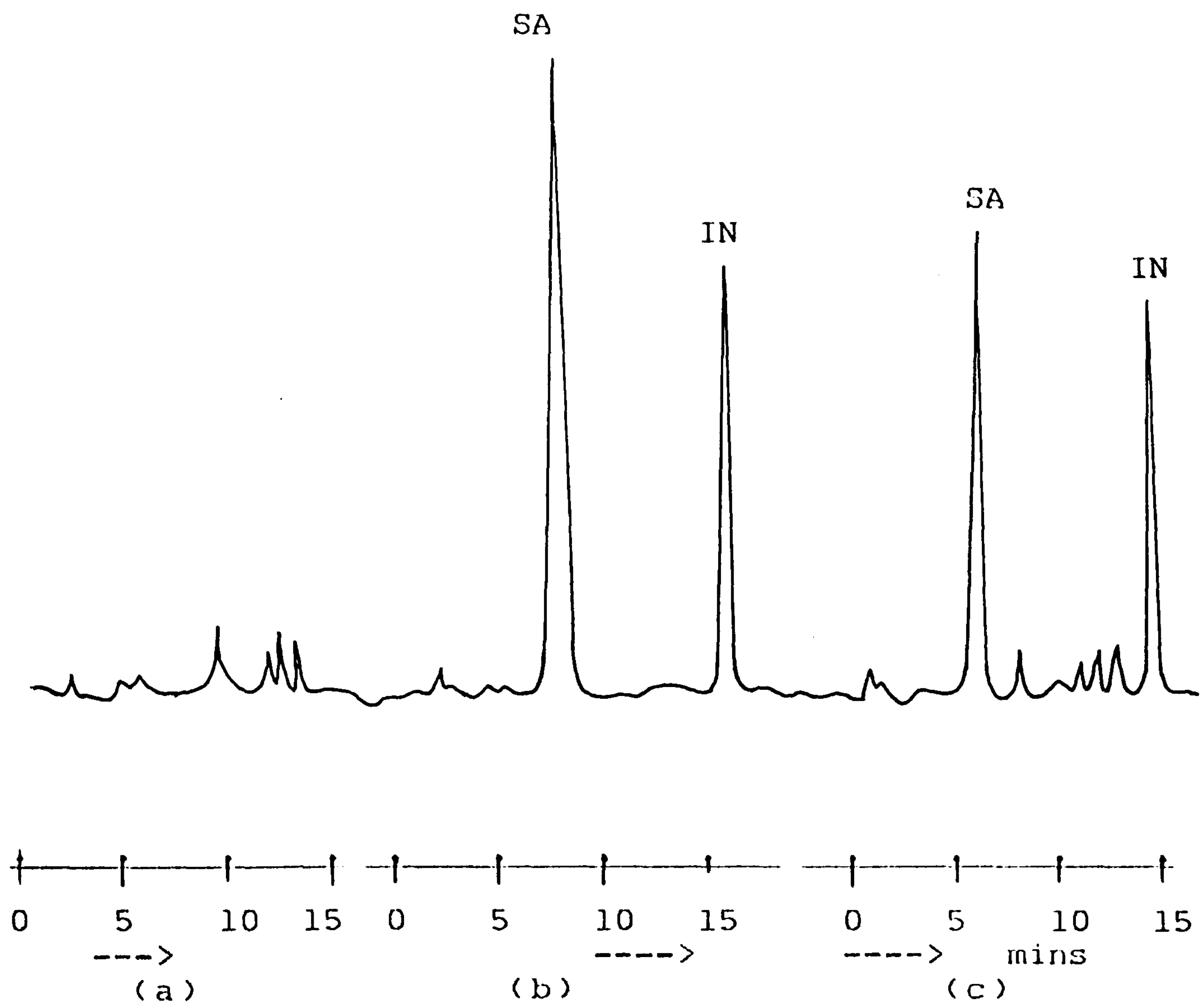


Figure 11 : Chromatogram of simultaneous determination of salicylic acid and indomethacin.
(a)Blank serum extract (b) standard compounds (c)extract of spiked serum

Concentration (µg/ml) \ Anal.No.	Peak Area (mm^2): Single assay 5	10	20	40	50	60
1.	20.6	39.6	67.0	138.2	163.2	188.0
2.	20.8	39.2	66.6	138.0	162.4	186.6
3.	21.0	39.0	67.4	139.2	163.0	187.8
4.	19.8	38.6	67.0	138.0	163.4	187.6
5.	20.2	39.2	66.4	138.4	163.0	187.4
6.	21.0	39.0	66.8	137.8	162.4	186.8
7.	20.0	38.9	67.4	138.6	162.6	187.0
8.	21.4	40.2	66.6	139.0	163.2	187.8
9.	20.4	39.0	67.4	138.0	162.0	186.4
10.	21.2	40.0	68.0	138.2	161.8	187.2
MEANS	20.8	39.4	67.1	138.3	162.8	187.3
S.E.M.	0.19	0.17	0.16	0.15	0.16	0.18
REGRESSION LINE	Peak area = 3.08 x Concentration + 7.63					
Correlation	0.998					

TABLE XI(a): Variations in the peak areas for concentrations of salicylic acid in elution solvent determined singly by gradient elution.

Concentration (µg/ml) \ Anal.No.	Peak Area (mm^2): Simultaneous assay					
	5	10	20	40	50	60
1.	20.8	40.2	67.2	139.2	163.0	187.0
2.	20.4	39.8	67.6	138.4	163.4	188.0
3.	19.8	41.0	68.0	139.2	162.6	187.6
4.	19.6	39.6	66.8	139.6	163.8	187.8
5.	20.6	40.0	67.2	138.6	162.4	187.4
6.	21.2	40.2	66.6	138.2	163.6	188.0
7.	19.8	40.4	67.0	139.4	163.8	187.0
8.	21.4	39.4	66.8	139.6	163.6	187.8
9.	20.8	40.0	67.4	139.2	162.6	187.4
10.	20.4	40.6	67.0	139.0	163.6	187.8
MEANS	20.5	40.1	67.2	139.0	163.6	187.6
S.E.M	0.18	0.16	0.15	0.16	0.15	0.12
REGRESSION LINE	Peak area = 3.09 x Concentration + 7.76					
Correlation	0.998					

TABLE XI Variations in the peak areas for concentrations of salicylic acid in elution solvent determined simultaneously with indomethacin by gradient elution. S.E.M. = standard error of mean.

Concentration (µg/ml) \ Anal.No.	Peak Area (mm^2): Single assay					
	5	10	20	40	50	60
1.	19.6	35.4	58.2	116.2	139.6	161.6
2.	18.6	36.0	59.0	116.4	139.4	162.0
3.	18.8	35.0	57.8	117.0	139.0	161.4
4.	19.4	35.8	58.0	116.4	138.8	161.6
5.	18.6	35.4	58.2	116.6	139.0	162.0
6.	19.4	35.6	58.4	116.8	139.2	161.4
7.	19.2	36.0	57.6	116.6	138.6	161.8
8.	19.2	36.0	57.6	116.6	138.8	161.0
9.	20.0	34.8	58.6	116.0	139.6	162.4
10.	18.8	35.2	58.4	116.2	139.2	161.4
MEANS	19.2	35.4	58.3	116.5	139.0	161.7
S.E.M.	0.15	0.15	0.14	0.10	0.12	0.13
REGRESSION LINE	Peak area = 2.61 x Concentration + 7.83					
Correlation	0.999					

TABLE XII (a): Variations in the peak areas for concentrations of salicylic acid in spiked serum determined singly for the construction of calibration curve.

Concentration (µg/ml) / Anal.No.	Peak area (mm^2): Simultaneous assay					
	5	10	20	40	50	60
1.	18.8	34.8	58.6	115.8	139.2	161.2
2.	19.0	35.8	57.8	116.6	138.8	160.6
3.	19.8	35.0	59.0	116.4	139.6	161.6
4.	18.6	36.0	58.2	115.6	138.0	161.4
5.	18.6	36.0	58.2	115.6	138.0	161.4
6.	19.0	35.4	58.8	117.0	139.4	161.8
7.	19.0	34.8	58.0	116.6	139.6	160.4
8.	20.0	36.4	58.6	116.2	139.2	161.6
9.	18.8	35.6	58.0	116.8	138.6	160.8
10.	19.4	35.8	57.8	115.8	139.0	161.6
MEANS	19.2	35.5	58.2	116.3	138.9	161.3
S.E.M.	0.16	0.17	0.17	0.16	0.17	0.16
REGRESSION LINE	Peak area = 2.60 x Concentration + 7.95					
Correlation	0.999					

XII (b): Variations in the peak areas for concentrations of salicylic acid in spiked serum extracted and used for the construction of calibration curve after simultaneous gradient elution with indomethacin.

Concentration (μg/ml) \ Anal.No.	Peak area (mm^2):Single assay					
	0.25	0.5	1.0	2.0	2.5	3.0
1.	7.4	20.0	46.4	83.6	108.2	129.4
2.	7.2	20.2	45.8	84.4	108.6	129.0
3.	7.0	19.8	46.2	83.8	107.8	128.4
4.	7.6	20.4	47.0	84.2	108.4	128.8
5.	6.8	20.0	45.8	84.0	108.8	128.0
6.	7.0	20.8	45.4	84.6	108.6	128.6
7.	7.2	19.8	46.0	83.4	108.8	129.0
8.	7.4	20.4	46.4	83.8	108.2	127.8
9.	6.2	20.6	46.0	84.6	108.2	128.6
10.	7.6	21.0	46.2	84.0	108.6	128.4
MEANS	7.2	20.3	46.1	84.0	108.4	128.6
S.E.M.	0.14	0.14	0.13	0.13	0.11	0.15
REGRESSION LINE	Peak area = 43.5 x Concentration - 1.34					
Correlation	0.999					

TABLE XIII : Variations in the peak areas for concentrations of indomethacin in eluent determined singly by gradient elution and used to construct calibration curve.

Concentration (μg/ml) / Anal.No.	Peak area (mm^2): Simultaneous assay					
	0.25	0.5	1.0	2.0	2.5	3.0
1.	7.6	19.8	45.6	84.2	108.4	129.0
2.	7.0	21.0	46.0	84.6	108.6	128.6
3.	6.8	20.4	44.8	83.6	107.8	129.4
4.	7.4	20.8	45.6	84.4	108.0	128.8
5.	6.6	20.6	45.0	84.0	107.4	129.4
6.	7.8	20.4	45.8	84.8	107.8	129.2
7.	7.4	20.2	45.6	83.8	108.2	128.6
8.	6.8	20.6	45.2	84.2	107.6	129.4
9.	7.2	19.8	45.4	84.6	108.4	129.6
10.	7.6	20.0	45.8	83.8	107.8	128.4
MEANS	7.2	20.4	45.5	84.2	108.0	129.0
S.E.M.	0.13	0.13	0.13	0.13	0.14	0.14
REGRESSION LINE	Peak area = 43.6 x Concentration - 1.56					
Correalation	0.999					

TABLE XIII (b) : Variations in the peak areas for concentrations of indomethacin in eluent determined simultaneously with aspirin by gradient elution and used to construct calibration curve.

Concentration (µg/ml) \ Anal.No.	Peak Area (mm^2) : Simultaneous assay					
	0.25	0.5	1.0	2.0	2.5	3.0
1.	6.4	17.2	38.8	71.2	91.0	110.6
2.	6.0	17.6	38.4	71.6	90.6	110.8
3.	6.8	17.8	37.8	71.0	91.2	109.6
4.	7.2	17.4	38.2	71.8	91.6	110.4
5.	6.4	16.8	37.6	70.6	90.8	110.6
6.	7.4	18.0	38.2	71.4	91.6	109.8
7.	6.2	17.8	38.4	70.8	91.4	110.6
8.	6.6	16.8	37.6	70.8	90.8	110.8
9.	6.4	17.6	37.8	71.6	91.6	110.4
10.	6.8	17.4	38.6	71.4	91.0	110.6
MEANS	6.6	17.4	38.4	71.2	91.2	110.4
S.E.M.	0.14	0.14	0.14	0.13	0.14	0.13
REGRESSION LINE	Peak area = 37.1 x Concentration - 0.27					
Correlation	0.999					

TABLE XIV : Variations of the peak areas for indomethacin concentrations in spiked serum extracted and determined singly for the construction of calibration curve.

Concentration (μg/ml) / Anal.No.	Peak area (mm^2) : Simultaneous assay					
	0.25	0.5	1.0	2.0	2.5	3.0
1.	6.6	17.8	38.2	71.4	91.8	110.4
2.	6.8	16.8	38.6	71.6	91.4	110.0
3.	6.2	17.0	37.8	70.8	90.6	109.8
4.	6.6	18.0	37.6	71.2	91.2	116.6
5.	7.0	17.6	37.8	70.6	91.0	110.2
6.	6.8	17.4	38.4	71.0	90.8	110.6
7.	7.4	16.8	38.6	71.4	91.6	109.8
8.	6.8	17.6	38.2	71.6	91.4	110.0
9.	6.0	18.0	37.8	70.8	90.8	110.2
10.	64.	17.4	38.4	71.0	91.0	110.6
MEANS	6.7	17.4	38.1	71.1	91.2	110.2
S.E.M.	0.13	0.15	0.13	0.12	0.14	0.11
REGRESSION LINE	Peak area = 37.0 x Concentration - 1.28					
Correlation	0.999					

TABLE XIV (b) : Variations in the peak areas for indomethacin concentrations in spiked serum extracted and determined simultaneously with salicylic acid by gradient elution for the construction of calibration curve.

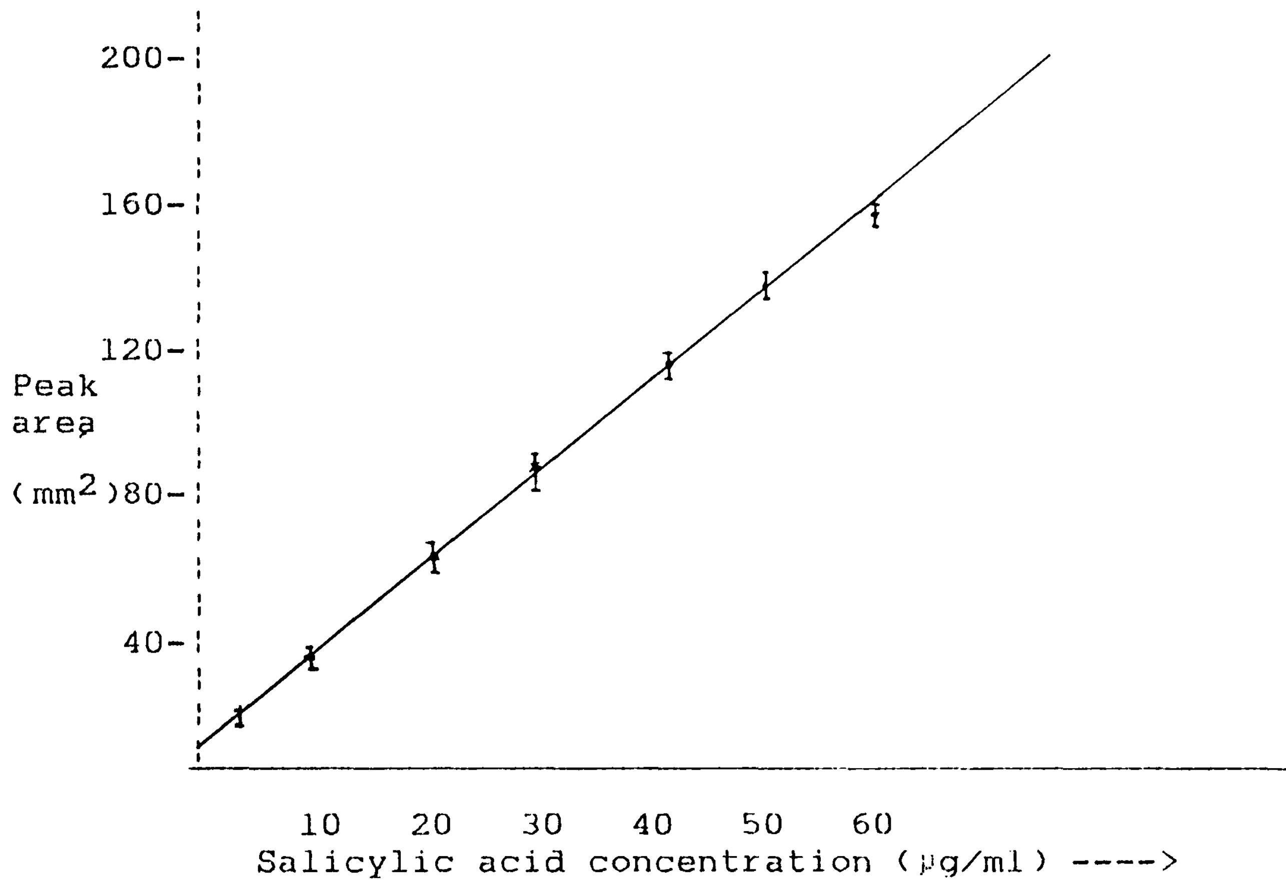


Figure 12 : Calibration curve for serum salicylic acid concentration determined singly by gradient elution

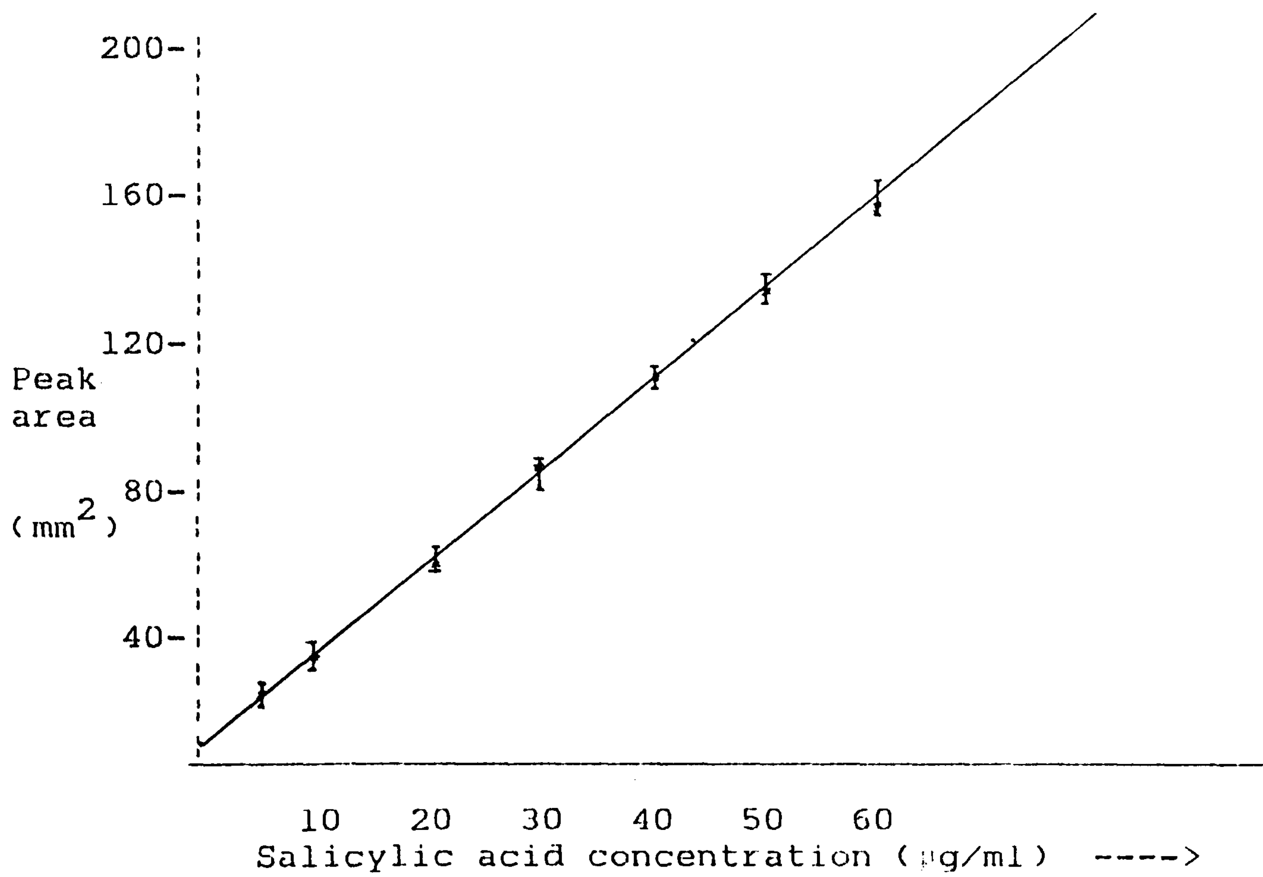


Figure 13 : Calibration curve for salicylic acid concentration determined simultaneously with indomethacin using gradient elution.

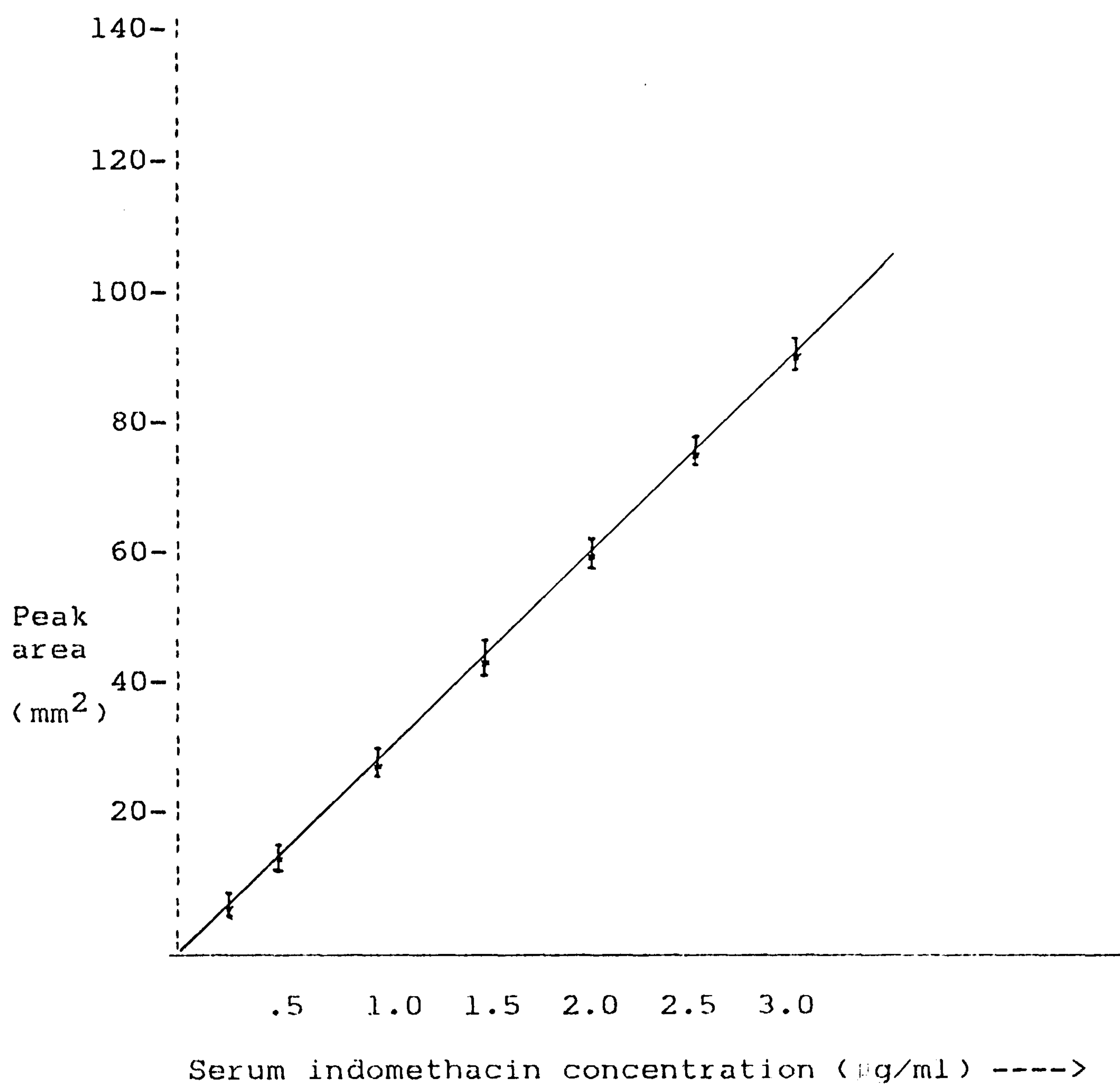


Figure 14 : Calibration curve for serum indomethacin concentration determined simultaneously with salicylic acid by gradient elution

5. PHARMACOKINETICS

5.1 INTRODUCTION

The changes in the concentration of salicylate in the blood with time after oral administration indicates the characteristics of its absorption,distribution,metabolism and excretion.These processes have been assessed by resolving the observed concentration-time profile into the different rate constants for the processes.

A general approach has been to conceptualise the body as comprised of compartments into which the drug enters and leaves at various rates in the direction of specified compartments.This is associated with the blood flow properties of the different organs and tissues of the body.That is the concept of disposition models.The physiological models also allow the relationship between the plasma concentrations and the amount of drug in the body to be defined in terms of time [118].This concept has been explored for application in this study.

5.2 CHARACTERISATION OF SALICYLATE CONCENTRATION-TIME PROFILE

5.2.1 MODEL DEFINITION

A model which defines the body as composed of two-compartments the first of which receives drug input and

from which the drug distributes to the second is a two-compartment model.When this allows the elimination of the drug through the first compartment (figure 15) it is a two-compartment open model [119].This model was assigned to aspirin following intravenous bolus administration [120].The intravenous bolus administration of a drug that fits this model results in a concentration-time plot described by the equation:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \qquad \ldots\ldots\ldots\ldots(1)$$

(definition of terms in 5.2.2).

Derivation of this equation was shown by Gibaldi and Perrier [121] and is obtained from the differentials of drug quantities determined by rate constants shown in Figure 15 :

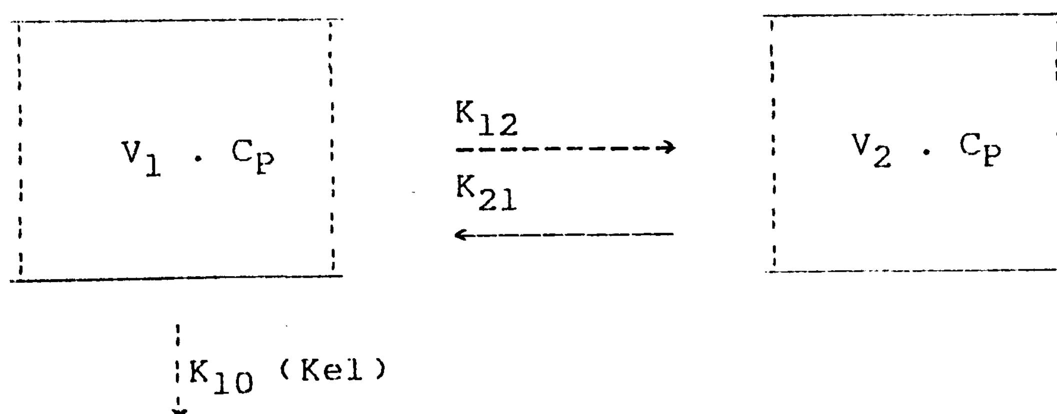


Figure 15 : Two-compartment open model for intravenous administration of drug.

Administration of aspirin by the oral route introduces a reservoir compartment and the absorption process depicted in figure 16 :

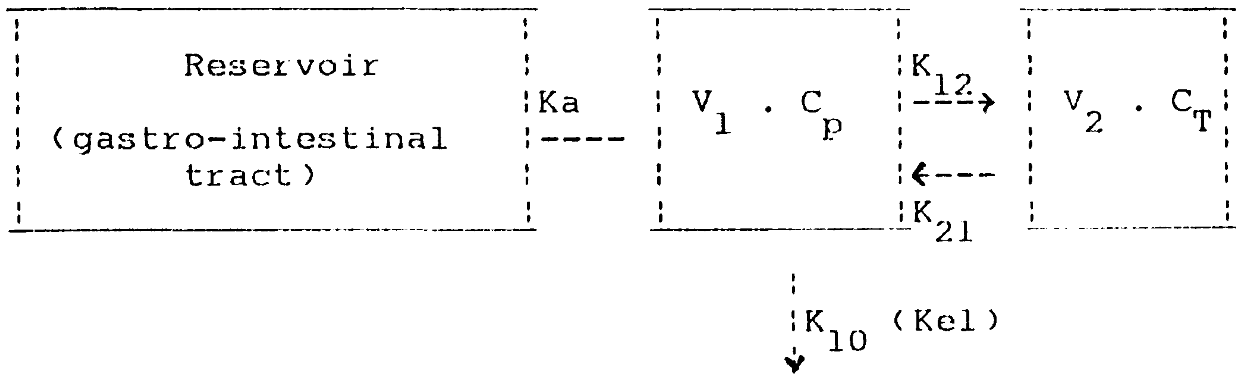


Figure 16 : Two-compartment open model for oral administration of a drug.

In this case the serum-concentration-time data may fit the equation :

$$C = Ae^{-\alpha t} + Be^{-\beta t} - (A + B)e^{-Kat} \qquad (2)$$

and

$$A + B = \left[\frac{F.Dose.Ka}{V} \right] \left[\frac{Ka - K_{21}}{(Ka-\alpha)(Ka-\beta)} \right] \qquad (3)$$

The curve described by this equation is :

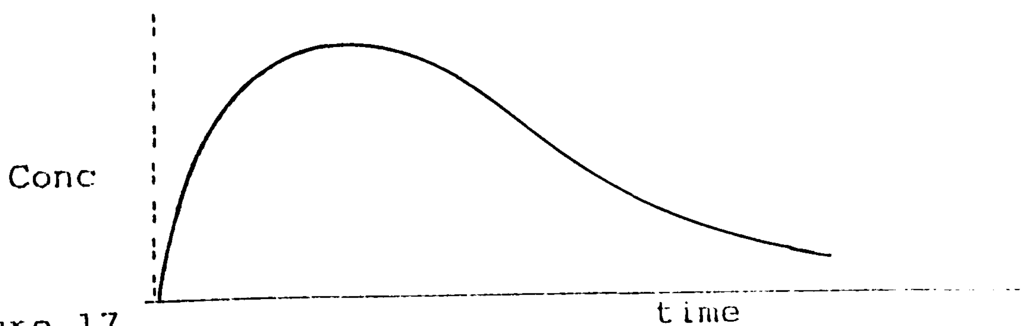


Figure 17

5.2.2 TERMS OF THE EQUATION : KINETIC PARAMETERS

5.2.2.1 Cp: the concentration of salicylate in serum. This is related to the amount of salicylate in the body by a proportionality constant, V, the amount being the dose given to the subject or fraction thereof remaining in the body at sampling time. Similarly, C_T is the salicylate concentration in the tissue.

5.2.2.2 V : the proportionality constant stated above for conceptual reasons is assumed to be equal to $V_1 + V_2$ and is often referred to as the volume of distribution.

5.2.2.3 F : the fraction of dose reaching systemic circulation upon drug administration. It is 1 for i.v. administration and for all drugs which are absorbed completely in unchanged form after oral administration. Aspirin *per se* has F less than 1. Since, as stated in 1.1.2, aspirin is rapidly hydrolysed during absorption (and also in the systemic circulation) to salicylic acid which is completely absorbed in the gastro-intestinal tract, F has been assumed 1 with respect to salicylic acid in this study.

5.2.2.4 Ka : the first order rate constant for absorption. This depends on the membrane permeability characteristics of aspirin and salicylic acid, the area of

absorbing surface and gastric emptying rate.The pKa of aspirin is incorporated in the permeability characteristics (see 1.7.1.1).

5.2.2.5 Kel (K_{10}):the elimination rate constant is the total rate at which drug is removed from the first compartment by metabolism and excretion. Kel is given by the equation :

$$K_{10} = \frac{A+B}{\frac{A}{\alpha} + \frac{B}{\beta}} \qquad \text{------------------- (eq. 4)}$$

5.2.2.6.1 K_{12}: the rate constant for distribution of drug from comparment 1 to 2. This assumes considerable importance at equilibrium distribution of salicylic acid between the two compartments.

5.2.2.6.2 K_{21}: the rate constant for distribution of drug from the tissue (compartment 2) back to systemic circulation.In health this constant is closely related to the molecular identity of salicylic acid.

5.2.2.7 t : is the time elapsed after drug administration. In this study it has been taken as the time through which drug reaches the sampling site and the sampling intervals.

5.2.2.8 A : the pre-equilibrium coefficient of distribution. This characterises the initial concentration of the drug in the first compartment during the fastest distribution phase, the α phase. A is an extrapolation term usually obtained from the residuals of logarithm of salicylic acid concentration-time plot as shown in figure 18.

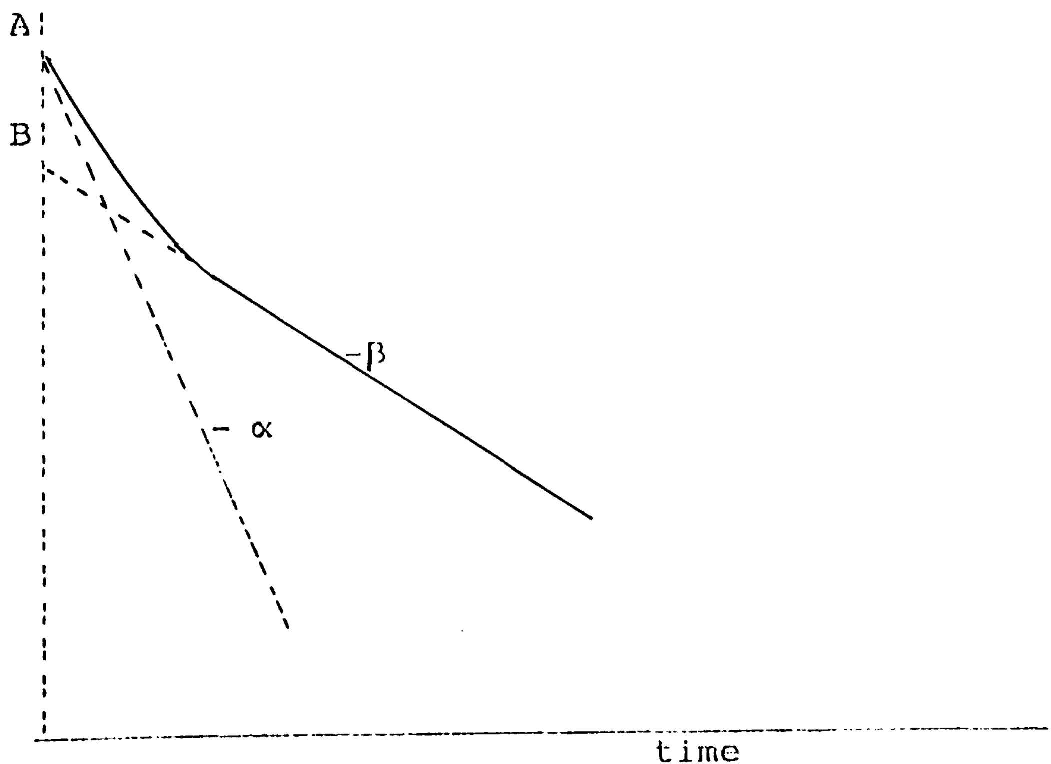


Figure 18 : Semi-logarithmic plot of serum concentration of a drug with a two-compartment model of disposition.

5.2.2.9 α : the rate constant shown in figure 18 for the fast distribution phase.When this phase is not apparent,for oral administration the body comforms to apparent one-compartment model,governed by the equation:

$$Cp = \left| \frac{F.Dose.Ka}{V(Ka-Kel)} \right| \left| e^{-Kelt} - e^{-Kat} \right| \quad \text{--- (eq.5)}$$

This equation describes the curve :

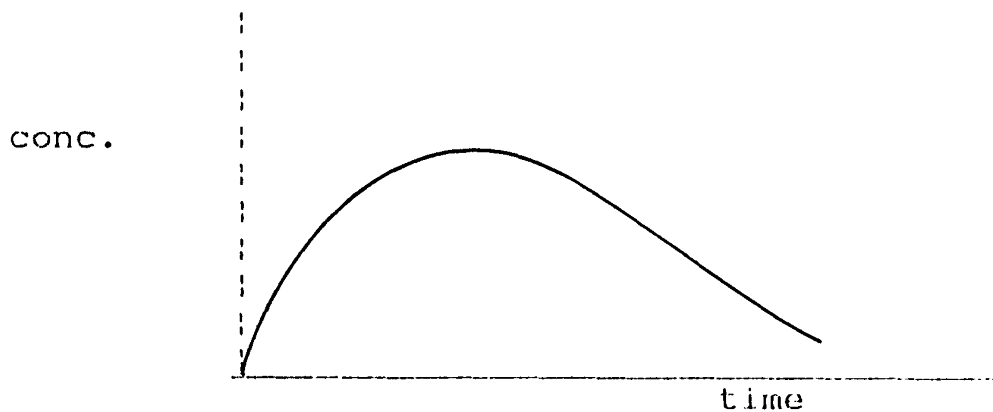


Figure 19

5.2.2.10 B : the equilibrium coefficient of distribution. This characterises the concentration in the first compartment after equilibrum with the second compartment is attained.It is the concentration at which elimination phase with respect to serum salicylic acid concentration sets in.

5.2.2.11 β : is the rate constant for elimination of drug at equilibrium distribution phase.This is also a pseudo-

distribution rate constant that characterises the elimination of the drug from the body.Alteration of β can occur with simultaneous administration of a drug that could compete with certain elimination pathways with aspirin.

When capacity-limited elimination occurs,as usually happens with anti-rheumatic dosage of aspirin,zero order kinetics operate before the β phase is attained [122].

5.2.2.12 DERIVED PARAMETERS

5.2.2.12.1 Maximum Serum Concentration (Cmax) is the highest concentration observed in a serum-concentration-time plot after oral administration of the drug.

5.2.2.12.2 T(max) is the time when salicylic acid reaches maximum serum concentration.

5.2.2.12.3 Area Under the Curve (AUC) : This is the integral of all changes in salicylate concentration or of any other drug with time.AUC is obtained by the summation of the area under the curve of equation (1) or (2).

For ethical and practical purposes this is often

calculated up to time of 8-12h or less in human experiments and according to anticipated frequency of administration. When the log-linear phase is attained then summation up to infinity could be achieved by extrapolating with $\frac{Cn + 1}{Kel}$. Using the trapezoidal rule :

$$AUCo\text{-}tn+1 = 1/2 \triangle t(Cn + Cn + 1)$$

Where n = number of small changes in time and Cn , Cn + 1 are corresponding concentrations at the specified time interval. Up to 8h at changes of 10 min ,Cn + 1 = concentration at 48th 10 minute interval and C = the concentration after the first 10-minute interval after oral administration of aspirin.

6 EXPERIMENTAL

6.1 DESIGN OF THE EXPERIMENT

The trial presented below is intended to test the hypothesis that absorption ,distribution and elimination of aspirin when given alone is the same as when given in combination with paracetamol or indomethacin.

The pharmacokinetic parameters for the absorption and disposition processes as defined in 5 are Ka,β,Vd,K_{10} , K_{12},K_{21},tmax,Cmax and AUC.

6.1.1 SUBJECTS AND DRUG ADMINISTRATION

Healthy volunteers,with informed consent,participated in the randomised cross-over studies.None of the subjects has history of hypersensitivity to non-steroidal anti-inflammatory drugs.Four were non-smokers. No subject consumed alcohol,paracetamol,aspirin or indomethacin 24h prior to the test.The volunteers attended fasting from 10 p.m. the night before.

Fine-particle suspension of 652mg of aspirin alone in 50ml glucose solution was administered to a group of 9 subjects in the first visit. A minimum of two-week period was allowed after the first visit. On a second visit fine-particle suspension of the same dose of aspirin and

1000mg of paracetamol in glucose solution was administered to the same subjects.After another period of not less than two weeks 7 of the subjects in the previous visits and an additional 3 subjects received 652mg of the same suspension of aspirin as in the previous studies and also 100mg of indomethacin concurrently.

6.1.2 COLLECTION AND ANALYSIS OF SERUM SAMPLES

On each of the visits indicated above 10ml of blood was withdrawn before drug administration and at 10, 20, 30, 40, 50, 60, 90, 120, 180, 240, 300 and 360 min. after drug intake. The sample withdrawn at each time was transferred into 10ml glass tube and allowed to clot. The samples were left for 0.5h - 1h and then centrifuged. Serum was decanted into 10ml glass tubes and frozen at -20°C until analysed for aspirin,salicylic acid, paracetamol and indomethacin.

The concentrations of paracetamol, indomethacin and/or salicylic acid at the sampling times were determined after extraction and high performance liquid chromatographic separation as described in 3.1 and 3.3.

6.2 PHARMACOKINETIC ANALYSIS

Logarithmic transforms of the raw data for each individual and on each occasion were used to compute the

test statistics for the processes of absorption,distribution and elimination. The computation employed linearised forms of equations (2,3,5) followed by linear regression of each phase of the serum salicylate concentration-time (C/t) profiles.

6.2.1 ABSORPTION

The rate constants for absorption of aspirin was determined when given alone and when given in combination with paracetamol and indomethacin.This involved the use of stable method for calculating the absorption rate constant for a drug whose disposition obeys equation (3) for a two-compartment model [123].

Thus the first three C/t points were fitted with a second degree polynomial :

$$C = a + bt + Ct^2$$

and the curve was extrapolated to a concentration value of 0 . This gives a time lag which was less than 2 min throughout the study data.Hence the 10 min concentration was not ignored on an hour time scale,2 min being relatively low and was negligible.

Another second degree plolynomial was fitted to the point with the highest value of c and to the point on either

side of it.The maximum point of the polynomial was therefore an estimate of maximum concentration and the time at which it was attained.The trapezoidal rule was used to calculate the area under the curve from time = 0 to time = tm corresponding to the maximum concentration point.

The absorption rate constant was then determined by iterative computer procedure.The starting point being an estimate of Ka (Ka estim) based on the equation :

$$\text{Ka estim} = 1.5/\text{tm} \quad \text{------------------} \quad (6)$$

Ka estim was substituted for Ka in the equation :

$$C + (A + B)e^{-Kat} = Ae^{-\alpha t} + Be^{-\beta t} \quad \text{----} \quad (7)$$

B and β as defined in 5.2.2.11 and 5.2.2.12 were determined from a linear regression of the last three data set of salicylic acid concentration. The starting value for (A + B) was based on the condition that Kel as defined in 5.2.2.5 is 2-5 times β , so A + B is estimated starting from the value given by the expression :

$$\lim_{\beta \to K_{10}} A + B = 4\beta.\text{Area} \quad \text{----------------} \quad (8)$$

This was used to calculate the left hand side of equation (7) called Y for each time

$$Y = C + (A + B)e^{-Kat} \text{-------------} \quad (9)$$

From eqution (7)

$$Y = Ae^{-\alpha t} + Be^{-\beta t}$$

and therefore a linear regression of $\ln(Y-Be^{-\beta t})/t$ gives a slope of $-\alpha$. A was then estimated from equation (2) with t = tm , C = Cm. At this point α was known to be less than 0.5Ka estim and therefore indicated that absorption rate and the fast disposition phase were such that the β-phase ensued at 4h, after both processes were over.

Taking natural logarithms of equation (4) the the absorption rate constant with respect to salicylic acid was determined by iterative computer regression analysis. The slope of the regression line was -Ka [124-126]. Since $\ln \frac{D}{Vd} \cdot \frac{1}{e^{(Ka-\alpha)t}-1} = \ln \frac{A.Ka}{Ka-\alpha} - Kat$ --(5)

6.2.2 DISTRIBUTION

The semi-logarithmic regression line of the last 3 data points (4h-6h) was a straight line with slope $-\beta$. The ratio of the administered dose to (A + B) (c.f. eq. (8)) was given by the equation :

$$Vd = \frac{Dose}{A + B} \text{------------} \quad (9)$$

where Vd is the volume of distribution.

Using the estimate of A and α from equation (1),when t = tm as the starting point a C/t curve was fit to the concentration data by iterative computer procedure.
the equilibrium coefficient of distribution, B, was the intercept on the ordinate axis of the regression line , as stated above , of the log-linear phase.The distribution rate constant K_{12} was calculated with the equation :

$$K_{12} = \alpha + \beta - K_{10} - K_{21} \quad \text{-------------------- (10)}$$

and the rate constant K_{21} was given by the

equation :

$$K_{21} = \alpha\beta/K \, . \quad \text{------------------------ (11).}$$

The pseudo-distribution rate constant for the elimination phase, β, was the negative slope of the regression line of the last three data points . The elimination rate constant , Kel, was calculated according to equation (4) (see 5.2.2.12).

The AREA UNDER THE CURVE (AUC) was calculated using the ratio of pre-equilibrium coefficient of distribution to the elimination rate constant.

Other parameters tested were the time to attain the maximum concentration (tmax) and the maximum concentration (Cmax).

6.3 STATISTICS

The salicylate kinetic parameters for the three occasions were tested for any satistical difference using paired t test and the Wilcoxon Sign Rank test.

7. RESULTS

7.1 SERUM SALICYLATE CONCENTRATIONS

7.1.1 ADMINISTRATION OF ASPIRIN ALONE

Salicylate concentrations in the samples were consistent with prompt absorption of aspirin for each occasion in the trial protocol. A 20-min test sample frozen immediately after sampling and determined shortly after showed the presence of intact aspirin (4μg/ml).In the rest of the samples frozen after 0.5 - 1h by design in this study no intact aspirin was detected.Figures 20 and 21 show the curves obtained with serial determinations of of salicylate up to 6h-samples of two subjects.The concentration indicate two distinct kinetic characteristics .Between these were the less-defined variates of individuals' salicylate kinetics that were apparent in this study.Table XV shows the salicylate concentrations determined in all the samples.

7.1.2 ADMINISTRATION OF ASPIRIN WITH PARACETAMOL

Both aspirin and paracetamol rapidly appeared within the first 10 mins of administration. The concentrations assumed the characteristic pattern of variations as in the lone administration of aspirin.Table XVII shows the

concentrations of paracetamol.No intact aspirin was detected in the samples.

7.1.3 ADMINISTRATION OF ASPIRIN WITH INDOMETHACIN

Aspirin was also detectable at 10 mins. when administered in combination with indomethacin. As in the previous studies , no aspirin was detected in these samples also frozen after 0.5 - 1h by design.No indomethacin was detected for most of the intervals for time of drug administration to 20mins.Table XVIII shows the concentrations of salicylic acid when given with indomethacin.The salicylic acid concentrations were the same for the same samples determined by either isocratic elution or by gradient elution.Table XIX shows the concentration of indomethacin.

Subject Time(min)	1	2	3	4	5	6	7	Mean ± S.E.M
10	8.8	6.2	5.6	7.5	5.0	9.2	6.0	6.9 ± 0.61
20	15.5	10.3	10.4	13.6	11.4	16.0	10.2	12.4 ± 0.95
30	22.4	14.2	14.0	20.0	13.6	21.3	13.4	16.9 ± 1.53
40	29.2	16.3	16.9	24.2	17.0	26.5	18.0	21.2 ± 2.02
50	31.8	20.5	20.9	30.0	20.0	30.0	21.0	24.9 ± 2.04
60	36.3	23.2	22.0	32.8	22.2	33.4	25.4	27.9 ± 2.29
90	41.8	27.6	27.4	40.5	26.7	40.7	29.8	33.5 ± 2.68
120	48.6	32.0	30.2	46.5	28.8	44.0	31.2	37.3 ± 3.25
180	45.0	32.7	28.6	45.5	29.0	43.1	28.3	36.0 ± 3.07
240	38.1	35.0	26.0	44.2	27.1	40.0	23.1	33.4 ± 3.03
300	35.0	31.4	24.0	42.0	24.5	31.8	21.0	29.9 ± 2.76
360	30.6	27.1	21.5	37.3	22.0	25.2	19.0	26.1 ± 2.36

TABLE XV : Concentrations (mg/L) of Salicylic acid when only aspirin 652mg is taken.

Subject / Time(min)	1	2	3	4	5	6	7	Mean ± S.E.M
10	9.1	7.6	8.4	15.0	8.4	8.5	9.8	9.54 ± 0.95
20	16.7	14.0	15.2	26.7	16.0	15.1	17.7	17.34 ± 1.62
30	24.3	19.0	21.0	32.6	21.4	20.4	24.2	23.27 ± 1.72
40	27.4	21.8	24.2	38.0	26.3	22.2	28.4	26.90 ± 2.08
50	30.1	27.1	26.7	43.1	30.1	25.6	31.5	30.60 ± 2.24
60	32.5	28.3	29.0	50.6	32.0	28.0	34.2	33.51 ±
90	41.0	31.4	33.2	54.0	39.0	31.0	40.0	38.51 ± 3.02
120	43.4	30.1	34.1	51.4	41.2	30.5	42.1	38.97 ±
240	40.1	28.2	28.6	40.1	36.0	27.5	35.2	36.93 ± 2.09
300	32.0	24.5	25.3	32.4	34.5	24.2	31.0	29.13 ± 1.63
360	28.0	22.4	21.0	26.4	31.0	21.8	28.1	25.53 ± 1.44

TABLE XIV : Concentrations of Salicylic acid when aspirin is taken with paracetamol.

Subject Time(min)	1	2	3	4	5	6	7	Mean ± S.E.M
10	3.4	3.0	2.4	2.0	2.2	1.6	7.6	2.60 ± 0.34
20	6.4	2.4	2.0	2.2	1.6	7.6	2.2	3.4 ± 0.91
30	7.2	3.0	3.0	3.2	3.0	7.2	3.6	4.3 ± 0.72
40	9.6	4.8	4.6	4.8	4.7	7.6	4.6	5.8 ± 0.76
50	16.0	5.0	7.8	5.4	6.0	7.2	4.5	7.4 ± 1.47
60	24.0	5.8	7.2	10.0	8.4	8.6	5.4	9.9 ± 2.42
90	16.0	3.8	6.8	6.2	7.2	8.0	6.6	7.8 ± 1.44
120	13.6	4.2	4.8	3.8	6.0	7.2	8.0	6.3 ± 1.29
180	8.0	3.4	4.0	4.4	5.0	5.8	3.0	4.8 ± 0.64
240	5.0	3.0	3.8	4.0	4.4	4.6	3.4	4.0 ± 0.26
300	3.0	1.5	2.0	2.5	2.0	3.0	1.8	2.2 ± 0.23
360	1.3	0.9	1.3	1.8	1.5	1.6	1.2	1.4 ± 0.11

TABLE XVII : Concentrations of paracetamol mg/L

Subject Time(min)	1	2	3	4	5	6	7	Mean ± S.E.M
10	11.6	8.3	10.4	13.6	14.1	9.0	10.7	11.10 ± 0.82
20	21.0	15.0	18.3	24.9	24.5	16.1	20.0	19.97 ± 1.45
30	27.1	20.5	24.1	34.1	29.8	21.7	28.0	26.47 1.80
40	33.2	23.1	28.4	41.7	33.5	26.1	33.7	31.39 ± 2.31
50	36.4	26.0	31.2	45.0	38.2	29.4	38.2	34.91 ± 2.31
60	39.5	27.6	33.0	48.2	41.3	31.9	40.1	37.37 ± 2.62
90	43.0	30.0	35.4	56.3	44.9	35.8	47.5	41.27 ± 3.23
120	41.0	28.0	32.1	60.1	42.8	36.4	50.0	41.23 ± 4.05
180	38.4	25.2	28.3	60.0	35.8	33.7	45.0	39.11 ± 4.79
240	34.2	21.3	25.0	58.1	29.6	29.6	41.0	35.40 ± 5.13
300	31.7	20.4	21.6	55.9	25.1	25.9	37.2	32.08 ± 4.98
360	27.8	18.5	20.0	48.0	20.0	22.0	32.0	28.19 ± 4.43

TABLE XVIII : Concentrations of salicylic acid (mg/L) when aspirin 652 mg is taken with indomethacin.

Subject Time(min)	1	2	3	4	5	6	7	Mean ± S.E.M
10	-	-	-	-	-	-	0.68	0.68 ±
20	-	-	-	0.33	0.94	_	0.94	0.73 ± 0.17
30	0.4	-	-	0.48	0.68	0.3	0.68	0.50 ± 0.07
40	0.3	0.2	0.25	0.65	1.24	0.5	1.24	0.62 ± 0.17
50	0.4	0.25	0.4	1.02	1.34	0.7	1.34	0.77 ± 0.17
60	0.85	0.60	0.30	1.40	1.21	1.0	1.21	0.93 ± 0.15
90	1.5	0.65	0.5	1.4	1.32	1.5	1.32	1.17 ± 0.16
120	1.0	1.15	0.8	2.0	0.87	1.5	1.32	1.23 ± 0.22
180	0.85	1.63	1.7	0.9	0.68	2.1	0.68	1.22 ± 0.22
240	0.70	2.0	2.5	1.5	0.73	-	0.75	1.17 ± 0.30
300	0.40	2.5	1.5	0.73	-	0.75	0.40	1.04 ± 0.31
360	0.50	1.30	0.9	0.50	-	0.6	-	0.76 ± 0.13

TABLE XIX Concentration of indomethacin (mg/L) When 100mg is taken with aspirin (652mg).

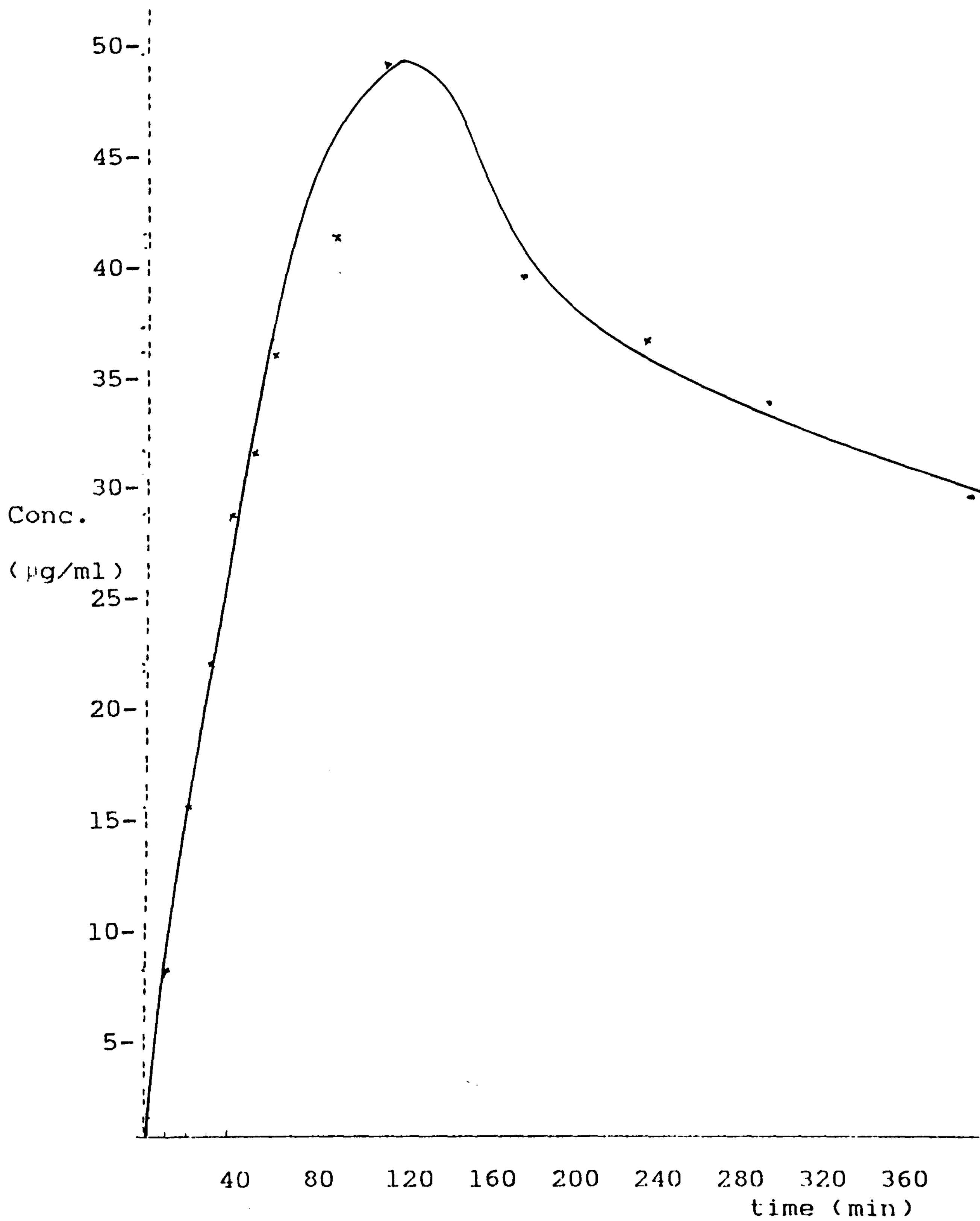


Figure 20 : Salicylic acid kinetic curve determined with samples from a subject showing high salicylic acid concentrations when aspirin was administered alone.

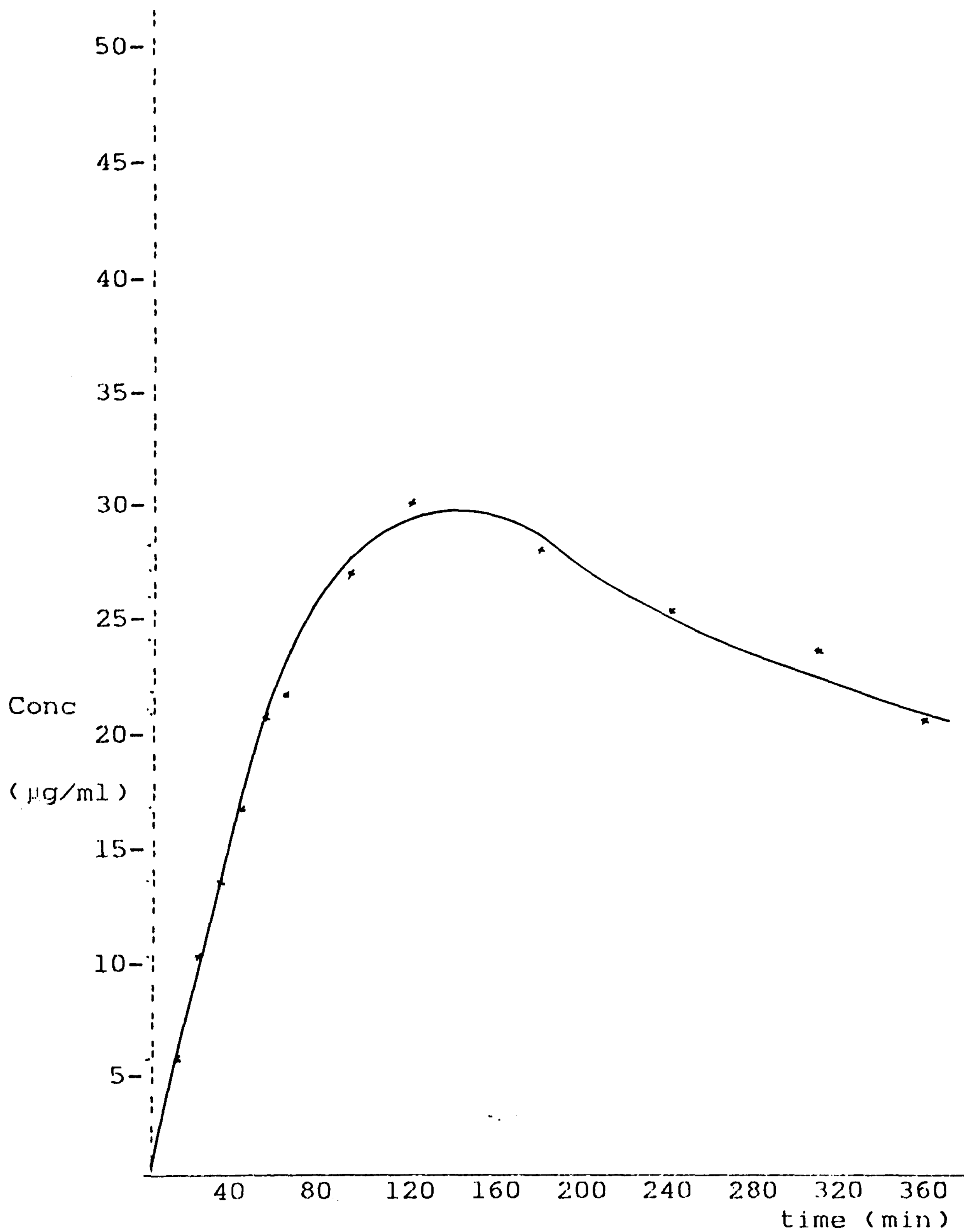


Figure 21 : Salicylic acid kinetic curve determined with samples from a subject showing low salicylic acid concentrations when aspirin was administered alone.

7.2 PHARMACOKINETICS

The serum salicylic acid concentrations up to 6h resolved into salicylic acid kinetic components were consistent with two-compartment disposition with simultaneous first order absorption kinetics. Tables XX - XXII show the kinetic constants for salicylic acid absorption and disposition for the three occasions of the administration of aspirin as stated below.

7.2.1 ADMINISTRATION OF ASPIRIN ALONE

7.2.1.1 ABSORPTION

The absorption rate constant was a mean ± S.E.M. 0.75 ± 0.03 hr^{-1}.

7.2.1.2 DISTRIBUTION

The volume of distribution was 8.6L ± 0.87L (mean ± S.E.M.)

The rate constant for transfer of salicylic acid from blood to tissues was a mean ± S.E.M. of 0.07 hr^{-1} ± 0.02hr^{-1}, while the rate of return of salicylic to blood was a mean ± S.E.M. of 0.15 ± 0.01hr^{-1}.

7.2.1.3 ELIMINATION

The pseudo-distribution rate constant for elimination was

a mean $\pm$ S.E.M. of 0.08 $\pm$ 0.02hr^{-1} .Simultaneously the rate constant for elimination from the central compartment was a mean $\pm$ S.E.M of 0.12 $\pm$ 0.01hr^{-1}.

7.2.1.4 AREA UNDER THE CURVE

The area under the curve was a mean $\pm$ S.E.M. of 434 $\pm$ 30.2 mghL^{-1}.

7.2.1.5 TIME TO REACH MAXIMUM SERUM CONCENTRATION

Peak salicylic acid concentration was attained in 2-4hr, mean $\pm$ S.E.M. of 2.4 $\pm$ 0.3hr.

7.2.1.6 MAXIMUM SERUM CONCENTRATION

The mean $\pm$ S.E.M. peak salicylic acid concentration was 37.5 $\pm$ 3.2 mgL^{-1}.

7.2.2 ADMINISTRATION OF ASPIRIN WITH PARACETAMOL

The serum salicylic acid concentration-time plots yielded curves resolvable according to a two-compartment disposition model with first order absorption.Table XXI shows the kinetic constants.

7.2.2.1 ABSORPTION

The absorption rate constant of aspirin with reference to salicylic acid was a mean $\pm$ S.E.M. of 0.95 $\pm$ 0.03hr^{-1}.

This was statistically different (P = 0.05) from that obtained when aspirin was administered alone but not different (P = 0.05) from that observed when aspirin was taken with indomethacin (see below).

7.2.2.2 DISTRIBUTION

Salicylic acid volume of distribution in this instance was a mean ± S.E.M.of 0.08 ± 0.5L. This was not statistically different (P = 0.05) from that obtained when aspirin was taken alone nor was it different (P = 0.05) when aspirin was combined with indomethacin.The mean ± S.E.M. rate of transfer of salicylic acid into the tissues was 0.11 ± 0.03hr^{-1} while the rate of return to the blood was 0.2 ± 0.02hr^{-1}.These rate constants were not satistically different (P = 0.05) from those calculated when aspirin was administered alone . Also the rate of transfer of salicylic acid into the tissues in this study was not statistically different (P = 0.05) from that calculated when aspirin was administered with indimethacin.The rate of return of salicylic acid to blood was also not different (P = 0.05) from that obtained when aspirin was administered in combination with indomethacin.

7.2.2.3 ELIMINATION

The pseudo-distribution constant for elimination was a mean $\pm$ S.E.M. of 0.08 $\pm$ 0.08hr while the rate constant for the elimination was a mean $\pm$ S.E.M. of 0.13 $\pm$ 0.02hr^{-1} . These constants were not statistically different (P = 0.05) from those obtained when aspirin was administered alone nor were they different when aspirin was combined with indomethacin (see below).

7.2.2.4 AREA UNDER THE CURVE

The area under the curve was a mean $\pm$ S.E.M. of 408 $\pm$ 30.7$mghL^{-1}$ .This was not statistically different (P = 0.05) from the area under the curve when aspirin was administered alone.It was also not statistically different (p = 0.05) from that obtained when aspirin was administered with indomethacin.

7.2.2.5 TIME TO REACH MAXIMUM SERUM SALICYLIC ACID CONCENTRATION (Tmax)

The mean $\pm$ S.E.M. tmax was 2.00 $\pm$ 0.19hr.The difference between this and that obtained when aspirin was administered alone was not statistically significant (P = 0.05). Also it was not different from the time observed when aspirin was administered with indomethacin.

7.2.2.6 MAXIMUM SERUM CONCENTRATION (Cmax)

The mean ± S.E.M. peak concentration of salicylic acid in serum was 39.54 ± 3.1 mgL^{-1} .This was not statistically different from the peak salicylic acid when aspirin was administered alone but it was statistically different (P = 0.05) from the Cmax when aspirin was administered with indomethacin.

7.2.3 ADMINISTRATION OF ASPIRIN WITH INDOMETHACIN

Serum salicylic acid concentrations in this study was in accordance with a two-compartment disposition model with simultaneous first order absorption kinetics.Table XXII shows the kinetic parameters .

7.2.3.1 ABSORPTION

The absorption rate constant was 1.14 ± $0.05hr^{-1}$ (mean ± S.E.M.).The difference between this and the absorption rate constant when aspirin was administered alone was statistically significant (P = 0.05).

7.2.3.2 DISTRIBUTION

The volume of distribution was a mean ± S.E.M. of 7.2 ± 0.44L. This was not statistically significant (P = 0.05) from the volume of distribution of salicylic acid administered alone.

The rate of transfer of salicylic acid into the the tissues was a mean ± S.E.M. of 0.13 ± 0.04hr^{-1}.This was not statistically different from those of the earlier studies.The rate of return of salicylic acid to the blood was a mean ± S.E.M. of 0.23 ± 0.03hr^{-1}. The difference between this value and those of the earlier studies was also not statistically significant (P = 0.05)

7.2.3.3 ELIMINATION

The mean ± S.E.M. pseudo-distribution constant for elimination was 0.09 ± 0.01hr^{-1}.This was not different (P = 0.05) from those calculated in the earlier studies. The rate constant from the central compartment was a mean ± S.E.M. of 0.13 ± 0.01 hr^{-1}. This represents no difference (P = 0.05) from the earlier studies.

7.2.3.4 AREA UNDER THE CURVE

The area under the curve was a mean ± S.E.M. of 470 ± 39.7 $mghL^{-1}$.It was not statistically different (P = 0.05) from ealier studies.

7.2.3.5 TIME TO ATTAIN MAXIMUM CONCENTRATION (Tmax)

Peak serum salicylic acid concentration was a mean ± S.E.M. of 1.7 ± 0.1hr.This was not statistically different (P = 0.05) from those of earlier studies.

7.2.3.6 MAXIMUM SERUM SALICYLIC ACID CONCENTRATION (Cmax)

The mean ± S.E.M. peak salicylic acid concentration was 42.8 ± 3.8 mgL^{-1} . This represents a statistical difference from the mean peak concentration obtained when aspirin was administered with paracetamol.

7.2.4 STATISTICS

Table XXIII summerises the outcome of statistical tests on the above presented parameters.The absorption rate of aspirin differed significantly (P =0.01) (see dicussion) between aspirin administered alone and the other two treatments.

7.2.5 CORRESPONDING KINETICS OF PARACETAMOL

7.2.5.1 The absorption of paracetamol was apparently first order.In the absorption phase of two subjects paracetamol concentrations tended towards saturation kinetic behaviour.This caused marked deviations from one-compartment and/or two-comparment linear dispositon models.With these subjects there was rapid rise in serum paracetamol concentrations approximately 40 mins after drug administration.The peak concentration in one of the subjects was 24.0 µg/ml and in the other it was 10µg/ml.The paracetamol kinetics in the subjects whose

peak paracetamol concentrations were below 6 μg/ml apparently were described by two-compartment linear disposition characteristics.These included simultaneous first order absorption.The other kinetic properties were:

7.2.5.2 DISTRIBUTION

The volume of distribution was 29.5 - 30L, apparently depending on the acting concentration of salicylic acid.

The rates of transfer of paracetamol into tissues were 0.06 - 0.04 hr^{-1} while the rates of return to the blood were o.69 - 0.7 hr^{-1}.

7.2.5.3 ELIMINATION

The pseudo- distribution rate constant for paracetamol elimination was 0.51 - 0.56hr^{-1} while the rate constant for elimination from the central compartment was 0.70 - 0.69hr^{-1}.

7.2.6 CORRESPONDING KINETICS OF INDOMETHACIN

The appearance of indomethacin in the blood was relatively slow in all but one subject.In general the kinetics tended to be eratic in the early intervals before the 30 min sample.

After 40 mins stable kinetics were attained in most subjects.The over-all concentrations determined up to 6h were apparently gaussian distributed.When pooled and resolved into the kinetic components the sample of the population disposition parameters were analysable by a two-comparment disposition model with simultaneous first order absorption kinetics.

7.2.6.1 ABSORPTION

The absorption rate constant was $1.14hr^{-1}$.

7.2.6.2 DISTRIBUTION

The volume of distribution was 11.5L.

7.2.6.3 ELIMINATION

The pseudo-distribution constant for elimination was $0.4hr^{-1}$ while the elimination rate from the central compartment was $0.37hr^{-1}$.

Subject / Parameter	1	2	3	4	5	6	7	Mean ± S.E.M
Ka hr^{-1}	0.85	0.73	0.72	0.61	0.76	0.82	0.76	0.75 ± 0.03
B hr^{-1}	0.11	0.04	0.06	0.14	0.04	0.09	0.05	0.08 ± 0.01
Vd L	7.2	12.3	9.2	5.6	9.4	7.9	9.1	8.6 ± 0.79
$K_{10} hr^{-1}$	0.15	0.06	0.11	0.17	0.08	0.13	0.11	0.12 ± 0.01
$K_{12} hr^{-1}$	0.04	0.03	0.09	0.01	0.14	0.04	0.14	0.07 ± 0.02
$K_{21} hr^{-1}$	0.17	0.08	0.18	0.17	0.18	0.14	0.16	0.15 ± 0.01
AUC $mghL^{-1}$	350	500	383	410	581	391	423	434 ± 30.11
tmax hr	2	4	2	2	3	2	2	2.4 ± 0.30
Cmax mgL^{-1}	48.6	33	30.2	46.6	29.0	44.0	31.2	37.5 ± 3.21

TABLE XX : Kinetic Parameters of Salicylic acid when only aspirin 652mg is taken.

Subject / Parameter	1	2	3	4	5	6	7	Mean ± S.E.M
Ka hr^{-1}	0.89	1.0	0.98	1.1	0.89	1.1	0.98	0.99 ± 0.03
B hr^{-1}	0.10	0.05	0.09	0.14	0.07	0.05	0.07	0.08 ± 0.01
Vd L	7.4	9.1	8.6	4.9	8.9	9.1	7.8	7.97 ± 0.57
K_{10} hr^{-1}	0.15	0.1	0.15	0.22	0.11	0.09	0.12	0.13 ± 0.02
K_{12} hr^{-1}	0.05	0.16	0.10	0.23	0.03	0.18	0.07	0.11 ± 0.03
K_{21} hr^{-1}	0.19	0.21	0.22	0.28	0.12	0.23	0.16	0.20 ± 0.02
AUC $mghL^{-1}$	349	465	278	381	406	522	455	408 ± 30.69
tmax hr	2	3	2	1.5	2	1.5	2	2.0 ± 0.19
Cmax mgL^{-1}	43.4	31.0	34.1	54.0	41.2	31.0	42.1	39.54 ± 3.12

TABLE XXI : Parameters of Salicylic acid when aspirin 652mg is taken with paracetamol 1000mg

Subject / Parameter	1	2	3	4	5	6	7	Mean ± S.E.M
Ka hr^{-1}	1.1	1.14	1.23	1.01	1.38	1.14	0.98	1.14 ± 0.05
B hr^{-1}	0.11	0.05	0.07	0.12	0.08	0.05	0.12	0.09 ± 0.01
Vd L	7.4	7.6	6.9	6.5	6.4	9.7	6.4	7.27 ± 0.45
K_{10} hr^{-1}	0.15	0.10	0.13	0.11	0.15	0.09	0.18	0.13 ± 0.01
K_{12} hr^{-1}	0.05	0.27	0.25	0.001	0.21	0.12	0.06	0.13 ± 0.01
K_{21} hr^{-1}	0.21	0.30	0.32	0.11	0.30	0.18	0.21	0.13 ± 0.04
AUC $mghL^{-1}$	339	622	518	527	472	487	328	470 ± 39.69
tmax hr	1.5	1.5	1.5	2.0	1.65	2.0	2.0	1.71 ± 0.10
Cmax mgL^{-1}	43.0	30.0	35.4	60.1	44.9	36.4	50.0	42.83 ± 3.83

TABLE XXII : Kinetic parameters of salicylic acid when aspirin 652mg is taken with indomethacin 100mg

DRUG	$Kahr^{-1}$			Bhr^{-1}			VdL		
ASA = 1	1			1			1		
ASA/PC = 2		2			2			2	
ASA/IND = 3			3			3			3
PARAMETERS									
Mean	0.75	0.99	1.14	0.08	0.08	0.09	8.7	8.0	7.3
W	14	15	7	12	12	12	28	18	10
P(W)	1.00	0.93	1.00	0.8	0.8	0.8	0.02	0.55	0.55
t2:1 ; t3:1	-	5.17	6.14	-	0.34	0.51	-	0.74	1.49
P(t)	-	.002	.009	-	0.74	0.63	-	0.49	0.19
t3:2	-	-	2.05	-	-	0.48	-	-	1.27
P(t3:2)	-	-	0.09	-	-	0.65	-	-	0.25
S 2:1	-	Y	-	-	N	-	-	-	-
S 3:1	-	-	Y	-	-	N	-	-	N
S 3:2	-	-	N	-	-	N	-	-	N
P Diff	-	0.05	0.05						

TABLE XXIII (a) Statistical analysis of absorption and distribution of aspirin/salicylic acid administered alone and in combination with paracetamol or indomethacin

W = Sum of signed rank deviations about mean
P(W) = two-sided Wilcoxon test for the parameters' probability distribution.

t = Students t value (single tail)

P(t)=one-sided t-test for the parameters' probability distribution.

DRUG PARAMETERS	$K_{10}hr^{-1}$			$K_{12}hr^{-1}$			$K_{21}hr^{-1}$		
	1	2	3	1	2	3	1	2	3
Mean	0.12	0.13	0.13	0.07	0.12	0.13	0.15	0.20	0.23
W	13	12	10	14	13	13	19	14	15
P(W)	0.8	0.8	1.0	1.0	0.93	0.93	0.45	1.0	0.93
t2:1 ; t3:1	-	1.01	0.81	-	1.19	1.32	-	1.86	2.31
P(t)	-	0.35	0.45	-	0.28	0.24	-	1.86	2.31
t3:2	-	-	0.21	-	-	0.38	-	-	0.73
P (t3:2)	-	-	0.84	-	-	0.32	-	-	0.49
S 2:1									
S 3:1			N			N			N
S 3:2			N			N			N
P Diff									

TABLE XXIII (b) Statistical analysis of elimination and distribution of aspirin/salicylic acid administered alone and in combination with paracetamol.Sybols are as defined in table XXIII(a)

DRUG PARAMETERS	AUC $mghL^{-1}$			tmax hr			Cmax mgL^{-1}		
	1	2	3	1	2	3	1	2	3
Mean	434	408	470	2.4	2.0	1.7	37.5	39.5	42.8
W	12	13	17	13	3	18	16	13	14
P(W)	0.80	0.93	0.67	0.93	1.0	0.55	0.8	0.93	1.0
t2:1	-	0.67	0.90	-	2.52	1.99	-	0.39	0.88
P(t)	-	0.53	0.40	-	0.05	0.09	-	0.71	0.41
t3:2	-	-	1.29	-	-	1.08	-	-	2.55
P(3:2)	-	-	0.24	-	-	0.32	-	-	0.04
S 2:1	-	-	-	-	-	-	-	-	-
S 3:1	-	-	N	-	-	N	-	-	N
S 3:2	-	-	N	-	-	N	-	-	N
P Diff									0.05

TABLE XXIII (c) Statistical analysis the disposition derived parameters of aspirin/salicylic acid administered alone and in combination with paracetamol or indomethacin. Symbols are as defined in table XXIII (a).

8 DISCUSSION AND CONCLUSION

Aspirin as a parent drug and the commonly used anti-inflammatory drugs presented in this thesis are antinociceptive when given alone or in combination. However,this is against the background of multifaceted interaction of salicylates with various autacoids,notably prostaglandins as mediators of pain and inflammation. The pharmacodynamic implications of combined administration of aspirin with paracetamol or indomethacin are therefore dependent on the peculiar pharmacokinetic characteristics of the individual drugs.This is apparently supported by the findings in this study (which see 8.2.)

8.1 DRUG DETERMINATION BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

HPLC was an efficient and convenient method for the simultaneous determination of aspirin and paracetamol or indomethacin.The sensitivity and reproducibility of drug analysis were adequate for the concentrations of these compounds as found in this study.

Periodic injections of extracted standards ensured in-process checks on the system from reagents to the detection properties by comparison with calibration values determined prior to the analysis of subject samples.Cross-contamination by previous injections was

continually monitored by injection of methanol.Rarely was any memory effect detected.When it occured it was after an injection of relatively high concentration of the analyte standard.The peak even in this case was so low and as such was of little effect.This was well before actual subject samples were analysed.Injection of methanol at the end of each set of plasma extracts showed no such effect.With the redissolution of the extracts in the elution solvent no trace of cross-contamination was found.

Paracetamol,aspirin and salicylic acid eluted well from the column under isocratic conditions,giving complete separation in 11 mins.Attempts to use the same eluent for other anti-inflammatories was not successful due to low sensitivity and/or long elution time (>25mins).After modification of the solvent system for use in the separation of ten of the most commonly used anti-inflammatories indomethacin was eluted in 15 mins but the sensitivity was low.Further alteration of the solvent to improve sensitivity prolonged the elution time.Also in the serum extracts an unknown endogenous compound interfered with the indomethacin peak.When the solvent mixture was altered to accommodate this interference and also with minimal work-up procedure the elution time was 25 mins.Even so, the peak tailed unacceptably.Only few

aliquots of the subject samples were analysed for salicylic acid for purposes of inter-analytical comparison.Gradient elution of indomethacin with aspirin was therefore developed and used for their analyses.

Simultaneous analyses of indomethacin and salicylic acid was achieved at calibration concentration range balanced between the sensitivities of both drugs at the concentrations found in the samples.At higher sensitivities of both drugs a multiplier effect increased the background noise.Addition of 0.5ml of distilled water to the serum prior to acidification and extraction reduced the noise with minimal dilutional effects on the extraction performance.

The problems of simultaneous determination of the co-administered drugs could be circumvented by analysing for each drug separately.However, the economy of sample volume would have been sacrificed for that alternative. The analytical protocol in this study obviated the necessity for excessive blood withdrawal thus allowing the trial to proceed within reasonable ethical limits. Furthermore,with the solvent system being of relative wide application to commonly used anti-inflammatory drugs concurrent taking of such drugs during the period of study would be observed.The overall performance of the

HPLC systems was a sustained drive during the drug analysis.

8.2 PHARMACOKINETICS

The effect of paracetamol or indomethacin on aspirin _in-vivo_ kinetics was biphasic.One phase represents the first concentration-time curve of the faster absorbed of the two concurrently administered drugs. Subsequent absorption of the second compound yielded concentration-time curves which when resolved into disposition phases indicate apparent reduction in the volume of distribution of aspirin. This could have led to higher serum concentrations manifested as an increase in the absorption rate with reference to salicylic acid. Possibly,the absorption rate of salicylic acid could have been increased by reduced salicylate residence time within the gastric mucosa. Both of these effects are related in as much as competition for sites of lodgement on proteins including enzymes could reduce the tissue distribution as well as enzyme contact time.Both of these are probably concentration-dependent.This is indicated by the rapid rise of paracetamol concentration when the concentration of salicylic acid was appreciably high in serum.The metabolic clearance of both compounds,perhaps via a common glucuronide pathway could be reduced under

these kinetic conditions.This pathway exhibits an appreciable degree of saturability [127-129] so that at appropriate concentration levels a reduced elimination of the parent compounds would manifest as inreased drug concentrations. For salicylic acid this effect may not be sustained for a long period since other competing parallel first order elimination processes occur simultaneously.Paracetamol on the other hand would be affected more than aspirin being relatively eliminated mainly by glucuronidation.The situations arise out of the <u>in-vivo</u> kinetic properties of aspirin and paracetamol as shown in figure 22 [130].

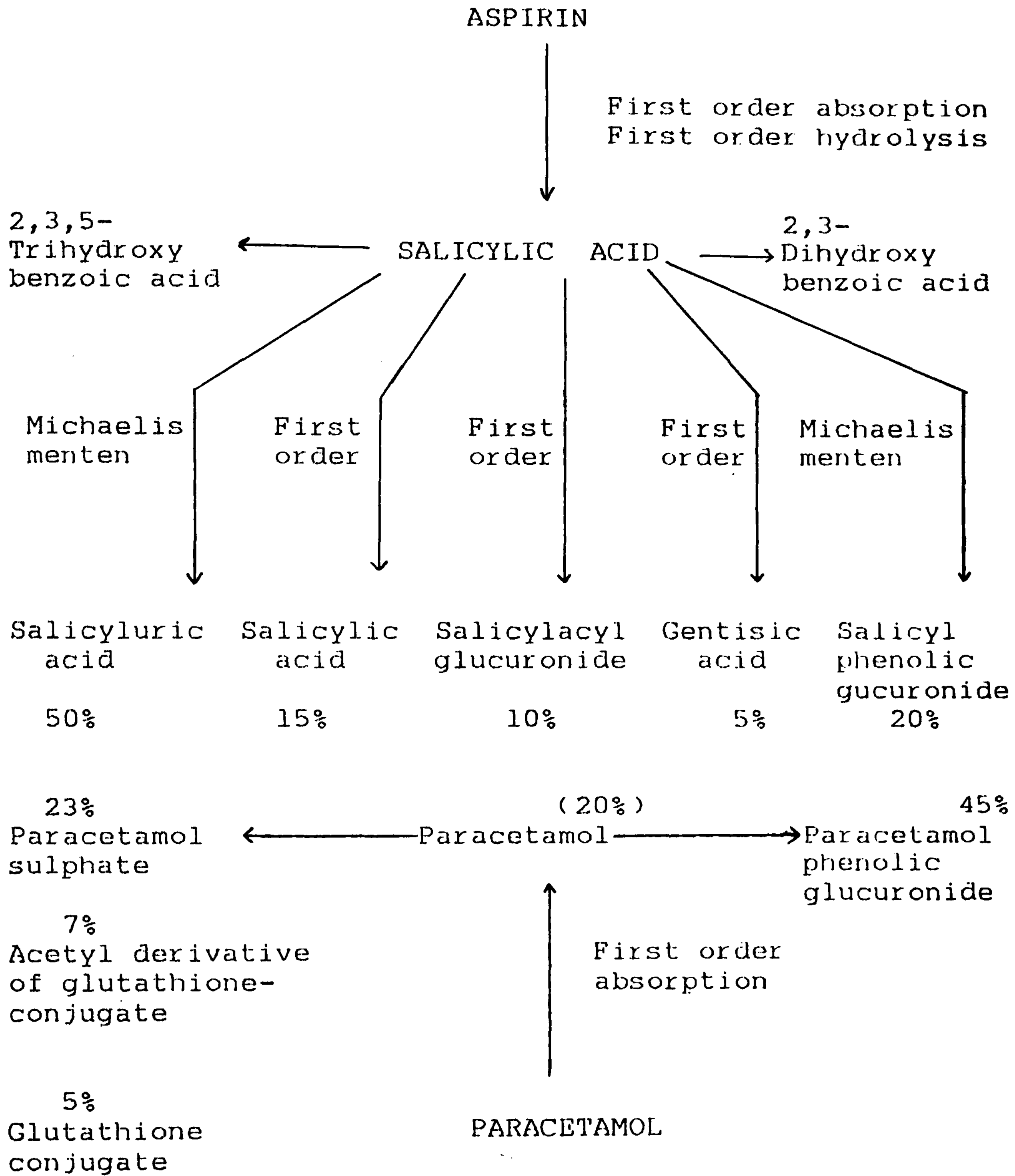


Figure 22 : Simultaneous Dispositon of Paracetamol and the Characterised Kinetics of Aspirin.

A similar situation also occurs during the glucuronidation of indomethacin metabolites,O-desmethyldeschlorobenzoyl indomethacin (DMBI), deschloro-benzoylindomethacin (DBI) and 4-chlorobenzoic acid. However,this would most likely occur at a period later than the case with paracetamol.This supports the relative higher concentrations of salicylic acid which was generally apparent when aspirin was administered with indomethacin.

More recently,evidence has been obtained for the lowering of cytochrome P450 activity by indomethacin in rats [131].If this situation is also true in man then it would be in agreement with the findings in this study i.e. reduced elimination of salicylic acid .

Some reports indicative that paracetamol or indomethacin inhibits the hydrolysis of aspirin was not supported by the findings in this study.In the study of Rylance and Wallace [132] very high concentrations (paracetamol 286μg/ml,indomethacin : 25μg/ml) inhibited aspirin esterase.In this study no intact aspirin was found after 30 mins in the presence of paracetamol (5-15μg/ml) or of indomethacin (0.3 - 2.5μg/ml) in serum.It would appear that the concentrations that are inhibitory on aspirin esterases approach toxic levels as in the therapy of

rheumatoid arthritis.Nevertheless,the concentration of paracetamol that exerted inhibitory effect on aspirin esterase was in the hepatotoxic range [133].

Levy and Regardh found no difference in the urinary elimination rates of either paracetamol or salicylic acid from the body after the administration of 1g salicylic acid at 2hr and 0.5g at 4hr in the presence of paracetamol [134].Of the three subjects who participated in that study one subject had an increase in the free paracetamol eliminated in urine while two did not.To this extent their study was at variance with this investigation.In our study there were some subjects whose serum salicylic acid concentrations did not differ between the administration of aspirin with paracetamol. More intriguing in this study is the comparatively lower dose of aspirin administered with paracetamol. Also the effect of paracetamol on salicylic acid kinetics was predominantly in the absorptive phase.In this respect the findings in this study agree with the study of Levy and Regardh.

A number of trials have been performed that report the effect of indomethacin on serum salicylate concentrations.Champion <u>et al</u> found a non-significant difference between the half-life of indomethacin alone

(1.82hr) and in combination with aspirin (2.24hr) at a dose of 3.6g of aspirin [58].The dose of aspirin is however characterised by saturable kinetics.Lindquist _et al_ administered indomethacin suppositories (100mg) and aspirin (3.6g) to 33 patients and found no effect of each compound on the serum concentrations of either [135]. Kaldestad _et al_ showed that indomethacin suppository did not affect the concentration of salicylic acid after concurrent oral administration of salicylate [136]. They found a statistically but not biologically significant difference between the serum half-lives of indomethacin administered as suppositories.These reports lend strong support to the indication of this study that prehepatic and hepatic elimination of salicylic acid generally tend to be reduced with the dosage form and dosages of the compounds employed in this study.The clinical significance of the study reported by Brooks _et al_ who administered concurrent oral doses of indomethacin (50mg) and aspirin (500mg) - doses a little less than those in our study - was indicative of increased systemic effects. These were manifested as headache or light-headedness [137].This, however,was not clinically prohibitive relative to the the administration of indomethacin alone. Moreover,they found no difference in indomethacin concentrations,salicylate concentrations incidentally was

not reported nor were kinetic constants.

8.3 THERAPEUTIC IMPLICATIONS OF THE FINDINGS IN THIS STUDY

8.3.1 ANTICIPATED FASTER ONSET OF ACTION : Analgesia,antipyresis.

Suppression of fever and pain with a faster onset of action appears to be a positive corollary of the hypothesis that apparent absorption rate (with reference to salicylic acid) is slightly higher with concurrent administration of aspirin with indomethacin. However, this cannot always be expected to occur in every individual.This is due to the anticipated variability in drug metabolising capacity of individuals.When dosage forms other than liquid preparations are administered the kinetics of the specific drugs would certainly differ. By reducing the perfusion of paracetamol or salicylic acid into hepatocytes or interhepatocellular spaces more of either compound would reach hyperalgesic foci, at a faster rate with accompanying dynamic action [79,81].This is in the sense that the compounds provide cyclo-oxygenase-inhibitory effects at different anatomical sites of inflammation.However,inflammatory conditions associated with rheumatism may not be suppressed without a high risk of toxicity.

8.3.1 NO INDICATION OF NEPHROTOXICITY - AS PER KINETIC DISPOSITION

The lack of difference in the rates of elimination observed in this study indicates no potentials for analgesic nephropathy.However,these studies have been performed under unsteady-state kinetics and as such imposes limitations on the inferences made therefrom [138].

8.3.3 REDUCED MUCOSAL DAMAGE

The indication of possible reduction in the residence time of aspirin within the gastric mucosa might favour the combined administration of both paracetamol and aspirin for the occasional suppression of fever and/or pains such as headache.

Paracetamol was shown to reduce the erosive activity of indomethacin and of aspirin in the rat stomach [139-140]. These actions of paracetamol were not due only to topical-physical protection. Rather , cyclo-oxygenase pathway was stated to be also involved.It would appear that the reduced tissue perfusion of salicylic acid in the absorption phase is also part of site-specific interplay of both compounds in dynamic action.Graf et al, using autoradiography showed that acidic non-steriodal

anti-inflammatory drugs accumulate in inflammed tissues as a pharmacokinetic process preceding the suppression of inflammation [141].The relative contributions of this process towards the observed,though less marked increase in serum concentrations during absorption when aspirin was concurrently administered with paracetamol or indomethacin is perhaps of additive consequence.This is clearly in concert with dynamic influences of both compounds,evident in the findings on the electropotential difference changes induced by a selection of such compounds [142].

8.3.3.1 EFFECTS ON ELECTROPOTENTIAL DIFFERENCE ACROSS THE GASTRIC MUCOSA

The study of Murray et al [142] showed that ethanol or aspirin or both (with additive effect) lowered electropotential differences across the gastric mucosa in man.Such effects were not demonstrated with phenylbutazone or indomethacin both of which are occasionally implicated in gastric injury.This observation supports the contra-distributive kinetics as observed in this study.Similar observations were also made with paracetamol and indomethacin each administered concurrently with aspirin [143].

8.3.3.2 CONCLUSION

The findings in this study indicate that the occasional user of aspirin would benefit from combined administration of the the drug, particularly when administered as suspension with paracetamol or indomethacin. An additive effect would be a likely outcome since the combination leads to elevation of serum levels sufficient to be within the therapeutic range for mild to moderate pains.However,any additive action would be short-lived as concentrations quickly return to normal levels.

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I.R. Tebbett, C.I. Omile and B. Danesh
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