# ASSESSMENT OF THE VALUE OF HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY FOR THE DETECTION AND CHARACTERISATION OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

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# DEDICATION

*These studies are dedicated to*

*Wendy, Sarah and Anna*

# *ASSESSMENT OF THE VALUE OF HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY FOR THE DETECTION AND CHARACTERISATION OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS*

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## *ABSTRACT*

A general screening method for the detection and characterisation of drugs and their metabolites in biological fluids based upon high-performance thin-layer chromatography (HPTLC) has been developed, and its potential value in drug research and development has been assessed.

Novel linear multiple development gradients using normal-phase chromatography on silica gel HPTLC plates were developed. The need for linear gradients was shown by an examination of the elutropic strength profiles of a number of "universal" Automated Multiple Development gradients, which each contained an abrupt change in elutropic strength at which many components of a complex mixture might cluster.

The hypothesis was proposed that particular metabolite types have a characteristic retention relative to their parent compound following development on linear normal-phase gradients. Model compounds were selected which were representative of the major metabolic pathways and included structural components, such as phenols, indoles, coumarins and biphenols, found in many drugs. A correlation was found between relative retention and metabolite type which supported the hypothesis described above.

Thus "rules" were proposed concerning the ranges of relative retention which were characteristic of particular metabolic changes. The rules showed the potential of HPTLC with linear multiple development for predicting the structures of unknown metabolites from their retention data.

Biological fluid samples required extracting prior to HPTLC analysis. A general solid-phase extraction procedure was developed using Bond Elut Certify ™ II cartridges with mixed reversed-phase and anion-exchange properties. Following their retention from plasma and urine, drugs and their metabolites were eluted in three different eluents which provided some discrimination between classes of compound. Non-acidic and some weakly acidic components were eluted first followed by weakly acidic components, including glucuronides, in the second eluent and finally strongly anionic components, including sulphates. Extraction efficiencies from plasma in excess of 90% were obtained for most model compounds examined, with limits of detection in the range 1 to 10$\mu$g/ml.

The proposed rules were tested by analysing samples of plasma and urine obtained following oral administration of ibuprofen and loxtidine to dogs and rats, and antipyrine and diazepam to rats. The data obtained, in terms of the extent of Phase I or Phase II metabolism which occurs, were in broad agreement with published data. Thus species differences in the metabolism of ibuprofen and loxtidine were detected. In a number of cases the type of metabolite formed was predicted correctly using relative retention data.

The screening method will provide data on the extent of drug metabolism and indicate the number of metabolites formed. The structures of metabolites can be proposed from their relative retention data and their properties during sample extraction. The present studies indicated that a reasonable likelihood of successful structural assignment can be expected. The method may be applied to the analysis of plasma and urine samples following administration of drugs to animals at high dose-levels, and may provide valuable early information in drug research and development programmes in the future.

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# *CHAPTER ONE*

# *GENERAL INTRODUCTION*

## *1.1 Introduction*

Considerable effort is expended to study the effects of chemicals upon biological systems in order, for example, to control the environment, improve quality of life, combat disease and increase productivity. Thus in the areas of ecology, medicinal research and agrochemical research, chemicals are sought for use as herbicides, fungicides, drugs and pesticides. The effect of chemicals on biological systems may be exerted by interaction with enzymes, genes or receptors resulting in a change to the biochemistry of the system. Highly selective and potent chemicals may be found which act specifically and effectively on the biological system to effect the control required.

In studying the effect of chemicals upon biological systems, the effect of the system on the chemical itself cannot be neglected. An active chemical may be rendered inactive by interaction with the metabolic pathways within the system. Thus an understanding of the means by which biological systems metabolise foreign chemicals is important to the development of effective products.

In this thesis, the application of drug metabolism studies within drug research and development is considered, and the value of high-performance thin-layer chromatography for the detection and characterisation of drugs and their metabolites in biological fluids is assessed.

## *1.2 Drug Metabolism*

The pharmacological action of a drug is related to the concentration of the drug at the site of action, which may be a receptor, an enzyme, a gene or an organism. The concentration of drug at the site of action cannot usually be measured directly, therefore it is estimated by measuring the concentrations of drug in the systemic circulation. Pharmacokinetic analysis is used to measure exposure to the drug, the extent to which drug is distributed throughout the body and the rate at which drug is removed from the systemic circulation. The processes of absorption, distribution, metabolism and excretion (ADME) describe the fate of a drug following administration. These processes involve input of the drug into the systemic circulation, transfer of the drug from the blood into other tissues within the body and elimination of the drug from the body. The processes of metabolism and excretion both contribute to elimination.

The most facile route for drug elimination is by excretion of the unchanged drug via the urine. Foreign compounds are removed from the blood by glomerular filtration, and flow down tubules in the kidney which are lined with epithelial cells packed closely together to form a lipid membrane between the blood and urine. The membrane allows transfer of lipid-soluble, non-ionised molecules, therefore lipid-soluble compounds which are present in the glomerular filtrate in the non-ionised form are re-absorbed into the bloodstream.

The physico-chemical properties of molecules, in particular whether they are acids or bases, their pKa and their lipophilicity determine whether they are subject to re-absorption from the kidney tubules. Strong acids and bases are highly ionised at

physiological pH and are therefore excreted in the urine readily without tubular re-absorption taking place. However, many drugs are weak organic acids or bases, and at physiological pH are present partly in the non-ionic form thus being subject to tubular re-absorption. Furthermore, the pH of urine is variable, usually in the range pH5 to pH8, and can therefore be a significant factor which determines whether a compound is excreted in the urine or re-absorbed.

Biliary excretion is another important means by which foreign compounds are removed from the body. The boundary between blood and bile is a porous membrane which allows the passage of most molecules and ions which are smaller than proteins. Thus drugs may appear in the bile in concentrations similar to those found in the plasma. However highly polar compounds, including conjugated metabolites (see Section 1.2.1), are excreted in the bile in much higher concentrations by a process of active transport.

The processes of excretion alone do not provide an efficient means of eliminating all drugs from the body. It is therefore necessary for the body to modify drugs chemically to form compounds which are excreted more readily in the urine and bile. Drug metabolism is the process by which drugs undergo chemical modification by living organisms with the principal purpose of eliminating them from that organism.

The chemical modifications that take place to form metabolites generally increase the water solubility and acidity of a substance. Metabolites are therefore generally less susceptible to tubular re-absorption than their parent drug because of their lower lipophilicity, and because the presence of anionic groups on acidic metabolites means that they are highly ionised at physiological pH. Thus metabolites are usually more easily excreted via the urine or bile than the original substance.

The important contribution provided by metabolism to the overall elimination of some drugs is evident because without the process of metabolism their rate of disappearance from the body would be changed drastically. For example, the half-life of thiopental (penthiobarbital), a short acting anaesthetic, would be about 100 years if its elimination was by excretion of the unchanged drug alone (Brodie, 1964) which would make it useless as an anaesthetic!

In the following sections, the routes and mechanisms of drug metabolism are described, and kinetic considerations are discussed.

### 1.2.1 Metabolic Transformations

The chemical and biochemical aspects of drug metabolism have been described by Testa and Jenner (1976) and Low and Castagnoli (1991) with detailed information about the routes and mechanisms involved. Knowledge of drug metabolism is increasing rapidly with advances in instrumentation and technology enabling minor metabolic pathways to be identified which were previously unknown. Thus Jenner and Testa (1978) reviewed novel pathways in drug metabolism, and reviews have been published in recent years (Hawkins, 1989; Hawkins, 1990; Hawkins, 1991; Hawkins, 1992).

Foreign molecules which enter the body are often referred to as xenobiotics, a term first used by Mason, North and Vanneste (1965) from the Greek, xenos and bios for "stranger to life". The metabolic reactions undergone by xenobiotics can be classified into two main types. Phase I reactions involve the introduction of a new chemical group into a molecule, examples being hydroxylation, dealkylation, hydrolysis, deamination, and

oxidation. Phase II reactions involve conjugation of a drug or a Phase I metabolite with an endogenous molecule to form for example, a glucuronide, an ethereal sulphate, a glycine, or an acetyl derivative. The metabolites formed by Phase II reactions are often referred to as conjugates. Williams (1974) represented the basic pattern of xenobiotic metabolism as follows:

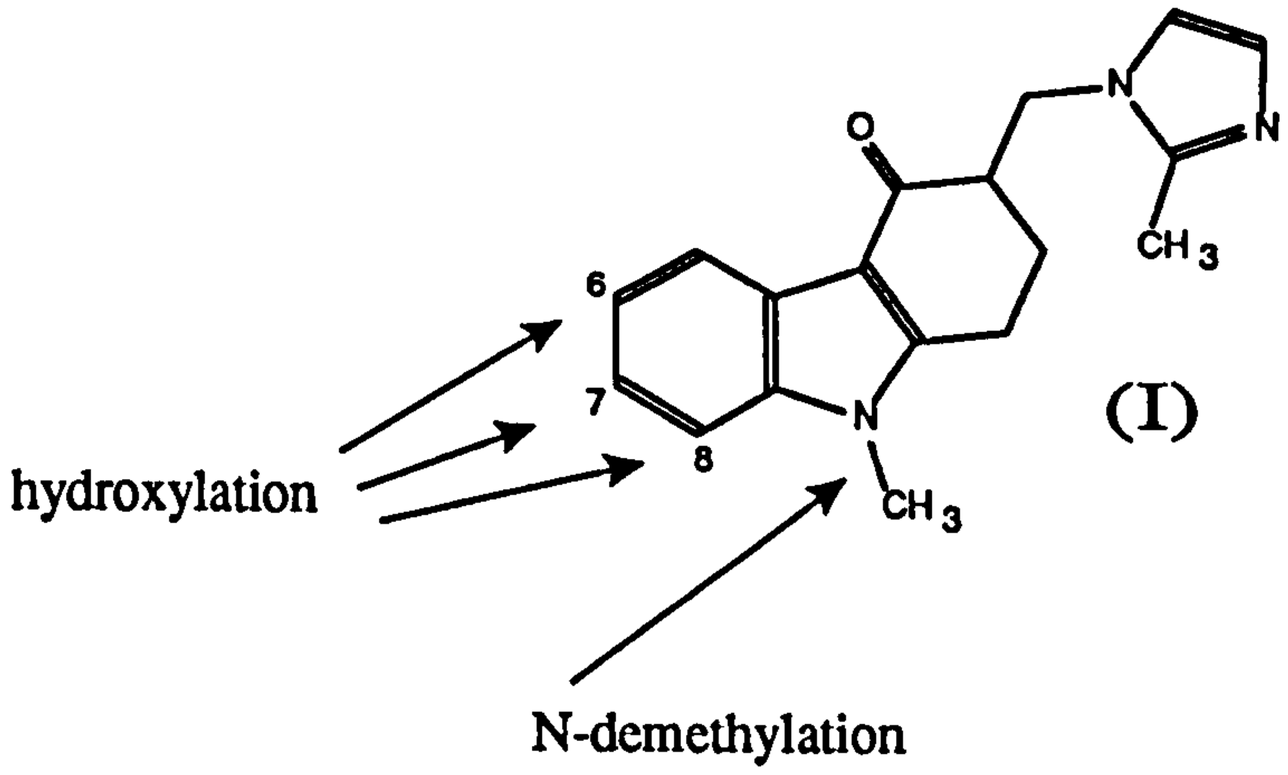
| | | | | |
|---|---|---|---|---|
| Xenobiotic | Phase I -------> enzymes | Oxidation, reduction, hydrolysis products | Phase II ------> enzymes | Synthetic or conjugation products |

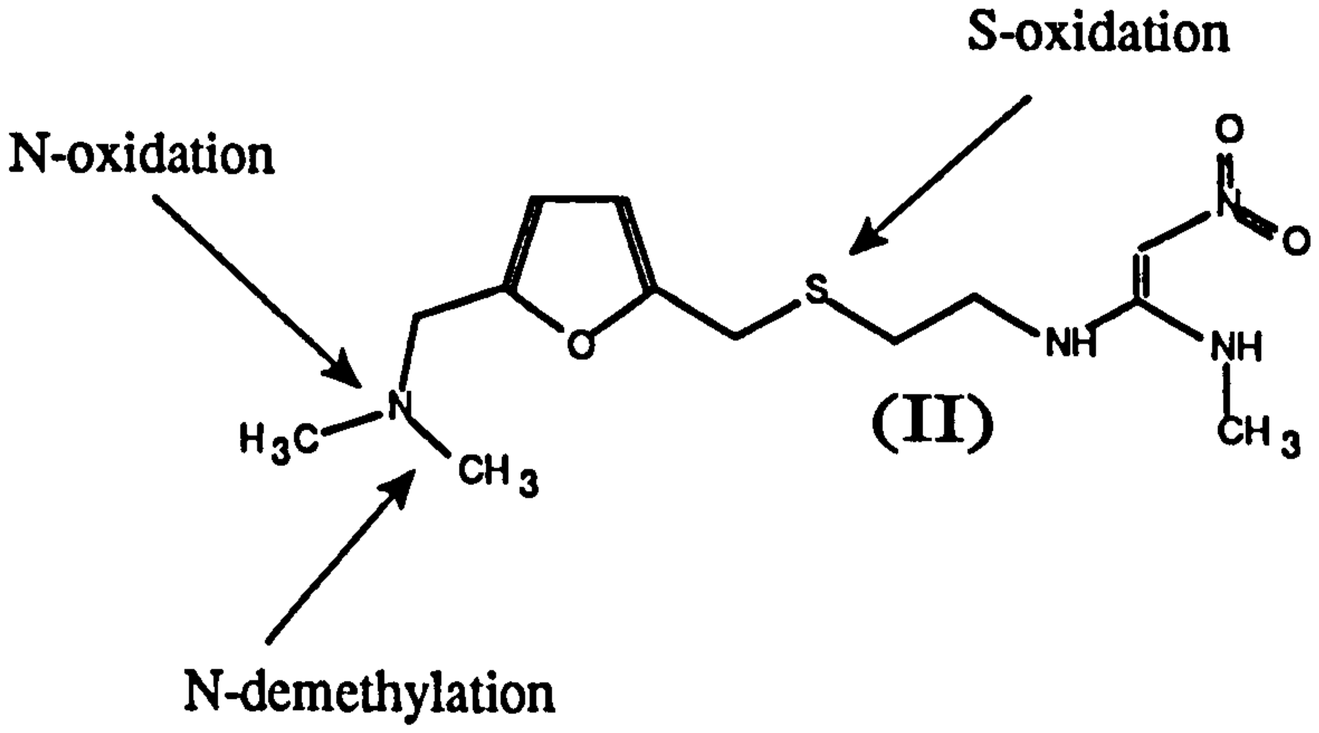
Examples of Phase I metabolic transformations are shown in **Figure 1.1.** Ondansetron (I), a 5-hydroxytryptamine$_3$ antagonist used in the treatment of chemotherapy and radiotherapy-induced nausea and vomiting (Stables, Andrews, Bailey, Costall, Gunning, Hawthorn, Naylor and Tyers, 1987) is metabolised by hydroxylation at the 6, 7 and 8 positions on the indole ring, and by N-demethylation of the indole nitrogen (Saynor and Dixon, 1989). Ranitidine (**II**), a histamine $H_2$-receptor antagonist used in the treatment of peptic ulcers (Brittain, Jack and Price, 1981) undergoes N-demethylation and is also oxidised to form N- and S-oxides (Bell, Dallas, Jenner and Martin, 1980). Salmeterol (**III**), a $\beta$-2 adrenoreceptor agonist used for the treatment of asthma (Ball, Coleman, Denyer, Nials and Sheldrick, 1987; Sandstrom, Frederiksen, Rosenhall and Sandstrom, 1989), is metabolised by O-dealkylation and aliphatic hydroxylation (Manchee *et al.*).

Examples of Phase II metabolic transformations are shown in **Figure 1.2.** Paracetamol, a widely used analgesic, is conjugated to form glucuronide and sulphate metabolites and is also metabolised via a reactive Phase I intermediate, N-acetyl-p-benzoquinoneimine

**Figure 1.1**

**Examples of Phase I metabolic transformations**



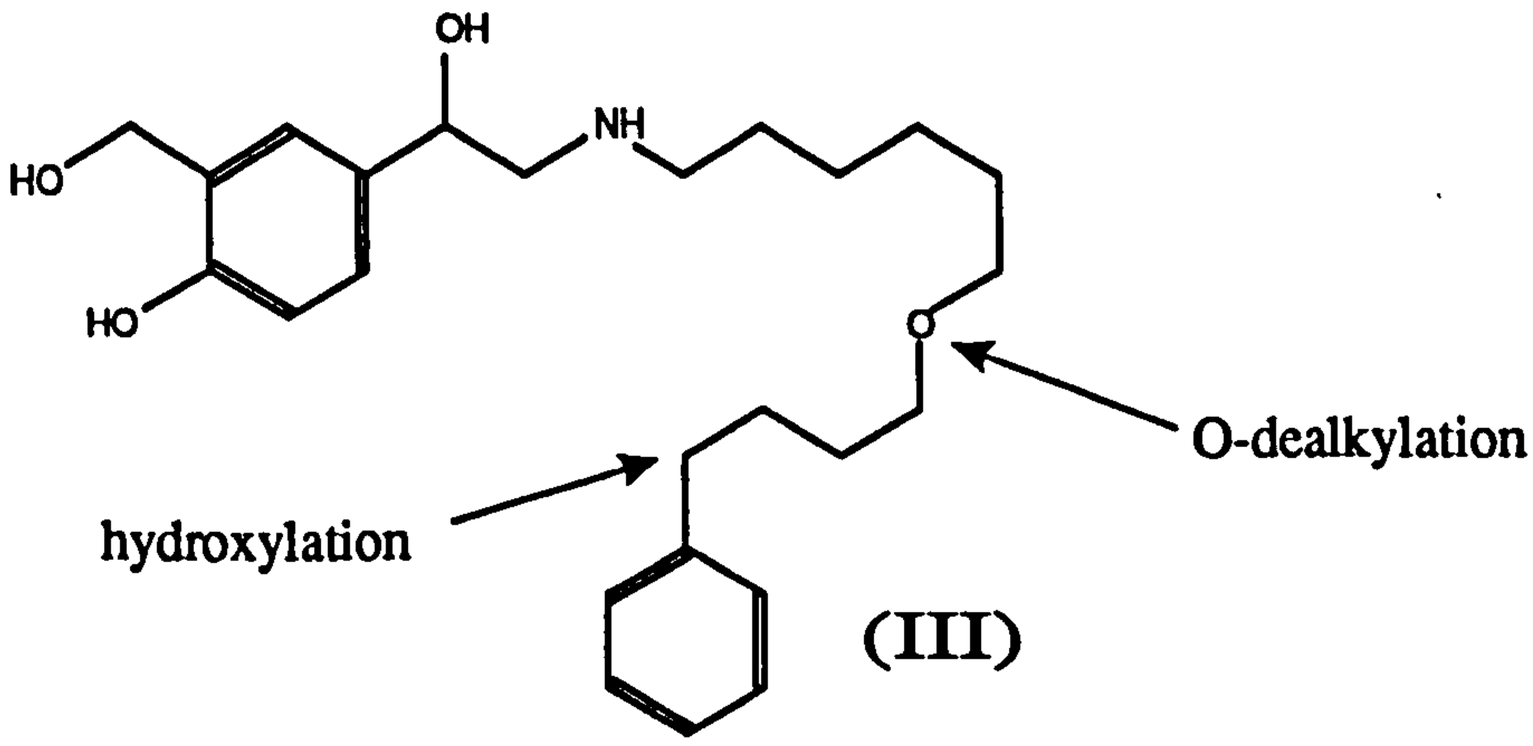
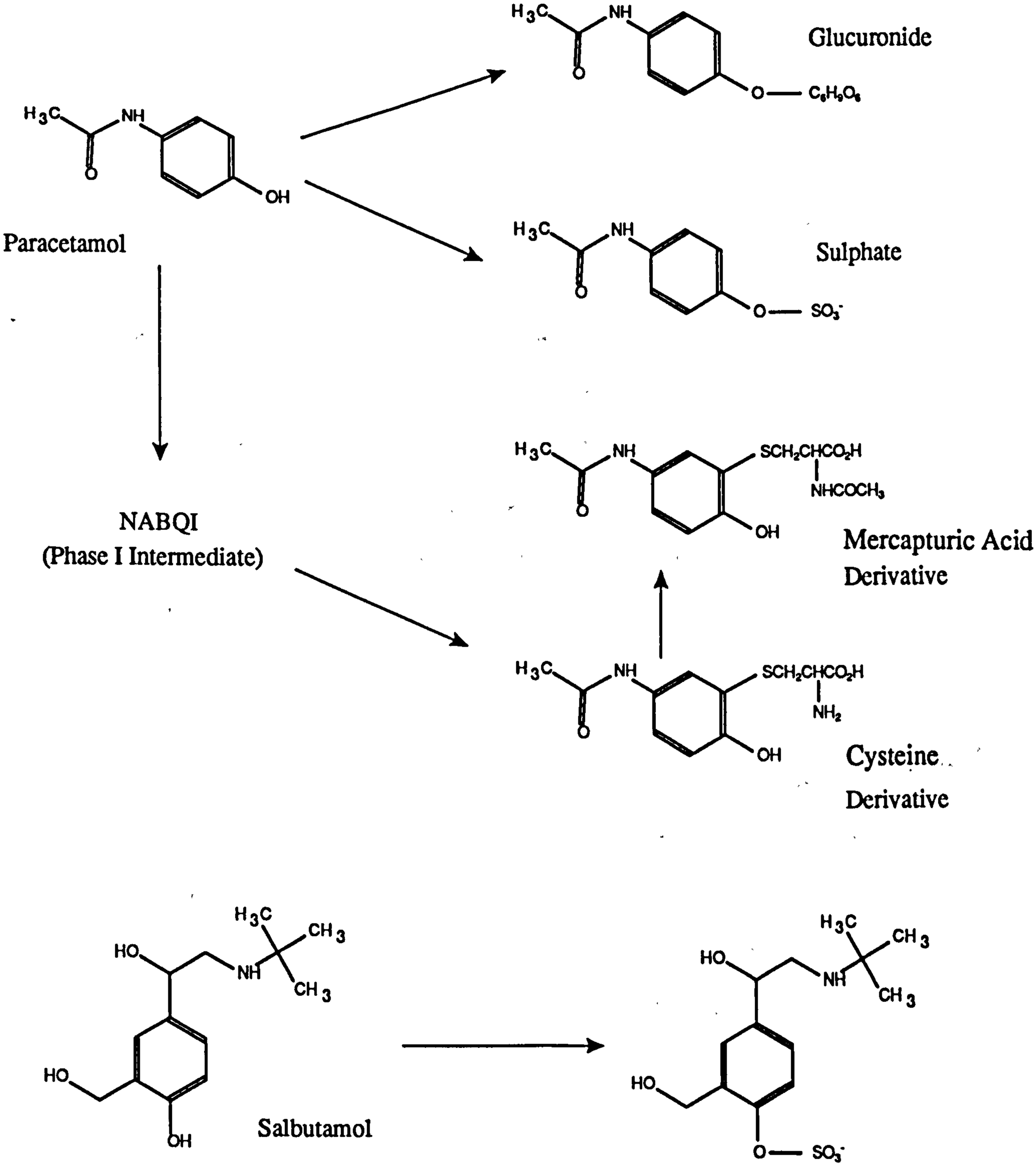



**Figure 1.2**

**Examples of Phase II metabolic transformations**



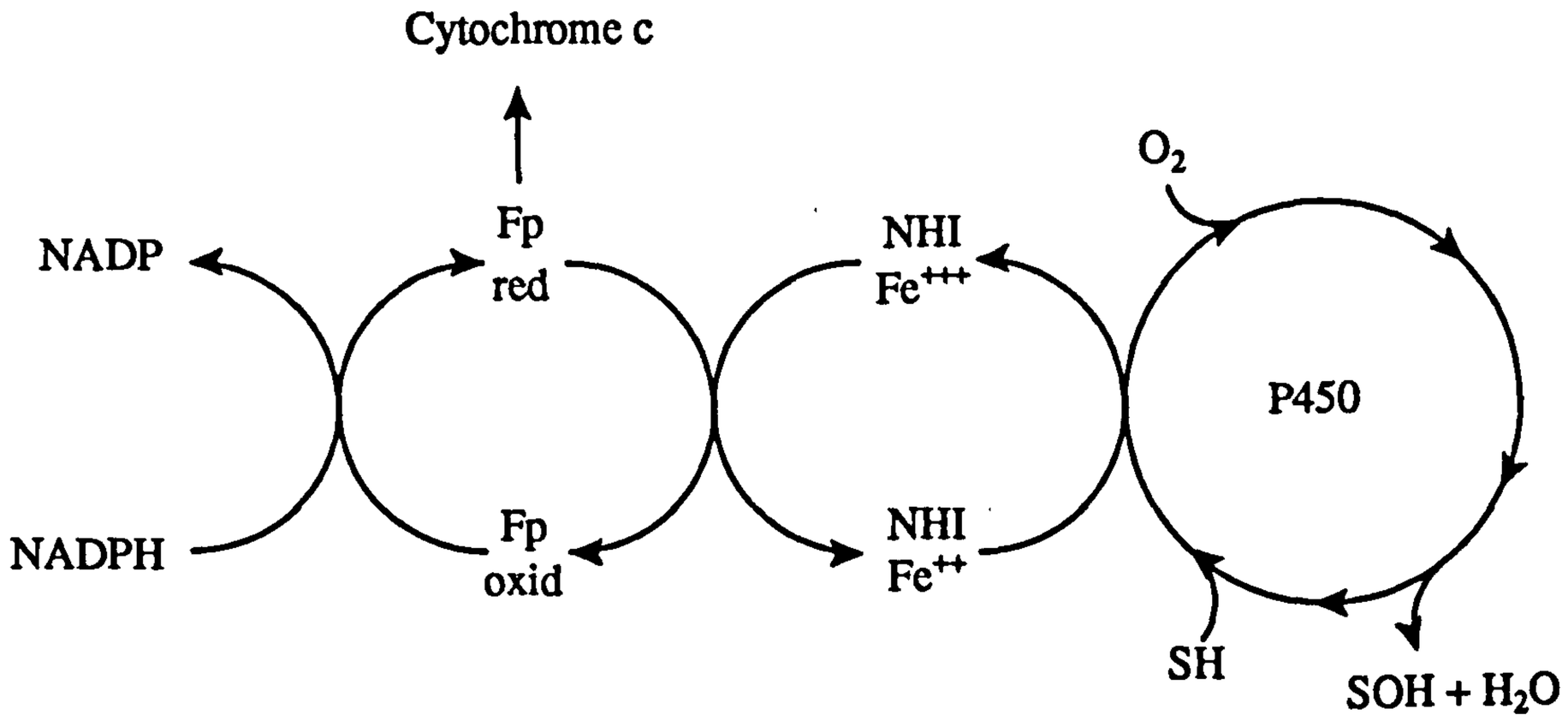
(NABQI) to form cysteine and mercapturic acid conjugates (Prescott, 1980). Salbutamol, a β-2 adrenoreceptor agonist widely used for the treatment of asthma, is metabolised to form a sulphate conjugate in man (Lin, Li, McGlotten, Morton and Symchowicz, 1977). The Phase I metabolites of ondansetron (**II, Figure 1.1**) are metabolised further by conjugation to form glucuronides and sulphates (Saynor and Dixon, 1989).

### 1.2.2 Mechanisms of Drug Metabolism

Metabolic transformations are catalysed by a variety of enzymes found particularly in the liver, but also in other tissues. The cytochromes P450 are a family of haemoproteins that catalyse the metabolism of a large number of diverse substrates by a variety of oxidative transformations. The reactions catalysed by the cytochromes P450 include aromatic and aliphatic hydroxylation, epoxidation, O-dealkylation, N-dealkylation and deamination, all of which involve the addition of an oxygen atom into the drug molecule (or the fragment which is lost in the case of N-dealkylation) to give a hydroxylated product. These have been reviewed recently (Guengerich, 1990). The electron transport systems involved in the oxidative processes carried out by these enzymes are shown in **Figure 1.3.** In recent years, numerous forms of cytochrome P450 have been isolated from humans and laboratory animals. A number of these have been sequenced and classified into families, for example CYP1, CYP2 and CYP3. It is now apparent that some P450s are specific for the metabolism of particular classes of substrate and some are implicated in the formation of reactive metabolites which may be precursors to chemical carcinogenicity. The current state of knowledge of the P450 metabolising enzymes has been reviewed recently (Gonzalez, 1992; Wrighton and Stevens, 1992; Soucek and Gut, 1992).

**Figure 1.3**

**The electron transport system involved in oxidations by P450 enzymes**




Fp: flavoprotein NADPH cytochrome c reductase

NHI: nonhaeme iron-containing protein

SH: substrate SOH: hydroxylated substrate (Product)

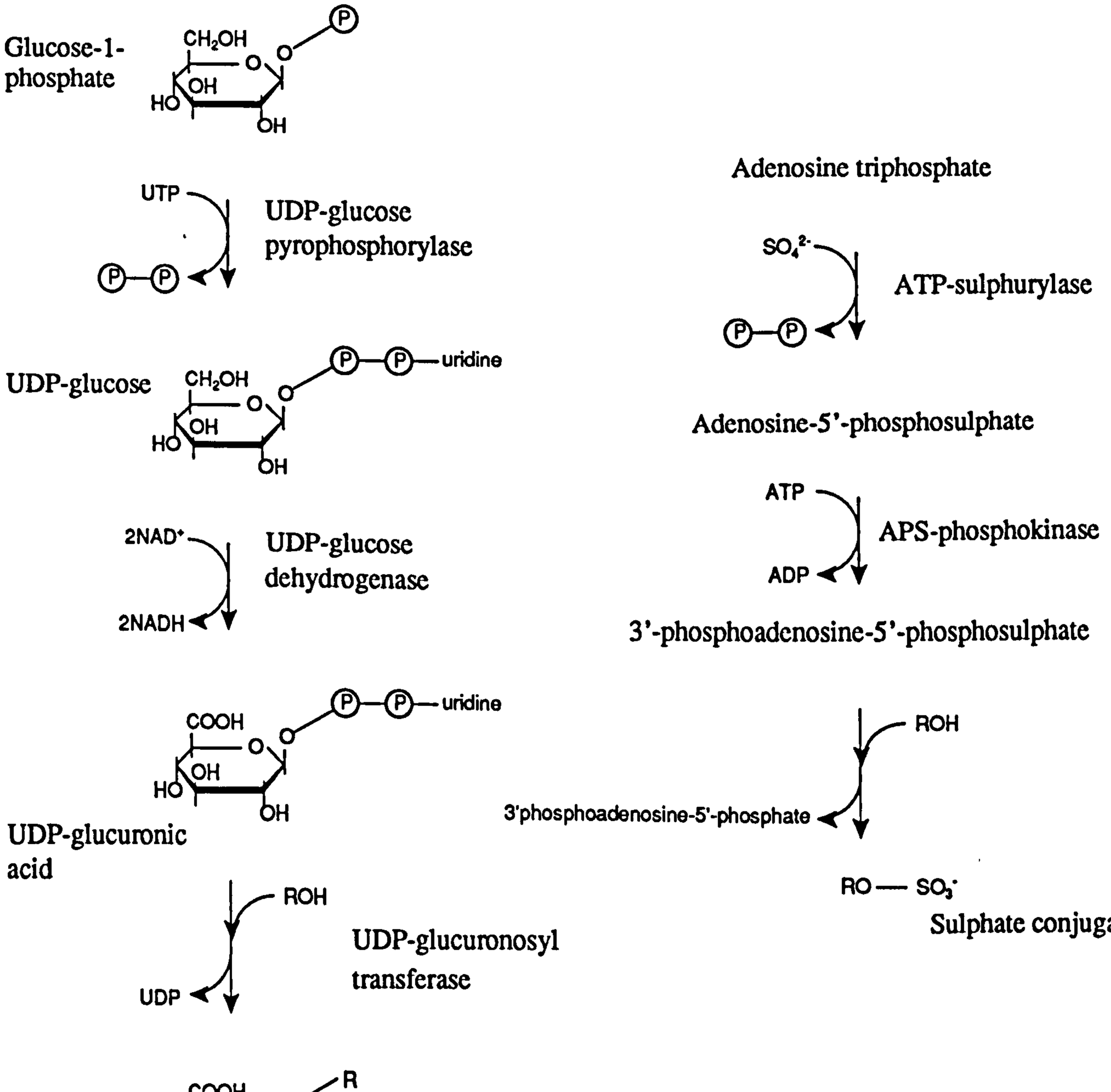
Xenobiotics are also metabolised by other enzyme systems, including monoamine oxidases (MAO), alcohol dehydrogenases and esterases. MAO catalyses the deamination of primary, secondary and tertiary aliphatic amines and is widely distributed throughout the body. Alcohol dehydrogenases are present in the liver, kidneys and lungs of many species, and catalyse the metabolism of primary alcohols to their corresponding aldehydes. Esterases are present in the blood plasma and the liver, and catalyse the hydrolysis of foreign esters and amides.

The intestinal microflora can play an important part in the metabolism of xenobiotics (Scheline, 1973). The importance of metabolism by the microflora should not be underestimated because many drugs are administered orally to man, and many drug metabolites are excreted via the bile into the gastrointestinal tract. Metabolic transformations which may be carried out by the intestinal microflora include O-dealkylation, N-dealkylation, a number of reductions and hydrolyses.

In Phase II or conjugation reactions, the endogenous substrate is transferred from a coenzyme onto the drug or Phase I metabolite substrate. Examples are shown in **Figure 1.4.** The formation of glucuronides is probably the most important conjugation mechanism and has been the subject of recent reviews (Tephly and Burchell, 1990; Kroemer and Klotz, 1992; Mulder, 1992). Glucuronide formation occurs in the liver and to a lesser extent in the kidney, gastrointestinal tract and the skin. The mechanism involves biosynthesis of the coenzyme donor uridine diphosphate glucuronic acid (UDPGA) followed by the transfer by uridine diphosphate transglucuronylases, of the glucuronyl moiety from UDPGA to the substrate. Another common class of conjugate is sulphate esters or "ethereal sulphates" (Mulder, 1988). Sulphate esters are biosynthesised

**Figure 1.4**

## Mechanisms of glucuronide and sulphate conjugate formation




UDP: Uridine diphosphate

NAD: Nicotinamide adenine dinucleotide

Ⓟ Phosphate

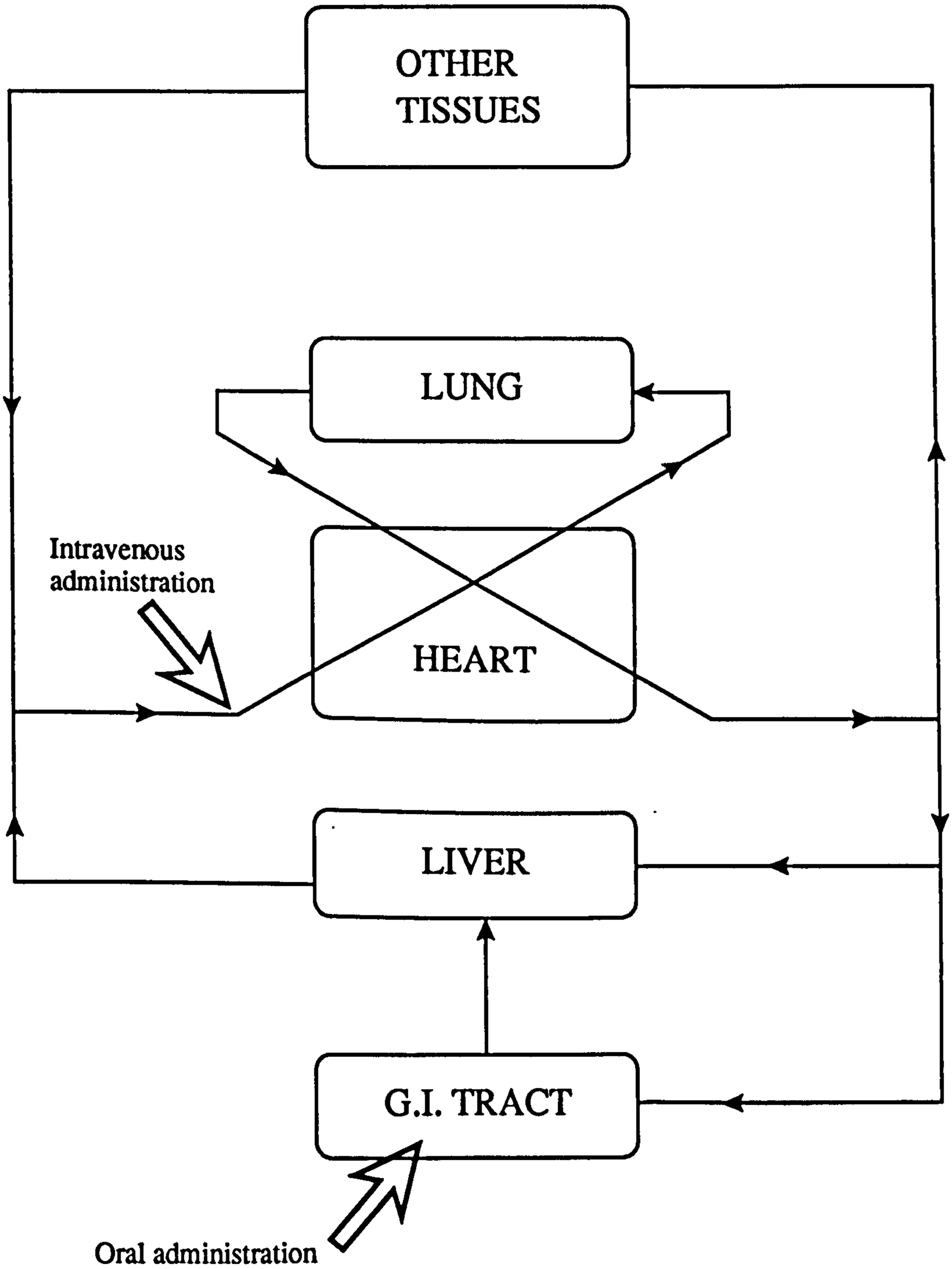
by the transfer of sulphate from adenosine-3'-5'-phosphosulphate (APPS) to a phenol, alcohol or amine by sulphate transferring enzymes known as sulphotransferases or sulphokinases. APPS is formed from adenosine-5'-triphosphate (ATP) in mammalian liver and other sites.

### 1.2.3 Kinetic Considerations

The route by which a drug is administered, for example intravenously or orally, can influence the extent to which it is metabolised (Cassidy and Houston, 1980). When a drug is administered intravenously all of the compound reaches the systemic circulation, and is completely (100%) bioavailable. The drug is then distributed rapidly throughout the systemic circulation and is metabolised, or excreted, as the blood in which it is dissolved passes through the organs such as the liver, lungs and kidneys which perform these functions. The process is illustrated in **Figure 1.5**. After oral administration, a drug is absorbed through the gastrointestinal tract into the portal vein, and passes through the liver before entering the systemic circulation. The liver has a very large capacity for metabolism, and a proportion of the dose may be metabolised during the first pass through this organ, thus not reaching the systemic circulation and leading to a reduction in the bioavailability of the drug with respect to that obtained following intravenous administration. This is referred to as first-pass metabolism, and is often the reason for a drug's low oral bioavailability, the other main reason being poor absorption through the gastrointestinal tract. Orally administered drugs may also be metabolised in the gastrointestinal tract, as discussed in Section 1.2.2, or during passage through the gut wall.

**Figure 1.5**

**Schematic representation of sites of metabolism with reference to intravenous and oral drug administration**




Adapted from Cassidy and Houston, 1980

## *1.3 The Role of Drug Metabolism in Drug Research and Development*

Drug metabolism studies have an important role in drug research and in drug development. In the following sections the objectives of the two functions are discussed, and the role of drug metabolism studies is described. Finally the current status of drug metabolism studies in drug research and development is discussed.

### 1.3.1 Drug Research

The broad objective of the research function is to discover potential new drugs which are specific in their action. More detailed objectives may require a compound with oral activity and a long duration of action. In drug research, a hypothesis is proposed that a particular compound will have therapeutic benefit in man. The evidence for the hypothesis is obtained from *in vitro* pharmacological studies which indicate whether the compound interacts with a particular receptor, enzyme, gene or organism, and from studies in animal models.

The role of drug metabolism studies in new drug discovery has been described recently (Humphrey and Smith, 1992), and the importance of drug metabolites in drug research has been discussed (Marten, 1985). Drug metabolism studies can provide answers to problems often encountered during drug research. During research, compounds may be found which are active *in vitro*, but inactive *in vivo*. Sometimes a compound is active when administered intravenously, but inactive following oral administration. Reasons for these findings may be that the compound is cleared from the body too rapidly either by renal excretion or by metabolism. Lack of oral activity may be due to extensive first-pass

metabolism, metabolism in the gastrointestinal tract or the gut wall, or because the drug is not absorbed from the gastrointestinal tract.

During drug research, knowledge of routes of metabolism can be used to build either metabolic stability or metabolic instability into a molecule. If the metabolism of a compound is known, its duration of action can often be increased by replacing metabolically unstable groups, or by adding metabolically stable groups at the site of the preferred reaction. This increases the metabolic stability of the drug and reduces the rate at which it is cleared from the body. It was known, for example, that isoprenaline was inactive as an oral bronchodilator because its 3-hydroxyl group was subject to rapid metabolism during absorption through the gut wall. Replacement of the 3-hydroxyl group of isoprenaline by a 3-hydroxymethyl group resulted in AH3021, which is less readily metabolised than isoprenaline and orally active (Hartley, Jack, Lunts and Ritchie, 1968). From this compound the bronchodilator salbutamol (AH3365) was developed (Brittain, Farmer, Jack, Martin and Simpson, 1968).

If a compound is metabolised rapidly by aromatic hydroxylation, addition of a fluorine atom or a trifluoromethyl group onto the aromatic ring can often render the molecule less susceptible to metabolism because the electron withdrawing properties of these substituents cause de-activation of the ring. An example of this is the antiobesity drug fenfluramine, a derivative of amphetamine, which has a trifluoromethyl group on the phenyl ring. Fenfluramine does not undergo aromatic hydroxylation (Bruce and Maynard, 1968), a common metabolic route for amphetamine derivatives, and is cleared slowly from the plasma in man with a half-life of about 20 hours (Campbell, 1971).

In contrast, metabolic instability can be advantageous in some cases. An inactive compound that when administered is metabolised readily to a pharmacologically active compound is referred to as a prodrug. The absorption of some compounds after oral dosing can be improved by administering prodrugs, an example being the antibiotic pivampicillin. Ampicillin is a penicillin derivative that, when administered orally, is incompletely absorbed because of its high water solubility. In order to increase the lipophilicity, the pivaloyloxymethyl ester is prepared which is more readily absorbed. The ester is hydrolysed rapidly to the active compound during passage from the gastrointestinal tract to the systemic circulation (Ehrnebo, Nilsson and Boreus, 1979). Another example is the antibiotic cefuroxime axetil which is metabolised rapidly after absorption from the gastrointestinal tract to yield the active cefuroxime moiety by hydrolysis of the ester (Harding, Williams and Ayrton, 1984).

### 1.3.2 Drug Development

The primary objective during the early stage of drug development is to test the hypothesis proposed during drug research that a compound will have particular efficacy in man. Therefore as soon as possible initial studies in human volunteers are carried out to investigate the safety and tolerance of the drug, and to demonstrate that the hypothesis for efficacy developed in animal models translates to man (Posvar and Sedman, 1989).

Although demonstration of a compound's efficacy is of vital importance during drug development, demonstration of its safety is no less important. It is not possible to use human volunteers to test the effects of lifetime exposure to a drug, or to assess the toxicity following administration at high doses. Furthermore, some evidence of safety in animals is required before a drug can be administered to man for the first time. Animals

are therefore used as models for man in which to perform the toxicity tests which are carried out as part of drug development.

The validity of the toxicity species as models for man depends upon the disposition of the drug and its metabolites in both animals and man, and upon whether the animals are exposed to the same metabolites as are formed in man. Pharmacokinetic and drug metabolism studies provide information which is used to validate these models. The information obtained from these studies is discussed in the following paragraphs.

During toxicity studies, the systemic exposure of animals to the drug is monitored and pharmacokinetic parameters are determined. These studies are referred to as toxicokinetic studies, and their importance and design have been reviewed recently (Smith, Humphrey and Charuel, 1990). During toxicokinetic studies, both acute and chronic exposure of animals to the drug are determined, and the pharmacokinetic parameters following single and repeated administration are obtained. Demonstration of exposure to the drug following oral administration is particularly important to prove that drug has been absorbed, and comparison of the concentrations of drug in the plasma following single and repeated administration show whether the drug accumulates in the plasma between doses. Differences in the pharmacokinetic parameters between single and repeated administration may indicate that the enzymes responsible for metabolising the drug are either induced (activity increased) or inhibited (activity decreased).

The acute and chronic exposure data obtained in animals during toxicity studies are compared with the exposure to man following clinical doses of the drug to determine the safety margin, or "cover", that has been achieved during the toxicity studies. These data

are required to help assess the risk associated with the clinical use of a drug against the therapeutic benefit to patients.

Metabolism studies are carried out to investigate the absorption, distribution, metabolism and excretion (ADME) of the drug in the animal species used for toxicity testing. Drug labelled with a radioactive isotope, usually $^{14}C$, is generally used for ADME studies to enable the fate of all drug-related material (drug and drug metabolites) to be determined. Absorption of the drug following oral administration is demonstrated by the presence of drug-related material in the plasma, and differences in the concentrations of unchanged drug and total drug-related material indicate the concentrations of metabolites in the circulation. It is important to compare the exposure of animals and man to metabolites as well as parent drug.

Distribution studies are carried out to identify the tissues and organs to which drug-related material is distributed following administration, and those which are exposed to drug or metabolites at high concentrations or for long periods of time. Generally compounds which are non-ionised at physiological pH are distributed widely because of their solubility in lipid membranes and fat. Thus weak bases are generally distributed widely throughout the body, whereas many acidic compounds and strong bases, which are largely ionised in the plasma, are not widely distributed. As discussed previously (Section 1.2), metabolites are generally less lipophilic and more acidic than their parent drug and are therefore generally less widely distributed.

The routes of metabolism of a drug are investigated by examining urine, faeces or bile to determine the nature and concentrations of drug and metabolites which these excreta contain. The metabolites are isolated and identified thus providing information about the

metabolism of the drug in animals which can be compared with the routes of metabolism of the drug in man.

The routes by which drug-related material is excreted are determined by comparing the levels of radioactive material in the various excreta; urine, faeces and bile. Similar information is obtained from man (except bile is not collected) allowing comparison of the rates and extent of excretion to be made between man and the animal species used in the toxicity testing.

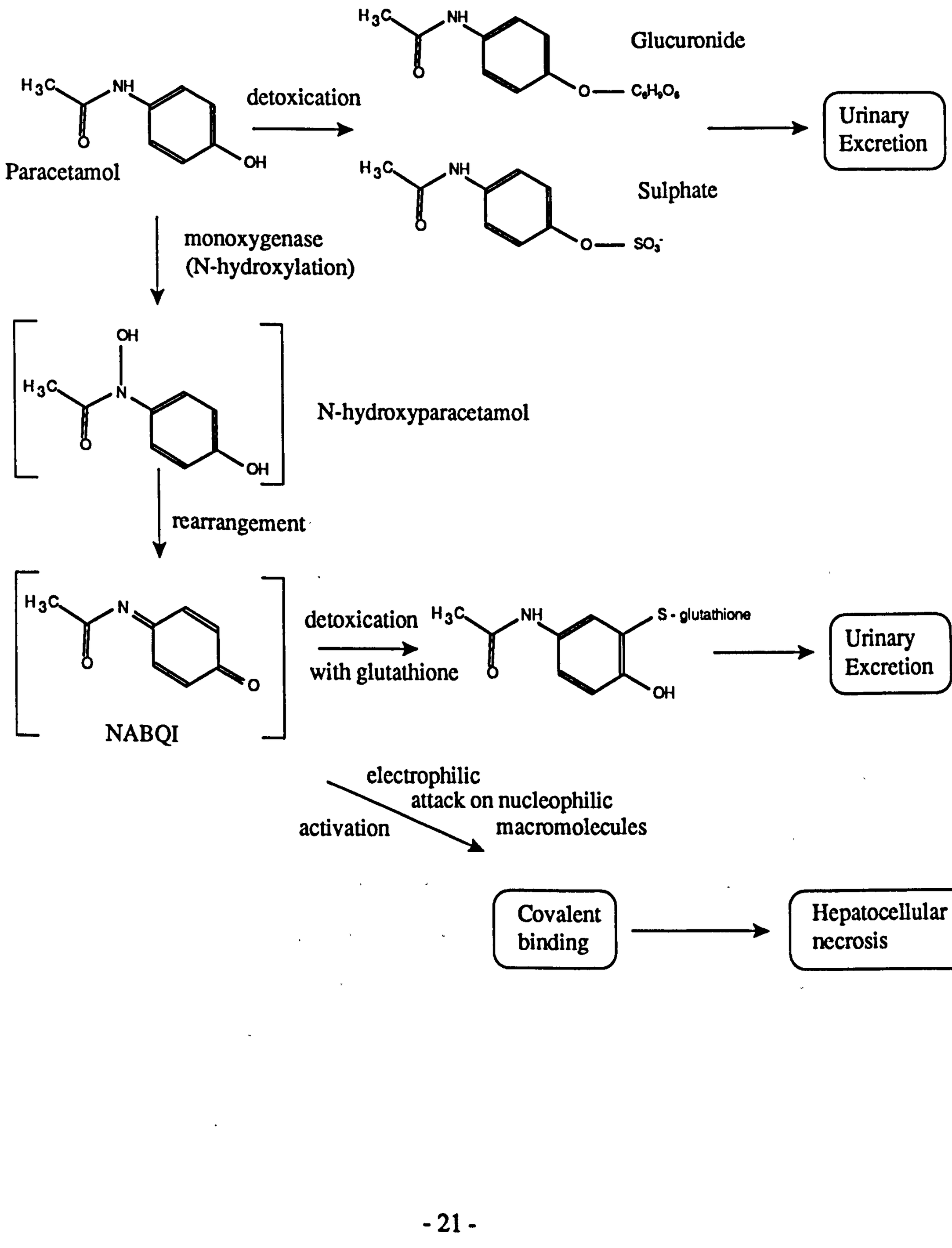
Drug metabolism studies may also assist in the interpretation of toxicity data in addition to providing validation of the studies themselves. In the majority of cases drug metabolism, particularly conjugation, produces metabolites which are less active pharmacologically than the parent drug. In some cases however, metabolism of an active drug molecule can result in the formation of metabolites which are themselves pharmacologically active, either with the same properties as the parent drug or different properties. An example of this is the tranquilliser diazepam which is metabolised via N-demethylation and hydroxylation to oxazepam an anticonvulsant drug (Ruelius, Lee and Alburn, 1965). Tamoxifen, a non-steroidal oestrogen inhibitor used in the treatment of early and advanced breast cancer, is metabolised by aromatic hydroxylation to form the 4-hydroxy metabolite which is one hundred times more potent than the parent drug (Robinson and Jordan, 1988). For a long time, glucuronylation has been considered a metabolic reaction that terminates the pharmacological activity of drugs. However this view is no longer tenable since it has been shown that the glucuronide conjugate formed at the 6-position of morphine is a much more potent agonist of the morphine receptor than morphine itself, and the glucuronide at the 3-position is a potent antagonist (Mulder, 1992).

In some cases the toxicity of a drug is attributable to metabolites rather than the drug itself. Thus identification of metabolites, particularly those in the systemic circulation, may provide interpretation of the toxicity data. Paracetamol is known to cause liver toxicity at high doses in both experimental animals and man (Mitchell, Jollow, Potter, Davies, Gillette and Brodie, 1973). This is thought to occur by an initial N-hydroxylation reaction and subsequent rearrangement of the hydroxylamine producing a reactive intermediate (NABQI), which following very high doses of paracetamol binds covalently to liver macromolecules leading to liver necrosis (**Figure 1.6**). However, at therapeutic doses NABQI is conjugated rapidly with glutathione and rendered non-toxic (Corcoran, Mitchell, Vaishnav and Horning, 1980).

Metabolic reactions which lead to the formation of epoxides often result in toxicity. The anticonvulsant carbamazepine, used in the treatment of epilepsy, undergoes aromatic hydroxylation which is thought to proceed via an arene oxide. In some patients, inability to convert this intermediate to the non-toxic hydroxylated metabolite has resulted in serious toxicity which is believed to have been caused by covalent binding of the arene oxide to cellular macromolecules leading to cytotoxic reactions (Shear and Speilberg, 1988). The polycyclic aromatic hydrocarbons are another class of compound from which toxic metabolites are formed. These compounds are metabolised to form diol-epoxide metabolites which are capable of covalent binding to nucleic acids and hence of initiating chemical carcinogenesis (Jerina and Daly, 1974). Potential drugs containing similar structures may also be associated with similar toxicity.

**Figure 1.6**

**The role of metabolism in the toxicological activation of paracetamol**



### 1.3.3 Current Status of Drug Metabolism Studies

Drug metabolism studies, particularly those comprising the "ADME package" required by regulatory authorities in support of a Product Licence Application, are well established. In addition, the use of pharmacokinetic and metabolism studies in support of drug research has increased during recent years (Humphrey and Smith, 1992).

Often radiolabelled drug is used during ADME studies to act as a tracer and to facilitate separation of drug-related and endogenous material. Synthesis of radiolabelled drug may be carried out early in the drug development programme and is rarely available when a compound is in drug research. Metabolic information has therefore rarely been obtained during drug research because of the technical difficulties associated with metabolite detection and isolation in the absence of radiolabelled drug. However early metabolic information may greatly aid drug research.

A general method to study the metabolism of compounds during drug research would provide information to complement the pharmacokinetic data which are being provided increasingly as part of drug research support. Identification of metabolic routes may help design compounds with improved bioavailability or longer duration of action.

Often little information is known about the nature or extent of metabolism of a compound at the start of drug development. If a compound is shown to be toxic during initial toxicity studies, radiolabelled drug may never be synthesised, or the metabolism of the compound may not be defined. It is therefore often the case that little information is obtained about the metabolism of compounds which are shown to be toxic early in drug development. However, metabolic information from toxic compounds might provide

some indication of why they are toxic while others are not, and may thus aid the process of drug design. Furthermore, early information on the metabolism of compounds which are successful in drug development might lead to the discovery of active metabolites which may themselves be drug candidates.

A method to screen for drugs and metabolites in plasma and urine which does not require customising for each compound, and does not rely upon radioactivity detection, would therefore be very valuable. In the following section, the methods used for the study of drug metabolism are discussed and their suitability as general screening methods is assessed.

## *1.4 The Study of Drug Metabolism*

There are a number of technical challenges encountered in the study of drug metabolism. The drug and metabolites have first to be separated from other material in the biological matrix that is being studied. Those studied most commonly, being plasma, urine, and bile, are complex matrices which vary in composition from species to species, between subjects, and from time to time. In addition to the complexity of the matrices, the concentration of drug and metabolites present is frequently small in comparison with the concentration of the endogenous material. Plasma is a more consistent matrix than urine, and generally contains less endogenous material which might interfere in an assay for drugs and metabolites. Offset against this however, for a drug which is cleared via the urine, concentrations of drugs and metabolites are usually higher in urine than in plasma.

The development of drugs with greater specificity and increased potency has meant that doses and hence concentrations of drug and metabolites in biological fluids are often very low. This is particularly true at the dose-levels used for ADME studies which often correspond to the lowest dose-level used in toxicity studies; a low multiple of the dose projected for clinical use in man. After oral administration of ondansetron to the rat at a dose-level of 1mg/kg, the maximum concentrations of drug and metabolites in the plasma (indicated by the levels of radioactive material) were approximately 200ng/ml while the maximum concentrations of parent drug were less than 10ng/ml (Saynor and Dixon, 1989). Similar results were obtained in the dog. An analytical method to determine the concentrations of drugs and metabolites in biological fluids must therefore have high sensitivity if the compounds of interest are to be detected. The challenges that have been described need to be overcome even if drug and known metabolites are being studied. If the metabolism is unknown, as is the case when novel compounds are being studied, the challenges are multiplied because an unknown number of unknown compounds is involved.

Techniques used to study drug metabolism can be divided broadly into indirect and direct methods. Indirect methods, which include chromatography and enzymic or chemical tests, rely upon circumstantial evidence such as co-chromatography with authentic standards or changes in the metabolic profile to provide information on the nature of metabolites. Direct methods comprise spectroscopic techniques such as mass spectrometry and nuclear magnetic resonance (nmr) spectroscopy, and provide unequivocal characterisation of metabolites.

In the following sections, chromatographic and spectroscopic techniques used in the study of drug metabolism are discussed, and the chromatographic methods are considered

alongside criteria for a general screening method for drugs and metabolites in biological fluids.

### 1.4.1 Chromatographic Techniques

Traditionally, techniques based upon chromatography have been used to separate drugs and metabolites from each other and from endogenous material. The history of chromatography is usually traced to Michael Tswett's separation of plant pigments during the early 1900s (Jupille, 1977). Chromatography is the separation of a mixture by differential migration resulting from the different distributions of its components between immiscible stationary and mobile phases. The three main chromatographic techniques are gas chromatography (GC), liquid chromatography (LC) and thin-layer chromatography (TLC).

In GC a sample (as a vapour) is carried by a moving gas through a column containing stationary material. The stationary material selectively retains different sample components either by adsorption on the solid surface or by partition between the gas and a stationary liquid coated onto the column walls or onto an inert solid support. In LC a sample in solution is carried by a moving liquid solvent through a column packed with stationary material. Selective retention of the components may occur by adsorption or partition as described for GC. LC can be operated in "normal-phase" in which the stationary phase is more polar than the mobile phase (as is the case with a silica column using adsorption chromatography), or "reversed-phase" when the stationary phase is less polar than the mobile phase as is often the case when using the partition mode. In TLC a sample in solution is carried by a flowing liquid through a thin bed of stationary material deposited on a rigid backing. The choice of stationary phases and separation mechanisms

for TLC and LC are similar. Compounds are separated by these chromatographic techniques as a function of their polarity, structure, and size or molecular weight. Drugs and their metabolites are usually molecules of low molecular weight, for which polarity and structural differences are the most important factors exploited to effect separation.

Early drug metabolism studies were carried out using paper chromatography and the technique was reviewed by Nestler (1979). Adams and Cliffe (1965) used paper chromatography to separate the metabolites of ibuprofen from parent compound in order to estimate the concentration of the drug in human serum. Thin-layer chromatography has been used extensively as a means of separating and isolating drug metabolites. More recently Wilson and Morden (1991) have successfully combined TLC with mass-spectrometry.

In recent years, high-performance liquid chromatography (HPLC) has emerged as a leading technique in the field of drug metabolism and analysis due to the ease with which it can be automated, and improvements in detector sensitivity and versatility. HPLC in conjunction with diode array detectors is able to provide both multi-wavelength and spectral information from a single injection of sample onto a HPLC column. This gives increased possibilities for identification in comparison with single wavelength detection, and the opportunity to test for peak purity. A diode array detector was used in conjunction with HPLC to screen bile for drug and metabolites following administration of butoprozine to man, and ticlopidine to rats (Overzet, Rurak, Van der Voet, Drenth, Ghijsen and De Zeeuw, 1983). The usefulness of diode array detection was demonstrated because metabolite peaks were detected which would not have been found by single wavelength detection. The authors commented, however, that further spectroscopic analysis was necessary for metabolite identification. Recently, HPLC-diode array

analysis of rat bile samples obtained following administration of a calcium antagonist Ro 40-5967, enabled the metabolites to be classified by reference to their ultraviolet spectra (Wiltshire, Harris, Prior, Kozlowski and Worth, 1992).

HPLC has also been combined successfully with mass-spectrometry, particularly using thermospray techniques (Rudewicz and Straub, 1987). This is discussed further in Section 1.4.2.

GC has been used less widely for metabolism studies, due largely to the need for derivatisation of many compounds before they will volatilise efficiently and chromatograph using this technique. However, with its high resolving power GC is particularly useful for the separation of drugs and known Phase I metabolites for assay purposes.

### 1.4.2 Spectroscopic Techniques

In recent years, nmr spectroscopy has been used to study drugs and metabolites in biological fluids (Nicholson, Sadler, Tulip and Timbrell, 1986; Nicholson and Wilson, 1987a; Nicholson and Wilson, 1987b; Everett, 1991). The basic theory of nmr spectroscopy is described by Nicholson and Wilson (1987b).

Advantages of nmr spectroscopy over traditional chromatographic methods are that it is a non-destructive technique, little or no sample preparation is required other than the addition of a small amount of deuterated water to the sample, sample throughput is fast (typically proton NMR spectra can be obtained in 5 minutes or less), and nmr

spectroscopy gives direct structural information on all the molecules it detects in a biological sample.

There are however a number of disadvantages to set against the benefits of the technique. Nmr spectroscopy is relatively insensitive and analytes must normally be present at concentrations of >50$\mu$M (equivalent to 15$\mu$g/ml for a drug with a molecular weight of 300). The sensitivity is greater for drugs containing fluorine atoms when $^{19}$F-nmr can be used (Ghauri, Blackledge, Wilson and Nicholson, 1990; Wade, Wilson, Troke and Nicholson, 1990; Gilbert, Hartley, Troke, Turcan, Vose and Watson, 1992) although relatively high dose-levels are often still required to achieve the concentrations of drugs and metabolites needed for detection.

Further disadvantages are that nmr spectrometers are expensive instruments requiring skilled operators.

Mass spectrometry is particularly useful for providing information which together with nmr data can often identify metabolites unequivocally. Usually samples need to be isolated from biological matrices before mass spectral data can be obtained. The isolation can be carried out by extraction, but in recent years HPLC-mass spectrometry (HPLC-MS) has been used very extensively for drug and metabolite screening.

Application of HPLC-MS in drug metabolite identification and in screening for drugs and metabolites has been reviewed (Niesson and Van der Greef, 1992). Following separation by HPLC, compounds are detected by mass, and therefore the instrument can be set up to detect specific molecular ions related to the molecular weight of the drug. Further mass spectral information can be obtained for the components of interest, and preliminary

identification of metabolites can often be made. In addition to providing specific structural information, HPLC-MS is a sensitive technique, and because of the selective detection afforded by the mass spectrometer, only limited sample preparation may be required. The main disadvantages of using HPLC-MS are the need for expensive mass spectrometers and skilled operators.

### 1.4.3 General Screen for Drugs and Metabolites

The spectroscopic techniques described in the previous section have a number of important advantages over chromatographic techniques, and could be used routinely to screen biological fluids for drugs and metabolites. However, the instrumentation required for these techniques is expensive, so consideration of an alternative screening method using chromatographic techniques which are relatively inexpensive, and therefore more widely available, is worthwhile.

There are a number of criteria necessary for a chromatographic technique to provide a general method for the detection of drugs and their metabolites in biological fluids. These are shown below:

1. The technique must have high resolution.
2. The technique must be sensitive.
3. It must be non-destructive.
4. It needs to be flexible both in terms of chromatography and detection.

These criteria largely preclude the use of GC. Derivatisation is often necessary before samples can be chromatographed by GC, and this can be a destructive process. In

addition, the application of sample into a hot injection port can cause breakdown of labile compounds. Although techniques now exist for cool injecting onto GC (Poy, Visani and Terrosi, 1981), these may be restricted to certain compound types and the behaviour of novel drugs and metabolites cannot be predicted easily.

HPLC and TLC are both non-destructive unless the compounds are unstable on the chromatographic medium or in the mobile phase used, and derivatisation is not necessary prior to separation by these techniques. A comparison of HPLC and TLC has been made recently (Sherma, 1991). The techniques have similar chromatographic capabilities; both can be operated under normal or reversed-phase conditions, isocratically or with a solvent gradient, and similar stationary phases are available for each technique. The ultraviolet and fluorescent modes of detection are common to both techniques, but each also has alternatives (HPLC : refractive index and electrochemical; TLC : fluorescence quenching). The detection limits are approximately the same ranging from picogram to nanogram amounts depending upon the mode of detection employed.

There are however some properties of TLC which make it a more flexible technique to be considered for the development of a general method for the detection of drugs and their metabolites in biological fluids. An important difference between the techniques is that whereas HPLC is dynamic, TLC is ultimately static. With HPLC, after the eluent has passed through the detector, it is lost unless a sample fractionating system is used for its collection. In the case of TLC, after development, the plate can be scanned and re-scanned, derivatised if necessary and scanned again. Post-chromatographic derivatisation on TLC is more flexible than with HPLC because reaction times are less important and heating can be used to encourage slow reactions. TLC is an archivable technique in terms of detection and for retrieval of separated components for identification. The open planar

chromatographic bed provides a degree of flexibility that the closed tubular columns of HPLC do not have. A further advantage of TLC, particularly when an unknown number of metabolites is present, is that at all times the whole of the sample that has been applied to the plate must be between the origin and the solvent front; two limits which are defined clearly. When a complex mixture of components with a wide range of polarities has been injected into an HPLC system one cannot be certain if the entire sample has been eluted at any time unless it is accounted for by radioactivity measurement. Thus in HPLC there can be loss of peaks, or unexpected appearance of peaks from previous samples.

A final point of comparison is that HPLC is a re-usable system whereas TLC is disposable. A TLC plate is used once therefore it can be destroyed in use, but the conditions under which HPLC can be operated are limited by the need for the column to be used repeatedly. This might be of importance in analysing for drugs and metabolites because it may be necessary to apply biological fluids directly to the chromatographic system or to apply crude extracts to ensure that all drug and metabolites are recovered. Similarly, the choice of solvent is not limited in TLC, whereas in HPLC it must be compatible with the column packing material. Furthermore, because the solvent is evaporated completely from the stationary phase in TLC before detection, the use of solvents with ultraviolet absorbing properties is feasible, whereas in HPLC their use would hamper detection of the components of interest.

It is concluded from the preceding discussion that thin-layer chromatography has a number of advantages which make it an appropriate technique to use for a general method to detect drugs and their metabolites in biological fluids.

## *1.5 Thin-Layer Chromatography*

The historical development of thin-layer chromatography (TLC) has been documented by Stahl (1969), and Jupille (1977) reviewed the principles, practice and potential of TLC with particular reference to high-performance (HP)TLC which was emerging as a technique with significant advantages over traditional TLC at about that time.

According to Kirchner (1973), the principle of TLC was first known in 1889 when the Dutch biologist Beyerinck allowed a drop of a mixture of hydrochloric and sulphuric acids to fall through a thin layer of gelatin. The hydrochloric acid travelled faster, forming a ring round the sulphuric acid. Meinhard and Hall (1949) were the first to use a binder to hold their stationary phase onto a microscope slide. Stahl first used the term "thin-layer chromatography" in 1956, and in 1958, recommended standard conditions for the technique were published following production of special grade silica by Merck, and the manufacture of basic equipment and accessories by Desaga (Stahl, 1983). TLC was used extensively up until, and during the 1970s. The instrumental development of gas chromatography occurred in parallel with further development of TLC, and the separating capacity of the fine particle size layers used in TLC stimulated attempts to pack tubular columns with these materials. Success with this led to the development of high-performance liquid chromatography (HPLC), a technique which lends itself readily to instrumentalisation and automation. The popularity of HPLC meant that in many areas it gradually replaced TLC during the 1980s in particular. This is reflected by the large increase in publications referring to HPLC during this period (Lochmuller, 1987), and the tendency of some authors to ignore the current value and potential of TLC.

However Zlatkis and Kaiser (1977) edited a book on high-performance thin-layer chromatography which helped to stimulate renewed interest in TLC. In an article entitled "HPTLC: Taking Off," Borman (1982) described the differences between conventional and high-performance TLC. These are listed in **Table 1.1**, and show that the main feature which resulted in the increased performance was the use of finer sized silica material with a much tighter particle size distribution. The greater resolution obtained meant that shorter development distances were required to effect separation, and hence the separation time was reduced. The thinner layer on HPTLC plates has a lower sample capacity than conventional TLC plates, thus to prevent overloading and to minimise spot spreading on sample application, small volumes are applied. However it has been shown that much larger volumes (up to 40$\mu$l) can be applied successfully if a solvent in which the sample does not migrate is selected in which to apply the samples (Colthup, 1988).

Various authors described the advances in instrumentation, mainly in sample application, plate development and quantitation which occurred around 1980 (Fenimore and Davis, 1981; Wall, 1982; Moi and Schmutz, 1982). These increased the sample throughput of HPTLC, and also the accuracy and precision of the technique. Many of these early reviews, and a recent review on modern TLC (Poole and Poole, 1989) emphasise the economy of HPTLC in terms of high sample throughput in comparison with HPLC. This may be true in cases where minimal sample preparation is required (for example Kreuzig, 1983), however for the routine analysis of drugs in biological matrices at low concentrations, where extensive sample preparation is required, HPLC may be more appropriate because it can be automated and allowed to run overnight. Although similar sensitivities (1ng/ml) were obtained with HPTLC (Colthup, 1988) and HPLC (Colthup, Felgate, Palmer and Scully, 1991) for the determination of ondansetron concentrations in plasma, there was a clear advantage for the use of HPLC in terms of sample throughput.

**Table 1.1**

**Comparison of conventional and high-performance TLC**

| Parameter | Conventional TLC | HPTLC |
|---|---|---|
| Sample volume | 1-5 μl | 0.1-0.2 μl |
| Starting spot diameter | 3-6mm | 1-1.5mm |
| Diameter of separated spots | 6-15mm | 2-5mm |
| Solvent migration distance | 10-15cm | 3-6cm |
| Separation time | 30-200min | 3-20min |
| Particle size (average) | 20 μm | 5 μm |
| Particle size (distribution) | 10-60 μm | Tight |
| Adsorbent layer thickness | 100-250 μm | 200 μm |
| Detection limits (absorption) | 1-5ng | 0.1-0.5ng |
| Detection limits (fluorescence) | 50-100pg | 5-10pg |

HPTLC has been used to measure the concentrations of drugs in plasma and urine, but in comparison with HPLC, applications are few. Imipramine, an antidepressant drug, and its desmethyl metabolite were quantified in human plasma down to 2ng/ml, and in urine down to 60 and 126ng/ml respectively following derivatisation with nitrous gases to form intense yellow derivatives (Sistovaris, Dagrosa and Keller, 1983). Chlorpromazine and thioridazine, drugs used in the treatment of psychotic disorders, were quantified in plasma down to 10ng/ml using ultraviolet absorption densitometry (Davies and Harrington, 1984). Tetrahydrocannabinol, the active principle in marijuana, was measured in plasma down to 1ng/ml following derivatisation and fluorescence detection (Vinson and Patel, 1984). Salbutamol was quantified in human plasma down to 1ng/ml and in urine down to 20ng/ml following derivatisation to form an indoaniline dye (Colthup, Dallas, Saynor, Carey, Skidmore, Martin and Wilson, 1985), and ondansetron was measured in plasma using fluorescence detection down to 1ng/ml (Colthup, 1988). These applications demonstrate that HPTLC has the sensitivity required to measure the concentrations of drugs in biological fluids.

There have also been a number of examples where HPTLC has been used to separate drugs and metabolites for quantitative purposes. Haefelfinger (1979) measured the 7-amino-metabolites of the benzodiazepine drugs flunitrazepam and clonazepam in plasma down to 1ng/ml. Mount, Nahlen, Patchen and Churchill (1987) described a method to determine concentrations of chloroquine, an antimalarial drug, and desethylchloroquine in urine down to 250ng/ml. Ifosfamide, a DNA-alkylating agent used in the treatment of tumours, and its five principal metabolites were measured by HPTLC in plasma, cerebrospinal fluid and urine down to concentrations of 1 $\mu$g/ml (Boddy and Idle, 1992). These applications show that HPTLC has the resolution required to separate drugs and their metabolites.

It is concluded from the preceding discussion that HPTLC has, in addition to the advantages of TLC, the resolution and sensitivity required of a general method to detect drugs and their metabolites in biological fluids. The perceived advantages of HPTLC warrant an assessment of the technique to determine whether it has value as a general method for drug and metabolite analysis which might provide valuable information about drug metabolism early in drug research and development programmes.

## *1.6 Aims of the Present Studies*

Consideration of the role of drug metabolism studies in drug research and development has shown that there is a place for a general method to detect drugs and their metabolites in biological fluids. HPTLC has a number of advantages which make it an appropriate analytical technique to use for this application, and has the resolution and sensitivity required. The aims of this thesis therefore include the following:

1. Assess the value of HPTLC for the detection and characterisation of drugs and metabolites in biological fluids.

2. Devise a general HPTLC development system to separate drugs and their metabolites.

3. Examine relationships between chromatographic properties and structural changes which occur during drug metabolism.

4. Develop general techniques to extract drugs and metabolites from plasma and urine as required.

5. Examine the utility of a general method for the detection of drugs and metabolites by analysing plasma and urine from animals collected after administration of drugs with known metabolism.

6. Assess the value of information provided by a general method in relation to drug research and development.

# *CHAPTER TWO*

# *SEPARATION OF DRUGS AND METABOLITES BY HPTLC WITH MULTIPLE DEVELOPMENT*

## *2.1 Introduction*

High-performance thin-layer chromatography (HPTLC) has a number of attributes which make it potentially useful for detecting drugs and metabolites in biological fluids, which have been discussed in the previous chapter. HPTLC itself provides two main chromatographic mechanisms and various modes of operation each of which has advantages in certain applications.

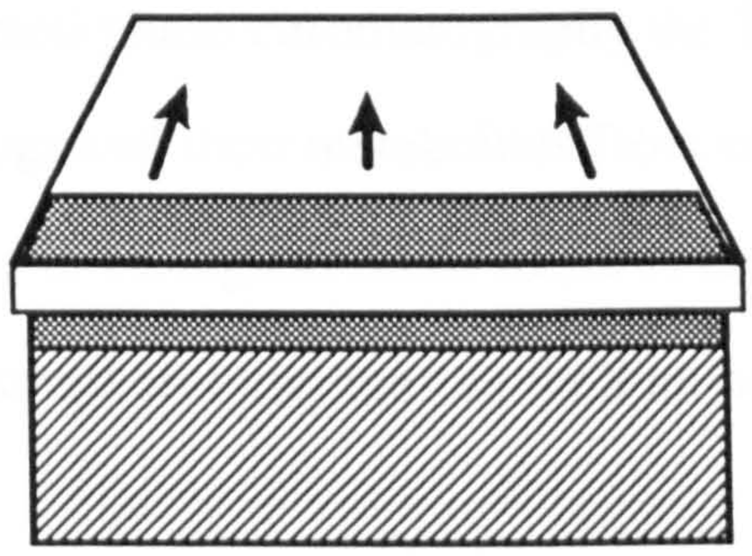
The modes of operation available for use in HPTLC are represented in **Figure 2.1.** The mode used most widely is linear development on either square or rectangular chromatographic plates. Other modes, are circular in which development is performed from the centre of a circle out towards the circumference, and anti-circular in which development occurs from the circumference towards the centre (Kaiser, 1988). Their use can improve resolution of compounds which are adsorbed strongly on the plate in the case of the circular mode, and those which are less strongly retained in the case of the anti-circular mode. Although there may be advantages in using the circular mode, particularly for the separation of strongly retained conjugated metabolites, both these techniques require specialist equipment, and may not be generally applicable. Linear development is thus considered to be the most suitable technique to use for a general screening method for drugs and metabolites.

Chromatographic mechanisms in HPTLC (and other liquid chromatographic techniques) can be divided broadly between normal-phase (adsorption) and reversed-phase (partition) chromatography. As discussed in Chapter 1, the metabolites formed during drug

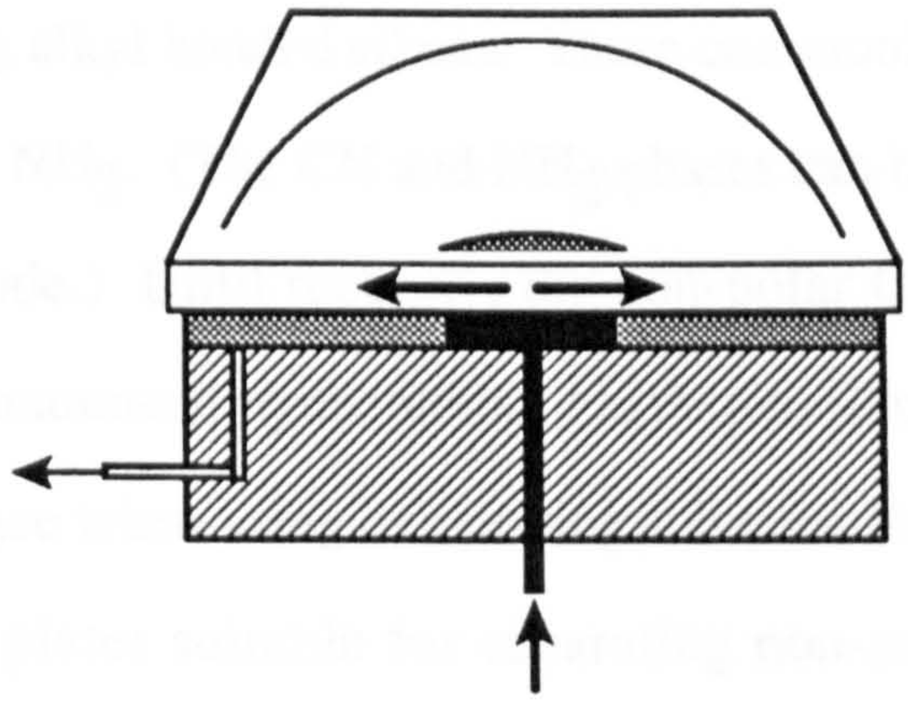
**Figure 2.1**

**Modes of operation available for development using HPTLC**

The Linear Mode



The Circular Mode



The Anti-circular Mode

metabolism generally have a greater polarity (are more water soluble) than the parent drug, and this property can be utilised to obtain a chromatographic separation. Chromatographic retention on TLC is described by the term Rf which is defined as the ratio of the spot and solvent front displacements from the origin after one development. Metabolites usually have a smaller Rf than the parent drug in normal-phase chromatography and in reversed-phase chromatography the Rf of metabolites is usually larger. The separation of drugs and their metabolites from other components of similar molecular weight and polarity in biological fluids requires that the chromatography used must be selective to small structural changes as well as being operable over a wide polarity range.

Reversed-phase chromatography embraces a wide range of different stationary phases, the most popular being alkyl bonded silicas. Those commonly available for HPTLC are $C_{18}$, $C_8$, $C_2$, CN, and $NH_2$. (The CN and $NH_2$ phases can be used in either the normal or reversed-phase mode.) Until recently, the non-polar $C_{18}$, $C_8$, and $C_2$ stationary phases have had a pronounced hydrophobic character. This has led to difficulties in wetting the plate surface when using developing solvents that contain greater than 35% water, and made the plates suitable for separating non-polar or only slightly polar substances (Brinkman and de Vries, 1982). Thus in 93 references to the use of reversed-phase TLC published in 1982 there were only a few applications to drug analysis, and these were mainly for fairly lipophilic molecules. For the same reason silica is still the stationary phase preferred by the large majority of workers, and reversed-phase TLC occupies a rather modest second place. In the last few years wettable $C_{18}$ plates have become available commercially and these could be of more use (Fischer, Hauck and Jost, 1988). It is probable that with alkyl bonded plates a gradient system with forced flow as

in over-pressure thin-layer chromatography (Tyihak and Mincsovics, 1991) would be required to achieve the separations required for drugs and metabolites.

Adsorption chromatography can be carried out on a number of stationary phases, but silica and alumina are used most commonly. Silica is used more widely than alumina, partly because silica HPTLC plates are commercially available, but also, alumina has the capacity to alter some analytes chemically (Neher, 1958). These factors make silica the most reasonable choice between the two phases for the present studies.

The interactions that occur between silica and molecules on its surface are polar, including hydrogen bonding, dipole-dipole, induced dipole-dipole, and pi-pi interactions (Unger, 1979). As a result of these interactions, the distribution of electrons between individual atoms in the functional groups is made unequal causing positive and negative polarity, and allowing interaction of the functional groups to occur with polar groups on the silica surface. Groups that exhibit this type of interaction are hydroxyls, amines, carbonyls, aromatic rings, double-bonds, and groups containing heteroatoms such as oxygen, nitrogen, sulphur, and phosphorus. Some of these functional groups are those that are involved in Phase I metabolic transformations, and their presence on or absence from a molecule can have a considerable effect on the retention of that molecule on silica. This makes silica a particularly useful chromatographic medium for the separation of drugs and metabolites. The suitability of silica may be enhanced further by using it with a solvent gradient.

The use of isocratic TLC to separate drugs and metabolites can often lead to a situation in which a polar mobile phase required for the separation of conjugated metabolites causes the drug and Phase I metabolites to migrate near the solvent front. Conversely a solvent

of lower strength used to give good separation of the Phase I metabolites leaves the conjugates on the origin. This is referred to as the "general elution problem" (Snyder and Kirkland, 1979), and one way in which it can be overcome is by use of gradient elution.

In this chapter methods which have been used for the formation of gradients on silica TLC plates are discussed. In particular the technique of Manual Multiple Development (MMD) is assessed in terms of its application to the separation of drugs and metabolites, and is compared with Automated Multiple Development (AMD). The retention data from model compounds and a number of drugs and metabolites are used to propose a relationship between relative retention and metabolite type. The application of the system to the direct analysis of plasma and urine is examined.

### 2.1.1 Gradient Formation in Normal-Phase TLC

According to Golkiewicz (1991), gradient elution was applied to column liquid chromatography in 1952 by Hagdahl, Williams and Tiselius. However, it was not until 1962 that gradient elution was applied to TLC when LDH-isozymes and nucleotides were separated on DEAE-Sephadex (Wieland and Determann, 1962). In contrast to column liquid chromatography, gradient development in TLC has been applied relatively rarely because of the complexity of the devices required for the production of reproducible gradients. Recently however, gradient development in TLC has become more popular as measured by the publication of papers on the theory and devices for gradient development. These have been reviewed recently (Golkiewicz 1991).

Gradient elution in column liquid chromatography operates by use of a continuous gradient as solvents are mixed in varying proportions, and the composition and elution

strength of the eluent is modified with time. Various devices have been proposed for the formation of continuous gradients on TLC using combinations of burettes and magnetic stirrers to modify the mobile phase composition with time, and wick arrangements to carry the solvent to the TLC plates. More recently Matysik and Soczewinski (1988) have described a miniaturised generator of continuous gradients for equilibrium sandwich chambers using a glass distributor to spread the solvent along the bottom edge of the TLC plate. A small volume of the weaker solvent (e.g. 2ml) was placed into a reservoir from which a syphon tube delivered solvent to the distributor plate in the sandwich chamber and hence onto the TLC plate. Meanwhile, the stronger solvent was fed into the reservoir by means of a syringe which was driven by a syringe pump, and the composition of the solvent delivered to the TLC plate thus changed with time. The slope of the gradient could be regulated by altering the speed of the syringe pump. A modification of this system, in which the reservoir was replaced by a coil of fine tubing, was used to obtain stepwise gradients (Soczewinski and Matysik, 1988). A number of pre-mixed solvents were drawn into the coil one after the other, separated by small air gaps, by means of the syringe, and were then delivered to the plate. Recently a further modification to the technique was reported in which the sandwich chamber was replaced by a horizontal chamber (Dzido, Matysik and Soczewinski, 1991). Although interesting data have been obtained in the hands of the group using these methods, the techniques are limited because some of the equipment is essentially "home made" and unavailable commercially.

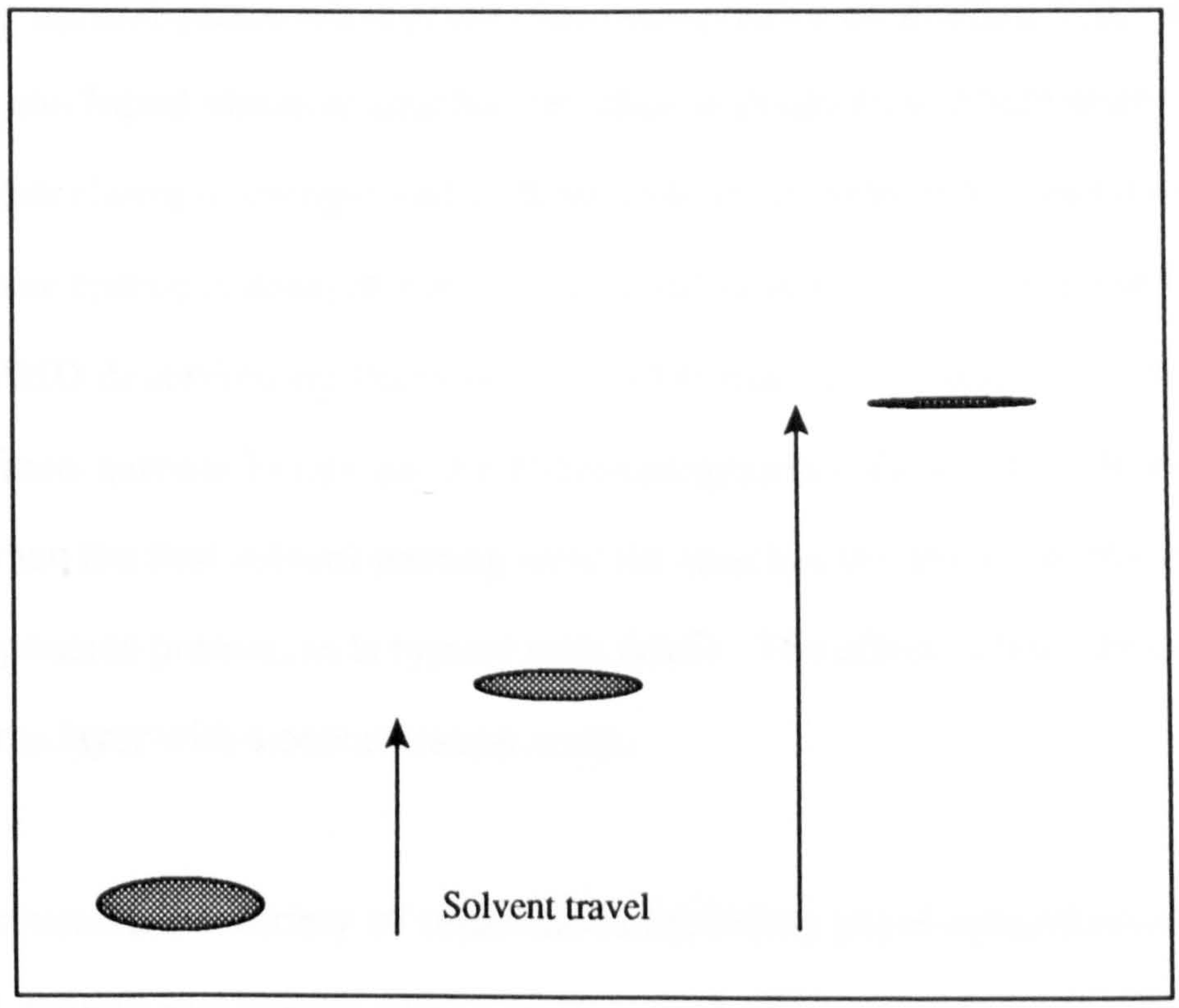
In practice, a continuous gradient is preferred in liquid chromatography (Snyder, 1979). However, stepwise gradients are easier to obtain in TLC, and it has been shown in column liquid chromatography that if a stepwise gradient comprises several steps then the gradient obtained is almost identical to a continuous gradient (Golkiewicz, 1981a;

Golkiewicz, 1981b). One method of obtaining stepwise gradients on TLC is by multiple development. The term programmed multiple development (PMD), in relation to TLC, was first used by Perry, Haag and Glunz (1973). In PMD, a TLC plate was developed repeatedly in the same direction with the same solvent, each subsequent development being longer than the previous one, and between developments the plate was dried by radiant heat, optionally assisted by a flow of gas. The lower edge of the plate remained in contact with the solvent in the reservoir at all times, and solvent migration distances were controlled by the length of intervals between heating cycles. During this process of repeated development the solvent passed over the material on the plate a number of times and caused spot concentration. This was achieved because on the second and subsequent passes, the solvent flowed over the trailing edge of the spot before reaching the leading edge, compressing the spot into a band of material thus increasing resolution and sensitivity (Jupille and Perry, 1975). The process is illustrated in **Figure 2.2.**

The principle of developing the chromatogram in the same direction over increasing solvent migration distances, as in PMD, was maintained by Burger (1984) when he introduced automated multiple development (AMD), however all other characteristics of AMD were different. Instead of developing the plate with the same solvent repeatedly, for at least a major portion of the total AMD process, a developing solvent different to that used for the preceding step was employed for each run. Whereas the plate was continuously in contact with the solvent in PMD, in AMD the solvent was removed completely from the developing device after each run to ensure that the solvent used in the next cycle had its intended composition and was not contaminated by traces of the previous solvent. After removal of the solvent, the plate was dried by vacuum in AMD -

**Figure 2.2**

**Representation of spot concentration on TLC by multiple development**



no heat was applied. This procedure has been automated by Camag (Muttenz, Switzerland) in an AMD instrument (Jaenchen, 1985) which is described in detail in Section 2.2.2.

The features of AMD described above allow a reproducible gradient elution to be achieved using normal-phase adsorption chromatography on a silica TLC plate. In contrast to column liquid chromatography, the elution gradient in AMD starts with the solvent of greatest elutropic strength and each successive development is carried out with a solvent of lower elutropic strength over an increased distance. In its original form, the technique of PMD described by Perry *et al.* (1973) had the tendency to concentrate sample spots into narrow bands on the chromatographic plate. This is enhanced dramatically when the first solvent passing over the spot has the strongest eluting power for all the components present, as is typical with AMD. The effect is thus similar to that which occurs in a layer with a concentration zone.

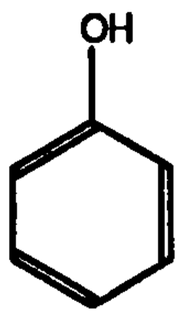
AMD has been used for a variety of separations including psychopharmaceutic drugs (Bigalke, Ebel, Ullrich and Voelkl, 1987), ecdysteroids (Wilson and Lewis, 1987), plant extracts (Trypsteen, Van Severen and De Spiegeleer, 1989), pesticides in water (Burger, 1984; Burger, Kohler and Jork, 1990; De La Vigne and Janchen, 1990; De La Vigne, Janchen and Weber, 1991) and PTH amino acids, analgesics, corticosteroids, dyes and barbiturates (Jaenchen, 1985). The technique has been found to be particularly useful for separating substances in those samples where the components differ appreciably in polarity (Burger *et al.*, 1990). This is particularly true in the field of drug metabolism research, thus AMD was selected as the separatory approach to investigate the usefulness of HPTLC for the detection and characterisation of drugs and metabolites in biological fluids in the present studies.

The term manual multiple development (MMD) has been used in two contexts. It has been used to describe the procedure by which the process of AMD is carried out manually as opposed to using the instrument which is commercially available (Wilson and Lewis, 1987). The term MMD has also been used to describe all multiple development procedures which were employed before PMD was proposed (Poole and Belay, 1991). In the present studies MMD refers to the manually operated AMD process.

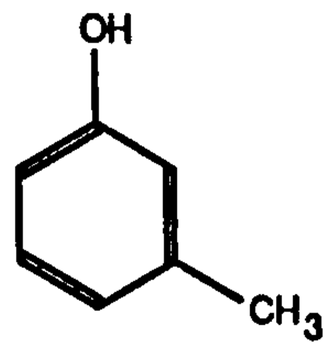
### 2.1.2 Assessment of Manual Multiple Development

Prior to assessing the usefulness of MMD for separating drugs and their metabolites, the MMD process itself was investigated. Five phenolic compounds; phenol, m-cresol, m-chlorophenol, resorcinol and m-hydroxybenzoic acid (m-HBA), were selected to investigate the MMD process. Their structures are shown below.

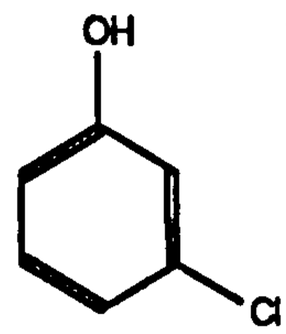
**Structures of phenolic compounds used to investigate MMD**



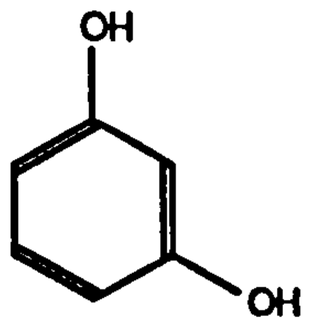

Phenol



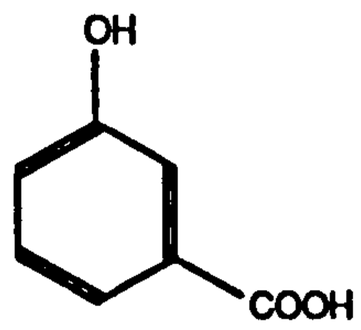

m-Cresol




m-Chlorophenol




Resorcinol




m-Hydroxybenzoic Acid

These compounds were selected because; (i) they cover a reasonable polarity range, (ii) phenol, m-cresol and m-chlorophenol are difficult to separate by isocratic TLC (Dietz, Traud, Koppe and Rubelt, 1976) and (iii) resorcinol and m-HBA differ from phenol by the addition of functional groups that are added in common Phase I metabolic transformations.

In order to assess fully the usefulness of MMD for separating drugs and metabolites the chromatographic properties of all known drugs and metabolites would need to be examined. Clearly this would be impractical, therefore model compounds were selected to carry out the investigation. The criteria for the selection of model compounds were as follows:

1. The compounds should be based upon simple chemical structures which form the molecular building blocks of many drugs.
2. Typical Phase I and Phase II metabolic transformations must be modelled by the compounds.
3. A number of "real" drugs and their metabolites must be included.

A series of phenols, indoles, coumarins and biphenyls (structures which form the molecular building blocks of many drugs) were selected to examine the changes in retention brought about by typical Phase I transformations. The structures of these compounds and the manner in which they correspond as models of metabolism are shown in **Figures 2.3-2.6**. Additional compounds; ondansetron, ranitidine, loxtidine and their metabolites were also chosen for examination by multiple development because they are examples of "real" drugs developed recently. Their structures are shown in **Figures 2.7-**

**2.9.** A series of glucuronides and sulphates was selected to examine the effect of conjugation on retention. These were selected because, as discussed in Chapter 1, glucuronylation and sulphation are the most common Phase II metabolic transformations. The structures of the compounds are shown in **Figures 2.10 and 2.11.**

**Figure 2.3**

**Phenolic models of Phase I transformations**

**Figure 2.4**

**Some potential Phase I metabolites of aspirin**

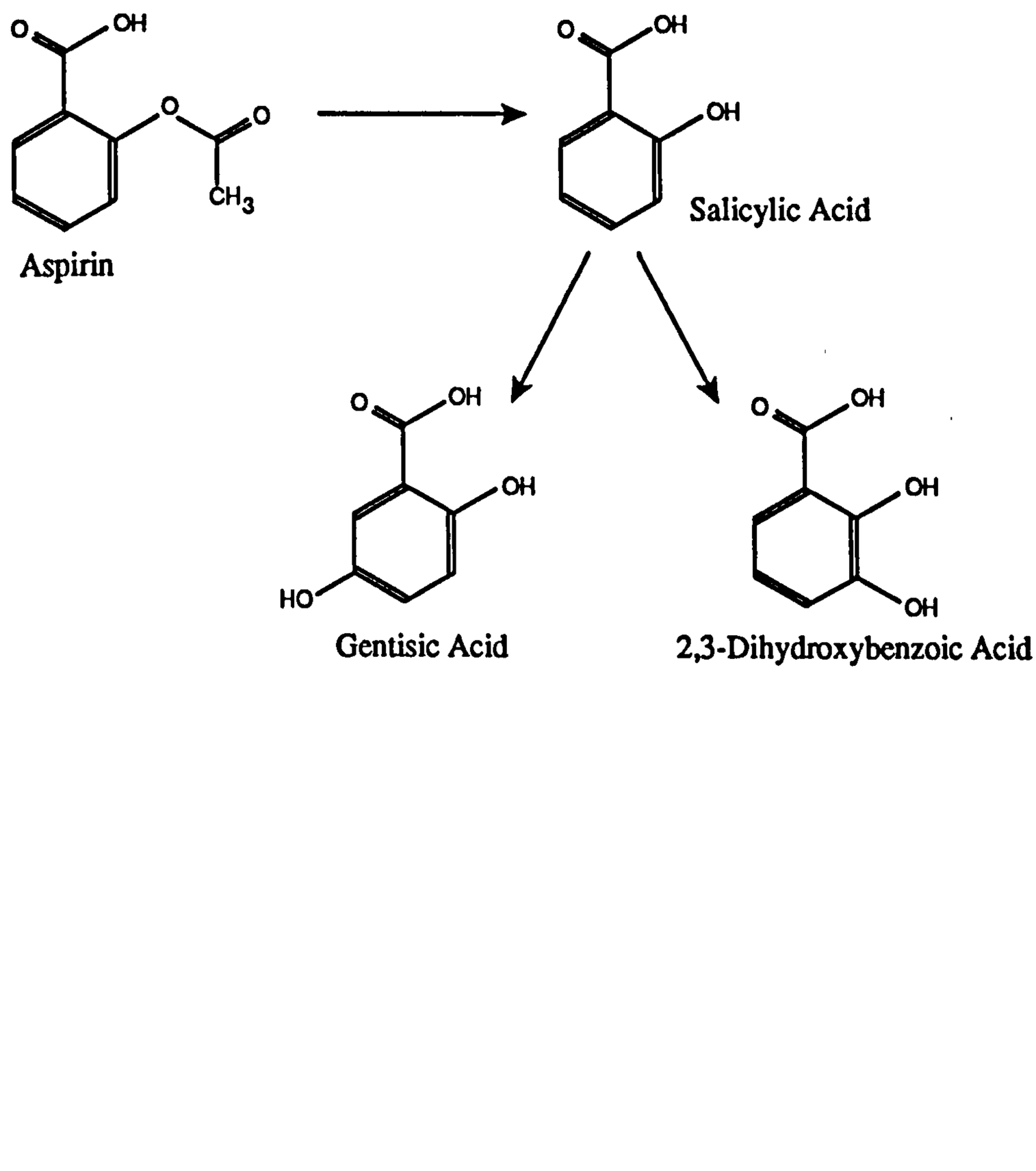
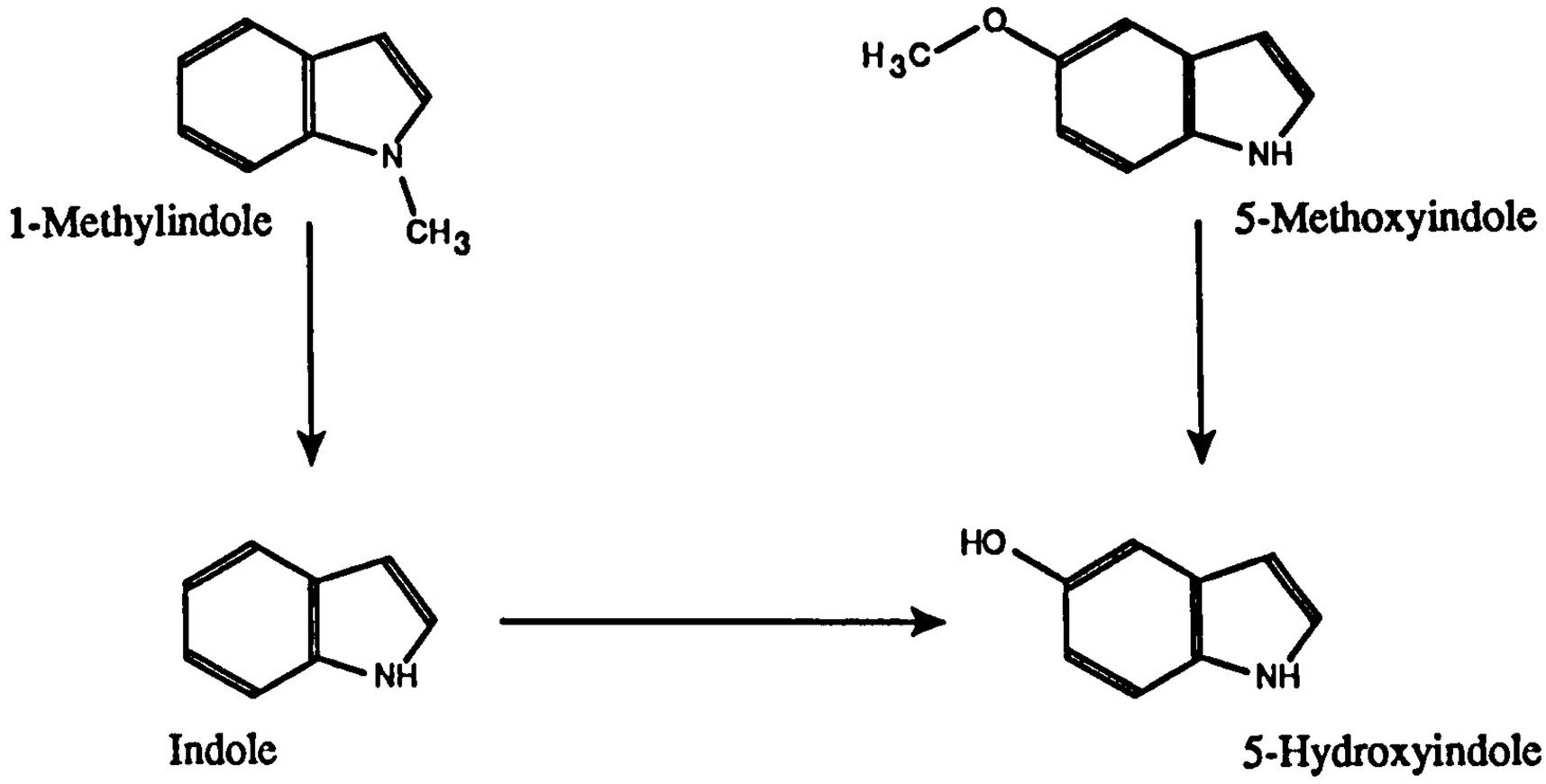


**Figure 2.5**

**Indole models of Phase I transformations**





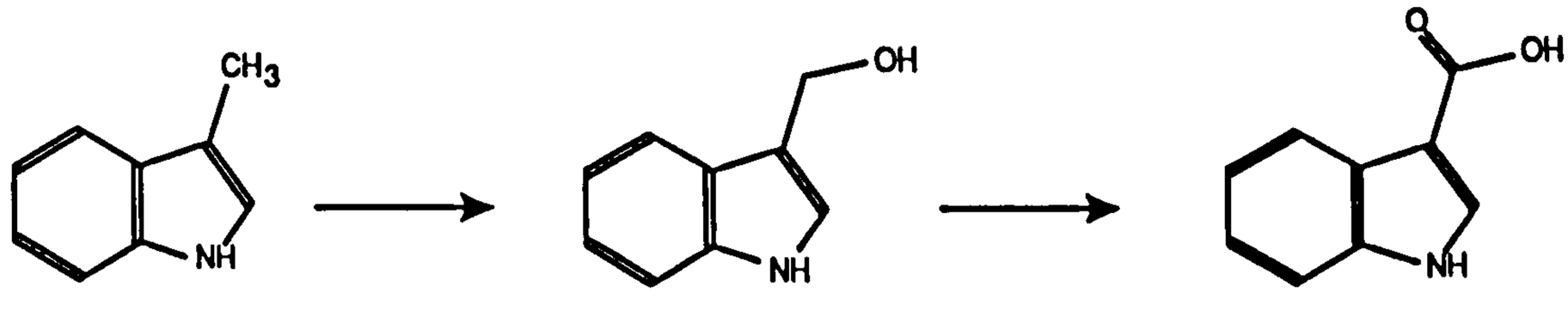


**Figure 2.6**

**Coumarin and biphenyl models of Phase I transformations**



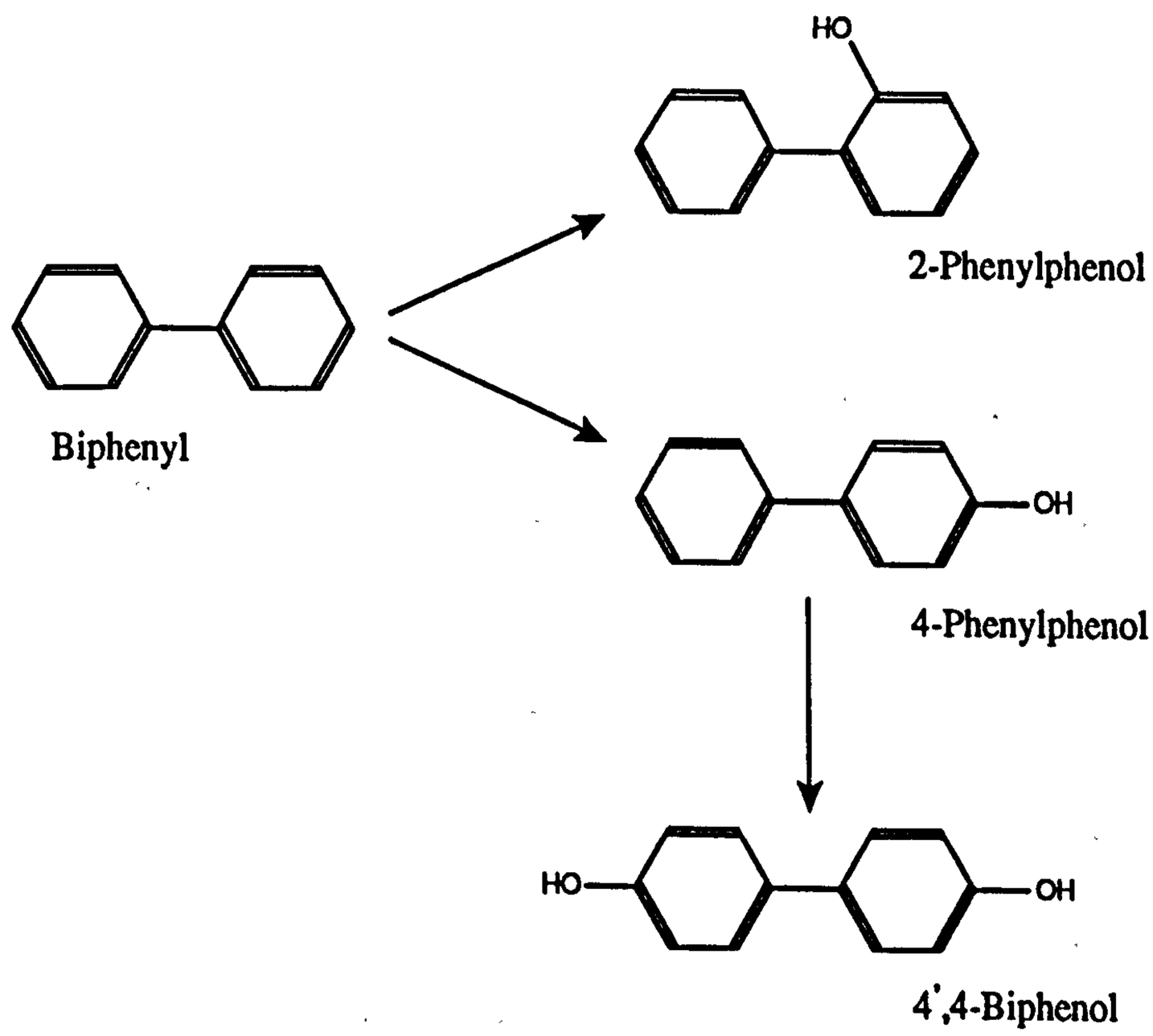
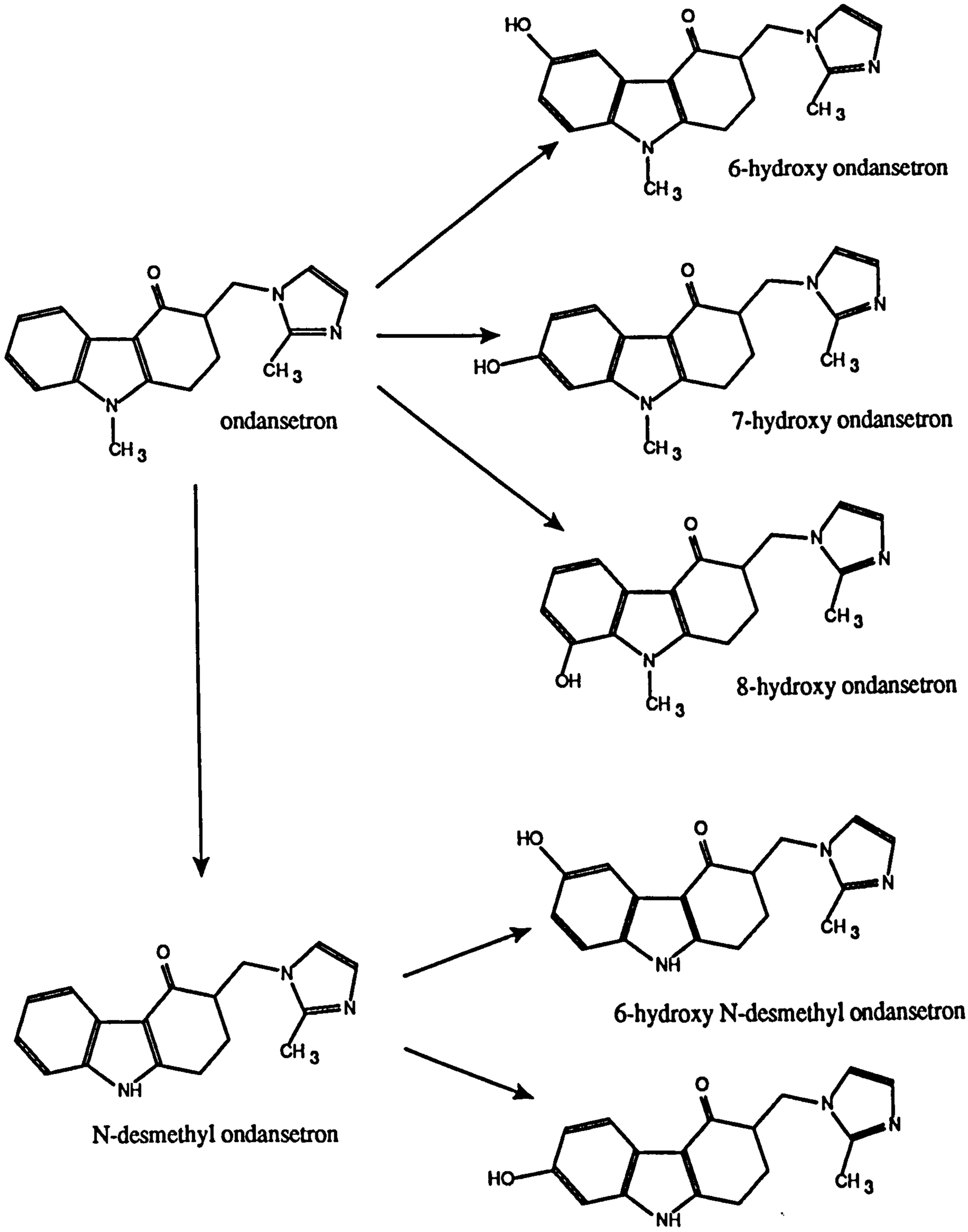



**Figure 2.7**

**Phase I metabolism of ondansetron (Saynor and Dixon, 1989)**




This is a schematic representation of the Phase I metabolism of ondansetron and the format is not intended to infer a reaction order.

**Figure 2.8**

**Metabolism of ranitidine (Bell *et al.*, 1980)**

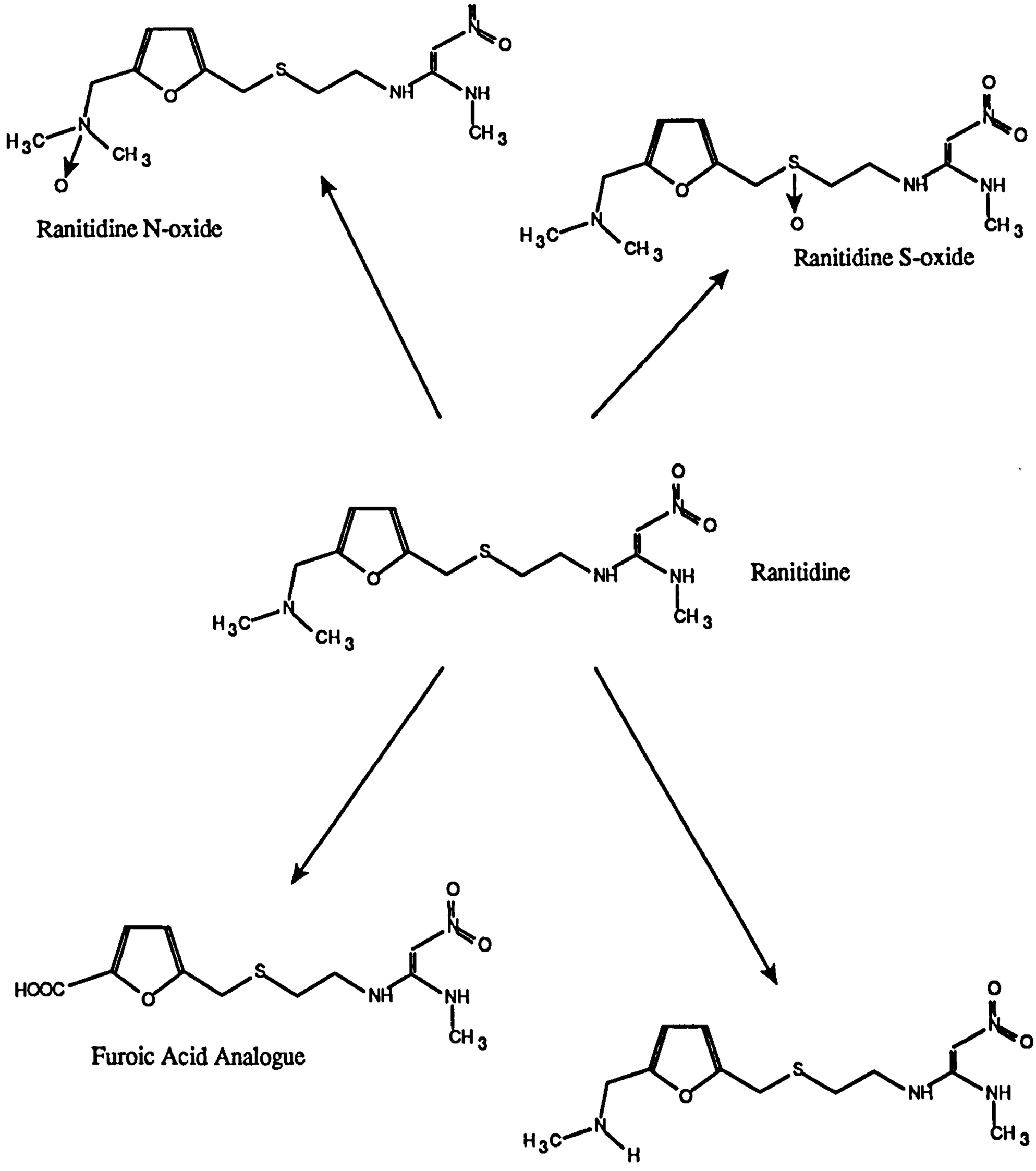


**Figure 2.9**

**Metabolism of loxtidine (Bell *et al.*, 1983)**

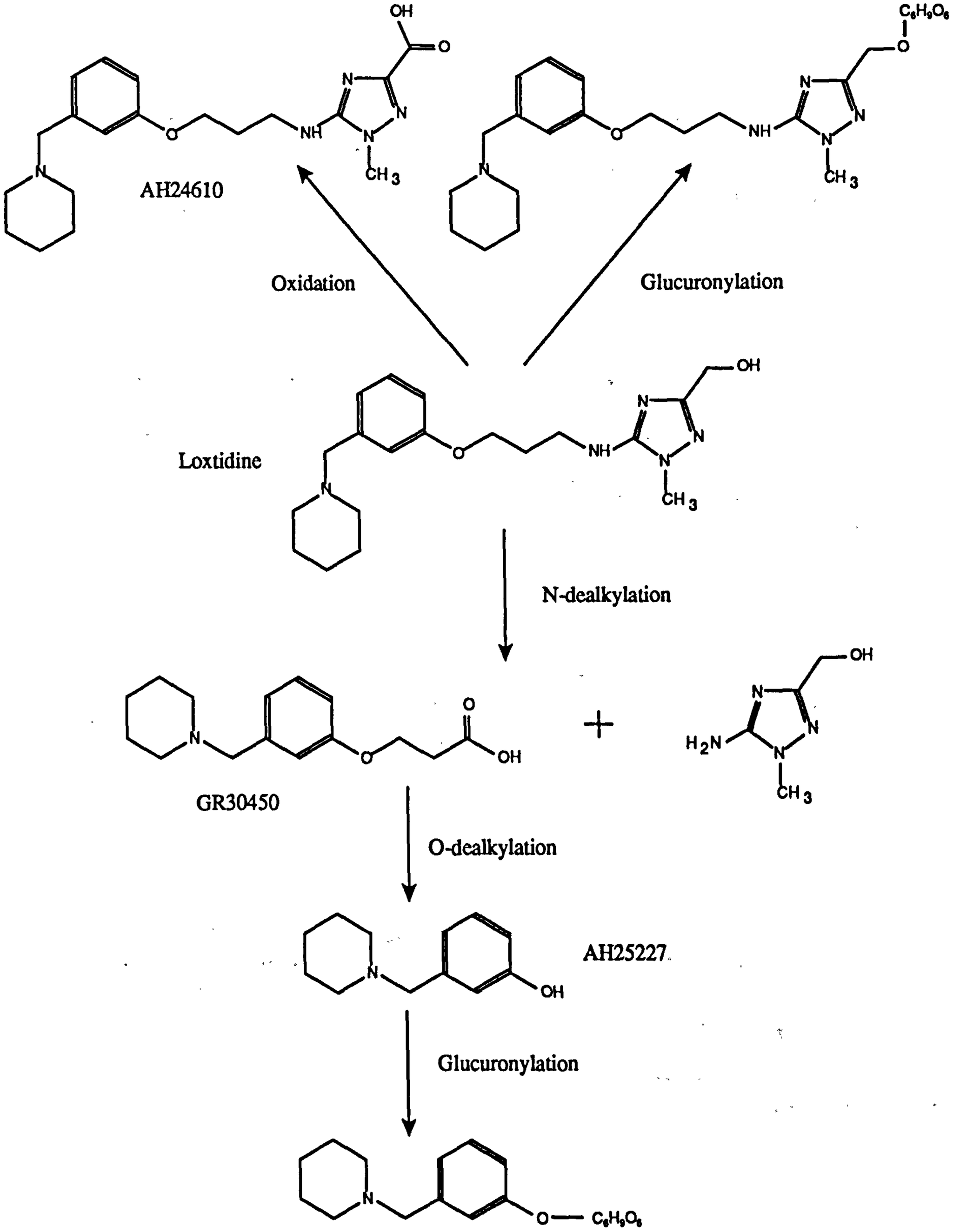


**Figure 2.10**

**Compounds used as models of Phase II metabolic transformations**

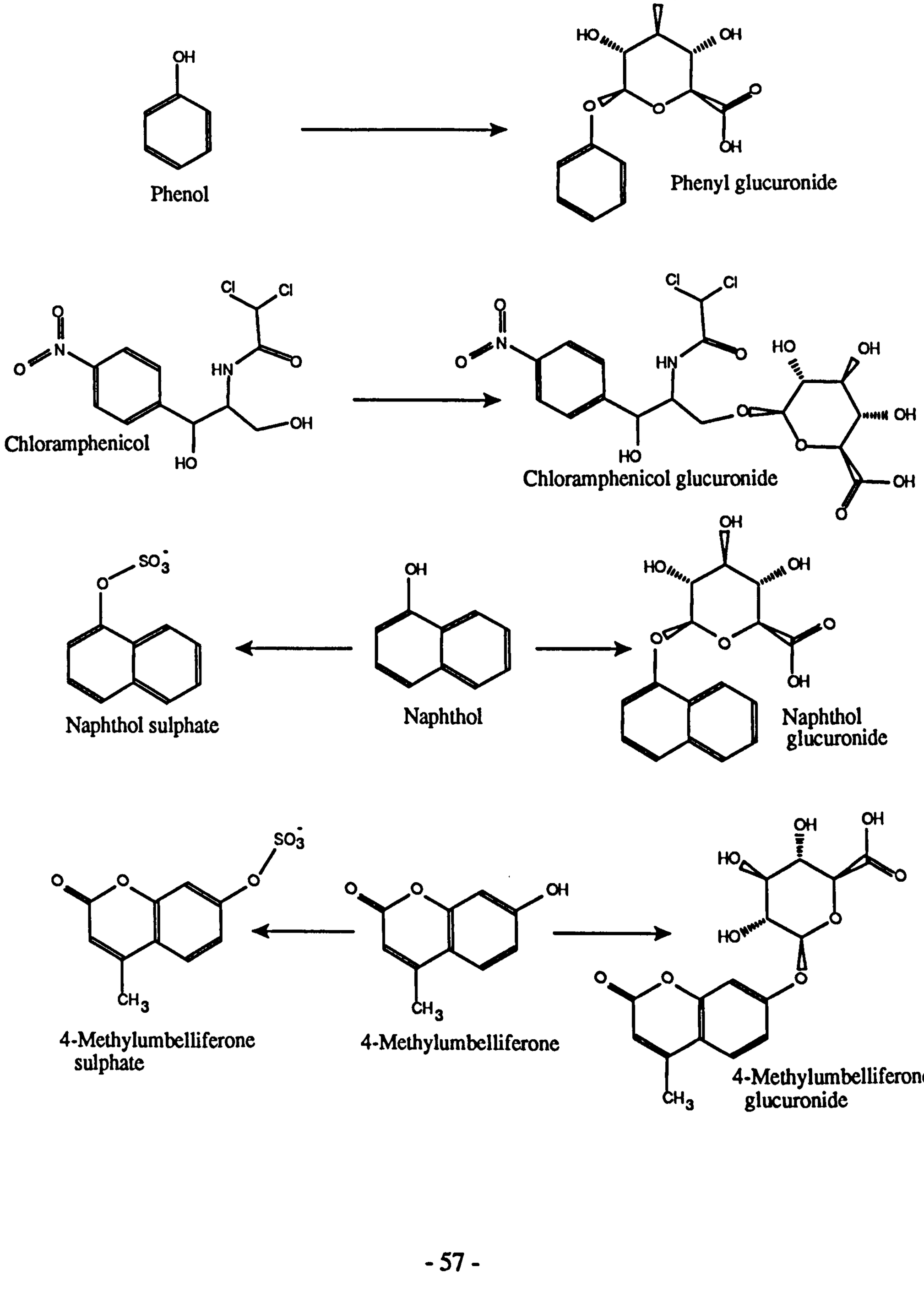
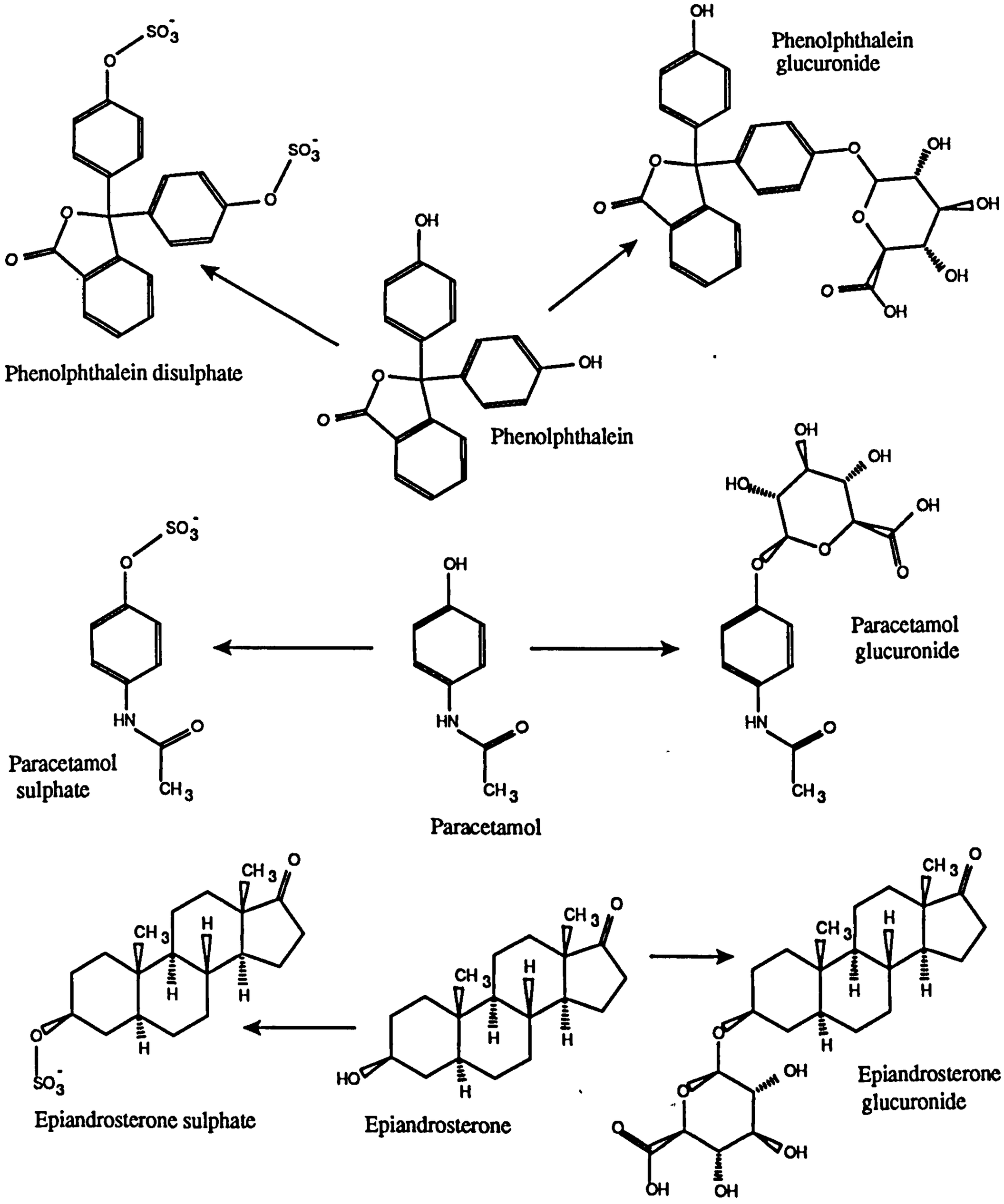


**Figure 2.11**

**Compounds used as models of Phase II metabolic transformations**



### 2.1.3 Aims of the present studies

The main aim of the studies described in this chapter is to assess the usefulness of multiple development techniques on normal-phase HPTLC for the separation of drugs and metabolites with a view to developing a generally applicable gradient. Further aims are to assess the sensitivity of HPTLC for detecting drugs and metabolites in biological fluids.

## *2.2 Methodology*

In this section the multiple development methods used are described in detail.

### 2.2.1 Manual Multiple Development

Chromatography was carried out on glass-backed silica gel 60 HPTLC plates (10x20cm or 10x10cm) without fluorescent indicator (Merck, Poole, UK). Prior to use, the plates were pre-developed with methanol to remove any material which may have been adsorbed to the surface, and were marked on the reverse side to indicate the development distance for each solvent mixture. Samples were applied as solutions in a suitable organic solvent (about 1mg/ml), 5mm from the bottom of the plates either as spots using micro-capillaries (1, 2, or 5$\mu$l), or as bands using a Camag Linomat IV sample applicator (BDH, Poole, UK).

Application of samples as spots using micro-capillaries is rapid, however, the sample volumes which can be applied are limited if compact spots are to be achieved. The size

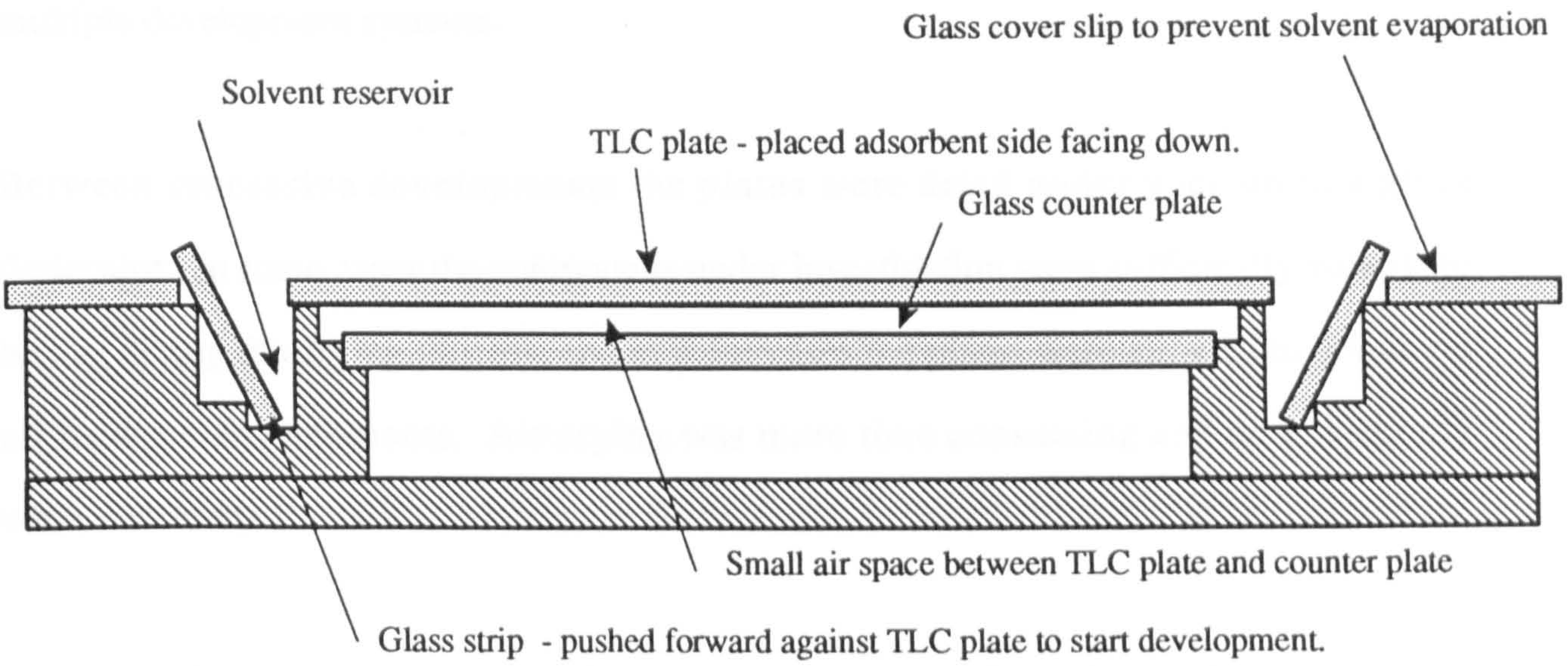
of initial spots can be minimised if solutions are prepared in the least polar solvent in which the compounds of interest will dissolve (Colthup, 1988). Compact spots are then achieved because although the solvent spreads out from the applicator, the dissolved material has an Rf of approximately zero in the solvent and hence remains at or near the origin; the point of application.

However, if samples contain many components it is unlikely that a non-polar solvent will dissolve all the components, and in this situation a compromise is necessary. Application of small volumes (e.g. 1$\mu$l) from micro-capillaries results in solvent spread to approximately 5mm and enables a reasonable amount of material to be applied (1$\mu$g if the solution is 1mg/ml). A spot of this size is reduced to a narrow band by the use of multiple development. The use of micro-capillaries for aqueous samples however is less successful, and when samples of this nature need to be applied use of a Camag Linomat IV sample applicator is more appropriate. The Linomat IV is a semi-automated instrument for applying samples to TLC plates as bands. The sample is loaded into a syringe and applied onto the plate in a stream of air or nitrogen through a fine nozzle as an aerosol. The aerosol droplets dry rapidly and hence band broadening on sample application is minimised.

Solvent mixtures (10ml) for MMD were prepared using Gilson pipettes (Anachem, Luton, UK) to dispense the required volumes accurately, and were stored prior to use in glass scintillation vials. The plates were then developed in a Camag horizontal development chamber (BDH, Poole, UK) for the prescribed distances. A schematic diagram of the chamber is shown in **Figure 2.12.** The use of a horizontal development chamber has two main advantages over conventional tank development. Firstly, because

**Figure 2.12**

**A schematic diagram of the Camag horizontal linear development chamber**



of the small air volume surrounding the plate surface, the atmosphere in contact with the plate rapidly becomes saturated with solvent vapour. This helps to maintain reproducible conditions from one plate to another. Secondly, the volume of solvent required for development of the plate is very small, 1 to 3ml, depending upon the development distance. This reduces solvent consumption which would otherwise be considerable with multiple development systems.

Between successive developments the plates were dried under vacuum in a glass desiccator. In some cases the compounds under investigation were sufficiently volatile to be lost during the drying process, and in these cases the plates were allowed to dry in the air between developments. Air drying was more time consuming and was used only when necessary.

When development was complete the plates were scanned from the origin to the solvent front using a Camag TLC Scanner II (BDH, Poole, UK), and material on the plate was detected by ultraviolet absorbance. The signal output was recorded on a Trilab 2000 Chromatography Data System (Trivector, Sandy, UK).

### 2.2.2 Automated Multiple Development

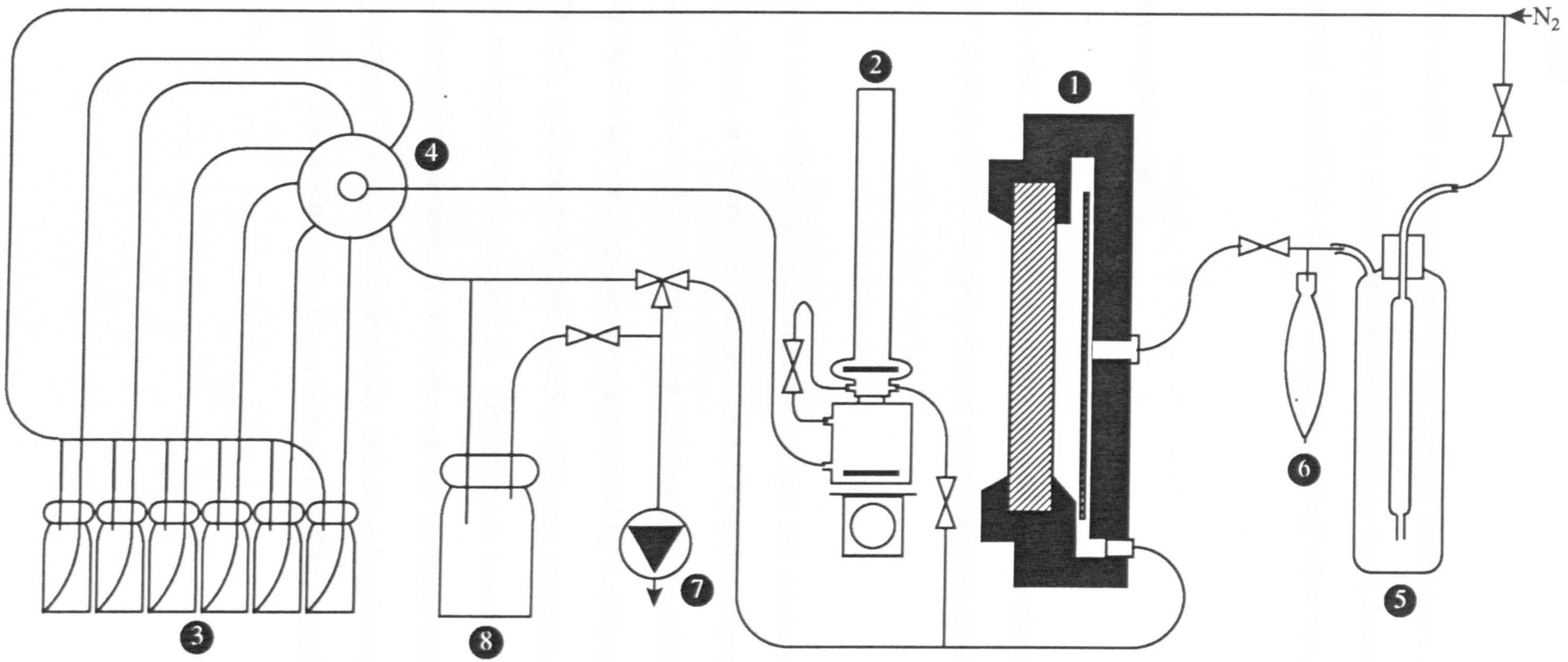
Chromatography was performed on glass-backed Silica Gel 60 HPTLC plates, as used for MMD, and samples were applied as solutions in methanol (about 1mg/ml) using a Camag Linomat IV sample applicator. Plates were then developed in a Camag AMD instrument (BDH, Poole, UK). The plates were scanned and the output recorded as described for MMD.

A simplified flow diagram of the AMD instrument is presented in **Figure 2.13.** Chromatographic development occurs in an enclosed chamber (1) which has inlet and outlet connections for supplying and withdrawing solvent, and for pumping gas phase in for plate conditioning and out for drying under vacuum between developments. Solvent mixtures are prepared in the gradient mixer (2) from up to six reservoirs (3) which contain the individual solvent components. The mixing chamber and reservoirs are connected by an electronic valve (4). Flow of liquids around the system is achieved by means of nitrogen gas pressure. Gas phase for plate conditioning is prepared by passing nitrogen through a wash bottle (5) which may contain sulphuric acid, ammonia solution or other reagents depending upon the conditioning required, into a bladder (6), from where it is released into the developing chamber at the appropriate time. The system is operated from a central microprocessor control unit.

A typical AMD step consists of the following sequence: the content (8ml) of the upper part of the gradient mixer (2) is transferred into the development chamber (1) which contains the chromatographic plate. Migration of the solvent starts immediately, and when the prescribed time (which determines the development distance) has elapsed, the remaining solvent is withdrawn from the chamber to the waste collection bottle (8). When all the liquid has been removed from the chamber, vacuum is applied by a pump (7) and the silica gel layer is dried for the required time which can be programmed into the sequence. Prior to starting the next development the plate is conditioned by introducing gas phase from the bladder (6) into the chamber. While the drying phase is in progress, solvent for the next step is prepared in the gradient mixer.

**Figure 2.13**

**A schematic diagram of the Camag automated multiple development instrument**




Adapted from Jaenchen, 1985

## *2.3 Experimental*

All MMD and AMD solvent schemes used in the present studies are presented in **Appendix I.** A summary of the schemes giving their elutropic strength ranges is shown in **Table 2.1.** Methanol and hexane were HPLC grade, and all other solvents were of AnalaR™ grade.

### 2.3.1 MMD Universal Gradients

Universal gradients in AMD describe systems which start with a very polar solvent which is varied via a "basis" solvent of medium polarity to a very non-polar solvent (Jaenchen and Issaq, 1988).

Initial experiments to study MMD were carried out using a test mixture comprising phenol, m-cresol, resorcinol, m-hydroxybenzoic acid (m-HBA) and m-chlorophenol. The mixture was developed using a modified version of a universal AMD gradient (Jaenchen, 1985). The solvents for the universal gradient were prepared by combining methanol, dichloromethane (DCM), and hexane to form a series of binary mixtures of gradually decreasing elutropic strength (MMD Scheme 1). Considerable bowing of the solvent front occurred with this system, particularly when the solvent mixtures contained DCM. This led to the solvent front having a concave profile and caused variation in retention across the HPTLC plate. The use of chloroform instead of DCM (MMD Scheme 2), overcame the problem.

**Table 2.1**

**Summary table of MMD and AMD Schemes used in the present studies**

| | Description | Elutropic Strength Range |
|---|---|---|
| MMD Scheme 1 | Modified Universal with DCM | 0.73 to 0.0 |
| MMD Scheme 2 | Modified Universal with $CHCl_3$ | 0.73 to 0.0 |
| MMD Scheme 3 | Linear Stepwise System | 0.73 to 0.0 |
| MMD Scheme 4 | Linear Stepwise System | 0.3 to 0.1 |
| MMD Scheme 5 | Linear Stepwise System | 0.49 to 0.0 |
| MMD Scheme 6 | Linear Stepwise System | 0.348 to 0.0 |
| MMD Scheme 7 | Linear Stepwise System | 0.73 to 0.598 |
| MMD Scheme 8 | Linear Stepwise System | 0.73 to 0.688 |
| MMD Scheme 9 | Linear Stepwise System | 0.73 to 0.51 |
| MMD Scheme 10 | Linear Stepwise System | 0.69 to 0.57 |
| MMD Scheme 11 | Linear Stepwise System | 0.654 to 0.604 |
| MMD Scheme 12 | Linear Stepwise System | 0.65 to 0.61 |
| AMD Scheme 1 | Linear Stepwise Approximation | 0.73 to 0.057 |

MMD Scheme 2 separated the phenols into two groups; resorcinol and m-HBA had Rfs of approximately 0.3, and the remaining phenols had Rfs of approximately 0.6. Previous work had indicated that although phenol, m-cresol and m-chlorophenol are difficult to separate by TLC, resorcinol and m-HBA should be resolved easily (Dietz *et al.*, 1976). As it was considered likely that the migration distance of analytes is a function of the elutropic strengths of the solvents forming the MMD gradient, the elutropic strengths of the solvents in MMD Scheme 2 were calculated using Snyder's equation (Snyder, 1968a).

$$E_{ab} = E_a + \frac{\log (N_b * 10^{\alpha n_b (E_b - E_a)} + 1 - N_b)}{\alpha n_b}$$

$E_{ab}$ = Solvent strength of binary mixture of solvents A & B.
$E_a$ = Solvent strength of solvent A.
$E_b$ = Solvent strength of solvent B.
$N_b$ = Mole fraction of solvent B in the binary solvent mixture.
$n_b$ = The effective molecular area of an absorbed solvent molecule B. (B is the stronger component of a binary mixture.)
$\alpha$ = Adsorbent surface activity function (0.7 for water deactivated silica).

The mole fraction of B in the solvent mixture is calculated as follows:

$$N_b = \frac{\text{No. molecules of B}}{\text{No. molecules of A + No.molecules of B}}$$

$$\text{No. molecules of A is proportional to } \frac{\text{Weight of A } (W_A)}{\text{Molecular Weight of A } (MW_A)}$$

and Weight A = Volume of A ($V_A$) * Density of A ($D_A$)

It therefore follows that

$$N_b = \frac{(D_B * V_B)/MW_B}{\{(D_A * V_A)/MW_A\} + \{(D_B * V_B)/MW_B\}}$$

The constants used for the calculations are shown in the table below (Snyder, 1968a).

**Constants used for the calculation of elutropic strengths of binary mixtures.**

| Solvent | Strength ($E_o$) | D/MW | Molecular Area (n) |
|---|---|---|---|
| n-Hexane | 0.00 | 0.87 | 10 |
| Chloroform | 0.26 | 1.26 | 10 |
| Dichloromethane | 0.32 | 1.57 | 10 |
| Ethyl Acetate | 0.38 | 1.02 | 5.7 |
| Acetonitrile | 0.50 | 1.91 | 10 |
| Propan-2-ol | 0.63 | 1.34 | 8 |
| Methanol | 0.73 | 2.49 | 8 |

A graph of elutropic strength against development distance for MMD Scheme 2 is shown in **Figure 2.14.** A large change in elutropic strength occurred at a position corresponding to Rf=0.3, thus explaining the results obtained with the phenols. Such an abrupt change in elutropic strength could cause a large number of compounds to cluster at the point of change, and might be a particular problem when screening for novel compounds in biological fluids. Other universal gradients have similar elutropic strength profiles **(Figure 2.15)**, so the separation of drugs and unknown metabolites might therefore be compromised by that feature of universal gradients. Wilson and Lewis (1987) found that six ecdysteroids migrated with an Rf of approximately 0.5 when developed with a universal gradient **(Figure 2.15)**, which is consistent with the abrupt change in solvent strength which occurs with the gradient used.

**Figure 2.14**

**Graph of elutropic strength against development distance in MMD Scheme 2**

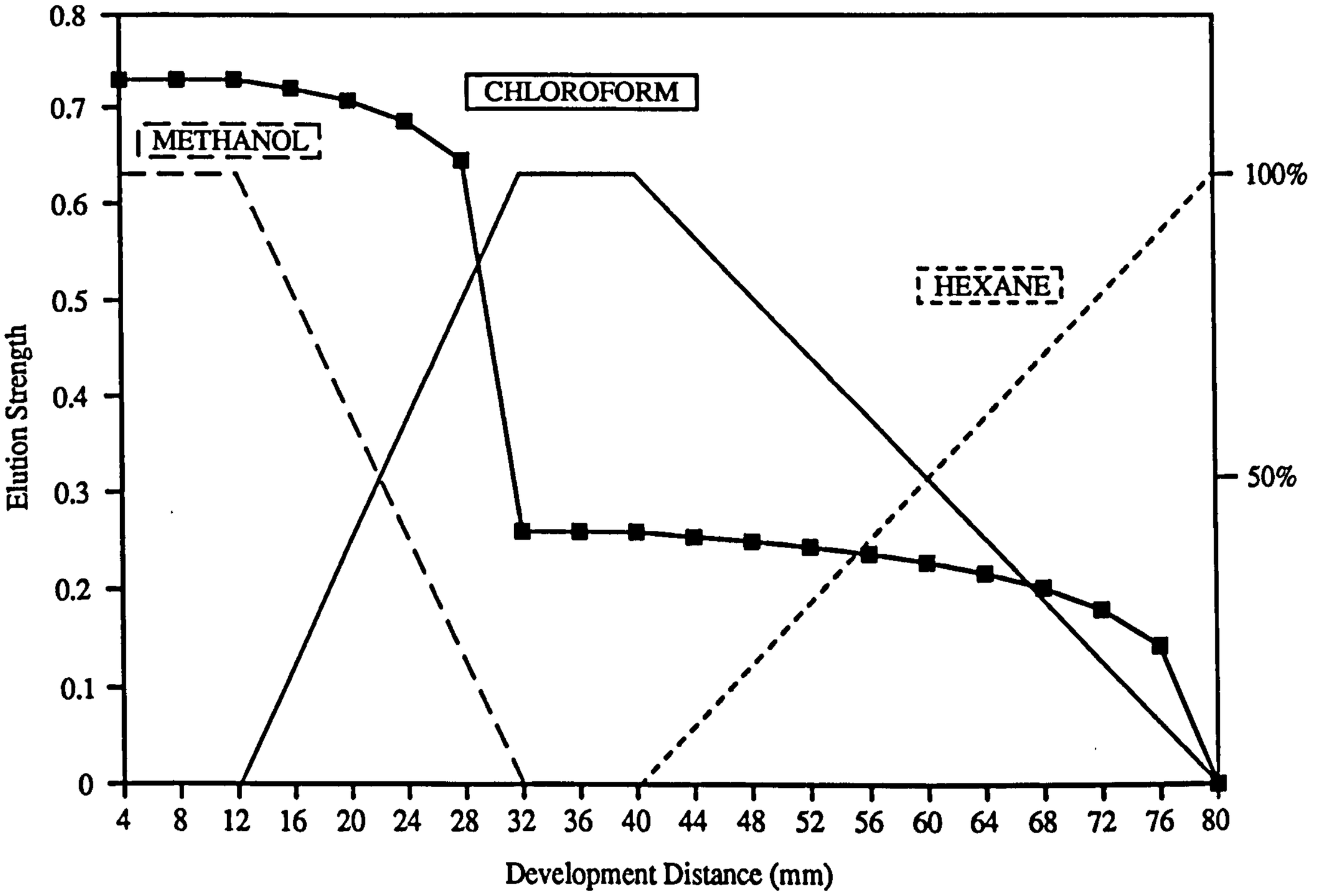
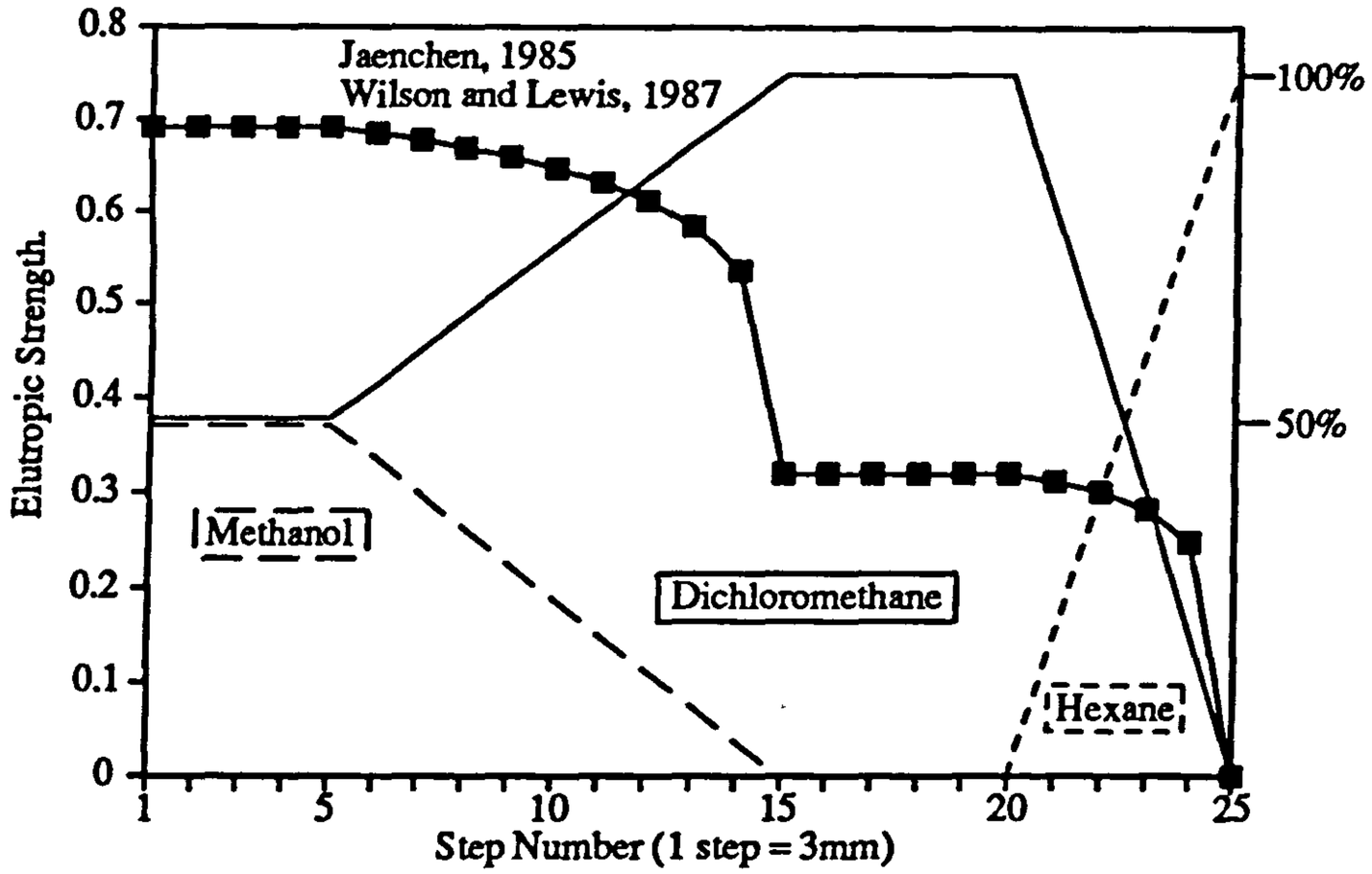
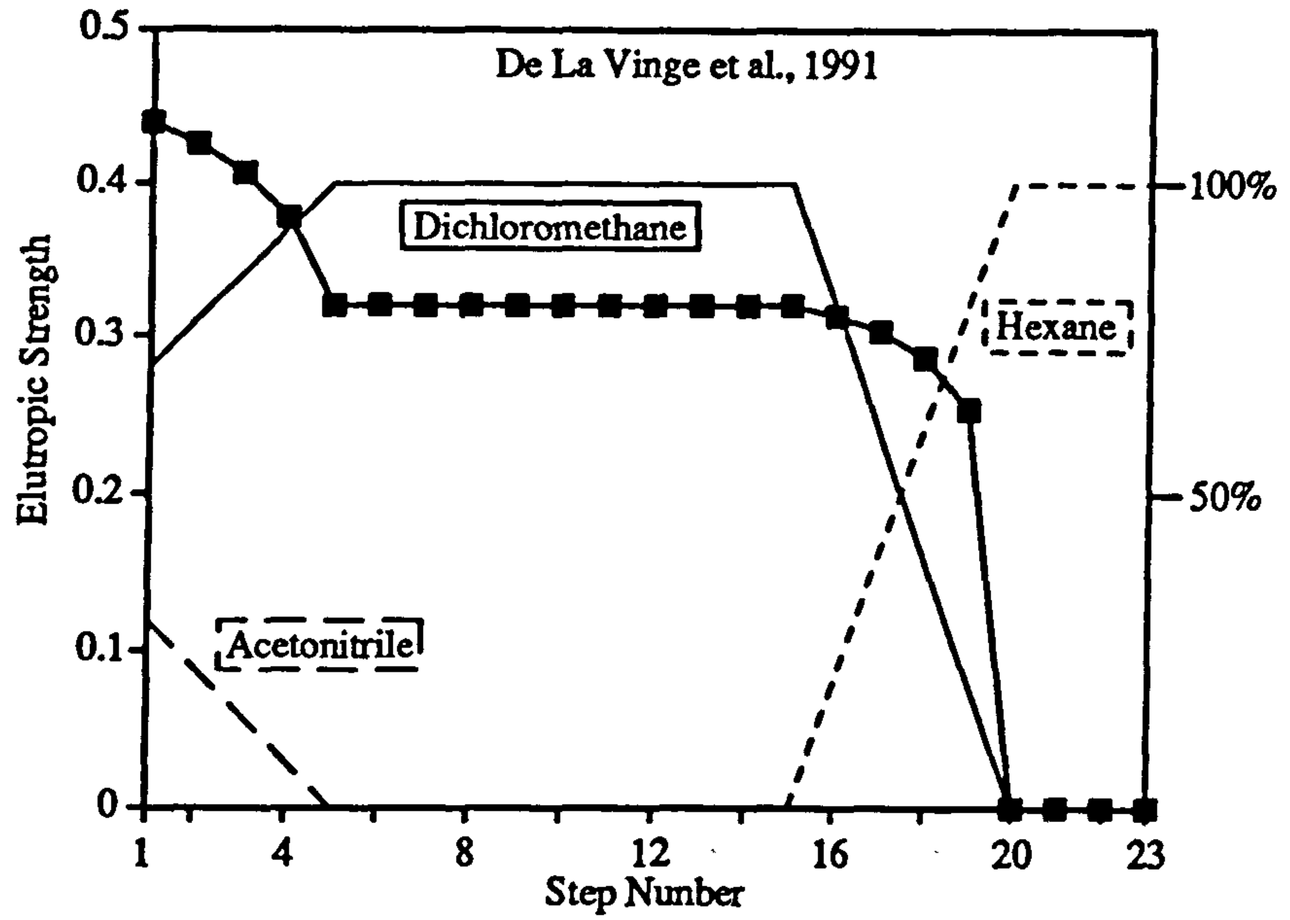
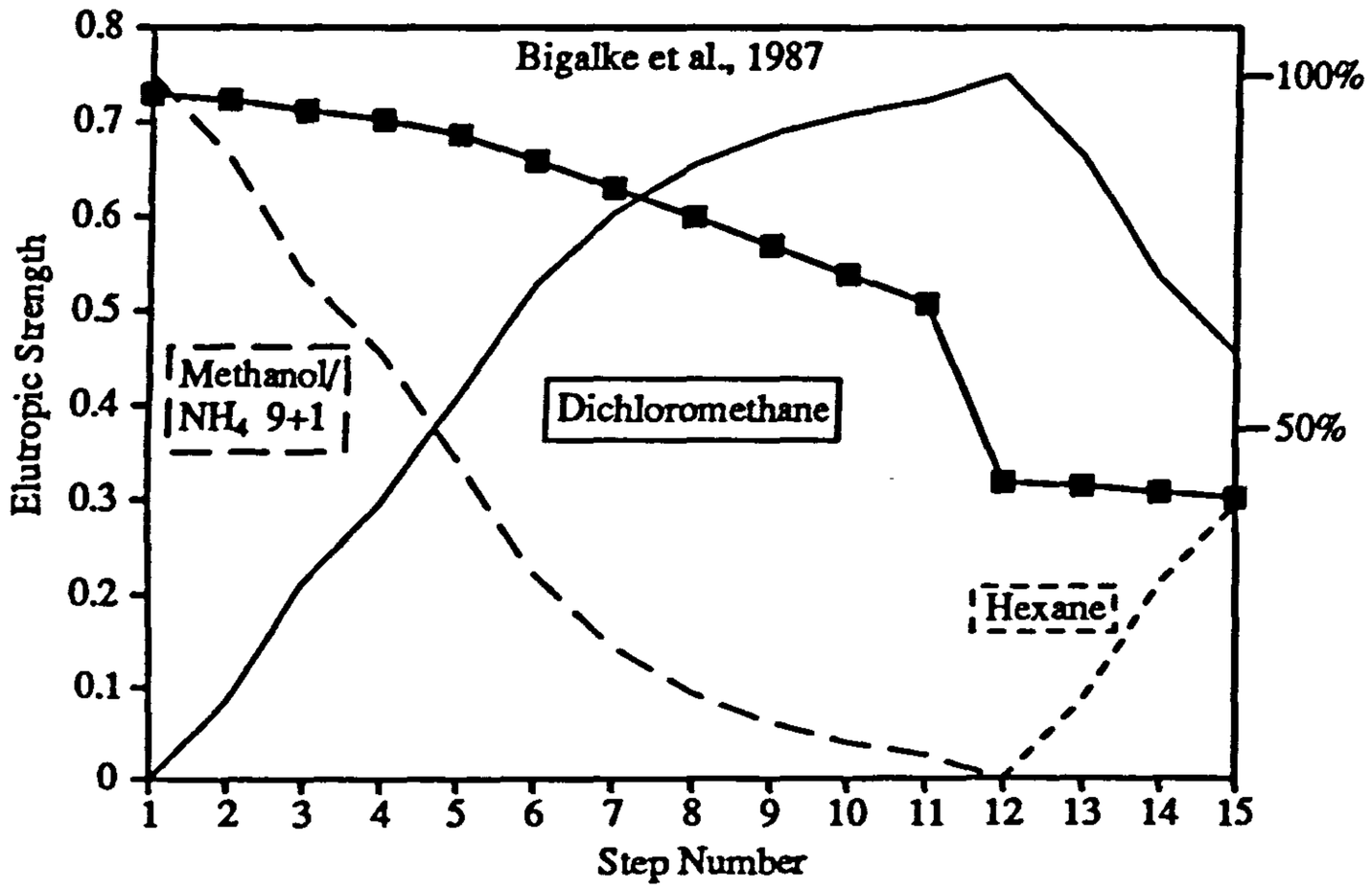


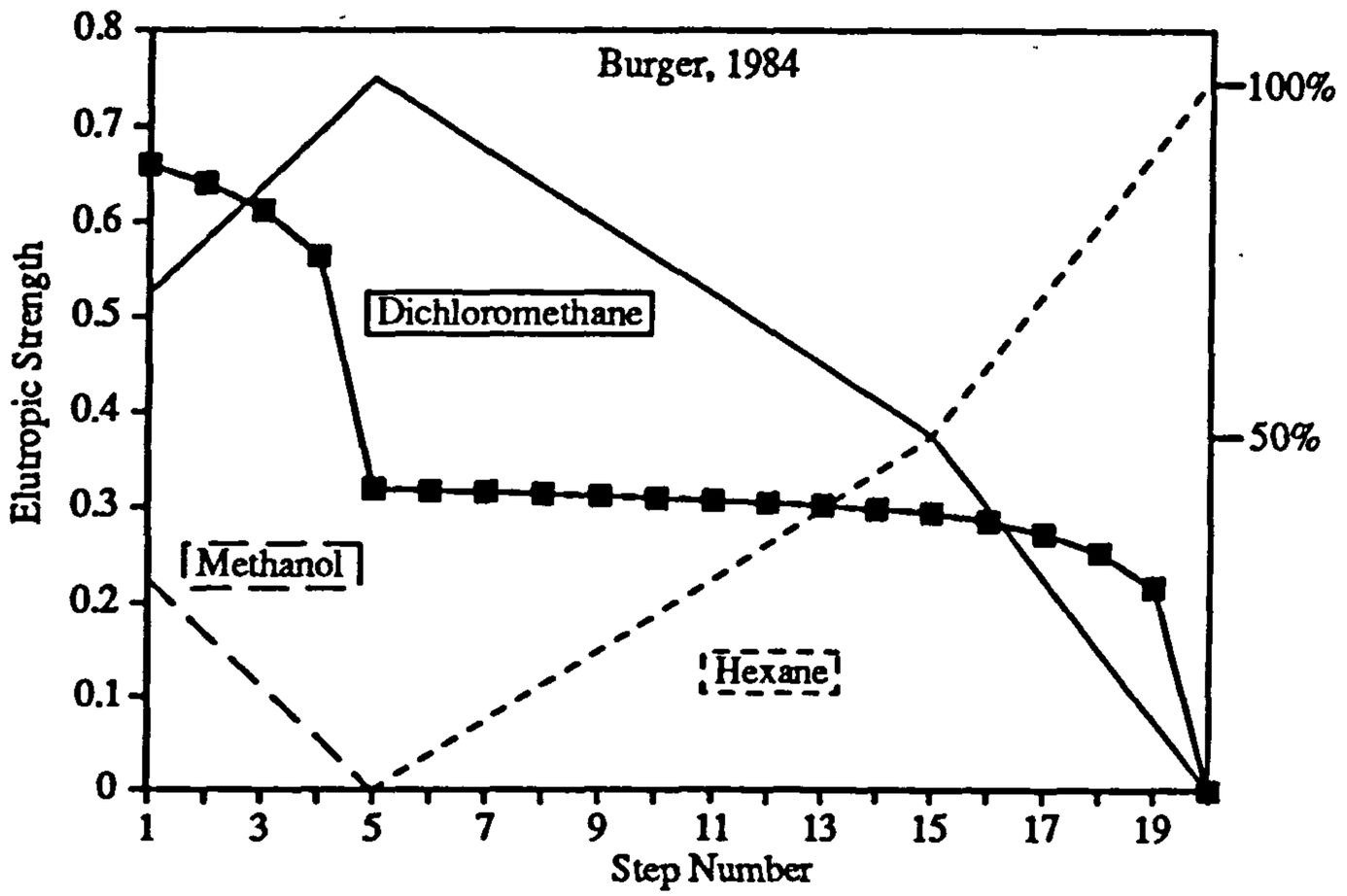
**Figure 2.15**

**Elutropic strength profiles of some universal gradients**



An ideal profile for multiple development would therefore be linear across the full elutropic strength range (0.73 - 0.00, methanol to hexane). In order to construct a linear profile, the elutropic strengths of binary solvent mixtures were calculated using Snyder's equation. The data are shown in **Figures 2.16 and 2.17.** The constants used in the calculations were as shown previously. From the data obtained a linear multiple development gradient comprising 12 steps with a regular series of elutropic strengths from methanol to hexane was constructed (MMD Scheme 3), **Figure 2.18.**

### 2.3.2 MMD Linear Gradients

The linear MMD gradient (MMD Scheme 3) was constructed by using four solvents instead of the three employed in universal gradients. Polar solvents were obtained with methanol and ethyl acetate mixtures, intermediate strength solvents were mixtures of ethyl acetate and chloroform and the non-polar solvents were chloroform and hexane mixtures.

#### 2.3.2.1 Mixture of Phenols

The test mixture of phenols was developed using the linear MMD gradient (MMD Scheme 3). An additional linear gradient over a reduced elutropic strength range using binary mixtures of chloroform and ethyl acetate, then chloroform and hexane, was constructed to improve further the separation obtained (MMD Scheme 4), **Figure 2.19.**

**Figure 2.16**

**Solvent strengths of binary solvent mixtures**

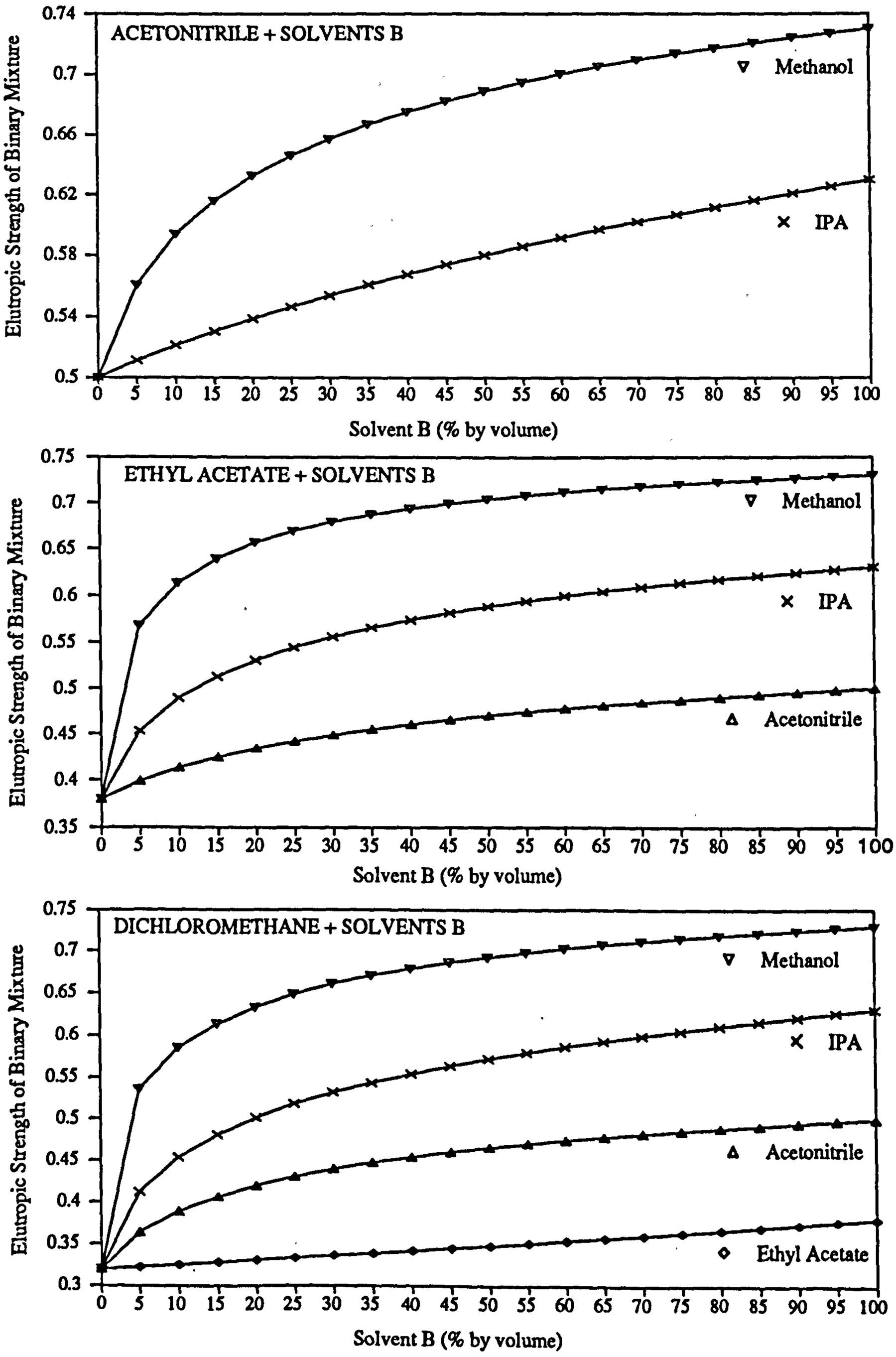


**Figure 2.17**

**Solvent strengths of binary solvent mixtures**

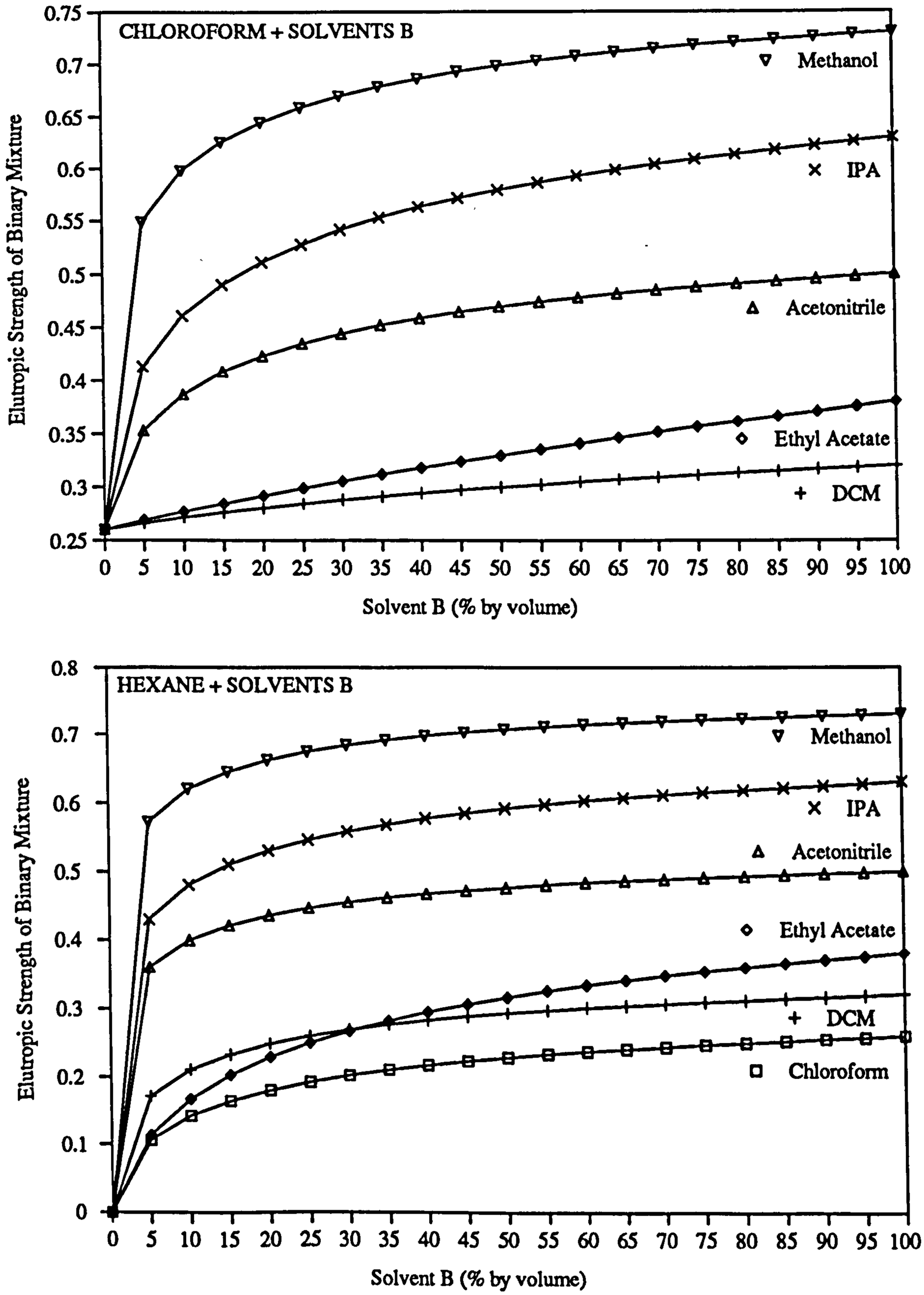


**Figure 2.18**

**Graph of elutropic strength against step number in linear MMD Scheme 3**

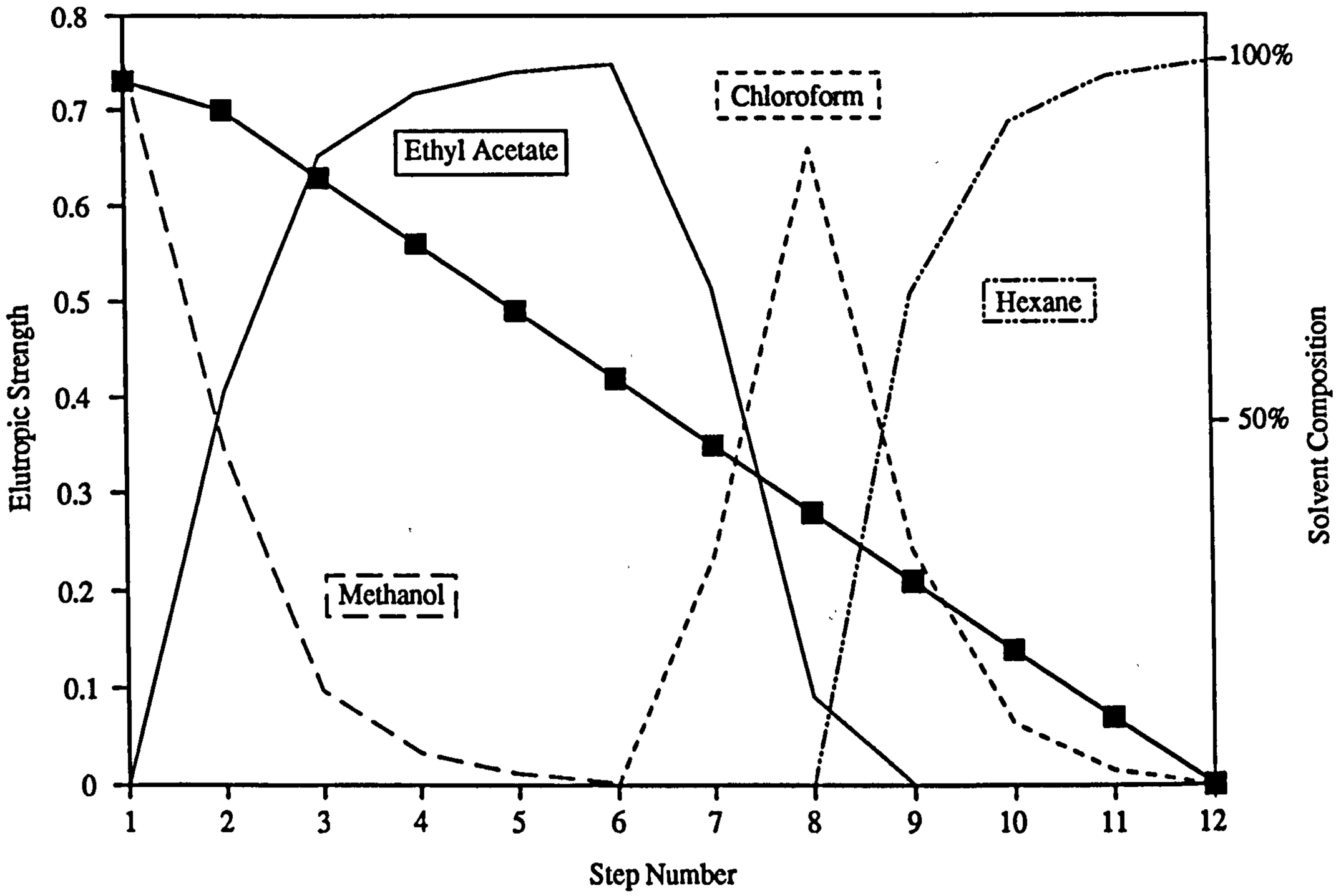
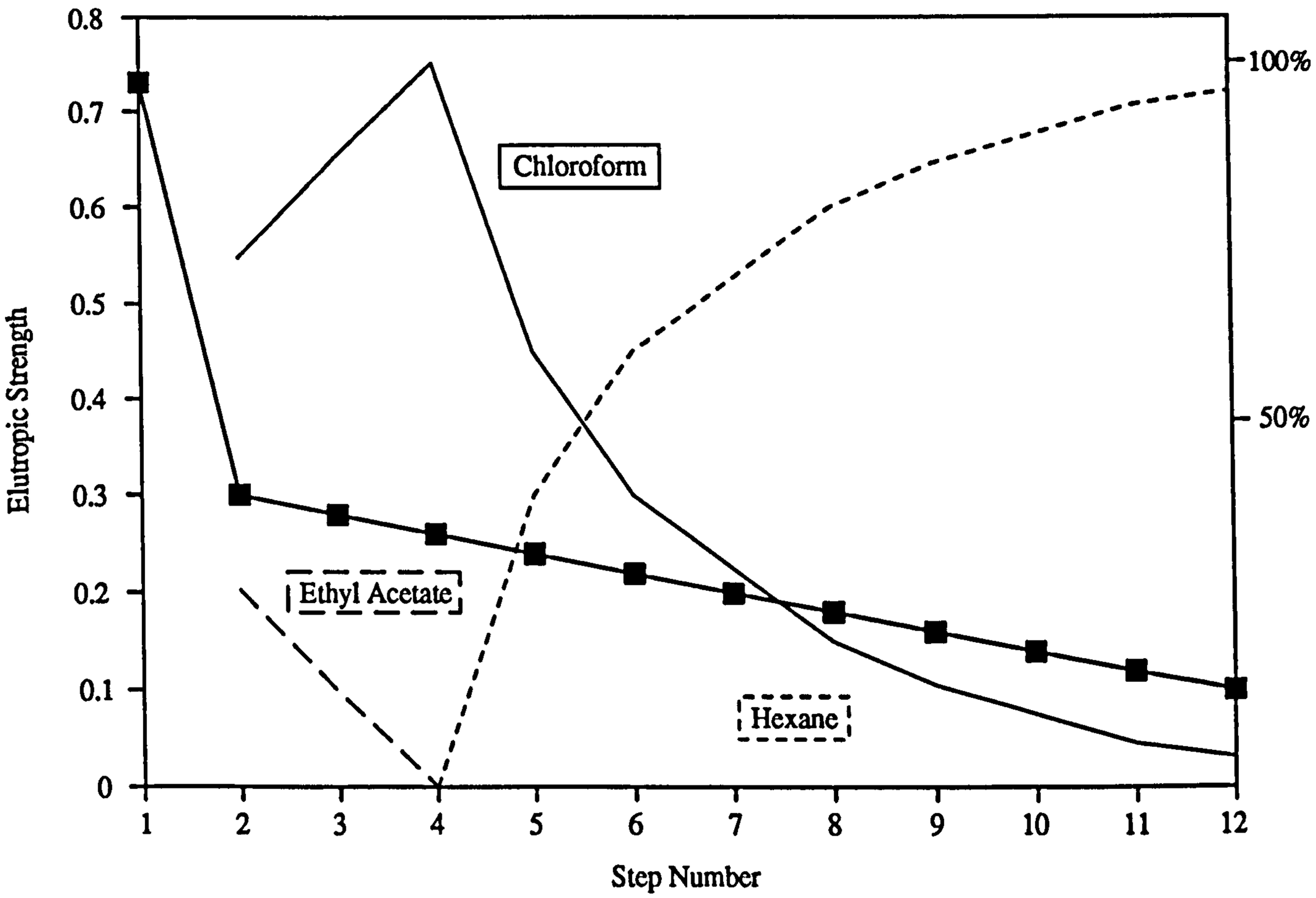


**Figure 2.19**

**Graph of elutropic strength against step number in linear MMD Scheme 4**



### 2.3.2.2 Compounds to model Phase I transformations

The series of phenols, indoles, coumarins and biphenyls which differ from each other by typical Phase I metabolic transformations (**Figures 2.3 to 2.6**) were applied to a HPTLC plate and developed using the full linear MMD gradient (MMD Scheme 3). Linear gradients over smaller elutropic strength ranges (MMD Schemes 5 and 6) were used to increase the separation in some cases.

Further compounds; ondansetron, ranitidine and loxtidine, and their Phase I metabolites (**Figures 2.7 to 2.9**) were applied to HPTLC plates and developed using the full linear gradient (MMD Scheme 3), and additional linear gradients over reduced elutropic strength ranges (MMD Schemes 7 to 12). The effect on migration and peak shapes of acidic and basic compounds was investigated when acetic acid, ammonia or triethylamine (TEA) were added to the solvent mixtures (1% v/v).

### 2.3.2.3 Compounds to model Phase II transformations

A series of compounds and their glucuronide and sulphate conjugates (**Figures 2.10 and 2.11**) were applied to HPTLC plates and developed using the full linear gradient (MMD Scheme 3). A mixture of paracetamol (Pa), Pa glucuronide and Pa sulphate was used to determine the reproducibility of retention from one track to another on a single HPTLC plate. Seven spots of the mixture were applied 1cm apart across a 10x10cm plate, the plate was developed using the full linear gradient, and the retention data were evaluated to determine the intra-plate reproducibility. The reproducibility of retention between plates was evaluated using the range of compounds and their glucuronide and sulphate conjugates. The mixtures were each applied 1cm apart to four different 10x10cm plates

which were then developed using the full linear gradient. The retention data were evaluated to determine the inter-plate reproducibility.

Linear gradients over reduced elutropic strength ranges (MMD Schemes 7 and 9) were used to increase the separation of the glucuronides and sulphates, and additional plates were developed with acetic acid and TEA (1% v/v) in the solvent mixtures to investigate the effect of these modifiers on the retention of the conjugated metabolites.

The epiandrosterone compounds do not contain ultraviolet absorbing chromophores, and were detected on the HPTLC plates by dipping the plates in 0.5% vanillin in sulphuric acid (98%) and ethanol (4:1) and heating at 100°C for 5 minutes. The compounds formed blue bands against a yellow background.

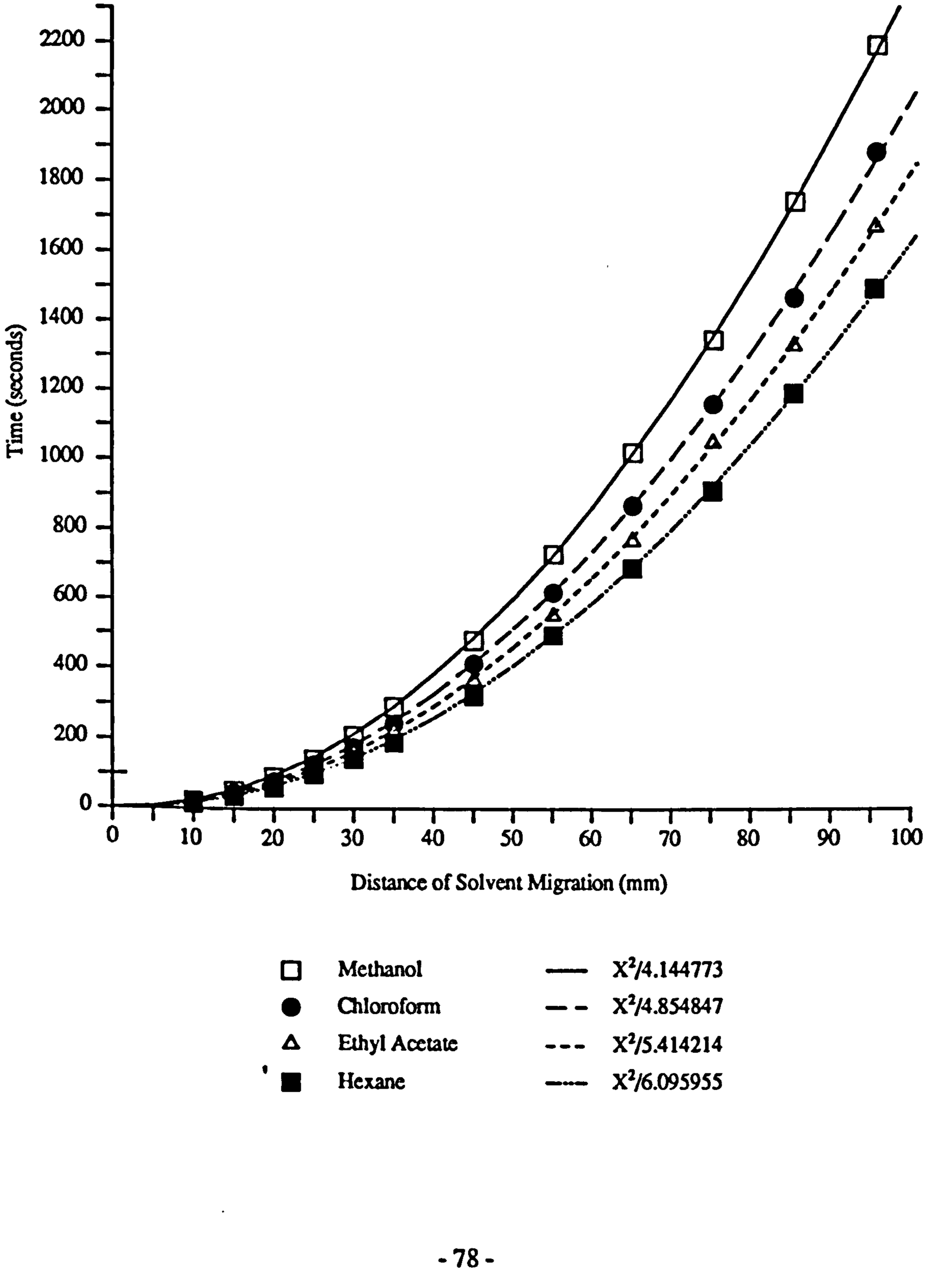
### 2.3.3 Automated Multiple Development

There are two major differences between the way in which the automated multiple development (AMD) instrument operates, and the MMD procedure. Firstly, development of each step is controlled by time instead of distance in AMD, and secondly the AMD instrument cannot be programmed to prepare solvent mixtures to any desired specification.

In order to estimate the times needed to run each step, the times taken for various solvents to migrate up a HPTLC plate were measured, and graphs of time against distance plotted, **Figure 2.20.** A curve for each solvent was obtained which fitted well to the equation $Y=X^2/k$. This is in agreement with Poiseuille's Law which states that capillary flow is

**Figure 2.20**

**Solvent migration times on silica gel HPTLC plates, and curves fitted to $Y = X^2 / k$**



inversely proportional to the length of a capillary (Perry *et al.*, 1973). In TLC, this means that the velocity of solvent advance is inversely proportional to the distance of solvent travel. Thus the time taken for solvent to advance increases as the square of that distance (if $v=dx/dt=k/X$, then $X\,dx = k\,dt$, and $t = (k/2)X^2$). The curves obtained were used to estimate the times needed for the various development distances that were required for AMD.

The AMD instrument prepares solvent mixtures by diluting the original solvent (i.e. that selected for Step 1) with solvents from subsequent reservoirs. Prior to Step 1 the mixer is filled with Solvent 1, and approximately 33% of the total volume present in the mixer is dispensed to the chamber for the first development. The solvent for Step 2 is prepared by filling the mixer while mixing the solvents by means of magnetic stirrers. Thus if the original solvent is methanol and the second reservoir contains ethyl acetate the solvent mixtures prepared by the instrument approximate to those shown in the table below.

**Approximate compositions of solvents prepared by AMD if the initial solvent methanol is diluted repeatedly with ethyl acetate**

| Step No | Methanol | Ethyl Acetate |
|---|---|---|
| 1 | 100 | 0 |
| 2 | 67 | 33 |
| 3 | 45 | 55 |
| 4 | 30 | 70 |
| 5 | 20 | 80 |
| 6 | 14 | 86 |
| 7 | 9 | 91 |
| 8 | 6 | 94 |
| 9 | 4 | 96 |
| 10 | 3 | 97 |

Thus the precise solvent mixtures required to automate the linear gradient could not be used, and in order to approximate the linear gradient a number of steps had to be "missed" by developing the plate for 0.1min only before removing the solvent and drying the plate. The unwanted solvent mixtures did not therefore migrate up the plate sufficient distance to affect the samples chromatographically. The gradient using methanol, ethyl acetate, chloroform and hexane (AMD Scheme 1) is shown in **Appendix I, Table 14.** Four AMD runs were carried out with the compounds selected to model Phase II transformations **(Figures 2.10 and 2.11)** to determine the reproducibility of the system between runs.

### 2.3.4 Detection Limits

The success of a method to screen for drugs and metabolites in biological fluids is dependent largely upon whether the components of interest can be detected in small enough quantity and against the background of biological matrices.

#### 2.3.4.1 Analyte against plate background

Whether HPTLC has sufficient sensitivity to detect drugs and metabolites in biological fluids was examined by determining the limits of detection for paracetamol and its glucuronide and sulphate metabolites on a HPTLC plate. Paracetamol was selected because it has relatively poor ultraviolet absorption with maximum absorbance (approximately 250nm) at a wavelength at which many other compounds absorb. Use of paracetamol would therefore test the system rigorously and not provide over optimistic data.

### 2.3.4.2 Analyte against control urine and plasma

An ideal system to screen for drugs and metabolites in biological fluids would comprise application of plasma or urine to the chromatographic system without prior preparation, followed by chromatographic separation with no further modification of the sample. In order to test whether HPTLC with MMD would fulfil these criteria, samples of control plasma (2μl diluted 1:1 with water) and urine (5μl) and of plasma and urine spiked with paracetamol and conjugates (1 and 10μg/ml) were applied as 5mm bands to 10x10cm silica gel HPTLC plates using a Camag Linomat IV sample applicator, and the plates were developed using the full linear gradient (MMD Scheme 3). The Linomat did not apply plasma satisfactorily when undiluted, hence samples were diluted 1:1 with water prior to application. If volumes of the diluted plasma in excess of 2μl were applied, the band of material on the plate did not wet when the solvent reached it and thus caused the solvent front to break up.

## *2.4 Results*

The results from the assessment of multiple development on HPTLC and the examination of detection limits are presented in the following sections.

### 2.4.1 Linear Gradients Using MMD

#### 2.4.1.1 Test mixture of phenols

The full linear gradient (MMD Scheme 3) separated m-HBA and resorcinol from each other, and from the remaining phenols (**Figure 2.21**), thus overcoming the compound clustering which occurred with the universal gradient at the point of abrupt elutropic strength change. Phenol, m-cresol and m-chlorophenol were not separated from each other which may have been due to their similar polarities or a lack of selectivity in the solvents used.

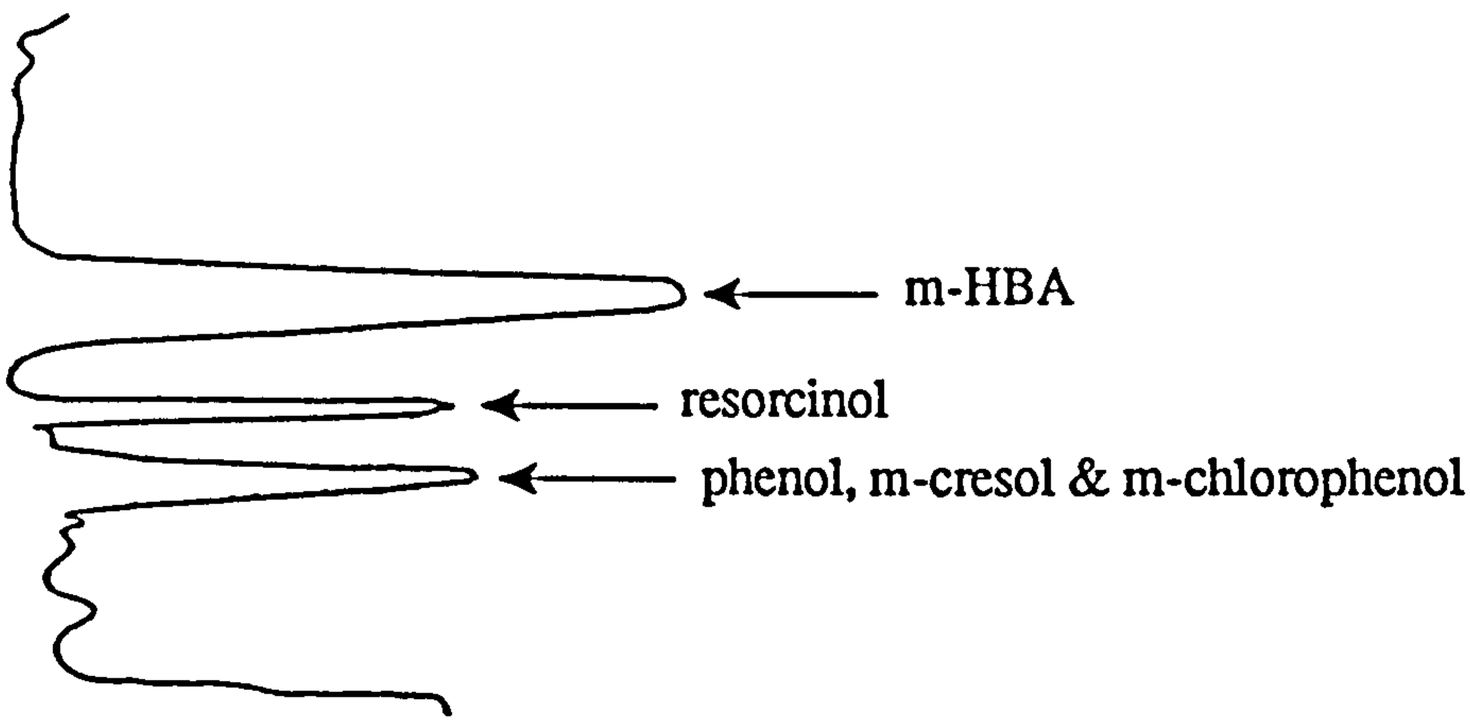
The expanded gradient (MMD Scheme 4) improved the separation of the previously unresolved phenols, **Figure 2.21.**

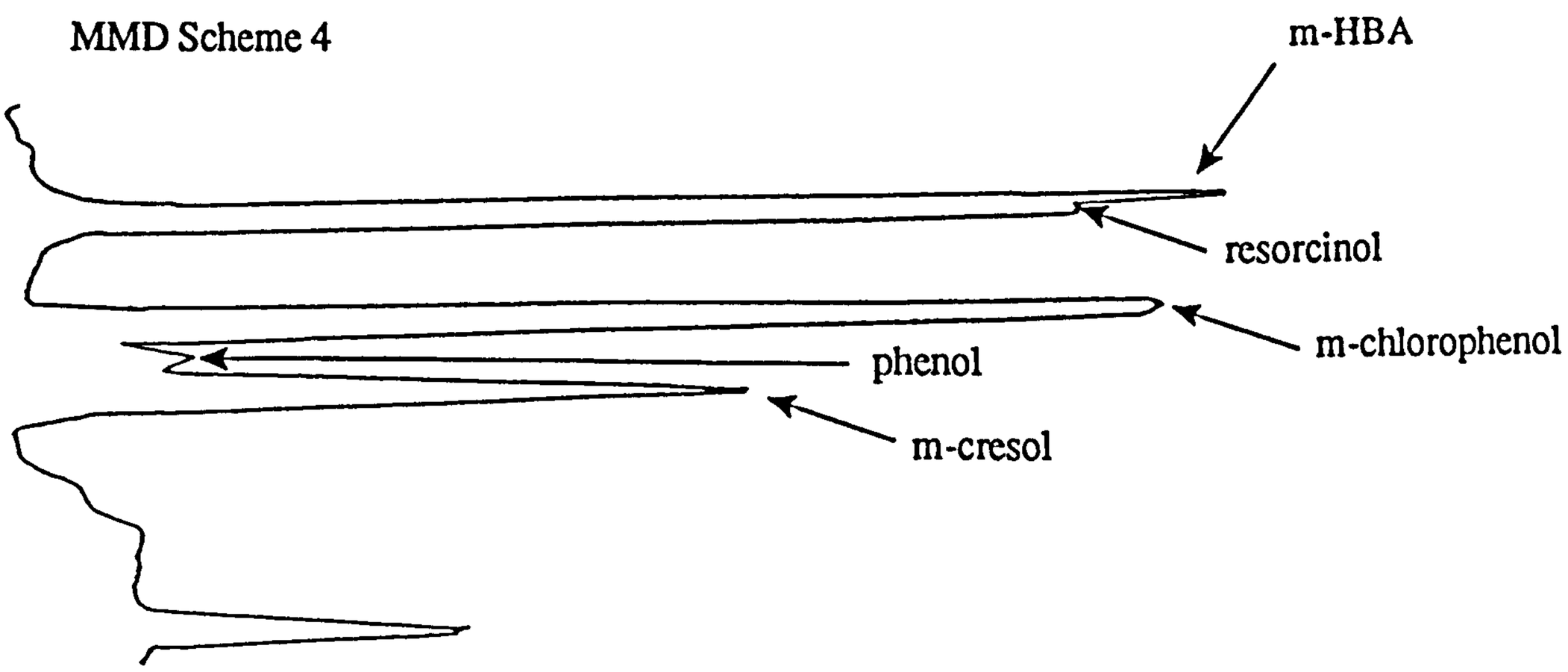
#### 2.4.1.2 Compounds to model Phase I transformations

Jupille and Perry (1975) showed that for PMD, the Rf value is not of great significance because all identical spots tend towards the same position on the plate. They stated that a better measure is distance travelled from the bottom of the plate or the elution distance (ED). In MMD or AMD, the concept of Rf is equally inappropriate because of the multiple development steps and the continually changing distances of solvent travel. Thus, chromatographic characteristics of compounds after MMD and AMD are described in the present studies as elution distances (ED), measured as distances travelled from the origin.

**Figure 2.21**

**HPTLC chromatograms of phenols after separation using MMD Scheme 3 and an expanded gradient MMD Scheme 4**



The EDs of indoles, coumarins, biphenyls and phenols obtained after development using the full linear gradient (MMD Scheme 3) are presented in **Table 2.2.** Most compounds migrated more than half way up the plate (ED>50mm) which would be expected of compounds with moderately low polarity. The metabolites of aspirin (salicylic acid, gentisic acid and 2,3-dihydroxybenzoic acid) migrated less far than the remaining compounds, and the peaks from these compounds were very broad. Other acidic compounds (indole-6-carboxylic acid, indole-3-carboxylic acid) also had broad peaks indicating the presence of the strong silanol interactions known to occur with acidic compounds on silica gel.

The elution distances of compounds which differed from others by typical Phase I transformations were calculated relative to the elution distances of the "parent" compounds. These relative elution distances (RED) are presented in **Table 2.2**, and ranged between 0.566 and 1.087. The RED data are summarised in **Table 2.3** showing the factors obtained for each type of Phase I transformation. REDs of compounds differing by aromatic hydroxylation, with the exception of 3 compounds, were greater than 0.9, whereas REDs of compounds differing by aliphatic hydroxylation were generally in the range 0.78 to 0.88. Oxidation to carboxylic acids resulted in REDs of approximately 0.8 with the formation of broad peaks on the chromatograms. O-dealkylation had a similar effect to aromatic hydroxylation which is consistent with the formation of phenolic compounds in the examples examined.

Separation of the indoles, coumarins and biphenyls was increased by the use of expanded linear gradients (MMD Schemes 5 and 6), **Table 2.4.** Thus the separation of indole and 5-hydroxyindole was increased from 6.4mm using MMD Scheme 3 to 12.4mm

**Table 2.2**

**Elution distances of indoles, coumarins, biphenyls and phenols following manual multiple development using a linear gradient (MMD Scheme 3) on silica gel 60 HPTLC plates**

| Compound | Peak Shape | ED(mm) | RED |
|---|---|---|---|
| 1-Methylindole | s | 69.2 | - |
| Indole | m | 75.2 | 1.087 |
| 5-Hydroxyindole | s | 68.8 | 0.915 |
| 5-Methoxyindole | s | 74.0 | - |
| 6-Methylindole | s | 76.0 | - |
| Indole-6-carboxylic acid | b | 61.2 | 0.805 |
| 3-Methylindole | s | 76.8 | - |
| Indole-3-carboxylic acid | b | 63.2 | 0.823 |
| 3-Indolemethanol | s | 61.6 | 0.802 |
| Coumarin | s | 72.0 | - |
| 4-Hydroxycoumarin | vb | 50.0 | 0.694 |
| 7-Hydroxycoumarin | s | 67.2 | 0.933 |
| 7-Methoxycoumarin | s | 70.4 | - |
| Aspirin | vb | 58.0 | - |
| Salicylic Acid | vb | 42.8 | 0.738 |
| Gentisic Acid | vb | 41.6 | 0.972 |
| 2,3-Dihydroxybenzoic acid | vb | 32.8 | 0.766 |
| Biphenyl | s | 76.4 | - |
| 2-Phenylphenol | m | 78.0 | 1.021 |
| 4-Phenylphenol | m | 74.0 | 0.969 |
| 4,4-Biphenol | b | 70.0 | 0.946 |
| 4-Pentylphenol | m | 68.8 | - |
| 1-[4-Hydroxyphenyl]pentan-1-ol | s | 56.8 | 0.826 |
| 4-Ethylphenol | m | 67.6 | - |
| 4-Hydroxyphenylethanol | s | 53.6 | 0.793 |
| 2-Ethylphenol | m | 69.6 | - |
| 2-[2-Hydroxyphenyl]ethanol | m | 60.4 | 0.868 |
| 2-Hydroxyphenylethan-1-ol | s | 54.8 | 0.787 |
| Phenol | m | 67.2 | - |
| Catechol | s | 60.8 | 0.905 |
| Resorcinol | s | 58.4 | 0.869 |
| Hydroquinone | s | 58.8 | 0.875 |
| o-Cresol | m | 68.0 | - |
| 2-Hydroxybenzyl alcohol | s | 59.2 | 0.871 |
| m-Cresol | m | 67.2 | - |
| 3-Hydroxybenzyl alcohol | s | 54.0 | 0.804 |
| p-Cresol | m | 66.0 | - |
| 4-Hydroxybenzyl alcohol | s | 54.0 | 0.818 |

s: sharp m: moderate b: broad vb: very broad

**Table 2.3**

**Summary table of transformation type and RED values obtained following development of model compounds on silica gel HPTLC plates using MMD scheme 3**

| N-Demethylation | Aromatic Hydroxylation | Aliphatic Hydroxylation | Oxidation to COOH | O-dealkylation |
|---|---|---|---|---|
| 1.087 | 0.915 | 0.802 | 0.805 | 0.930 |
| | 0.933 | 0.694 | 0.823 | 0.954 |
| | 0.972 | 0.828 | | |
| | 0.766[a] | 0.793 | | |
| | 1.021[b] | 0.868 | | |
| | 0.969 | 0.787 | | |
| | 0.946 | 0.871 | | |
| | 0.905 | 0.804 | | |
| | 0.869[c] | 0.818 | | |
| | 0.875[d] | | | |
| **Mean** | 0.917 | 0.807 | | |
| **S.D.** | 0.070 | 0.052 | | |

[a] 2,3-Dihydroxybenzoic acid from salicylic acid
[b] 2-Phenylphenol from biphenyl
[c] Resorcinol from phenol
[d] Hydroquinone from phenol

**Table 2.4**

**Elution distances of indoles, coumarins and biphenyls following manual multiple development on silica gel 60 HPTLC plates with expanded linear gradients**

| Compound | MMD Scheme 5 | | MMD Scheme 6 | |
|---|---|---|---|---|
| | Peak Shape | ED(mm) | Peak Shape | ED(mm) |
| 1-Methylindole | s | 50.0 | m | 77.6 |
| Indole | m | 60.0 | m | 56.4 |
| 5-Hydroxyindole | s | 47.6 | s | 32.4 |
| 5-Methoxyindole | s | 54.4 | s | 45.6 |
| 6-Methylindole | s | 61.2 | s | 58.0 |
| Indole-6-carboxylic acid | m | 39.2 | m | 18.4 |
| 3-Methylindole | s | 62.8 | s | 60.4 |
| Indole-3-carboxylic acid | m | 41.2 | m | 18.8 |
| 3-Indolemethanol | s | 46.8 | s | 38.4 |
| Coumarin | s | 53.2 | s | 44.4 |
| 4-Hydroxycoumarin | m | 29.2 | m | 14.4 |
| 7-Hydroxycoumarin | s | 45.2 | s | 28.4 |
| 7-Methoxycoumarin | s | 50.0 | s | 39.6 |
| Biphenyl | | ND | s | 87.6 |
| 2-Phenylphenol | m | 59.6 | m | 57.2 |
| 4-Phenylphenol | m | 52.0 | m | 42.4 |
| 4,4-Biphenol | m | 45.6 | m | 26.8 |

ND: Not detected
s: sharp m: moderate

with MMD Scheme 5 and 24.0mm with MMD Scheme 6. Similarly the separation of coumarin and 7-hydroxycoumarin increased from 4.8mm using MMD Scheme 3 to 8.0mm and 16.0mm with MMD Schemes 5 and 6 respectively.

Inclusion of acetic acid (1%v/v) in each solvent mixture increased the migration of some acidic compounds (4-hydroxycoumarin, aspirin, salicylic acid, gentisic acid and 2,3-dihydroxybenzoic acid) and generally improved the peak shapes of all acidic compounds examined, **Table 2.5.** The retention of the non-acidic compounds was unaffected by the addition of acetic acid to the solvent mixtures. Thus the ED of aspirin increased from 58.0mm to 64.0mm, salicylic acid increased from 42.8mm to 65.6mm, gentisic acid increased from 41.6mm to 58.0mm and 2,3-dihydroxybenzoic acid increased from 32.8mm to 55.2mm.

The EDs obtained for ondansetron, ranitidine, loxtidine and their metabolites following development with the full gradient (MMD Scheme 3) are shown in **Table 2.6.** The EDs were very small, probably due to cation-exchange interactions between the silanol groups on the silica and the amine functions on the molecules. EDs were increased by use of expanded linear gradients (MMD Schemes 7 and 8), **Table 2.6**, but the separation between ondansetron and its metabolites in particular was not increased. Addition of ammonia solution (1%v/v) to each solvent mixture in both the full gradient and an expanded gradient did not change the retention of the compounds, and had only minimal effect on their separation, **Table 2.7.** The use of TEA (1% v/v), however, increased migration of the compounds on the full gradient, **Table 2.8**, and expanded gradients with TEA in the solvent mixtures (MMD Schemes 9-12) increased migration further. The use of very shallow gradients designed to increase the separation of the ondansetron

**Table 2.5**

**Elution distances of selected acidic indoles, coumarins, and phenols following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3 with and without 1% glacial acetic acid**

| Compound | Without Acid | | With Acid | |
|---|---|---|---|---|
| | Peak Shape | ED(mm) | Peak Shape | ED(mm) |
| Indole | m | 75.2 | m | 72.8 |
| 6-Methylindole | s | 76.0 | s | 73.6 |
| Indole-6-carboxylic acid | b | 61.2 | s | 62.8 |
| 3-Methylindole | s | 76.8 | s | 75.2 |
| Indole-3-carboxylic acid | b | 63.2 | m | 62.8 |
| Coumarin | s | 72.0 | s | 69.2 |
| 4-Hydroxycoumarin | vb | 50.0 | s | 58.4 |
| 7-Hydroxycoumarin | s | 67.2 | s | 63.2 |
| Aspirin | vb | 58.0 | s | 64.0 |
| Salicylic Acid | vb | 42.8 | s | 65.6 |
| Gentisic Acid | vb | 41.6 | b | 58.0 |
| 2,3-Dihydroxybenzoic acid | vb | 32.8 | b | 55.2 |

s: sharp m: moderate b: broad vb: very broad

**Table 2.6**

**Elution distances of ondansetron, ranitidine, loxtidine and metabolites following manual multiple development on silica gel 60 HPTLC plates**

| | MMD Scheme 3 | | Scheme 7 | Scheme 8 |
|---|---|---|---|---|
| **Compound** | **ED(mm)** | **RED** | **ED(mm)** | **ED(mm)** |
| Ondansetron | 11.2 | - | 44.0 | 72.0 |
| N-desmethyl ondansetron | 13.6 | 1.214 | 48.4 | 76.8 |
| 5-OH ondansetron | 12.8 | 1.143 | 47.2 | - |
| 6-OH ondansetron | 11.2 | 1.000 | 42.4 | 71.2 |
| 7-OH ondansetron | 11.2 | 1.000 | 41.6 | 72.0 |
| 8-OH ondansetron | 13.2 | 1.179 | 47.6 | 77.6 |
| 6-OH,N-desmethyl ondansetron | 12.8 | 1.143 | 45.6 | 75.6 |
| 7-OH,N-desmethyl ondansetron | 13.2 | 1.179 | 44.4 | 71.6 |
| Ranitidine | 5.6 | - | - | 46.0 |
| N-desmethyl ranitidine | 4.0 | 0.714 | - | 20.8 |
| Ranitidine N-oxide | 2.8 | 0.500 | - | 16.4 |
| Ranitidine S-oxide | 2.8 | 0.500 | - | 22.8 |
| Loxtidine | 5.2 | - | - | - |
| AH25227 | 15.2 | 2.923 | - | - |
| GR30450 | 6.0 | 1.154 | - | - |
| AH24610 | 3.2 | 0.615 | - | - |

**Table 2.7**

**Elution distances of ondansetron, ranitidine, and metabolites following manual multiple development on silica gel 60 HPTLC plates using MMD Schemes 3 and 7 with 1% ammonia solution**

| Compound | Scheme 3 ED(mm) | Scheme 3 with $NH_3$ ED(mm) | Scheme 7 ED(mm) | Scheme 7 with $NH_3$ ED(mm) |
|---|---|---|---|---|
| Ondansetron | 11.2 | 13.2 | 44.0 | 43.6 |
| N-desmethyl ondansetron | 13.6 | 15.2 | 48.4 | 46.4 |
| 5-OH ondansetron | 12.8 | 14.0 | 47.2 | 45.2 |
| 6-OH ondansetron | 11.2 | 12.0 | 42.4 | 42.0 |
| 7-OH ondansetron | 11.2 | 12.0 | 41.6 | 42.0 |
| 8-OH ondansetron | 13.2 | 14.8 | 47.6 | 45.6 |
| 6-OH,N-desmethyl ondansetron | 12.8 | 14.0 | 45.6 | 44.0 |
| 7-OH,N-desmethyl ondansetron | 13.2 | 13.6 | 44.4 | 43.2 |
| Ranitidine | 5.6 | 6.4 | - | 21.2 |
| N-desmethyl ranitidine | 4.0 | 4.8 | - | 11.6 |
| Ranitidine N-oxide | 2.8 | 3.2 | - | 12.8 |
| Ranitidine S-oxide | 2.8 | ND | - | 6.4 |

ND: Not detected

**Table 2.8**

**Elution distances of ondansetron, loxtidine and metabolites following manual multiple development on silica gel 60 HPTLC plates using MMD Schemes with 1% TEA**

| Compound | Scheme 3 | Scheme 9 | Scheme 10 | Scheme 11 | Scheme 12 |
|---|---|---|---|---|---|
| | ED(mm) | ED(mm) | ED(mm) | ED(mm) | ED(mm) |
| Ondansetron (ond) | 21.2 | 51.6 | 51.2 | 59.2 | 63.6 |
| N-desmethyl ond | 17.6 | 44.8 | 50.0 | 56.8 | 59.2 |
| 6-OH ondansetron | 17.2 | 47.2 | 49.2 | 55.6 | 59.6 |
| 7-OH ondansetron | 14.8 | 42.0 | 46.4 | 50.8 | 55.6 |
| 8-OH ondansetron | 18.0 | 40.4 | 50.0 | 56.4 | 61.6 |
| 6-OH,N-desmethyl ond | 15.6 | 41.6 | 46.4 | 52.0 | 55.2 |
| 7-OH,N-desmethyl ond | 14.0 | 36.8 | 42.8 | 44.4 | 50.0 |
| Ranitidine | - | 40.8 | | | |
| N-desmethyl ranitidine | - | 27.2 | | | |
| Ranitidine N-oxide | - | 6.0 | | | |
| Ranitidine S-oxide | - | 24.8 | | | |
| Loxtidine | 20.0 | 42.0 | | | |
| AH25227 | 31.6 | 76.0 | | | |
| GR30450 | 7.6 | 16.8 | | | |
| AH24610 | 5.2 | 10.4 | | | |

metabolites was unsuccessful; increased focusing of the gradient did not increase separation. The presence of TEA as the strongest component in these systems may have reduced the effect of small changes in the other solvents.

Isocratic development of ondansetron and its metabolites was carried out to determine whether multiple development could be avoided. Under these conditions, separation of some components was increased, but the peaks obtained were much broader than after multiple development so the potential increased resolution was lost, **Table 2.9.** This demonstrated the advantage of multiple development in focusing sample bands on the chromatograms.

### 2.4.1.3 Compounds to model Phase II transformations

The EDs obtained from model compounds and their glucuronide and sulphate conjugates after development with the full linear gradient (MMD Scheme 3) are presented in **Tables 2.10 and 2.11.** The relative elution distances of the conjugates (relative to the EDs of the parent compounds) are summarised in **Table 2.12** and presented graphically in **Figure 2.22.** In general the mean REDs of the glucuronides were <0.3 (range 0.127 to 0.275), whereas those of the sulphates were >0.3 (range 0.326 to 0.354) with the exception of epiandrosterone sulphate (0.225). Phenolphthalein disulphate had a mean RED value more typical of a glucuronide (0.263), however a disulphate might be expected to behave in a different manner chromatographically to a mono-sulphate.

Analysis of variance was applied to the conjugate RED data to test the null hypothesis that the samples (compounds) came from populations with equal means. The hypothesis

**Table 2.9**

**Elution distances of ondansetron and metabolites on silica gel 60 HPTLC plates using isocratic development for 9.0cm**

System 1: Methanol (1.8) + Ethyl Acetate (8.2) + 1% TEA

System 2: Methanol (2.55) + Ethyl Acetate (7.45) + 1% TEA

| Compound | System 1 ED(mm) | System 2 ED(mm) |
|---|---|---|
| GR38032 | 49.6 | 74.0 |
| GR41352 : N-desmethyl | 42.4 | 72.0 |
| GR60661 : 6-OH | 42.4 | 66.8 |
| GR63418 : 7-OH | 35.2 | 58.8 |
| GR60317 : 6-OH, N-desmethyl | 35.6 | 62.4 |
| GR65826 : 7-OH, N-desmethyl | 26.8 | 49.6 |

**Table 2.10**

**Elution distances of conjugated glucuronide and sulphate metabolites following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

| | PLATE 1 | | PLATE 2 | |
|---|---|---|---|---|
| **Compound** | **ED(mm)** | **RED** | **ED(mm)** | **RED** |
| Naphthol | 70.0 | - | 64.4 | - |
| N. glucuronide | 14.0 | 0.200 | 13.6 | 0.211 |
| N. sulphate | 21.2 | 0.303 | 22.0 | 0.342 |
| Paracetamol | 45.6 | - | 44.0 | - |
| Pa. glucuronide | 9.6 | 0.211 | 10.4 | 0.236 |
| Pa. sulphate | 15.6 | 0.342 | 16.4 | 0.373 |
| Phenol | 67.2 | - | 62.8 | - |
| Ph. glucuronide | 10.8 | 0.161 | 11.6 | 0.185 |
| Phenolphthalein | 55.2 | - | 54.8 | - |
| Pp. glucuronide | 12.8 | 0.232 | 14.4 | 0.263 |
| Pp. disulphate | 13.6 | 0.246 | 15.2 | 0.277 |
| 4-MeU | 58.8 | - | 62.0 | - |
| 4-MeU glucuronide | 9.6 | 0.163 | 9.6 | 0.155 |
| 4-MeU sulphate | 22.8 | 0.388 | 20.0 | 0.323 |
| Epiandrosterone | 66.0 | - | 68.5 | - |
| Ep. glucuronide | 8.0 | 0.121 | 8.0 | 0.117 |
| Ep. sulphate | 14.0 | 0.212 | 15.0 | 0.219 |
| Chloramphenicol | 47.6 | - | 47.2 | - |
| Chl. glucuronide | 11.6 | 0.244 | 12.4 | 0.263 |

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Table 2.11**

**Elution distances of conjugated glucuronide and sulphate metabolites following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

| | PLATE 3 | | PLATE 4 | |
|---|---|---|---|---|
| **Compound** | **ED(mm)** | **RED** | **ED(mm)** | **RED** |
| Naphthol | 65.6 | - | 65.6 | - |
| N-glucuronide | 14.4 | 0.220 | 10.4 | 0.159 |
| N-sulphate | 22.0 | 0.335 | 21.2 | 0.323 |
| Paracetamol | 45.6 | - | 44.4 | - |
| Pa-glucuronide | 13.6 | 0.298 | 8.2 | 0.185 |
| Pa-sulphate | 16.4 | 0.360 | 15.2 | 0.342 |
| Phenol | 64.8 | - | 64.0 | - |
| Ph-glucuronide | 13.2 | 0.204 | 10.0 | 0.156 |
| Phenolphthalein | 56.0 | - | 55.6 | - |
| Pp-glucuronide | 14.4 | 0.257 | 11.2 | 0.201 |
| Pp-disulphate | 15.2 | 0.271 | 14.4 | 0.259 |
| 4-MeU | 58.4 | - | 54.0 | - |
| 4-MeU glucuronide | 9.6 | 0.164 | 10.4 | 0.193 |
| 4-MeU sulphate | 19.2 | 0.329 | 20.0 | 0.370 |
| Epiandrosterone | 64.5 | - | 59.0 | - |
| Ep. glucuronide | 8.0 | 0.124 | 8.5 | 0.144 |
| Ep. sulphate | 15.0 | 0.233 | 14.0 | 0.237 |
| Chloramphenicol | 43.2 | - | 42.0 | - |
| Chl. glucuronide | 12.0 | 0.278 | 13.2 | 0.314 |

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Table 2.12**

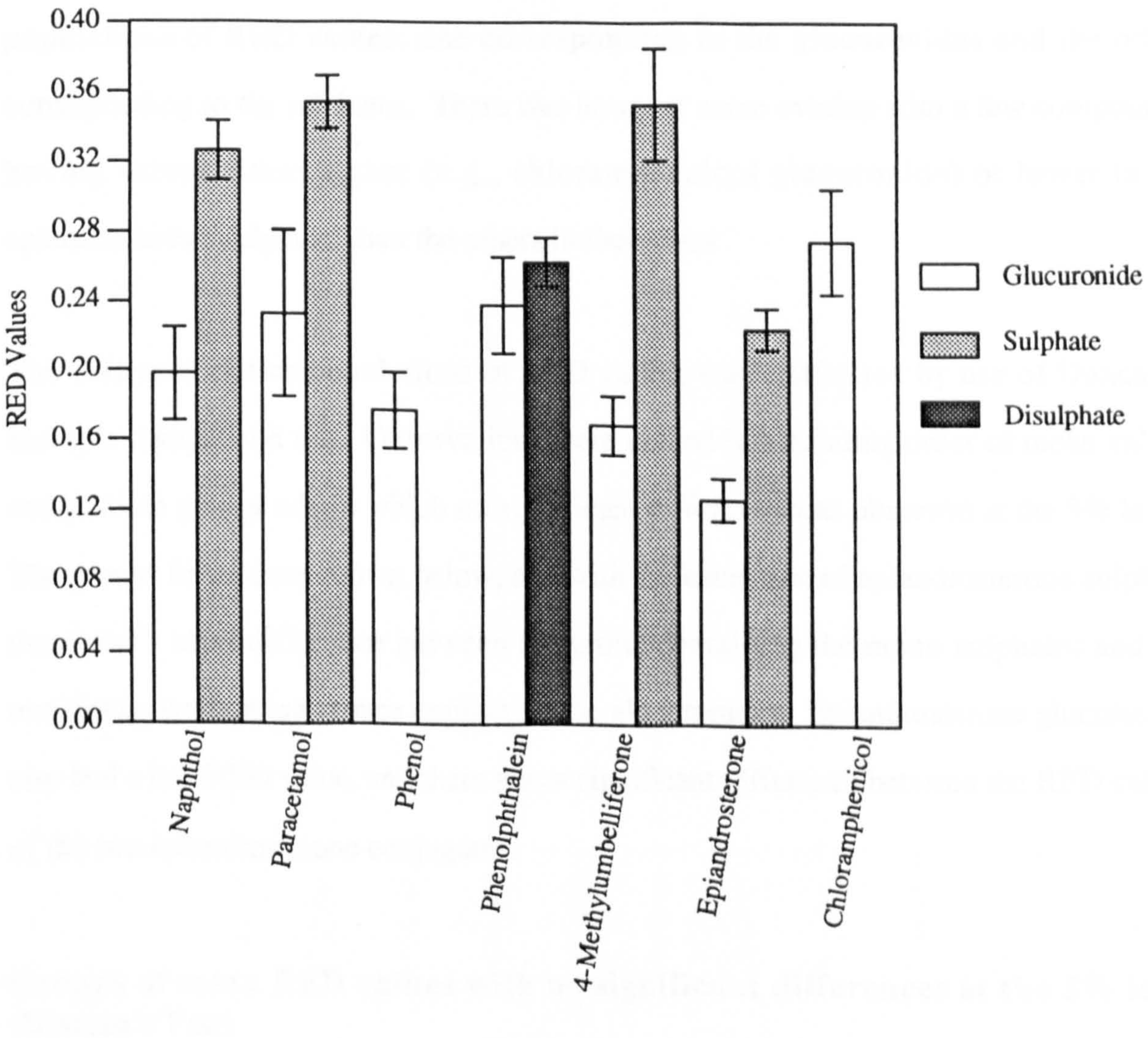
**Relative elution distances of conjugated glucuronide and sulphate metabolites following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

| Compound | Plate 1 | Plate 2 | Plate 3 | Plate 4 | Mean | S.D. | C of V |
|---|---|---|---|---|---|---|---|
| N gluc. | 0.200 | 0.211 | 0.220 | 0.159 | 0.198 | 0.027 | 13.6% |
| Pa gluc. | 0.211 | 0.236 | 0.298 | 0.185 | 0.233 | 0.048 | 20.8% |
| Ph gluc. | 0.161 | 0.185 | 0.204 | 0.156 | 0.177 | 0.022 | 12.6% |
| Pp gluc. | 0.232 | 0.263 | 0.257 | 0.201 | 0.238 | 0.028 | 11.8% |
| 4-MeU gluc. | 0.163 | 0.155 | 0.164 | 0.193 | 0.169 | 0.017 | 9.9% |
| Ep gluc. | 0.121 | 0.117 | 0.124 | 0.144 | 0.127 | 0.012 | 9.5% |
| Chl gluc. | 0.244 | 0.263 | 0.278 | 0.314 | 0.275 | 0.030 | 10.8% |
| N sulph. | 0.303 | 0.342 | 0.335 | 0.323 | 0.326 | 0.017 | 5.2% |
| Pa sulph. | 0.342 | 0.373 | 0.360 | 0.342 | 0.354 | 0.015 | 4.3% |
| 4-MeU sulph. | 0.388 | 0.323 | 0.329 | 0.370 | 0.353 | 0.032 | 9.0% |
| Ep sulph. | 0.212 | 0.219 | 0.233 | 0.237 | 0.225 | 0.012 | 5.2% |
| Pp disulph. | 0.246 | 0.277 | 0.271 | 0.259 | 0.263 | 0.014 | 5.2% |

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Figure 2.22**

**Relative elution distances (RED) of glucuronide and sulphate conjugates after MMD on silica gel 60 HPTLC plates using MMD Scheme 3**



was shown to be incorrect (p=0) indicating that the mean RED values came from different populations. A simultaneous group comparison test was carried out and the results are shown in **Figure 2.23.** The error bars from four out of the seven glucuronides tested did not overlap with the grand average error bounds indicating that their means were significantly below the average. Three out of the four mono-sulphates had means significantly above the average. This demonstrated that there were essentially two populations of RED values, one corresponding to the glucuronides and the other corresponding to the sulphates. There was however some overlap with a few compounds having values either higher (e.g., chloramphenicol glucuronide) or lower (e.g., epiandrosterone sulphate) than the others in their class.

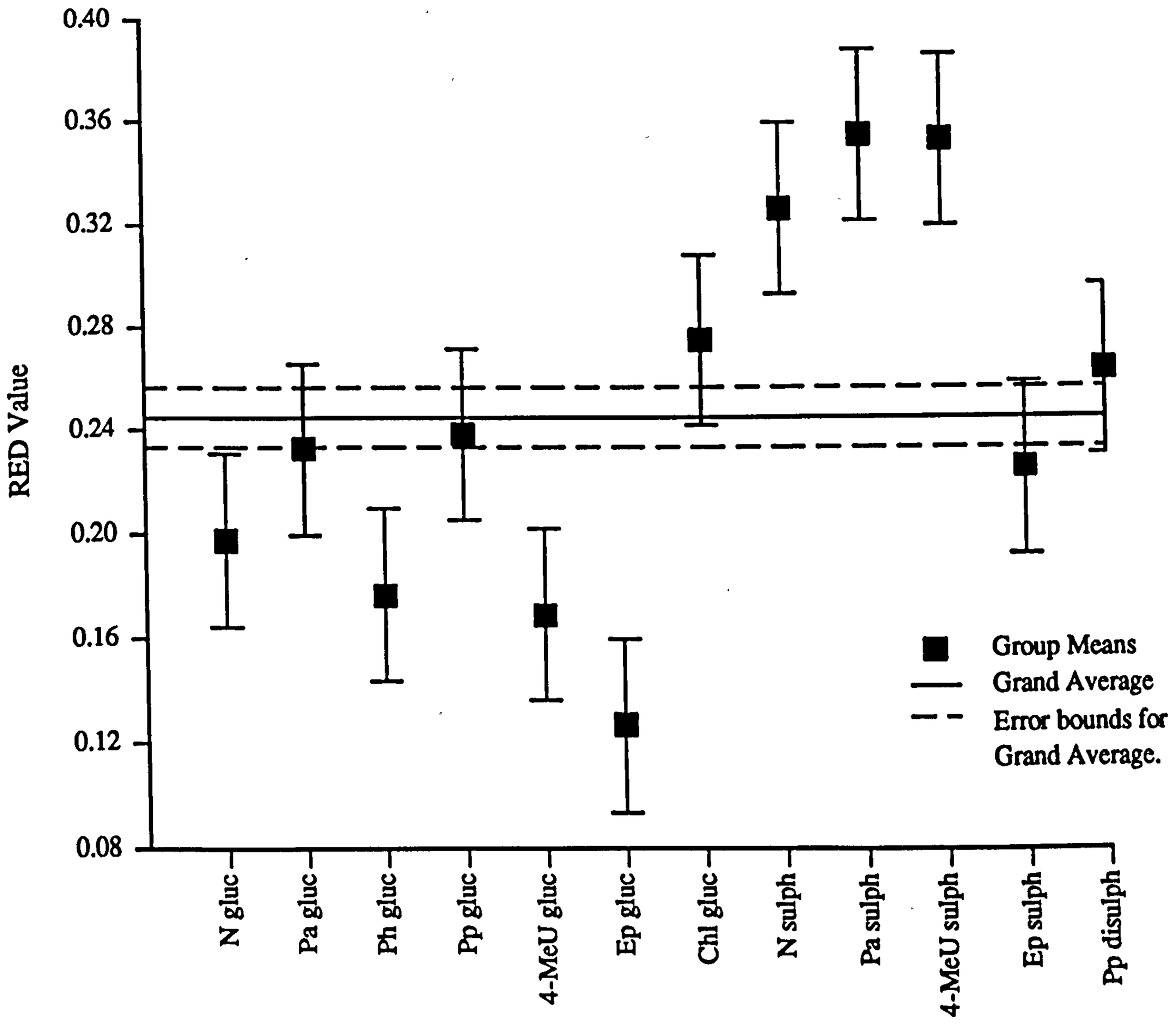
The existence of two populations of RED values was confirmed by use of Duncan's multiple comparison test. Observations were ranked in ascending order of mean values and put into groups within which no significant difference was observed at the 5% level. The groups formed are shown below, and with the exception of epiandrosterone sulphate there was a clear difference between the group containing the mono-sulphates and the remaining overlapping groups containing the glucuronides. Epiandrosterone glucuronide also had a low RED value, and there was a significant difference between the RED values of the two epiandrosterone conjugates.

**Groups of mean RED values with no significant differences at the 5% level (Duncan's Test)**

| Ep-g | 4MeU-g | Ph-g | N-g | Ep-s | Pa-g | Pp-g | Pp-ds | Ch-g | N-s | 4MeU-s | Pa-s |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | |
| | | | | | | | | | | | |

**Figure 2.23**

**Simultaneous comparisons between groups of RED values following MMD of conjugated metabolites on silica gel 60 HPTLC plates using MMD Scheme 3**




Error bars overlap if the difference between two means is not significant at the 5 percent level. If the error bars for the group mean overlap the dashed line, the group mean is not significantly different from the grand average.

The data obtained to determine the intra-plate variation in retention are shown in **Table 2.13.** Excellent reproducibility was obtained for the REDs (0.89 and 1.65%) indicating that it is valid to compare EDs of different compounds when chromatographed on the same plate, but on different tracks. The inter-plate variation, shown in **Table 2.12,** was considerably greater than the intra-plate variation. In general, the variation in the REDs of the glucuronides was greater than that of the sulphate REDs. This can be explained because migration of the glucuronides took place primarily in the first two or three steps of the MMD procedure, and the extent of migration was therefore controlled largely by the accuracy to which the first step was developed to 4mm beyond the origin. The sulphates migrated further, and therefore their migration and that of parent compounds was controlled by subsequent elution steps.

The full linear gradient provided excellent separation between parent compounds and the glucuronide and sulphate conjugates. However although the conjugates were resolved from each other, resolution was not complete in all cases. In biological matrices where endogenous conjugated material is present, additional separation might be required in order to separate drug-related material from endogenous components. The EDs obtained from the glucuronide and sulphate conjugates after development on expanded linear gradients (MMD Schemes 7 and 9) are presented in **Table 2.14.** Greater separation of the conjugated metabolites was achieved with both systems, however the parent compounds migrated with the solvent front so RED values could not be obtained.

The EDs obtained after development of the conjugates with full linear gradients containing either glacial acetic acid (1% v/v) or TEA (1% v/v) are presented in **Table 2.15** and are compared with the data obtained without modifier. In all cases the REDs of

**Table 2.13**

**Intra-plate variation in relative elution distances following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

| Track No. | Paracetamol Glucuronide | Paracetamol Sulphate |
|---|---|---|
| 1 | 0.212 | 0.363 |
| 2 | 0.212 | 0.354 |
| 3 | 0.212 | 0.363 |
| 4 | 0.212 | 0.363 |
| 5 | 0.211 | 0.360 |
| 6 | 0.219 | 0.360 |
| 7 | 0.219 | 0.360 |
| **Mean** | 0.214 | 0.360 |
| **S.D.** | 0.004 | 0.003 |
| **C of V** | 1.65% | 0.89% |

**Table 2.14**

**Elution distances of conjugated glucuronide and sulphate metabolites following manual multiple development on silica gel 60 HPTLC plates using expanded linear MMD Schemes 7 and 9**

| Compound | MMD Scheme 7 | MMD Scheme 9 |
|---|---|---|
| | ED(mm) | ED(mm) |
| Naphthol | ND | ND |
| N. glucuronide | 30.8 | 18.8 |
| N. sulphate | 64.4 | 55.2 |
| Paracetamol | ND | ND |
| Pa. glucuronide | 24.0 | 15.6 |
| Pa. sulphate | 52.0 | 35.6 |
| Phenolphthalein | ND | ND |
| Pp. glucuronide | 30.0 | 18.8 |
| Pp. sulphate | 44.0 | 55.2 |
| 4-MeU | ND | ND |
| 4-MeU glucuronide | 26.4 | 17.2 |
| 4-MeU sulphate | 61.6 | 51.6 |
| Epiandrosterone | ND | ND |
| Ep. glucuronide | 26.0 | 15.5 |
| Ep. sulphate | 50.0 | 34.5 |
| Chloramphenicol | ND | ND |
| Chl. glucuronide | 34.8 | 22.0 |

ND Not detected - migrated with solvent front.

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Table 2.15**

**Effect of modifiers on the elution distances of conjugated glucuronide and sulphate metabolites following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

| | NORMAL | | TEA | | ACETIC ACID | |
|---|---|---|---|---|---|---|
| | ED(mm) | RED | ED(mm) | RED | ED(mm) | RED |
| Naphthol | 66.4 | - | 66.8 | - | 70.4 | - |
| Na glucuronide | 13.1 | 0.198 | 16.8 | 0.251 | 14.4 | 0.205 |
| Na sulphate | 21.6 | 0.326 | 24.0 | 0.359 | 28.8 | 0.409 |
| Phenolphthalein | 55.4 | - | 61.6 | - | 61.6 | - |
| Pp glucuronide | 13.2 | 0.238 | 16.8 | 0.273 | 15.6 | 0.253 |
| 4-Me Umbelliferone | 58.3 | - | 60.8 | - | 61.6 | - |
| 4-Me U glucuronide | 9.8 | 0.169 | 16.4 | 0.270 | 13.2 | 0.214 |
| 4-Me U sulphate | 20.5 | 0.353 | 23.6 | 0.388 | 25.2 | 0.409 |
| Paracetamol | 44.9 | - | 52.0 | - | 52.8 | - |
| Pa glucuronide | 10.5 | 0.233 | 16.4 | 0.315 | 12.4 | 0.235 |
| Pa sulphate | 15.9 | 0.354 | 20.4 | 0.392 | 18.0 | 0.341 |
| Chloramphenicol | 45.0 | - | 49.2 | - | 51.6 | - |
| Chl glucuronide | 12.3 | 0.275 | 18.8 | 0.382 | 15.2 | 0.295 |

the glucuronides were increased more with acetic acid as the modifier than with TEA, and conversely in all cases except one (paracetamol sulphate) the sulphate REDs were increased more with TEA than with acetic acid in the solvent mixtures. This could provide an additional means of conjugate identification in conjunction with the RED data.

### 2.4.3 Automated Multiple Development (AMD)

The EDs obtained after developing the conjugated metabolites and their parent compounds using AMD are presented in **Tables 2.16 and 2.17.** The RED values are summarised in **Table 2.18** and presented graphically in **Figure 2.24.** The AMD inter-plate data were more reproducible than those obtained using the MMD procedure, and the difference in RED values between the glucuronide and sulphate conjugates was more pronounced. Using AMD, all the glucuronide RED values were <0.2 with the exception of chloramphenicol glucuronide (0.268), and the sulphate RED values were all >0.25. The disulphate had a lower RED value than the mono-sulphates (0.226) as was found with MMD. These data showed the benefit of automation with this complex development procedure.

### 2.4.4 Detection Limits

#### 2.4.4.1 Analyte against plate background

The detection limits for paracetamol and its conjugates applied as pure solutions to a HPTLC plate were determined. The ultraviolet absorbance maxima of the compounds on a silica HPTLC plate were measured by scanning spots of the compounds *in situ* with a Camag TLC scanner II.

**Table 2.16**

**Elution distances of conjugated glucuronide and sulphate metabolites following automated multiple development on silica gel 60 HPTLC plates using AMD Scheme 1**

| | PLATE 1 | | PLATE 2 | |
|---|---|---|---|---|
| **Compound** | **ED(mm)** | **RED** | **ED(mm)** | **RED** |
| Naphthol | 58.8 | - | 58.4 | - |
| N. glucuronide | 8.4 | 0.143 | 9.2 | 0.158 |
| N. sulphate | 19.6 | 0.333 | 19.2 | 0.329 |
| Paracetamol | 44.4 | - | 43.2 | - |
| Pa. glucuronide | 6.8 | 0.153 | 8.4 | 0.194 |
| Pa. sulphate | 12.4 | 0.279 | 12.8 | 0.296 |
| Phenol | 57.2 | - | 57.2 | - |
| Ph. glucuronide | 3.6 | 0.063* | 7.2 | 0.126 |
| Phenolphthalein | 54.0 | - | 53.6 | - |
| Pp. glucuronide | 8.4 | 0.153 | 9.2 | 0.172 |
| Pp. disulphate | 11.6 | 0.215 | 11.6 | 0.216 |
| 4-MeU | 53.2 | - | 53.2 | - |
| 4-MeU glucuronide | 6.4 | 0.120 | 7.6 | 0.143 |
| 4-MeU sulphate | 19.6 | 0.368 | 19.2 | 0.361 |
| Epiandrosterone | 58.0 | - | 59.0 | - |
| Ep. glucuronide | 8.0 | 0.138 | 9.0 | 0.153 |
| Ep. sulphate | 14.5 | 0.250 | 14.5 | 0.246 |
| Chloramphenicol | 45.2 | - | 42.4 | - |
| Chl. glucuronide | 10.4 | 0.230 | 10.8 | 0.255 |

* Not used in the calculation of means and standard deviations (Table 29) because this data point is inconsistent with the remaining data.

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Table 2.17**

**Elution distances of conjugated glucuronide and sulphate metabolites following automated multiple development on silica gel 60 HPTLC plates using AMD Scheme 1**

| | PLATE 3 | | PLATE 4 | |
|---|---|---|---|---|
| **Compound** | **ED(mm)** | **RED** | **ED(mm)** | **RED** |
| Naphthol | 57.2 | - | 57.2 | - |
| N. glucuronide | 9.2 | 0.161 | 9.2 | 0.161 |
| N. sulphate | 17.6 | 0.308 | 17.2 | 0.301 |
| Paracetamol | 42.0 | - | 42.4 | - |
| Pa. glucuronide | 8.0 | 0.190 | 8.0 | 0.189 |
| Pa. sulphate | 12.4 | 0.295 | 12.8 | 0.302 |
| Phenol | 56.0 | - | 56.4 | - |
| Ph. glucuronide | 6.8 | 0.121 | 7.6 | 0.135 |
| Phenolphthalein | 52.0 | - | 51.6 | - |
| Pp. glucuronide | 9.2 | 0.177 | 8.8 | 0.171 |
| Pp. disulphate | 12.0 | 0.231 | 12.4 | 0.240 |
| 4-MeU | 51.6 | - | 51.2 | - |
| 4-MeU glucuronide | 8.0 | 0.155 | 8.0 | 0.156 |
| 4-MeU sulphate | 17.2 | 0.333 | 17.2 | 0.336 |
| Epiandrosterone | 59.0 | - | 58.0 | - |
| Ep. glucuronide | 9.5 | 0.161 | 9.5 | 0.164 |
| Ep. sulphate | 15.5 | 0.263 | 15.0 | 0.259 |
| Chloramphenicol | 38.8 | - | 37.6 | - |
| Chl. glucuronide | 11.2 | 0.289 | 11.2 | 0.298 |

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Table 2.18**

**Relative elution distances of conjugated glucuronide and sulphate metabolites following automated multiple development on silica gel 60 HPTLC plates using AMD Scheme 1**

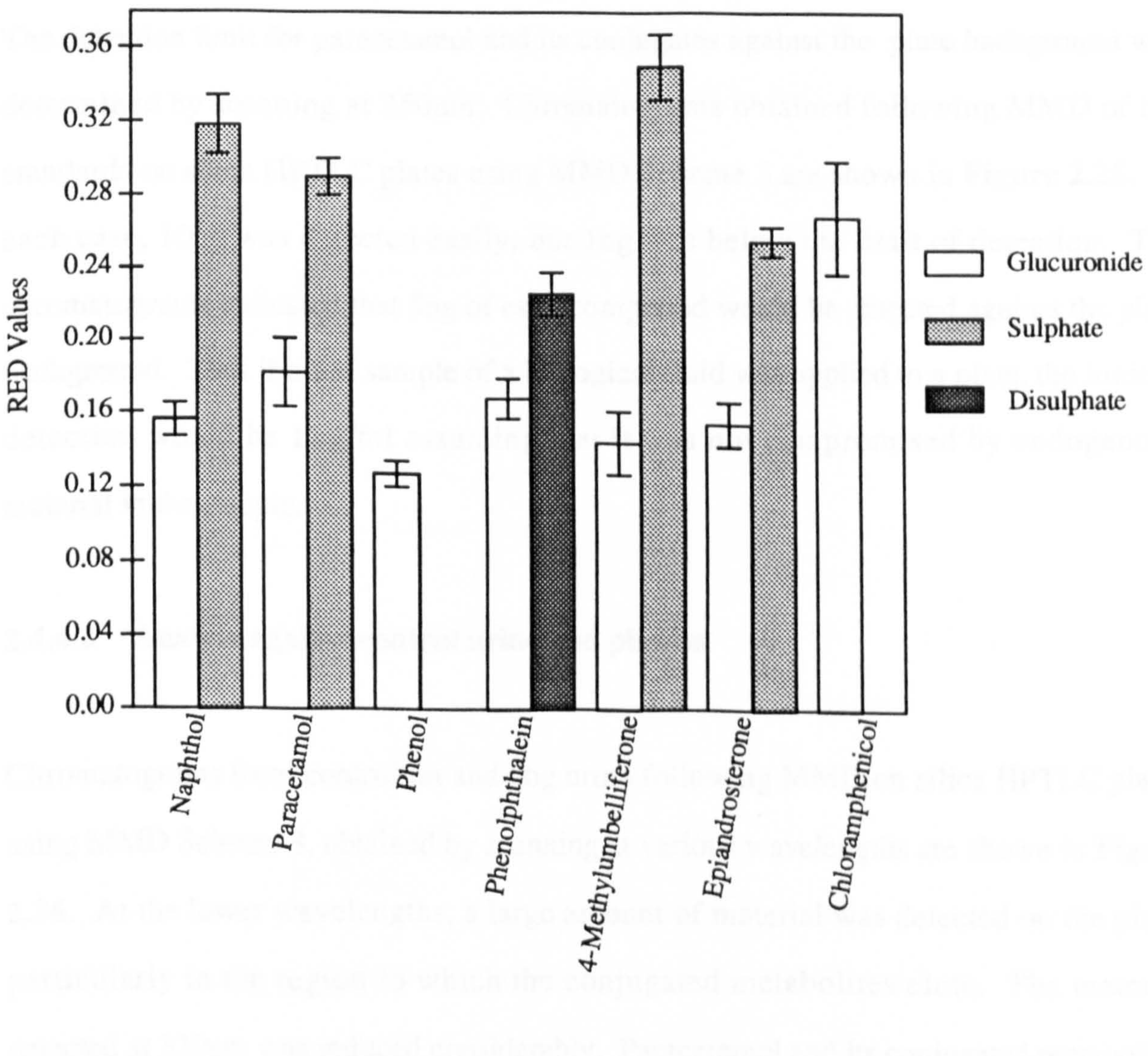
| Compound | Plate 1 | Plate 2 | Plate 3 | Plate 4 | Mean | S.D. | C of V |
|---|---|---|---|---|---|---|---|
| N gluc. | 0.143 | 0.158 | 0.161 | 0.161 | 0.156 | 0.009 | 5.5% |
| Pa gluc. | 0.153 | 0.194 | 0.190 | 0.189 | 0.182 | 0.019 | 10.5% |
| Ph gluc. | 0.063* | 0.126 | 0.121 | 0.135 | 0.127 | 0.007 | 5.6% |
| Pp gluc. | 0.153 | 0.172 | 0.177 | 0.171 | 0.168 | 0.011 | 6.2% |
| 4-MeU gluc. | 0.120 | 0.143 | 0.155 | 0.156 | 0.144 | 0.017 | 11.7% |
| Ep gluc. | 0.138 | 0.153 | 0.161 | 0.164 | 0.154 | 0.012 | 7.6% |
| Chl gluc. | 0.230 | 0.255 | 0.289 | 0.298 | 0.268 | 0.031 | 11.7% |
| N sulph. | 0.333 | 0.329 | 0.308 | 0.301 | 0.318 | 0.016 | 4.9% |
| Pa sulph. | 0.279 | 0.296 | 0.295 | 0.302 | 0.293 | 0.010 | 3.4% |
| 4-MeU sulph. | 0.368 | 0.361 | 0.333 | 0.336 | 0.350 | 0.018 | 5.0% |
| Ep sulph. | 0.250 | 0.246 | 0.263 | 0.259 | 0.255 | 0.008 | 3.1% |
| Pp disulph. | 0.215 | 0.216 | 0.231 | 0.240 | 0.226 | 0.012 | 5.4% |

* Not used in the calculation of means and standard deviations because this data point is inconsistent with the remaining data.

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Figure 2.24**

**Relative elution distances (RED) of glucuronide and sulphate conjugates after AMD on silica gel 60 HPTLC plates using AMD Scheme 1**



The UV maxima were :

| | |
|---|---|
| Paracetamol: | 251nm |
| Paracetamol glucuronide | 247nm |
| Paracetamol sulphate | 245nm |

The detection limit for paracetamol and its conjugates against the plate background was determined by scanning at 250nm. Chromatograms obtained following MMD of the standards on silica HPTLC plates using MMD Scheme 3 are shown in **Figure 2.25.** In each case, 10ng was detected easily, but 1ng was below the limit of detection. The chromatograms indicated that 5ng of each compound would be detected against the plate background. Thus if a 5$\mu$l sample of a biological fluid was applied to a plate, the limit of detection would be 1$\mu$g/ml assuming that it was not compromised by endogenous material in the sample.

### 2.4.4.2 Analyte against control urine and plasma

Chromatograms from control rat and dog urine following MMD on silica HPTLC plates using MMD Scheme 3, obtained by scanning at various wavelengths are shown in **Figure 2.26.** At the lower wavelengths, a large amount of material was detected on the plate, particularly in the region to which the conjugated metabolites elute. The material detected at 320nm was reduced considerably. Paracetamol and its conjugated metabolites were not detected on the plate against the endogenous background at concentrations of either 1 or 10$\mu$g/ml in urine.

**Figure 2.25**

**HPTLC chromatograms of paracetamol and its glucuronide and sulphate conjugates following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

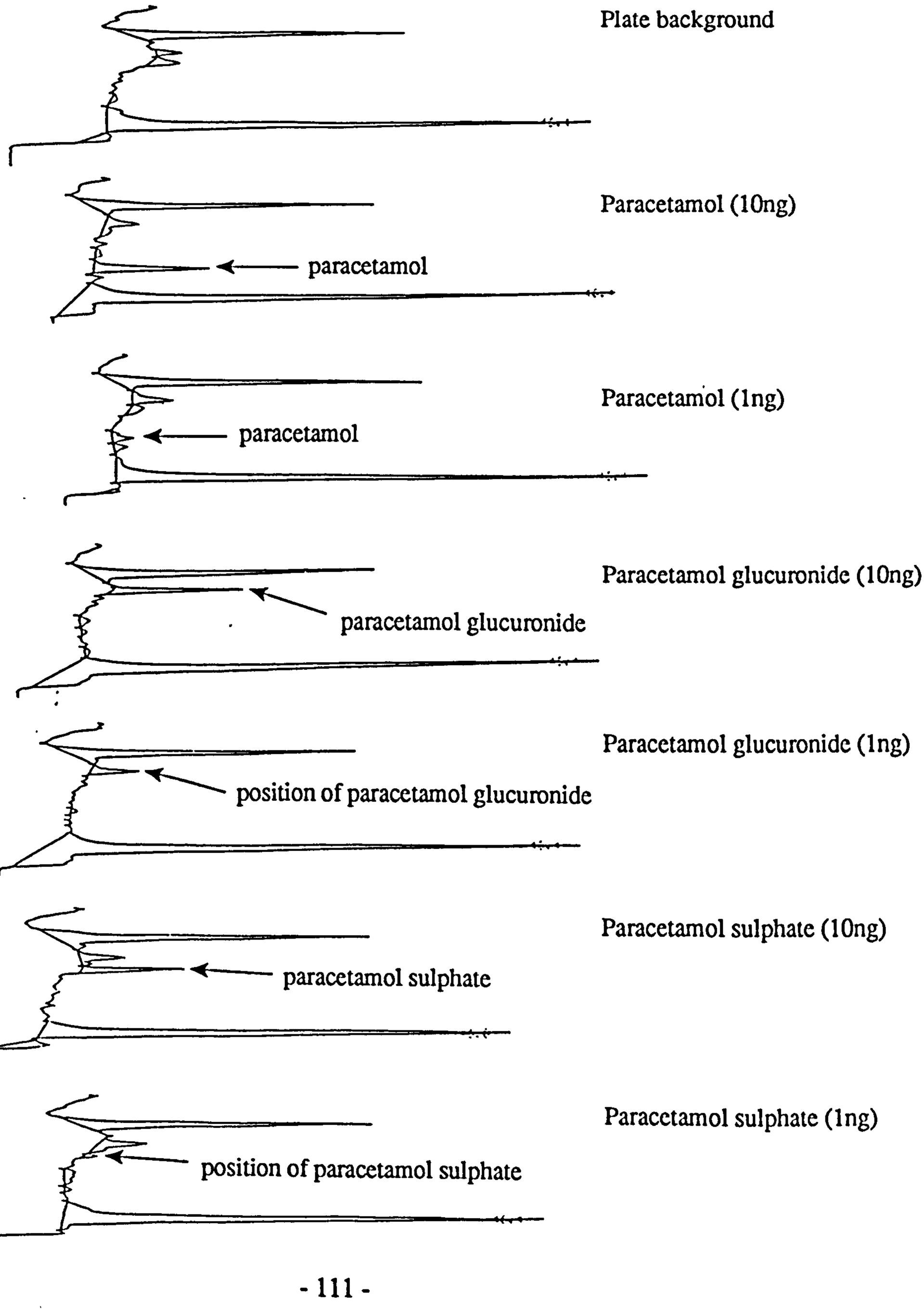
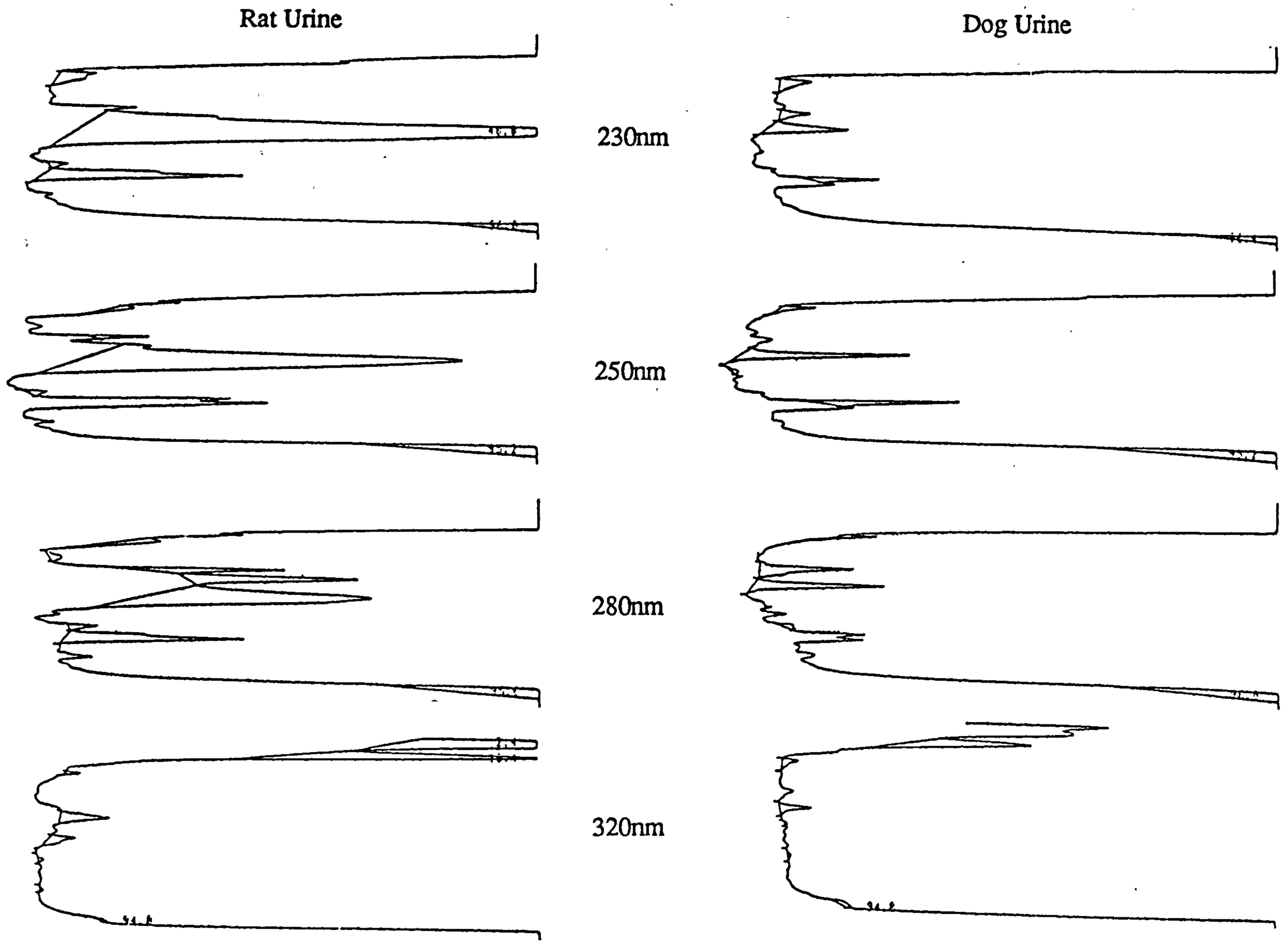


**Figure 2.26**

**HPTLC chromatograms of control rat and dog urine following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**



Chromatograms from control rat and dog plasma following MMD on silica HPTLC plates using MMD Scheme 3, obtained by scanning at various wavelengths are shown in **Figure 2.27.** There was much less material detected on the plate than was found with urine, and at 320nm the material detected was reduced considerably. Chromatograms from plasma spiked with paracetamol and its conjugated metabolites at 1 and 10$\mu$g/ml after MMD on silica HPTLC plates using MMD Scheme 3 are shown in **Figure 2.28.** Paracetamol and its conjugated metabolites were not detected on the plate against the endogenous background at a concentration of 1$\mu$g/ml, but at 10$\mu$g/ml there was evidence of paracetamol and the sulphate metabolite. The glucuronide was not detected.

## *2.5. Discussion*

The main aim of the studies described in this chapter was to assess the usefulness of multiple development techniques on normal-phase HPTLC for the separation of drugs and metabolites with a view to developing a generally applicable gradient. Further aims were to assess the sensitivity of HPTLC for detecting drugs and metabolites in biological fluids.

High-performance thin-layer chromatography with multiple development has been shown to be useful for the separation of a wide range of compounds with large differences in polarity. The elution distances (EDs) obtained from a multiple development gradient comprising solvent mixtures having a linear decrease in elutropic strengths from methanol (0.73) to hexane (0.0) are summarised in **Figure 2.29.** The compounds were separated over most of the HPTLC plate and only a few compounds failed to elute more

**Figure 2.27**

**HPTLC chromatograms of control rat and dog plasma following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

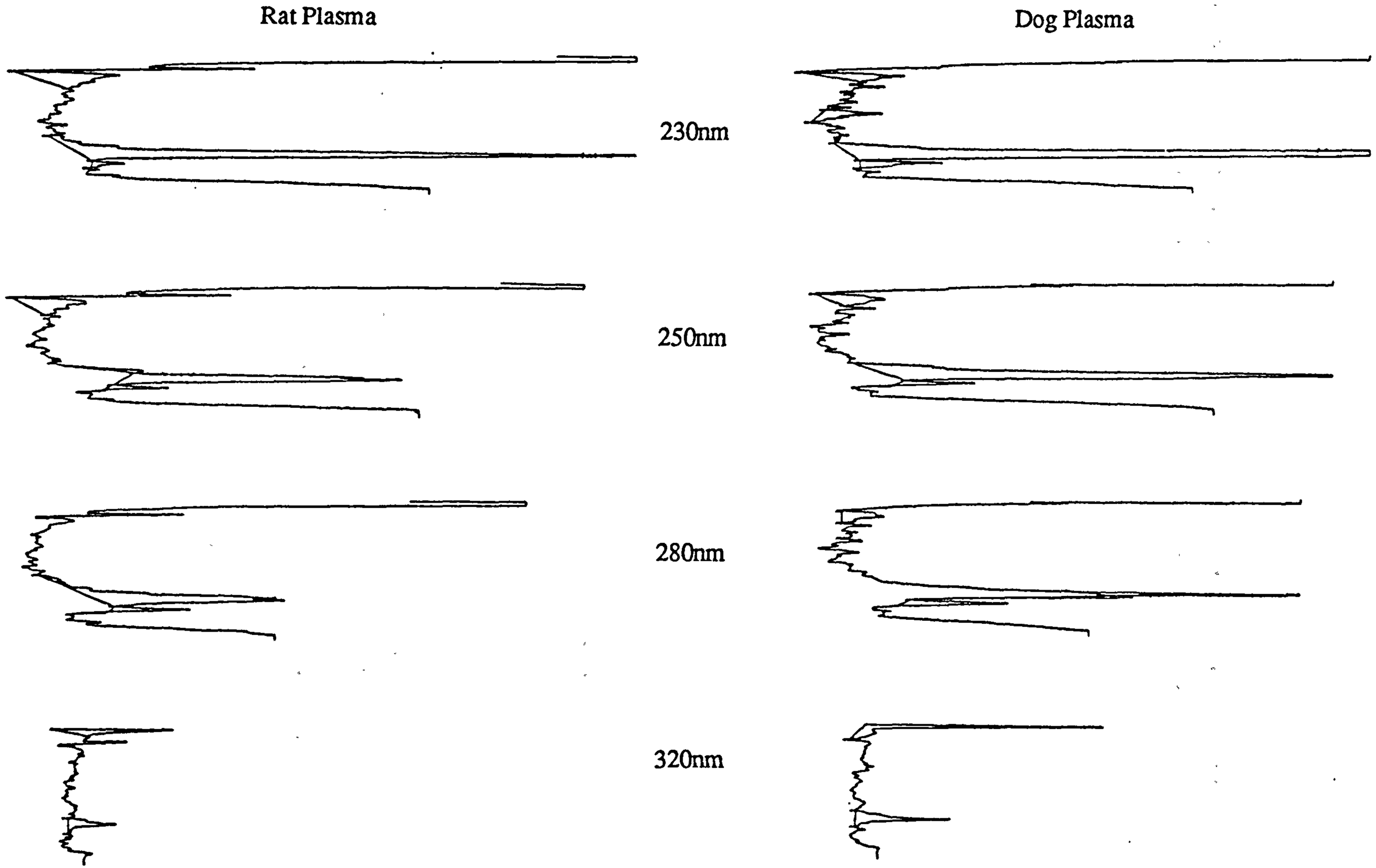


**Figure 2.28**

**Detection of paracetamol and conjugated metabolites in plasma after direct spotting of plasma and manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**

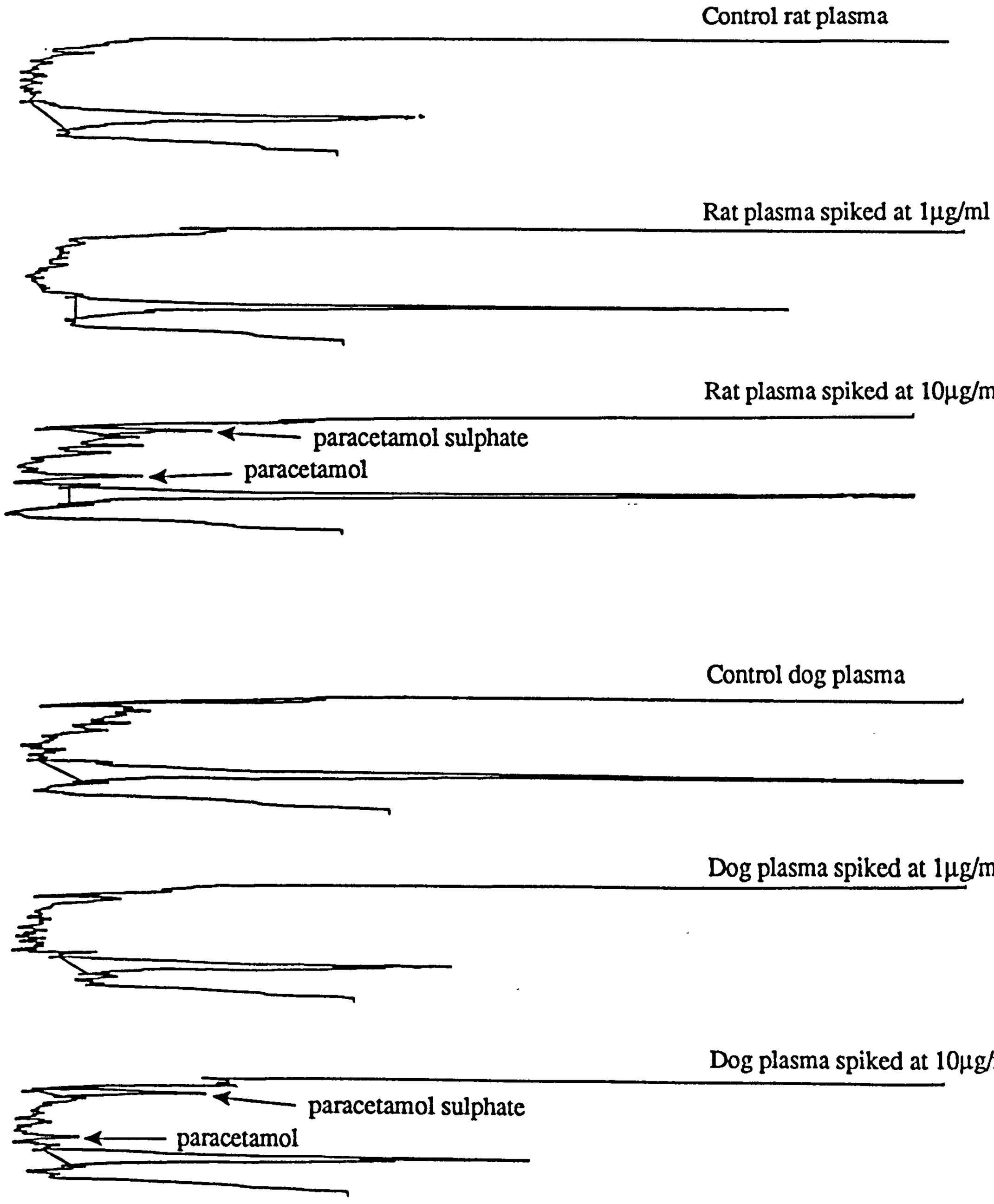
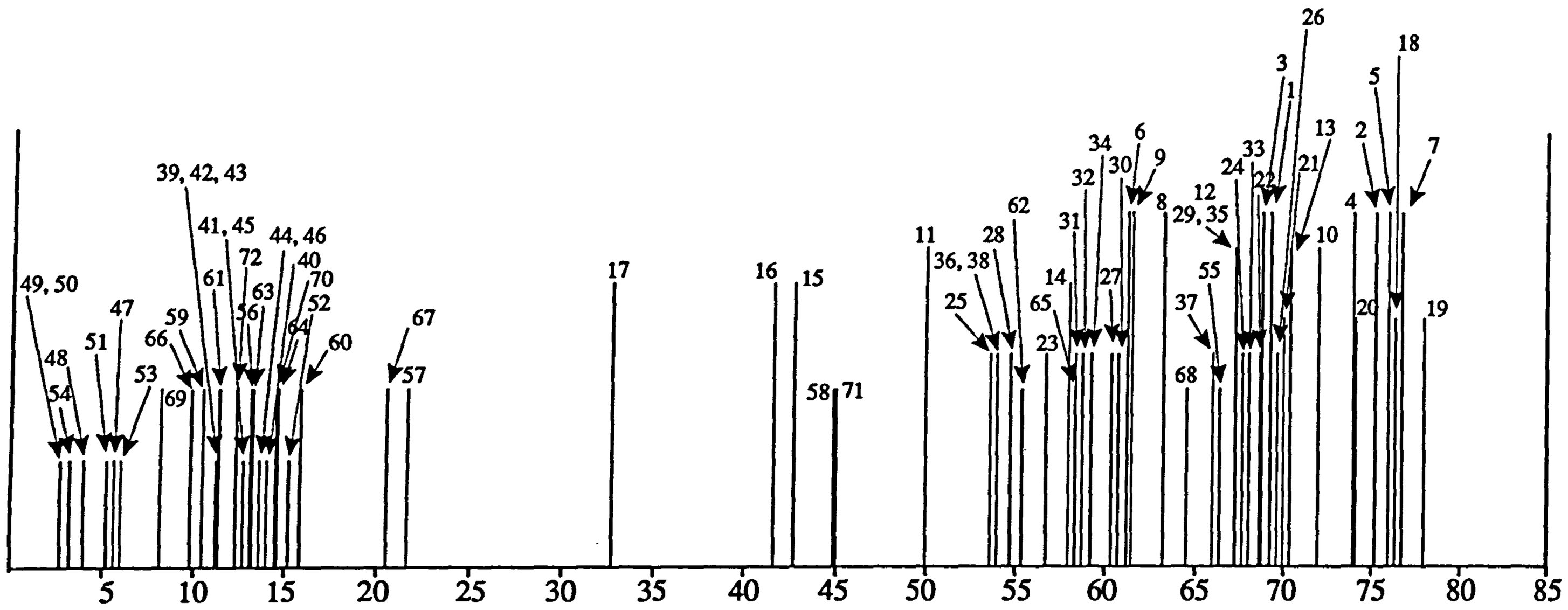


**Figure 2.29**

**Elution distances of model compounds following manual multiple development on silica gel 60 HPTLC plates using MMD Scheme 3**




| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1-Methylindole | 13 | 7-Methoxycoumarin | 25 | 4-Hydroxyphenylethanol | 37 | p-Cresol | 49 | Ranitidine N-oxide | 61 | Phenol glucuronide |
| 2 | Indole | 14 | Aspirin | 26 | 2-Ethylphenol | 38 | 4-Hydroxybenzyl alcohol | 50 | Ranitidine S-oxide | 62 | Phenolphthalein |
| 3 | 5-Hydroxyindole | 15 | Salicylic Acid | 27 | 2-[2-Hydroxyphenyl]ethanol | 39 | Ondansetron | 51 | Loxtidine | 63 | Phenolphthalein glucuronide |
| 4 | 5-Methoxyindole | 16 | Gentisic Acid | 28 | 2-Hydroxyphenylethan-1-ol | 40 | N-desmethyl ondansetron | 52 | AH25227 | 64 | Phenolphthalein sulphate |
| 5 | 6-Methylindole | 17 | 2,3-Dihydroxybenzoic acid | 29 | Phenol | 41 | 5-Hydroxy ondansetron | 53 | GR30450 | 65 | 4-Methylumbelliferone (4-MeU) |
| 6 | Indole-6-carboxylic acid | 18 | Biphenyl | 30 | Catechol | 42 | 6-Hydroxy ondansetron | 54 | AH24610 | 66 | 4-MeU glucuronide |
| 7 | 3-Methylindole | 19 | 2-Phenylphenol | 31 | Resorcinol | 43 | 7-Hydroxy ondansetron | 55 | Naphthol | 67 | 4-MeU sulphate |
| 8 | Indole-3-carboxylic acid | 20 | 4-Phenylphenol | 32 | Hydroquinone | 44 | 8-Hydroxy ondansetron | 56 | Naphthol glucuronide | 68 | Epiandrosterone |
| 9 | 3-Indolemethanol | 21 | 4'4-Biphenol | 33 | o-Cresol | 45 | 6-Hydroxy-N-desmethyl ondansetron | 57 | Naphthol sulphate | 69 | Epiandrosterone glucuronide |
| 10 | Coumarin | 22 | 4-Pentylphenol | 34 | 2-Hydroxybenzyl alcohol | 46 | 7-Hydroxy-N-desmethyl ondansetron | 58 | Paracetamol | 70 | Epiandrosterone sulphate |
| 11 | 4-Hydroxycoumarin | 23 | 1-[4-Hydroxyphenyl]pentan-1-ol | 35 | m-Cresol | 47 | Ranitidine | 59 | Paracetamol glucuronide | 71 | Chloramphenicol |
| 12 | 7-Hydroxycoumarin | 24 | 4-Ethylphenol | 36 | 3-Hydroxybenzyl alcohol | 48 | N-desmethyl ranitidine | 60 | Paracetamol sulphate | 72 | Chloramphenicol glucuronide |

than 10mm using this gradient. The linear gradient thus had an advantage over previously used universal gradients which tended to include abrupt changes in elutropic strength which were likely to cause clustering of compounds at the point where the change occurred.

Most of the compounds selected as model Phase I metabolites had smaller EDs than the "parent" compounds as would be predicted. The exceptions were indole when compared with N-methyl indole, some metabolites of ondansetron when developed with gradients not containing TEA, and the relatively non-polar metabolite of loxtidine formed after N- and O-dealkylation. The loxtidine metabolite is formed through loss of the relatively polar triazole and carboxylic acid groups, so an increase in retention would be expected in this case. The separation between "parent compound" and "metabolite" on the full linear gradient was small in a large number of cases with RED values being greater than 0.9 particularly after aromatic hydroxylation and O-dealkylation to form aromatic hydroxylated compounds. In contrast, aliphatic hydroxylation resulted in greater separation with a mean RED value of about 0.8. This can be explained because a hydroxyl group has a high electron density, tending to release electrons, and by this means interacts with the silica surface. The electron density remains with the hydroxyl group on aliphatic alcohols which thus interact strongly with the silica surface. However, an aromatic system is unsaturated electronically, so a hydroxyl group attached to an aromatic ring is able to share its electron density with that system. There is therefore a reduced electron density on an aromatic hydroxyl group compared with an aliphatic hydroxyl, and its interaction with the silica surface is accordingly weaker. The three phenolic compounds which had lower RED values (2,3-dihydroxybenzoic acid, resorcinol and hydroquinone) also had other electron releasing hydroxyl groups on the

ring which may have reduced the extent to which delocalisation could occur when a second hydroxyl group was added.

Oxidation to form carboxylic acids resulted in retention changes similar to aliphatic hydroxylation, and the accompanying characteristic broad peaks obtained with these compounds were indicative of their presence. The presence of acidic groups may be confirmed further by development with a gradient containing acetic acid. The acetic acid suppresses the ionisation of acidic compounds, causing increased migration of some acidic compounds with an improved peak shape in all cases. Insufficient examples of compounds formed by N-demethylation were obtained for this transformation to be examined adequately with compounds which migrated on the full gradient system.

Ondansetron, ranitidine, loxtidine and their metabolites were retained very strongly using MMD Scheme 3. These compounds are able to interact with silica by a cation-exchange mechanism, hence they were only eluted reasonable distances with solvent mixtures containing high proportions of methanol, or with a modifier such as TEA which competes for the sites on the silica surface which are able to partake in cation-exchange. Ondansetron, ranitidine and loxtidine all required separation on gradients over a narrow elutropic strength range because of their strong interactions with silica, and although separation was achieved, the RED values obtained could not be compared directly with the values obtained from the other compounds using the full gradient.

Separation of Phase I model compounds was increased when gradients covering reduced elutropic strength ranges were used. Use of these shallow gradients may be considered as being analogous to increasing the magnification of a microscope when studying a specimen. As more detail is observed with greater magnification under a microscope, so

a more shallow gradient increases the resolution on the HPTLC plate. The RED values increased accordingly as the "magnification" was increased, but could not be compared with the values obtained using the full gradient.

The full linear gradient gave excellent separation of conjugated metabolites from their parent compounds, and the RED values obtained for the conjugated metabolites were generally within two ranges which were significantly different for glucuronides and sulphates. This gave a means for the tentative characterisation of conjugated metabolites, which may be strengthened by investigation of the behaviour of the component of interest with the addition of acetic acid (greater increase in RED of a glucuronide) and TEA (greater increase in RED of a sulphate). The full gradient separated the glucuronides from the sulphates, but increased separation was obtained when gradients covering reduced elutropic strength ranges were used. However, this again posed the problem of comparing retention with that of parent compound on the full gradient.

The previous discussion has indicated the difficulty that exists in obtaining RED values when comparing elution distances of two compounds on different gradients. In the following sections, a method to overcome this problem is proposed.

A more appropriate parameter than elution distances for comparing retention between different linear gradients may be obtained by calculating "elution indices". If elutropic strength is plotted against the mid-point of the difference in distance between adjacent developments on linear MMD gradients, linear plots are obtained, and equations for these lines can be calculated by linear regression analysis. An "elution index" can be interpolated from the regression equation for each distance from the origin to which a

substance might elute, as shown in **Figure 2.30.** The equations for the full linear gradient (MMD Scheme 3), and expanded gradients (MMD Schemes 7 and 9) are shown in **Table 2.19.** The elution distances obtained for the compounds developed using each system can be interpolated from the equation for the system concerned and the values calculated for the glucuronide and sulphate conjugates are shown in **Table 2.19.**

The elution indices calculated from the expanded gradients (MMD Schemes 7 and 9) were in very good agreement with each other and with the values from the full gradient. If the elution indices from the expanded systems are then substituted into the equation for the full gradient, the elution distances to which the compounds would have run on that system can be obtained. These values are shown in parentheses in **Table 2.19** and are also in good agreement with the actual distances to which the compounds eluted with the full gradient. Relative elution distances can then be computed using the elution distances calculated from the elution indices obtained on the expanded gradients and the elution distance of the parent compound from the full gradient. The values obtained are shown in **Table 2.20.**

Very good agreement was found between the estimated RED values and those obtained experimentally on the full gradient. These data show that expanded gradients can be used to increase resolution, and also that the elution data can be related back to the full gradient by use of elution indices. Meaningful RED values can thus be calculated. This method can be applied to the linear gradients because the linearity provides a function to relate different gradients to each other. This would be much more difficult to achieve on universal gradients and thus gives another advantage to the linear gradients.

**Figure 2.30**

**Interpolation of elution indices from graphs of elutropic strength against development distance for three linear MMD gradients**

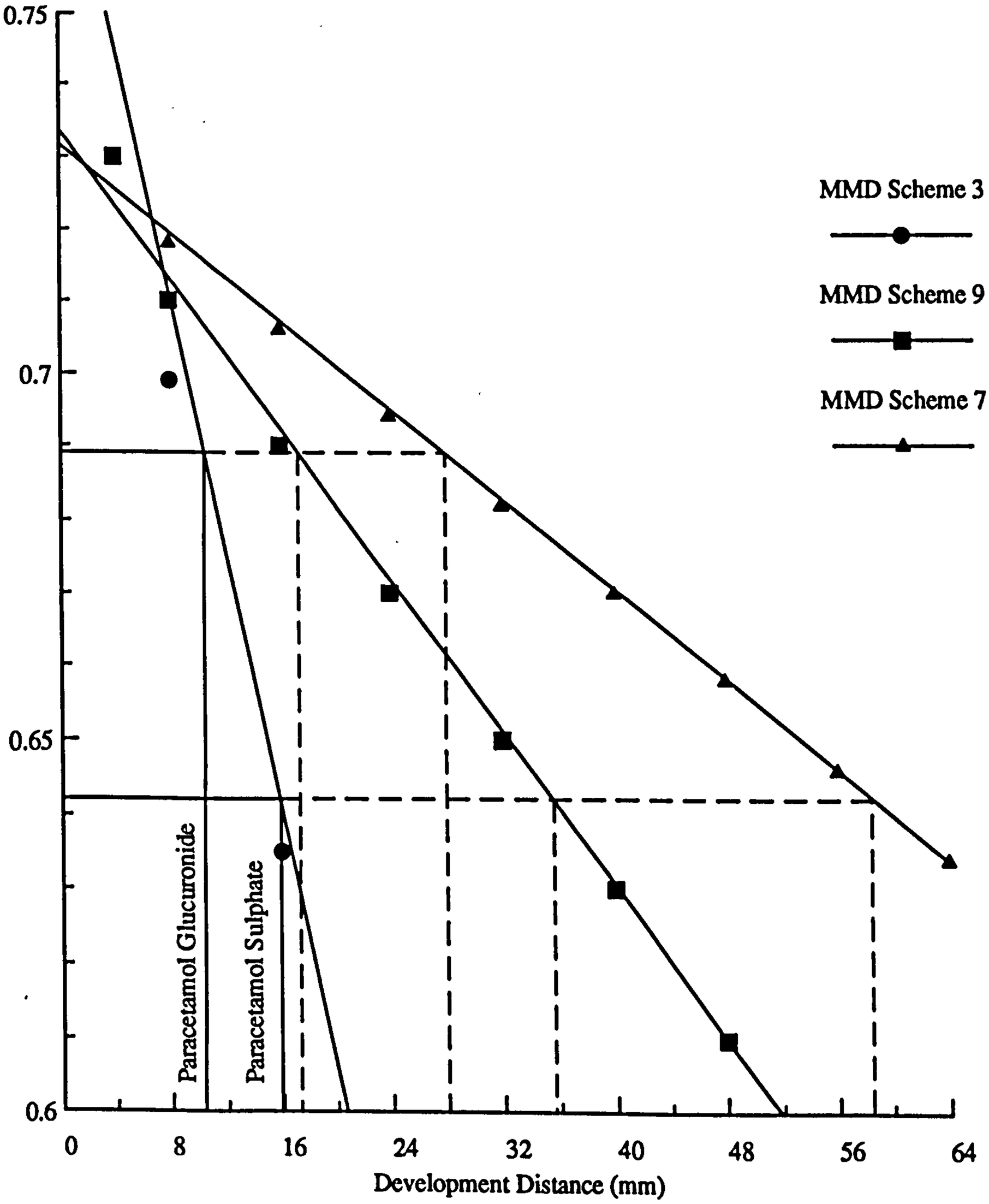


**Table 2.19**

**Elution indices calculated from linear MMD gradients for glucuronide and sulphate metabolites**

| Gradient | Linear Regression Equation |
|---|---|
| MMD Scheme 3 | Y = -0.00873X + 0.780510 |
| MMD Scheme 7 | Y = -0.00152X + 0.731717 |
| MMD Scheme 9 | Y = -0.00254X + 0.732861 |

Y = Elution Index
X = Distance (mm)

**Elution Index (Predicted Distance (mm) on MMD Scheme 3)**

| Compound | MMD Scheme 3 | MMD Scheme 7 | MMD Scheme 9 |
|---|---|---|---|
| N glucuronide | 0.666 (13.1) | 0.685 (10.9) | 0.685 (10.9) |
| N sulphate | 0.592 (21.6) | 0.633 (16.9) | 0.592 (21.6) |
| Pa glucuronide | 0.689 (10.5) | 0.695 (9.8) | 0.693 (10.0) |
| Pa sulphate | 0.642 (15.9) | 0.652 (14.7) | 0.642 (15.9) |
| Pp glucuronide | 0.670 (12.7) | 0.686 (10.8) | 0.685 (10.9) |
| Pp disulphate | 0.653 (14.6) | 0.665 (13.2) | 0.662 (13.6) |
| 4-MeU glucuronide | 0.697 (9.6) | 0.691 (10.2) | 0.689 (10.5) |
| 4-MeU sulphate | 0.606 (20.0) | 0.638 (16.3) | 0.602 (20.2) |
| Ep glucuronide | 0.710 (8.1) | 0.679 (10.1) | 0.677 (10.0) |
| Ep sulphate | 0.654 (14.5) | 0.655 (14.4) | 0.645 (15.5) |
| Chl glucuronide | 0.672 (12.4) | 0.679 (11.6) | 0.677 (11.9) |

N : Naphthol
Pa : Paracetamol
Ph : Phenol
Pp : Phenolphthalein
4-MeU : 4-Methylumbelliferone
Ep : Epiandrosterone
Chl : Chloramphenicol

**Table 2.20**

**RED values for conjugated metabolites estimated from elution indices on expanded linear MMD gradients compared with full gradient values**

| Compound | RED Values MMD Scheme 3 | MMD Scheme 7 | MMD Scheme 9 |
|---|---|---|---|
| N glucuronide | 0.197 | 0.164 | 0.164 |
| N sulphate | 0.325 | 0.255 | 0.325 |
| Pa glucuronide | 0.234 | 0.218 | 0.223 |
| Pa sulphate | 0.354 | 0.327 | 0.354 |
| Pp glucuronide | 0.229 | 0.195 | 0.197 |
| Pp disulphate | 0.264 | 0.238 | 0.245 |
| 4-MeU glucuronide | 0.163 | 0.173 | 0.179 |
| 4-MeU sulphate | 0.340 | 0.297 | 0.340 |
| Ep glucuronide | 0.126 | 0.157 | 0.155 |
| Ep sulphate | 0.225 | 0.223 | 0.240 |
| Chl glucuronide | 0.261 | 0.244 | 0.250 |

The elution distances from indoles, coumarins and biphenyls obtained using expanded gradients, and the data from ondansetron and its metabolites on an expanded gradient with TEA were treated in the same way as the conjugate data, and predicted elution distances were computed. These are compared with the measured values in **Table 2.21.**

The data from the expanded gradients for the indoles, coumarins and biphenyls were in good agreement with each other, but tended to predict smaller elution distances than were obtained in practice. The reason for this might be that for these analyses the HPTLC plates could not be placed in a vacuum to remove the solvent after each step because some of the compounds under examination would have evaporated under those conditions. This would have meant that traces of the more polar solvents (in particular methanol) would still have been present on the plate and might have caused an increase in migration of the compounds. The expanded gradients only used a single step containing methanol to concentrate the sample spots initially, thus on these systems limited contamination with methanol would have occurred, whereas MMD Scheme 3 used methanol for six steps.

The predicted elution distances for ondansetron and its metabolites were in broad agreement with those obtained experimentally. The data therefore support the use of elution indices to predict elution distances on the full gradient particularly for compounds that are retained strongly. The use of expanded gradients for the less polar compounds may be limited to obtaining increased resolution in order to determine the number of components present in areas of the chromatogram where a number of peaks elute close to each other.

**Table 2.21**

**Elution distances calculated from elution indices compared with measured elution distances for indoles, coumarins, biphenyls and ondansetron metabolites**

| Gradient | Linear Regression Equation |
|---|---|
| MMD Scheme 5 | Y = -0.00612X + 0.539 |
| MMD Scheme 6 | Y = -0.00312X + 0.373 |

Y = Elution Index X = Distance (mm)

| Compound | MMD Scheme 3 | MMD Scheme 5 | MMD Scheme 6 |
|---|---|---|---|
| 1-Methylindole | 69.2 | 62.7 | 74.4 |
| Indole | 75.2 | 69.7 | 66.8 |
| 5-Hydroxyindole | 68.8 | 61.1 | 58.2 |
| 5-Methoxyindole | 74.0 | 65.8 | 67.4 |
| 6-Methylindole | 76.0 | 70.6 | 67.4 |
| Indole-6-carboxylic acid | 61.2 | 55.1 | 53.2 |
| 3-Methylindole | 76.8 | 71.7 | 68.3 |
| Indole-3-carboxylic acid | 63.2 | 56.5 | 53.4 |
| 3-Indolemethanol | 61.6 | 60.5 | 60.4 |
| Coumarin | 72.0 | 65.0 | 62.6 |
| 4-Hydroxycoumarin | 50.0 | 48.2 | 51.8 |
| 7-Hydroxycoumarin | 67.2 | 59.4 | 56.9 |
| 7-Methoxycoumarin | 70.4 | 62.7 | 60.9 |
| Biphenyl | 76.4 | Not Detected | 78.0 |
| 2-Phenylphenol | 78.0 | 69.4 | 67.2 |
| 4-Phenylphenol | 74.0 | 65.2 | 61.8 |
| 4'4-Biphenol | 76.4 | 59.6 | 56.3 |

| Compound | MMD Scheme 3 with 1% TEA | MMD Scheme 9 with 1% TEA |
|---|---|---|
| Ondansetron | 21.2 | 20.4 |
| N-desmethyl ondansetron | 17.6 | 18.5 |
| 6-OH ondansetron | 17.2 | 19.2 |
| 7-OH ondansetron | 14.8 | 17.8 |
| 8-OH ondansetron | 18.0 | 17.8 |
| 6-OH,N-desmethyl ondansetron | 15.6 | 17.6 |
| 7-OH,N-desmethyl ondansetron | 14.0 | 16.2 |

The elution distances predicted for ondansetron, ranitidine and their metabolites were used to calculate RED values for the metabolites which are shown in **Table 2.22.** The data for the hydroxylated metabolites of ondansetron were in general agreement with the data obtained from the other compounds undergoing aromatic hydroxylation, however the data showed that the position of substitution relative to other groups within the ring system affects the magnitude of the RED value. When the hydroxyl group was in closer proximity to the indole nitrogen it appeared to cause a greater change in the retention relative to ondansetron. This may have been due to the electron donating properties of the basic indole nitrogen suppressing the delocalisation of the hydroxyl group electrons when the two groups were in close proximity as was the case for the 8-hydroxy metabolite.

N-demethylation of ondansetron had little effect on the elution distance (RED=0.907), whereas the N-demethylated metabolite of ranitidine had a RED value of 0.67. This difference was due to the differing extents to which the molecular structures can accommodate the increase in basicity caused by N-demethylation. In the case of ondansetron, the electrons of the basic indole nitrogen are delocalised within the aromatic ring structure, whereas in ranitidine the amine is essentially aliphatic in nature and its electrons are therefore available for interaction with the silica surface.

The data obtained in the present studies have shown that certain metabolic transformations caused characteristic changes in elution distance with linear MMD systems in the rank order aromatic hydroxylation and dealkylation < aliphatic hydroxylation and aromatic oxidation to carboxylic acids << sulphate conjugation < glucuronide conjugation. Manual multiple development proved reliable and fairly reproducible, however the technique is very time consuming and tedious for the operator.

**Table 2.22**

**RED values from predicted elution distances for metabolites of ondansetron and ranitidine**

| Compound | RED Value |
|---|---|
| N-desmethyl ondansetron | 0.907 |
| 6-OH ondansetron | 0.941 |
| 7-OH ondansetron | 0.872 |
| 8-OH ondansetron | 0.843 |
| 6-OH,N-desmethyl ondansetron | 0.862 |
| 7-OH,N-desmethyl ondansetron | 0.794 |
| N-desmethyl ranitidine | 0.664 |
| Ranitidine N-oxide | 0.718 |
| Ranitidine S-oxide | 0.670 |

Automated multiple development was more reproducible because human error was removed, and was far less tedious to operate than MMD. The main disadvantage of the AMD system is its inability to mix solvents to order. The mode by which it operates makes it ideal for universal systems, however it is difficult to produce linear gradients, and the need to miss out steps which were not required made it cumbersome to use. A version of the instrument which mixes solvents to any specification would be most useful.

HPTLC has been shown to have the sensitivity required to detect very small amounts of material, however the background from plasma, and urine especially, was high. The high background from the biological matrices coupled with the limited volumes of material which could be applied to the plate meant that paracetamol and its metabolites could not be detected in urine up to a concentration of 10$\mu$g/ml, and in plasma only two components were detected. Thus the ideal screening method comprising application of the biological fluid followed by chromatography and detection would be impractical for the majority of compounds. The exception to this may be compounds which have ultraviolet absorption at wavelengths in excess of 300nm or which are fluorescent. However, it would need to be assumed that these properties of parent compound were preserved in metabolites which may not be the case. Compounds present at higher concentrations may be detected after direct application of the biological matrix, but relatively large components may be missed, and the large salt deposit at the origin of the HPTLC plate is likely to affect the chromatography and possibly invalidate some of the elution distance data obtained.

It is therefore necessary to produce a purer and more concentrated solution of drug and metabolites from the biological fluids which can be applied to the HPTLC plate. This

could take the form of an extract which might be prepared in a number of ways. However if the aims of this thesis are to be achieved it is necessary for the extraction to be of a general nature so that all drug-related material is made available for subsequent HPTLC analysis.

# *CHAPTER THREE*

# *EXTRACTION OF DRUGS AND METABOLITES FROM BIOLOGICAL FLUIDS*

## *3.1 Introduction*

An ideal method for the detection of all drug-related material in biological fluids following administration of a drug to animals would require no sample pre-treatment or clean-up prior to the separation and detection of the compounds of interest. It has been found however, that a clean-up step is required in order to remove interfering endogenous material and to concentrate samples of plasma and urine before analysis by HPTLC/MMD (Chapter 2). There are a number of criteria required of a general clean-up procedure. These criteria are listed below.

1. The clean-up procedure must be sufficiently general to provide recovery of all drug-related material.
2. The procedure must be simple and robust.
3. The procedure must not use conditions under which drug or metabolites might decompose.
4. Proteins and low molecular weight components which may interfere with chromatography and with the detection of drug-related material must be removed.
5. The sample must be collected in a form which facilitates concentration of the sample and application to a HPTLC plate for analysis.
6. The procedure should provide some discrimination between classes of compound.

In designing a strategy for a general extraction procedure, the physical and chemical properties of drugs and their metabolites must be considered alongside the extraction techniques available.

In this chapter, a strategy for the development of a general extraction procedure is proposed, and the various options are assessed to determine whether they fulfil the requirements for a general extraction procedure. Paracetamol and its glucuronide and sulphate conjugates are used as model compounds to carry out the assessment. The extraction scheme selected is examined for its general applicability by extracting a range of model compounds from plasma, and estimates of detection limits are made by comparing chromatograms of extracts from spiked samples with chromatograms from controls. Finally, the extraction procedure is assessed for its likely applicability to metabolism studies.

### 3.1.1 Sample Preparation Methods Used in Metabolism Studies

It has been suggested that sample preparation strategy and tactics have to be learned largely by experience and "lore" (Martin and Reid, 1981). There has, however, been significant development in sample preparation technology in the last decade. With this development, the view that robust procedures to isolate components of interest from complex matrices can be devised successfully by considering the chemistry of the interactions involved has gained greater acceptance (McDowall, 1989). Various reviews on sample preparation techniques for biomedical analysis or metabolite isolation have been published (Reid, 1976; Martin and Reid, 1981; Lim, 1988; McDowall, 1989): nevertheless, in considering a strategy for the development of a general extraction procedure it is pertinent to review the sample preparation techniques that have been used

previously in drug metabolism studies and biomedical analysis, and to assess them against the criteria for the general procedure. The methods available can be classed generally as protein precipitation, liquid-liquid extraction and liquid-solid extraction. The suitability of these methods are considered in the following sections.

### 3.1.1.1 Protein Precipitation

The most simple approach to sample preparation is protein precipitation, and this may be applicable to the analysis of plasma. Proteinacious material can be precipitated from plasma by the addition of water miscible solvents such as methanol, acetonitrile, and acetone (Woollard ,1984). Acids such as perchloric acid, trichloroacetic acid and tungstic acid form insoluble protein salts with proteins at low pH, and proteins may also be precipitated by forming insoluble salts with cations such as $Cu^{2+}$ and $Zn^{2+}$ in alkaline solutions. The benefits and problems which might be associated with these methods are considered below.

The use of acids might result in hydrolysis of labile metabolites, and addition of inorganic salts would increase the salt concentration of the samples and might further exacerbate interference with the solvent front found during chromatography of untreated plasma and urine samples (Chapter 2). These methods are therefore considered to be inappropriate. The use of organic solvents might provide a simple approach to sample preparation (i.e. the removal of proteins may be sufficient to overcome the problems encountered with HPTLC/MMD of untreated plasma). In this case however, low molecular weight compounds such as salts would not be removed, and the process would result in dilution rather than concentration of the sample unless the organic solvent was subsequently removed by evaporation. It has also been found that some compounds adsorb onto or

co-precipitate with the protein precipitate causing a reduction in recovery. The technique, although simple, would not provide discrimination between classes of compound, and would not assist in the analysis of urine. In view of the problems perceived, protein precipitation is not considered to be an appropriate method of sample preparation to pursue for the present studies.

### 3.1.1.2 Liquid-liquid Extraction

Liquid-liquid extraction (LLE) has been the method used most widely for extracting drugs and metabolites from biological fluids for many years, and it is only in the last decade that its dominance has been challenged. In LLE, the biological fluid is mixed with an immiscible organic solvent, and drug-related material is concentrated in either the aqueous or organic layer by partition. Ionised molecules tend to remain in the aqueous layer whereas non-ionised molecules partition into the organic solvent. The distribution of molecules between the aqueous and organic layers is influenced strongly by the pH at which the extraction is carried out and the $pk_a$ of ionisable groups on the molecules. Acids are non-ionised (i.e. protonated) at pH values below their $pk_a$ and are present as anions at pH values above their $pk_a$. Conversely, bases are present as cations below their $pk_a$ and are non-ionised above their $pk_a$. Thus, at low pH, acidic compounds tend not to be ionised and would therefore be concentrated in the organic layer, whereas bases which would be ionised at low pH would remain in the aqueous layer. The converse would be true at high pH.

Use of LLE as a general procedure for the isolation of drugs and unknown metabolites from biological fluids is complicated by wide polarity differences between parent drugs, which may be lipophilic in nature, and conjugated metabolites which are usually

hydrophilic. Often, drug and its Phase I metabolites can be extracted into a fairly non-polar solvent, assuming that the pH conditions are correct, but this may be complicated if an ionisable group is added to the molecule during Phase I metabolism. As discussed in Chapter 1 (Section 1.2), the groups which are added during drug metabolism are generally anionic, so extraction of samples containing acidic drugs and their metabolites is generally straight forward. The complications arise with basic drugs because addition of anionic groups leads to the formation of zwitterions. Zwitterions are molecules containing an acidic and a basic group which cannot exist in a completely non-ionised form at any pH. Thus, a basic molecule which would be extracted into a non-polar solvent at high pH, might be oxidised during metabolism to form a carboxylic acid which would require a low pH for extraction. An example of this is the metabolite formed by oxidation of loxtidine in the rat (Bell, Bradbury, Jenner, Manchee and Martin, 1983). Similarly, oxidative deamination of a basic drug can give rise to an acidic metabolite as is the case with mescaline in the rat and man (Schreiber, 1970).

A molecule in its ionised form, despite its high polarity, would not necessarily remain in the aqueous layer, but may be sufficiently lipophilic to extract into an organic layer. Some sulphate metabolites, although highly polar, are fairly lipophilic and can be extracted by LLE (Martin and Reid, 1981).

Although many schemes have been devised in which drugs and metabolites have been extracted from biological fluids using LLE, these have often been specific for the particular drug and metabolites under investigation. In most cases conjugated metabolites have been identified by the increases in Phase I metabolites observed following enzyme or acid hydrolysis of the samples rather than by isolation of the intact conjugated metabolites. Thus Kumbs, Genin-Ramakers and Mardens (1985) extracted

anticonvulsant drugs and their Phase I hydroxylated and epoxide metabolites from plasma into ethyl acetate at pH 6.7, Covey, Crowther, Dewey and Henion (1985) isolated desmethyl imipramine, imipramine N-oxide, promazine N-oxide, and promazine N-oxide sulphoxide by LLE from horse urine into dichloromethane/2-propanol (3/1 v/v) under basic conditions and Sistovaris (1983) extracted nomifensine and its Phase I metabolites from urine into ethyl acetate at pH 10 after enzyme hydrolysis of the N-glucuronides present. Covey *et al.* (1985) also extracted hydroxy metabolites using the procedure described above after enzyme hydrolysis of the glucuronide conjugates.

If a drug and its Phase I metabolites are extracted successfully, conjugated metabolites can often be extracted into less polar solvents such as 1-butanol, ethyl acetate or ethanol-ether after acidification of the aqueous layer and addition of inorganic salts such as ammonium sulphate. Thus Becket, Beaven and Robinson (1963) used n-butanol to isolate N-dedimethylchlorpromazine glucuronide from urine and Irving, Wiseman and Hill (1967) used ether-ethanol to extract the O-glucuronide of N-hydroxy-2-acetylaminofluorene from bile.

LLE is a versatile technique with an extensive range of pH conditions and solvents available, giving a large number of possible conditions for extracting drugs and metabolites from biological fluids. In seeking a general extraction procedure however, the diversity of schemes possible becomes a limitation because for each class of compound the choice of solvent is important. Furthermore, LLE does not always provide good recoveries, particularly for conjugated metabolites (Tomasic, 1978).

Although specific LLE techniques used in drug metabolism studies do not lead towards a general extraction procedure, the field of toxicological and clinical drug screening may

provide schemes which would be of use for drug and metabolite screening. Horning, Gregory, Nowlin, Stafford, Lertratanangkoon, Butler, Stillwell and Hill (1974) described an isolation procedure, which was a modification of alcohol-potassium carbonate salting out procedures developed by Bastos, Kananen, Young, Monforte and Sunshine (1970), in which a range of basic and neutral drugs was extracted into ethyl acetate with recoveries in the range 84-104% from plasma and urine saturated with ammonium carbonate. Ammonium carbonate was used instead of potassium carbonate because it dissolves endothermically causing the solution to cool, whereas the potassium salt dissolves exothermically producing heat which could cause breakdown of labile metabolites. Acidic drugs and metabolites, including glucuronides, were extracted into ethyl acetate or chloroform from urine acidified with sulphuric acid to pH 1. This method provided good recoveries and some discrimination between classes of compound. It may therefore fulfil most of the criteria for a general extraction procedure.

### 3.1.1.3 Liquid-Solid Extractions

Liquid-solid extractions (LSE) can be carried out with a wide range of different materials. In LSE, biological fluid samples are passed through a solid matrix onto which molecules in solution adsorb, and are thus extracted. The main interactions which occur depend upon the nature of the solid matrix, and range from non-polar Van der Waals forces to electrostatic interactions. The principles of method development using LSE have been documented clearly by Van Horne (1985), and the use of these materials in biomedical analysis has been reviewed recently by McDowall (1989).

A wide range of different solid support materials has been used to isolate drugs and metabolites from biological fluids either for quantitative analysis by chromatographic or

other techniques, or for the purpose of identification. Classical adsorbents such as carbon and alumina have been used for many years and have had wide application. Jack, Dean, Kendall and Laugher (1980) extracted antihypertensive drugs and their metabolites from urine under basic conditions onto a charcoal slurry which was subsequently washed with water and then mixed with methanol. The methanol supernatant was analysed by TLC. Activated charcoal was also used by Fujimoto and Way (1957) to isolate morphine glucuronide from human urine and by Arcos and Lieberman (1965) to extract glucuronide conjugates of steroids from urine. Alumina has been used traditionally for the isolation of catecholamines because of its selectivity for analytes with vicinal hydroxyl groups (Anton and Sayre, 1962). Alumina therefore has specialised uses, and neither alumina or charcoal are available currently in a form convenient for routine use in a manner which would ensure reproducibility from one extraction to the next. They are not therefore considered as possible materials upon which to base a general extraction strategy.

Non-ionic resins made from high surface area styrene-divinylbenzene copolymers, have been found to be useful for the adsorption of organic compounds. The materials have a large surface area and seem to be generally applicable as adsorbents for moderately hydrophilic organic compounds. One of the earliest uses of this material to isolate metabolites was by Fujimoto and Haarstad (1969) when they isolated morphine ethereal sulphate from the urine of the chicken and cat, and morphine-3-glucuronide from the urine of the rabbit. Fujimoto and Wang (1970) showed that Amberlite XAD-2 resin was useful for the extraction of a range of narcotic analgesics from human urine, and Weissman, Lowe, Beattie and Demetriou (1971) showed that metabolites of narcotics, analgesics, symphatomimetics, hypnotics and sedatives can be isolated using XAD-2 resin. Recently, Szumilo and Dzido (1992) used XAD-2 resin to isolate tryptamide (a compound with potent anti-inflammatory and analgesic activity) and its metabolites,

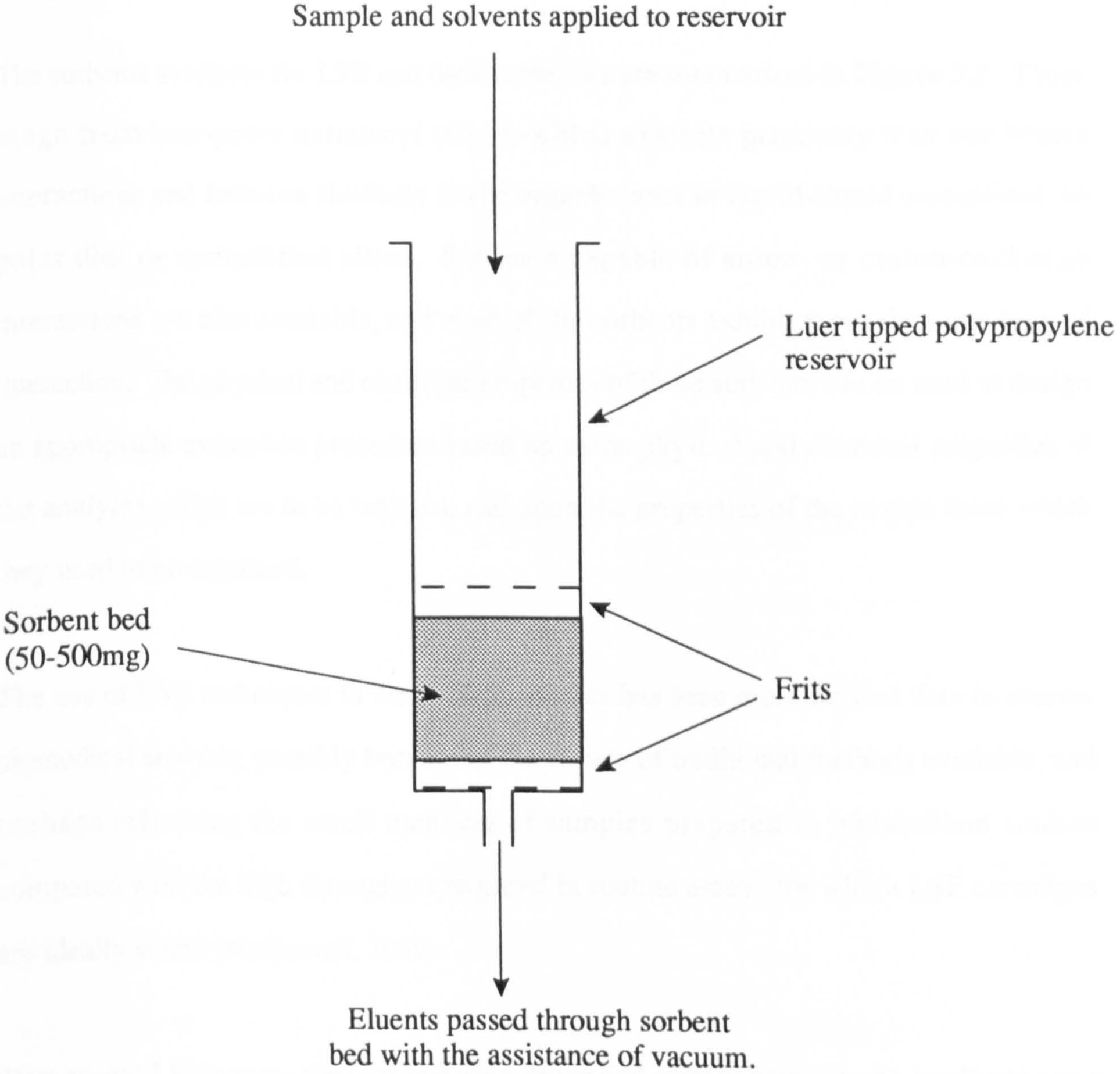
including conjugates, from rat urine. However, the conjugates were identified by comparing TLC profiles of extracts before and after enzyme hydrolysis. Non-ionic resins have been used extensively to isolate a wide range of drugs and metabolites, particularly from urine. The advantage is almost quantitative recovery of organic material, however its lack of selectivity makes it essentially a procedure to remove salt and concentrate the sample. Thus most of the criteria specified for a general extraction procedure would be fulfilled by a non-ionic resin, but there would be no easy discrimination between classes of compound.

Some of the non-specificity of the non-ionic resins could be overcome by use of ion-exchange resins, particularly the basic resins, which would preferentially retain conjugated metabolites by an anion-exchange mechanism (Tomasic, 1978). The trend over the last decade, however, has been towards the use of chemically modified silicas for LSE, such as are used for high-performance liquid chromatography (HPLC). These materials have the advantage that they are readily available commercially from a number of suppliers packed in a form convenient for routine use. The bonded phases available have a range of chemical properties which can be utilised to obtain either specific or general extractions, and the quality control of the material is such that reproducibility from one batch of material to another is obtained.

The bonded silica material used for sample preparation by LSE is generally based upon silica particles with a mean diameter of 40$\mu$m compared with 3 or 5$\mu$m material that is used for packing HPLC columns. The material, 50-500mg, is dry packed into Luer tipped polypropylene reservoirs to form a sorbent bed, and held in place between two frits as shown in **Figure 3.1.** For use, the LSE cartridges are mounted on a vacuum manifold

**Figure 3.1**

**Liquid-solid extraction cartridge**



which can hold up to 50 cartridges (Bland, Harrison, Martin and Tanner, 1984). Solvent and sample are passed through the sorbent bed by use of the vacuum. The equipment used is described more fully in Section 3.2.2.

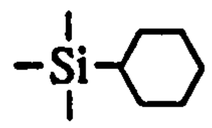
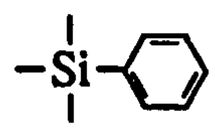
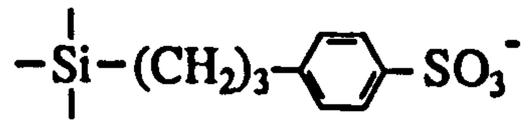
The sorbents available for LSE and their properties are summarised in **Figure 3.2.** These range from non-polar octadecyl ($C_{18}$), which exhibits primarily Van der Waals interactions and behaves similarly to the organic layer in liquid-liquid extractions, to polar diol or unmodified silica. Sorbents capable of anion- or cation-exchange interactions are also available, and most of the sorbents exhibit more than one type of interaction. The physical and chemical properties of these sorbents can be used to design an appropriate extraction procedure based upon the physical and chemical properties of the analytes which are to be isolated, and upon the properties of the matrix from which they need to be separated.

The use of LSE techniques in metabolism studies has been more limited than in routine biomedical analysis, possibly because of the variety of traditional methods available, and perhaps reflecting the small numbers of samples prepared in metabolism studies compared with the high throughput required in routine assays for which LSE cartridges are ideally suited (McDowall, 1989).

Two recent LSE approaches for isolating drugs and metabolites may be applicable to a general extraction. These are based upon; (i) use of ion-pairing to retain anionic metabolites on a non-polar sorbent followed by sequential elution via an anion-exchange material to obtain separate elution of anions of different strength and (ii) use of ion-suppression by acidifying the sample to retain anionic components on a non-polar sorbent

**Figure 3.2**

**Physical and chemical properties of commercially available sorbents**

| | | | Interactions | |
|---|---|---|---|---|
| NON-POLAR | | | Primary | Secondary |
| $C_{18}$ | Octadecyl | $-\overset{\vert}{\underset{\vert}{Si}}-C_{18}H_{37}$ | Non-polar | Polar/CE |
| $C_8$ | Octyl | $-\overset{\vert}{\underset{\vert}{Si}}-C_8H_{17}$ | Non-polar | Polar/CE |
| $C_2$ | Ethyl | $-\overset{\vert}{\underset{\vert}{Si}}-C_2H_5$ | Non-polar/Polar | CE |
| CH | Cyclohexyl |  | Non-polar | Polar/CE |
| PH | Phenyl |  | Non-polar | Polar/CE |
| POLAR | | | | |
| CN | Cyanopropyl | $-\overset{\vert}{\underset{\vert}{Si}}-CH_2CH_2CH_2CN$ | Non-polar/Polar | CE |
| 2OH | Diol | $-\overset{\vert}{\underset{\vert}{Si}}-(CH_2)_3OCH_2\underset{\vert}{\overset{\vert}{CH}}-\underset{\vert}{\overset{\vert}{CH_2}}$ (OH OH) | Non-polar/Polar | CE |
| Si | Silica | $-\overset{\vert}{\underset{\vert}{Si}}-OH$ | Non-polar/Polar | CE |
| ION-EXCHANGE | | | | |
| CBA | Carboxymethyl | $-\overset{\vert}{\underset{\vert}{Si}}-CH_2COO^-$ | CE | Non-polar/Polar |
| PRS | Sulphonylpropyl | $-\overset{\vert}{\underset{\vert}{Si}}-(CH_2)_3SO_3^-$ | CE | Non-polar/Polar |
| SCX | Benzenesulphonylpropyl |  | CE/Non-polar | Polar |
| $NH_2$ | Aminopropyl | $-\overset{\vert}{\underset{\vert}{Si}}-(CH_2)_3NH_2$ | Polar/AE | Non-polar/CE |
| PSA | N-propylethylenediamine | $-\overset{\vert}{\underset{\vert}{Si}}-(CH_2)_3NH(CH_2)_2NH_2$ | Polar/AE | Non-polar/CE |
| DEA | Diethylaminopropyl | $-\overset{\vert}{\underset{\vert}{Si}}-(CH_2)_3\overset{+}{N}H(C_2H_5)_2$ | Polar/AE | Non-polar/CE |
| SAX | Trimethylaminopropyl | $-\overset{\vert}{\underset{\vert}{Si}}-(CH_2)_3\overset{+}{N}(CH_3)_3$ | AE | Polar/Non-polar/CE |

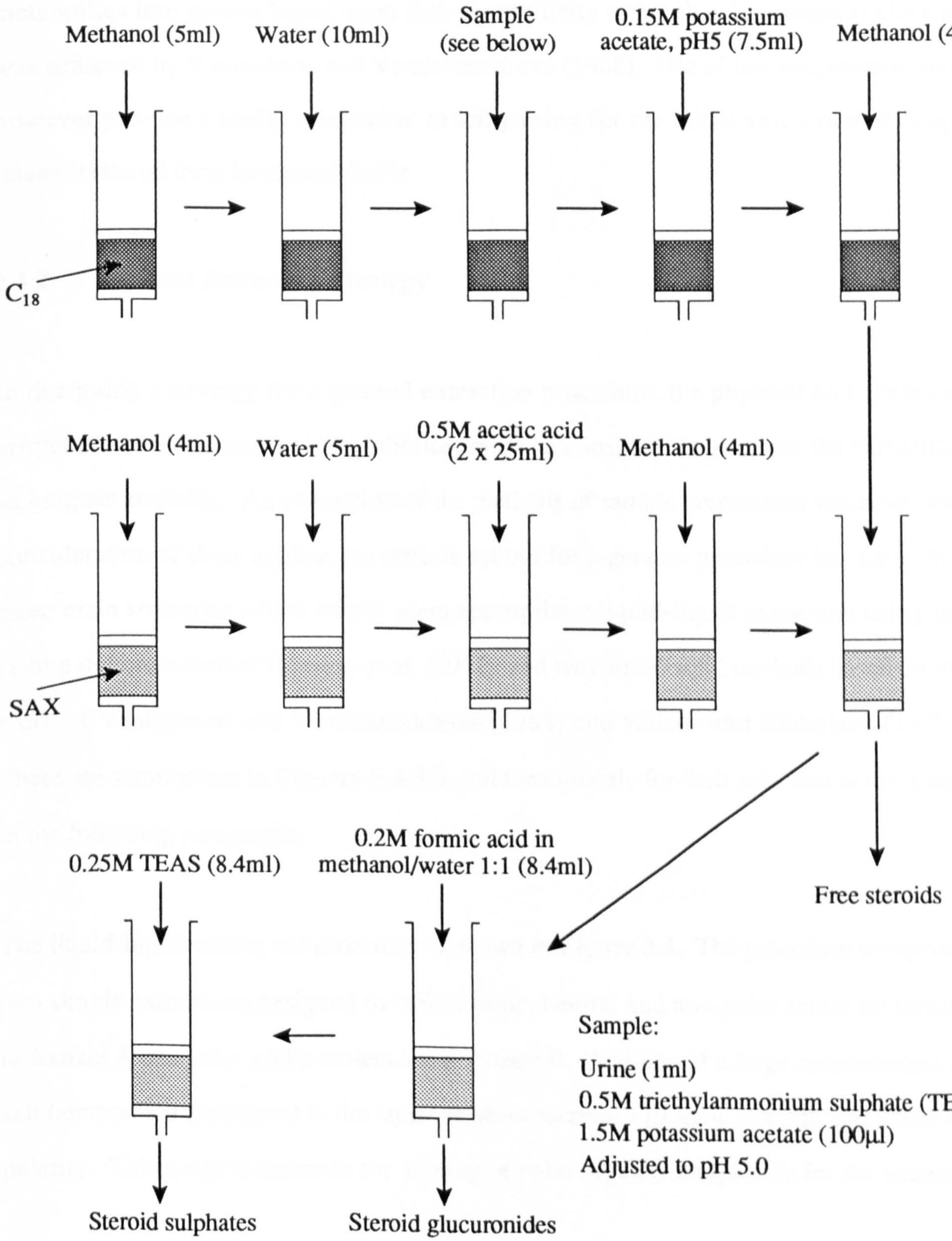
followed by sequential elution with varying proportions of methanol-water to elute components in groups based upon their lipophilicity. These are discussed in more detail below.

Vanluchene and Vandekerckhove (1983) developed a method to isolate free and conjugated steroids from serum, tissues, and peritoneal fluids. The fluids were buffered to pH 5, and triethylammonium sulphate (TEAS) was added to form ion-pairs with the anionic glucuronide and sulphate metabolites. The mixture was then extracted using Sep-Pak™ $C_{18}$ cartridges which were washed with buffer prior to eluting the steroids and conjugates with methanol. The free and conjugated steroids were then separated from each other by a variety of fairly complex liquid-liquid extraction procedures. Thus this method provided recovery of all material, but no discrimination between classes of compound. In 1988, the same workers used an approach to isolate free and conjugated steroids using a combination of $C_{18}$ and SAX (strong anion-exchange) cartridges (Vanluchene and Vandekerckhove, 1988) which is summarised in **Figure 3.3.** This approach afforded recovery of all compounds from the biological fluids, but had the additional advantage that the free and conjugated steroids were eluted separately. Furthermore the difference in strength of the glucuronide and sulphate anions was exploited to elute these two classes of compound separately. Thus discrimination between classes of compound was achieved.

The second approach was used by Wilson and Nicholson (1987). Urine containing ibuprofen and its metabolites was acidified with formic acid prior to extraction on a $C_{18}$ cartridge. Acidification of the sample suppressed the ionisation of ibuprofen, its acidic Phase I metabolites and glucuronide conjugates, making them more lipophilic and able to

**Figure 3.3**

**Schematic representation of $C_{18}$/SAX extraction procedure (Vanluchene and Vandekerckhove, 1988)**




Sample:

Urine (1ml)
0.5M triethylammonium sulphate (TEAS, 250µl)
1.5M potassium acetate (100µl)
Adjusted to pH 5.0

interact non-ionically with the octadecyl groups on the sorbent. The metabolites were then recovered from the cartridge in fractions by eluting sequentially with methanol-water mixtures containing increasing proportions of methanol. This approach separated metabolites into groups based upon their lipophilicity rather than by chemical class as was achieved by Vanluchene and Vandekerckhove (1988). Use of ion-suppression may however provide a useful alternative to ion-pairing for the initial extraction of drug-related material from biological fluids.

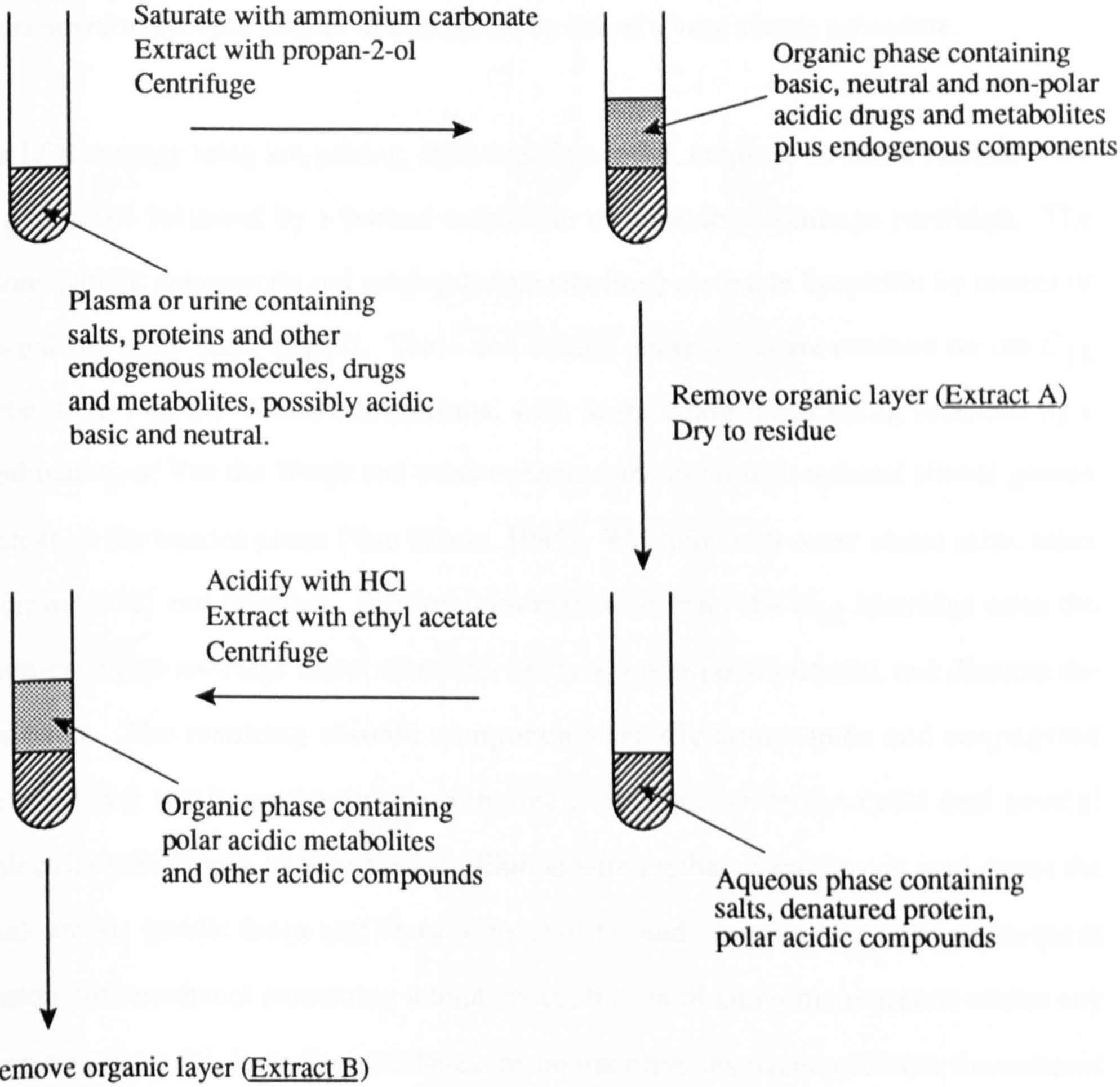
### 3.1.2 General Extraction Strategy

In designing a strategy for a general extraction procedure, the physical and chemical properties of drugs and their metabolites must be considered alongside the extraction techniques available. An evaluation of the methods of sample preparation available, and consideration of these against the criteria set out for a general procedure has identified three main strategies which would seem appropriate; liquid-liquid extraction using the salting out procedure of Horning *et al.* (1974), and two liquid-solid methods based on the work of Vanluchene and Vandekerckhove (1988) and Wilson and Nicholson (1987). These are summarised in **Figures 3.4-3.6**, and the rationale for their selection is discussed in the following paragraphs.

The liquid-liquid salting out procedure is shown in **Figure 3.4.** The procedure comprises two simple extractions designed to isolate basic, neutral and non-polar acidic molecules in Extract A and polar acidic molecules in Extract B. Addition of a large concentration of salt (ammonium carbonate) to the aqueous phase increases its ionic strength and hence its polarity. This tends to decrease the affinity of polar organic compounds for the aqueous

**Figure 3.4**

**Liquid-liquid salting out procedure**

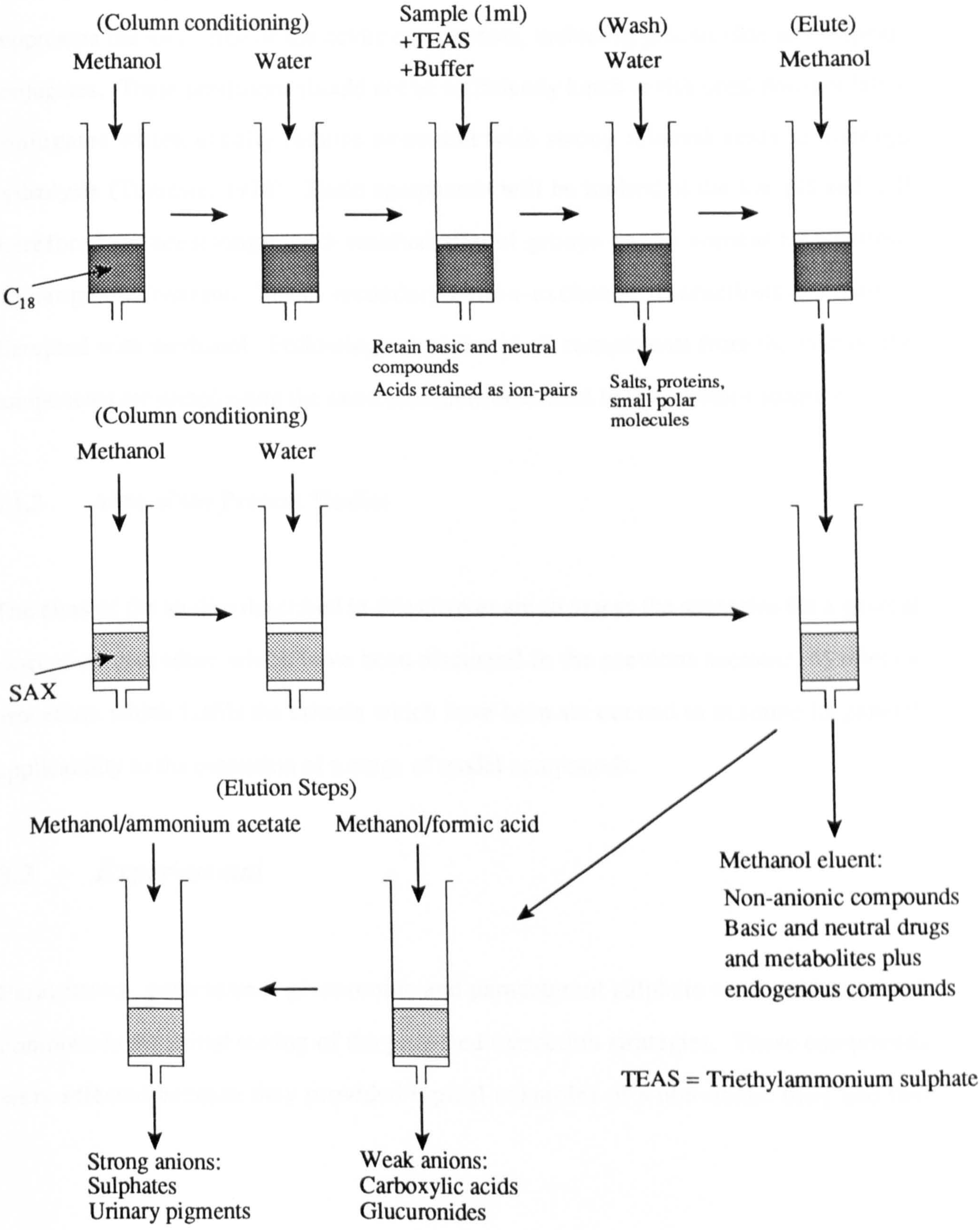


phase and thus allows them to be extracted into the organic phase. All except the most polar metabolites are extracted under these conditions. After the initial extraction, acidification of the sample suppresses the ionisation of any acidic compounds or conjugated metabolites present, increasing their lipophilicity and allowing them to be extracted into the organic phase. Thus two extracts are obtained with some discrimination between classes of compound by use of a very simple procedure.

The LSE strategy using ion-pairing, shown in **Figure 3.5**, employs an initial extraction on $C_{18}$ material followed by a second extraction on an anion-exchange cartridge. The anions (acidic compounds and conjugated metabolites) are made lipophilic by means of ion-pairing with TEAS at pH5. These and neutral compounds are retained on the $C_{18}$ sorbent by Van der Waals interactions, with basic compounds being retained by a combination of Van der Waals and weak cationic interaction with residual silanol groups present on the bonded phase (Van Horne, 1985). Washing with water elutes salts, other polar material and proteins. Elution with methanol from the $C_{18}$ cartridge onto the anion-exchange cartridge elutes all except extremely non-polar material, and disrupts the ion-pairs. The resulting anionic components (acidic compounds and conjugated metabolites) retain on the anion-exchange cartridge, while the basic and neutral molecules pass though to be collected. Elution with methanol and formic acid elutes the weak anions (acidic drugs and Phase I metabolites and glucuronides), and subsequent elution with methanol containing a high concentration of ammonium acetate elutes any remaining material, including sulphates, by competitive displacement from the cationic sites on the sorbent by the high concentration of anions present.

**Figure 3.5**

**Liquid-solid extraction procedure using ion-pairing**




TEAS = Triethylammonium sulphate

The second liquid-solid strategy using ion-suppression, shown in **Figure 3.6**, employs an initial extraction on $C_{18}$ material at a low pH, followed by further extraction on an anion-exchange cartridge. Addition of formic acid to the biological fluid reduces the pH and suppresses the ionisation of the acidic components, including glucuronide and sulphate conjugates. These conditions should not be sufficiently harsh to risk breakdown of labile conjugates which usually require treatment with strong mineral acids to undergo hydrolysis (Tomasic, 1978). Basic compounds will be ionised at the low pH and will therefore interact strongly with residual silanol groups on the sorbent by a cation-exchange mechanism. These secondary cation-exchange interactions are readily disrupted with methanol. Following extraction of all components from the matrix, the components are eluted using the same procedure described in the previous strategy.

### 3.1.3 Aims of the Present Studies

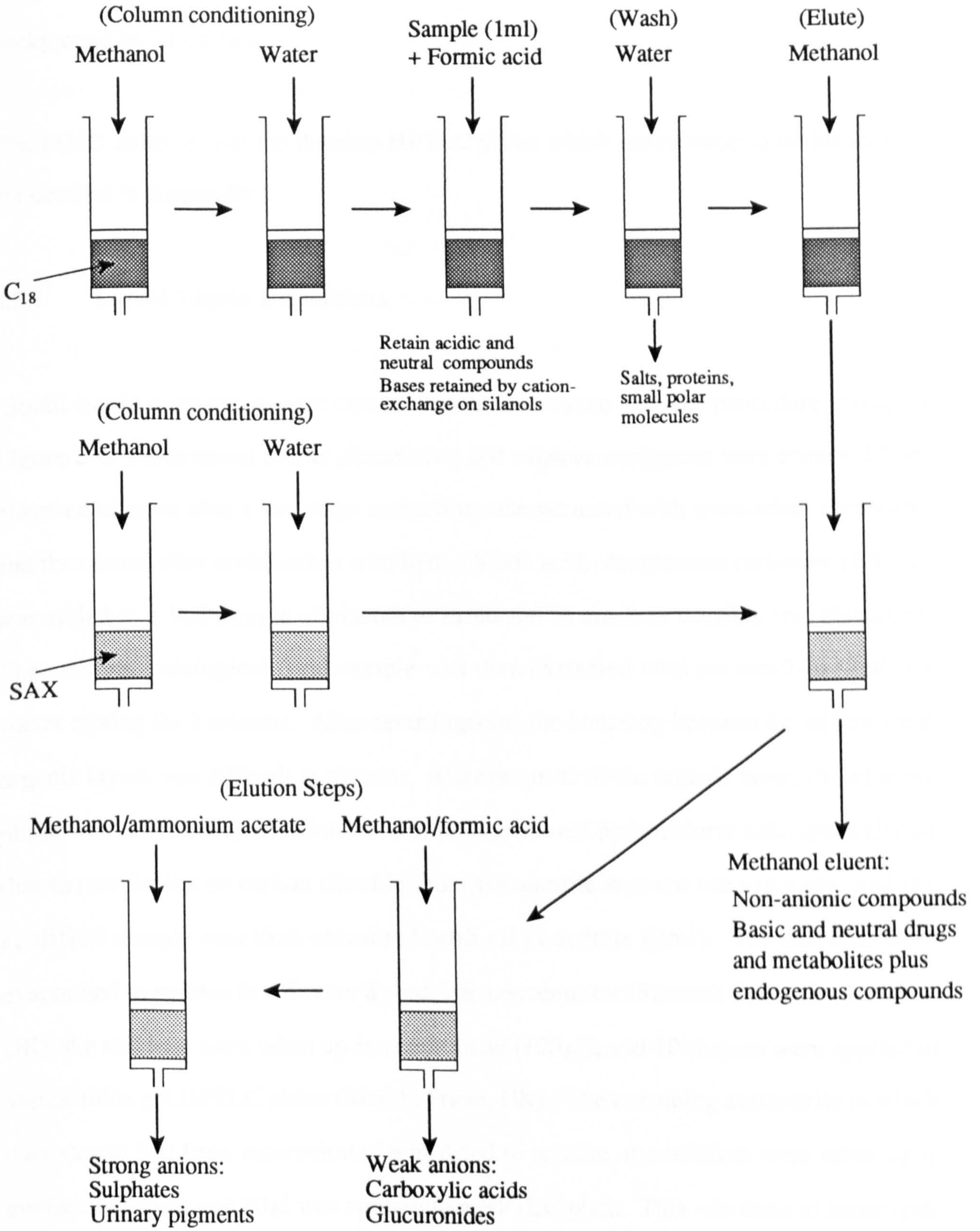
The aims of the studies described in this chapter are to assess the strategies for a general extraction procedure which have been discussed in the previous sections, to select a procedure which fulfils the criteria which have been set out and to examine its general applicability to the extraction of a range of model compounds.

## *3.2 Experimental*

Paracetamol, paracetamol glucuronide and paracetamol sulphate were used as model compounds for initial testing of the proposed extraction strategies. These compounds were selected because they provided typical examples of a non-acidic drug and two

**Figure 3.6**

**Liquid-solid extraction procedure using ion-suppression**



anionic conjugated metabolites. This combination was chosen to determine whether the extraction strategies would discriminate between classes of compound, and in addition, indicate whether drugs and metabolites would be detected against the endogenous background in the extracts.

The MMD Schemes used to develop HPTLC plates which are referred to in this section are detailed in **Appendix I.**

### 3.2.1 Liquid-Liquid Extractions

Liquid-liquid extractions were carried out in accordance with the procedure shown in **Figure 3.4.** Paracetamol and its glucuronide and sulphate conjugates were extracted from plasma and urine after a two stage extraction; one saturated with ammonium carbonate, and the second after acidification with hydrochloric acid. Ammonium carbonate (200mg) was added to a 1ml sample of plasma or urine and as much as possible was dissolved. The saturated biological fluid sample was then extracted with propan-2-ol (2ml) by vortex mixing for 2 minutes. After centrifugation, the boundary between the aqueous and organic layers was difficult to discern. After removal of the organic layer, the aqueous phase was acidified by addition of 300$\mu$l concentrated hydrochloric acid, gradually so that the evolution of carbon dioxide from the sample was not too vigorous, and the acidified sample was then extracted with ethyl acetate (2ml). The extracts were evaporated to residue in a Savant Speed Vac concentrator (Stratech Scientific, London, UK), the residues were taken up in acetonitrile (100$\mu$l), and 10$\mu$l spots were applied to Merck silica gel HPTLC plates (Merck, Poole, UK). The remaining acetonitrile in which the extracts had been reconstituted was dried to residue, the residues were taken up in methanol (100$\mu$l) and 10$\mu$l was applied to a HPTLC plate. This was done to investigate

whether use of acetonitrile instead of methanol to reconstitute the residues would reduce the amount of interfering material taken up without reducing the recovery of drug-related material. The extracts were analysed by HPTLC using a reduced MMD procedure which had been shown to separate paracetamol and its glucuronide and sulphate conjugates. The development steps used are shown below:

1. Methanol for 1cm
2. Methanol (4.5) + Ethyl acetate (5.5) for 2.5cm
3. Methanol (1.4) + Ethyl acetate (8.6) for 4cm
4. Ethyl acetate (7) + Chloroform (3) for 5.5cm

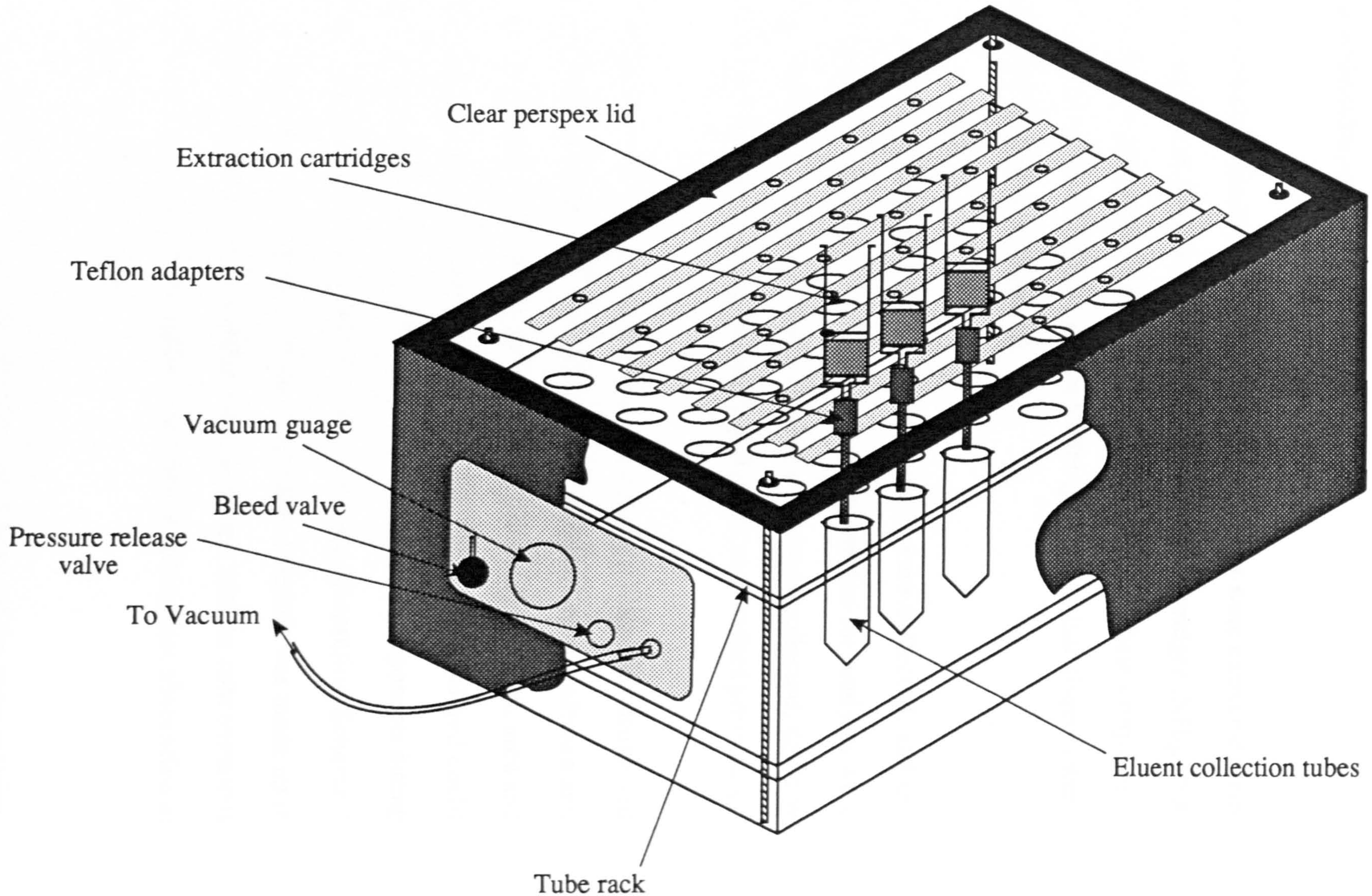
After development, the HPTLC plates were scanned from origin to solvent front using a Camag TLC Scanner II (BDH, Poole, UK), and material on the plate was detected by ultraviolet absorbance at 254nm. The signal output was recorded on a Trilab 2000 Chromatography Data System (Trivector, Sandy, UK).

### 3.2.2 Liquid-Solid Extractions

Liquid-solid extractions were carried out using Bond Elut™ extraction cartridges (Jones Chromatography, Hengoed, UK). Conditioning, washing and eluting solvents, and samples, were passed through the sorbent material by use of a vacuum manifold underneath which was placed either a waste tray to collect unwanted eluent, or sample tubes in which to collect eluents that were required for analysis. The equipment used was described by Bland *et al.* (1984) and is shown schematically in **Figure 3.7.**

**Figure 3.7**

**Diagram of vacuum box used for liquid-solid extractions**



### 3.2.2.1 Reversed-phase/ion-exchange combinations

Initially, paracetamol and its glucuronide and sulphate conjugates were extracted from solutions using reversed-phase ($C_{18}$) and various anion-exchange cartridges ($NH_2$, PSA and SAX). Solutions of paracetamol and its glucuronide and sulphate conjugates (10$\mu$g/ml) were prepared in 10mM sodium acetate buffer (pH6), and 1ml aliquots were extracted using Extraction Scheme 1, **Table 3.1.**

The eluents from sample application and the aqueous wash were collected, and the ultraviolet absorption was measured at 254nm to determine whether the analytes were present in these solutions. The organic washes and eluents were collected, dried to residue, reconstituted in 50$\mu$l methanol and analysed by HPTLC as described previously.

A number of molecules which might interfere in a screening method for drugs and metabolites are present as endogenous components of plasma or urine (Altman and Dittmer, 1974). Some components such as creatinine, hippuric acid, uric acid, urea and allantoin may be present in urine at concentrations greater than 10mg/ml and could therefore represent major interferences. The fate of these endogenous components during extraction on $C_{18}$ and PSA cartridges placed in tandem using Extraction Scheme 2 **(Table 3.1)** was investigated. A solution of each compound (1mg/ml) was made up in 10mM sodium acetate buffer (pH6), the solutions were extracted, and the concentrations of each compound in washes and eluents were measured by ultraviolet absorption at 220nm against control washes and eluents.

Control human plasma and control dog urine samples, and samples of the same matrices

**Table 3.1**

## Extraction Schemes 1 and 2

### Extraction Scheme 1

100mg Bond Elut™ Extraction Cartridges ($C_{18}$, $NH_2$, PSA or SAX)

| | |
|---|---|
| Wet | Methanol (2ml) |
| Wet | 10mM sodium acetate buffer, pH6 (2ml) |
| Apply | Sample in buffer (1ml) |
| Wash | 10mM buffer (2ml) |
| Wash | Hexane (1ml) |
| Elute | 3% ammonium acetate in methanol (2ml) |

### Extraction Scheme 2

100mg Bond Elut™ $C_{18}$ and PSA cartridges placed in tandem

| | |
|---|---|
| Wet Independently | Methanol (2ml) |
| Wet Independently | Water (2ml) |
| Wet Independently | 10mM sodium acetate buffer, pH6 (2ml) |

Place cartridges in tandem ($C_{18}$ on PSA)

| | |
|---|---|
| Apply | Sample (1ml + 5ml buffer) |
| Wash | Buffer (2ml) |
| Wash | Hexane (2ml) |
| Elute | Methanol (2ml) |
| Elute | 3% ammonium acetate in methanol (2ml) |

spiked with paracetamol and paracetamol conjugates (1, 10 and 100$\mu$g/ml) were extracted on $C_{18}$/PSA cartridges using Extraction Scheme 2. Similar samples were also extracted on $C_{18}$ cartridges using an ion-pair extraction based on the work of Vanluchene and Vandekerckhove (1988) (Extraction Scheme 3, **Table 3.2**). Eluents were analysed by HPTLC/MMD using MMD Scheme 3. Samples of hippuric acid and urea were also applied to the plate to examine whether they would be likely to interfere with the detection of drugs and metabolites.

### 3.2.2.2 Development of a solid-phase extraction scheme

It was envisaged that analysis of relatively large numbers of samples would be required in order to develop a robust general extraction procedure, therefore a HPLC system, specific for paracetamol and its conjugated metabolites, was used to analyse extracts prepared from plasma and urine. HPLC was chosen for this work because samples could be analysed automatically, whereas HPTLC/MMD required manual operation. An HPLC system to separate paracetamol, its glucuronide and sulphate was developed based on a method used by Wilson, Slattery, Forte and Nelson (1982). Chromatography was carried out at 40°C, using a 5$\mu$m Ultrasphere™ ODS column (150 x 4.6mm) and a mobile phase of 0.1M potassium dihydrogen phosphate containing methanol (4%) and glacial acetic acid (0.75%) at a flow rate of 1ml/min. The compounds were detected by ultraviolet absorbance at 248nm. Baseline resolution of the three components was achieved with retention times of 222, 342, and 549 seconds for the glucuronide, sulphate and paracetamol respectively.

Initially, the extracts which had been prepared and analysed previously by HPTLC (Sections 3.2.1 and 3.2.2.1) were re-assayed using HPLC.

**Table 3.2**

## Extraction Schemes 3 and 4

### Extraction Scheme 3

100mg Bond Elut™ $C_{18}$ Cartridges

| | |
|---|---|
| Wet | Methanol (5ml) |
| Wet | Water (10ml) |
| Wet | 0.15M potassium acetate buffer, pH5.0 (7.5ml) |
| Apply | Sample (1ml) + 0.5M triethylammonium sulphate (250µl) + 1.5M potassium acetate (100µl) |
| Wash | 0.15M potassium acetate buffer, pH5.0 (7.5ml) |
| Elute | Methanol (4ml) |

### Extraction Scheme 4

100mg Bond Elut™ $C_{18}$ and SAX cartridges

| | |
|---|---|
| Wet Independently | Methanol (2ml) |
| Wet Independently | Water (2ml) |
| Wet Independently | 10mM sodium acetate buffer, pH6 (2ml) |

Place cartridges in tandem ($C_{18}$ on SAX)

| | |
|---|---|
| Apply | Sample (1ml + 5ml buffer) |
| Wash | Buffer (2ml) |
| Wash | Hexane (2ml) |
| Elute | Methanol (2ml) |
| Elute | 3% ammonium acetate in methanol (2ml) |

Samples of plasma and urine spiked with paracetamol and conjugates at 10 and 100$\mu$g/ml were extracted using tandem $C_{18}$/PSA and $C_{18}$/SAX cartridges (Extraction Schemes 2 and 4). The extracts were analysed by HPLC.

The effect of sample dilution on the extraction efficiency was investigated. Plasma and urine samples spiked with paracetamol and conjugates at 10$\mu$g/ml, and control samples, undiluted and diluted 1:1 or 1:5 with buffer, were extracted using Extraction Scheme 2. The extracts were analysed by HPLC.

One reason for the difficulty experienced with extracting glucuronides from biological matrices by ion-exchange methods may be the high concentrations of competitive strong anions in plasma and urine such as citrates, succinates, fumarates, and maleates. These strong anions can be precipitated from solution by the formation of insoluble lead salts when lead acetate is added, and thus be removed from solution. A solution of lead acetate (284mg/ml) was prepared based on estimates of the anion concentrations likely in plasma and urine respectively, and was added to plasma and urine in 10$\mu$l aliquots. The samples were mixed and centrifuged between addition of aliquots, the supernatant was removed and the process was repeated until no further precipitate was formed. Volumes required for complete precipitation of the anions were 70$\mu$l and 40$\mu$l for plasma and urine respectively.

Plasma and urine samples spiked with paracetamol and metabolites at 10 and 100$\mu$g/ml were treated with lead acetate and extracted using Extraction Scheme 2. The extracts were assayed by HPLC. The urine extracts were re-analysed separately with 30 minute run times because of late eluting peaks.

Plasma and urine samples spiked with paracetamol and metabolites at 10 and 100$\mu$g/ml were extracted, with and without precipitation of the anions, on $C_{18}$ cartridges using ion-suppression based upon the work of Wilson and Nicholson (1987) (Extraction Scheme 5, **Table 3.3**). The extracts were analysed by HPLC.

Bond Elut Certify™ II, a combined non-polar and anion-exchange phase, is a new mixed mode sorbent material recently introduced by Analytichem International (Harbor City, US), and has been used for the isolation of 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid from human urine (Dixit and Dixit, 1990). A series of extractions using ion-exchange and ion-suppression strategies were carried out on Bond Elut Certify™ II cartridges using plasma and urine spiked with paracetamol and metabolites at 10$\mu$g/ml (Extraction Schemes 6-9, **Tables 3.3-3.5**). The extracts were analysed by HPLC.

### 3.2.2.3 Analysis of extracts by HPTLC/MMD

Bond Elut Certify™ II extracts of plasma and urine were analysed using HPTLC/MMD. Spots (5$\mu$l, 5% of the extract) were applied to the plate and development was carried out using MMD Scheme 3. Further analyses were carried out using MMD Scheme 9 (1$\mu$l, 1% of the extract applied) and MMD Scheme 7 (0.5$\mu$l, 0.5% of the extract applied).

Plasma spiked with paracetamol and metabolites at 10$\mu$g/ml was extracted and eluted with; (i) methanol (2ml), (ii) methanol containing 0.5% formic acid (2ml) and (iii) 5% ammonium acetate in methanol (2ml) (Extraction Scheme 10, **Table 3.5**). The extracts were analysed by HPTLC/MMD using MMD Scheme 3 and were compared with extracts from control plasma prepared using the same conditions.

**Table 3.3**

## Extraction Schemes 5 and 6

### Extraction Scheme 5

100mg Bond Elut™ $C_{18}$ Cartridge

| | |
|---|---|
| Wet | Methanol (2ml) |
| Wet | Water (2ml) |
| Apply | Sample + 10$\mu$l formic acid |
| Wash | Water (1ml) |
| Elute | Methanol (2ml) |

### Extraction Scheme 6

130mg Bond Elut Certify™ II Cartridge

| | |
|---|---|
| Wet | Methanol (4ml) |
| Wet | Water (4ml) |
| Wet | 10mM sodium acetate buffer, pH 6 (2ml) |
| Apply | Sample (1ml) |
| Wash | 10mM sodium acetate buffer, pH 6 (2ml) |
| Elute | Methanol (2ml) |
| Elute | 5% ammonium acetate in methanol (2ml) |

**Table 3.4**

## Extraction Schemes 7 and 8

### Extraction Scheme 7

130mg Bond Elut Certify™ II Cartridge

| | |
|---|---|
| Wet | Methanol (4ml) |
| Wet | Water (4ml) |
| Wet | 10mM sodium acetate buffer, pH 6 (2ml) |
| Apply | Sample (1ml) previously treated with lead acetate |
| Wash | 10mM sodium acetate buffer, pH 6 (2ml) |
| Elute | Methanol (2ml) |
| Elute | 5% ammonium acetate in methanol (2ml) |

### Extraction Scheme 8

130mg Bond Elut Certify™ II Cartridge

| | |
|---|---|
| Wet | Methanol (4ml) |
| Wet | Water (4ml) |
| Apply | Sample (1ml) + 10μl formic acid |
| Wash | Water (2ml) |
| Elute | Methanol (2ml) |
| Elute | 5% ammonium acetate in methanol (2ml) |

**Table 3.5**

## Extraction Schemes 9 and 10

### Extraction Scheme 9

130mg Bond Elut Certify™ II Cartridge

| | |
|---|---|
| Wet | Methanol (4ml) |
| Wet | Water (4ml) |
| Apply | Sample (1ml) + 10μl formic acid |
| Wash | 0.25M hydrochloric acid (2ml) |
| Elute | Methanol (2ml) |
| Elute | 5% ammonium acetate in methanol (2ml) |

### Extraction Scheme 10

130mg Bond Elut Certify™ II Cartridge

| | |
|---|---|
| Wet | Methanol (4ml) |
| Wet | Water (4ml) |
| Apply | Sample (1ml) + formic acid (10μl for urine, 20μl for plasma) |
| Wash | Water (2ml) |
| Elute | Methanol (2ml) |
| Elute | 0.5% formic acid in methanol (2ml) |
| Elute | 5% ammonium acetate in methanol (2ml) |

Four control human plasma samples, a control dog plasma sample and a control rat plasma sample were extracted using Extraction Scheme 8 to check for interference from endogenous material. Samples of control urine were also extracted, with and without precipitation of anions with lead acetate. The extracts were analysed by HPTLC/MMD using MMD Scheme 3.

Control urine was extracted using Extraction Scheme 10 and the methanol/formic acid and methanol/ammonium acetate extracts were analysed by HPTLC/MMD using MMD Scheme 9. The plate was scanned at different wavelengths to investigate the extent of endogenous interference.

Model compounds, selected from those used in previous studies to investigate multiple development (Chapter 2), were spiked into control plasma (1 or 5 $\mu$g/ml) and extracted on Bond Elut Certify™ II cartridges using Extraction Scheme 10. (Extracts were not carried out using compounds which were either volatile and would be lost during drying to residue, or were labile.) Control plasma and urine samples were also extracted. The extracts were dried to residue and reconstituted in methanol (200 $\mu$l). Spots (1 $\mu$l) were applied to HPTLC plates for analysis, and development was carried out using MMD Scheme 3.

## *3.3 Results*

During the present studies to investigate extraction strategies, and to develop a procedure for a general screening method, single experiments were carried out. Use of single experiments was considered appropriate for this work because each experiment was

designed to provide guidance for the next without definitive data being required. When a suitable system was developed, this would be tested for applicability and reproducibility.

### 3.3.1 Liquid-Liquid Extractions

The recoveries of paracetamol and its glucuronide and sulphate conjugates obtained from plasma and urine by liquid-liquid extractions, and analysed by HPTLC/MMD from acetonitrile and methanol solutions, are shown in **Table 3.6.** Recovery of paracetamol from plasma into propan-2-ol ranged from 45% at 1μg/ml to 29% at 10μg/ml and 7% at 100μg/ml on the acetonitrile plate. Some of the remainder was extracted into the ethyl acetate; 13% at 10μg/ml and 5% at 100μg/ml. Similar results were obtained on the methanol plate. No glucuronide was detected on the acetonitrile plate, but a small amount was detected on the methanol plate from the acid extract of the 100μg/ml plasma sample. No glucuronide was detected in any other extracts. The sulphate metabolite was extracted from plasma and detected on the acetonitrile plate, but was not detected on the methanol plate. Recovery of paracetamol sulphate ranged from 31% at 1μg/ml to 18% at 10μg/ml and 6% at 100μg/ml. Some of the remainder was extracted into the ethyl acetate; 5% at 10μg/ml and 1% at 100μg/ml. Most of the sulphate conjugate was extracted into propan-2-ol even though it would be ionised at the pH of the extraction. It is therefore possible that the sulphate was extracted by an ion-pair mechanism. Both paracetamol and paracetamol sulphate were present in both the propan-2-ol and ethyl acetate extracts, so the extraction provided no discrimination between compound classes. Recovery of all components from urine was poor.

**Table 3.6**

**Recoveries of paracetamol and conjugates from plasma and urine by liquid-liquid extraction**

**Extracts reconstituted in acetonitrile**

| | Paracetamol | Paracetamol Sulphate | Paracetamol Glucuronide |
|---|---|---|---|
| Plasma (1µg/ml, IPA) | 45% | 31% | 0 |
| Plasma (10µg/ml, IPA) | 29% | 18% | 0 |
| Plasma (100µg/ml, IPA) | 7% | 6% | 0 |
| Plasma (1µg/ml, EtAc) | 0 | 0 | 0 |
| Plasma (10µg/ml, EtAc) | 13% | 5% | 0 |
| Plasma (100µg/ml, EtAc) | 5% | 1% | 0 |
| | | | |
| Urine (1µg/ml, IPA) | 0 | 0 | 0 |
| Urine (10µg/ml, IPA) | 9% | 0 | 0 |
| Urine (100µg/ml, IPA) | 5% | 0 | 0 |
| Urine (1µg/ml, EtAc) | 0 | 0 | 0 |
| Urine (10µg/ml, EtAc) | 13% | 0 | 0 |
| Urine (100µg/ml, EtAc) | 4% | 0 | 0 |

**Extracts reconstituted in methanol**

| | Paracetamol | Paracetamol Sulphate | Paracetamol Glucuronide |
|---|---|---|---|
| Plasma (1µg/ml, IPA) | 40% | 0 | 0 |
| Plasma (10µg/ml, IPA) | 31% | 0 | 0 |
| Plasma (100µg/ml, IPA) | 7% | 0 | 0 |
| Plasma (1µg/ml, EtAc) | 0 | 0 | 0 |
| Plasma (10µg/ml, EtAc) | 13% | 4% | 0 |
| Plasma (100µg/ml, EtAc) | 6% | 2% | 3% |
| | | | |
| Urine (1µg/ml, IPA) | 0 | 0 | 0 |
| Urine (10µg/ml, IPA) | 6% | 0 | 0 |
| Urine (100µg/ml, IPA) | 3% | 0 | 0 |
| Urine (1µg/ml, EtAc) | 0 | 0 | 0 |
| Urine (10µg/ml, EtAc) | 10% | 0 | 0 |
| Urine (100µg/ml, EtAc) | 1% | 0 | 0 |

Chromatograms from liquid-liquid extracts of control plasma and urine samples following HPTLC/MMD after reconstitution in methanol or acetonitrile are shown in **Figure 3.8.** These show that use of acetonitrile instead of methanol to dissolve the residues and apply the samples to the HPTLC plate resulted in cleaner chromatograms. Thus detection of the sulphate was improved using acetonitrile, however in one extract on the methanol plate there was evidence of the glucuronide being present which did not appear on the acetonitrile plate. It was not clear therefore whether acetonitrile would dissolve all components of interest, particularly glucuronide conjugates.

### 3.3.2 Liquid-Solid Extractions

The data obtained from liquid-solid extractions are presented in the following sections.

#### 3.3.2.1 Reversed-phase/ion-exchange combinations

Recoveries of paracetamol and its glucuronide and sulphate conjugates obtained from buffer solutions on reversed-phase and anion-exchange Bond Elut™ cartridges using Extraction Scheme 1 are presented in **Table 3.7.** Paracetamol and its sulphate conjugate were retained on the $C_{18}$ cartridge, but the glucuronide was not. The $NH_2$ cartridge did not retain any drug-related material, however the other anion-exchange cartridges retained the conjugates but not paracetamol. Good recovery of all material retained on the cartridges, with the exception of paracetamol sulphate on the SAX cartridge, was obtained with 3% ammonium acetate in methanol. The data indicate that a combination of $C_{18}$ and PSA cartridges would be suitable to provide an extraction of these compounds with discrimination between compound classes, this being possible if a methanol elution was added to remove paracetamol before eluting the conjugates.

**Figure 3.8**

**Chromatograms from liquid-liquid extracts of control plasma and urine samples following HPTLC/MMD after reconstitution in methanol or acetonitrile**

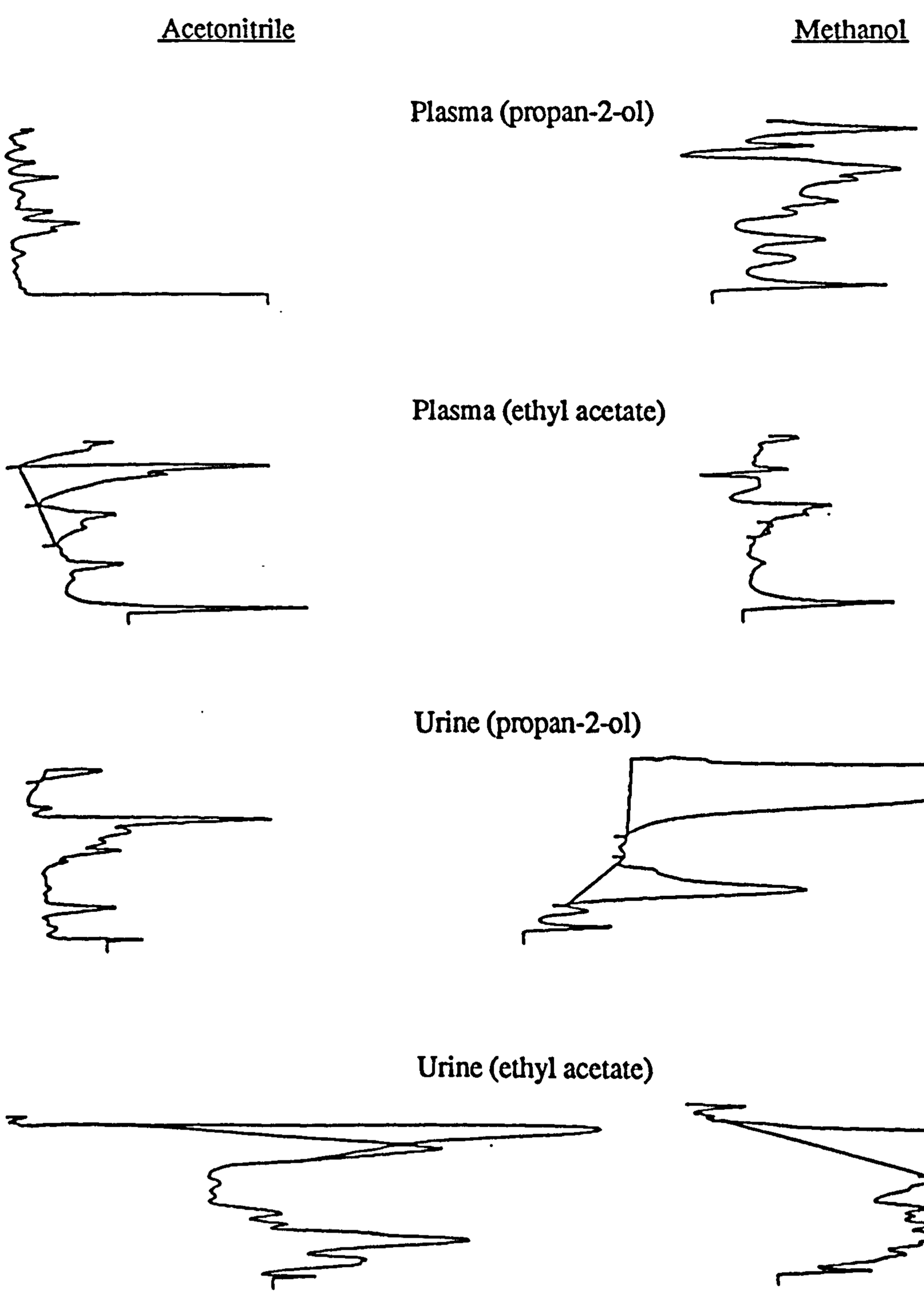


**Table 3.7**

**Recoveries of paracetamol and conjugates (10$\mu$g/ml) from buffer solutions (pH6) on reversed-phase and anion-exchange Bond Elut™ cartridges using Extraction Scheme 1**

| | $C_{18}$ | $NH_2$ | PSA | SAX |
|---|---|---|---|---|
| Paracetamol (apply)† | 7 | 53 | 57 | 42 |
| Conjugates (apply)† | 24 | 34 | 0 | 0 |
| Paracetamol (aq wash)† | 0 | 2 | 0 | 0 |
| Conjugates (aq wash)† | 0 | 0 | 0 | 0 |
| Paracetamol (hex wash)‡ | 0 | 0 | 0 | 0 |
| Conjugates (hex wash)‡ | 0 | 0 | 0 | 0 |
| Paracetamol (elute)‡ | 56% | 0 | 5% | 0 |
| Glucuronide (elute)‡ | 0 | 0 | 89% | 72% |
| Sulphate (elute)‡ | 48% | 0 | 105% | 0 |

† Measurement by UV absorbance (arbitrary units)

‡ Measurement by HPTLC analysis

Elution was carried out with 3% ammonium acetate in methanol

Ultraviolet absorbance measurements of eluents obtained following extraction of endogenous plasma and urine components from buffer solutions using Extraction Scheme 2 are shown in **Table 3.8.** Creatinine, uric acid and allantoin were not retained on the cartridges because they were present in the sample breakthrough and washes. Small amounts of uric acid also appeared in the eluent. Urea could not be traced by use of ultraviolet absorption measurements because of its very poor ultraviolet absorbance. However, for the same reason, it would not interfere with detection on HPTLC/MMD, although its presence on the plate might affect the chromatography of other compounds. Hippuric acid was not detected in the sample breakthrough or washings, but about 20% of the amount applied was detected in the methanol/ammonium acetate eluent. Thus the endogenous compound most likely to be extracted from plasma and urine by this procedure is hippuric acid.

Extracts from plasma and urine samples prepared using Extraction Schemes 2 and 3 were analysed by HPTLC/MMD, and although drug and conjugates were detected in the standards, no evidence of drug-related material was observed in the samples. Chromatograms from the control plasma and urine samples extracted using Extraction Schemes 2 and 3 are shown in **Figure 3.9.** There was very little interference on the chromatograms of the control plasma extracts from both extraction schemes. The chromatograms from the urine extracts had interfering material in the conjugate region of the chromatograms particularly, but there was more general interference from the Scheme 3 extracts.

Hippuric acid was detected by HPTLC/MMD using MMD Scheme 3 (ED=13.6mm), and would therefore interfere with conjugated metabolites. Urea was not detected, which was expected because of its poor ultraviolet absorbance.

**Table 3.8**

**Ultraviolet absorbance measurements of eluents from liquid-solid extraction of endogenous components from sodium acetate buffer (pH6)**

**(Extraction Scheme 2)**

| | Creatinine | Hippuric Acid | Urea | Uric Acid | Allantoin |
|---|---|---|---|---|---|
| Std (0.1mg/ml) | 0.406[a] | 0.515[a] | 0.058[b] | 0.492 | 0.632 |
| Sample + washes | 0.403[c] | 0.026 | 0.012 | 0.531 | 0.628 |
| Eluents | 0.001 | 0.101 | -0.008 | 0.020 | -0.005 |

[a] 0.01mg/ml

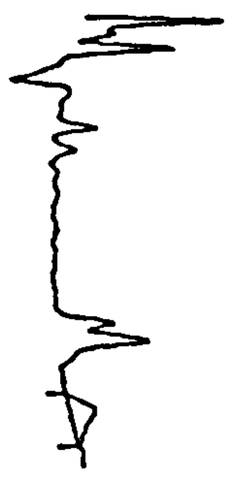
[b] 1mg/ml

[c] 10-fold dilution

**Figure 3.9**

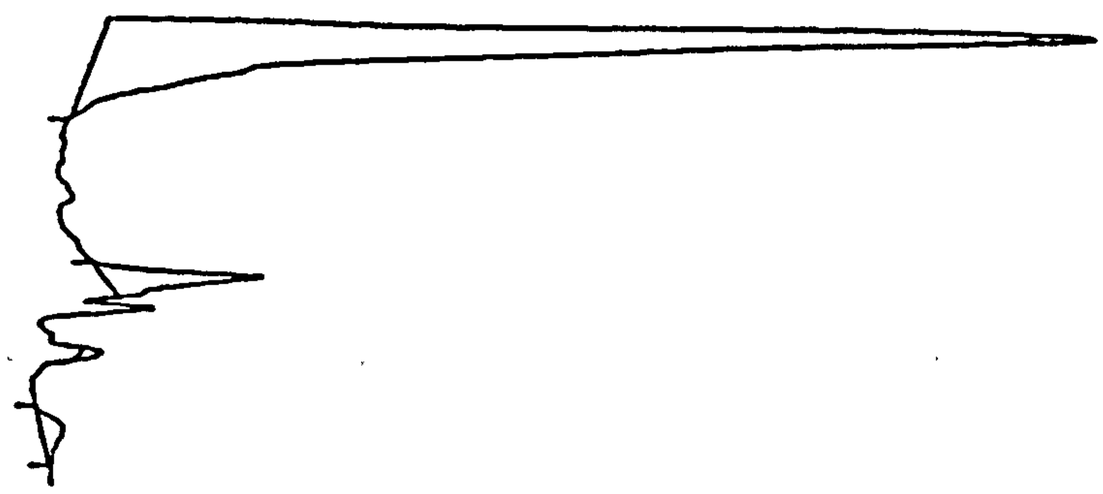
**HPTLC/MMD chromatograms from liquid-solid extractions of control plasma and urine using Extraction Schemes 2 and 3**

Control plasma (Extraction Scheme 2)

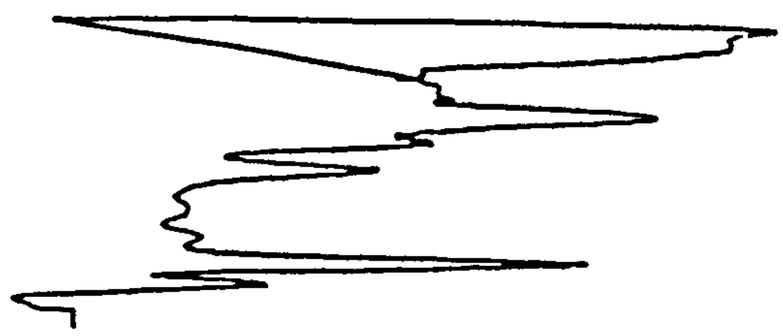


Control plasma (Extraction Scheme 3)

Control urine (Extraction Scheme 2)



Control Urine (Extraction Scheme 3)



### 3.3.2.2 Development of a solid-phase extraction scheme

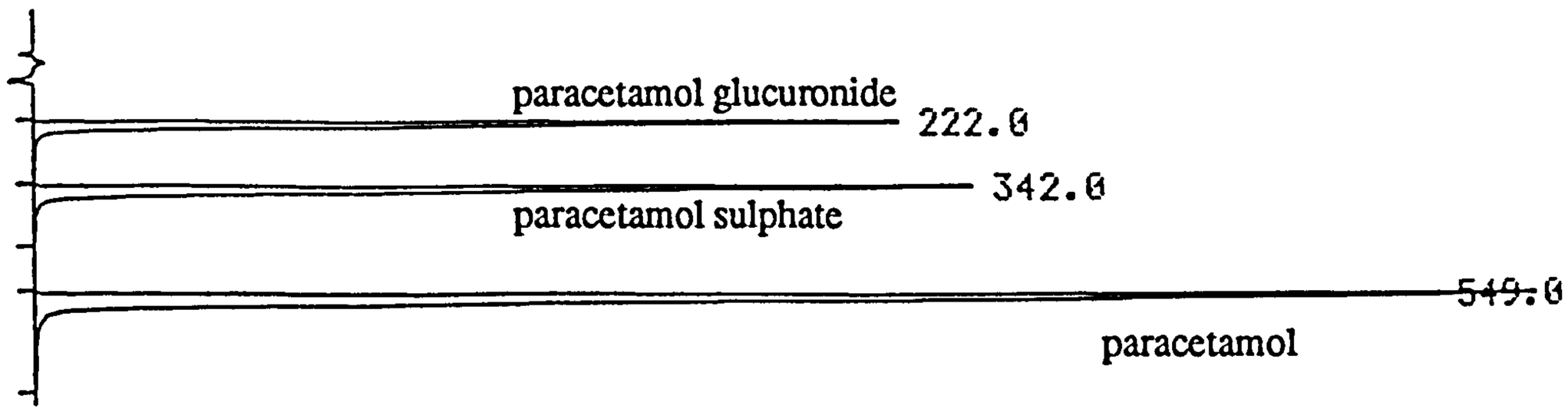
A typical chromatogram recorded after HPLC analysis of paracetamol, paracetamol sulphate and paracetamol glucuronide standards is shown in **Figure 3.10.** Good resolution of the three components was obtained. The extracts previously analysed by HPTLC/MMD were re-analysed by HPLC and no drug-related material was detected. These results confirmed those obtained by HPTLC.

The recoveries of paracetamol and its glucuronide and sulphate conjugates from plasma and urine following extraction on tandem $C_{18}$/PSA and $C_{18}$/SAX cartridges using Extraction Schemes 2 and 4 are shown in **Table 3.9.** The recovery of paracetamol from plasma ranged from about 39% at 10$\mu$g/ml to about 28% at 100$\mu$g/ml, and was independent of the matrix and the anion-exchange strength of the extraction cartridges. The recovery of paracetamol sulphate was variable, ranging between 39% at 10$\mu$g/ml and 9% at 100$\mu$g/ml from plasma on PSA cartridges. Similar recoveries were obtained on SAX cartridges, therefore it was not clear whether either anion-exchange phase was preferable. The glucuronide was extracted poorly from plasma, recoveries ranging between 9% and 12%, and was not detected in the urine extracts.

The data obtained from extractions on tandem $C_{18}$/PSA cartridges to examine the effect of sample dilution on recoveries of paracetamol and its conjugated metabolites are shown in **Table 3.10.** Recoveries of paracetamol from plasma ranged between 71% when undiluted to 26% when diluted 1:5 with buffer. Similar results were obtained from urine. Recoveries of the sulphate also decreased with dilution of the sample. Paracetamol glucuronide was not detected in any extracts. A reason for the failure of paracetamol

**Figure 3.10**

**HPLC chromatogram of paracetamol and its glucuronide and sulphate metabolites**




HPLC Conditions

5µm Ultrasphere™ ODS Column (150 x 4.6mm)
Mobile phase : 0.1M potassium dihydrogen phosphate containing methanol (4%)
and glacial acetic acid (0.75%)
Flow rate : 1ml/min
Temperature 40°C

**Table 3.9**

**Recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on tandem $C_{18}$/PSA and $C_{18}$/SAX cartridges**

**(Extraction Schemes 2 and 4)**

| Sample | Paracetamol | Sulphate | Glucuronide |
|---|---|---|---|
| Plasma 10μg/ml (Scheme 2, PSA) | 38% | 39% | 12% |
| Plasma 100μg/ml (Scheme 2, PSA) | 28% | 9% | 9% |
| Plasma 10μg/ml (Scheme 4, SAX) | 40% | 16% | 12% |
| Plasma 100μg/ml (Scheme 4, SAX) | 28% | 20% | 11% |
| Urine 10μg/ml (Scheme 2, PSA) | 38% | 0 | 0 |
| Urine 100μg/ml (Scheme 2, PSA) | 30% | 8% | 0 |
| Urine 10μg/ml (Scheme 4, SAX) | 39% | 0 | 0 |
| Urine 100μg/ml (Scheme 4, SAX) | 27% | 36% | 0 |

**Table 3.10**

**Effect of sample dilution on recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on tandem $C_{18}$/PSA and $C_{18}$/SAX cartridges**

**(Extraction Scheme 2. Samples spiked at 10μg/ml)**

| Sample | Paracetamol | Sulphate | Glucuronide |
|---|---|---|---|
| Plasma (undiluted) | 71% | 22% | 0 |
| Plasma (diluted 1:1) | 50% | 13% | 0 |
| Plasma (diluted 1:5) | 26% | 12% | 0 |
| Urine (undiluted) | 57% | 15% | 0 |
| Urine (diluted 1:1) | 44% | 15% | 0 |
| Urine (diluted 1:5) | 22% | 10% | 0 |

Diluent was 10mM sodium acetate buffer, pH6.

glucuronide to extract, might have been the high concentrations of competitive strong anions present in plasma and urine. The anions present would include citrates, succinates, fumarates and maleates, and removal of these prior to extraction may be advantageous.

The recoveries of paracetamol and its conjugated metabolites obtained from extractions on tandem $C_{18}$/PSA cartridges following precipitation of the strong anions with lead acetate are presented in **Table 3.11.** Recoveries of paracetamol were similar to those obtained with no anion precipitation (**Table 3.10**). The recoveries of the glucuronide and sulphate were improved considerably by use of the precipitation step prior to extraction of the samples, and there was less difference in efficiencies between plasma and urine than had been observed previously. These data confirmed that extraction efficiencies were greater when plasma and urine were applied to the cartridges undiluted as was shown previously.

The recoveries of paracetamol and its glucuronide and sulphate conjugates from plasma and urine following extraction with ion-suppression on $C_{18}$ cartridges using Extraction Scheme 5 are shown in **Table 3.12.** Recoveries of paracetamol ranged from 63% to 87% at 10$\mu$g/ml and from 45% to 56% at 100$\mu$g/ml, and were not affected by anion precipitation. Very little conjugated material was retained on the $C_{18}$ cartridges, with or without anion precipitation. It may be that the wash should have been carried out with acidified water to maintain the ion-suppression. The results from these and previous extractions have indicated that the 100mg Bond Elut™ cartridges did not have sufficient capacity for this application because recoveries from the samples with high concentrations were often less than those from the samples with low concentrations.

**Table 3.11**

**Effect of anion precipitation on recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on tandem $C_{18}$/PSA cartridges**

**(Extraction Scheme 2)**

| Sample | Paracetamol | Sulphate | Glucuronide |
|---|---|---|---|
| Plasma 10μg/ml (undiluted) | 73% | 73% | 56% |
| Plasma 100μg/ml (undiluted) | 54% | 45% | 31% |
| Plasma 10μg/ml (diluted 1:5) | 28% | 27% | 15% |
| Plasma 100μg/ml (diluted 1:5) | 21% | 26% | 16% |
| | | | |
| Urine 10μg/ml (undiluted) | 85% | 80% | 102% |
| Urine 100μg/ml (undiluted) | 40% | 45% | 18% |
| Urine 10μg/ml (diluted 1:5) | 42% | 33% | 47% |
| Urine 100μg/ml (diluted 1:5) | 21% | 24% | 11% |

Diluent was 10mM sodium acetate buffer, pH6.

**Table 3.12**

**Recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on tandem $C_{18}$/PSA cartridges**

**(Extraction Scheme 5 with and without anion precipitation)**

| Sample | Paracetamol | Sulphate | Glucuronide |
|---|---|---|---|
| Plasma 10μg/ml | 87% | 0 | 0 |
| Plasma 100μg/ml | 56% | 5% | 0 |
| Plasma 10μg/ml (Ppt) | 86% | 0 | 0 |
| Plasma 100μg/ml (Ppt) | 54% | 4% | 0 |
| | | | |
| Urine 10μg/ml | 75% | 0 | 0 |
| Urine 100μg/ml | 51% | 4% | 0 |
| Urine 10μg/ml (Ppt) | 63% | 0 | 0 |
| Urine 100μg/ml (Ppt) | 45% | 0 | 0 |

The recoveries of paracetamol and its glucuronide and sulphate conjugates from plasma and urine following extractions on Bond Elut Certify™ II cartridges are presented in **Table 3.13.** The ion-exchange mode gave some recovery of all components from plasma when the anion precipitation step had been carried out (Extraction Scheme 7), but no extraction of the glucuronide, and limited recovery of the sulphate occurred without prior anion precipitation (Extraction Scheme 6). The extraction efficiencies were similar from urine with the exception that glucuronide was not detected with or without anion precipitation.

The ion-suppression mode gave good recovery of all components from both plasma and urine when the water wash was used (Extraction Scheme 8). The acid wash used in Extraction Scheme 9 reduced the recovery of the conjugated metabolites. Recoveries of paracetamol sulphate were greater from urine using ion-suppression, which may have been a function of the pH at which the extraction was carried out. Plasma may require addition of more formic acid to reach the same pH obtained by adding 10$\mu$l formic acid to urine.

### 3.3.2.3 Analysis of extracts by HPTLC/AMD

The recoveries of paracetamol and its conjugated metabolites from plasma and urine obtained by HPTLC/MMD analysis of Bond Elut Certify™ II extracts using ion-exchange (Extraction Scheme 7) and ion-suppression (Extraction Scheme 8) are shown in **Table 3.14.** During the early MMD development steps using MMD Scheme 3, the extract material applied to the plate did not wet, leading to the formation of irregular solvent fronts. This was particularly severe with the methanol/ammonium acetate

**Table 3.13**

**Recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on Bond Elut Certify™ II cartridges using Extraction Schemes 6 to 9**

(Samples spiked with paracetamol and conjugates at 10$\mu$g/ml)

**Analysis by HPLC**

**Plasma**

| Extraction Scheme | Paracetamol | | Sulphate | | Glucuronide | |
|---|---|---|---|---|---|---|
| | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ |
| 6 | 50% | 0 | 4% | 14% | 0 | 0 |
| 7 | 52% | 0 | 0 | 45% | 2% | 19% |
| 8 | 53% | 0 | 0 | 72% | 0 | 52% |
| 9 | 49% | 0 | 5% | 19% | 12% | 0 |

**Urine**

| Extraction Scheme | Paracetamol | | Sulphate | | Glucuronide | |
|---|---|---|---|---|---|---|
| | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ |
| 6 | 61% | 0 | 5% | 0 | 0 | 0 |
| 7 | 56% | 0 | 6% | 75% | 0 | 0 |
| 8 | 56% | 0 | 0 | 95% | 0 | 53% |
| 9 | 33% | 0 | 0 | 16% | 3% | 0 |

**Table 3.14**

**Recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on Bond Elut Certify™ II cartridges using Extraction Schemes 6 to 9**

(Samples spiked with paracetamol and conjugates at 10$\mu$g/ml)

**Analysis by HPTLC/MMD**

**Plasma**

| Extraction Scheme | Paracetamol | | Sulphate | | Glucuronide | |
|---|---|---|---|---|---|---|
| | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ |
| 7 | 92% | 0 | 0 | 0 | 0 | 0 |
| 8 | 94% | 0 | 0 | 80% | 0 | 58% |

**Urine**

| Extraction Scheme | Paracetamol | | Sulphate | | Glucuronide | |
|---|---|---|---|---|---|---|
| | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ |
| 7 | 105% | 0 | 0 | NQ | 0 | NQ |
| 8 | NQ | 0 | 0 | 0 | 0 | 0 |

NQ Evidence of material present, but not quantifiable because of interference.

extracts, and may have been due to local overloading of the silica layer. Nevertheless, paracetamol was detected at the concentrations expected from all extracts except the urine extract from the ion-suppression extraction (Extraction Scheme 8). The conjugates were detected in the ion-suppression extracts from plasma, but not in the ion-exchange extracts. No conjugates were detected in the urine extracts. The plasma control extracts contained little interfering material, but the urine extracts contained more endogenous material, especially the extracts obtained using the ion-suppression mode.

The recoveries of paracetamol and its conjugated metabolites obtained from the same samples following HPTLC/MMD analysis using MMD Schemes 9 and 7 are shown in **Table 3.15.** There was little disturbance of the solvent front when 1% of the extract was applied, and no disturbance when 0.5% was applied. MMD Scheme 9 allowed paracetamol and paracetamol sulphate to be detected in all the extracts, and MMD Scheme 7 enabled paracetamol glucuronide to be detected in extracts obtained using the ion-suppression mode. These data demonstrated that further HPTLC/MMD analyses using shallow gradients may be required to detect drugs and metabolites in urine.

Chromatograms from different eluents obtained following extraction of plasma spiked with paracetamol and its conjugated metabolites on Bond Elut Certify™ II cartridges showed that paracetamol was eluted in the methanol eluent, paracetamol glucuronide was eluted in the formic acid/methanol eluent, and paracetamol sulphate was eluted in the ammonium acetate/methanol eluent.

Examples of HPTLC/MMD chromatograms obtained from extracts of control human, dog and rat plasma on Bond Elut Certify™ II cartridges are shown in **Figure 3.11.**

**Table 3.15**

**Recoveries of paracetamol and conjugates from plasma and urine by liquid-solid extraction on Bond Elut Certify™ II cartridges**

(Samples spiked with paracetamol and conjugates at 10$\mu$g/ml)

**Analysis by HPTLC/MMD**

**MMD Scheme 9**

| Extraction Scheme | Paracetamol | | Sulphate | | Glucuronide | |
|---|---|---|---|---|---|---|
| | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ |
| Plasma (7) | 74% | 0 | 0 | 63% | 0 | 0 |
| Plasma (8) | 90% | 0 | 0 | 109% | 0 | 67% |
| Urine (7) | 47% | 0 | 0 | 61% | 0 | 0 |
| Urine (8) | NQ | 0 | 0 | 62% | 0 | 0 |

**MMD Scheme 7**

| Extraction Scheme | Paracetamol | | Sulphate | | Glucuronide | |
|---|---|---|---|---|---|---|
| | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ | MeOH | MeOH/$NH_4Ac$ |
| Plasma (7) | SF | SF | 0 | 61% | 0 | 0 |
| Plasma (8) | SF | SF | 0 | 111% | 0 | 65% |
| Urine (7) | SF | SF | 0 | NQ | 0 | 0 |
| Urine (8) | SF | SF | 0 | 0 | 0 | 65% |

NQ Evidence of material present, but not quantifiable because of interference.

SF Material on solvent front.

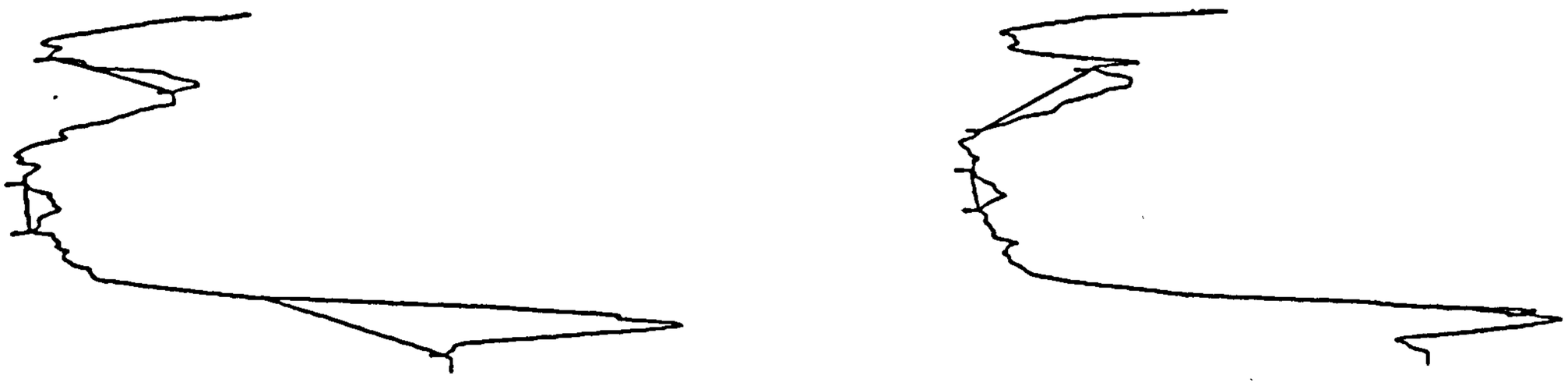
**Figure 3.11**

**HPTLC/MMD chromatograms from Bond Elut Certify™ II extracts of control plasma**

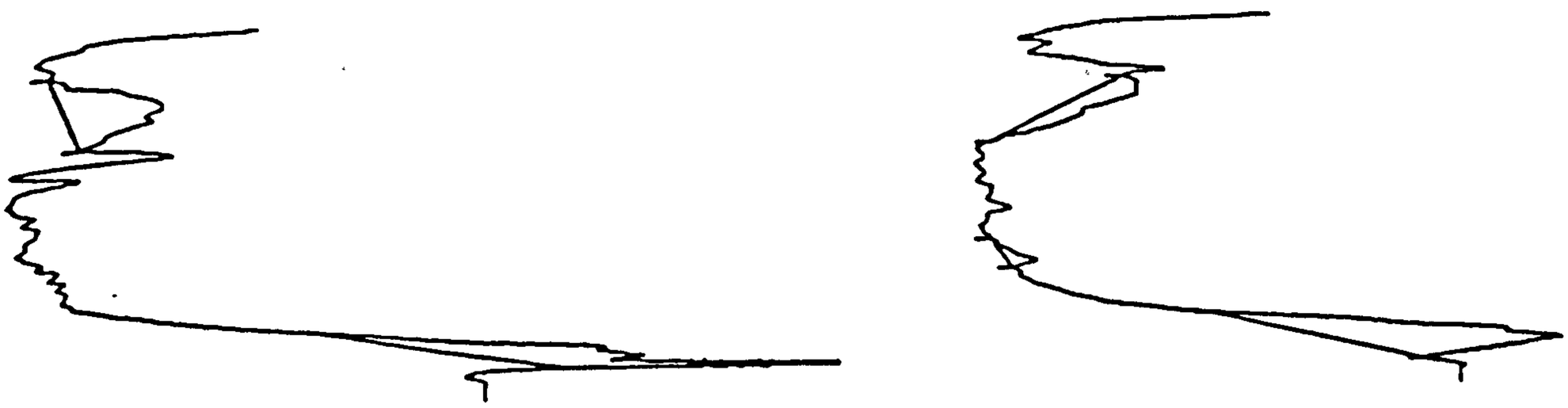
Methanol eluent

Methanol/ammonium acetate eluent

Human plasma 1



Human plasma 2



Rat plasma

Dog plasma

The extracts of control plasma samples gave similar chromatograms with no species having endogenous components which were not present in the others except for one component in the dog plasma methanol/am acetate eluent. Chromatograms from control urine are shown in **Figure 3.12**, and show that urine contained greater amounts of interfering material than plasma, however no advantage was obtained by precipitating the anions with lead acetate prior to extracting the samples.

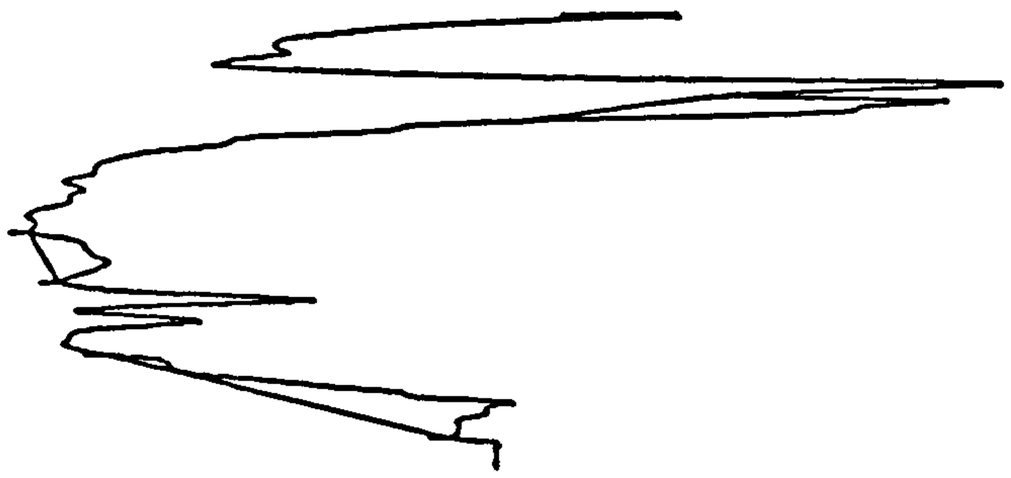
Chromatograms obtained after analysis of methanol/formic acid and methanol/ammonium acetate eluents from Bond Elut Certify™ II extracts of urine by HPTLC/MMD using MMD Scheme 9 are shown in **Figures 3.13 and 3.14.** The majority of the interfering material was eluted in the methanol/formic acid and would therefore compromise the detection of glucuronide metabolites if they also eluted in the same eluent. Use of higher wavelengths, especially 280nm or above, reduced the amounts of interfering material detected, but whether these wavelengths could be used would depend upon the nature of the compounds being examined.

The recoveries of model compounds from plasma obtained using Extraction Scheme 10 on Bond Elut Certify™ II cartridges are shown in **Table 3.16**, and chromatograms are presented in **Figures 3.15-3.19.** All compounds tested were extracted from plasma with recoveries of 45% or greater (except phenolphthalein disulphate which was not detected), and most compounds had recoveries in excess of 90%. Weakly acidic compounds containing carboxylic acid groups such as may be present on acidic drugs or Phase I metabolites, and glucuronide conjugates were eluted in methanol/formic acid with the exception of indole-3-carboxylic acid, and sulphates were eluted in methanol/ammonium acetate. This provided discrimination between classes of compound as required by the criteria for a general extraction procedure.

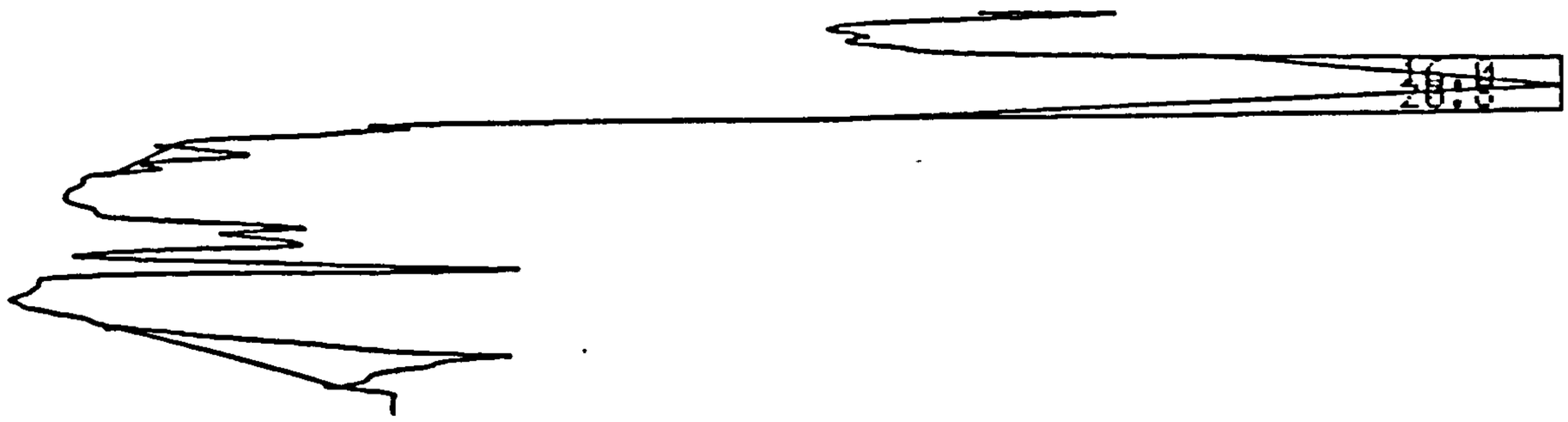
**Figure 3.12**

**HPTLC/MMD chromatograms from Bond Elut Certify™ II extracts of control urine**

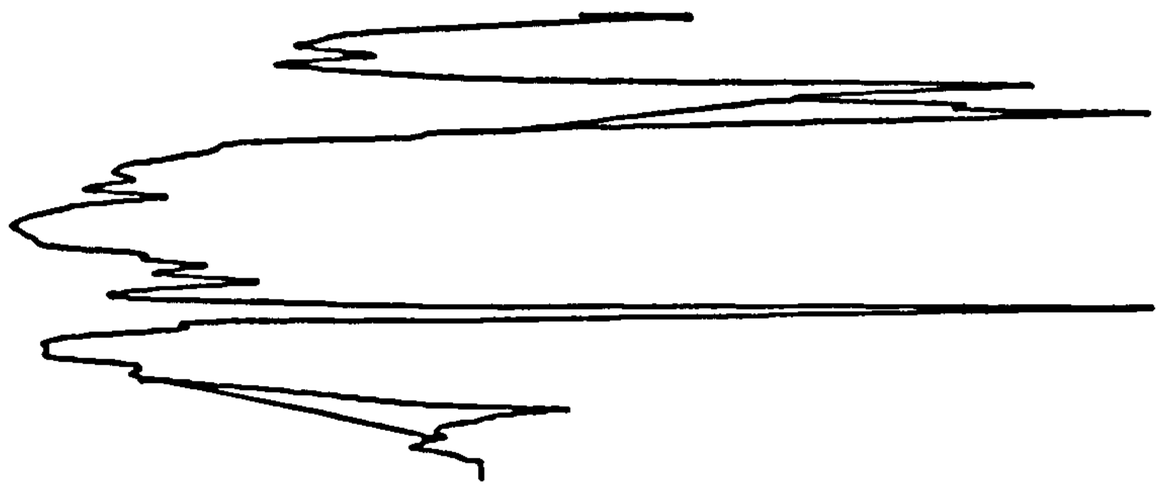
Methanol eluent (without anion precipitation)



Methanol/ammonium acetate eluent (without anion precipitation)



Methanol eluent (with anion precipitation)



Methanol/ammonium acetate eluent (with anion precipitation)

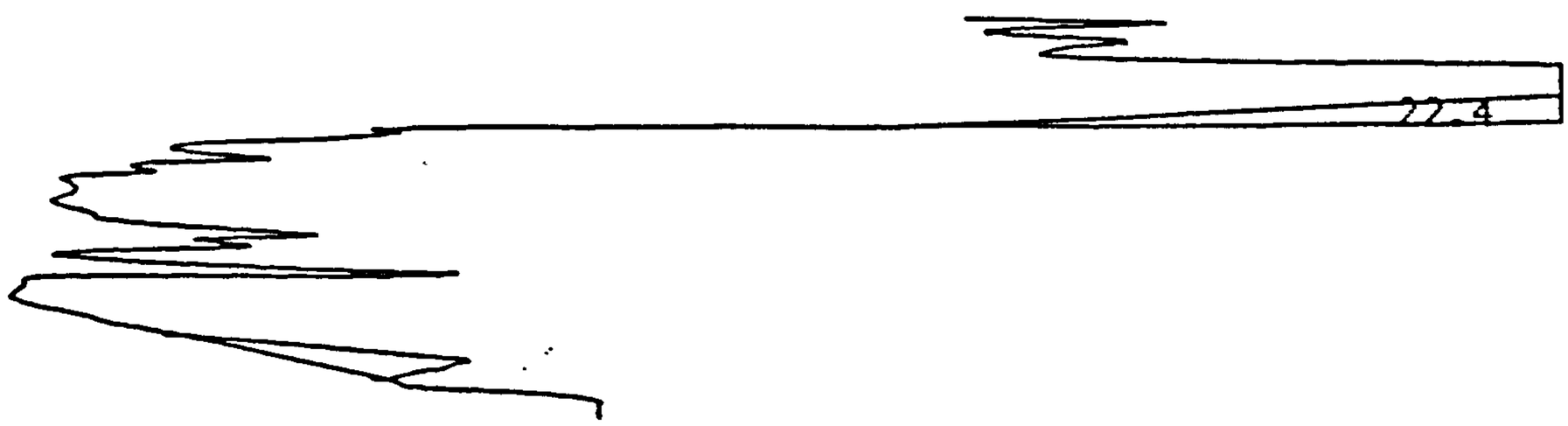
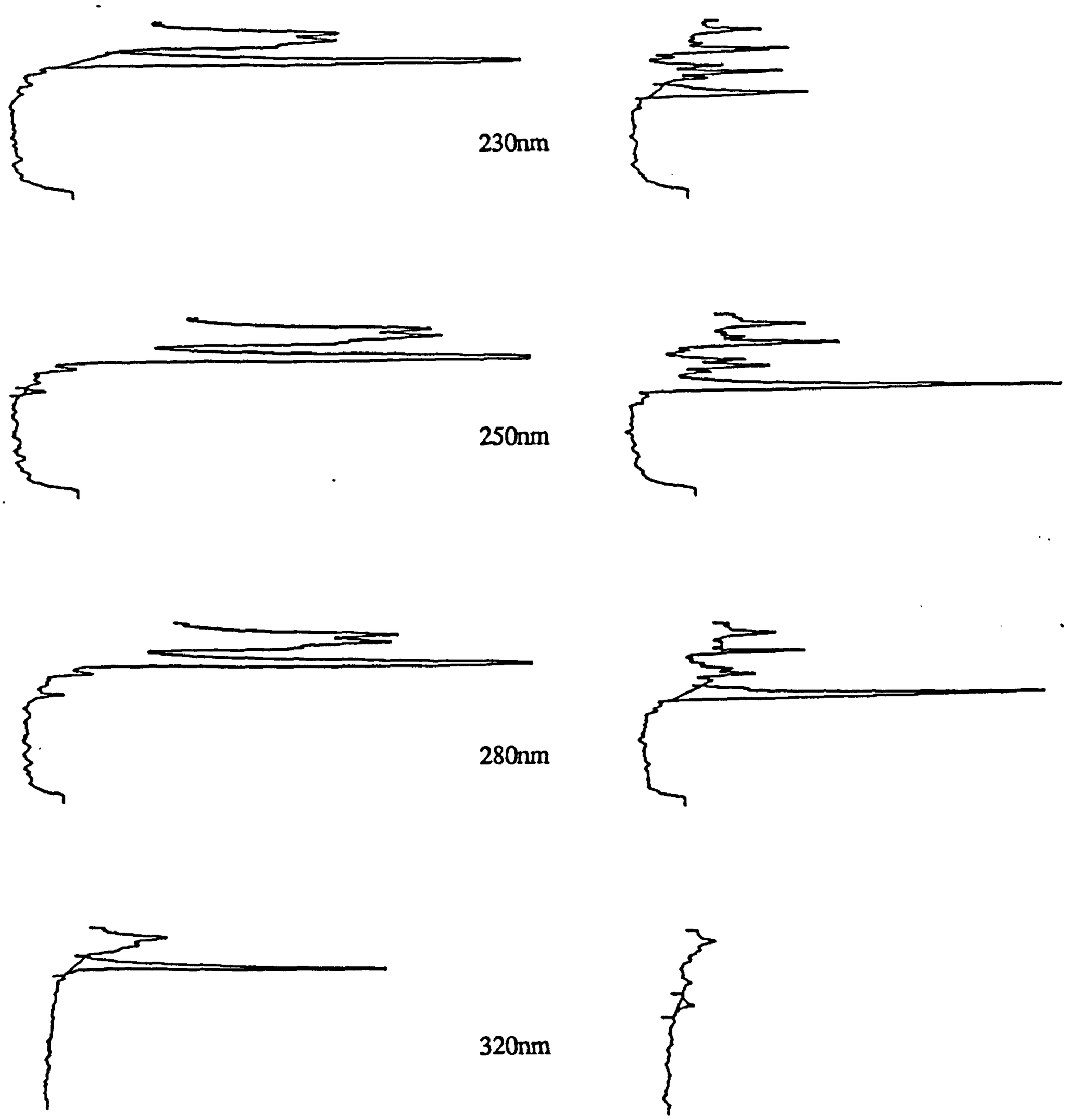


**Figure 3.13**

**HPTLC/MMD chromatograms from Bond Elut Certify™ II extracts of control dog urine**




Paracetamol glucuronide and sulphate (1µg), equivalent to 200µg/ml sample (230nm)

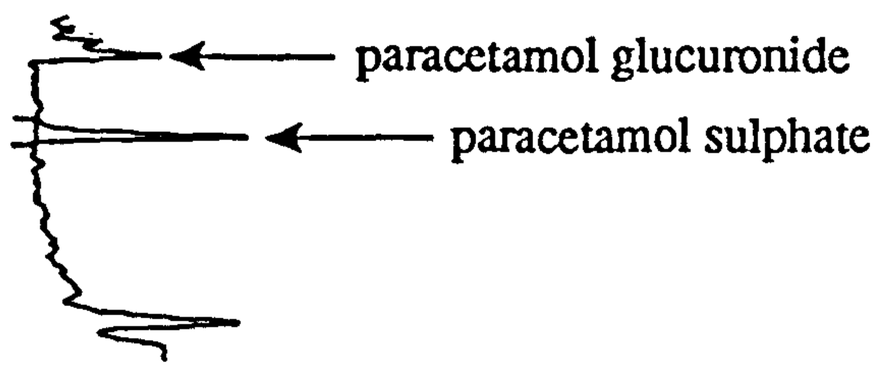
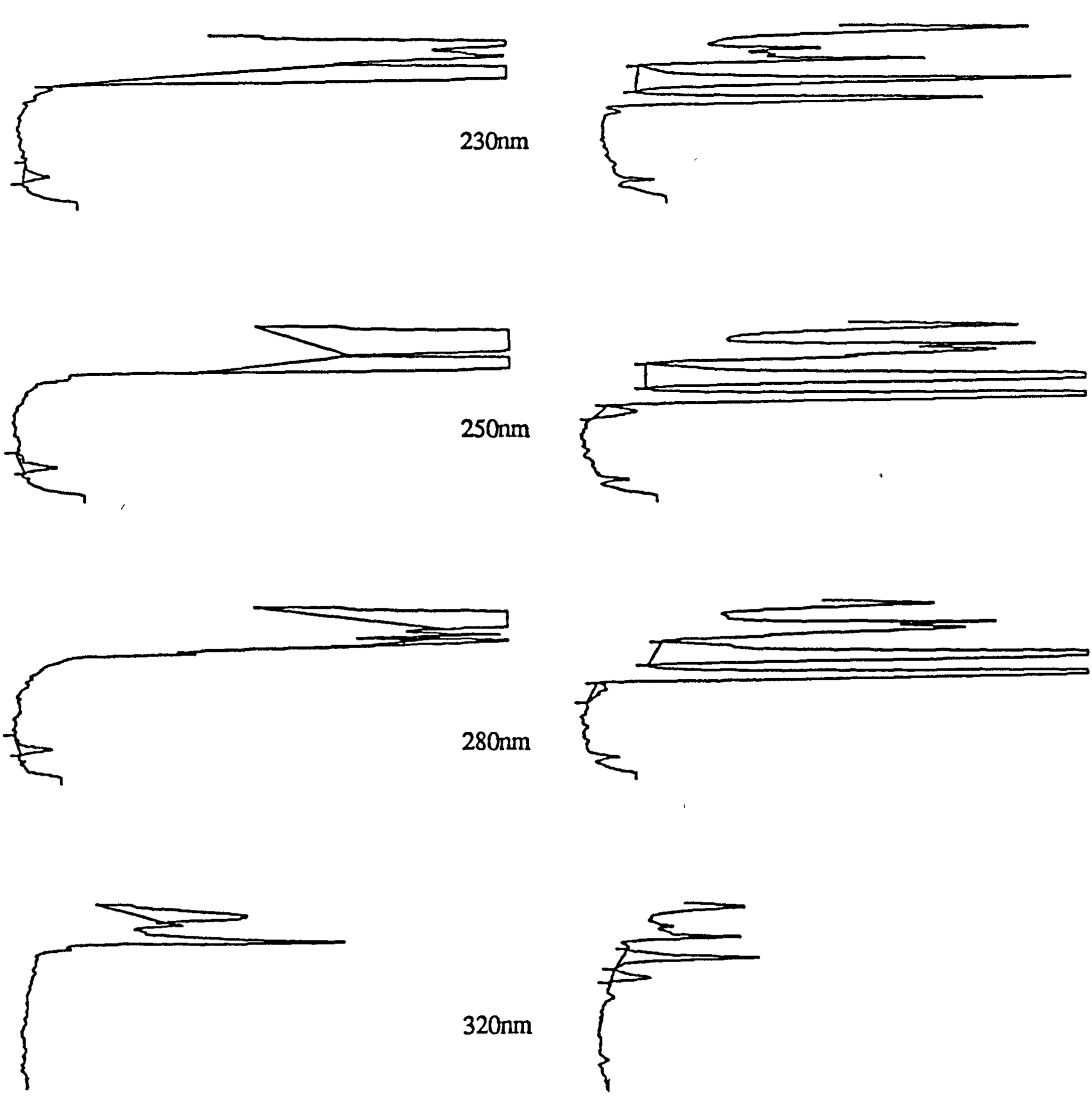


**Figure 3.14**

**HPTLC/MMD chromatograms from Bond Elut Certify™ II extracts of control rat urine**




Paracetamol glucuronide and sulphate (1µg), equivalent to 200µg/ml sample (230nm)

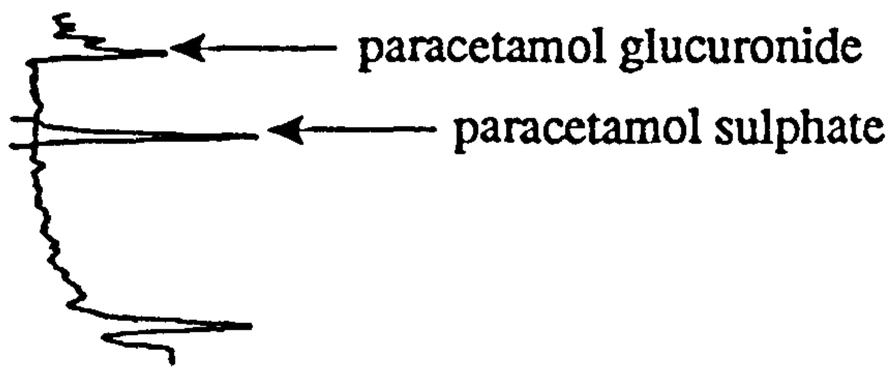


**Table 3.16**

**Recoveries of model compounds from plasma by liquid-solid extraction on Bond Elut Certify™ II cartridges and analysis by HPTLC/MMD**

(Compounds above break were spiked at 50μg/ml, those below were spiked at 10μg/ml)

| Compound | Methanol Elution | MeOH/Formic Acid Elution | MeOH/$NH_4Ac$ Elution |
|---|---|---|---|
| 4-Hydroxycoumarin | - | 119 | - |
| 7-Hydroxycoumarin | 109 | - | - |
| 4'4-Biphenol | 96 | - | - |
| Aspirin | - | NQ | - |
| Salicylic Acid | - | 129 | - |
| Gentisic Acid | - | 45 | - |
| 5-Hydroxyindole | 72 | - | - |
| Indole-3-carboxylic acid | 95 | - | - |
| 3-Indolemethanol | 87 | - | - |
| Ondansetron | 96 | - | - |
| N-desmethyl ondansetron | 62 | - | - |
| 7-Hydroxy-N-desmethyl ondansetron | 128 | - | - |
| 4-Methylumbelliferone (4MeU) | 109 | - | - |
| 4-MeU glucuronide | - | 74 | - |
| 4-MeU sulphate | - | - | 109 |
| Chloramphenicol | 101 | - | - |
| Chloramphenicol glucuronide | - | 101 | - |
| Naphthol | 173 | - | - |
| Naphthol glucuronide | - | 103 | - |
| Naphthol sulphate | - | - | 68 |
| Paracetamol | 66 | - | - |
| Paracetamol glucuronide | - | 78 | - |
| Paracetamol sulphate | - | - | 76 |
| Phenolphthalein | 103 | - | - |
| Phenolphthalein glucuronide | - | 97 | - |
| Phenolphthalein disulphate | - | - | - |

NQ: Not quantifiable because of interference in control plasma chromatogram.

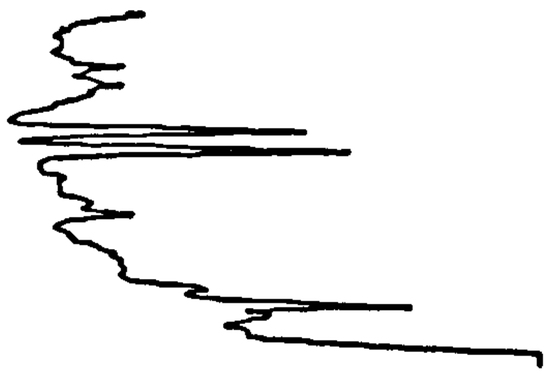
**Figure 3.15**

**HPTLC/MMD chromatograms of methanol eluents from Bond Elut Certify™ II extracts of model compounds from plasma**

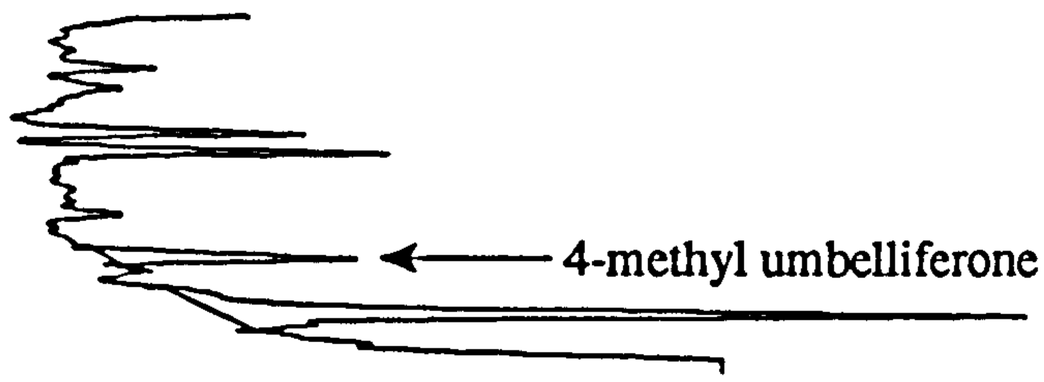
**Figure 3.16**

**HPTLC/MMD chromatograms of methanol eluents from Bond Elut Certify™ II extracts of model compounds from plasma**

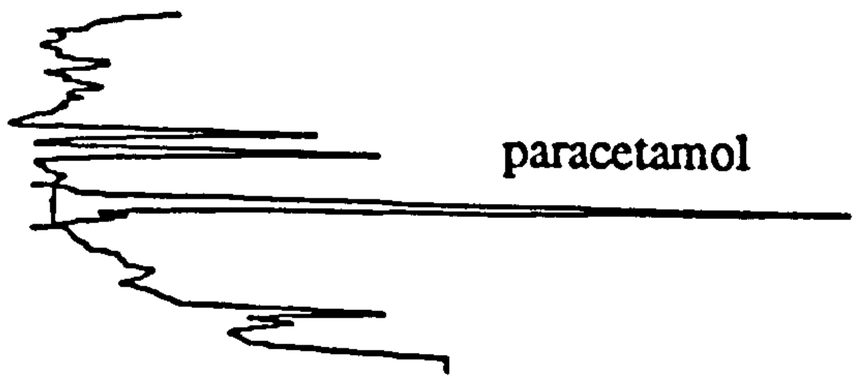
Control plasma



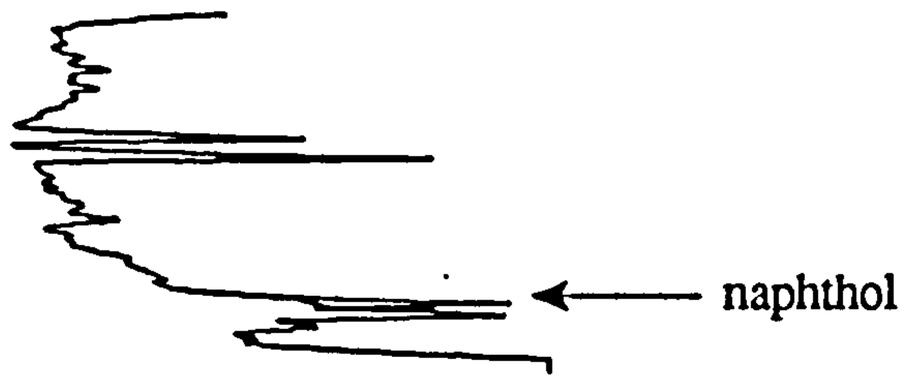
4-Methyl umbelliferone (10µg/ml)



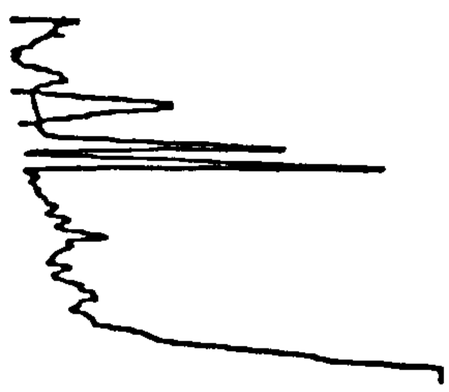

Paracetamol (10µg/ml)



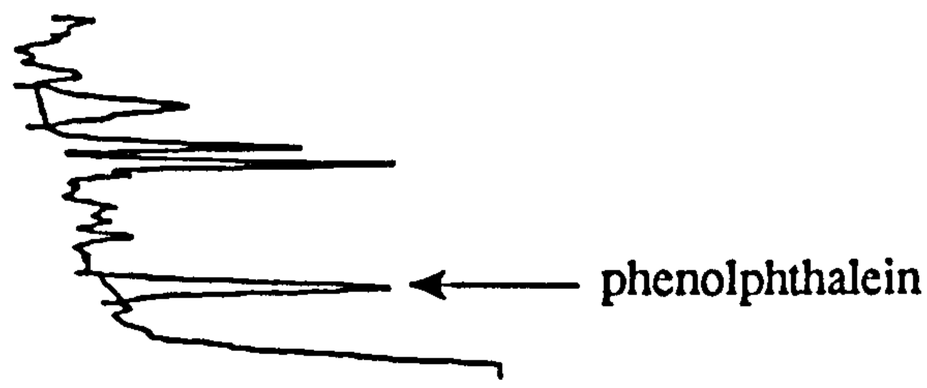

Naphthol (10µg/ml)




Control plasma



Phenolphthalein (10µg/ml)




Chloramphenicol (10µg/ml)

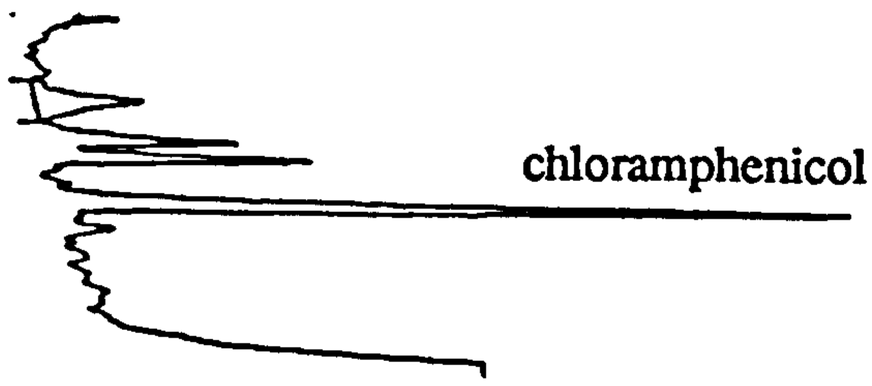


**Figure 3.17**

**HPTLC/MMD chromatograms of methanol and methanol/formic acid eluents from Bond Elut Certify™ II extracts of model compounds from plasma**

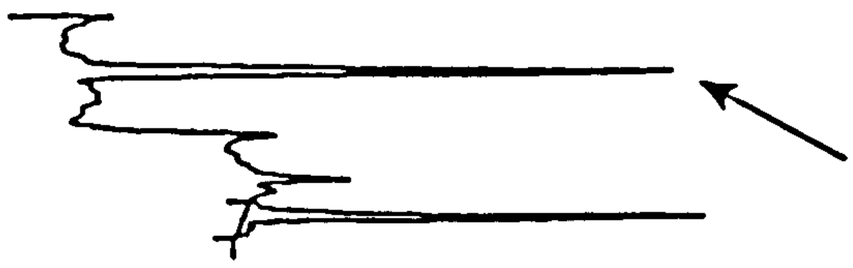
Control plasma (300nm)



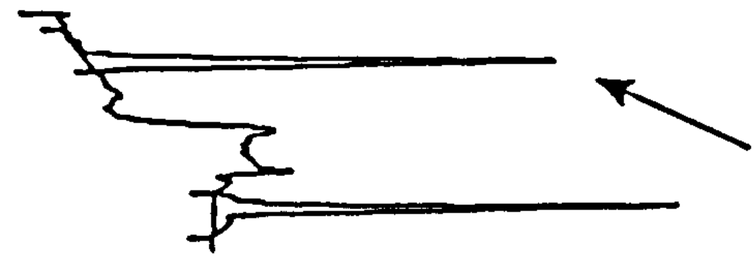
Ondansetron (10µg/ml)



N-desmethyl ondansetron (10µg/ml)



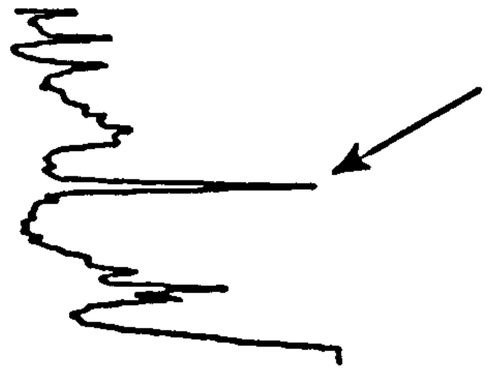
7-Hydroxy-N-desmethyl ondansetron (10µg/ml)



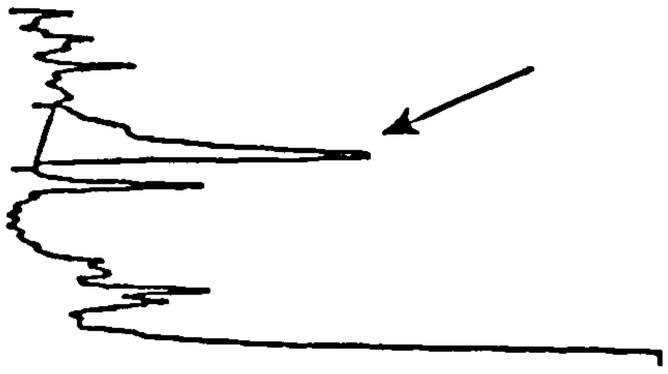
<u>Methanol/formic acid eluents</u>

Control plasma

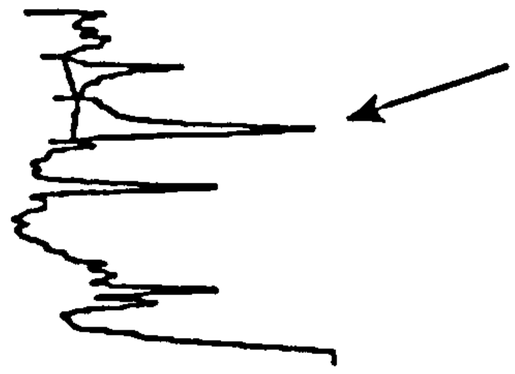
Aspirin (50µg/ml)



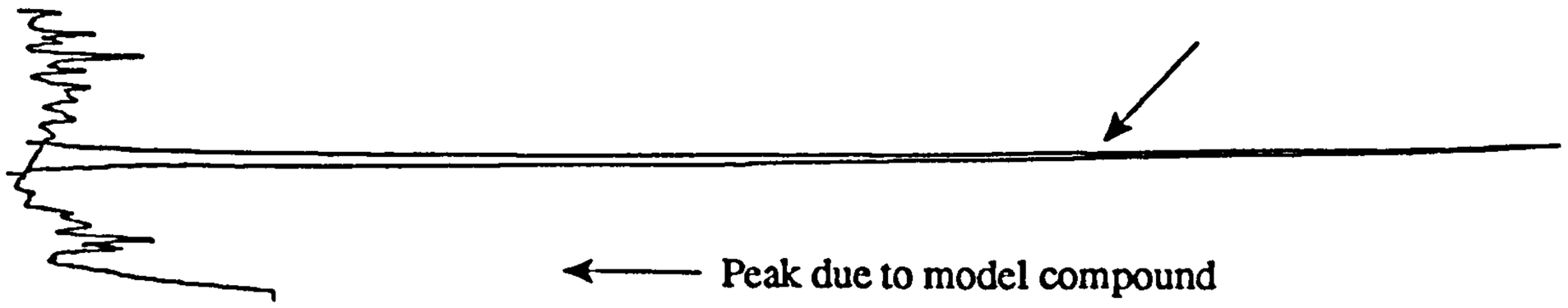
Salicylic acid (50µg/ml)



Gentisic acid (50µg/ml)



4-Hydroxy coumarin (50µg/ml)



← Peak due to model compound

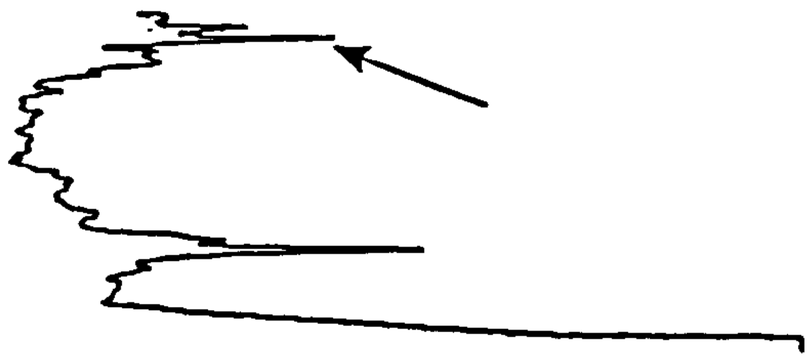
**Figure 3.18**

**HPTLC/MMD chromatograms of methanol/formic acid eluents from Bond Elut Certify™ II extracts of model compounds from plasma**

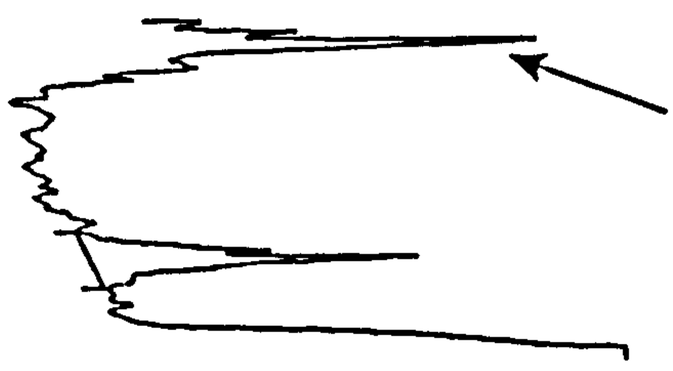
Control plasma (human)

Control plasma (rat)

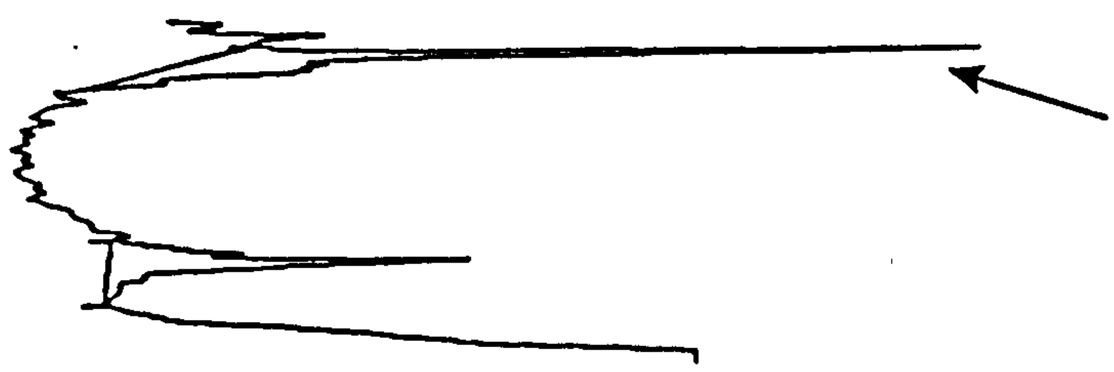
4-Methylumbelliferone glucuronide (10µg/ml)



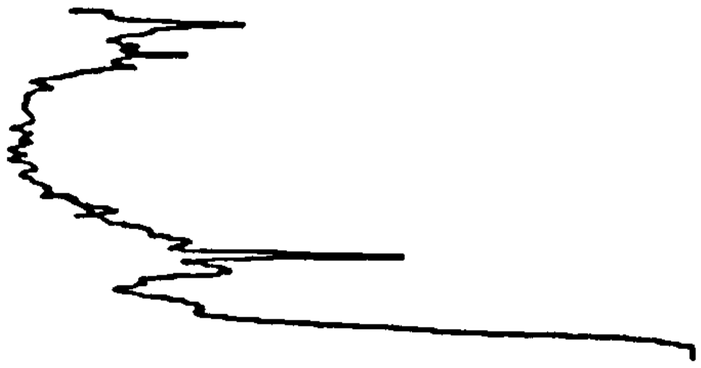
Naphthol glucuronide (10µg/ml)



Chloramphenicol glucuronide (10µg/ml)

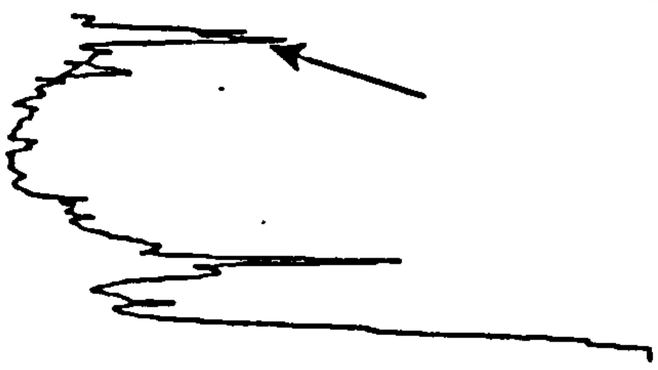


Control plasma (human)

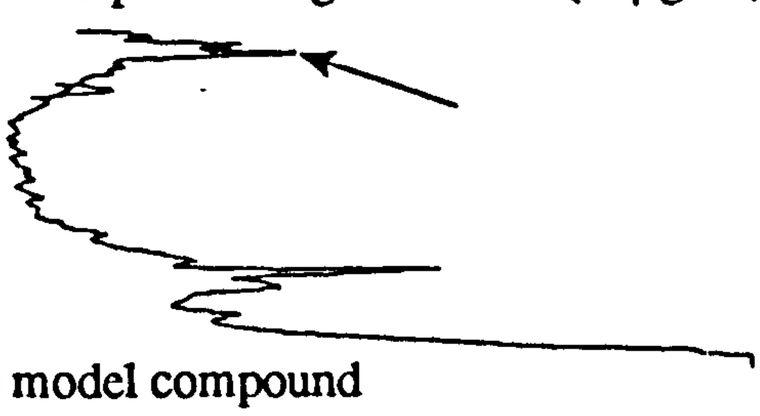


Control plasma (dog)

Paracetamol glucuronide (10µg/mml)



Phenolphthalein glucuronide (10µg/ml)

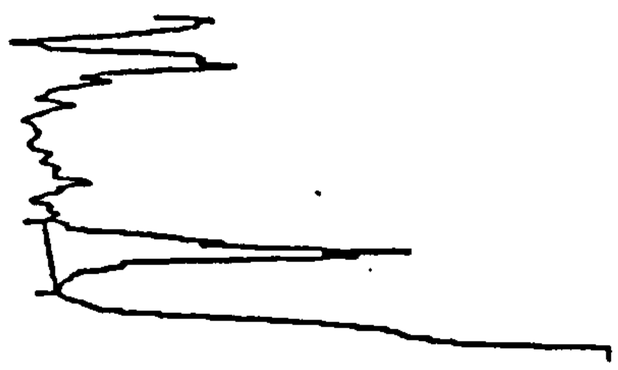


← Peak due to model compound

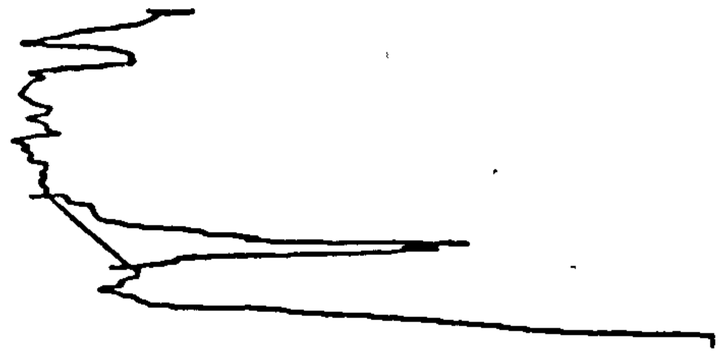
**Figure 3.19**

**HPTLC/MMD chromatograms of methanol/ammonium acetate eluents from Bond Elut Certify™ II extracts of model compounds from plasma**

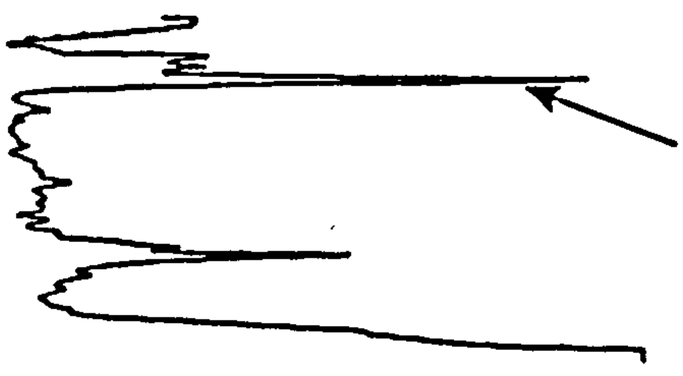
Control plasma (human)



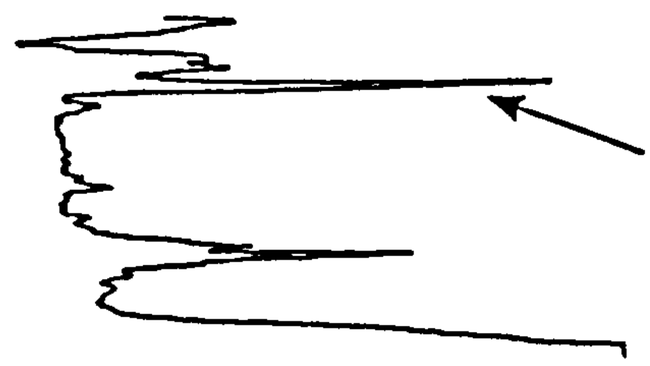
Control plasma (rat)



4-Methylumbelliferone sulphate (10µg/ml)



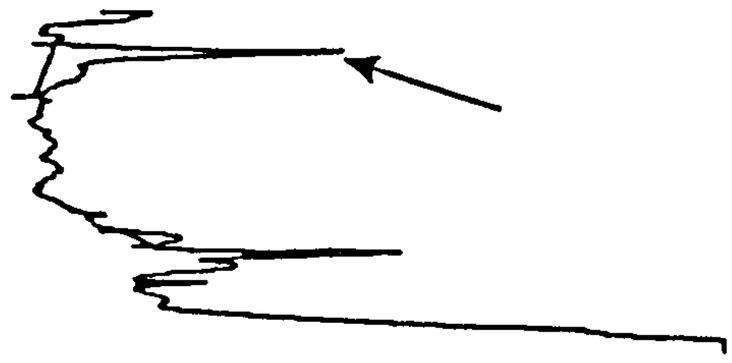
Naphthol sulphate (10µg/ml)



Control plasma (human)

Control plasma (dog)

Paracetamol sulphate (10µg/mml)



← Peak due to model compound

Comparison of the elution distances obtained in these studies with those obtained from analysis of solutions (Chapter 2) showed that they were broadly similar. This indicated that there was no interference with the chromatography caused by any endogenous material co-extracted from the plasma.

Estimated limits of detection for the compounds extracted from plasma are shown in **Table 3.17.** The peak areas of the analytes were calculated by subtraction of the area from the plasma control if the analyte co-eluted with an endogenous component. The limits of detection were in the range 1-50$\mu$g/ml, but most compounds would have been detected between 1 and 10$\mu$g/ml. The background interference from urine was much greater than from plasma therefore the limits of detection from urine would be higher.

## *3.4 Discussion*

The aims of the studies described in this chapter were to assess strategies for a general extraction procedure, to select a procedure, and to examine its general applicability.

A general procedure for the extraction of drugs and metabolites from biological fluids in a form suitable for analysis by HPTLC/MMD has been developed. A general liquid-liquid extraction method and two liquid-solid strategies have been examined to determine their applicability measured against criteria required of a general extraction procedure.

The data from the liquid-liquid extraction based upon the work of Horning *et al.* (1974) showed that the model compounds were recovered only moderately well from plasma and

**Table 3.17**

**Estimated detection limits of model compounds from plasma after liquid-solid extraction on Bond Elut Certify™ II cartridges and analysis by HPTLC/MMD**

| Compound | Estimated limit of detection (μg/ml) |
|---|---|
| 4-Hydroxycoumarin | 5 |
| 7-Hydroxycoumarin | 10 |
| 4 4-Biphenol | 4 |
| Aspirin | 50 |
| Salicylic Acid | 20 |
| Gentisic Acid | 25 |
| 5-Hydroxyindole | 10 |
| Indole-3-carboxylic acid | 5 |
| Ondansetron | <1 |
| N-desmethyl ondansetron | <1 |
| 7-Hydroxy-N-desmethyl ondansetron | <1 |
| 4-Methylumbelliferone (4MeU) | 5 |
| 4-MeU glucuronide | 10 |
| 4-MeU sulphate | 3 |
| Chloramphenicol | 2 |
| Chloramphenicol glucuronide | 2 |
| Naphthol | 10 |
| Naphthol glucuronide | 5 |
| Naphthol sulphate | 3 |
| Paracetamol | 2 |
| Paracetamol glucuronide | 10 |
| Paracetamol sulphate | 5 |
| Phenolphthalein | 5 |
| Phenolphthalein glucuronide | 10 |

poorly from urine. Paracetamol glucuronide did not appear to be recovered from either matrix. This was inconsistent with the results reported by Horning *et al.* (1974) where recoveries of 84-104% were obtained for all drugs and metabolites extracted using ethyl acetate/ammonium carbonate, and where propan-2-ol/ammonium carbonate was reported as being an excellent salt-solvent pair as far as recovery was concerned. The recovery obtained with paracetamol and its metabolites was dependent upon the solvent in which the extract was reconstituted for application to the HPTLC plate, and was lower in the case of methanol which would be expected to dissolve drug-related material more readily. This suggested that the methanol extracts contained so much material that compounds were held up on the origin of the plate due to local overloading, if they were in fact present in the extracts. It was concluded from the investigation that the liquid-liquid approach using salting out, although simple, was inappropriate for use in combination with HPTLC/MMD largely because of the high background interference. Furthermore, because of incomplete extraction in the first stage, this approach did not discriminate between classes of compound, although this may have been improved by repeated extractions. No attempt was made to develop liquid-liquid extraction further because although many combinations of solvent and pH were available, it was considered that there was no rational approach based upon robust chemical interactions which could be applied in pursuit of a general extraction by this technique. In contrast however, liquid-solid extraction provided possibilities for an approach based upon chemical interactions.

Initial studies with liquid-solid extraction using model compounds in buffer solutions, showed that the reversed-phase and ion-exchange interactions discussed in the proposed strategy did occur with paracetamol and its sulphate and glucuronide metabolites. It was also shown that paracetamol sulphate was retained very strongly by the strong anion-exchange (SAX) sorbent due to the strength of the sulphate anion, and that its elution

from that sorbent would be difficult. This confirmed that it would be possible to exploit the difference in the anionic strengths of glucuronides and sulphates to separate them within the extraction procedure as performed by Vanluchene and Vandekerckhove (1988).

First attempts to extract the model compounds from plasma and urine using reversed-phase/ion-exchange combinations were unsuccessful. Extracts analysed by both HPTLC/MMD and HPLC were shown to contain no drug-related material. Subsequent extracts, prepared by the same extraction procedure and analysed by HPLC only, however, showed drug-related material to be present, and in further extracts paracetamol and paracetamol sulphate were found but paracetamol glucuronide was not. These variable data demonstrated the delicate nature of the ion-exchange process when being used with matrices of variable ion content and ionic strength such as biological fluids, and urine in particular.

The use of dilution with buffer at pH6, designed to create solutions of uniform and low ionic strength, was shown to decrease the extraction efficiency. This indicated that the major mechanism by which the model compounds were being retained was not ion-exchange, but non-polar, even in the case of paracetamol sulphate. This non-polar interaction was enhanced by the high ionic strength of the undiluted biological fluids, probably due to a salting out effect. This assumption was given strength because when endogenous anions were removed from the biological fluids prior to extraction by precipitation as lead salts, the recovery of the conjugated metabolites was increased. This indicated that a different retention mechanism, presumably anion-exchange which was previously saturated by the high concentration of endogenous anions present in the matrix, had been made available.

Thus, it was shown that for an extraction procedure to be robust for conjugated metabolites in addition to drugs and their Phase I metabolites, it was necessary to remove endogenous anions before the anionic drug-related components reached the anion-exchange material. This could be achieved by precipitating the anions with lead acetate, but its use may also cause precipitation of glucuronide conjugates (Kamil, Smith and Williams, 1952; Mitchell, Idle and Smith, 1982) and would therefore be inappropriate for a general extraction procedure. Removal of endogenous anions was also found to be achieved by temporarily retaining conjugated metabolites by non-polar interaction either as ion-pairs or in protonated form at low pH (ion-suppression) while the endogenous anions were washed through the extraction cartridge.

Initial examination of the ion-pair approach was not successful, and because it was considered that this approach would be less robust than ion-suppression, further investigations were not carried out. Use of ion-suppression on Bond Elut Certify™ II sorbent material was successful, and had the advantage over both ion-exchange and ion-pairing of relying upon robust chemical interactions. When the pH of biological fluids was reduced to approximately 2.5 by the addition of formic acid, the glucuronide conjugates (pKa 3-4) (Martin and Reid, 1981) and endogenous anions such as citrate, fumarate and succinate were protonated. Glucuronides were then retained by non-polar interaction with the sorbent whereas the strongly hydrophilic endogenous acids remained in the aqueous phase and passed through the cartridge. Thus the relatively weak glucuronide anions were temporarily retained while the potential competitors for the anion-exchange sites on the sorbent material were removed. When the pH was raised during the water wash, conjugated metabolites reverted to their anionic state and were

retained by an anion-exchange interaction in the absence of competition, and hence were not eluted with methanol.

Incorporation of an acid wash after initial retention of the conjugates, designed to make the extraction more robust, eluted the conjugates from the cartridge. The reason for this may have been that retention of these polar compounds by non-polar interactions from the high ionic strength biological fluids occurred by salting out, but when the ionic strength was reduced during washing with dilute hydrochloric acid, they were eluted because of their hydrophilicity, and were not retained by anion-exchange because their ionisation was suppressed by the low pH conditions.

The use of HPLC to examine extracts during the development of the general extraction procedure enabled a distinction to be made between problems arising because of the extractions and those due to HPTLC/MMD such as local overloading of the HPTLC plate. Use of HPLC also decreased the time taken to analyse the extracts because automation allowed analyses to be carried out overnight. Furthermore, the use of a HPLC system designed specifically to separate the compounds of interest reduced the likelihood of interference from endogenous components. Although HPLC proved most appropriate for this specific application, use of HPTLC/MMD for the general case would still be favoured for the reasons discussed in Chapter 1.

The coupling of the general extraction procedure with HPTLC/MMD was hampered initially by problems caused by local overloading of the HPTLC plate at the origin. This was overcome by reducing the proportion of the extract that was applied to the plate. The optimum application was 0.5% of the extract, equivalent to 5$\mu$l from a 1ml biological

fluid sample, which provided a fivefold increase in the amount which could be applied in comparison with untreated plasma (Chapter 2).

The data obtained from HPTLC/MMD of Bond Elut Certify™ II extracts showed that the model compounds were detected more easily in those extracts prepared using the ion-suppression mode than in those prepared using the ion-exchange mode. This was in agreement with the data obtained by HPLC. The Bond Elut Certify™ II extraction based upon ion-suppression was therefore selected to be tested for general applicability using a range of compounds. The recovery data from these compounds showed that the extraction procedure which had been developed (Extraction Scheme 10) provided a general method suitable for the extraction of a range of different compounds, and for their detection by HPTLC/MMD. The method also provided clear and reproducible discrimination between classes of compound which was based upon robust chemical interactions. Basic and neutral compounds and some weak acids were eluted in methanol, glucuronides and carboxylic acids were eluted in methanol/formic acid and sulphates were eluted in methanol/ammonium acetate. The only compound which was not detected, presumably because it was not eluted, was a disulphate which may have required a stronger anion than acetate to displace it from the anion-exchange material.

All the compounds tested were detected by HPTLC/MMD and estimated limits of detection varied between 1 and 50$\mu$g/ml, with most compounds having detection limits in the range 1-10$\mu$g/ml from plasma. Concentrations of drug-related material in excess of these concentrations would be expected in the plasma of laboratory animals after oral administration of drugs at high dose-levels. This can be considered simplistically using the rat as an example. If a drug is administered at a dose-level of 50mg/kg to a rat (200g), and is absorbed completely and rapidly from the gastrointestinal tract, the plasma

concentration of drug-related material (assuming a blood volume of 10% body weight and even distribution of drug-related material between the plasma and red blood cells) would be 500$\mu$g/ml if distribution was limited to the blood. If the drug-related material was generally and evenly distributed throughout the whole body, the concentration would be 50$\mu$g/ml. If a blood sample is taken soon after maximum concentrations occur, and before much material has been excreted, concentrations of drug-related material in plasma should be in the range 50-500$\mu$g/ml. These concentrations are likely to be detected by HPTLC/MMD.

The background endogenous material from urine was greater than from plasma, particularly in the methanol/formic acid and methanol/ammonium acetate eluents making the detection of conjugated metabolites in urine difficult. The detection limits for drugs and metabolites in urine would be between 10 and 50 times greater than in plasma, but in the case of drugs for which renal excretion is a major route, the concentrations of drug-related material in urine would be expected to be high. The concentrations which might be found in urine can also be estimated using the rat as an example. If a drug is administered orally to a rat (200g) at a dose-level of 50mg/kg, is completely absorbed and 50% of the dose is excreted via the urine (5-10ml in 24h), the concentration of drug-related material would be 1-2mg/ml. It is therefore likely under these circumstances that drug-related material would be detected in the urine by HPTLC/MMD if the number of different metabolites formed was not too great.

A general extraction procedure has been developed which enables drugs and metabolites to be extracted from plasma and be detected by HPTLC/MMD down to concentrations of 1-10$\mu$g/ml in most cases. The procedure is also applicable to urine, but the limits of detection would be higher than for plasma. Furthermore, the extraction procedure

provides discrimination between classes of compound, which when combined with HPTLC/MMD increases the capability of the screening method to predict the nature of metabolites, particularly glucuronides and sulphates. An assessment of the likely applicability of the system to drug metabolism studies has indicated that it is likely to be applicable for the analysis of plasma and urine after administration of drugs to animals at high dose-levels. The system may be more sensitive for compounds which absorb ultraviolet radiation at wavelengths in excess of 280nm, but the metabolites formed may not retain the characteristics of the parent compound.

The screening method, combining the extraction procedure developed during the present studies and HPTLC/MMD (Chapter 2), requires testing by analysing samples of plasma and urine from animals after administration of drugs at high dose-levels.

# *CHAPTER FOUR*

# *METABOLISM OF IBUPROFEN AND LOXTIDINE IN THE RAT AND DOG, AND OF ANTIPYRINE AND DIAZEPAM IN THE RAT*

## *4.1 Introduction*

It has been shown previously (Chapter 2) that high-performance thin-layer chromatography (HPTLC) in combination with manual multiple development (MMD) provides a useful chromatographic system for the separation of drugs and drug metabolites. In addition, it was shown that the elution distance of a metabolite relative to the parent drug may be used to indicate the chemical structure of the metabolite.

An extraction procedure has been developed (Chapter 3) which gave good recovery from plasma of the model compounds tested, and differentiation of metabolite types through selective elutions. Analysis of spiked plasma samples by HPTLC/MMD, following extraction, indicated that limits of detection in plasma and urine would be low enough to detect drugs and drug metabolites in samples which may be obtained following high doses of drugs to animals. In combination, HPTLC/MMD and the extraction procedure may provide a method suitable for use as a general screen for drugs and drug metabolites in biological fluids.

The studies described in this chapter were designed to investigate the utility of the screening method by analysing samples of plasma and urine obtained following administration of drugs to animals at high dose-levels.

### 4.1.1 Criteria for the Selection of Test Drugs

The following criteria were considered before selecting drugs which would be suitable to use as test substances.

1. The metabolism of the drug must be known and documented.
2. High oral dose-levels of the drug must be tolerated in animals.
3. The Phase I and II metabolic transformations used in the work with model compounds (Chapters 2 and 3) must be encompassed by the range of drugs selected.
4. The drug should not be metabolised via a large number of routes which might result in very low concentrations of many compounds in plasma and urine.
5. Following oral administration of the drug, a reasonable proportion of the dose must be excreted via the urine.
6. Species differences in metabolism must occur in some of the selection.
7. Not all the compounds selected should interact strongly with silica.
8. At least one acidic, basic and neutral drug should be selected.
9. Ideally, the drug should be available radioisotopically labelled with $^{14}C$ to provide an independent means of testing the results obtained from the screening method.

### 4.1.2 Test Drugs

Four drugs; ibuprofen, loxtidine, antipyrine and diazepam were selected using the criteria described above. Ibuprofen and loxtidine were selected for administration to rats and dogs, and the other compounds for administration to rats only. The oral route of administration was used so that metabolism would be maximised by the first-pass effect and thus provide the highest concentrations of drug metabolites in plasma and urine possible. The rationale for selecting these drugs is described in the following sections.

### 4.1.2.1 Ibuprofen

Ibuprofen (2-(4-isobutylphenyl)propionic acid) is a nonsteroidal compound with anti-inflammatory, analgesic and antipyretic properties (Adams, Cliffe, Lessel and Nicholson, 1967), which has value in the treatment of rheumatoid arthritis and other rheumatic conditions (Busson, 1986) and is presently available "over the counter" as a general pain relieving remedy.

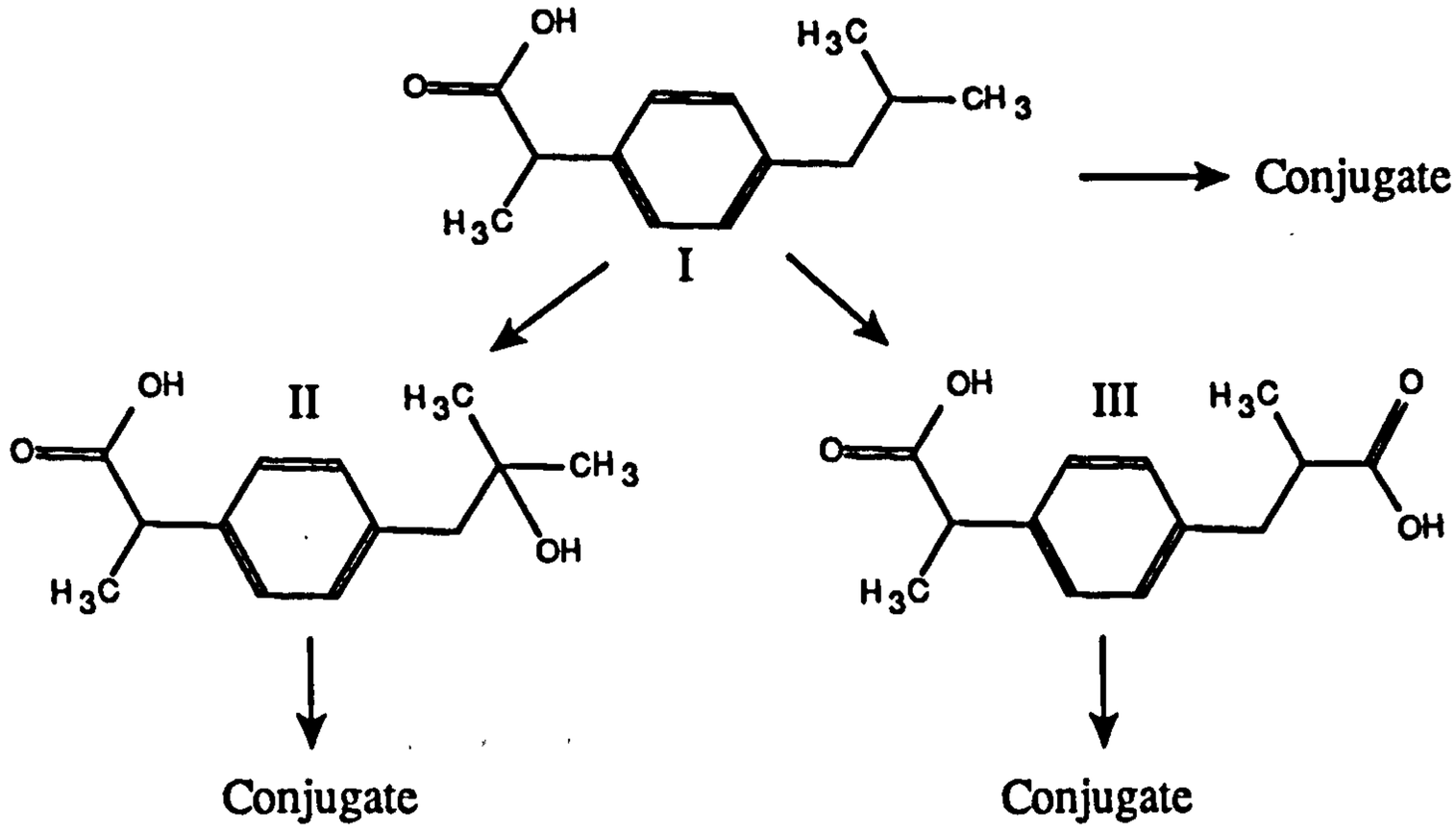
The toxicity of ibuprofen in various animal species was described by Adams, Bough, Cliffe, Lessel and Mills, (1969). The oral LD50 in the rat is 1600mg/kg, and repeated oral dosing for 6 months at a dose-level of 180mg/kg produces only moderate toxicity. In the dog, no ill effects are observed following single oral doses at dose-levels of 20 and 50mg/kg, and dogs given ibuprofen orally at a dose-level of 16mg/kg daily for 30 days show no gross or clinical signs of toxicity. Thus, ibuprofen can be administered orally as a single dose to both rats and dogs at high dose-levels.

The metabolism of ibuprofen is shown in **Figure 4.1.** Metabolism studies in various species showed differences in the metabolism of ibuprofen between the rat and dog (Mills, Adams, Cliffe, Dickinson and Nicholson, 1973).

Following oral administration of ibuprofen to Wistar rats at a dose-level of 20mg/kg, the major component in the plasma at 1.5 hours after dosing is ibuprofen (I) with the hydroxylated metabolite (II) and the carboxylic acid metabolite (III) also present. In dog plasma, only ibuprofen is detected at 1.5 hours following oral administration to Beagle dogs at a dose-level of 8mg/kg.

**Figure 4.1**

**The metabolism of ibuprofen**




Both the rat and dog excrete more than 50% of the dose via the urine (Mills *et al.*, 1973), but different metabolites are excreted by each. Rat urine contains primarily Phase I metabolites of ibuprofen, whereas dog urine contains predominantly glucuronide conjugates.

Ibuprofen is an acidic drug, and from its structure, would not be expected to interact very strongly with silica. The compound is not available radioisotopically labelled, however it was considered to fit the remaining criteria and was therefore selected. It was decided to administer ibuprofen to both the rat and dog to assess whether the differences in metabolism would be detected using the screening method.

### 4.1.2.2 Loxtidine

Loxtidine (1-methyl-5-[3-[3-[(1-piperidinyl)methyl]phenoxy]propyl]amino-1H-1,2,4-triazole-3-methanol) is a potent, long acting histamine $H_2$-receptor antagonist which was investigated as a potential anti-ulcer drug (Brittain and Jack, 1983). The drug can be administered orally to both rats and dogs at dose-levels up to 50mg/kg (Bell *et al.*, 1983).

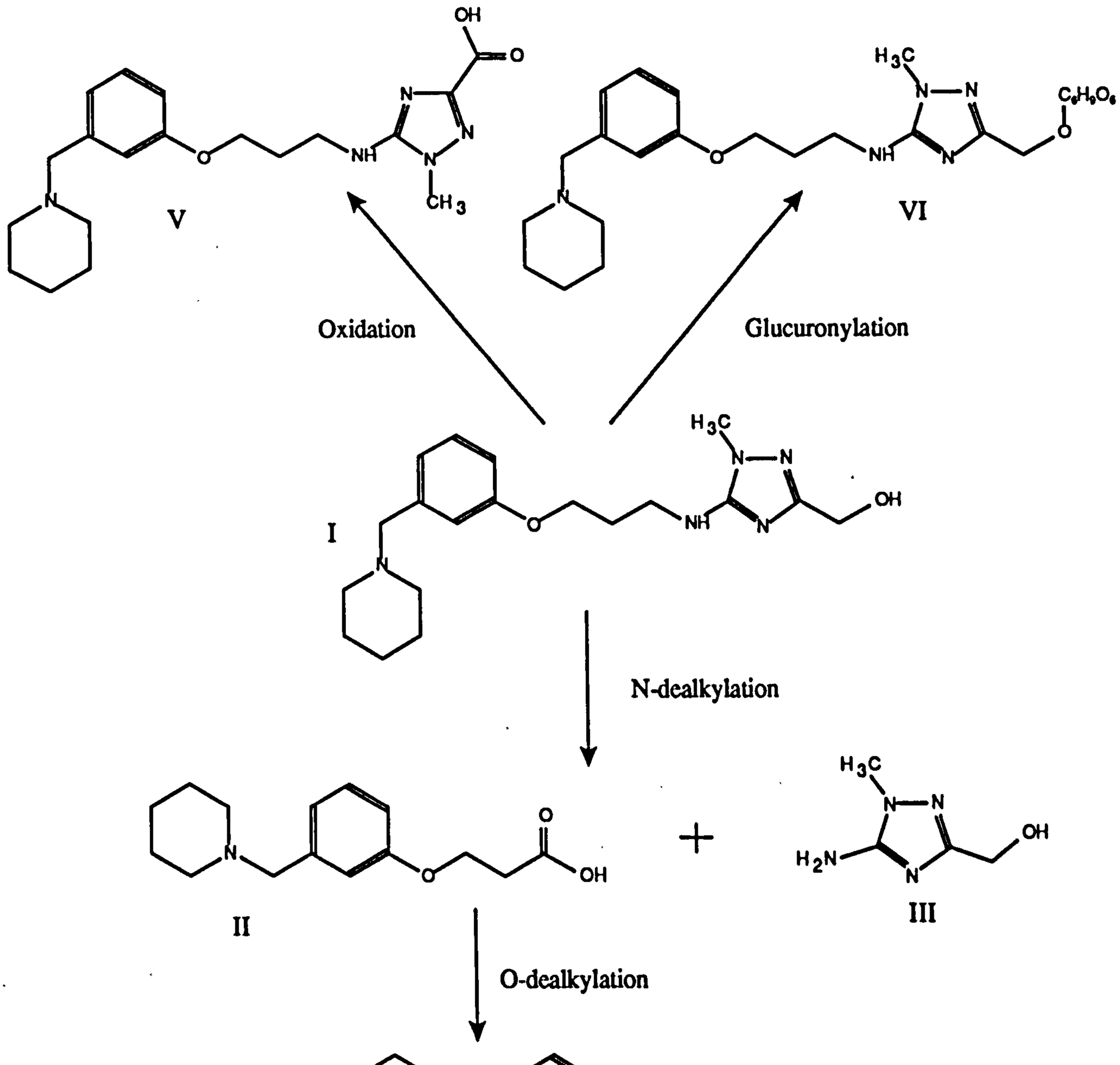
The metabolism of loxtidine is different in the rat and dog (Bell *et al.*, 1983). The metabolic scheme is shown in **Figure 4.2.** In the rat, Phase I metabolism predominates, whereas in the dog, Phase I metabolism is limited and loxtidine is extensively conjugated with glucuronic acid. The concentration of unchanged loxtidine in the plasma of male rats at 1 hour after oral administration at a dose-level of 50mg/kg is about 5$\mu$g/ml (Jenner, 1988). The identity of any other drug-related material circulating in the plasma has not been examined. In the dog, following the same oral dose, levels of radioactive material in the plasma are about 50$\mu$g/ml at 1 hour after dosing (Bradbury, Rosendale and Bell, 1983). The concentrations of unchanged loxtidine are about 15$\mu$g/ml, the majority of the radioactive material comprising polar material.

In both species following oral administration of $^{14}$C-loxtidine at a dose-level of 50mg/kg, urinary excretion of radioactive material accounts for 50 to 75% of the dose.

Loxtidine is a basic molecule which interacts strongly with silica as shown by the low elution distances achieved during multiple development (Chapter 2). The compound is

**Figure 4.2**

**The metabolism of loxtidine**



available radioisotopically labelled with $^{14}C$. Loxtidine fits the criteria and was therefore selected. It was decided to administer the drug to both the rat and dog to assess whether the differences in metabolism would be detected using the screening method.

#### 4.1.2.3 Antipyrine

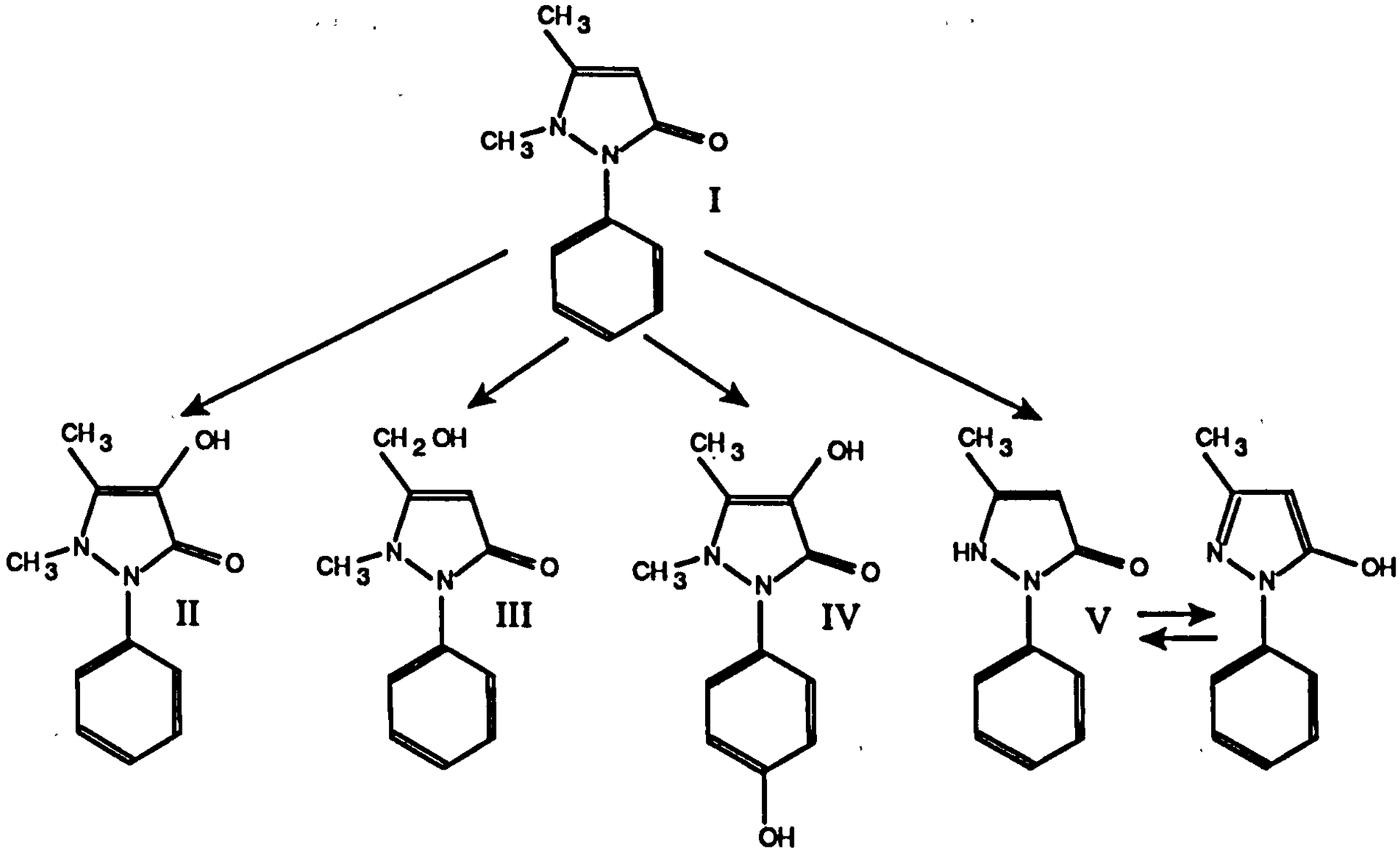
Antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one) is an analgesic which is used extensively as a probe for assessing mixed function oxygenase activity in man and animals (Vesell, 1979). The compound can be administered orally to animals at high dose-levels, the LD50 in the rat being 1.8 g/kg (Hart, 1947).

Antipyrine has a number of pharmacokinetic properties which make it a suitable model compound for studying metabolism. It is rapidly and completely absorbed from the gastrointestinal tract, has a low volume of distribution equivalent to total body water which results in high concentrations in the plasma, and has negligible binding to plasma proteins (Vesell, 1979). In addition, antipyrine is metabolised extensively to both Phase I and Phase II metabolites, almost all of which are excreted in the urine. The metabolic scheme for antipyrine in the rat is shown in **Figure 4.3.**

Antipyrine is a weakly basic compound, and is available labelled with $^{14}C$. This compound fits the criteria and was selected for administration to rats.

**Figure 4.3**

**The metabolism of antipyrine in the rat**



### 4.1.2.4 Diazepam

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) is used clinically as an anticonvulsant, skeletal muscle relaxant, antidepressant and anxiolytic agent (Svenson and Gordon, 1965). The drug can be administered to rats at high oral doses, the LD50 being 700mg/kg (Goldenthal, 1971).

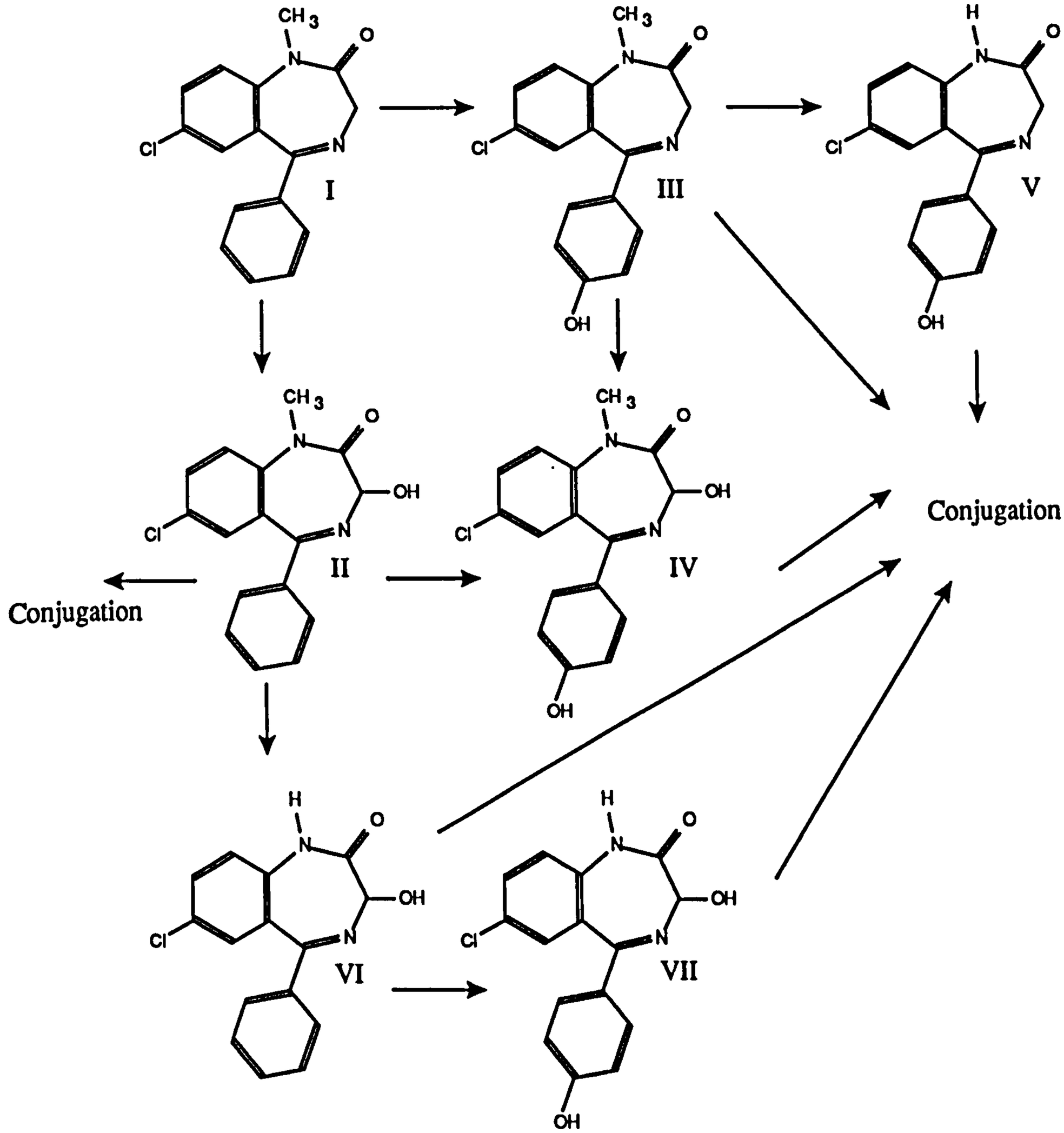
A general scheme for the metabolism of diazepam in the rat is shown in **Figure 4.4.**

Various studies have been carried out to investigate the metabolism of diazepam in animals and man (Schwartz, Koechlin, Postma, Palmer and Krol, 1965; Schwartz, Bommer and Vane, 1967; Andrews and Griffiths, 1984; Trennery and Waring, 1985). The results obtained in these studies differed quantitatively, however the general pattern shows that diazepam undergoes extensive Phase I metabolism by hydroxylation and demethylation followed by conjugation with glucuronic acid. Some evidence of sulphation was found at high dose-levels in the rat. Excretion occurs primarily via the bile in the faeces with the remainder of the dose being excreted in the urine.

Diazepam is a fairly lipophilic, basic molecule, available labelled with $^{14}C$, which is metabolised extensively and excreted predominantly in the faeces. Despite its low excretion in urine, diazepam was considered a useful compound to use for testing the screening method because compounds similar in properties to diazepam will need to be studied in the future. It was thus important that the method was tested with a more difficult compound. Diazepam was selected for administration to rats.

**Figure 4.4**

**The metabolism of diazepam in the rat**



## *4.2 Materials and Methods*

### 4.2.1 Animals

Adult male AHA rats (190 ± 12g) and adult male Beagle dogs (10.1 and 10.8kg) were bred and supplied by the Animal Services Department, Glaxo Group Research Limited, Ware, Herts.

### 4.2.2 Chemicals

Ibuprofen (Lot No. 117F0797), antipyrine (Lot No. 37F0643), 3-$^{14}$C antipyrine, specific activity 5.7MBq (154$\mu$Ci)/mg, (Lot No. 021H9229) and diazepam (Lot No. 105F0451) were supplied by Sigma Chemical Company, Poole, UK.

$^{14}$C-Diazepam, specific activity 6.9MBq (187$\mu$Ci)/mg, (Batch 20) was supplied by Amersham International plc, Aylesbury, UK.

Loxtidine hemisuccinate (AH23844A) (Batch No. C215/153) was synthesised by the Chemical Research Department, Glaxo Group Research Limited, Ware.

$^{14}$C-Loxtidine hemisuccinate (AH23844D), specific activity 0.5MBq (14.0$\mu$Ci)/mg salt, 0.6MBq (16.3$\mu$Ci)/mg base, Batch No. C782/58/1, was supplied by the Radioisotope Laboratory, Process Research Department, Glaxo Group Research Limited, Ware.

Non-radiolabelled loxtidine was analysed by nuclear magnetic resonance spectroscopy and was greater than 99% pure. Other non-radiolabelled drugs were received as >98% pure standards.

All other chemicals and solvents were of AnalaR™ grade or equivalent.

### 4.2.3 Dosing Solutions

#### 4.2.3.1 Dogs

For ibuprofen administration: 750mg of ibuprofen was formulated as a solution containing sodium carbonate (420mg) and distilled water (15.0ml) to give a final concentration of 50mg/ml.

For loxtidine administration: approximately 4.5mg $^{14}$C-AH23844D (equivalent to 3.9mg $^{14}$C-AH23844 base) and 869mg AH23844A (equivalent to 746mg AH23844 base) were dissolved in distilled water (15ml) to give a final concentration of 50mg base/ml, approximately 0.155MBq (4.2$\mu$Ci)/ml.

#### 4.2.3.2 Rats

For ibuprofen administration: 200mg of ibuprofen was formulated as a solution containing sodium carbonate (120mg) and distilled water (10ml) to give a final concentration of 20mg/ml.

For loxtidine administration: approximately 4.2mg $^{14}$C-AH23844D (equivalent to 3.6mg $^{14}$C-AH23844 base) and 54mg AH23844A (equivalent to 46.4mg AH23844 base) were dissolved in distilled water (5ml) to give a final concentration of 10mg base/ml, approximately 0.372MBq (10$\mu$Ci)/ml.

For antipyrine administration: approximately 0.3mg 3-$^{14}$C antipyrine and 500mg antipyrine were dissolved in distilled water (10ml) to give a final concentration of 50mg/ml, approximately 0.372MBq (10$\mu$Ci)/ml.

For diazepam administration: approximately 0.3mg $^{14}$C-diazepam and 50mg diazepam were dissolved in 1ml 0.5M hydrochloric acid and made up to 5ml with distilled water. The excess acid was neutralised by adding 150$\mu$l of 2.5M sodium hydroxide solution, and the solution was made up to 10ml with distilled water to give a final concentration of 5mg/ml, approximately 0.186MBq (5$\mu$Ci)/ml.

#### 4.2.3.3 Analysis

Aliquots of each dosing solution (1$\mu$l) were analysed by HPTLC to determine their purity. Analyses were carried out on silica gel 60 HPTLC plates (Merck, Poole, UK) using the solvent systems shown in **Table 4.1.** After development, the plates were dried at room temperature. The material on the HPTLC plates was quantified by ultraviolet absorption (ibuprofen 220nm, others 250nm) to determine the chemical purity, and using a TLC-linear analyser (Isomess IM3016 linked to a 68000 data system, Raytest Limited, Sheffield, UK) to examine the radiochemical purity where appropriate.

**Table 4.1**

**Thin-layer chromatography systems used to determine the purity of the dosing solutions**

| Drug | System | Components | Proportion By Volume | Distance (cm) |
|---|---|---|---|---|
| Ibuprofen | Plate Pre-wash | Methanol:Acetic Acid | 9:1 | 10 |
| | Development 1 | Methanol:Acetic Acid | 9:1 | 1 |
| | Development 2 | Toluene:Acetic Acid | 9:1 | 8 |
| Loxtidine | Plate Pre-wash | Methanol | | 10 |
| | Development 1 | Methanol | | 1 |
| | Development 2 | Ethyl Acetate:Propan-2-ol: Water:Ammonia (33%) | 5:3:1.6:0.4 | 8 |
| Antipyrine | Plate Pre-wash | Methanol | | 10 |
| | Development 1 | Methanol | | 1 |
| | Development 2 | Chloroform:Ethanol | 9:1 | 9 |
| Diazepam | Plate Pre-wash | Methanol | | 10 |
| | Development 1 | Methanol | | 1 |
| | Development 2 | Hexane:Chloroform:Ethanol | 5:5:0.5 | 8 |

### 4.2.4 Animal Experimentation

#### 4.2.4.1 Dogs

Two male Beagle dogs were denied access to food overnight and housed in individual stainless steel metabolism cages to collect control urine. The dogs were weighed prior to dosing and control blood samples (10ml) were taken. One dog was dosed orally with ibuprofen (50mg/kg) and the other was dosed orally with $^{14}$C-loxtidine (50mg/kg). The dog which received ibuprofen was fed immediately after dosing and the other was denied access to food for a further 1.5 hours. Blood samples (10ml) were taken into heparinised containers from each dog at 1.5 hours after dosing. The dogs remained in metabolism cages for 24 hours after dosing and urine was collected during this period. The blood samples were centrifuged and the plasma was removed and stored at approximately -20°C prior to analysis. The urine was collected into containers cooled with dry ice in order to minimise degradation of metabolites, and was stored at approximately -20°C prior to analysis.

#### 4.2.4.2 Rats

Twenty male AHA rats were starved overnight. Groups of four rats were dosed orally with ibuprofen (100mg/kg), $^{14}$C-loxtidine (50mg/kg), $^{14}$C-antipyrine (250mg/kg) or $^{14}$C-diazepam (50mg/kg). The remaining animals were used to collect control plasma and urine. Two animals from each group were placed into stainless steel metabolism cages immediately after dosing in order to collect urine over a 0-24 hour period. Terminal blood samples were taken into heparinised containers from the remaining

animals at approximately 1.5 hours after dosing. The blood and urine samples were treated as described above.

### 4.2.5 Liquid Scintillation Counting

Samples of each dosing solution (50μl) were diluted to 10ml with distilled water and aliquots of the diluted solutions (50μl) were mixed with scintillation cocktail (8ml, Picofluor 30, Canberra Packard Limited, Pangbourne, UK) and the level of radioactivity was determined by liquid scintillation counting (Packard 2200A liquid scintillation system, Canberra Packard Limited, Pangbourne, UK). The levels of radioactivity in the dosing solutions were used to determine the specific activity; i.e. the number of counts which corresponded to 1μg drug.

Aliquots of plasma and urine samples taken following administration of radiolabelled drug (100μl) were mixed with scintillation cocktail (8ml) and the level of radioactivity was determined as described above. The counts obtained were used to calculate the levels of radioactive material in the samples and final results were expressed as μg drug equivalents/ml of sample.

### 4.2.6 Sample Preparation

Plasma and urine samples, controls, and standards (10μg/ml in plasma and 100μg/ml in urine) were prepared by solid-phase extraction on 130mg Bond Elut Certify™ II extraction cartridges prior to analysis by HPTLC/MMD. The cartridges were conditioned with methanol (4ml) followed by water (4ml), during which, the cartridges were not allowed to dry. Plasma or urine samples (1ml) mixed with 98% formic acid (20μl for

plasma and 10μl for urine) were applied to the cartridges and allowed to pass through at approximately one drip every 3 seconds with the aid of vacuum. The cartridges were then dried under vacuum, washed with water (2ml) and dried again. Three separate elutions were then carried out using methanol (2ml), then 0.5% formic acid in methanol (2ml) and 5% ammonium acetate in methanol (2ml). Each eluent was collected separately and dried to residue in a Savant speed vac concentrator (Stratech Scientific Limited, London, UK). The dried eluents were stored at approximately -20°C prior to analysis.

The portion of sample which passed through the cartridge, and the water wash from samples containing radioactive material were collected together and analysed by liquid scintillation counting. Portions of the eluents (100μl) from the same samples were also taken for analysis by liquid scintillation counting before the remainder of the eluents were dried to residue. The counts obtained were used to determine the extraction efficiencies and the amount of radioactive material recovered in each eluent.

### 4.2.7 HPTLC/MMD Analysis

Residues obtained from the methanol and methanol/formic acid eluents were dissolved in methanol (100μl) by sonication for 5 minutes followed by vortex mixing. The residues from the methanol/ammonium acetate eluents contained more material and were dissolved in 200μl methanol so that the proportion of the sample applied to the HPTLC plate was less, and disturbance of the solvent front during plate development was avoided. Portions of each sample, control or spiked sample (1μl) were applied to the HPTLC plates (silica gel 60 without fluorescent indicator, Merck, Poole, UK) and

allowed to dry prior to development. MMD was performed as described previously (Chapter 2, Section 2.2.1).

Portions of 1mg/ml drug solutions in methanol (1$\mu$l) were analysed by HPTLC/MMD using MMD Scheme 3 (**Appendix I, Table 3**) to define the elution distances of the drugs and enable suitable MMD gradients to be designed for analysis of the samples. The elution distances of the standards obtained using MMD Scheme 3 are shown below.

**Elution distances of standards following development on silica gel 60 HPTLC plates using MMD Scheme 3**

Ibuprofen : 12mm (broad peak)

Loxtidine : 6mm

Antipyrine : 27mm

Diazepam : 51mm

These data indicated that MMD Scheme 3 would not be a suitable gradient for analysis of the samples from this study with the exception of those obtained following administration of diazepam. This conclusion was drawn because drug metabolites are generally more strongly retained than the parent drug, being more polar. Thus the majority of peaks due to drug-related material in samples obtained following administration of ibuprofen, loxtidine and antipyrine would probably be concentrated in the bottom 25-30% of the plate. In the case of ibuprofen, an acidic compound, a poor peak shape was obtained with MMD Scheme 3, indicating that addition of acetic acid to the developing solvents would be beneficial. It had been shown previously (Chapter 2) that addition of triethylamine

(TEA) was required for loxtidine to migrate a reasonable distance up the plate. The following MMD gradients were therefore used to analyse the samples.

**MMD gradients used for analysis of plasma and urine samples**

| | | |
|---|---|---|
| Ibuprofen | : | MMD Scheme 3 + 1% acetic acid |
| Loxtidine | : | MMD Scheme 7 + 1% TEA |
| Antipyrine | : | MMD Scheme 13 (Elutropic strength range 0.73 - 0.48) |
| Diazepam | : | MMD Scheme 3 |

The compositions of the solvents and development distances are shown in Appendix I, Tables 3, 7, 13 and 3 respectively.

### 4.2.8 Detection

Following MMD, the HPTLC plates were scanned using a Camag Scanner II (BDH, Poole, UK). Material on the plates was detected by ultraviolet absorbance, and each plate was scanned at 230, 250 and 280nm. The ibuprofen plates were scanned at 220nm only because of the limited ultraviolet absorbing properties of the drug. The scanner settings used are shown in the table overleaf.

**HPTLC scanner settings used for detection following HPTLC/MMD**

| Parameter | | Setting |
|---|---|---|
| Wavelength | : | 220, 230, 250 or 280nm (as described above) |
| Band width | : | 30nm |
| Slit width | : | 0.2mm |
| Slit height | : | 3mm |
| Scanning speed | : | 1mm/sec |
| Sensitivity | : | 250 (220 or 225 for urines) |
| Span | : | 250 (220 or 225 for urines) |
| Offset | : | 50 |

The analog signal from the scanner was captured using a Trilab 2000 Chromatography Data System (Trivector, Sandy, UK) from which chromatograms were printed and peak areas calculated. A signal filter box (MH Scientific, Aylesbury, UK) was placed between the scanner and the integrator to remove electrical noise.

## *4.3 Results*

### 4.3.1 Analysis of Dosing Solutions

The dosing solution purity data obtained are shown in **Table 4.2.**

The chemical purities of the dosing solutions ranged from 90.4% to 99.0%. With the exception of the ibuprofen solution used for administration to the dog, all purities were 94% or greater. The radiochemical purities of the dosing solutions ranged from 95.7% to 100%. These levels of purity were considered acceptable to enable the objectives of the study to be met.

### 4.3.2 Liquid Scintillation Counting

#### 4.3.2.1 Dosing solutions

The levels of radioactive material measured in the dosing solutions, and the specific activities of the solutions are shown below.

| Sample | Level of Radioactive Material in Solution ($\mu$Ci/ml) | Specific Activity per $\mu$g drug (dpm) |
|---|---|---|
| Loxtidine (Dog) | 4.0 | 173.6 |
| Loxtidine (Rat) | 11.5 | 2532 |
| Antipyrine | 24.7 | 1089 |
| Diazepam | 7.4 | 3248 |

**Table 4.2**

**Dosing solution purities determined by HPTLC after animal experimentation was complete**

| Dosing Solution | Purity | |
|---|---|---|
| | Chemical | Radiochemical |
| Ibuprofen 50mg/ml (Dog) | 90.4% | - |
| Ibuprofen 20mg/ml (Rat) | 94.0% | - |
| Loxtidine 50mg/ml (Dog) | 98.9% | 95.7% |
| Loxtidine 10mg/ml (Rat) | 99.0% | 96.4% |
| Antipyrine 50mg/ml | 96.0% | 100.0% |
| Diazepam 5mg/ml | 93.9% | 99.6% |

The levels of radioactive material in the dosing solutions were approximately equal to, or higher than the levels expected. The higher levels obtained for antipyrine and diazepam result from the inaccuracies of weighing small amounts of material experienced by the suppliers. The differences between the target and actual levels achieved did not conflict with the aims of the study.

#### 4.3.3.2 Plasma and urine samples

The levels of radioactive material measured in the plasma and urine samples are shown in **Table 4.3.** All the plasma samples from animals which received radiolabelled drug contained radioactive material, and thus showed that drug was absorbed from the gastrointestinal tract into the systemic circulation. The levels of radioactive material in the plasma ranged from 5.8 to 7.2$\mu$g equivalents/ml in the rat after administration of loxtidine and diazepam, to 50.6$\mu$g/ml in the dog after dosing with loxtidine and 171 to 207$\mu$g/ml in the rat after administration of antipyrine.

The levels of radioactive material in the 0 to 24 hour urine samples were approximately 10 to 70 times those found in the plasma. Levels ranged from about 107$\mu$g/ml in the rat after administration of diazepam (corresponding to between 12 and 14% of the dose) to about 500$\mu$g equivalents/ml in the rat after dosing with loxtidine (44 to 56% of the dose), 1150$\mu$g equivalents/ml in the dog after dosing with loxtidine (50% of the dose) and about 2600$\mu$g equivalents/ml in the rat after administration of antipyrine (62 to 75% of the dose). In all cases except after dosing with diazepam, a high proportion of the dose was excreted in the urine in the first 24 hours. Lower levels of radioactive material were

**Table 4.3**

**Levels of radioactive material in plasma and urine samples following oral administration of $^{14}$C-loxtidine, $^{14}$C-antipyrine or $^{14}$C-diazepam to AHA rats or Beagle dogs**

| Drug | Species | Animal Number | Dose-level (mg/kg) | Sample | Time After Dose (h) | Level of Radioactive Material (μg equiv./ml) |
|---|---|---|---|---|---|---|
| Loxtidine | Dog | 0LK5 | 50 | Plasma | 1.5 | 50.6 |
| | | | | Urine | 0 - 24 | 1150 |
| Loxtidine | Rat | 99 | 50 | Plasma | 1.5 | 7.2 |
| | | 100 | 50 | Plasma | 1.5 | 6.9 |
| | | 97 | 50 | Urine | 0 - 24 | 615 |
| | | 98 | 50 | Urine | 0 - 24 | 479 |
| Antipyrine | Rat | 103 | 250 | Plasma | 1.5 | 171 |
| | | 104 | 250 | Plasma | 1.5 | 206 |
| | | 101 | 250 | Urine | 0 - 24 | 2690 |
| | | 102 | 250 | Urine | 0 - 24 | 2540 |
| Diazepam | Rat | 107 | 50 | Plasma | 1.5 | 5.8 |
| | | 108 | 50 | Plasma | 1.5 | 7.0 |
| | | 105 | 50 | Urine | 0 - 24 | 106 |
| | | 106 | 50 | Urine | 0 - 24 | 107 |

expected in the urine following administration of diazepam, and those obtained in the present studies (12 to 14%) were similar to the 17% reported previously (Andrews and Griffiths, 1984).

#### 4.3.2.3 Extraction washes and eluents

The levels of radioactive material in the washes and eluents from the solid-phase extractions carried out to prepare the samples for analysis by HPTLC/MMD were determined, and used to calculate the efficiencies of the extractions. The rat plasma and urine samples containing the highest levels of radioactive material were analysed. All samples were analysed on two occasions separated by approximately four weeks. The results obtained are shown in **Table 4.4.**

The overall recoveries were all in excess of 94% with only small amounts being lost either by break-through on sample application or in the washes. The total amounts of radioactive material in the washes and eluents were approximately equal to the amounts applied in the samples to the cartridges, indicating that essentially all of the material had been eluted. In addition to the high recoveries obtained, the repeat extractions demonstrated that the system was reproducible both in terms of overall recovery and in specificity between eluents.

Previous studies (Chapter 3) indicated that following extraction of drugs and drug metabolites from biological fluids on Bond Elut Certify™ II cartridges, the eluent in which a compound eluted from the cartridge was indicative of its chemical nature. Thus, non-acidic or weakly acidic drugs and Phase I metabolites eluted in methanol, acidic

**Table 4.4**

**Extraction efficiencies on Bond Elut Certify™ II cartridges following extraction of drugs and drug metabolites from plasma and urine after oral administration of $^{14}$C-loxtidine to rats and dogs and $^{14}$C-antipyrine and $^{14}$C-diazepam to rats**

**Samples Following Administration of Loxtidine**

| Sample | Washes | Methanol | Methanol/FA | Methanol/$NH_4Ac$ | Recovery |
|---|---|---|---|---|---|
| Rat Plasma 1 | 4.2% | 93.0% | 1.2% | 1.7% | 95.8% |
| Rat Plasma 2 | 1.9% | 95.2% | 1.3% | 1.7% | 98.2% |
| Rat Urine 1 | 3.6% | 92.1% | 2.4% | 1.9% | 96.4% |
| Rat Urine 2 | 3.3% | 92.1% | 4.0% | 0.6% | 96.7% |
| Dog Plasma 1 | 3.2% | 92.7% | 3.7% | 0.4% | 96.8% |
| Dog Plasma 2 | 3.5% | 92.1% | 2.4% | 2.0% | 96.5% |
| Dog Urine 1 | 4.0% | 92.2% | 3.1% | 0.7% | 96.0% |
| Dog Urine 2 | 3.3% | 92.1% | 4.0% | 0.6% | 96.7% |

**Samples Following Administration of Antipyrine**

| Sample | Washes | Methanol | Methanol/FA | Methanol/$NH_4Ac$ | Recovery |
|---|---|---|---|---|---|
| Rat Plasma 1 | 1.1% | 86.4% | 3.0% | 9.4% | 98.9% |
| Rat Plasma 2 | 0.7% | 85.8% | 3.0% | 10.6% | 99.4% |
| Rat Urine 1 | 5.7% | 36.9% | 14.1% | 43.3% | 94.3% |
| Rat Urine 2 | 4.7% | 44.7% | † | 50.6% | 95.3% |

**Samples Following Administration of Diazepam**

| Sample | Washes | Methanol | Methanol/FA | Methanol/$NH_4Ac$ | Recovery |
|---|---|---|---|---|---|
| Rat Plasma 1 | 5.8% | 46.2% | 9.0% | 39.0% | 94.2% |
| Rat Plasma 2 | 4.9% | 43.7% | 8.8% | 42.6% | 95.1% |
| Rat Urine 1 | 0.4% | 10.7% | 14.3% | 74.7% | 99.6% |
| Rat Urine 2 | 0.3% | 12.8% | 13.3% | 73.6% | 99.7% |

† Methanol and methanol/formic acid washes were counted together.

drugs or Phase I metabolites and glucuronide conjugates eluted in methanol/formic acid, and sulphate conjugates eluted in methanol/ammonium acetate. These observations can be used to predict the general nature of the material which eluted from the extraction cartridges in the present studies.

In the samples obtained following administration of loxtidine, most of the radioactive material was eluted in the methanol eluents from both the plasma and urine samples from the rat and dog. Thus the results obtained previously (Chapter 3) suggest that the material comprised either non-acidic or weakly acidic compounds, probably Phase I metabolites.

The plasma and urine samples obtained following administration of antipyrine to the rat had different elution patterns. From the plasma, most of the radioactive material eluted in the methanol (about 86%) with low amounts in the methanol/formic acid (3%) but higher levels in the methanol/ammonium acetate eluent (about 10%). This suggests that the majority of the drug-related material in the plasma comprised Phase I metabolites, and that sulphate conjugates may have been present. In urine, the highest levels of radioactive material were eluted in the methanol/ammonium acetate eluent (about 50%) with about 37% in the methanol and 14% in the methanol/formic acid. These data suggest that drug-related material in the urine comprised sulphate conjugates with lower concentrations of Phase I metabolites and glucuronides also present.

Following administration of diazepam, plasma and urine again showed different elution patterns. Similar amounts of radioactive material in plasma were eluted in the methanol (about 45%) and methanol/ammonium acetate (about 41%), and a small amount (9%) was eluted in the methanol/formic acid. This suggests that drug-related material in the

plasma comprised largely Phase I metabolites and sulphate conjugates with some glucuronide conjugates also present. In urine the majority of the drug-related material (about 74%) was eluted in the methanol/ammonium acetate with similar amounts of the remaining material being eluted in the other two eluents. This suggests that the urine contained mainly sulphate conjugates with lower concentrations of Phase I metabolites and glucuronide conjugates also present.

### 4.3.3 HPTLC/MMD

Chromatograms obtained following HPTLC/MMD of the plasma and urine extracts were studied to identify peaks which were present in sample extracts, but absent in controls. All extracts from both extractions were analysed. The analyses of samples from each set of extractions were carried out on different occasions separated by approximately four weeks. The results of these analyses are described in detail for each test substance in the following sections.

The peak areas of drug-related material were obtained either directly from the chromatograms, or by subtracting the area of the control sample from the sample area when peaks co-eluted with endogenous material. Estimated concentrations were obtained by comparing peak areas of drug-related components with those of standards, assuming linearity of response and that the extinction coefficients of the parent drug and metabolites are the same.

### 4.3.3.1 Ibuprofen

The peaks detected following HPTLC/MMD analysis of the samples obtained after administration of ibuprofen are shown in **Table 4.5** and are summarised graphically in **Figures 4.5 and 4.6.** Typical chromatograms are shown in **Figures 4.7 and 4.8.** The letters in the following description of the results correspond with those on **Figures 4.7 and 4.8.**

In rat plasma, three peaks were detected in sample extracts which were not in controls and were therefore assumed to be drug-related components. The major component (A) had the same elution distance as parent drug and was present at a concentration in excess of 100$\mu$g/ml. The second component (B) had a relative elution distance (relative to the elution distance of parent compound, RED) of 0.909 to 0.924 and its concentration was about 70 to 80$\mu$g/ml assuming a similar extinction coefficient to ibuprofen. These major components were eluted in the methanol eluent. A third component (C), present at about 15 to 20$\mu$g/ml had a RED of 0.939 to 0.955 and was eluted in the methanol/formic acid eluent. No peaks were detected in the methanol/ammonium acetate eluent.

In rat urine, no component was observed with the same elution distance as ibuprofen. A major component (D) with a RED of 0.890 to 0.897 was eluted in the methanol, and was present at a concentration in excess of 1mg/ml. A second major component (E) with a RED of 0.658 to 0.706 was eluted in both the methanol and methanol/formic acid eluents. The concentration of this component was also in excess of 1mg/ml. A third component (F) with a RED of 0.918 to 0.926 was eluted in the methanol/formic acid, and had a

**Table 4.5**

**Peaks detected by HPTLC/MMD of plasma and urine samples from rats and dogs after oral administration of ibuprofen**

| Sample | Peak Elution Distances, mm (Area) | |
|---|---|---|
| | First Analysis | Second Analysis |
| Spiked Plasma | (10μg/ml) 66 (30) | (100μg/ml) 66 (535) |
| Rat Plasma (MeOH) | 60 (443); 66 (789) | 61 (386), 66 (835) |
| Rat Plasma (MeOH/FA) | 62 (77) | 63 (93) |
| Rat Plasma (MeOH/$NH_4Ac$) | No peaks detected | No peaks detected |
| Spiked Urine (100μg/ml) | 73 (28) | 68 (132) |
| Rat Urine (MeOH) | 48 (598); 65 (707) | 48 (563); 61 (1873) |
| Rat Urine (MeOH/FA) | 24 (64); 48 (353); 67 (89) | 48 (701); 63 (265) |
| Rat Urine (MeOH/$NH_4Ac$) | No peaks detected | No peaks detected |
| Spiked Plasma (10μg/ml) | 66 (30) | 66 (80) |
| Dog Plasma (MeOH) | 60 (22); 66 (516) | 64 (485); 66 (760) |
| Dog Plasma (MeOH/FA) | 21 (62); 31 (385) | 32 (445) |
| Dog Plasma (MeOH/$NH_4Ac$) | 28 (66) | 28 (42) |
| Spiked Urine (100μg/ml) | 73 (28) | 67 (92) |
| Dog Urine (MeOH) | 48 (1190); 64 (379); 73 (62) | 48 (2320); 58 (817); 66 (131) |
| Dog Urine (MeOH/FA) | 47 (255) | 47 (155) |
| Dog Urine (MeOH/$NH_4Ac$) | 15 (1528) | 16 (1738) |

**Figure 4.5**

**Summary of peaks detected by HPTLC/MMD of rat plasma and urine samples obtained after oral administration of ibuprofen at a dose-level of 100mg/kg**

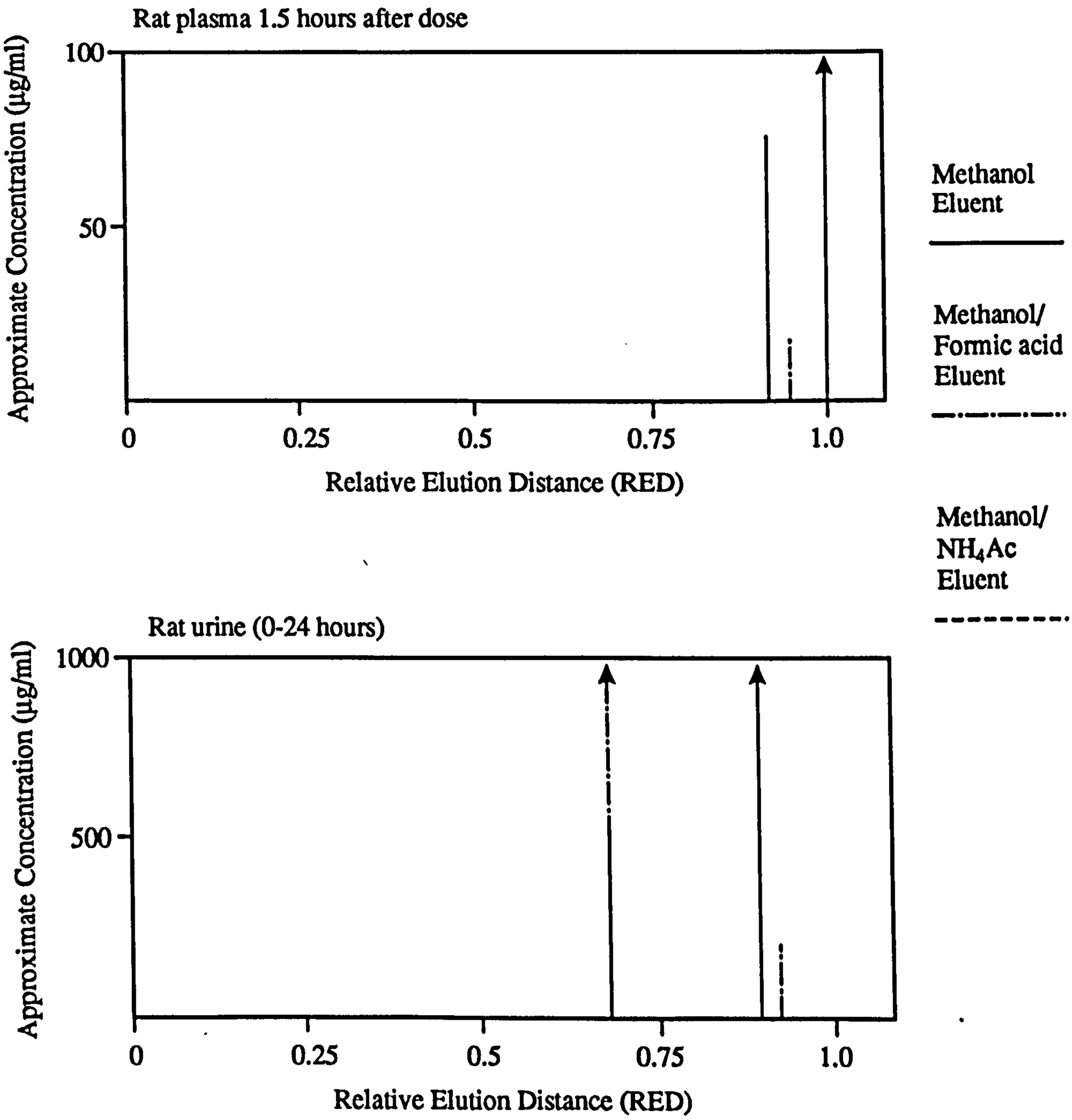


**Figure 4.6**

**Summary of peaks detected by HPTLC/MMD of dog plasma and urine samples obtained after oral administration of ibuprofen at a dose-level of 50mg/kg**

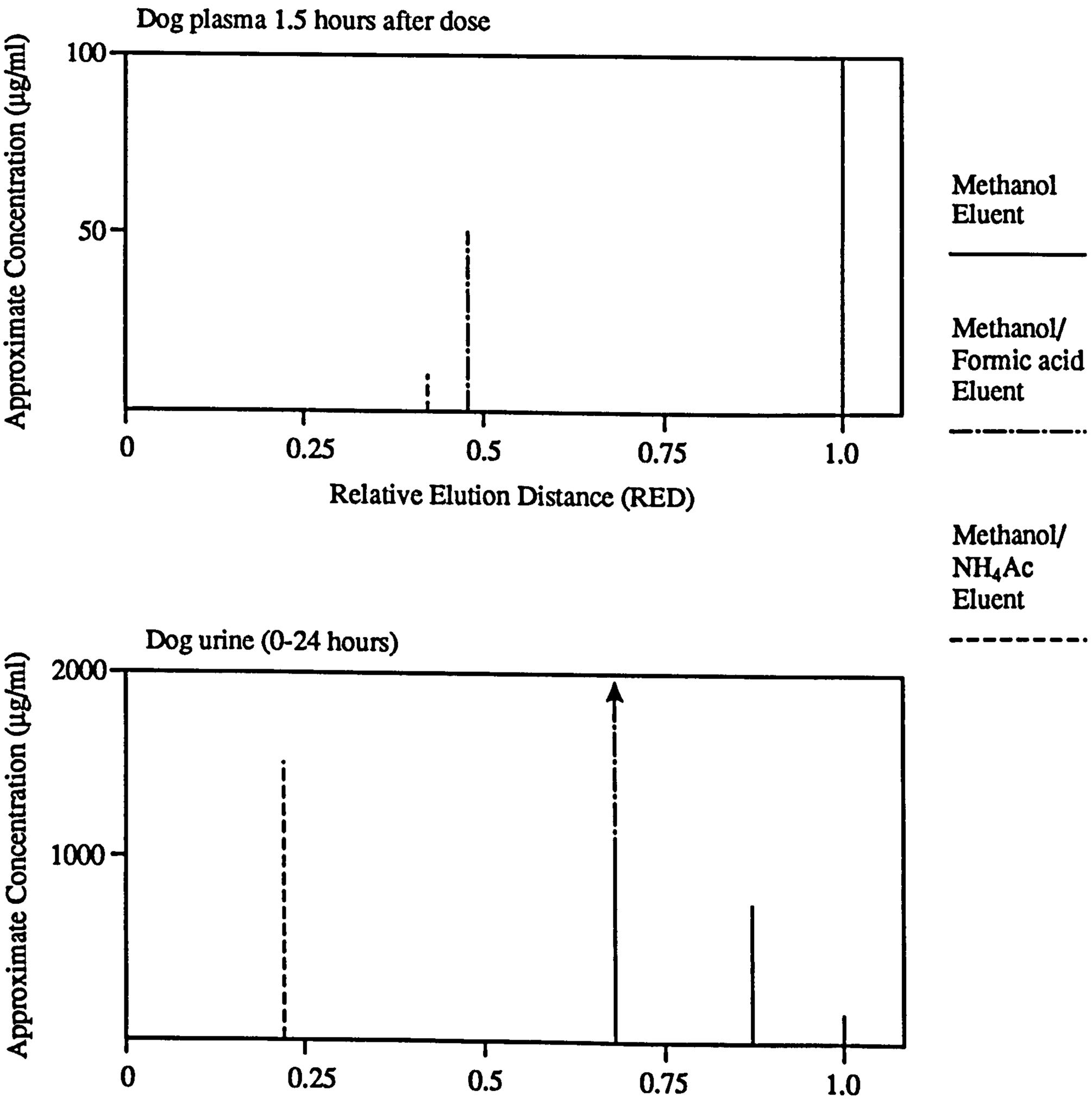


**Figure 4.7**

**Typical HPTLC/MMD chromatograms obtained from rat plasma and urine after oral administration of ibuprofen at a dose-level of 100mg/kg**

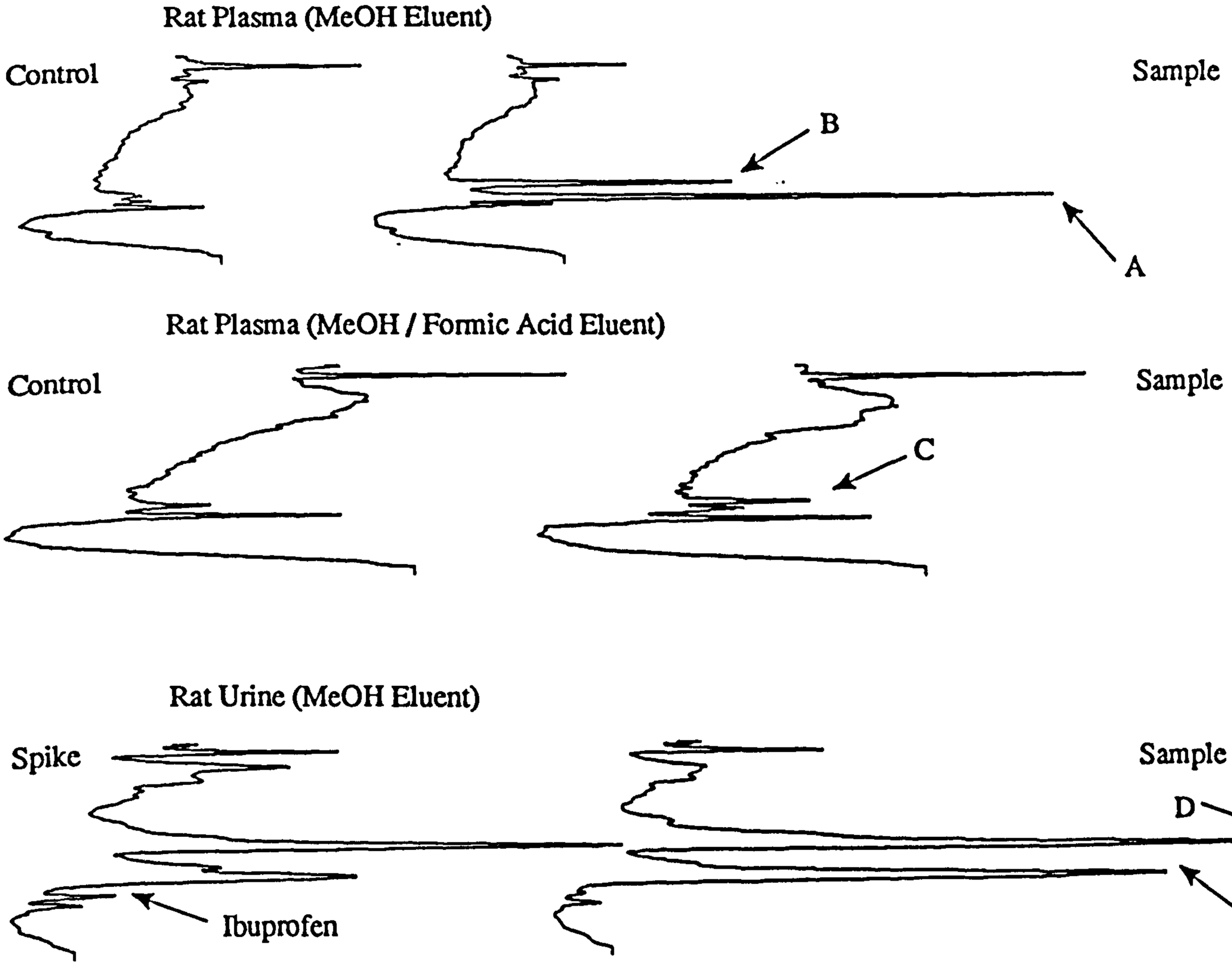
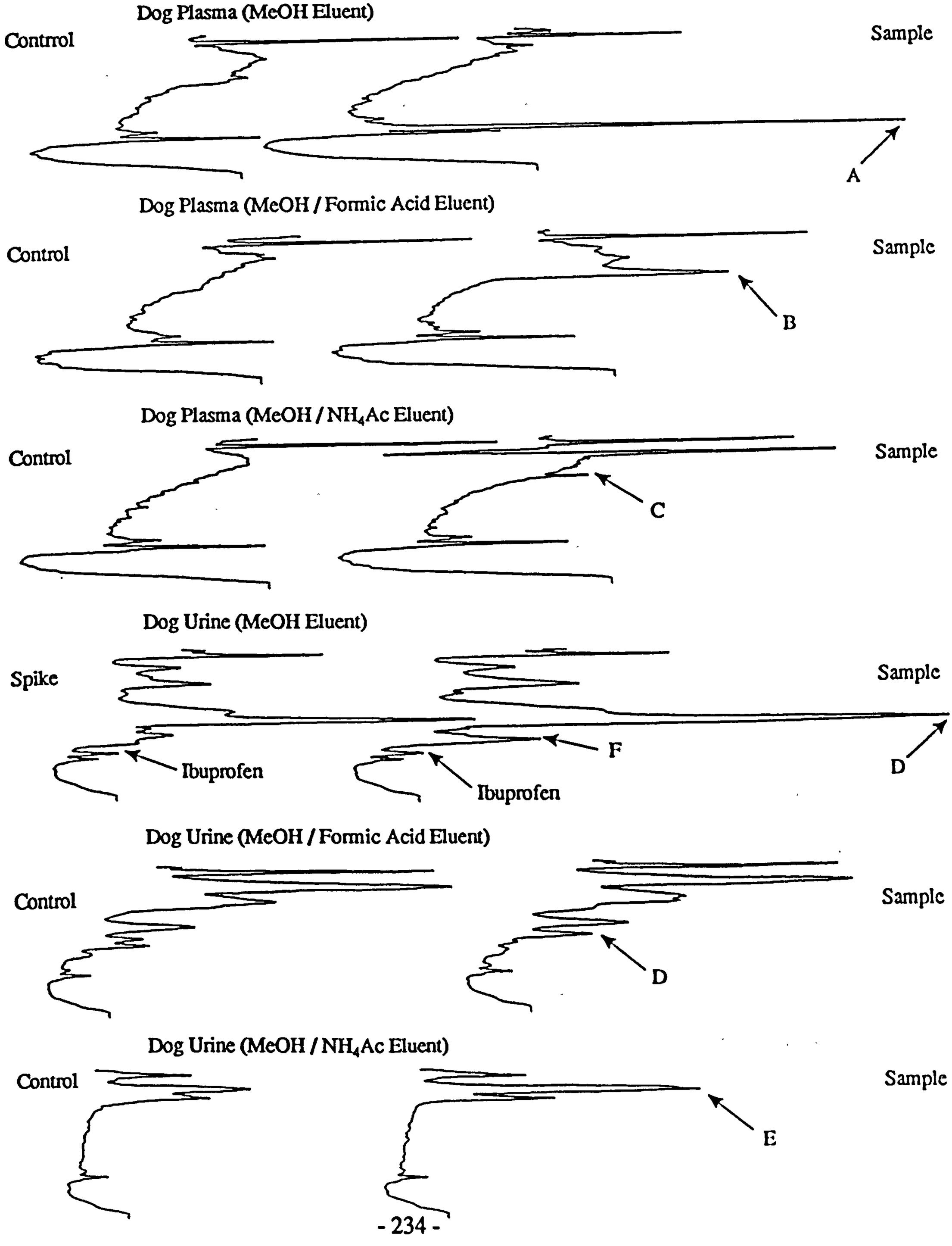


**Figure 4.8**

**Typical HPTLC/MMD chromatograms obtained from dog plasma and urine after oral administration of ibuprofen at a dose-level of 50mg/kg**



concentration of about 200$\mu$g/ml. No peaks were detected in the methanol/ammonium acetate eluent. An additional peak in the methanol/formic acid eluent in the first analysis was not detected in the second analysis.

In dog plasma, two major peaks were detected in the first analysis, but one of the peaks split, giving three components in the second analysis. The major peaks common to both analyses were one with an elution distance equal to ibuprofen (A) and a concentration of about 100$\mu$g/ml, and a second (B) with a RED of 0.470 to 0.485, present at about 50$\mu$g/ml. The first component eluted in the methanol eluent and the second in the methanol/formic acid. In addition to two major components observed in both analyses, a minor component (C) was observed in the methanol/ammonium acetate eluent with a RED of 0.424, at a concentration of about 10$\mu$g/ml.

In the second analysis, a component was detected in the methanol eluent with a RED of 0.970, at a concentration of about 50$\mu$g/ml which was absent in the first analysis. It seemed unlikely that this component was drug-related, and apparently being present at such high concentrations, was not observed in the first analysis. The peak may have been due to a defect in the surface of the HPTLC plate or to a change in the constituents of plasma with time. A peak with a RED of 0.318 was observed in the methanol/formic acid eluent in the first analysis at a low concentration, however was not detected in the second analysis.

The major component in dog urine (D) had a RED of 0.658 to 0.706 (the same as component E in rat urine) and was eluted in the methanol and methanol/formic acid eluents. The concentration of this component was in excess of 2mg/ml. A second component (E) with a RED of 0.205 to 0.239 was eluted in the methanol/ammonium

acetate and was present at a concentration of 1400 to 1600μg/ml. Another component (F) with a RED of 0.866 to 0.877 was eluted in the methanol and was present at a concentration of 700 to 800μg/ml. A small amount of material with the same elution distance as ibuprofen was detected at a concentration of 100 to 200μg/ml. This was eluted in the methanol.

There was broad agreement in the results obtained between the two analyses. All of the major components would have been characterised similarly on both occasions. The only differences were some additional peaks which may have been due to plate irregularities or sample matrix changes.

### 4.3.3.2 Loxtidine

The peaks detected following HPTLC/MMD analysis of the samples obtained after administration of loxtidine are shown in **Table 4.6** and are summarised graphically in **Figures 4.9 and 4.10.** Typical chromatograms are shown in **Figure 4.11.** The letters in the following description of the results correspond with those on **Figure 4.11.**

In rat plasma, two components were detected in the methanol eluent during the first analysis, and three during the second. The components found in the first analysis were both present at concentrations of less than 5μg/ml as would be expected from the radioactivity measurement (7.2μg/ml). The components had RED values of (A) 1.0 and (B) 0.681. In the second analysis, the same components were detected (REDs 1.0 and 0.723), but at much higher concentrations which may indicate that interfering

**Table 4.6**

**Peaks detected by HPTLC/MMD of plasma and urine samples from rats and dogs after oral administration of loxtidine**

| Sample | Peak Elution Distances, mm (Area) | |
|---|---|---|
| | First Analysis | Second Analysis |
| Spiked Plasma (10μg/ml) | 47 (290) | 47 (65) |
| Rat Plasma (MeOH) | 32 (105); 47 (103) | 21 (375); 34 (700)<br>47 (53) |
| Rat Plasma (MeOH/FA) | No peaks detected | No peaks detected |
| Rat Plasma (MeOH/$NH_4Ac$) | No peaks detected | No peaks detected |
| Spiked Urine (100μg/ml) | 45 (88) | 45 (60) |
| Rat Urine (MeOH) | 31 (11)*; 46 (251) | 31 (30)*; 45 (539) |
| Rat Urine (MeOH/FA) | No peaks detected | No peaks detected |
| Rat Urine (MeOH/$NH_4Ac$) | 17 (124)*; 24 (96) | 25 (74) |
| Spiked Plasma (10μg/ml) | 47 (290) | 45 (65) |
| Dog Plasma (MeOH) | 13 (180); 46 (137) | 17 (16); 22 (113);<br>45 (17) |
| Dog Plasma (MeOH/FA) | No peaks detected | No peaks detected |
| Dog Plasma (MeOH/$NH_4Ac$) | No peaks detected | No peaks detected |
| Spiked Urine (100μg/ml) | 45 (88) | 45 (73) |
| Dog Urine (MeOH) | 16 (689); 46 (170) | 17 (749); 46 (336) |
| Dog Urine (MeOH/FA) | No peaks detected | No peaks detected |
| Dog Urine (MeOH/$NH_4Ac$) | No peaks detected | No peaks detected |

* Peaks detected after scanning at 250nm, sens/span 220.

**Figure 4.9**

**Summary of peaks detected by HPTLC/MMD of rat plasma and urine samples obtained after oral administration of loxtidine at a dose-level of 50mg/kg**

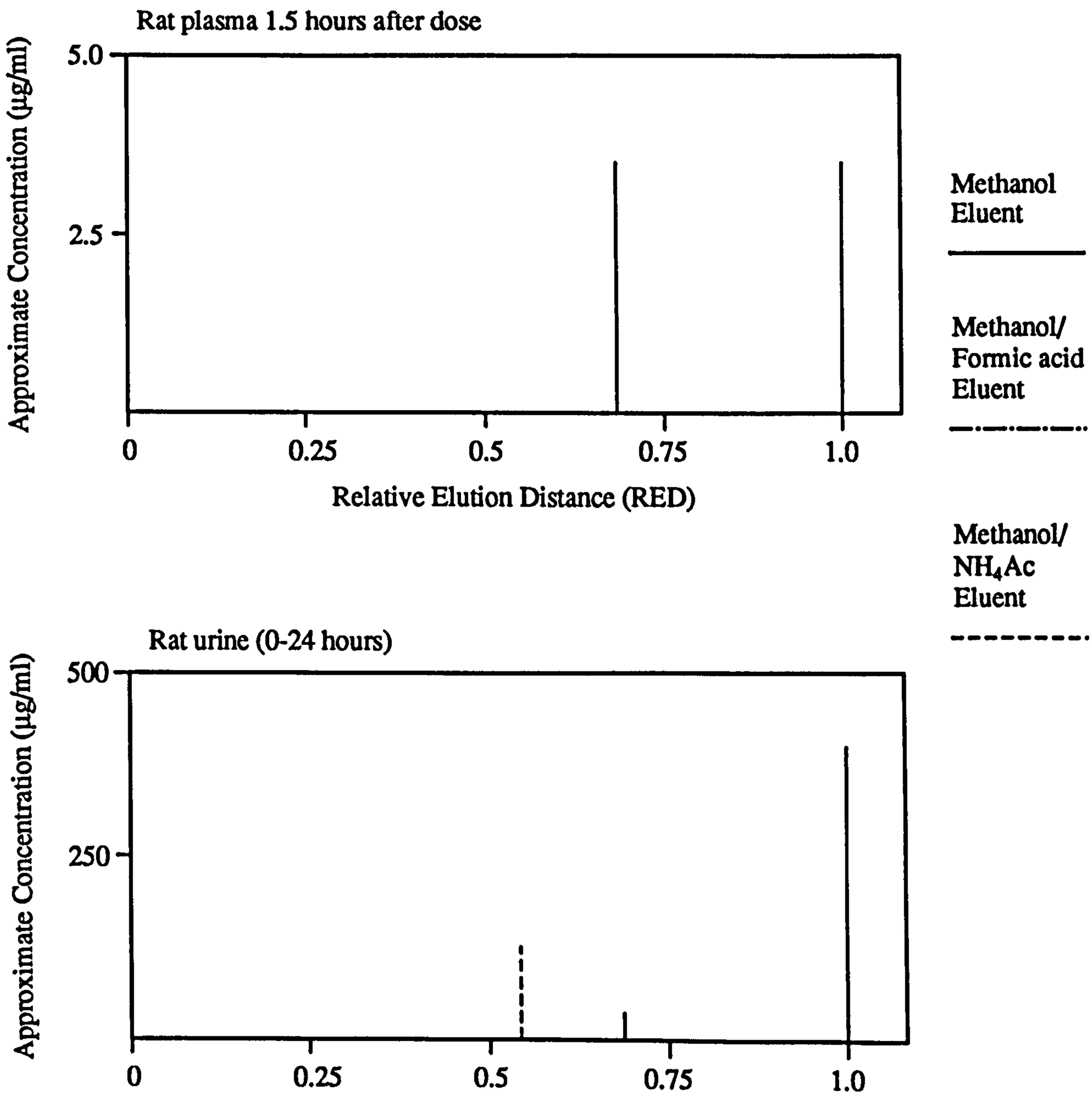


**Figure 4.10**

**Summary of peaks detected by HPTLC/MMD of dog plasma and urine samples obtained after oral administration of loxtidine at a dose-level of 50mg/kg**

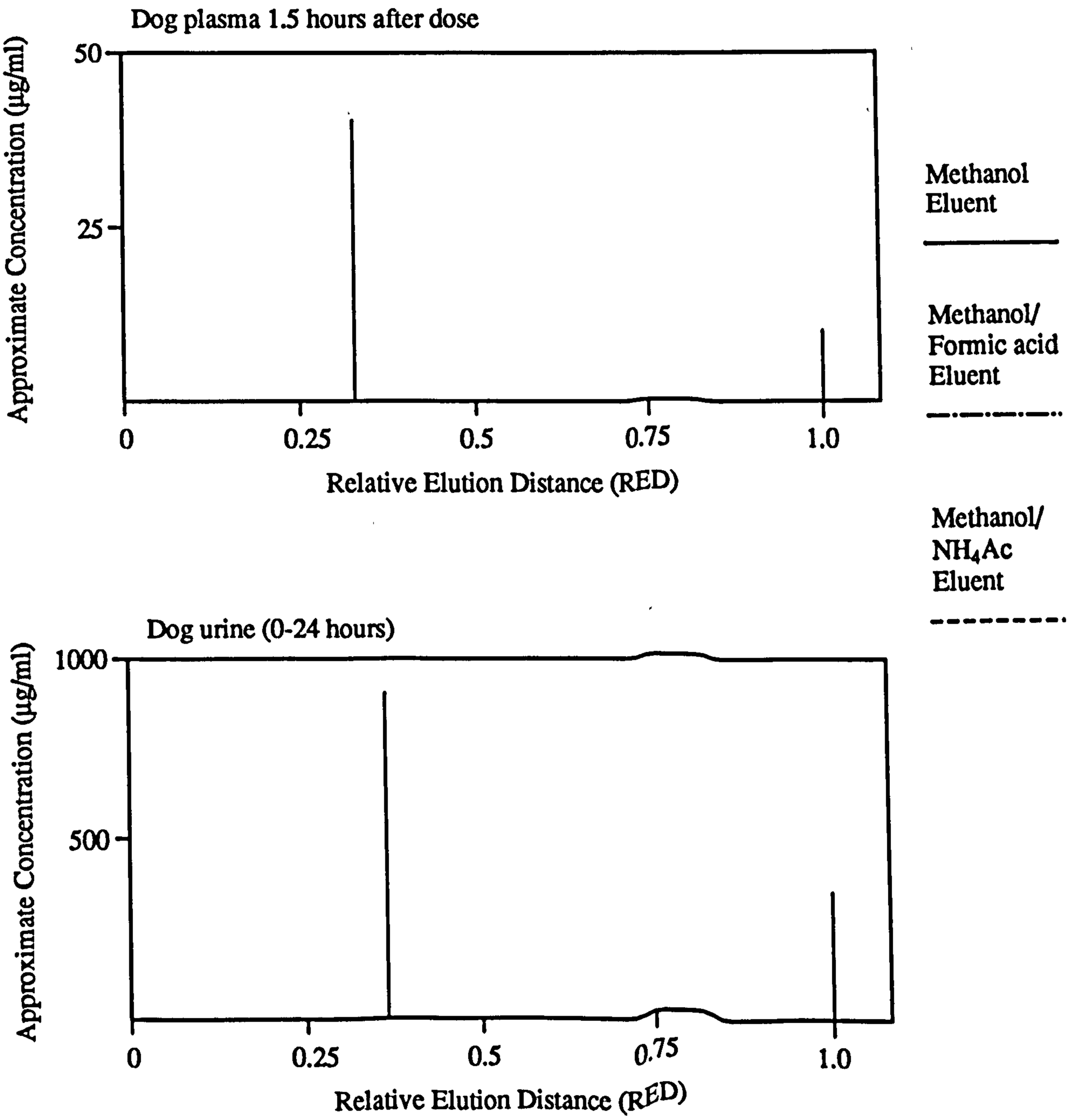


**Figure 4.11**

**Typical HPTLC/MMD chromatograms obtained from rat and dog plasma and urine after oral administration of loxtidine at a dose-level of 50mg/kg**

components were also present. A third component was detected in the second analysis with a RED of 0.447 which was not detected during the first analysis. Nothing was detected in the methanol/formic acid or methanol/ammonium acetate eluents.

The major component of rat urine had an RED of 1.0 and was present at a concentration of about 300 to 500μg/ml. This component eluted in the methanol. A small amount of material with a RED of 0.689 also eluted in the methanol at a concentration of less than 50μg/ml, which was only detected at 250nm. No peaks were detected in the methanol/formic acid eluent. A component (C) eluted in the methanol/ammonium acetate eluent with a RED of 0.522 to 0.566 at a concentration of 100 to 120μg/ml. Another peak was detected in the methanol/ammonium acetate eluent (RED 0.378) during the first analysis, but was not detected in the second analysis.

In dog plasma and urine two components were detected. These had REDs of 1.0 and 0.277 to 0.378, and were both eluted in the methanol eluent. In plasma, the concentration of the component with the same elution distance as loxtidine was 5 to 10μg/ml which is consistent with previous data (Bradbury *et al.*, 1983). The concentration of the other component (D) was greater. In urine, the major component (D) had a RED of 0.356 to 0.378 and a concentration of 800μg to 1mg/ml. The concentration of the component, co-eluting with loxtidine, was 200 to 500μg/ml

In dog plasma a third component was detected during the second analysis which was not present during the first analysis. The same material was also detected in rat plasma during the second analysis but not during the first analysis. This compound could therefore have been a breakdown product of an endogenous component formed either during storage or freeze-thaw cycles.

There was broad agreement in the results obtained between the two analyses, although there was some difference in absolute areas obtained between analyses which may have been due to the detector lamp alignment or inaccuracies in positioning the spots onto the HPTLC plates. All of the major components would have been characterised similarly on both occasions.

#### 4.3.3.3 Antipyrine

The peaks detected following HPTLC/MMD analysis of the samples obtained after administration of antipyrine are shown in **Table 4.7** and are summarised graphically in **Figure 4.12.** Typical chromatograms are shown in **Figure 4.13.** The letters in the following description of the results correspond with those on **Figure 4.13.**

In rat plasma, two components were eluted in the methanol. These had REDs of (A) 1.020 to 1.065 and (B) 0.824 to 0.870, being present at concentrations of 60 to 70 $\mu$g/ml and about 20 $\mu$g/ml respectively. Two components eluted in the methanol/formic acid with REDs of (E) 0.235 to 0.304 and (D) 0.314 to 0.413. The concentrations of these components were about 5 $\mu$g/ml and <1 $\mu$g/ml respectively. Two components were also eluted in the methanol/ammonium acetate eluent with REDs of (C) 0.588 to 0.644 and 0.765 to 0.866, and concentrations of 4 to 10 $\mu$g/ml, the latter having the same RED as component B. A component with a RED of 1.059 to 1.087 was detected in the methanol/ammonium acetate eluent at a concentration of about 3 $\mu$g/ml in the first

**Table 4.7**

**Peaks detected by HPTLC/MMD of plasma and urine samples from rats after oral administration of antipyrine**

| Sample | Peak Elution Distances, mm (Area) | |
|---|---|---|
| | First Analysis | Second Analysis |
| Spiked Plasma (10 $\mu$g/ml) | 51 (316) | 46 (544) |
| Rat Plasma (MeOH) | 42 (930); 52 (3535) | 40 (1003); 49 (3651) |
| Rat Plasma (MeOH/FA) | 12 (320); 16 (30) | 14 (280); 19 (20) |
| Rat Plasma (MeOH/$NH_4Ac$) | 30 (112); 39 (386)<br>54 (92) | 31 (148); 38 (293);<br>50 (672) |
| Spiked Urine (100 $\mu$g/ml) | 51 (316) | 47 (455) |
| Rat Urine (MeOH) | 10 (907); 31 (50);<br>43 (774); 50 (272);<br>55 (20) | 11 (2900); 32 (127);<br>42 (2127); 48 (946) |
| Rat Urine (MeOH/FA) | 11 (828); 17 (91) | 13 (700); 19 (183) |
| Rat Urine (MeOH/$NH_4Ac$) | 22 (46); 33 (1036);<br>42 (1710); 56 (1668) | 34 (1098); 41 (2050);<br>53 (2224); 61 (99) |

**Figure 4.12**

**Summary of peaks detected by HPTLC/MMD of rat plasma and urine samples obtained after oral administration of antipyrine at a dose-level of 250mg/kg**

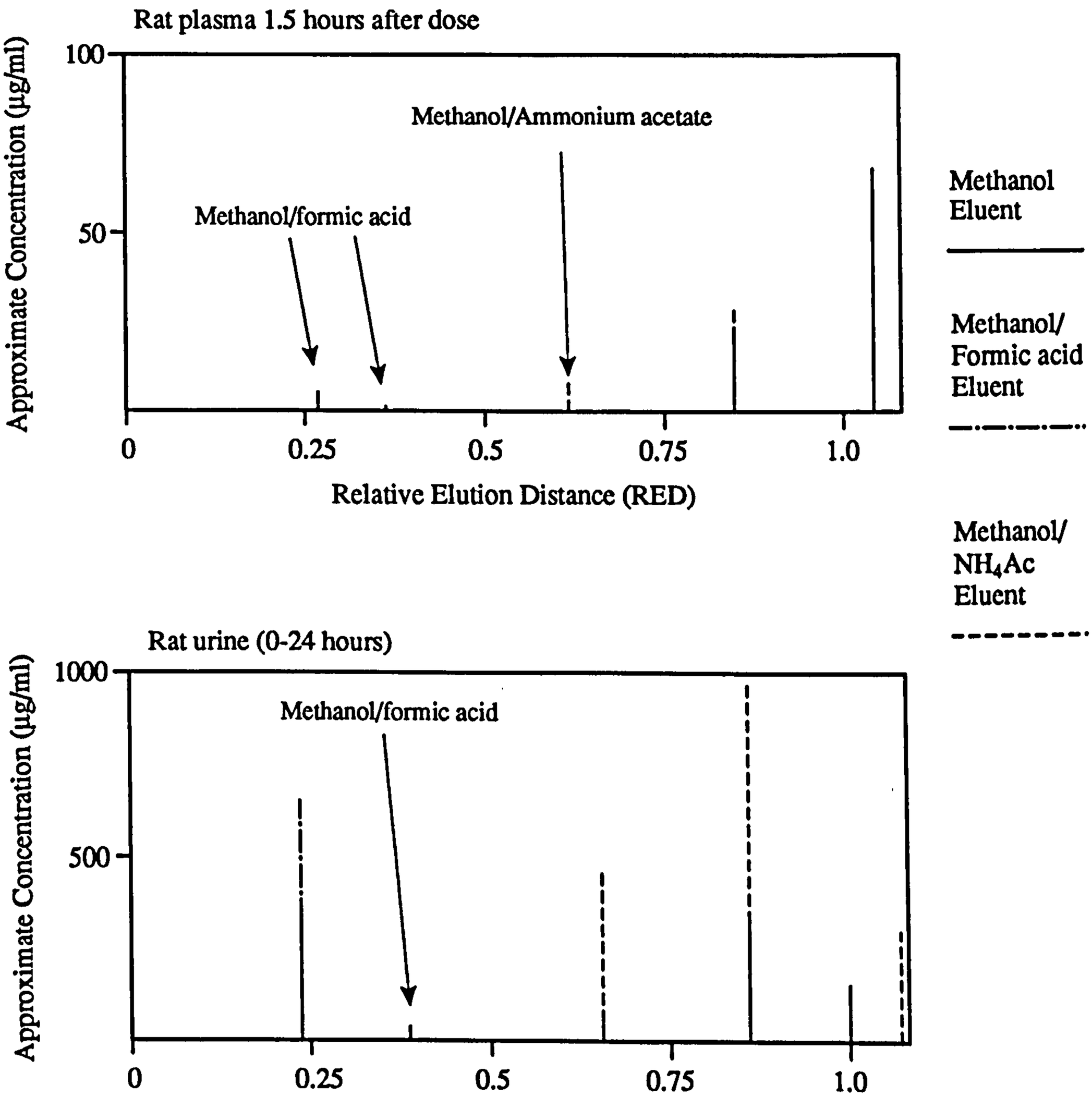
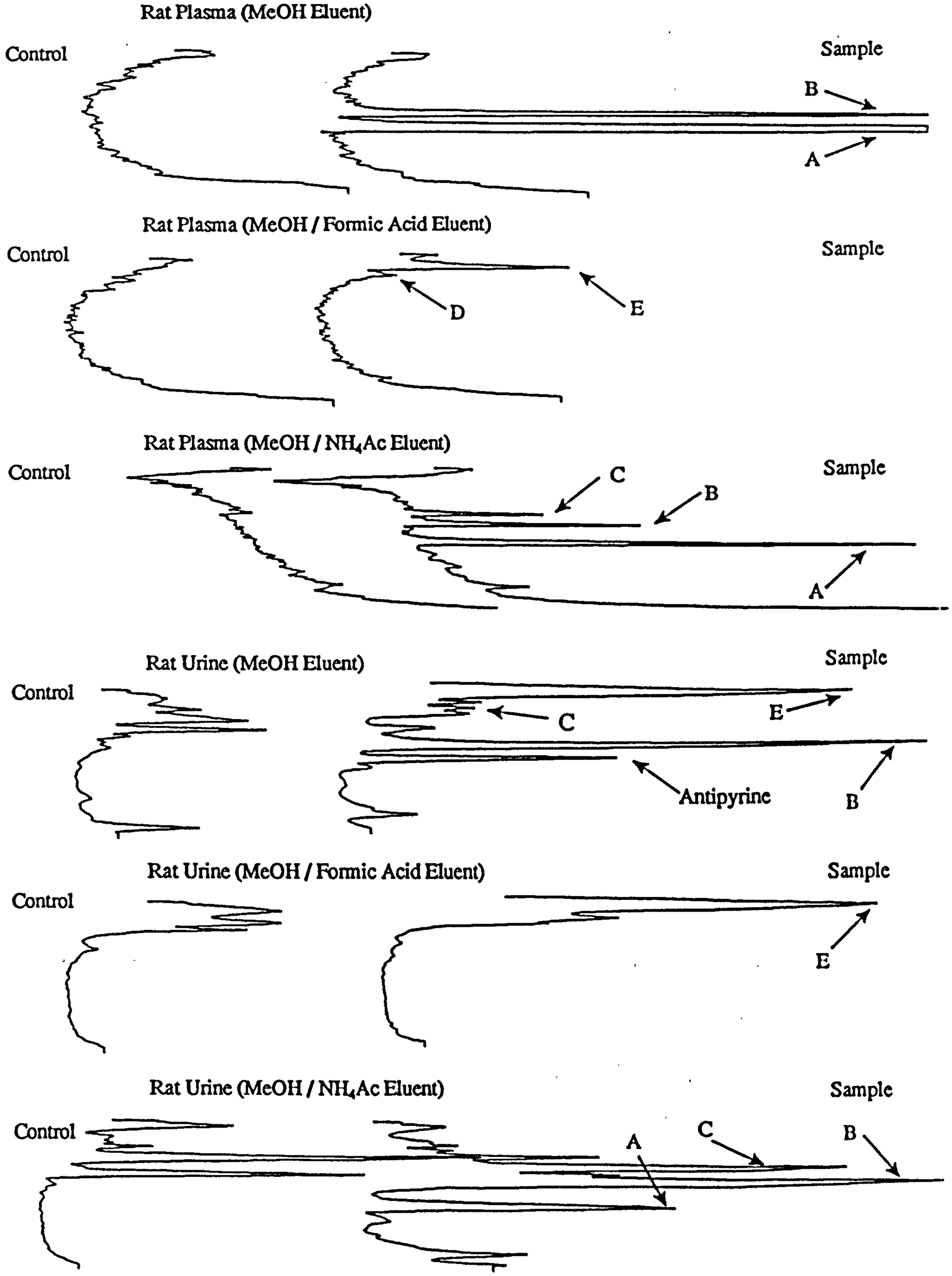


**Figure 4.13**

**Typical HPTLC/MMD chromatograms obtained from rat plasma and urine after oral administration of antipyrine at a dose-level of 250mg/kg**



analysis and 20μg/ml in the second analysis. This had the same RED as component A.

In rat urine, six components were detected, some of which were eluted in more than one eluent. The major components had REDs of (E) 0.196 to 0.277 and (B) 0.824 to 0.894 and were present at concentrations of 700 to 1000μg/ml respectively. These components were both eluted in two eluents, the first in methanol and methanol/formic acid and the second in methanol and methanol/ammonium acetate. Two other components with concentrations of 400 to 800μg/ml had REDs of (C) 0.608 to 0.703 and (A) 1.098 to 1.128. The first of these was eluted in the methanol and methanol/ammonium acetate eluents, and the second was eluted in methanol/ammonium acetate only. A further component with a RED of 0.98 to 1.021 was eluted in methanol at a concentration of 100 to 200μg/ml, and the final component with a RED of 0.333 to 0.444 was eluted in methanol/formic acid at a concentration of <50μg/ml.

There was good agreement in the results obtained between the two analyses. All of the major components would have been characterised similarly on both occasions. One additional peak was detected during the second analysis in the methanol/ammonium acetate eluent but was smaller than the remaining peaks.

#### 4.3.3.4 Diazepam

The peaks detected following HPTLC/MMD analysis of the samples obtained after administration of diazepam are shown in **Table 4.8** and are summarised graphically in **Figure 4.14.** Typical chromatograms are shown in **Figure 4.15.** The letters in the following description of the results correspond with those on **Figure 4.15.**

**Table 4.8**

**Peaks detected by HPTLC/MMD of plasma and urine samples from rats after oral administration of diazepam**

| Sample | Peak Elution Distances, mm (Area) | |
|---|---|---|
| | First Analysis | Second Analysis |
| Spiked Plasma (10μg/ml) | 55 (885) | 55 (788) |
| Rat Plasma (MeOH) | 19 (186); 32 (34);<br>47 (152); 50 (16);<br>55 (89) | 18 (109); 34 (53);<br>46 (244); 50 (33);<br>56 (75) |
| Rat Plasma (MeOH/FA) | 12 (93) | 12 (40) |
| Rat Plasma (MeOH/$NH_4Ac$) | 19 (197) † | 18 (159) † |
| Spiked Urine (100μg/ml) | 55 (895) | 55 (723) |
| Rat Urine (MeOH) | 18 (101) | 18 (144) |
| Rat Urine (MeOH/FA) | No peaks detected | No peaks detected |
| Rat Urine (MeOH/$NH_4Ac$) | 18 (990) † | 18 (576) † |

† Single peak also detected following development using MMD Scheme 7

**Figure 4.14**

**Summary of peaks detected by HPTLC/MMD of rat plasma and urine samples obtained after oral administration of diazepam at a dose-level of 50mg/kg**

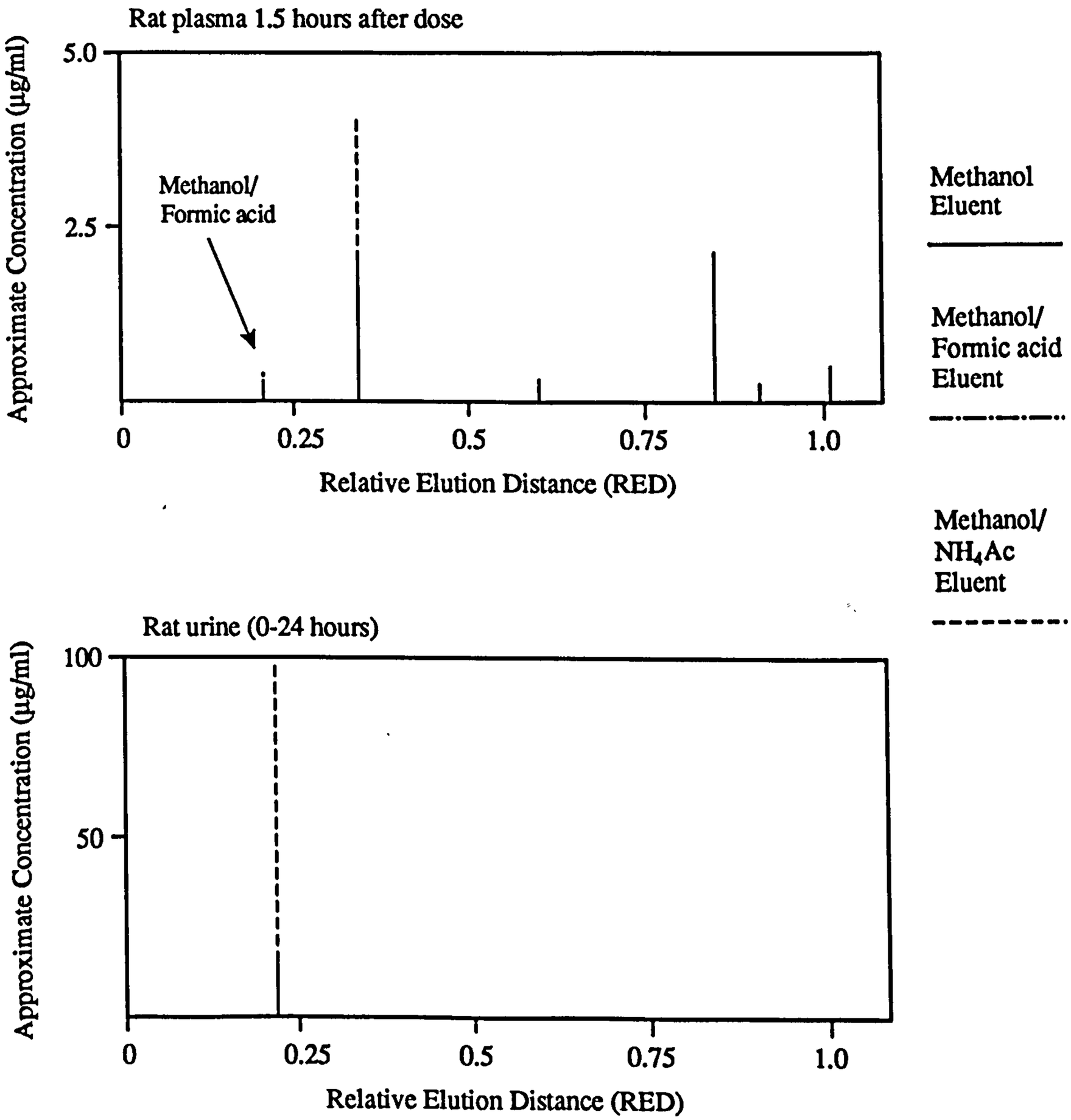


**Figure 4.15**

**Typical HPTLC/MMD chromatograms obtained from rat plasma and urine after oral administration of diazepam at a dose-level of 50mg/kg**

In rat plasma, the majority of the material detected was in the methanol eluent, five components being found on both occasions. The relative proportions varied slightly on each occasion, but all five components were present at concentrations of less than 5 μg/ml with most being less than 1 μg/ml. The major component during the first analysis (E) had a RED of 0.345 and was eluted in the methanol/ammonium acetate as well as the methanol eluent. In the second analysis this component was present at a lower concentration, but one other component (C) with a RED of 0.836 to 0.855 was present at a correspondingly higher concentration. This may indicate that a conjugated metabolite was present which was hydrolysed to a Phase I metabolite in plasma on storage or during the freeze-thaw process. Other components, all present at concentrations of less than 1 μg/ml had REDs of (A) 1.0 to 1.018, (B) 0.909 and (D) 0.582 to 0.618. One component (F) eluted in the methanol/formic acid with a RED of 0.218 at a concentration of less than 1 μg/ml.

In rat urine, only one peak (E) was observed at a RED of 0.327, which eluted partially in the methanol, but predominantly in the methanol/ammonium acetate eluent. The concentration was about 100 μg/ml which was in agreement with the levels of radioactivity measured in the urine. A single peak was detected after samples containing this component were analysed using an expanded linear gradient (MMD Scheme 7). Samples containing component E were developed using MMD Scheme 3 with 1% acetic acid or 1% TEA. The RED values were 0.323 and 0.292 respectively.

There was good agreement in the results obtained between the two analyses. All of the major components would have been characterised similarly on both occasions.

## *4.4 Discussion*

The aim of the studies described in this chapter was to investigate the utility of the method developed to screen for drugs and drug metabolites in biological fluids by analysing plasma and urine samples obtained after administration of drugs to animals. The results have shown that the method detected drugs and drug metabolites in all of the samples analysed. In the following discussion, the results from the present studies are interpreted using the data from Chapters 2 and 3 on relative elution distances (REDs) and solid-phase extraction with selective elution. The routes of metabolism predicted by the screening system are then compared with published data.

### 4.4.1 Ibuprofen

In the following discussion, the letters describing drug-related components correspond with those on **Figures 4.7 and 4.8.** The Roman numerals refer to the chemical structures in **Figure 4.1.**

Ibuprofen had an elution distance of 66mm and a sharp peak when MMD Scheme 3 with 1% acetic acid was used. This was a suitable elution distance to allow detection of the more polar metabolites. Being acidic, ibuprofen might have been expected to be eluted from the extraction cartridges in the methanol/formic acid eluent rather than the methanol eluent in which it was found. However, indole-3-carboxylic acid, one of the acidic compounds tested previously, was eluted in methanol so the presence of a carboxylic acid group does not guarantee retention on the extraction cartridge by an anion-exchange mechanism.

In the rat, circulating drug-related material comprised components with mean REDs of; (A) 1.0, (B) 0.917 and (C) 0.947. Components A and B were eluted in methanol and component C was eluted in methanol/formic acid. The concentrations showed that the relative abundance of these components was A > B > C. The system predicted that A was unchanged ibuprofen, both from its RED and it being eluted in methanol. The RED of B was consistent with a metabolite formed by aromatic hydroxylation, and this was supported by the compound's elution in methanol. Component C was eluted in methanol/formic acid and was therefore more strongly anionic than the parent compound. The RED did not suggest that the compound was a glucuronide conjugate, and the value was not as small as would have been expected for a metabolite formed by oxidation to a carboxylic acid. The structural change predicted by the RED value was aromatic hydroxylation, possibly in a position which caused the carboxylic acid group present on the molecule to be more strongly anionic.

Unchanged ibuprofen was not detected in rat urine. Three main components detected had mean REDs of (D) 0.894, (E) 0.682, (F) 0.922 the relative concentrations being E > D >> F. Component E was incompletely eluted by the methanol eluent, the remainder being eluted in methanol/formic acid. This suggested that it was a stronger anion than ibuprofen. The RED of the compound was consistent with a metabolite formed by oxidation to a carboxylic acid, probably aliphatic because the value was lower than would have been expected for an aromatic carboxylic acid. Intuitively from the structure of ibuprofen the most likely compound to be formed is that obtained by oxidation of the isopropyl group. Component D had a RED consistent with a metabolite formed by hydroxylation, probably aromatic, but was close to the range of REDs associated with aliphatic hydroxylation. The compound was eluted in the methanol which suggested that

the change in structure did not markedly alter the anionic strength of the carboxylic acid group. A likely position for hydroxylation was on the tertiary butyl group especially as two aromatic hydroxylations had already been postulated for components found in the plasma. The relatively large RED could in that case be explained by the steric hindrance around the hydroxyl group caused by the tertiary butyl. This would reduce interaction with the silica layer. The third component (F) had a RED consistent with a metabolite formed by aromatic hydroxylation and its elution in the methanol/formic acid indicated that hydroxylation occurred at a position which increased the anionic strength of the carboxylic acid group.

The extraction data combined with the RED values indicated that components B and D may have been the same and that C and F may have been also. The RED values differed slightly, which could have been due to the different endogenous components present in the plasma and urine extracts affecting the chromatography. The overall metabolic pattern for ibuprofen in the rat indicated by the method was therefore Phase I metabolism by aliphatic hydroxylation of the tertiary butyl group and oxidation of the tertiary butyl group to a carboxylic acid, with a small amount of aromatic hydroxylation also occurring. The three metabolites formed were found in the urine, but no unchanged ibuprofen was detected. The drug-related material circulating in the plasma comprised unchanged ibuprofen primarily with the aliphatic and aromatic hydroxylated metabolites also present. There was no evidence of the carboxylic acid metabolite in the plasma.

These data compared favourably with the published data (Mills *et al.*, 1973). Following oral administration of ibuprofen to Wistar rats at a dose-level of 20mg/kg, the major component in the plasma at 1.5 hours after dosing was ibuprofen (I, 24$\mu$g/ml) with the hydroxylated metabolite (II, 15$\mu$g/ml) and the carboxylic acid metabolite (III, 3.6$\mu$g/ml)

also present. Rat urine contained the hydroxylated metabolite (II, 42% of dose), the carboxylic acid metabolite (III, 18%) and small amounts of ibuprofen glucuronide (6%).

Thus, in rat plasma, unchanged ibuprofen was the major component with the aliphatic hydroxylated metabolite also present at high concentrations. The carboxylic acid metabolite was detected by Mills *et al.*, but at low concentrations, and they found no evidence for aromatic hydroxylation. The major urinary components found by Mills *et al.* were the aliphatic hydroxylated metabolite and the carboxylic acid metabolite which were also detected in the present studies. A small amount of ibuprofen glucuronide was found in the urine by Mills *et al.*, but was not detected in the present studies. Neither studies showed evidence for unchanged ibuprofen in the urine. In the present studies an aromatic hydroxylated metabolite was found which was not detected previously.

In the present studies, the pattern of metabolism in the dog was different to that found in the rat although there were some similarities. Three circulating drug-related components were detected with mean REDs of; (A) 1.0, (B) 0.478 and (C) 0.424, the relative concentrations being A > B > C. The method indicated that A was unchanged ibuprofen, both from its RED and it being eluted in methanol. Component B was eluted in methanol/formic acid and was therefore more strongly anionic than ibuprofen, so could have been a glucuronide conjugate. The RED value was greater than would have been expected of a glucuronide conjugate relative to a phenolic precursor, however larger REDs might be expected for a glucuronide relative to a carboxylic acid precursor. The reason for this is that the carboxylic acid would have a smaller elution distance than its corresponding alcohol or phenol. Component C was eluted in methanol/ammonium acetate which indicated that it was strongly anionic, possibly a sulphate. The RED

although a little larger than expected could have been indicative of a sulphate in this case for the reasons discussed above.

A small amount of ibuprofen was detected in dog urine. Three other components were present with REDs of; (D) 0.682, (E) 0.222 and (F) 0.872, the relative concentrations being D > E > F. Components D and F corresponded to the metabolites formed by oxidation of the tertiary butyl group to a carboxylic acid and aliphatic hydroxylation of the same group respectively. The reasons for these assignments were as described for the rat samples. Component E had a RED which was characteristic of a glucuronide conjugate, however it was eluted in methanol/ammonium acetate which suggested it may have been a sulphate. The method was not clear in this case because either a glucuronide or sulphate of the tertiary butyl alcohol could have been formed theoretically. In the case of a glucuronide with a carboxylic acid also present the strongly anionic nature might mean that formic acid was not a sufficiently strong anion for the compound to be eluted in methanol/formic acid. From this and the RED value, the most likely structure of this component was a glucuronide.

Thus, the screening method indicated that unchanged ibuprofen was the major circulating drug-related component and that glucuronides were also present. The urinary data showed that both Phase I and Phase II metabolism occurred in the dog, unlike the rat.

These data are in broad agreement with the published data (Mills *et al.*, 1973). Following oral administration of ibuprofen to Beagle dogs at a dose-level of 8mg/kg, only ibuprofen (35$\mu$g/ml) was detected in the plasma at 1.5 hours after dosing. Dog urine contained predominantly glucuronide conjugates of II (23% of dose) and III (13%) with some non-conjugated II (9%) and ibuprofen glucuronide (7%).

HPTLC/MMD of plasma and urine obtained after administration of ibuprofen detected the main metabolites of ibuprofen. In addition the method showed that whereas Phase I metabolism predominated in the rat resulting in high concentrations of circulating Phase I metabolites, in the dog Phase II metabolism also occurred. There were no circulating Phase I metabolites in the dog, but a component considered to be ibuprofen glucuronide was found at high concentrations. The data from the present studies were in broad agreement with reported data, and an additional metabolite was found in the rat. This may have been formed following administration of ibuprofen at a high dose-level when the routes of metabolism which occur at lower dose-levels may be saturated. The dose-level in the present studies was 100mg/kg compared with 8mg/kg in the published data.

### 4.4.2 Loxtidine

In the following discussion, the letters describing drug-related components correspond with those on **Figure 4.11.** The Roman numerals refer to the chemical structures in **Figure 4.2.**

Loxtidine is a basic molecule which interacts strongly with silica and hence a MMD gradient with TEA was required for it to migrate a reasonable distance on a HPTLC plate. An elution distance of 47mm was achieved with the gradient used, which enabled separation from metabolites to occur and the more polar metabolites to be detected. Loxtidine was eluted from the extraction cartridge in the methanol eluent as would be predicted from its structure which contains no anionic groups.

In the rat, the concentration of circulating drug-related material was low, nevertheless two components were detected at concentrations of less than 5 $\mu$g/ml. These components had mean REDs of (A) 1.0 and (B) 0.702. Component A was considered to be unchanged loxtidine from its elution distance and it being eluted in the methanol eluent. The RED of component B suggested that it was a carboxylic acid metabolite, however its elution in methanol indicated that it did not have a very strong anionic group. An acidic metabolite of loxtidine would however be zwitterionic, possessing both anionic and cationic groups, which might combine to prevent retention by an anion-exchange mechanism. By inspection of the loxtidine molecule it is apparent that carboxylic acid formation can take place either by N-dealkylation to form the propionic acid derivative, or by oxidation of the methanol group. Oxidation of a methanol group was shown to produce REDs of about 0.8 (Chapter 2), however the RED of component B was 0.7. Thus, N-dealkylation was considered the more likely route as it would result in a greater change in the polarity of the molecule than would be achieved by methanol oxidation.

The major drug-related component in rat urine was considered to be loxtidine from its elution distance and it being eluted in methanol. Other components were detected with REDs of (C) 0.689 and (D) 0.544. Component C was considered to be the propionic acid metabolite formed by N-dealkylation, corresponding to component B in the plasma. Component D eluted in the methanol/ammonium acetate and therefore had greater anionic strength than the propionic acid derivative, possibly being a sulphate. The RED did not suggest that the compound was a sulphate metabolite because it was larger than would be expected, however on a molecule such as loxtidine which itself interacts strongly with silica, sulphation may result in smaller changes in retention. The identity of component D could not therefore be predicted unambiguously by the screening method from the data obtained.

In summary, the screening method indicated that Phase I metabolism of loxtidine predominated in the rat, with the propionic acid metabolite formed by N-dealkylation being the primary metabolite. Small amounts of a more anionic metabolite were also found in the urine.

In previous studies, the concentration of unchanged loxtidine in the plasma of male rats at 1 hour after oral administration at a dose-level of 50mg/kg was 5.2μg/ml (Jenner, 1988), however the identity of any other drug-related material circulating in the plasma has not been examined. The concentration of unchanged loxtidine measured in rat plasma during the present studies was thus consistent with previous data, and whereas the identity of the circulating metabolites had not been identified previously, the present studies indicated that the main component was the propionic acid derivative.

The major components of 0-24 hour urine following oral administration of loxtidine to rats at a dose-level of 50mg/kg are loxtidine (75% of urinary radioactive material) and the propionic acid derivative (II) formed by N-dealkylation of loxtidine (15%). Small amounts of the carboxylic acid metabolite (V, <5%), a phenolic metabolite (IV, <1%), loxtidine glucuronide (VI, <5%) and the phenolic glucuronide (VII, <5%) are also present (Bell *et al.*, 1983). The data from the present studies were in broad agreement with previous data, although the detailed metabolic information determined by radiolabelled studies was not shown by the screening method.

In the present studies, only two components were found in dog plasma and urine, one of which was considered to be unchanged loxtidine. The other component was present in plasma and urine in greater amounts than loxtidine, and had a mean RED of 0.328. The

RED suggested that the compound was either a glucuronide or sulphate conjugate, however it was eluted in methanol and therefore did not have strong anionic characteristics. As discussed previously however, a glucuronide or sulphate metabolite of loxtidine would be zwitterionic and may not be retained by anion-exchange. A sulphate group is a very strong anion, and would be unlikely to show no anionic character, therefore it is more likely that this compound was a glucuronide. The method could not indicate more specifically the precise glucuronide metabolite formed.

The data from the present studies were similar to previous results. Following oral administration of loxtidine to dogs at a dose-level of 50mg/kg, levels of radioactive material in the plasma at 1 hour after dosing were about 50$\mu$g/ml (Bradbury *et al.*, 1983). The concentrations of unchanged loxtidine were about 15$\mu$g/ml, the majority of the radioactive material comprising polar material. The major components of 0 to 24 hour urine following oral administration of loxtidine to dogs at a dose-level of 50mg/kg were loxtidine (20% of urinary radioactive material) and loxtidine glucuronide (up to 75%).

In summary, the screening method indicated that loxtidine was metabolised in the dog primarily by conjugation to a glucuronide. The data obtained from the dog samples were in agreement with the published metabolism of loxtidine in the dog (Bell *et al.*, 1983). The concentrations of loxtidine measured in the plasma were similar to previous concentrations measured (Bradbury *et al.*, 1983), and the screening method confirmed that the metabolite circulating in dog plasma was the same as that excreted in the urine.

### 4.4.3 Antipyrine

In the following discussion, the letters describing drug-related components correspond with those on **Figure 4.13.** The Roman numerals refer to the chemical structures in **Figure 4.3.**

Antipyrine eluted to a distance of between 46 and 51mm using MMD Scheme 13, which was a suitable distance to allow more polar metabolites to be separated and detected. The drug was eluted from the extraction cartridges in methanol as would be predicted from its structure which contains no anionic groups.

Unchanged antipyrine was not detected in rat plasma. This may have been due to poor separation from a large component (A) which had a mean RED of 1.043. Four other components were present in the plasma at lower concentrations than A, and had REDs of; (B) 0.847, (C) 0.616, (D) 0.364 and (E) 0.270. Component A was eluted mostly in methanol although some was detected in the methanol/ammonium acetate eluent. The RED of this component, being greater than 1.0, suggested that it was an N-demethylated metabolite (norantipyrine). Component B was also eluted in methanol and is therefore likely to have been a Phase I metabolite. Its RED of 0.847 was characteristic of aliphatic hydroxylation and it could therefore have been the 3-hydroxymethyl metabolite. Component C was eluted in methanol/ammonium acetate indicating that it may have been a sulphate metabolite. Its RED however did not support it being a sulphate, but could indicate that it was a metabolite formed by more than one Phase I oxidation. This did not explain why the compound was eluted in methanol/ammonium acetate unless it was present in plasma as a sulphate which was decomposed prior to analysis either on the HPTLC plate or during the drying down stage. Components D and E were present in

very small amounts and were eluted in methanol/formic acid which indicated that they were glucuronide conjugates. The RED of component E supported it being a glucuronide, but the RED of component D was more characteristic of a sulphate.

In rat urine, most components detected were eluted in more than one eluent, some in the methanol suggesting that they were Phase I metabolites and then also in methanol/ammonium acetate indicating that they were sulphates. The REDs of these components, however, were characteristic of Phase I metabolites. This gave further support to the suggestion that the sulphate metabolites of antipyrine were unstable, and subsequent to their extraction from biological fluids as sulphate conjugates, were hydrolysed to the Phase I metabolites from which they were formed.

The components detected in urine had similar REDs to those found in plasma, and in addition, unchanged antipyrine was also detected. The most abundant component in urine had a RED of 0.859, which was characteristic of a metabolite formed by aliphatic hydroxylation and may therefore have been the 3-hydroxymethyl metabolite, corresponding with component B in plasma. This was present in urine as the sulphate as well as the free Phase I metabolite. A component of similar magnitude had a RED of 0.270, and being eluted partly in methanol with the remainder in methanol/formic acid, may have been a glucuronide metabolite, corresponding to component E in plasma. The component in next abundance corresponded with component C in plasma, and may have been a metabolite formed by more than one Phase I transformation. This was present both as the free metabolite and the sulphate conjugate. The component predicted to be N-demethylated antipyrine was detected with a RED similar to the free metabolite found in plasma, but was eluted in methanol/ammonium acetate. This indicated that it was present in urine as the sulphate metabolite.

The screening method indicated that antipyrine was metabolised in the rat by Phase I metabolism to norantipyrine and 3-hydroxymethyl antipyrine primarily, with other Phase I transformations also taking place. The Phase I metabolites then underwent further metabolism to form mainly sulphate conjugates but also some glucuronides.

This was in broad agreement with published data on the metabolism of antipyrine in the rat, although some variation was found within the literature.

The metabolism of antipyrine in the rat has been studied following intraperitoneal administration to Wistar rats at a dose-level of approximately 50mg/kg (Bottcher, Bassmann and Schuppel, 1982a). The concentrations of drug and metabolites in plasma were not determined in these studies, but the urinary metabolic profile was examined. More than 60% of the dose was excreted in the urine within 24 hours of dosing. The principal metabolite was 4-hydroxyantipyrine (II), representing up to 31% of the dose. Two other Phase I metabolites, 3-hydroxymethyl antipyrine (III, up to 28% of the dose) and 4,4'-dihydroxy antipyrine (IV, up to 18% of the dose) were present in the urine. The majority of the three hydroxylated metabolites appeared in the urine as sulphate conjugates (Bottcher, Bassmann and Schuppel, 1982b) although some glucuronides were also formed. Norantipyrine (V) was not quantified in these studies.

In further studies, following oral administration of 3-$^{14}$C antipyrine to the rat, approximately 93% was recovered in the 0-24 hour urine (Huetter, Albert, Bayer, Zeller and Hartmann, 1987). This material was identified mainly as 3-hydroxymethyl antipyrine (III, 21.7%), norantipyrine (V) sulphate (20%), 4-hydroxyantipyrine (II)

sulphate (14%), 4, 4'-dihydroxyantipyrine (IV) sulphate (15%), 4-hydroxyantipyrine (II) glucuronide (9.2%) and 3-hydroxymethyl antipyrine (III) glucuronide (12.8%).

In more recent studies, the metabolism and pharmacokinetics of antipyrine was studied in various animal species including the rat (Witkamp, Lohuis, Nijmeijer, Kolker, Noordhoek and Van Miert, 1991). Following administration of antipyrine intraperitoneally at a dose-level of 50mg/kg to Wistar rats, concentrations of unchanged drug in the plasma ranged from about 60 to 20$\mu$g/ml during the first 4 hours after dosing. The predominant metabolite in urine from male rats, following hydrolysis of conjugates, was 3-hydroxymethyl-antipyrine (III, 75.6%) with 4-hydroxyantipyrine (II, 19.3%) and norantipyrine (V, 11.1%) also present. The 4, 4'-dihydroxy metabolite (IV) was not detected in male rat urine, but was detected in urine from female rats in small amounts (2.2%).

Witkamp *et al.* (1991) found that 3-hydroxymethyl antipyrine and norantipyrine were the major components of rat urine which was in agreement with the present studies. The presence of 4-hydroxyantipyrine was reported previously, however it was not detected in the present studies which may have been due to the different strain of rat used. The present studies showed that conjugation occurred to form mostly sulphate metabolites which was in agreement with the studies carried out by Bottcher *et al.* (1982b). The screening method thus indicated the main routes by which antipyrine is metabolised but missed some detail.

### 4.4.4 Diazepam

In the following discussion, the letters describing drug-related components correspond with those on **Figures 4.15.** The Roman numerals refer to the chemical structures in **Figure 4.4.**

Diazepam had an elution distance of 55mm using MMD Scheme 3. This was a suitable distance to allow more polar metabolites to be separated and detected. The drug was eluted from the extraction cartridges in methanol as would be predicted from its structure which contains no anionic groups.

Six drug-related components were detected in rat plasma with REDs of; (A) 1.009, (B) 0.909, (C) 0.846, (D) 0.60, (E) 0.345 and (F) 0.218. Component A was one of the minor components present, and was considered to be unchanged diazepam from its RED and it being eluted in methanol. Components B, C and D were eluted in methanol and were therefore likely to be Phase I metabolites. The RED of component B, being 0.909, was consistent with a metabolite formed by aromatic hydroxylation, which is most likely to occur on the phenyl ring of the diazepam molecule. Component C had a RED of 0.846 which was characteristic of a metabolite formed by aliphatic hydroxylation. This transformation can occur on the benzodiazepine ring of diazepam between the ketone and the unsaturated nitrogen. Component D, with a RED of 0.60, was likely to be a metabolite formed by more than one Phase I transformation, possibly aromatic and aliphatic hydroxylation. Component E had a RED characteristic of a sulphate and was eluted partially by methanol with the remainder in methanol/ammonium acetate. This was also consistent with a sulphate metabolite which was perhaps incompletely retained by anion-exchange because of its lipophilic character. Component F was eluted in

methanol/formic acid which indicated that it was a glucuronide. The RED of this component was also consistent with it being a glucuronide.

In urine, only one component was detected which was partially eluted in methanol, but mainly in methanol/ammonium acetate. The RED of this component was characteristic of a sulphate metabolite, corresponding to component E in plasma. The RED of component E was unchanged when a gradient with acetic acid was used, but was reduced by the addition of TEA. This did not assist the assignment of component E as a sulphate from the HPTLC/MMD data obtained previously (Chapter 2, Section 2.4.1.3).

The screening method predicted that diazepam was metabolised in the rat by aromatic and aliphatic hydroxylation to Phase I metabolites which were conjugated to form glucuronide and sulphate conjugates. Only one sulphate metabolite was excreted in the urine.

The results obtained in plasma from the present studies indicated the main routes of diazepam metabolism, and were in general agreement with published data, however the urinary results differed in the assignment of the main metabolite in urine.

Following intraperitoneal administration of diazepam to rats at a dose-level of 100mg/kg (Schwartz *et al.*, 1967), the intestinal contents were examined by thin-layer chromatography, and the metabolites characterised by mass spectroscopy and nuclear magnetic resonance. Four metabolites (II - V) of diazepam (I) were identified in the intestinal contents as either glucuronide or sulphate conjugates.

In studies carried out by Andrews and Griffiths (1984), male Wistar rats received 2-$^{14}C$ diazepam orally at a dose-level of approximately 13mg/kg. Only 17% of the dose was excreted in the urine and over 70% was present as glucuronic acid conjugates as shown by enzyme hydrolysis. The major metabolites were glucuronide conjugates of 4'-hydroxyoxazepam (VII, 26.8%), 4'-hydroxydiazepam (III, 16.2%), oxazepam (VI, 15.6%), 4'-hydroxytemazepam (IV, 11.9%), 4'-hydroxydesmethyldiazepam (V, 5.7%) and temazepam (II, 4.2%). Very low concentrations of Phase I metabolites were found. Similar results were obtained by Trennery and Waring (1985) except the major metabolite in urine was oxazepam (VI) glucuronide (38.0%) with glucuronides of 4'-hydroxydesmethyldiazepam (V, 22.7%), 4'-hydroxydiazepam (III, 19.2%), 4'-hydroxytemazepam (IV, 5.0%), 4'-hydroxyoxazepam (VII, 3.9%) and temazepam (II, 2.9%) also present.

The screening method indicated which metabolites of diazepam were present in the systemic circulation of the rat which had not been determined in the previous studies cited. In urine, a sulphate metabolite was detected, however previous studies have reported various glucuronide metabolites being excreted in the urine. In this case, the data from the screening method may have led to the incorrect assignment of the urinary metabolite being made. Possible other reasons for the difference between the present and previous studies include the dietary status of the animals, the strain and the higher dose-level used in the present studies.

In the present studies the animals were fasted overnight prior to administration of diazepam which may have resulted in low blood glucose concentrations and a depletion of the substrate UDPGA required for glucuronylation. This, in combination with the higher dose-level of diazepam administered may have led to a greater proportion of

sulphation occurring. However, only a low proportion of the dose (12 to 14%) was excreted in the urine, therefore the sulphate metabolite may have been a minor metabolite with glucuronides being excreted in the bile.

### 4.4.5 Overall Conclusions

The present studies showed that the data obtained from the screening method were in broad agreement with published data on the metabolism of ibuprofen, loxtidine, antipyrine and diazepam. The species differences which occur in the metabolism of ibuprofen and loxtidine were detected, although in both cases the precise nature of the metabolites formed may not have been elucidated from the data obtained without prior knowledge. The general patterns of metabolism elucidated from the data obtained for all four compounds were generally consistent with published data in terms of the extent of Phase I or Phase II metabolism which occurs. Thus the method was found to be useful in broad terms and was also able to provide some detailed information. There were, however, a number of cases where the "rules" proposed from the work carried out with model compounds were not upheld in the analysis of real samples.

The screening method has been shown to provide data on the extent of drug metabolism and the number of metabolites formed. The structures of the metabolites formed have been proposed by using relative elution data and the behaviour of the compound during sample preparation. The data obtained during the present studies indicated that a reasonable likelihood of successful structural assignment can be expected.

# *CHAPTER FIVE*

# *GENERAL DISCUSSION*

## 5. *General Discussion*

The general aim of the present studies was to assess the value of high-performance thin-layer chromatography (HPTLC) for the detection and characterisation of drugs and metabolites in biological fluids. The assessment comprised investigations of gradient development techniques on HPTLC plates and of sample preparation techniques suitable for extracting drugs and metabolites from plasma and urine. These investigations resulted in the development of novel linear normal-phase gradients which separated drugs and metabolites, and an efficient general extraction procedure. The gradient development system and extraction were combined to provide a method which in principle met criteria for a general screening method for drugs and metabolites in biological fluids.

The hypothesis was proposed that particular metabolite types have a characteristic retention relative to their parent compound following development using linear normal-phase gradients. The existence of a correlation between metabolite type and relative retention would thus enable prediction of the structure of a metabolite to be made from its retention data. A full investigation of the correlation between metabolite type and retention may be considered, however because of the wide range of metabolic transformations which occur, and the structural diversity of drugs, this would be impractical. Thus in the present studies, model compounds were selected which were representative of the major metabolic pathways and included structural components found in many drugs. The correlation between metabolic change and retention was investigated using these models and was found to support the hypothesis. This showed the potential of the method to predict the structures of unknown metabolites from their retention data.

The utility of the method was examined by analysing samples of plasma and urine obtained from animals following oral dosing with four drugs which have documented metabolic data; ibuprofen, loxtidine, antipyrine and diazepam. The aim of these studies was to determine how much information about the metabolism of a drug would be provided by analysing plasma and urine samples using the method which had been developed. Major themes of this thesis were, therefore, an assessment of how much information about the nature and extent of metabolism of a drug could reasonably be expected to be provided by the screening method, and identification of its limitations. In addition, Quantitative Structure Retention Relationships (QSRR) of drugs and metabolites following development on linear gradients were examined as a means of obtaining information about the nature of metabolites.

The potential utility of a general screening method to detect drugs and metabolites in plasma and urine of animals during drug research or at an early stage in the drug development process was discussed in Chapter 1. HPTLC was considered to have a number of advantages which made it an appropriate technique to use as the basis for such a method, and consideration of the chromatographic options and modes of development available using HPTLC led to the opinion that normal-phase chromatography on silica would provide the interactions required to separate drugs and metabolites. It was considered that gradient elution would be needed to overcome the general elution problem (Snyder and Kirkland, 1979) which is often associated with isocratic chromatographic separation of compounds with widely differing polarities.

Formation of normal-phase gradients on silica HPTLC plates was carried out by an adaptation of the automated multiple development (AMD) method described by Burger (1984) which was originally derived from the programmed multiple development

technique proposed by Perry *et al.* (1973). The main changes to the AMD method in the present studies were the use of manual rather than automated procedures, and use of gradients with linear elutropic strength : distance profiles in contrast to those used in the previously employed universal gradients which were described by Jaenchen and Issaq (1988). The method used was therefore referred to as manual multiple development (MMD).

Initially the resolution and sensitivity of HPTLC/MMD were assessed using a series of compounds selected to model typical drug metabolism transformations. These studies, described in Chapter 2, showed that HPTLC/MMD provided the resolution required to separate drugs and metabolites. Furthermore, the chromatographic data provided evidence of a correlation between metabolite type and retention relative to parent compounds. Thus "rules" were proposed concerning the ranges of relative retention which were characteristic of particular metabolic changes. The inherent sensitivity of HPTLC was also shown to be sufficient for the purpose of these studies, with 5ng of paracetamol being detected against the plate background. This was not a particularly low limit of detection, and reflected the low ultraviolet absorbing properties of paracetamol. However it was a rigorous test for the technique and demonstrated that the method would be suitable for most compounds rather than only those with high extinction coefficients or with fluorescent properties. The inherent sensitivity of HPTLC is great as shown by analytical methods which quantify drugs in plasma down to 1ng/ml, however the endogenous background obtained from untreated plasma and urine was found to be so great that detection of small amounts of drug in these matrices, following direct application to the HPTLC plate, was not possible. This indicated that some sample preparation would be required prior to HPTLC/MMD analysis.

The methods available for preparing biological fluid samples prior to chromatographic analysis in general were reviewed and a number of approaches, consistent with criteria for a general extraction procedure, were evaluated. From the studies described in Chapter 3, a general extraction procedure based on methods described by Vanluchene and Vandekerckhove (1988) and Wilson and Nicholson (1987) was developed. The method used Bond Elut Certify™ II solid-phase extraction cartridges, a new product which had been used previously for the isolation of 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid from human urine (Dixit and Dixit, 1990). The mixed reversed-phase and anion-exchange properties of the Certify™ II bonded phase were utilised to retain drugs and metabolites, and subsequently to elute them in stages depending upon their anionic strength. Thus compounds with no, or only weak anionic character were eluted in the first eluent, glucuronides and weak acids were eluted in the second eluent and sulphates were eluted in a third eluent. This provided a means for discriminating between metabolite type during sample preparation. Extractions of model compounds from plasma resulted in extraction efficiencies of greater than 45% for all compounds tested, and in excess of 90% for most of the compounds when extracts were analysed by HPTLC/MMD. During these studies it was found that the HPTLC plates were easily overloaded if too much of a plasma or urine extract was applied. This led to disruption of the chromatography in the early stages of the MMD procedure due to failure of the sample spots to wet with methanol in particular. It was thus necessary to apply small proportions of the sample extracts and rely on the inherent sensitivity of HPTLC to detect the resulting small amounts of material on the plates. Despite the need to limit the amount of sample analysed by HPTLC/MMD, limits of detection for the model compounds ranged between 1 and 50μg/ml with most compounds being detected in the range 1 to 10μg/ml.

It was established that HPTLC/MMD in combination with an extraction using Bond Elut Certify™ II cartridges would meet the criteria for a general screening method in principle. An assessment was was then carried out to determine how much information about the metabolism of a drug the method would provide from plasma and urine samples obtained from animals. Criteria were established prior to selecting suitable drugs for administration to animals. The objective was to select drugs which would provide a reasonable likelihood of success, ensure that a variety of compound classes was studied, provide an independent means of establishing reasons for failure should that occur and provide species differences to test whether they would be predicted by the method. The drugs selected were ibuprofen, loxtidine, antipyrine and diazepam. Plasma and urine samples were collected following oral administration of all four compounds to rats and ibuprofen and loxtidine to dogs.

The studies, described in Chapter 4, showed that the data obtained from the screening method were in broad agreement with published data on the metabolism of the compounds. The species differences which occur in the metabolism of ibuprofen (Mills *et al.*, 1973) and loxtidine (Bell *et al.*, 1983) were detected, although in both cases the precise nature of the metabolites formed may not have been elucidated from the data obtained without prior knowledge. The general patterns of metabolism elucidated from the data obtained for all four compounds were generally consistent with published data in terms of the extent of Phase I or Phase II metabolism which occurs. Thus the method was found to be useful in broad terms and was also able to provide some detailed information. There were, however, a number of cases where the "rules" proposed from the work carried out with model compounds were not upheld in the analysis of real samples.

In the following sections, the differences observed between model compounds and "real" drugs are discussed with respect to the MMD procedure and the extraction. The possible impact on this thesis of recent work in the fields of multiple development on HPTLC, QSRRs and sample preparation are also discussed. An assessment of the utility of the screening method is made and the value of the information which it may provide is placed into the context of drug research and development processes. Finally, future directions are proposed for the further development and use of the technique.

## *5.1 Separation of Drugs and Metabolites by HPTLC/MMD*

In a recent review by Poole and Belay (1991), Automated Multiple Development (AMD) was described as a technique still in its infancy with optimisation strategies for method development remaining difficult. AMD method development strategies based on trial and error experiments have been employed (Menziani, Tosi, Bonora, Reschiglian and Lodi, 1990) and the use of preliminary isocratic developments of selected standards with binary solvent mixtures has been proposed as a means of obtaining preliminary data to aid gradient design (Lodi, Betti, Kahie and Mahamed, 1991; Lodi, Betti, Menziani, Brandolini, and Tosi, 1991). In the latter approach, retention data obtained from isocratic developments were expressed as plots of $R_m$ values ($R_m = \log\{(1-Rf)/Rf\}$) against solvent composition and used to predict the optimum solvents for the gradient and the composition of the start and end points. Both the trial and error, and $R_m$ plot approaches required a significant amount of experimental work, the latter using up to 20 isocratic developments before an initial gradient was defined. Furthermore, standards were needed to carry out the preliminary experimentation, a requirement which could not be fulfilled when attempting the separation of a novel drug and its unknown metabolites. Except in

one case, where the gradient was formed using two solvents only, all the applications reported used universal gradients.

In the studies described in Chapter 2 of this thesis, an example was found where two components (resorcinol and m-hydroxybenzoic acid) were not resolved following development using a universal MMD gradient, despite previous examples of their separation by isocratic TLC (Dietz *et al.*, 1976). Their co-elution was attributed to the large change in solvent elutropic strength which occurred during the universal gradient when use of the strong solvent was discontinued and solvent mixtures with the non-polar solvent were started. Similar large changes in elutropic strength occur in many universal gradients, therefore it was concluded that biological fluids would be likely to contain a number of components, including drugs and metabolites, which might co-chromatograph under these conditions. Alternative multiple development systems were therefore developed which had linear elutropic strength profiles from the beginning to the end of the gradient. It was found that the linear gradients provided conditions under which a large number of compounds with widely differing polarity were separated. The use of a single linear gradient also overcame the need for optimisation. A single multiple development run provided data to indicate the elutropic strength range over which development was required, and a subsequent run over a reduced elutropic strength range increased the separation if required.

## *5.2 Quantitative Structure-Retention Relationships*

Quantitative Structure-Retention Relationships (QSRR) describe the process by which chromatographic system and solute properties are used for precalculation of retention,

and have been the subject of a recent report (Kaliszan, 1992). QSRR is analogous to the methodology used for quantitative structure-biological activity relationships (QSARs) used in drug design (Hansch and Fujita, 1964). It would be more useful with respect to the aims of this thesis if the QSRR concept could be inverted so that chromatographic system and retention data could be used to predict solute structure. This possibility has been examined recently for a series of benzodiazepines (Dimov and Moskovkina, 1991), however has not previously been applied to drugs and their metabolites in general.

In the present studies, the chromatographic properties of a number of compounds, selected to model typical metabolic transformations, were examined using the linear multiple development gradient described in this thesis (Chapter 2). A correlation was observed between metabolite type and retention relative to the parent compound. Thus addition of a hydroxyl group, a glucuronic acid moiety or a sulphate group resulted in a characteristic change in retention of the new compound relative to the parent. The potential impact of this finding is that the chemical structures of metabolites may be predicted from their retention on linear MMD gradients.

In the following paragraphs, the development and principles of QSRR are reviewed, and the data from the present studies are discussed and placed into context with conclusions drawn by other workers.

Correlation between the $R_f$ of compounds on partition chromatographic systems (paper chromatography and reversed-phase TLC) and their partition coefficients has been established (Consden, Gordon and Martin, 1944; Bate-Smith and Westall, 1950; Green and Marcinkiewicz, 1963). Consden *et al.* (1944) proposed the following mathematical relationship between the partition coefficient (P) and $R_f$:

$$P = \frac{A_m}{A_s}\left(\frac{1}{R_f} - 1\right)$$

where $A_m/A_s$ is the effective ratio of the cross sectional areas of the mobile and stationary phases. They showed that for six amino acids, the partition coefficients calculated by means of the above relationship agreed well with those measured directly.

Denoting log[(1-Rf)/Rf] as $R_m$, the equation may be rewritten as follows:

$$\log P = \log(A_m/A_s) + R_m$$

Thus $R_m$ is proportional to the partition coefficient. It was shown further that addition of a group X to a substance A should change the partition coefficient by a given factor depending upon the nature of the group and the pair of phases employed, but not upon the rest of the molecule. It thus follows that when a compound A is substituted with **m**X groups and **n**Y groups to give compound B, the following correlation exists between the $R_m$ values of A and B in the same chromatographic system:

$$R_{m,B} = R_{m,A} + m.R_{m,X} + n.R_{m,Y}$$

The validity of this relationship has been studied by paper chromatography and reversed-phase thin-layer chromatography using numerous compound types. The results of these investigations were summarised by Tomlinson (1975) who concluded that the $R_m$ change caused by the introduction of a particular group into a parent structure is only of constant value providing that its substitution into the parent structure does not result in any intramolecular interactions with other functions in the structure. In practice,

intramolecular interactions occur often and therefore a range of $R_m$ values will correspond with the substitution or addition of any particular group.

Knowledge of the group $R_m$ values and the $R_f$ value of the original compound in a given chromatographic system, therefore, theoretically permits the possibility of drawing conclusions about the structures of unknown derivatives from their chromatographic behaviour.

The expressions derived for partition chromatography were later applied to adsorption chromatography (Sporer and Trueblood, 1959). It was postulated that the standard free energy of the adsorption process (the energy required for one mole of solute to pass from solution to the adsorbed state) is, to a first approximation, expressible as the sum of the free energy changes of the component groups of atoms which comprise the molecule. The $R_f$ of a compound under given chromatographic conditions is related to the adsorption energy, thus a relationship between retention and structure can in principle be expected in adsorption chromatography as was described for partition chromatography.

Group adsorption affinities have been calculated for various functional groups and the theory has been tested with a number of groups of compounds. The data were summarised by Snyder (1968b). The group adsorption energies on silica are ranked as follows:

$Al\text{-}CONH_2 > Al\text{-}NH_2 > Al\text{-}COOH > Ar\text{-}CONH_2 > Ar\text{-}COOH > Al\text{-}OH > Ar\text{-}NH_2 > Ar\text{-}OH >> Ar\text{-}CH_3$ and $Al\text{-}CH_3$

where Al = Aliphatic and Ar = Aromatic.

The relative elution data obtained in the present studies (Chapter 2) were in agreement with the order of the group adsorption energies. Thus, the smallest changes in retention were found following aromatic hydroxylation with aliphatic hydroxylation resulting in larger changes. Greater changes in elution distance were obtained following oxidation to carboxylic acids, and the change observed with the aliphatic acid metabolite of ibuprofen was greater than that observed with the aromatic acid derivatives of indole. Evidence for the strong adsorption energies of unsaturated amines was obtained from the low elution distances achieved with ranitidine and loxtidine. The low elution found with ondansetron showed that the imidazole group exhibits a high adsorption energy on silica.

It has been shown that the additive nature of adsorption energies is true for some compounds. However there are numerous cases where the theory breaks down, and the reasons for this have been discussed in detail (Snyder, 1968b). Principally, there are two causes for deviation from the theoretical situation, these being the properties of the stationary phase and intramolecular interactions within the solute itself. These are discussed in the following two paragraphs.

The surface of silica is covered with discrete sites (free silanol groups) where interaction, principally by hydrogen bonding, can take place. A series of compounds containing only one group capable of interacting with the silica surface is likely to obey the additivity theory because sites for adsorption will be found easily. In more complex molecules containing a number of groups which may hydrogen bond with sites on the silica, it may not be possible for each group to be held at the optimum distance from the site of adsorption for the process to occur. The reason for this is the finite distances between the silanol groups on the silica surface and the inflexibility of the medium.

Similarly, in the case of a molecule containing only one group capable of hydrogen bonding, intramolecular interactions will not occur. When a molecule contains a number of groups it is possible for intramolecular hydrogen bonding to take place. This would have the effect of apparently weakening the adsorption energy of that particular molecule in comparison with that predicted by adding the adsorption energies of the individual groups. Whether or not intramolecular interactions occur depends upon the position of the groups within the molecule and the flexibility of the molecule in terms of bond rotation. It is thus difficult to predict differences in adsorption energies (and hence retention) accurately in adsorption chromatographic systems.

In recent studies (Dimov and Moskovkina, 1991) the structures of fifteen benzodiazepines were related to chromatographic retention using QSRRs. The present problems with modelling in QSRR studies were stated as; (i) uncertainty regarding the correct representation of solute structures, (ii) absence of adequate mathematical handling, and (iii) lack of reproducibility of the experimental retention data for compounds of interest. Empirical linear equations were derived by Dimov and Moskovkina to describe quantitatively the retention data of the benzodiazepines. This was achieved by selecting the fragments of the solute molecules which were responsible for the chromatographic retention; these were termed chromatophores. The contributions of the chromatophores were additive, but in some instances the fragment contributions differed from unity depending upon the influence of neighbouring atoms so the equations were derived by data interpolation. The requirement for interpolation of data from known compounds meant that the equations would not be useful in their present form to assist in structure elucidation of unknown compounds from retention data alone.

In a recent report on QSRR (Kaliszan, 1992) it was concluded that the QSRR approach has been useful with retention data obtained by partition chromatography, however the modelling of solute behaviour in more structurally sensitive chromatographic systems (i.e. adsorption chromatography) is much more difficult and has been reported only occasionally. The main problem was cited as the inadequacy of the available mathematical descriptors for representing the structural features that determine retention. A major reason for this difference between partition and adsorption systems is the fluidity of solutions versus the rigidity of solid surfaces. In solution, solvent molecules are free to adjust their relative positions for optimum interaction regardless of the shape of the molecule, whereas for adsorption onto a solid surface the situation is quite different as described previously. Thus intramolecular and sorbent effects are more important in adsorption chromatography.

Studies have been undertaken to minimise the problem stated by Dimov and Moskovkina that variation in the chromatographic conditions has lead to imprecise measurements of $R_f$. One approach taken to increase the precision of $R_f$ measurement was use of circular thin-layer chromatography by Bidlo-Igloy (1978) who studied aniline derivatives using a normal-phase system. This method provided control of the adsorbent layer properties, constant development velocities and consistent chamber saturation. Good reproducibility of $R_f$ values was obtained, however compounds such as drugs and metabolites with wide polarity differences may be difficult to separate isocratically because of the general elution problem. In the present studies, it has been shown that multiple development (particularly when automated) is able to provide reproducible elution distances (Chapter 2). The controlled conditions under which the development is carried out in terms of solvent mixing, development time and chamber saturation are ideal for ensuring reproducibility. In addition modern HPTLC plates are manufactured to high standards

ensuring reproducible layer thickness and tight control of the stationary phase particle size distribution. The use of gradient development enables drugs and their metabolites to be separated.

The retention data obtained in the present studies from model compounds (Chapter 2) and also drugs and metabolites (Chapter 4) have shown the difficulties in attempting to correlate discrete retention changes in adsorption chromatography with the addition of particular groups. The ranges of relative elution distances (REDs) obtained from model compounds following hydroxylation, oxidation to carboxylic acids, glucuronylation and sulphation were broad, but generally separable. This may have been due in part to the relative simplicity of the compounds used and their having only one functional group in most cases. It is pertinent to note that the RED values for aromatic hydroxylation which were at the extremes of the range found (Section 2.4.1) were those from compounds which contained two or more groups capable of forming hydrogen bonds. Thus it would seem that retention was either enhanced due to efficient alignment of interacting groups with the silanols on the silica surface, or reduced by intramolecular interactions or poor alignment with the silica surface.

The RED values of the metabolites detected following administration of drugs to animals (Chapter 4) showed the need to consider the total structure of molecules when interpreting the data. Glucuronylation of a carboxylic acid was shown to result in a larger RED (i.e. a smaller change in retention) than glucuronylation of a phenol. This is rational with respect to the additivity rule of free energies of adsorption if the order of free energies is:

glucuronic acid > carboxylic acid > aromatic OH

Thus, addition of the free energy for glucuronic acid at the expense of carboxylic acid results in a smaller free energy change than addition of glucuronic acid at the expense of the aromatic hydroxyl group.

Similarly, loxtidine glucuronide had a larger RED than would have been predicted from the work with model compounds. Loxtidine itself however has functional groups capable of strong hydrogen bonding. In this case, intramolecular interactions may have been responsible for the higher RED.

The present studies have shown that caution is needed when using changes in retention as a means of predicting chemical structure. It has been shown however that retention data from the method described in this thesis can be used successfully as a guide to structure, and the use of essentially one MMD system based on the same solvent mixtures decreases the possibility of large variations arising because of changes in selectivity. If the data generated are used in conjunction with knowledge of the types of intramolecular interactions that are likely to occur, and with reference to functional groups present on the molecule, the method is able to provide useful information. Sufficient data have been obtained to show the potential utility of the method, and as more data are generated in future, the data base from which to draw conclusions will enlarge and the likelihood of correct structural assignment being made may increase.

## *5.3 Sample Preparation Strategies*

There have been recent advances in the techniques used to prepare biological samples for analysis. These include development of new chemically bonded resins with anion-exchange properties, increased use of microdialysis as a means of sample preparation and use of immunochemical extraction procedures with non-specific antibodies. These are discussed in the following sections and compared with the approach taken in the present studies.

### 5.3.1 Bond Elut Certify™ II Extractions

The general sample preparation strategy using solid-phase extraction on Bond Elut Certify™ II cartridges adopted for the present studies provided essentially complete recovery of drug-related material when used for analysis of plasma and urine samples from animals (Chapter 4), and demonstrated discrimination between classes of compound when model compounds were investigated (Chapter 3).

A number of cases were found during the analysis of plasma and urine samples when the class discriminating properties of the extraction appeared to fail. In some cases this apparent failure was explained, as with the zwitterionic character of glucuronides of basic molecules. In other cases elution of the same compound occurred in two eluents, this being particularly noticeable when it involved the first (methanol) and third (methanol/ammonium acetate) eluents. This occurred in the cases of sulphate metabolites of both antipyrine and diazepam, and the most likely explanation is that the cartridges were overloaded. The number of sites available for anion-exchange (the mechanism by

which sulphates were expected to be retained) is finite, and may have been saturated because of the amount of material that was applied. The problem was most apparent during the analysis of urine which is known to contain large amounts of anionic material. Plasma and urine volumes of 1ml were extracted in all cases, but only 0.5 or 1% of the extract was applied to the HPTLC plate for analysis. It is therefore possible that the same sensitivity would be obtained if smaller sample volumes were used such as 100 to 200μl, and the residues from the extraction were reconstituted in correspondingly smaller volumes of methanol prior to analysis. Extraction of smaller samples would reduce the likelihood of overloading the column, and would also mean that smaller volumes of biological fluid would be needed for the assay to be carried out. This would have important ethical considerations because smaller blood samples could be obtained from rodents by tail bleeding without the need for exsanguination. Thus more than one blood sample would be obtained from each animal and the number of animals used would be reduced.

### 5.3.2 Chemically modified resins

The packings most widely used for liquid-solid extraction are chemically bonded silicas. Other materials are available such as porous polystyrene resins, but these are difficult to wet with aqueous samples and hence poor sample-surface contact can lead to low extraction efficiencies. Recently however, resins chemically modified by the addition of acetyl or hydroxymethyl groups have been shown to give extraction efficiencies superior to those obtained on chemically bonded silica packings (Sun and Fritz, 1992). Addition of the polar groups rendered the resins less hydrophobic and therefore able to be wetted by aqueous samples. The increased extraction efficiency was attributed to the greater surface area of the resins compared with silica. If this technology is pursued in future,

and wettable resins with anion-exchange properties are developed commercially, these materials may provide a useful alternative to silica based media. Possible advantages would be the lack of secondary interactions such as the weak cationic activity of silanol groups which causes mixed modes of extraction and leads to variability, and the increased surface area described previously.

### 5.3.3 Microdialysis

Advances have been made in the field of microdialysis. This has been the subject of a recent review (Lunte, Scott and Kissinger, 1991) and its use in bioanalytical sample preparation has been discussed (Linhares and Kissinger, 1992). A microdialysis sampling probe comprises a dialysis capillary through which low molecular weight substances can diffuse easily. Perfusion medium matched in ionic strength and pH to the sample is pumped through the capillary and collects the molecules which pass through from the sample. Microdialysis probes can be implanted into tissue or veins of living animals and thus provide a rapid means of sampling without withdrawing fluid from the animal with a concurrent clean-up of the biological matrix. More work needs to be carried out to assess the general applicability of such sampling, however it may provide a useful alternative as a general extraction procedure in the future. A disadvantage compared with the extraction method developed in the present studies is that no discrimination between sample type would be achieved. The extent of this disadvantage would depend upon how much endogenous material was present with analytes in the microdialysates.

### 5.3.4 Immunochemical Extraction

The strategies for sample preparation discussed thus far have relied upon the physicochemical properties of drugs and metabolites to provide discrimination from endogenous components. An alternative strategy based upon molecular structure might be appropriate for isolating drugs and metabolites from biological fluids. As was discussed in Chapter 1, during metabolism the chemical structure of a drug is modified to form metabolites which are generally more polar in nature than the parent drug. Often however, the main structure of the parent molecule remains intact during metabolism, and thus the molecular shape of the drug and its metabolites are similar. Immunochemistry provides a method of selecting molecules based upon their chemical structure and has been utilised in an approach to sample preparation.

Immunochemistry has been used for drug analysis by radio-immuno assay (RIA) for many years. In RIA, antisera to the drug of interest are raised by inoculating animals with a foreign protein to which the drug has been bound covalently. The animal produces antisera which have the ability to recognise the drug and bind it in solution. A specific antisera is able to bind the drug but not its metabolites, and is thus able to provide a specific RIA, however less specific antisera can be raised which might have the ability to bind a drug and its metabolites. If the antisera are then covalently bound to a support medium such as Sepharose gel, the resulting material can be used as a structure-specific solid-phase extraction medium. This technique has been used for preparing human plasma samples for salbutamol analysis by high-performance liquid chromatography (Ong, Adam, Perreault, Marleau, Bellemare, Du Souich and Beaulieu, 1989). The resulting chromatograms were free from endogenous background, however the sulphate

metabolite of salbutamol was present, indicating the possibility of using the technique for extracting both parent drug and metabolites.

The immunochemical approach has the potential of providing very specific extractions, however there are a number of disadvantages. Preparation of antisera typically takes three to six months, therefore the advantage of early data which the screening method was designed to provide would be lost. (However if non-specific antisera could be developed for a range of compounds with similar structures, the disadvantage would be overcome.) The status of the antisera in terms of specificity would not be known therefore it would not be possible to be certain that all drug-related material had been extracted. Finally, there would be no means of discriminating between metabolite types.

## *5.4 Utility of the Screening Method*

The screening method was reliable in providing information about the extent and complexity of metabolism of a drug following administration to animals at high dose-levels. A reliable indication of whether a drug is cleared renally was also obtained from analysis of urine. The reliability of the method to provide data on the nature of metabolites was dependent upon the level of specificity required and the structure of the parent drug.

In the following discussion, the utility of the screening method is considered. The strengths and weaknesses of the method are discussed and its limitations are defined. Use of the method for plasma and urine analysis is discussed, and implications of the

requirement for samples following administration of drug at high dose-levels, as defined in Chapter 3, are considered.

### 5.4.1 Overall utility of the method

The present studies have shown that the extraction method developed is efficient, reproducible and able to provide some discrimination between metabolite types. The linear MMD gradients have been shown to provide adequate separation of drugs and their metabolites, with relative retention giving an indication of metabolite structure. The method has been applied to the analysis of "real" samples, and patterns of metabolism shown were in broad agreement with previous studies.

The method was able to show whether parent drug was present in plasma and urine, and indicate whether the drug was present in higher or lower concentrations than metabolites. It was apparent from the chromatograms whether only one metabolite was present, as was found in the dog following administration of loxtidine. It was also clear when metabolism occurred by a number of different pathways as was found following administration of diazepam to the rat. The method was therefore able to indicate how complex the metabolism of a drug might be.

There are two levels of information which can be obtained about the nature of metabolites formed. At one level it is possible to determine whether the metabolites present are Phase I or Phase II. At the second level, the identity of the metabolite is defined more precisely as for example a hydroxylated metabolite, a glucuronide or a sulphate. The screening method has been shown to provide reliable information at the level of discriminating between Phase I and Phase II metabolites in most circumstances. Thus all Phase I

metabolites had RED values of >0.5 by HPTLC/MMD, and during the extraction procedure all except some carboxylic acids were eluted with methanol. By contrast, RED values of conjugated metabolites were <0.5, with most RED values being <0.4. During the extraction procedure conjugated metabolites were eluted in either the second or third eluent, except for glucuronides of basic compounds with strong silanol interacting groups, or as a result of possible cartridge overload. Elution in the later eluents confirmed the anionic nature of the conjugated metabolites.

The screening method provided data which enabled a number of metabolites to be assigned to a particular class with a high degree of confidence. There were other instances where precise assignments could not be made, and one or two cases where the data led to incorrect assignments. This level of interpretation is concerned with QSRR, which as discussed previously is currently at best empirical in its application. Nevertheless, the high proportion of correct assignments which were made during the present studies indicate that the method is useful in this respect, and that its utility may increase with use as the data base increases.

### 5.4.2 Plasma Analysis

The method was highly applicable to the analysis of plasma samples for drugs and metabolites. The reasons for this conclusion and implications for the overall utility of the method are discussed below.

The method was sensitive, with diazepam and its metabolites being detected at concentrations of <1 μg/ml, and loxtidine and a metabolite being present at concentrations of 2.5 to 5 μg/ml in plasma. In samples obtained following administration of ibuprofen

and antipyrine, the major drug-related components would still have been detected if dose-levels had been 25% of those used in the present studies. The sensitivity in plasma was due largely to the low level of interfering material in the plasma extracts which were obtained following solid-phase extraction.

The determination of drugs and metabolites in the plasma is of great importance toxicologically and pharmacologically. Systemic toxicity of a compound may be due to a metabolite rather than the parent drug, therefore the identification of circulating metabolites may assist interpretation of toxicological data. In general, the intensity and duration of drug action is proportional to the concentration of the drug at the site of action and the length of time it remains there. As drugs often reach their site of action via the systemic circulation, it is important to know whether any metabolites are present in the plasma in addition to parent drug. It is of further benefit to know whether any metabolites present are Phase I or Phase II because generally Phase I metabolites are more likely to be active pharmacologically.

### 5.4.3 Urine Analysis

The method was less useful for detecting metabolites in urine than in plasma. The reasons for this conclusion and implications for the utility of the method are discussed below.

Generally, the sensitivity of the method for detecting drugs and metabolites in urine was lower than in plasma. This was expected from the studies described in Chapter 3, and was not considered too important because the concentrations of drug-related material in urine tend to be higher than in plasma except where renal excretion is very low. This was

confirmed by the levels of radioactive material determined in 0-24 hour urine from the present studies (Chapter 4) which were between 14 and 77 times higher than the levels in plasma at 1.5 hours after dosing. The main reason for the decreased utility of the method for urine analysis was the large variation in concentrations of endogenous material in urine. The majority of endogenous urinary components eluted in the bottom 25% of the HPTLC plate when MMD was carried out using the full gradient, and although resolution of the components could be increased by using expanded systems, close or co-elution of drug-related material with endogenous components still occurred in some cases. It was therefore more difficult to be certain whether a peak which was present in a sample chromatogram, and either absent or much smaller in the control sample, related to a metabolite unless it was present at high concentrations.

During drug research or early stages of development, knowledge about the drug-related material in urine is less important than corresponding knowledge in plasma. The reasons for this are twofold; firstly relating to the importance of circulating drug and metabolites discussed previously, and secondly because the extent of urinary excretion relative to excretion via the faeces is not known until studies with radiolabelled drug are undertaken. The urinary profile can therefore at best be only a guide to the overall metabolic fate of a drug in the absence of faecal data. In cases where urinary excretion is low, metabolites in the urine may be minor in terms of the overall metabolic profile as was found following administration of diazepam in the present studies, and may give an incorrect indication of the major metabolic routes of a drug.

### 5.4.4 Dose-Levels

It was necessary to administer drugs at high dose-levels in order to obtain plasma and urine samples in which drugs and metabolites would be detected by the screening method. The implications of this requirement to the utility of the method are discussed below.

The dose-levels used in the present studies ranged from 50 to 250mg/kg, although as discussed in Section 5.4.2, these could have been reduced by up to fourfold in some cases without affecting the level of information that was obtained. Nevertheless, the most appropriate animals from which to obtain samples for analysis by this method would be those in the high dose groups of toxicity studies. Analysis of these samples would give a useful indication of the extent of metabolism and the nature of metabolites formed, however different data may be obtained from subsequent studies at lower dose-levels using radiolabelled drug. Reasons for this are that the processes of absorption, distribution, metabolism and excretion may be capacity-limited such that at high doses the amount of absorption, the extent and pathways of metabolism or the route and rate of elimination may be changed significantly. Conversely however, ADME studies which are carried out at low dose-levels may not provide appropriate data to assist interpretation of toxicological findings at high dose-levels. Furthermore, analysis of samples obtained from high dose-level studies might lead to the identification of a metabolite which was either not formed or too minor for detection at low dose-levels. Reasons for this might be saturation of a particular metabolic pathway or metabolic switching at high dose-levels. If the metabolite concerned happened to be one which was later found to be formed in man, the information could be vital in assisting to validate the toxicity studies.

## *5.5 Utility with respect to other techniques*

The screening method developed in the present studies has been shown to be useful for detecting drugs and metabolites in biological fluids, and indicating the nature and extent of drug metabolism. A number of other techniques are used for metabolite screening, such as HPLC with diode array detection, HPLC-mass spectrometry and nmr. As described in Chapter 1 (Sections 1.4.1 and 1.4.2) these techniques can be classified broadly as those which provide indirect or direct evidence of metabolite structures. In addition, studies with radiolabelled drug are carried out. The screening method is compared with these techniques in the following discussion.

HPLC with diode array detection has been used to screen for drugs and metabolites, however reliance was placed upon ultraviolet spectra for classifying metabolites. Ultraviolet absorption spectra alone do not generally provide structural information, therefore chromatographic properties would also need to be considered. Thus development of a HPLC system capable of separating drug-related components and eluting them in a structurally related order would be necessary. It is considered that this would be more difficult to achieve using HPLC than HPTLC for the reasons discussed in Chapter 1. The screening method developed in the present studies has advantages, therefore, in terms of metabolite identification over HPLC with diode array detection.

Mass spectrometry and nmr spectroscopy have the advantage that they provide direct characterisation of metabolites. A study was carried out using nmr which provides a comparison with the screening method described in this thesis. Wilson, Nicholson, Ghauri and Blackledge, (1991) described the use of $^{1}$H-nmr for the analysis of human

urine following administration of ibuprofen. Most of the known metabolites of ibuprofen could be assigned from the nmr traces using authentic standards, however the authors considered that less information would have been obtained had their study been the first on a new compound. It would have been ascertained that urine was a major route of excretion for drug-related material, a number of different metabolites were excreted and conjugation to form glucuronides occurred. In the present studies, at least the level of information reported for ibuprofen would have been obtained using the screening method developed.

The lack of structural information obtained from the indirect chromatographic methods compared with that obtained with the direct spectroscopic techniques is evident. The screening method described in this thesis, although based upon a chromatographic technique may be consided to lie between techniques which provide indirect and direct evidence of metabolite structure. The method may thus have served to narrow the gap between chromatographic and spectroscopic techniques in this respect.

The use of radiolabelled drug simplifies the study of drug metabolism. It provides a selective method of detecting drug-related material and means that all except minor components are detected. The presence of the radioisotope facilitates easier analysis of samples by HPLC because recovery can be calculated by collecting and counting the eluent. The chromatographer therefore knows whether components remain on the HPLC column at the end of a run, and if necessary can adjust the conditions to ensure complete elution. When radiolabelled drug is available, an element of uncertainty is removed, and different techniques become more appropriate. There would be little benefit in using the screening method developed in the present studies once radiolabelled drug is available.

## *5.6 Future Directions*

In this section the future directions for use of the screening method as it has been developed to date are discussed, and future studies which may build upon what has been achieved are proposed.

In its current form the screening method can be used to analyse plasma and urine samples from studies with novel compounds in both the drug research and development environments. As the method is used in practice, information will be obtained which will prove either true or false when definitive metabolism studies with the drugs concerned are performed. A data base of information will thus be built up, and after a number of applications, a re-evaluation of the method's performance may be made. This may provide an indication of the types of compound to which the method is most suited, or show that for certain others the method is not applicable. From the larger data base of relative elution distances, construction of some more robust structure-retention relationships may be possible.

Recently, dihydroxyalkyl-bonded silica gel ("diol") has been introduced into the range of phases available for HPTLC. Studies with diol plates have shown that it behaves like a deactivated silica gel and that it has some interesting differences in selectivity compared with silica (Witherow, Thorp, Wilson and Warrander, 1990; Martin, Taberner, Thorp and Wilson, 1992). It is possible that the diol phase would provide different structure-retention relationships and therefore be a useful alternative to silica for use with linear gradients. Further studies could be carried out to investigate the utility of the diol phase.

The general extraction procedure developed as part of the screening method may be of use for extracting samples from studies with radiolabelled drug. The class discriminating properties of the procedure may assist in assigning structures to metabolites which are isolated from these samples. Use of the extraction procedure to extract samples from these studies will enable a larger data base to be built up, and the relationships between structure and elution order proposed in the present studies may be refined further.

In some extractions carried out during the present studies, cartridge overloading appeared to occur as discussed in Section 5.3.1. The sensitivity of the method and the small proportion of the sample that was applied to the HPTLC plates for analysis mean that the volumes of samples analysed could be reduced. Smaller blood samples would therefore be required, so use of tail bleeding for rodents could be considered. In future studies the use of smaller sample volumes for analysis could therefore be investigated.

The MMD procedure used for the present studies was very labour intensive, and open to errors being made in the accuracy of development distances particularly. Studies carried out with Automated Multiple Development (AMD) showed that automation improves the reproducibility of the system, but that the current AMD instrument does not easily perform linear gradients. Studies to automate linear gradients would be beneficial in the future.

The performance of the screening method would be increased significantly if proof of the structural assignments of metabolites made could be obtained easily. In recent years, the development of a new generation of smaller, high performance mass spectrometers, combined with renewed attention to methods of ionisation, and source and interface engineering have made possible a viable combination of TLC with mass spectrometry

(Busch, 1992a; Busch, 1992b). The use of laser desorption techniques (Zhu and Yeung, 1989) in the future may also enhance the interface between TLC and mass spectrometry. Mass spectrometry has been combined successfully with TLC using an off-line procedure in recent studies (Wilson and Morden, 1991) in which polar molecules and conjugates were identified successfully from TLC plates by their mass spectra. Future work could include investigation of TLC-mass spectral techniques to confirm the data obtained using the screening method.

Use of *in vitro* methods to study the metabolism of drugs has increased over the past few years particularly within academic research groups, but recognition of the potential benefits of these techniques is increasing within the industrial setting (Bell and Tarbit, 1992). Analysis of incubation media from *in vitro* studies with microsomes, hepatocytes and liver slices during drug research when radiolabelled drug is not available could be carried out using the screening method developed in the present studies. *In vitro* studies enable useful information to be obtained early about the metabolism of drugs in different species and may thus aid the selection of toxicology species and decrease the use of animals. Investigation of the applicability of the screening method to the analysis of incubation media could form the basis of some future studies.

## *5.7 Concluding Remarks*

The screening method described in this thesis has a place in drug research and the early development programme of a drug. The information which the system might provide is summarised and its potential value is discussed below. Other areas of study to which the

advances in understanding of linear gradients on HPTLC, QSRR and general extraction strategies described in this thesis may be applied are also considered.

The screening method will provide data on the extent of drug metabolism and indicate the number of metabolites formed. The structures of the metabolites formed can be proposed by using relative elution data and the behaviour of the compound during sample preparation. The data obtained during the present studies indicated that a reasonable likelihood of successful structural assignment can be expected.

The value of such information increases the earlier it is obtained. During research, identification of circulating metabolites might lead to the discovery of a better drug than the compound under evaluation, and may also provide a valuable commercial advantage if a new chemical entity is patented as a result of the studies. Even if metabolites are not potential drugs, knowledge of the routes of metabolism of a compound may assist in the design of a drug with a better pharmacokinetic profile, providing greater bioavailability and a longer duration of action.

The information from the method would be less valuable once a compound has entered development because data from studies with radiolabelled compound would be available. However, the method may be of use in assisting the interpretation of toxicological findings if a drug is toxic at high dose-levels. Use of the method to obtain data following administration of drug at high dose-levels may result in identification of metabolites which are not formed, or are undetectable, at the lower ADME dose-levels. Data may therefore be provided which would assist in validating the toxicology species with respect to man.

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The methods described in this thesis have the advantage of requiring only relatively inexpensive equipment and may provide a means of screening in fields other than drug metabolism research which have been hampered previously by equipment cost and the requirement for specialised scientists. There are numerous areas of scientific study where samples are analysed to screen for an unknown number of unidentified components and where application of linear MMD gradients and a general extraction procedure may be useful. Examples include agrochemical research, the food industry, examination of plants for novel drugs, water analysis and examination of samples for degradation products. In many cases a screen for a large number of known components is required, and the methods described in this thesis may prove advantageous over methodology currently in use.

In conclusion, the preceding discussion has shown that the major objectives of this thesis have been achieved. High-performance thin-layer chromatography has been assessed, and found to be of value as an analytical tool for the detection and characterisation of drugs and metabolites in biological fluids. Generally applicable separation and extraction methods have been developed, and the method has been tested by analysing samples of plasma and urine following administration of drugs to animals. A general screening method for the detection and characterisation of drugs and metabolites had been devised which may provide valuable early information in drug research and development programmes in the future.

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**Table 1**

**Manual Multiple Development Scheme 1**

| Step | Distance (mm) | Solvent Composition |
|---|---|---|
| 1 | 4 | Methanol |
| 2 | 8 | Methanol |
| 3 | 12 | Methanol |
| 4 | 16 | Methanol (9) + Dichloromethane (1) |
| 5 | 20 | Methanol (8) + Dichloromethane (2) |
| 6 | 24 | Methanol (7) + Dichloromethane (3) |
| 7 | 28 | Methanol (6) + Dichloromethane (4) |
| 8 | 32 | Methanol (5) + Dichloromethane (5) |
| 9 | 36 | Methanol (4) + Dichloromethane (6) |
| 10 | 40 | Methanol (3) + Dichloromethane (7) |
| 11 | 44 | Methanol (2) + Dichloromethane (8) |
| 12 | 48 | Methanol (1) + Dichloromethane (9) |
| 13 | 52 | Dichloromethane |
| 14 | 56 | Dichloromethane |
| 15 | 60 | Dichloromethane |
| 16 | 64 | Dichloromethane (8) + Hexane (2) |
| 17 | 68 | Dichloromethane (6) + Hexane (4) |
| 18 | 72 | Dichloromethane (4) + Hexane (6) |
| 19 | 76 | Dichloromethane (2) + Hexane (8) |
| 20 | 80 | Hexane |

**Table 2**

**Manual Multiple Development Scheme 2**

| Step | Distance (mm) | Solvent Composition |
|---|---|---|
| 1 | 4 | Methanol |
| 2 | 8 | Methanol |
| 3 | 12 | Methanol |
| 4 | 16 | Methanol (8) + Chloroform (2) |
| 5 | 20 | Methanol (6) + Chloroform (4) |
| 6 | 24 | Methanol (4) + Chloroform (6) |
| 7 | 28 | Methanol (2) + Chloroform (8) |
| 8 | 32 | Chloroform |
| 9 | 36 | Chloroform |
| 10 | 40 | Chloroform |
| 11 | 44 | Chloroform (9) + Hexane (1) |
| 12 | 48 | Chloroform (8) + Hexane (2) |
| 13 | 52 | Chloroform (7) + Hexane (3) |
| 14 | 56 | Chloroform (6) + Hexane (4) |
| 15 | 60 | Chloroform (5) + Hexane (5) |
| 16 | 64 | Chloroform (4) + Hexane (6) |
| 17 | 68 | Chloroform (3) + Hexane (7) |
| 18 | 72 | Chloroform (2) + Hexane (8) |
| 19 | 76 | Chloroform (1) + Hexane (9) |
| 20 | 80 | Hexane |

**Table 3**

**Manual Multiple Development Scheme 3**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.73 |
| 2 | 12 | Methanol (4.6) + Ethyl Acetate (5.4) | 0.70 |
| 3 | 20 | Methanol (1.3) + Ethyl Acetate (8.7) | 0.63 |
| 4 | 28 | Methanol (0.44) + Ethyl Acetate (9.56) | 0.56 |
| 5 | 36 | Methanol (0.15) + Ethyl Acetate (9.85) | 0.49 |
| 6 | 44 | Methanol (0.03) + Ethyl Acetate (9.97) | 0.42 |
| 7 | 52 | Ethyl Acetate (6.86) + Chloroform (3.14) | 0.35 |
| 8 | 60 | Ethyl Acetate (1.21) + Chloroform (8.79) | 0.28 |
| 9 | 68 | Chloroform (3.5) + Hexane (6.5) | 0.21 |
| 10 | 76 | Chloroform (0.95) + Hexane (9.05) | 0.14 |
| 11 | 84 | Chloroform (0.22) + Hexane (9.78) | 0.07 |
| 12 | 92 | Hexane | 0.00 |

**Table 4**

**Manual Multiple Development Scheme 4**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.73 |
| 2 | 12 | Ethyl Acetate (2.7) + Chloroform (7.3) | 0.30 |
| 3 | 20 | Ethyl Acetate (1.3) + Chloroform (8.7) | 0.28 |
| 4 | 28 | Chloroform | 0.26 |
| 5 | 36 | Chloroform (6.0) + Hexane (4.0) | 0.24 |
| 6 | 44 | Chloroform (4.0) + Hexane (6.0) | 0.22 |
| 7 | 52 | Chloroform (3.0) + Hexane (7.0) | 0.20 |
| 8 | 60 | Chloroform (2.0) + Hexane (8.0) | 0.18 |
| 9 | 68 | Chloroform (1.4) + Hexane (8.6) | 0.16 |
| 10 | 76 | Chloroform (1) + Hexane (9) | 0.14 |
| 11 | 84 | Chloroform (0.6) + Hexane (9.4) | 0.12 |
| 12 | 92 | Chloroform (0.4) + Hexane (9.6) | 0.10 |

**Table 5**

**Manual Multiple Development Scheme 5**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (0.145) + Ethyl Acetate (9.855) | 0.490 |
| 3 | 20 | Methanol (0.055) + Ethyl Acetate (9.945) | 0.441 |
| 4 | 28 | Methanol (0.008) + Ethyl Acetate (9.992) | 0.392 |
| 5 | 36 | Ethyl Acetate (6.19) + Chloroform (3.81) | 0.343 |
| 6 | 44 | Ethyl Acetate (2.16) + Chloroform (7.84) | 0.294 |
| 7 | 52 | Chloroform (6.87) + Hexane (3.13) | 0.245 |
| 8 | 60 | Chloroform (2.45) + Hexane (7.55) | 0.196 |
| 9 | 68 | Chloroform (0.965) + Hexane (9.035) | 0.147 |
| 10 | 76 | Chloroform (0.37) + Hexane (9.63) | 0.098 |
| 11 | 84 | Chloroform (0.114) + Hexane (9.886) | 0.049 |
| 12 | 92 | Hexane | 0.000 |

**Table 6**

**Manual Multiple Development Scheme 6**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol (1.0) + Ethyl Acetate (9.0) | 0.613 |
| 2 | 12 | Ethyl Acetate (6.67) + Chloroform (3.33) | 0.348 |
| 3 | 20 | Ethyl Acetate (4.41) + Chloroform (5.59) | 0.323 |
| 4 | 28 | Ethyl Acetate (2.45) + Chloroform (7.55) | 0.298 |
| 5 | 36 | Ethyl Acetate (0.77) + Chloroform (9.23) | 0.273 |
| 6 | 44 | Chloroform (7.38) + Hexane (2.62) | 0.248 |
| 7 | 52 | Chloroform (4.22) + Hexane (5.78) | 0.223 |
| 8 | 60 | Chloroform (2.54) + Hexane (7.46) | 0.198 |
| 9 | 68 | Chloroform (1.575) + Hexane (8.425) | 0.173 |
| 10 | 76 | Chloroform (0.985) + Hexane (9.015) | 0.148 |
| 11 | 84 | Chloroform (0.61) + Hexane (9.39) | 0.123 |
| 12 | 92 | Chloroform (0.37) + Hexane (9.63) | 0.098 |

**Table 7**

**Manual Multiple Development Scheme 7**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (7.08) + Ethyl Acetate (2.92) | 0.718 |
| 3 | 20 | Methanol (5.27) + Ethyl Acetate (4.73) | 0.706 |
| 4 | 28 | Methanol (4.06) + Ethyl Acetate (5.94) | 0.694 |
| 5 | 36 | Methanol (3.19) + Ethyl Acetate (6.81) | 0.682 |
| 6 | 44 | Methanol (2.55) + Ethyl Acetate (7.45) | 0.670 |
| 7 | 52 | Methanol (2.07) + Ethyl Acetate (7.93) | 0.658 |
| 8 | 60 | Methanol (1.69) + Ethyl Acetate (8.31) | 0.646 |
| 9 | 68 | Methanol (1.39) + Ethyl Acetate (8.61) | 0.634 |
| 10 | 76 | Methanol (1.15) + Ethyl Acetate (8.85) | 0.622 |
| 11 | 84 | Methanol (0.95) + Ethyl Acetate (9.05) | 0.610 |
| 12 | 92 | Methanol (0.79) + Ethyl Acetate (9.21) | 0.598 |

**Table 8**

**Manual Multiple Development Scheme 8**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (8.85) + Ethyl Acetate (1.15) | 0.726 |
| 3 | 20 | Methanol (7.88) + Ethyl Acetate (2.12) | 0.722 |
| 4 | 28 | Methanol (7.07) + Ethyl Acetate (2.93) | 0.718 |
| 5 | 36 | Methanol (6.38) + Ethyl Acetate (3.62) | 0.714 |
| 6 | 44 | Methanol (5.79) + Ethyl Acetate (4.21) | 0.710 |
| 7 | 52 | Methanol (5.27) + Ethyl Acetate (4.73) | 0.706 |
| 8 | 60 | Methanol (4.81) + Ethyl Acetate (5.19) | 0.702 |
| 9 | 68 | Methanol (4.41) + Ethyl Acetate (5.59) | 0.698 |
| 10 | 76 | Methanol (4.05) + Ethyl Acetate (5.95) | 0.694 |
| 11 | 84 | Methanol (3.73) + Ethyl Acetate (6.27) | 0.690 |
| 12 | 92 | Methanol (3.45) + Ethyl Acetate (6.55) | 0.688 |

**Table 9**

**Manual Multiple Development Scheme 9**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (5.8) + Ethyl Acetate (4.2) | 0.710 |
| 3 | 20 | Methanol (3.75) + Ethyl Acetate (6.25) | 0.690 |
| 4 | 28 | Methanol (2.55) + Ethyl Acetate (7.45) | 0.670 |
| 5 | 36 | Methanol (1.8) + Ethyl Acetate (8.2) | 0.650 |
| 6 | 44 | Methanol (1.3) + Ethyl Acetate (8.7) | 0.630 |
| 7 | 52 | Methanol (0.95) + Ethyl Acetate (9.05) | 0.610 |
| 8 | 60 | Methanol (0.7) + Ethyl Acetate (9.3) | 0.590 |
| 9 | 68 | Methanol (0.52) + Ethyl Acetate (9.48) | 0.570 |
| 10 | 76 | Methanol (0.38) + Ethyl Acetate (9.62) | 0.550 |
| 11 | 84 | Methanol (0.28) + Ethyl Acetate (9.72) | 0.530 |
| 12 | 92 | Methanol (0.20) + Ethyl Acetate (9.80) | 0.510 |

**Table 10**

**Manual Multiple Development Scheme 10**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (3.75) + Ethyl Acetate (6.25) | 0.690 |
| 3 | 20 | Methanol (2.95) + Ethyl Acetate (7.05) | 0.678 |
| 4 | 28 | Methanol (2.375) + Ethyl Acetate (7.625) | 0.666 |
| 5 | 36 | Methanol (1.93) + Ethyl Acetate (8.07) | 0.654 |
| 6 | 44 | Methanol (1.58) + Ethyl Acetate (8.42) | 0.642 |
| 7 | 52 | Methanol (1.30) + Ethyl Acetate (8.70) | 0.630 |
| 8 | 60 | Methanol (1.08) + Ethyl Acetate (8.92) | 0.618 |
| 9 | 68 | Methanol (0.895) + Ethyl Acetate (9.105) | 0.606 |
| 10 | 76 | Methanol (0.745) + Ethyl Acetate (9.255) | 0.594 |
| 11 | 84 | Methanol (0.62) + Ethyl Acetate (9.38) | 0.582 |
| 12 | 92 | Methanol (0.52) + Ethyl Acetate (9.48) | 0.570 |

**Table 11**

**Manual Multiple Development Scheme 11**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (1.93) + Ethyl Acetate (8.07) | 0.654 |
| 3 | 20 | Methanol (1.775) + Ethyl Acetate (8.225) | 0.649 |
| 4 | 28 | Methanol (1.63) + Ethyl Acetate (8.37) | 0.644 |
| 5 | 36 | Methanol (1.505) + Ethyl Acetate (8.495) | 0.639 |
| 6 | 44 | Methanol (1.385) + Ethyl Acetate (8.615) | 0.634 |
| 7 | 52 | Methanol (1.28) + Ethyl Acetate (8.72) | 0.629 |
| 8 | 60 | Methanol (1.185) + Ethyl Acetate (8.815) | 0.624 |
| 9 | 68 | Methanol (1.095) + Ethyl Acetate (8.905) | 0.619 |
| 10 | 76 | Methanol (1.01) + Ethyl Acetate (8.99) | 0.614 |
| 11 | 84 | Methanol (0.935) + Ethyl Acetate (9.065) | 0.609 |
| 12 | 92 | Methanol (0.865) + Ethyl Acetate (9.135) | 0.604 |

**Table 12**

**Manual Multiple Development Scheme 12**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (1.805) + Ethyl Acetate (8.195) | 0.650 |
| 3 | 20 | Methanol (1.685) + Ethyl Acetate (8.315) | 0.646 |
| 4 | 28 | Methanol (1.58) + Ethyl Acetate (8.42) | 0.642 |
| 5 | 36 | Methanol (1.48) + Ethyl Acetate (8.52) | 0.638 |
| 6 | 44 | Methanol (1.385) + Ethyl Acetate (8.615) | 0.634 |
| 7 | 52 | Methanol (1.30) + Ethyl Acetate (8.70) | 0.630 |
| 8 | 60 | Methanol (1.22) + Ethyl Acetate (8.78) | 0.626 |
| 9 | 68 | Methanol (1.145) + Ethyl Acetate (8.855) | 0.622 |
| 10 | 76 | Methanol (1.075) + Ethyl Acetate (8.925) | 0.618 |
| 11 | 84 | Methanol (1.01) + Ethyl Acetate (8.99) | 0.614 |
| 12 | 92 | Methanol (0.95) + Ethyl Acetate (9.05) | 0.610 |

**Table 13**

**Manual Multiple Development Scheme 13**

| Step | Distance (mm) | Solvent Composition | Strength |
|---|---|---|---|
| 1 | 4 | Methanol | 0.730 |
| 2 | 12 | Methanol (5.39) + Ethyl Acetate (4.61) | 0.707 |
| 3 | 20 | Methanol (3.38) + Ethyl Acetate (6.62) | 0.685 |
| 4 | 28 | Methanol (2.213) + Ethyl Acetate (7.787) | 0.662 |
| 5 | 36 | Methanol (1.503) + Ethyl Acetate (8.497) | 0.639 |
| 6 | 44 | Methanol (1.044) + Ethyl Acetate (8.956) | 0.616 |
| 7 | 52 | Methanol (0.744) + Ethyl Acetate (9.256) | 0.594 |
| 8 | 60 | Methanol (0.525) + Ethyl Acetate (9.475) | 0.571 |
| 9 | 68 | Methanol (0.37) + Ethyl Acetate (9.63) | 0.548 |
| 10 | 76 | Methanol (0.258) + Ethyl Acetate (9.742) | 0.525 |
| 11 | 84 | Methanol (0.181) + Ethyl Acetate (9.819) | 0.503 |
| 12 | 92 | Methanol (0.122) + Ethyl Acetate (9.878) | 0.480 |

**Table 14**

**Automated Multiple Development Scheme 1**

| Step | Methanol | Ethyl Acetate | Chloroform | Hexane | Distance (mm) | Time (min) | Strength |
|---|---|---|---|---|---|---|---|
| 1 * | 100 | - | - | - | 6.0 | 0.2 | 0.73 |
| 2 | 81.4 | 18.6 | - | - | 0.1 | | |
| 3 * | 52.6 | 47.4 | - | - | 14.0 | 0.7 | 0.706 |
| 4 | 34.0 | 66.0 | - | - | 0.1 | | |
| 5 | 22.0 | 78.0 | - | - | 0.1 | | |
| 6 * | 14.2 | 85.8 | - | - | 22.0 | 1.5 | 0.635 |
| 7 | 9.2 | 90.8 | - | - | 0.1 | | |
| 8 * | 5.9 | 94.1 | - | - | 30.0 | 2.8 | 0.579 |
| 9 | 3.8 | 96.2 | - | - | 0.1 | | |
| 10 * | 2.5 | 97.5 | - | - | 38.0 | 4.5 | 0.523 |
| 11 | 1.6 | 98.4 | - | - | 0.1 | | |
| 12 * | 1.0 | 99.0 | - | - | 46.0 | 6.5 | 0.469 |
| 13 * | 0.8 | 80.6 | 18.6 | - | 54.0 | 10.0 | 0.362 |
| 14 | 0.5 | 52.1 | 47.4 | - | 0.1 | | |
| 15 | 0.3 | 33.7 | 66.0 | - | 0.1 | | |
| 16 | 0.2 | 21.8 | 78.0 | - | 0.1 | | |
| 17 * | 0.1 | 14.1 | 85.5 | - | 62.0 | 13.2 | 0.283 |
| 18 | - | - | 100.0 | - | 0.1 | | |
| 19 | - | - | 81.4 | 18.6 | 0.1 | | |
| 20 | - | - | 52.6 | 47.4 | 0.1 | | |
| 21 * | - | - | 34.0 | 66.0 | 70.0 | 15.1 | 0.213 |
| 22 | - | - | 22.0 | 78.0 | 0.1 | | |
| 23 | - | - | 14.2 | 85.8 | 0.1 | | |
| 24 * | - | - | 9.2 | 90.8 | 78.0 | 16.9 | 0.144 |
| 25 | - | - | 5.9 | 94.1 | 0.1 | | |
| 26 * | - | - | 3.8 | 96.2 | 86.0 | 20.5 | 0.099 |
| 27 | - | - | 2.5 | 97.5 | 0.1 | | |
| 28 * | - | - | 1.6 | 98.4 | 94.0 | 24.5 | 0.057 |

* Steps used for multiple development