A STUDY OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN THE URINE OF THE RACING GREYHOUND

by

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LIST OF ABBREVIATIONS

BPC	bonded phase column
BSA	N,O-bis(trimethylsilyl)acetamide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
ECD	electron capture detection
FID	flame ionisation detection
GC	gas chromatography
GC-MS	combined gas chromatography mass spectrometry
HMDS	hexamethylsilazane
HPLC	high performance (pressure) liquid chromatography
MTBSTFA	N-methyl-N-(tertiary-butyldimethylsilyl)-trifluoracetamide
mu	methylene unit
N.G.R.C.	National Greyhound Racing Club
NPD	nitrogen-phosphorus detection
NSAIDs	non-steroidal anti-inflammatory drugs
ODS	octadecylsilane
PLOT	porous layer open-tubular column
RI	retention indice
SCOT	support coated open-tubular column
SID	selected ion detection
SIMS	secondary ion mass spectrometry
SIR	selected ion recording
TCD	thermal conductivity detection
TIC	total ion current
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TMSI	N-trimethylsilylimidazole
UV	ultra-violet
WCOT	wall coated open-tubular column

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SUMMARY

The purpose of this study was to develop a screening procedure for the detection of non-steroidal anti-inflammatory drugs (NSAIDs) in the urine of the racing greyhound. The study was divided into three parts, dealing with the evaluation and selection of an appropriate analytical methodology, the investigation of acidic endogenous components of greyhound urine and the analysis of selected NSAIDs in urine following administration at therapeutic levels to greyhounds.

(1) In the first part of the study, the analytical methods examined were isocratic high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection, capillary gas chromatography (GC) with temperature programming and flame ionisation detection and gas chromatography-mass spectrometry (GC-MS). Twelve NSAIDs in common use in the UK were selected as test compounds for the evaluation of the separating ability, selectivity and sensitivity of detection of the three methods.

(a) HPLC : For one compound, phenylbutazone, the HPLC capacity factor (k') was measured in seven reversed-phase solvent systems using an octadecylsilane column. The remaining compounds were examined on two selected systems. In addition, fluorescence spectrometry and electrochemical detection (ECD) were examined to assess their value as additional HPLC detection systems which might increase the selectivity or sensitivity of the

method. All of the test compounds were eluted from the column and could be detected by UV spectrometry at a sample size of 5 nanograms on-column. Five of the test compounds could be detected by fluorescence spectrometry and seven by ECD. However, none of the solvent systems examined resolved all of the test compounds from each other.

(b) GLC : Three chemical derivatives were evaluated to increase the thermal stability and improve the chromatographic behaviour of the test compounds - the methyl, trimethylsilyl and tertiary-butyldimethylsilyl derivatives. The retention indices of the three derivatives for each substance were recorded using a glass capillary column coated with dimethylsilicone (non-polar) stationary phase. Three compounds, all pyrazolidine diones, could be chromatographed without derivatisation but produced a mixture of products following reaction with the methylating agent, diazomethane. These were further examined by mass spectrometry.

(c) GC-MS : Gas chromatography with mass spectrometric detection was carried out using similar GC conditions to those described above. Electron impact spectra were recorded at 70 eV for each of the derivatives of the test substances and possible fragmentation reactions giving rise to the most prominent ions in the spectra were proposed. The reaction products of pyrazolidinedione NSAIDs produced by reaction with diazomethane were characterised by GC-MS and tentative structures were

proposed in which methylene addition had occurred at the enol-oxygen atom and at the carbonyl group, to give enol ethers and oxiranes respectively.

It was concluded that GC-MS was the only method which would have sufficient selectivity and separating ability for use in a screening procedure and this method was used in the rest of the study.

(2) The second part of the study established the normal pattern of organic acids present in greyhound urine, which would be co-extracted with any NSAIDs present and might lead to interference in the detection and quantitation of the drugs.

Three different chemical derivatisation methods (methylation, trimethylsilylation and tert-butyl-dimethylsilylation) were evaluated using standards of acids normally present in human urine. Both gas chromatographic retention data and mass spectra were collected. The latter were examined to establish the main fragmentation pathways for each type of derivative.

Urine samples were collected from 3 dogs and 1 bitch, kept in registered kennels, at different times of the day and on successive days. Acid extracts were prepared for GC-MS analysis using a solid-phase method based on cross-linked polystyrene resin (XAD-2). A pooled extract was subdivided into portions and derivatised by four procedures including those listed above and also by combined methylation-trimethylsilylation. The four derivatives were analysed by GC-MS and revealed complex

mixtures of acids, of which more than seventy components were identified.

Although the tertiary-butyldimethylsilyl derivatives gave excellent diagnostic ions, little data was available in the literature to assist in the identification of unknown acids. The methylated extract presented the least complex chromatogram and methylation was used for subsequent analysis. The urinary acid pattern varied between animals, according to the time of day and from day to day.

(3) In the third part of the study five test compounds (ibuprofen, naproxen, ketoprofen, mefenamic acid and phenylbutazone) were administered at therapeutic levels to greyhounds. Blood and urine samples were collected serially in the ibuprofen and mefenamic acid experiments. Urine samples only were collected for the remaining three compounds. Urinary creatinine concentrations were measured for each sample.

The urinary components were extracted as before with XAD-2 resin and glucuronide and sulphate conjugates present in the samples were hydrolysed by incubation with helix-pomatia extract. Plasma samples were extracted using heptane:ethyl acetate. All samples were methylated using diazomethane.

The five test compounds were detected and quantified in the urine and plasma samples collected. Plasma drug concentration/time curves and excretion profiles were obtained for ibuprofen and mefenamic acid.

Metabolites of ibuprofen and naproxen were observed in urine samples. The importance of parallel creatinine measurements to correct for variation in the urinary volume was shown in the naproxen study. The implications of the results with respect to the detection of these five drugs and the interpretation of their concentrations during a screening procedure at a race meeting are discussed.

CHAPTER ONE

INTRODUCTION

1.1 DRUGS IN SPORT

The abuse of drugs in sport is believed to have occurred as far back as the Third Century B.C., when athletes, competing in the Olympic Games, used any means possible to improve their performances. In 1879, in the Six Day Cycle Race, French competitors are reported to have used caffeine, cocaine and strychnine, and Belgians, sugar soaked in ether, in order to improve their performance. Whereas in amateur sporting events such as the Olympic Games, honour and prestige are at stake, greyhound and horse racing offer the incentive of prize money.

In 1967 the cyclist Tommy Simpson died while competing in the Tour de France, Simpson had over-exerted himself and put tremendous strain on his heart. Post-mortem findings revealed that he had taken amphetamine, methamphetamine and alcohol. This was to be a final stimulus to the International Olympic Committee prior to the beginning of drug testing in human sport in the Mexico Olympics in 1968.

Greyhound racing is a major spectator sport in Great Britain, second only to football in the number of participants. It is also a popular gambling sport accounting for approximately 25% of the £4 billion staked in Britain each year. Two types of greyhound tracks exist

in the U.K; 37 tracks under the aegis of the National Greyhound Racing Club (N.G.R.C.), which corresponds to the Jockey Club of horse racing, and the independent "flapping tracks".

At N.G.R.C. tracks such as Shawfield in Glasgow and Powderhall in Edinburgh, dogs run under official rules and are subject to drug testing. The "flapping tracks" do not carry out drug testing procedures.

Pre-race drug screening of urine samples is carried out in a laboratory at the track on all competing greyhounds two hours before a race. Samples found to be positive at this stage are submitted for further examination to the Department of Forensic Medicine and Science at Glasgow University and the animal involved would be immediately withdrawn from the race. About 80,000 pre-race samples are analysed each year. In 1988, 622 samples were assessed as positive at the track laboratories and were submitted to Glasgow University for further analysis. Of these samples 37 were found to contain a banned substance. Table 1.1.1 lists the frequency and the drugs detected.

The list of prohibited substances used internationally by many racing authorities, including the N.G.R.C., is comprehensive, and the intention is to ban the use of any drug in racing greyhounds at the time of competition. By contrast the stated intention of the Medical Commission of the International Olympic Committee is to ban those drugs which are likely to be harmful when

TABLE 1.1.1: Drugs reported to be present in the urine of racing greyhounds in Britain in 1988.

D R U G	Number of Occasions Detected
Caffeine	13
Chlorpromazine	4
Cyclizine	4
Quinalbarbitone	4
Quinine/Quinidine	3
Primidone and Phenobarbitone	1
Amphetamines	1
Ibuprofen	1
Caffeine, Theophyline and Diazepam	1
Millophylline	1
Diazepam and Diphenhydramine	1
P-Aminobenzoic Acid	1
Procaine	1
Glutethimide	1

misused, but with the minimum of interference with the normal therapeutic use of drugs. Non-steroidal anti-inflammatory drugs for instance, are banned in greyhound racing but are permitted to athletes.

Drugs may be used to try to increase or decrease the performance of a greyhound but the desired effect is never guaranteed. A wide range of drugs has been detected in the urine of racing greyhounds, inclucing stimulants such as amphetamine and caffeine, sedatives such as barbiturates, phenothiazines, benzodiazepines and chlorbutanol, and also non-steroidal anti-inflammatory drugs such as phenylbutazone.

The preferred body fluid used for analysis in all species is urine. The collection of urine is non-invasive and is easy to obtain. Greyhounds readily provide a urine sample when released from their transporter. The authenticity of the urine sample must be ensured. Therefore, samples must be correctly labelled and contamination, or sample-switching must be avoided.

In the case of greyhound racing, the urine sample is divided into two portions; one is analysed at the track and the second portion is analysed at Glasgow University if the pre-race screening test is positive. The racing manager at the track can also request that a post-race sample is analysed at the University if he suspects that an animal is not performing as he would have predicted.

1.2 PHARMACOKINETICS AND DRUG METABOLISM

1.2.1 PHARMACOKINETICS

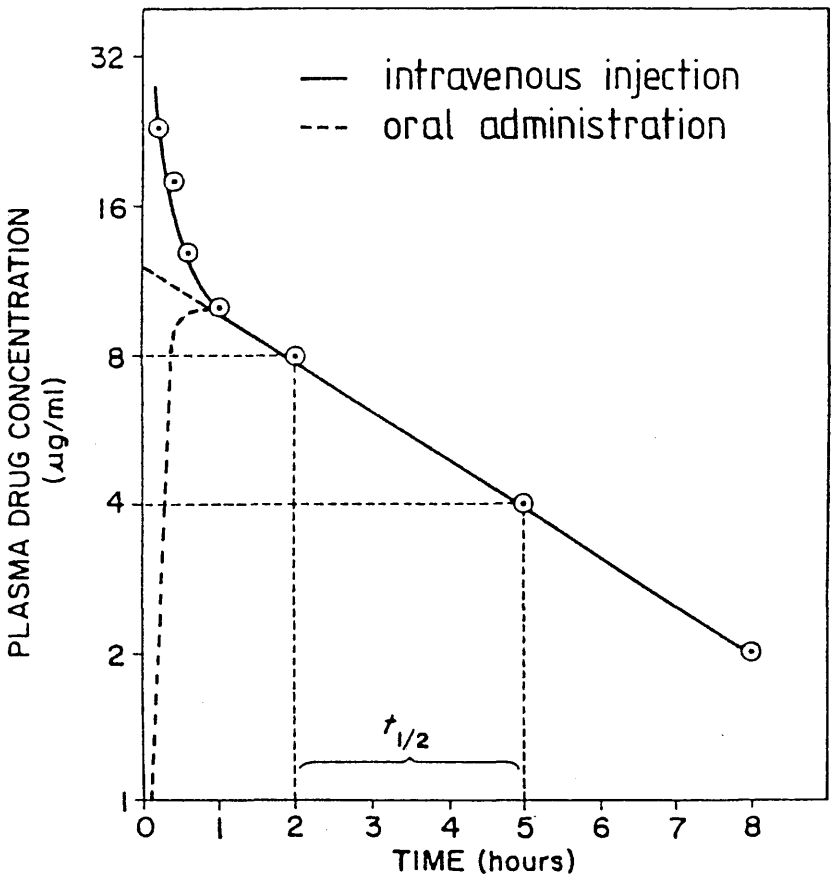
The disposition of a drug is controlled by three major processes: (i) **absorption** into the blood (ii) **distribution** from the blood to peripheral tissues and (iii) **elimination** by both metabolism and excretion. Pharmacokinetics is a mathematical expression of these rate processes.

Figure 1.2.1 illustrates a typical plot of plasma concentration (Cp) versus time for a drug given by intravenous injection and orally. The terminal elimination rate of plasma concentration is the same irrespective of the route of administration. For most drugs the phase of elimination follows first order kinetics.

Drugs, metabolites and their conjugates are removed from the body by various physiological channels of excretion including the urine, bile and faeces. Successful clearance depends on physiological mechanisms of transport (heart, vessels, blood and circulation) and of excretion (kidney and bowel functions) and is related to the physico-chemical properties of the drug molecule, in particular water solubility and protein binding.

The main route of excretion is in the urine. Drugs appear in the glomerular filtrate but if they are lipid-soluble they are readily reabsorbed in the renal tubules by passive diffusion. Metabolism of a drug often results in a less lipid-soluble compound, aiding renal

FIGURE 1.2.1: Plot of plasma concentration versus time for a drug given by intravenous injection and orally.



excretion (see Section 1.2.2). The pH of the urine will affect ionisation of weak acids or bases. Unionised drug is reabsorbed; ionised drug is cleared. Manipulation of the urine pH is sometimes useful in increasing renal excretion. For example, bicarbonate administration makes the urine alkaline; this ionises aspirin making it less lipid-soluble and increasing its rate of excretion.

Bile is also a route of clearance. Once excreted the drug is cleared in the faeces, but some drugs are reabsorbed to a significant extent through entero-hepatic circulation (Figure 1.2.2). The effect is to prolong the presence of a drug in the body.

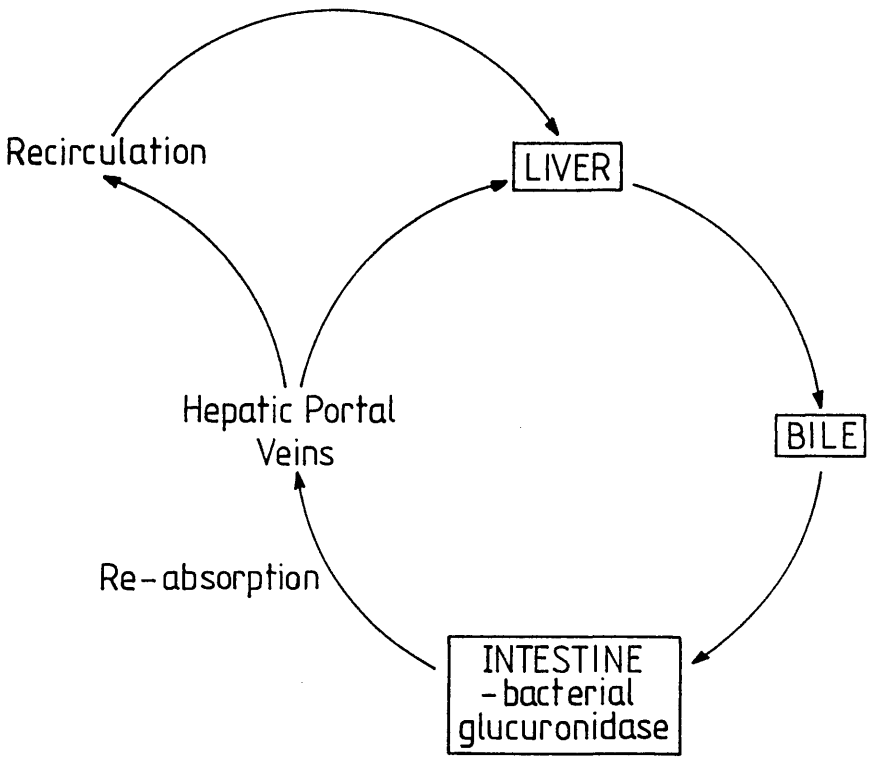
Some of the terms used in pharmacokinetics which are relevant to later discussion are outlined briefly below. Reviews of all aspects of pharmacokinetics are discussed elsewhere [1-3].

(a) Half-Life: the half-life ($t_{1/2}$) is the time taken for the concentration of a drug in blood to fall by half its original value (Figure 1.2.1). Measurement of the $t_{1/2}$ allows calculation of the elimination rate constant (k) from the formula:

$$k = 0.693/t_{1/2}$$

(b) Elimination Rate Constant: the elimination rate constant (k) is the fraction of drug present at any time that would be eliminated in unit time. For example, $k = 0.02\ min^{-1}$ means that 2% of the drug present is eliminated in 1 minute.

FIGURE 1.2.2: The entero-hepatic circulation of a drug which is excreted in the bile.



(c) Volume of Distribution: the volume of distribution (V_D) is the apparent volume into which a drug is distributed.

(d) Clearance: Clearance is the volume of blood or plasma cleared of drug in unit time. Plasma clearance (Clp) is given by the expression: $Clp = V_D k$ and the rate of elimination = Clp x Cp. Clearance is the sum of individual clearances, including metabolic and renal clearance.

1.2.2 DRUG METABOLISM

Since in greyhound racing the analyst is generally supplied with a urine sample for analysis he must be aware of the rates and products of metabolism of any drugs which might be present.

The metabolism of drugs (that is, the effect that an organism has on drugs), encompasses a wide range of chemical reactions. Drug metabolism is generally divided into two phases: Phase I (reactions on functional groups) and Phase II (conjugation reactions)[4]. Phase I reactions include oxidation, reduction, hydrolysis and hydration and Phase II reactions include glucuronidation, sulphation, methylation and amino acid conjugation.

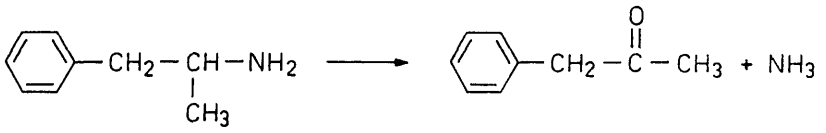
Drugs are often lipophilic in nature and in order to eliminate them from the body they have to be metabolised to a more water-soluble form.

Figure 1.2.3 gives a few examples of the type of reactions involved in the metabolism of drugs. Oxidation reactions can be divided into two types depending on the

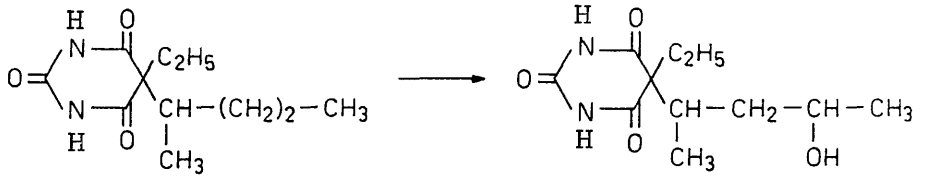
FIGURE 1.2.3:

I. Oxidation involving cytochrome P-450

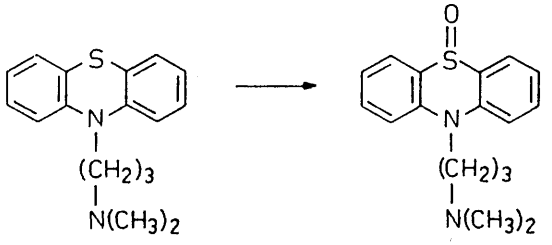
(a) The oxidative deamination of amphetamine



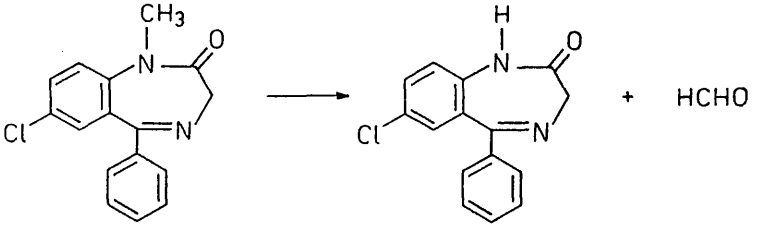
(b) The side-chain hydroxylation of pentobarbital



(c) The S-oxidation of chlorpromazine

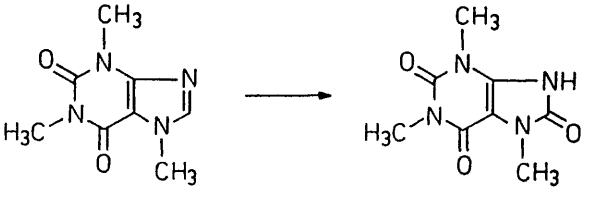


(d) The N-demethylation of diazepam



II Other enzyme systems e.g. xanthine oxidase

(a) The oxidation of caffeine



enzymes involved; oxidation performed by the microsomal mixed-function oxidase system (cytochrome P-450-dependent) and other oxidation systems. The mixed-function oxidase system performs many different functionalisation reactions; four examples are given in Figure 1.2.3. The other oxidation reactions are performed by a number of enzymes in the body not related to mixed-function oxidases, including alcohol dehydrogenase, xanthine oxidase and amine oxidase.

The main function of Phase I metabolism is to prepare a compound for Phase II metabolism and not to prepare the drug for excretion. Phase II is the true 'detoxification' of drugs and gives products that are generally water-soluble and easily excreted.

In most cases the end products of Phase I metabolism is a chemically reactive functional group, such as -OH, $-NH_2$, -SH and -COOH. Drugs containing these functional groups can then be conjugated in Phase II reactions with compounds such as glucuronic acid, glycine, glutathione and sulphates.

1.3 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

The non-steroidal anti-inflammatory drugs (NSAIDs) form part of a group of drugs used in the treatment of arthritis and have analgesic, anti-inflammatory and antipyretic properties. Their abuse in greyhound racing may be with the intention to alleviate the pain, swelling and stiffness of injured joints and may help a dog which is not quite at its peak to run a little better. They may also enable a lame dog to run. The use of NSAIDs is banned by the N.G.R.C. in greyhounds during competition.

The non-steroidal anti-inflammatory group of drugs contains a diverse range of structural types, but most are acidic and can be classified under three main headings: arylcarboxylic, arylalkanoic and enolic acids. Examples of each group are shown in Table 1.3.1. Representative members of each group were selected for inclusion in this study (indicated with *); these are the compounds most widely prescribed in Great Britain in the treatment of arthritis.

Despite the diverse structural range within the NSAID group their pharmacological properties are very similar. Their mode of action is complex but generally speaking they all inhibit the biosythesis and release of prostaglandins which are involved in the pathogenesis of inflammation and fever. The pharmacology of these drugs is well reviewed elsewhere [5] and will be discussed in subsequent chapters.

TABLE 1.3.1: Non-steroidal anti-inflammatory drugs used in the treatment of arthritis.

ARYLCARBOXYLIC ACIDS

Salicylic Acids

Acetylsalicylate
Diflunisal

Anthranilic Acids (Fenamates)

Etafenamic Acid
Flufenamic Acid
Meclofenamic Acid
*Mefenamic Acid

ARYLALKANOIC ACIDS

Arylpropanoic Acids

*Fenbufen
Fenprofen
*Flurbiprofen
*Ibuprofen
Indoprofen
*Ketoprofen
*Naproxen
Tiaprofenac

Arylacetic Acids

Alclofenac
Diclofenac
Fenclofenac
Fentaizac

Heteroaryl Acetic Acids

Tolmetin
Zomepirac

Idene and Indole Acetic Acids

*Indomethacin
*Sulindac

ENOLIC ACIDS

Pyrazolidinediones

*Azapropazone
Feprazone
Kebuzone
*Phenylbutazone
*Sulphinpyrazone

Oxicams

*Piroxicam

* representative NSAIDs included in this study.

1.4 PROJECT AIMS

The purpose of this study was to develop a screening procedure to detect and identify non-steroidal anti-inflammatory drugs in the urine of the racing greyhound. In order to do this it was necessary to establish a method for systematic metabolism studies.

Since many NSAIDs and their metabolites resemble endogenous substances screening for NSAIDs in urine is a difficult problem requiring relatively sophisticated methodology.

Although the metabolism of NSAIDs has been extensively studied in rats, rabbits and humans, it was expected that due to the low fat and high muscle mass of the greyhound, the metabolism of these compounds may be quite different from other animals, both in terms of pharmacokinetics and the nature and relative amounts of the metabolites formed. A knowledge of these sets of information for each drug of interest is a necessary pre-requisite for the creation of an efficient drug screening procedure.

CHAPTER TWO

ANALYTICAL METHODS

2.1. INTRODUCTION

The purpose of this study was to develop a method to screen qualitatively and quantitatively for non-steroidal anti-inflammatory drugs (NSAIDs) in the urine of the racing greyhound. The twelve drugs which were chosen for study, as the most commonly prescribed representatives of the class of drugs in the U.K., were ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen, mefenamic acid, indomethacin, sulindac, phenylbutazone, sulphinpyrazone, azapropazone and piroxicam.

Most NSAIDs can be quantified using spectrophotometric and/or spectrofluorometric techniques. These methods, however, were not suitable for this study because they are not specific due to interferences from metabolites, other drugs and endogenous co-extracted material from body fluids. Immunological methods also were not considered. It would be impossible to raise one antibody against a group of drugs with such diverse chemical structures. Screening would require sets of antibodies for each chemical class (when screening for drugs of abuse, for instance, radioimmunoassays are run for opiates, cannabinoids and amphetamines). Only chromatographic techniques will be considered here.

The literature contains many specific methods for

the analysis of analgesics in various matrices including pharamaceuticals and body fluids but few studies include more than about ten NSAIDs [6-14] and even fewer assay several of the drugs in urine [6,7,15-17]. The methods which assay the drugs in urine will be considered first.

Hunt and co-workers [6] developed a gas chromatographic screening procedure for a limited number of NSAIDs in horse urine. The drugs were solvent extracted from urine and were detected by gas chromatography (GC) with flame ionisation detection (FID) after on-column methylation using trimethylanilinium hydroxide as the methylating reagent. Fifteen NSAIDs were studied. Eight of the drugs were detected in horse urine (including mefenamic acid, ibuprofen, indomethacin, naproxen and ketoprofen) after the animal had been administered with the drug. The other seven drugs (including flurbiprofen) detected by this method were simply added to the horse urine. Hunt and his colleagues used a packed GC column (equivalent to SE-30) and a temperature programmed run. The methylation procedure was found not to be sufficiently reproducible and could not therefore be used for quantitation. No metabolites were detected using this method. However, the authors commented that to detect the metabolites hydrolysis of the urine would most likely be necessary.

The method of Hunt and co-workers was further developed by Chalmers and colleagues in 1984 [7] and employed a combination of thin layer chromatography (TLC),

high-performance liquid chromatography (HPLC), GC and gas-chromatography mass spectrometry (GC-MS). TLC was carried out using Kieselgel plates and a mobile phase of ethylacetate:methanol:0.88 ammonia (8:1:1, v/v/v) and the plates were examined under UV light before overspraying the plates with acidic spray reagents. GC was effected with a packed column as before [6] but used both FID and nitrogen-phosphorus detection (NPD). The HPLC system was reversed-phase and consisted of a pre-column (Co Pell C_{18}, 5cm x 4.6mm) and an analytical column of octadecyl silica (ODS) (10μm, 22cm x 4.6mm), UV detection was at 280nm. Gradient elution was carried out with an acetonitrile/0.1M acetic acid mix. The TLC and GC methods were successful in detecting the standard drugs, but the HPLC method failed to detect ibuprofen and a few of the other test compounds. The chromatographic systems were less effective when used to assay the drugs in the presence of urine. Twenty-three out of the twenty-eight drugs assayed were detected using a combination of the three methods but the other five compounds were difficult to distinguish from co-extracted material present in the horse urine.

Each HPLC method in the literature which assays NSAIDs in urine can only resolve a limited number of drugs. Three such methods have been developed by Upton and co-workers [15], Thomas and co-workers [16] and Ng [17] and are discussed below.

In 1980 Upton and co-workers [15] reported a

sensitive HPLC method for five NSAIDs (including ketoprofen and naproxen) in plasma and urine. The drug conjugates were hydrolysed in 1M sodium hydroxide before analysis and extracted by solvent extraction. The method of detection was HPLC-UV (at 262nm) using a reversed-phase system consisting of an ODS column (5μm, 4cm x 4.6mm) protected by a 4cm x 3.2mm pre-column and a mobile phase of 0.05M phosphate buffer (pH7) containing between 6 and 8 percent (v/v) acetonitrile. This system could resolve naproxen, ketoprofen, ibuprofen, fenoprofen and probenecid.

Thomas and colleagues reported an HPLC method for the analysis of six NSAIDs (including indomethacin, ketoprofen, naproxen and phenylbutazone) in plasma or urine. Diluted urine, protein-free plasma or ether extracts were injected onto the ODS column (5cm x 5mm). The mobile phase was aqueous methanol adjusted to pH3. The methanol content and detection wavelength had to be altered to optimise the system for each drug.

Ng used a simple sample preparation of salt precipitation of proteins followed by HPLC-UV to analyse four NSAIDs (including indomethacin, phenylbutazone and sulindac). The HPLC consisted of an ODS column (30cm x 3.9mm) and the solvents used as mobile phases were aqueous mixtures of acetonitrile and methanol acidified with phosphoric acid to pH2.2. Similar to Thomas and co-workers' method, Ng's mobile phase composition varied depending on the drug of interest.

Other workers who have analysed ten or more

NSAIDs have developed methods to analyse the drugs in blood [8] or plasma [9,13,14] or simply drug standards [10,11,12].

Recently Sharp reported [8] a GC-FID method which involved a single extraction with ethyl acetate for sixty acidic or neutral drugs in blood (including nine of those of interest in the present study). The samples were assayed either without derivatisation or with on-column methylation (using flash-heater methylation) on a fused silica capillary column with a split injector. A temperature programmed run was used. Out of the nine drugs of interest which were examined, indomethacin and piroxicam were found to have poor chromatographic properties, ketoprofen, naproxen and phenylbutazone were found to give multiple peaks on methylation and sulindac did not chromatograph at all.

Two of the methods which assayed several NSAIDs in plasma were HPLC methods [9,14]. The HPLC assay of Nielsen-Kudsk [14] assayed fifteen NSAIDs, including nine of interest in this study. An ODS column (30cm x 3.9mm) with a methanol-phosphate buffer was used, the methanol content needing to be altered to obtain optimal separation and reasonable elution times as with other methods already discussed [16,17]. Owen and co-workers recently reported a rapid HPLC assay for the simultaneous determination of nine NSAIDs [9]. The system consisted of an ODS column (30cm x 3.9mm) and a phosphoric acid 0.03%, pH2.5 - acetonitrile (45:55, v/v) mobile phase. Ketoprofen

and naproxen were not sufficiently separated with this system and the detection wavelength had to be altered to provide sufficient sensitivity to measure ibuprofen. The authors noted that the metabolites of phenylbutazone, present in the plasma, would interfere in the elution of naproxen and ketoprofen.

Giachetti reported [13] a solvent extraction method from plasma of ten NSAIDs (including four of interest in the present study) followed by capillary GC analysis of the methyl esters. The methyl esters were formed using anhydrous diazomethane in diethyl ether and a temperature programmed run and FID detection were used. This system was able to resolve all the standards examined, but some of the later-eluting peaks (including indomethacin) were broad.

Some methods have presented retention data for ten or more compounds when considering interferences with specific assays. Two assays of this type have been reported; the analysis of ibuprofen in dog serum [11] and the determination of naproxen in plasma [12]. The other NSAIDs mentioned in these two papers were therefore non-extracted drug standards. Kearns and Wilson [11] found indomethacin to interfere in their ibuprofen assay which consisted of a solvent extraction followed by HPLC analysis on an ODS column with a mobile phase of methanol-water-glacial acetic acid (pH3.4, 75:25:1, v/v/v). In the naproxen HPLC assay of Broquaire and co-workers [12]

oxyphenbutazone and sulindac were reported to interfere with the analysis. In this assay an ODS column was also used and the mobile phase was acetonitrile-aqueous orthophosphoric acid (pH3, 45:55, v/v).

In 1986 Stevens and Gill [10] reported HPLC retention data for over forty analgesics, including nine of those of interest. The authors reported that initial experiments with various eluents revealed that the analgesic drugs showed a very wide range of retention properties. Stevens and Gill found that a series of isocratic eluents were necessary to elute the compounds of interest with suitable retention characteristics. An ODS silica stationary phase was employed and three different isocratic eluents were used. The eluents were prepared from isopropanol, formic acid and an aqueous phosphate buffer. Nine of the compounds of interest were eluted with the mobile phase isopropanol - potassium hydrogen phosphate (0.1M) - formic acid (154:1000:1, v/v/v).

There are other methods available which assay between three and ten NSAIDs. The majority of those methods have been based on HPLC and are similar to the ones already mentioned in that they all employ a reversed-phase system using an ODS column and an acidic mobile phase. Some of these methods are isocratic [18-23] while others employ gradient elution [24] or some modification of wavelength and/or mobile phase to enable detection of all the drugs examined [25,26].

Ford and co-workers [27] reported a rapid

extraction method for acidic drugs in blood employing GC-NPD and GC-MS. The only drugs of interest which were examined were ibuprofen and phenylbutazone.

In this study it was decided to examine HPLC, GC and GC-MS as possible methods for the analysis of the twelve NSAIDs. It was realised that isocratic HPLC alone would not be selective enough for the drugs of interest (a gradient system was not available) so different methods of detection, such as UV, fluorescence and electrochemical detection were examined. It was decided that a reversed-phase system with an acidic mobile phase would be necessary to elute the compounds and this has been demonstrated in the literature already mentioned.

A capillary GC method would also be examined. It was thought that a capillary column would be necessary to provide sufficient resolution of the twelve compounds. As it would be necessary to derivatise some of the drugs of interest, different methods of derivatisation would be examined.

Finally it was thought that GC-MS would be necessary to give absolute identificaton of the twelve compounds, so GC-MS of the standards and their derivatives would be considered.

2.2 CHROMATOGRAPHY : GENERAL INTRODUCTION

2.2.1 HISTORY

Mikhail Semenovich Tsvet, a Russian botanist, was the pioneer of chromatography; he first described the method in 1903 [28] and gave a full account of it in German in 1906 [29]. Tsvet employed the technique to separate various plant pigments such as chlorophylls and xanthophylls by passing a solution of their compounds through a glass column packed with finely divided calcium carbonate. The separated species appeared as coloured bands on the column, which accounts for the name Tsvet chose for the method. Although the technique had been used occasionally between 1910 and 1930 [30] it was not until 1931 when work was published by Kuhn and co-workers [31] that chromatography came into general use.

Martin & Synge published their first paper on liquid-liquid partition chromatography in 1941 [32]. It was here that they first introduced the concept of gas-liquid chromatography. This idea, however, lay dormant until 1952 when James and Martin published the first report devoted to the subject of gas-liquid chromatography [33]. They separated a series of fatty acids using a titration procedure as a detector employing a microburette. The microburette was eventually automated providing a very effective on line detector with an integral response [34].

High pressure or high performance liquid chromatography (HPLC) was subsequently developed from work

on the theory of partition chromatography by Martin and Synge [32] and from experience in gas-liquid chromatography. Giddings [35] provided a major theoretical influence on the advance of HPLC, but instrumental problems delayed its realization. The major experimental advances which brought about practical high pressure liquid chromatography systems, were made by Lipsky in 1967 [36], Huber in 1967 [37] and Kirkland in 1969 [38].

The applications of chromatography have grown exponentially in the last forty years and the tremendous impact which these methods have had on science is attested by the 1952 Nobel Prize that was awarded to Martin and Synge for their discoveries in the field.

2.2.2 TYPES OF CHROMATOGRAPHY

Chromatography is essentially a technique for the separation of the components of mixtures by a continuous distribution of the components between two phases, one of which (the mobile phase) is moving past the other (the stationary phase). The technique relies upon intermolecular interactions in the mobile and stationary phases to cause differences in the migration rates of the components thus achieving a separation. Table 2.2.1 lists the types of interactions which are possible.

Chromatography is generally divided into four types: adsorption, partition, ion exchange and molecular exclusion, depending on the primary interactions operating in the system. However, the four types of chromatography cannot be so easily compartmented as described here. In

TABLE 2.2.1: Types of interactions involved in chromatography

System	Primary Interaction	Secondary Interaction
Adsorption	Hydrogen Bonding Dipole-Dipole	Ion-Ion Interaction Van Der Waals
Partition	Van Der Waals	Hydrogen Bonding Ion-Ion Interaction
Ion-Exchange	Ion-Ion Interaction	Hydrogen Bonding Van Der Waals
Molecular Exclusion	Steric Exclusion	

reality there are no distinct boundaries and several different mechanisms, or secondary interactions, often operate simultaneously. In gas-liquid chromatography, for instance, active sites may be available on the support material to allow adsorptive processes to occur as well as partition. Ion exchange chromatography involves hydrophobic interactions and specific adsorption processes as well as electrostatic ion interactions.

The choice of mobile phase in gas chromatography is fairly limited, an inert gas such as helium or nitrogen or argon/methane (for nitrogen-phosphorus detection, see Section 2.4.1(c)) is normally used. In liquid chromatography the number of mobile phases available is extensive, the choice depending on many factors. The total interaction of a solvent molecule with a sample molecule is a result of four major interactions: dispersion, dipole, hydrogen bonding (both proton donors and proton acceptors) and dielectric interactions. These interactions in combination determine a solvent's polarity. The polarity of solvents is typically expressed in an elutropic series in which the solvents are arranged in order of increasing polarity. Solvent strength increases with solvent polarity in normal-phase partition liquid chromatography and in adsorption liquid chromatrography, whereas in reversed-phase liquid chromatography solvent strength decreases with increasing polarity. Snyder and Kirkland [39] describe solvent selection in their book on modern liquid chromatography

and give a table of solvent polarities.

Recently liquefied gases such as carbon dioxide, neon or ammonia have been used as a mobile phase: this is known as supercritical fluid chromatrography [40].

(a) ADSORPTION CHROMATOGRAPHY

In adsorption chromatography the solute (the component to be isolated or measured) and the solvent (or mobile phase) compete for active sites on the stationary phase. Separations are achieved by the retention of a component resulting from its affinity for a particular site. The stationary phase is a solid and the mobile phase is gaseous or liquid. Adsorption chromatography, therefore, includes gas-solid and liquid-solid chromatography. Liquid-solid chromatography incorporates column chromatography, thin layer chromatography and high performance (pressure) liquid chromatography.

Gas-solid chromatography is useful for the analysis of gases; carbon disulphide [41], carbon dioxide [42], propellants [43], solvents [43] and volatile organic compounds [44], such as acetonitrile, benzene, carbon tetrachloride and styrene. The mobile phase is an inert gas such as helium or nitrogen and the stationary phase is an active solid. These solids may be inorganic materials, for example, synthetic zeolite molecular sieve, carbon molecular sieve, silica gel or graphitised carbon, or they may be organic polymers. The organic polymers include divinylbenzene cross-linked polystyrene co-polymers (the

Chromasorb and Porapak series) and Tenax-GC, a porous polymer of 2,6-diphenyl-p-phenylene oxide.

In liquid-solid chromatography, either as column or thin layer, silica, alumina or cellulose are commonly used as the adsorbent. These adsorbents may have water on their surfaces, allowing partition and adsorptive processes to occur. The mobile phase can be a pure liquid or a mixture of solvents. The choice of solvent is determined by the sorption process employed and by the nature of the sample components.

Liquid solid chromatography has been employed in the analysis of many compounds including insecticides [45], antibiotics [46], porphyrins [47], flavanoids [48] and organometallic isomers [49].

Thin layer chromatography is used extensively by both synthetic and analytical chemists. It is a fast method of checking a reaction's progression and a simple method to assess a compound's purity and identity [50].

As discussed later in the theory of chromatography (Section 2.2.3), chromatographic efficiency increases with decreasing particle size of the sorbent. In order to achieve a practical flow rate under these conditions a positive pressure must be applied to the top of the column, this is known as high pressure liquid chromatography.

(b) PARTITION CHROMATOGRAPHY

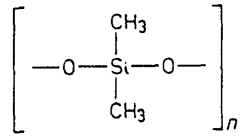
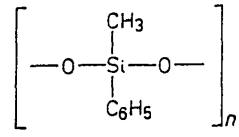
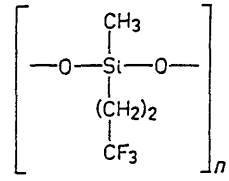
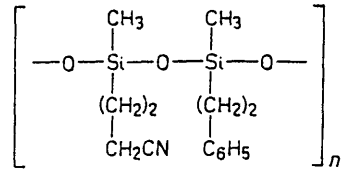
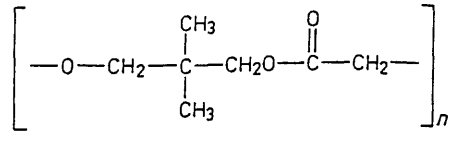
Another form of chromatography is partition chromatography. In this case the solute is distributed

between two immiscible phases according to its partition coefficient. Separations are achieved when the components of a mixture have different partition coefficients and so take different times to travel through the stationary phase. The stationary phase is a liquid and the mobile phase is gaseous or liquid. Partition chromatography includes gas-liquid and liquid-liquid chromatography. Liquid-liquid chromatography includes paper, column chromatography and high-pressure liquid chromatography.

In gas-liquid chromatography (GC) the mobile phase is an inert gas such as helium or nitrogen and the liquid stationary phase is coated on a solid support material for packed column chromatography. For capillary column chromatography the stationary phase may be located directly onto the walls of the column or onto a support which is bonded to the glass walls. The most common support material is calcined diatomaceous earth which is acid or base washed to remove impurities and sieved to remove fine particles. It can also be treated with a silanising reagent which reacts with the surface hydroxyl groups and reduces adsorption effects. There are a great number of liquid stationary phases available for GC covering a wide range of polarity. Some examples of stationary phases used in GC are given in Table 2.2.2.

In general, non-polar compounds chromatograph best on non-polar phases and polar compounds on polar phases. Gas-liquid chromatography has been applied to the analysis of many compounds including antifungals [51], amino

TABLE 2.2.2: Examples of GC stationary phases

Class	Structure	Character	Name
Methylsilicone		Non-polar	OV1 SE30 SP2100
Methylphenyl-silicone		Medium-polar	OV17 SP2250
Trifluoropropyl-silicone		Medium-polar	QF1 OV210 SP2401
Cyanopropyl-methylphenyl-silicone		Medium-polar	OV225
Neopentylglycol succinate		Polar	NPGS
Polyethylene glycol	$CH_2OH[CH_2OCH_2]_nCH_2OH$	Polar	Carbowax

acids [52], fatty acids [53] and there are numerous applications of GC to drug analysis; Clarke's "Isolation and Identification of Drugs" is an excellent reference source [50]. (Gas-liquid chromatography columns will be examined in more detail in Section 2.4.1).

Paper chromatography might appear to be a form of liquid-solid chromatography, however, due to water molecules associated with the cellulose fibres; it is in fact an example of liquid-liquid chromatography. Many applications of paper chromatography also call for the creation of an immobilised solvent layer within the paper fibres by dipping the paper in a suitable solvent mixture. As in liquid-solid chromatography a range of solvents with varying polarities can be employed in the elution of paper chromatography.

Liquid-liquid chromatography exists in two forms: normal (or straight-phase) and reversed-phase. The normal phase mode utilises a non-polar mobile phase and a polar stationary phase. This type of system would retain polar solutes and non-polar solutes would elute first. The reversed-phase system employs a polar mobile phase and a non-polar stationary phase, when polar components elute first.

Classical liquid-liquid partition systems (in either column chromatography or HPLC) require a column of more or less inert packing material that has been coated with a stationary phase. A typical normal phase system would use triethylene glycol coated on to a wide pore

silica gel as stationary phase and triethylene-glycol-saturated hexane as mobile phase. This system has been used to separate phenols [54], steroids [54] and aromatic alcohols [55]. This type of system remains stable as long as the eluent is saturated with the stationary phase. Reversed-phase systems, where, for example, squalene (a non-polar stationary phase) is coated onto a silica based support and the samples eluted with a water/alcohol mobile phase are no longer used. The squalene, being non-polar, is not held strongly on the silica and results in a low bleed from the column.

The most widely used column packings for modern liquid chromatography are chemically-bonded packings prepared by bonding an organic moiety to the surface of an adsorbent. Halasze and Sebastian were the first to introduce bonded-phase materials in 1969 [56]. A wide range of functional groups allows both normal and reversed-phase chromatography. Polar bonded-phase packings (for example, silica) are used for normal-phase separations. The most popular reversed-phase stationary material is octadecyl silane (ODS) in which the C_{18} group is bonded to a silica support via a silyl ether (siloxane) linkage. The mobile phase is a polar solvent such as methanol-water. Other chemically-bonded stationary phases, for example, have C_8 hydrocarbon chains, substituents containing aromatic moieties such as phenyl groups, or polar groups such as cyanopropyl and nitropropyl.

The literature covering separations of drugs, pesticides and many other compounds by bonded-phase materials, is now vast: examples are given in several books on HPLC [50,57-60]. Further details of liquid-liquid chromatography can be found in the text by Snyder and Kirkland [39].

Types of HPLC columns will be discussed in section 2.3.1.

(c) ION EXCHANGE

Ion exchange chromatography is based on the principle that oppositely charged ions are attracted to each other. The functional groups on the packing material are groups such as amines or quarternary amines (for anion separation) and sulphonic acid or carboxylic acid (for cation separation). For example, an ion exchange column may contain sulphonate residues fixed to the stationary phase along with oppositely charged counter-ions, Na^+. The counter-ions are also normally present in the mobile phase as a salt, NaCl, for instance. The ionic sample molecules, in this case, would be positively charged and are retained by exchange with the Na^+ ions. Ion exchange chromatography has been used to analyse many biologically important substances [57,59].

(d) Molecular Exclusion

Molecular exclusion chromatography can be carried out in both gas chromatography and liquid chromatography, where it is also referred to as gel chromatography, gel filtration or gel permeation chromatography. This method

separates molecules on the basis of molecular weight and size. The column packing in gel chromatography is porous with pores of a certain size. The larger molecules are eluted more quickly since the smaller ones diffuse further into the pores of the stationary phase and take a longer time to be eluted. Molecular exclusion is used, for instance, in the analysis of carbon monoxide by GC [61] but is mostly used in liquid chromatography of naturally occurring and synthetic polymers.

2.2.3 THEORY

(a) THEORY OF SEPARATION AND RETENTION CHARACTERISTICS

In most chromatography systems the stationary phase is retained inside a column. This is true for classical column, gas-solid, gas-liquid and high-pressure liquid chromatography. In the case of thin layer and paper chromatography, the stationary phase is an open bed. The theory discussed here refers to column chromatography but may also be applied to open bed chromatography.

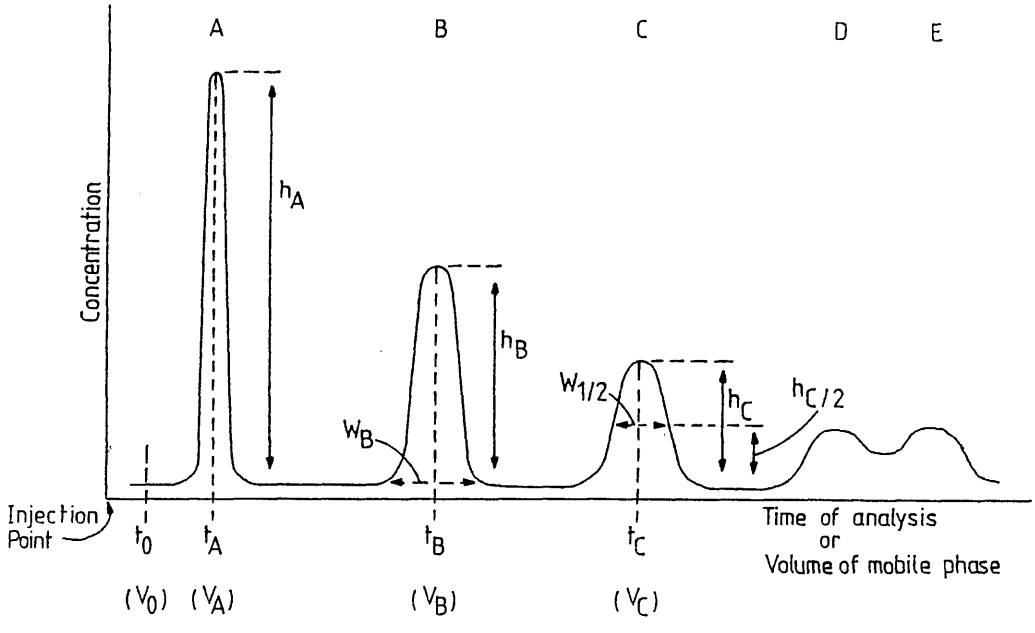
In elution chromatography the sample is applied to the top of the column and immediately the components of the sample distribute themselves between the mobile and stationary phases. Introduction of additional mobile phase (or eluent) forces the solvent containing a part of the sample down the column, where further partitioning between the mobile phase and fresh portions of the stationary phase occurs. Simultaneously, partitioning between the fresh solvent and the stationary phase takes place at the site of the original sample. Continued

additions of solvent carry solute molecules through the stationary phase in a continuous series of transitions between the mobile and the stationary phases. Since solute movement can only occur in the mobile phase, the average rate at which a solute migrates depends on the fraction of time it spends in that phase. If the solute has a strong affinity for the stationary phase it will be retained longer. If, however, the solute has a low affinity for the stationary phase it will be eluted more quickly. Provided that the column is long enough these differences in affinity will result in the components of a mixture separating into bands. Theoretically the solute molecules should migrate through the stationary phase as symmetrical zones. If a detector that responds to solute concentration is placed at the end of the column and its signal plotted as a function of time (or volume of eluent) a series of symmetrical peaks would be obtained.

A plot of time (or volume) versus signal is known as a chromatogram and can be useful for both qualitative and quantitative analysis. The position of the peaks can help to identify the component and the areas under the peaks are related to concentration of the components (See Figure 2.2.1).

It is apparent that movement down a column increases the distance between two components. At the same time broadening of both bands occurs which lowers the efficiency of the column as a separating device. This band broadening is unavoidable, but fortunately occurs

FIGURE 2.2.1: Chromatogram illustrating some of the terms used in chromatography (explained in more detail in the text)



t_0 retention time of unretained solute peak

V_0 retention volume of unretained solute peak

t_A retention time of peak A

V_A retention volume of peak A

$t'_A = t_A - t_0$ adjusted retention time of peak A

$V'_R = V_R - V_0$ adjusted retention volume of peak A

W_B peak width of peak B

$W_{½}$ peak width at half peak height

h_A peak height of peak A

$h_{c/2}$ half peak height of peak c

more slowly than band separation. A useful theory of chromatography therefore must account for both the rate at which solutes migrate and the rate of band broadening. The rate or kinetic theory of chromatography describes the effects of variables on the band retention and band spreading characteristics of a system. Thermodynamics govern the speed at which any band of solute moves along the column and kinetics govern the rate at which a band broadens as it moves down the column.

During the chromatography process, therefore, the sample components quickly distribute themselves between the mobile and stationary phases as soon as the sample is applied to the top of a column. Shortly after the commencement of migration an equilibrium is achieved between the solute in the mobile phase, Xm and the solute in the stationary phase, Xs:

$$Xm \rightleftharpoons Xs$$

This equilibrium can be expressed quantitatively by means of a temperature-dependent constant, the partition coefficient, K:

$$K = \frac{[Xs]}{[Xm]}$$

where

[Xs] is the concentration of the solute in the stationary phase, and,

[Xm] is the concentration of the solute in the mobile phase.

K is only a constant at low concentration ranges such as those generally found in chromatography. For wide

concentration ranges the variation of K with solute concentration must be taken into account. Chromatography carried out under conditions such that K is constant is called linear chromatography.

During chromatography, molecules move while in the mobile phase at the mean linear speed of the mobile phase, V, and when in the stationary phase they do not move at all. The velocity of the centre of each band is therefore determined by the partition coefficient. It follows that the speed of the band of solute molecules, Vs, relative to the mobile phase is given by:

$$\frac{Vs}{V} = \text{fraction of solute in mobile phase at equilibrium}$$

The fraction of molecules in the mobile phase is given by:

$$\frac{Qm}{Qm + Qs}$$

where

Qm is the quantity of the solute in the mobile phase, and,

Qs is the quantity of the solute in the stationary phase.

Therefore,

$$\frac{Vs}{V} = \frac{Qm}{Qm + Qs}$$

rearranging the above equation gives:

$$\frac{Vs}{V} = \frac{1}{1 + Qs/Qm} = \frac{1}{1 + k'}$$

The ratio Qs/Qm is known as the capacity factor k'. It is affected by the polarity of the solvent and the packing and is a measure of sample retention. The capacity

factor, k', is a more practical quantity than the partition coefficient, K, and can be determined from the chromatogram (Figure 2.2.1), thus:

$$k' = \frac{V_A - V_0}{V_0}$$

or,

$$k' = \frac{t_A - t_0}{t_0}$$

where,

V_0 is the retention volume of an unretained peak

V_A is the retention volume of peak A

t_0 is the retention time of an unretained peak

and,

t_A is the retention time of peak A

V_0 (or t_0) can be measured by injection, for instance, of a dilute solution of sodium nitrite, in the case of HPLC, or methane for GC.

The two constants K and k' which describe the distribution of a sample component between the mobile and stationary phases can be related since,

Quantity = Concentration x Volume

Hence,

$$k' = Qs/Qm = \frac{[Xs]Vs}{[Xm]Vm} = K\,\frac{Vs}{Vm}$$

Since exact volumes of mobile and stationary phases for columns are not known absolute retention values are seldom reported. It is also difficult to obtain exact duplicates of systems reported in the literature. Relative retention values are therefore used since individual column characteristics cancel out when retention values are

divided. The relative retention value, alpha, is defined as the ratio of capacity factors for any two given peaks. Alpha is often referred to as the selectivity or separation factor and is affected by the chemistry of the whole system.

Instead of reporting absolute retention values, in GC Kovats Indices [62] are used as a measure of a compound's retention. This system is based on a linear relation between the log of the partition coefficient of an aliphatic hydrocarbon in the stationary phase and the carbon number in a homologous series of test standards. The indices are normally used for isothermal chromatography. The expression for the Kovats index is given by:-

$$I^{\text{temperature}}_{\text{stationary -phase}} = \left[\frac{\log tr - \log tr(n)}{\log tr(n+1) - \log tr(n)} + n \right] \times 100$$

where

tr is the retention time of an unknown

tr(n) is the retention time of a normal alkane having n carbon atoms, and,

tr(n + 1) is the retention time of a normal alkane having (n + 1) carbon atoms.

Another retention index system, which normally is expressed in methylene Units (M.U) [63] is used with temperature programming and is given by an analogous expression:

$$M.U. = \frac{tr - tr(n)}{tr(n+1) - tr(n)} + n$$

The standardisation of HPLC retention data using relative retention times or capacity ratios is particularly difficult as the data can be highly irreproducible due to differences in nominally identical column packing materials or in environmental conditions. Attempts have been made to standardise HPLC systems with similar methods to the Kovats retention indices used in GC. Instead of using a series of alkanes, as used in GC, the system was based on 2-ketoalkanes [64] and more recently on a series of alkyl-arylketones [65,66].

(b) COLUMN EFFICIENCY

The efficiency of a separation depends on the differences in the migration rates of the solutes and on the spreading of the solute zone. The rates at which the solutes travel depend on their partition coefficients and the resultant profile approximates to a normal Gaussian distribution profile.

The peak maxima represent the k' values of each component and approximate to the average rate of travel of the solute molecules. Ideally a peak would be sharp and symmetrical. However, peak broadening occurs when molecules with the same k' value migrate at different velocities. Chromatographic peaks are generally broadened by three kinetically controlled processes; eddy diffusion, longitudinal diffusion and non-equilibrium mass transfer. The magnitudes of these effects are determined by such controllable variables as flow rate, particle size of the packing material and the thickness of the stationary phase.

A quantitative measure of efficiency is given by two expressions; N, the number of theoretical plates and H, the height equivalent to a theoretical plate. This concept of theoretical plates was originally developed by Martin and Synge [32]. The theory envisages a chromatographic column as being composed of a series of discrete but continuous, narrow, horizontal layers called theoretical plates. At each plate, equilibration of the solute between the mobile and the stationary phase is assumed to take place. Movement of the solute and solvent is then viewed as a series of stepwise transfers from one plate to the next.

The number of theoretical plates, N, is given by:-

$$N = 16\left(\frac{t'}{W}\right)^2$$

where,

t' is the adjusted retention time, and,

W is the peak width.

The height equivalent to a theoretical plate, H, is given by:-

$$H = \frac{L}{N} = \frac{L}{16}\left(\frac{W}{t'}\right)^2$$

where L is the length of the column.

The number of theoretical plates, then, can be calculated from the two time measurements t' and W; to obtain H we must also know the length of the column.

The quantity H measures the efficiency of a given column (operated under a specific set of operating

conditions) per unit length of column. The efficiency of a chromatographic column as a separation device improves as the number of equilibrations increases - that is, as the number of theoretical plates increases or the plate height decreases. The sharpness of a peak reflects the plate height.

Generally speaking, for a gas chromatographic packed column the efficiency would be about 500-2000 theoretical plates, for a gas chromatographic capillary column it would be about 10,000-100,000 theoretical plates and for a 25cm reversed-phase high performance liquid chromatographic column the efficiency would be in the range 6,000-12,000 theoretical plates.

(c) BAND BROADENING

As previously mentioned chromatographic peaks are generally broadened by kinetically controlled processes. These processes are eddy diffusion, longitudinal diffusion and non-equilibrium mass transfer. A number of equations have been developed that relate efficiency to these band broadening factors. The earliest of these is known as the van Deemter equation and was derived for gas liquid chromatography. The van Deemter equation relates plate height to diffusion and average linear velocity, U,

$$H = A + (B/U) + CU$$

In this equation, A, B and C are associated with eddy diffusion, longitudinal diffusion and non-equilibrium mass transfer respectively.

Eddy diffusion arises from the numerous ways by

which a molecule can find its way down a column. The lengths of these paths can differ and so two particles of the same species can actually take different times to emerge, thus contributing to band broadening. The quantity A includes factors such as particle size, column geometry and uniformity of the packing material. Band broadening due to eddy diffusion can be minimised by careful packing using small spherical particles with a limited size range.

Longitudinal diffusion occurs as molecules tend to diffuse from regions of high solute concentration to those of low solute concentration and this can occur in both the mobile and stationary phases. Longitudinal diffusion is more significant when the mobile phase is a gas since diffusion rates in the gas phase are several orders of magnitude greater than those in the liquid phase. Diffusion increases with time, thus broadening increases with decreased flow rate. The constant B in the van Deemter equation is related to the diffusion coefficient and is inversely proportional to the flow rate. Broadening due to longitudinal diffusion can be reduced by decreasing the temperature (thus reducing the diffusion coefficient) and increasing the flow rate.

The third process contributing to the van Deemter equation, non-equilibrium mass transfer, arises because true equilibrium between phases cannot be achieved due to the flow of the mobile phase. At the front of a band, where the mobile phase encounters fresh stationary phase,

equilibrium is not instantly achieved and solute is carried further down the column than it would be had a true equilibrium been established. At the rear of a band solutes encounter fresh mobile phase and since equilibrium is not instant the tail of the band is drawn out. As a result, there is broadening at both ends of the bands. If the flow rate is decreased there is more time for equilibrium to be achieved and so the effects of non-equilibrium mass transfer become smaller. If the channels through which the mobile phase flow and the stagnant pools of mobile phase on the stationary phase are as thin as possible, equilibrium will be more easily achieved. Equilibrium is also more closely approached at high temperatures and with low solvent viscosities.

Figure 2.2.2 illustrates the contribution of each term in the van Deemter equation as a function of the mobile phase velocity as well as their net effect on H. It can be seen that the optimum efficiency corresponds to the flow rate at the minimim point in the Net Effect curve.

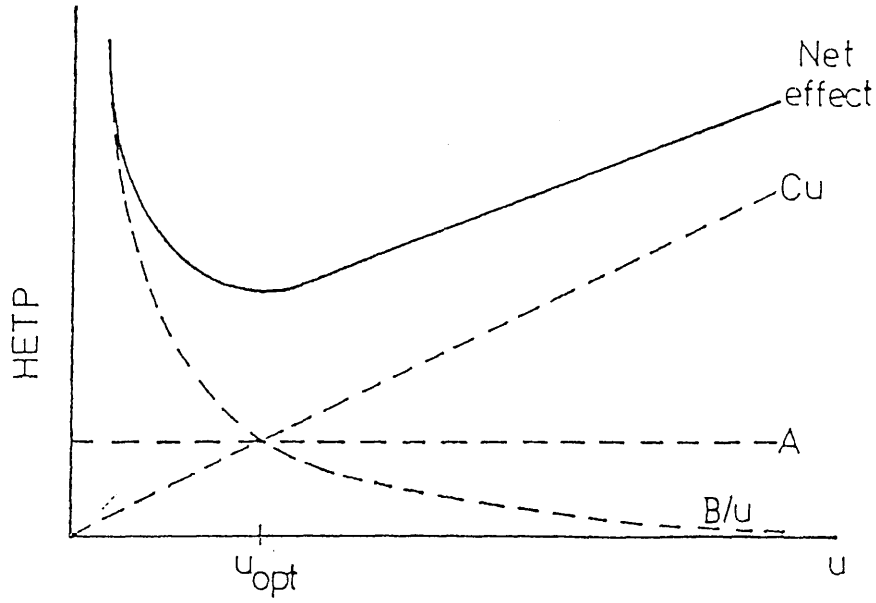
(d) RESOLUTION

Resolution is the term used to describe the separating power of a column. Resolution, Rs, is defined as the distance between two adjacent solute zone centres divided by the average peak width, thus

$$Rs = \frac{2(t'_B - t'_A)}{W_A + W_B}$$

Resolution depends on the efficiency of a column, the relative retention characteristics of the components and

FIGURE 2.2.2: Plot of the van Deemter equation showing the contribution of each term and the optimum mobile phase velocity.



the capacity factor. These three terms can be related to resolution in the following equation:

$$Rs = \frac{1}{4}\frac{(alpha - 1)}{alpha}\sqrt{N}\,\frac{k'}{1 + k'}$$

Adjacent peaks which are incompletely resolved can be separated by reducing the band broadening, by using a longer column or one with more theoretical plates, or by increasing the separation of the peaks by varying the mobile or stationary phase to give different relative partition characteristics. Generally an increase in N, alpha or k' will give better resolution. However, as k' increases so does the transit time for the solutes through the system giving increased band broadening. In practice a good efficient column is used and then either the mobile or stationary phase is varied to achieve better resolution.

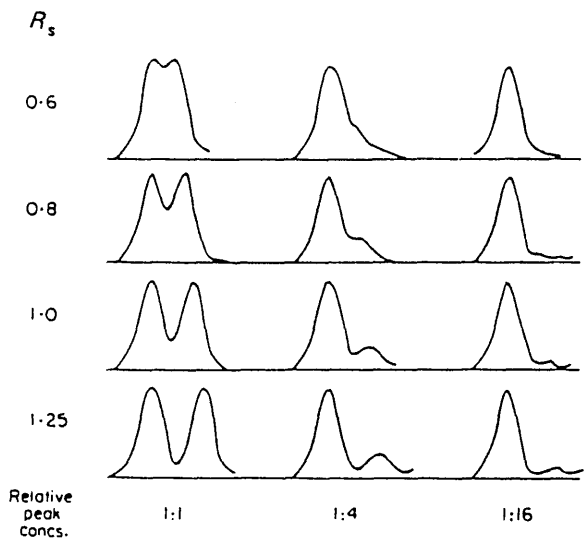
Another factor affecting peak shape and resolution is related to the capacity of the stationary phase and sample size. Exceeding the sample capacity causes overloading and therefore results in unsymmetrical peaks and loss of resolution.

It is difficult to quantify the criteria for resolving two peaks but in general the peak separation should be greater than $2W_{1/2}$ apart with better than a 10% valley between the peaks.

In quantitative chromatography based on peak area measurement, values of $R_s < 1$ should be avoided, for example, peaks D and E in Figure 2.2.1. A value of Rs = 1 corresponds to about a 3-5% overlap of peaks.

Higher values of Rs represent progressively smaller overlaps. Values of Rs > 1.2 are desirable (for example, peaks A and B in Figure 2.2.1). Larger values of Rs then indicate better separations and smaller values of Rs indicate poor separations. For a given value of Rs, band overlap becomes more serious when one of the two bands is much smaller than the other, see Figure 2.2.3.

FIGURE 2.2.3: Separation achieved and appearance of eluted peaks at various levels of resolution R_s and differing peak intensities



2.3. HIGH PRESSURE LIQUID CHROMATOGRAPHY

2.3.1 INTRODUCTION

The theory of high pressure liquid chromatography (HPLC) has already been discussed in Section 2.2; this section will deal with some of the practical aspects of HPLC.

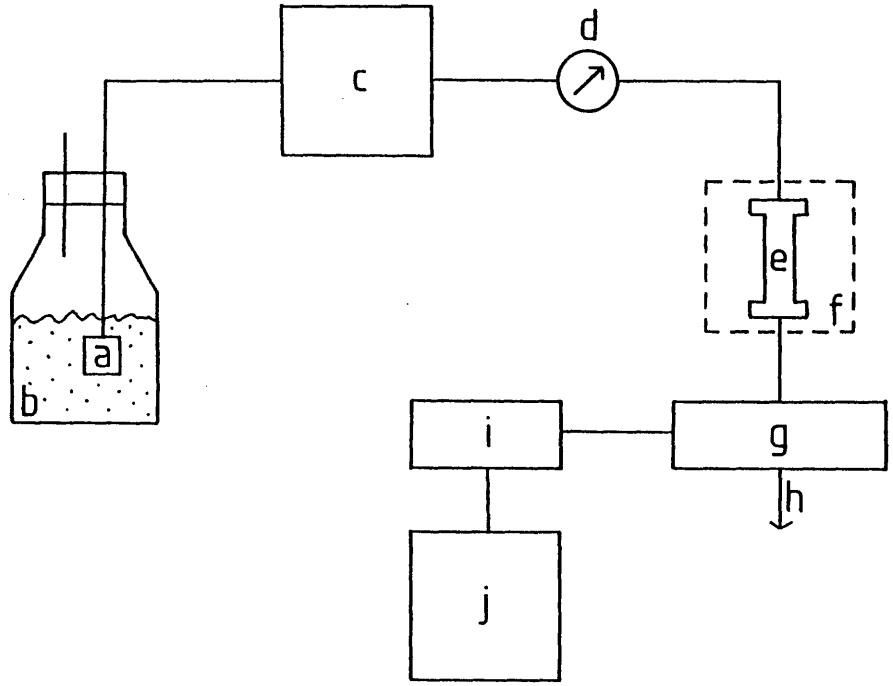
HPLC is a complementary technique to gas chromatography (GC) in that HPLC can be used to analyse compounds which are polar and/or thermally labile. One of the limitations of HPLC is that the sample components must be soluble in a suitable solvent that can be employed as a mobile phase. A schematic diagram of a typical isocratic HPLC system is given in Figure 2.3.1.

Firstly, the mobile phase is degassed: this can be done by ultrasonication, purging with helium or refluxing. The solvent is then pumped through a filter, the injection system, the column, the detector and then goes to waste or to a fraction collector.

The sample is flushed onto the column from the inlet system and the various components travel through the column at different rates, depending on their partition coefficients, and are separated. As in GC, the sample reaches the detector and generates an electrical signal which is amplified and recorded as a chromatogram.

Injections of samples onto an HPLC column can either be made with a syringe or more commonly using a multiport valve with a sample loop. The column can be left at ambient laboratory temperature or housed in a

Figure 2.3.1: Typical isocratic HPLC system



Key

a – Filter
b – Solvent Reservoir
c – Pump
d – Sample Inlet Valve
e – Column
f – Oven
g – Detector
h – Waste
i – Amplifier
j – Recorder

column oven to maintain a constant temperature.

(a) TYPES OF COLUMN

Conventional HPLC columns are made of stainless steel, are about 10 to 30cm in length and have internal diameters of 3 to 5mm. They are generally packed with 5 to 10μm particles. In order to improve chromatographic performance particles of smaller diameters have been employed. Stout [67] has demonstrated the use of columns with 3μm particles. Since reducing the particle size increases efficiency, the shortening of columns is also possible. Smaller columns of 5cm or less in length, with 3 to 5mm diameters and packed with 3 to 5μm materials are now commercially available. Another trend in column technology is the reduction of internal diameters and hence the development of microbore columns. Four types of microcolumns are recognised: open tubular [68,69]; packed microcapillaries [70]; microbore [71] and narrow bore [72]. The principle features of these columns are outlined in Table 2.3.1.

Microbore columns offer several advantages over the conventional columns such as low solvent consumption (flow rates of 50μl min^{-1} with microbore columns as opposed to 1ml min^{-1} with conventional columns), greater sensitivity and efficiencies and rapid separations. Scott et al. [74] reported the separation of seven compounds (phenylundecane, benzene, benzyl acetate, acetophenone, dimethylphenyl-carbinol, α-phenyl-ethyl alcohol and benzyl alcohol) in 30 seconds. Several other

Table 2.3.1 Characteristics and operational parameters for microbore columns (from ref.73)

Column Types		Material	Column i.d. µm	Particle Size µm	Column Length m	Flow Rate µl/min
Open-tubular column		Soft Glass Fused-silica glass	10-300	-	0.5 - 35	0.01 - 1
MICROBORE PACKED COLUMN	Microcapillary packed column	Soft Glass Pyrex Glass	50 - 200	10 - 100	10 - 60	0.01 - 5
	Small-bore packed column	Stainless steel Teflon	500 - 1000	5 - 20	0.1 - 1	30 - 100
	Narrow-bore packed column	Pyrex Glass PTFE Fused-silica Glass Stainless steel	100 - 500	3 - 10	0.1 - 2	0.1 - 20

applications have also been reported by Scott and co-workers [75-77]. Apparatus for microbore columns has to be specially designed since amplifier time constant and detecter response become critical. Equipment of this type is not yet widely available and was not used in the present study. A review of advances in HPLC has been given by Majors [78].

The mobile phase is generally a mixture of solvents, since it is difficult to achieve the correct polarity for elution with a single solvent. If the solvent composition remains the same throughout the analysis this is termed 'isocratic' elution and is used for simple mixtures of compounds. If a more complex mixture, for instance, one containing drugs and metabolites, was being analysed a gradient system could be employed. Gradient elution involves changing the proportions of the various solvents in a controlled manner with time: for this type of elution two pumps and a mixing chamber are necessary. Isocratic elution is the equivalent of isothermal analysis by GC and gradient elution can be compared with temperature programming by GC.

(b) DETECTORS

As for GC, there are a variety of detectors available for HPLC [79]. The most commonly used detector is the UV-visible spectrophotometer which can detect compounds that absorb in the UV-visible wavelengths. Fluorescing compounds can be determined with a fluorescence detector. The electrochemical detector is

also available. This is suitable for use with compounds which can be readily oxidised or reduced within the voltage range of the electrode. The development of this technique has been reviewed by Bratin [80]. Radiochemical detectors and atomic absorption detectors can also be used with HPLC.

The mass spectrometer can also be used as an HPLC detector, but first the technical problem of interfacing the HPLC with the mass spectrometer has to be overcome. An interface design is required which allows only a small portion of the mobile phase into the source without disturbing the vacuum system. Reviews of various interfaces which have been tried have been given by Arpino and Guiochon [81,82] and McFadden [83]. Takeuchi and co-workers [84] and Herion [85] have demonstrated the direct coupling of micro HPLC with a mass spectrometer.

(c) DERIVATISATION

HPLC, generally negates the need to derivatise compounds but in some instances derivatisation can be used to increase the sensitivity and selectivity of a detector [86]. Common examples include the formation of 2,4-dinitrophenyl hydrazones of carbonyl compounds or dansyl derivatives of amines to increase the UV-extinction coefficient or provide a fluorescing derivative. Pre- and post-column derivatisation have both been used in a dynamic manner.

2.3.2 EXPERIMENTAL

(a) REAGENTS

1. Anhydrous di-sodium hydrogen orthophosphate, Analar (BDH Chemicals Limited, Poole, England).
2. Orthophosphoric acid, Analar (BDH Chemicals Limited, Poole, England).
3. Glacial acetic acid, Analar (BDH Chemicals Limited, Poole, England).
4. Acetonitrile, HPLC grade (Rathburn Chemicals Limited, Walkerburn, Scotland).
5. Methanol, HPLC grade (Rathburn Chemicals Limited, Walkerburn, Scotland).

(b) DRUG STANDARDS

Drug standards were kindly supplied by the following manufacturers -

1. Ibuprofen (The Boots Company plc).
2. Flurbiprofen (The Boots Company plc).
3. Naproxen (Syntex Pharmaceuticals Limited).
4. Ketoprofen (May and Baker Limited).
5. Fenbufen (Lederle Laboratories).
6. Mefenamic Acid (W.R. Warner and Company Limited).
7. Indomethacin (Berk Pharmaceuticals Limited).
8. Sulindac (Merck Sharp and Dohme Limited).
9. Phenylbutazone(Ciba-Giegy Pharmaceuticals Division).
10. Oxyphenbutazone (Ciba-Giegy Pharmaceuticals Division).
11. Sulphinpyrazone (Ciba-Giegy Pharmaceuticals Division).
12. Azapropazone (A.H. Robins Company Limited).

13. Piroxicam (Pfizer Limited).

(c) APPARATUS

1. A Kratos Spectroflow 400 dual piston pump fitted with a Rheodyne 7120 injection valve with a 20ul sample loop.

2. A stainless steel column (250 x 4.6mm) packed with Hypersil 5µm ODS (HPLC Technology).

3. A Kratos Spectroflow 757 variable wavelength detector (set at 230 nm).

4. A Bioanalytical Systems LC-4A amperometric detector with a TL-5 vitrified carbon working electrode, operating at +1 volt.

5. A Farrand Mark 1 Spectrofluorimeter.

6. A Hewlett Packard diode array UV spectrometer was used to record the UV spectrum of each drug before HPLC was carried out.

(d) HPLC ELUENTS

The HPLC eluents used are listed in Table 2.3.2 -

Table 2.3.2: HPLC eluents

NUMBER	C O M P O S I T I O N (v/v)
1	Acetonitrile/Buffer*:70/30
2	Methanol/Buffer:70/30
3	Methanol/Aqueous Acetic Acid#:80/20
4	Methanol/Aqueous Acetic Acid:70/30
5	Methanol/Aqueous Acetic Acid:65/35
6	Methanol/Aqueous Acetic Acid/Acetonitrile:70/20/10
7	Methanol/Aqueous Acetic Acid/Acetonitrile:40/30/30

*Buffer - 0.1M di-sodium hydrogen orthophosphate adjusted to pH3 with orthophosphoric acid
#Aqueous Acetic Acid - 2% v/v glacial acetic acid in distilled water.

(e) PREPARATION OF STOCK SOLUTIONS

Stock solutions (1mg/ml) of each drug were prepared by dissolving the drug in mobile phase. Solutions for analysis were then prepared by making appropriate dilutions of the stock solutions in mobile phase.

2.3.3 RESULTS

Table 2.3.3 compares the effects of using several HPLC mobile phases on the capacity factors (k' values) of three drugs; phenylbutazone, indomethacin and ibuprofen.

Initially seven mobile phases were examined with phenylbutazone. All of the mobile phases used eluted the drug; capacity ratios (k') ranged from 0.83 (with mobile phase 3) to 3.55 (with mobile phase 1). A complete list is given in Table 2.3.3.

The analysis was extended to indomethacin and ibuprofen for selected mobile phases as shown in Table 2.3.3. Indomethacin and ibuprofen were also eluted with all the mobile phases tried.

Table 2.3.4 lists retention data following the use of mobile phase 1 and mobile phase 4 to analyse a wide range of NSAIDs. In both systems all of the drugs could be eluted within 5 minutes with mobile phase 1 and 10 minutes with mobile phase 4.

UV detection was useful for all of the drugs examined (Table 2.3.5). Full spectra are given in Figures 2.3.2-2.3.5. With the exception of ibuprofen all of the drugs contain chromophores with high extinction coefficients. Typically detection limits were about 5ng of drug on-column.

Fluorescence detection was found to be suitable only for ibuprofen, flurbiprofen, indomethacin, mefenamic acid and naproxen (Table 2.3.6) with detection limits similar to these obtained with the UV detector.

Electrochemical detection was applicable to seven of the test drugs as illustrated in Table 2.3.6. The detector was extremely sensitive to four of the drugs namely mefenamic acid, sulphinpyrazone, azapropazone and oxyphenbutazone. Limits of detection for these compounds were less than 1 ng on-column.

TABLE 2.3.3: Comparison of k' values for phenylbutazone, indomethancin and ibuprofen with several different mobile phases, recorded with a UV detector at 230nm. Column was 250 x 4.6mm (i.d.) packed with 5um Hypersil ODS.

MOBILE PHASE	k'		
	Phenylbutazone	Indomethacin	Ibuprofen
3	0.83	1.16	1.41
6	0.97	-	-
4	1.81	2.82	3.56
2	1.83	-	-
5	2.02	3.76	4.55
7	2.03	2.47	2.94
1	3.55	2.45	-

TABLE 2.3.4: HPLC data for some NSAIDs recorded with a UV detector at 230nm. Column was 250 x 4.6mm (i.d.) packed with 5um Hypersil ODS.

D R U G	k'	
	Mobile Phase 1	Mobile Phase 4
Sulindac	0.68	1.03
Ketoprofen	1.08	1.06
Oxyphenbutazone	1.26	0.70
Azapropazone	1.40	0.42
Sulphinpyrazone	2.20	1.86
Flurbiprofen	2.22	2.82
Indomethacin	2.45	2.82
Phenylbutazone	3.55	1.81
Mefanamic Acid	4.09	2.36
Ibuprofen	4.45	3.56
Naproxen	-	1.27
Fenbufen	-	2.42
Piroxicam	-	1.05

TABLE 2.3.5: UV absorbance data for some NSAIDs.

D R U G	UV Max in nm ($A^{1\%}_{1cm}$)#
Mefenamic Acid	236 (417), 280 (314), 354 (274)
Ibuprofen	234 (46)
Flurbiprofen	248 (768)
Naproxen	240 (667)
Ketoprofen	258 (651)
Fenbufen	230 (157), 286 (887)
Indomethacin	238 (477), 258 (481)
Sulindac	236 (434), 286 (369), 330 (324)
Phenylbutazone	240 (467)
Sulphinpyrazone	252 (519)
Azapropazone	250 (1117)
Piroxicam	244 (368), 340 (462)

#FOOTNOTE $A^{1\%}/1cm$: the absorbance of 1g of drug in 100 ml of solvent measured in a 1cm cell. The solvent in this case was mobile phase 4.

TABLE 2.3.6: Comparison of UV, fluorescence and electrochemical detection of some NSAIDs in mobile phase 1.

D R U G *	M E T H O D O F D E T E C T I O N		
	UV (230nm)	Fluorescence (nm)	Electrochemical (+1 volt)
Ibuprofen	*	** λex 270 λemm 290	N D
Flurbiprofen	**	** λex 290 λemm 320	N D
Naproxen	*	** λex 320 λemm 410	-
Ketoprofen	**	N D	N D
Fenbufen	**	N D	-
Mefenamic Acid	**	** λex 365 λemm 400	**
Indomethacin	**	** λex 305 λemm 375	*
Sulindac	**	N D	*
Phenylbutazone	**	N D	*
Oxyphenbutazone	**	N D	**
Sulphinpyrazone	**	N D	**
Azapropazone	**	N D	**
Piroxicam	**	N D	-

** good response
* poor response
N D not detected
λex wavelength of excitation
λemm wavelength of emission

FIGURE 2.3.2: UV spectra of azapropazone, fenbufen and flurbiprofen.

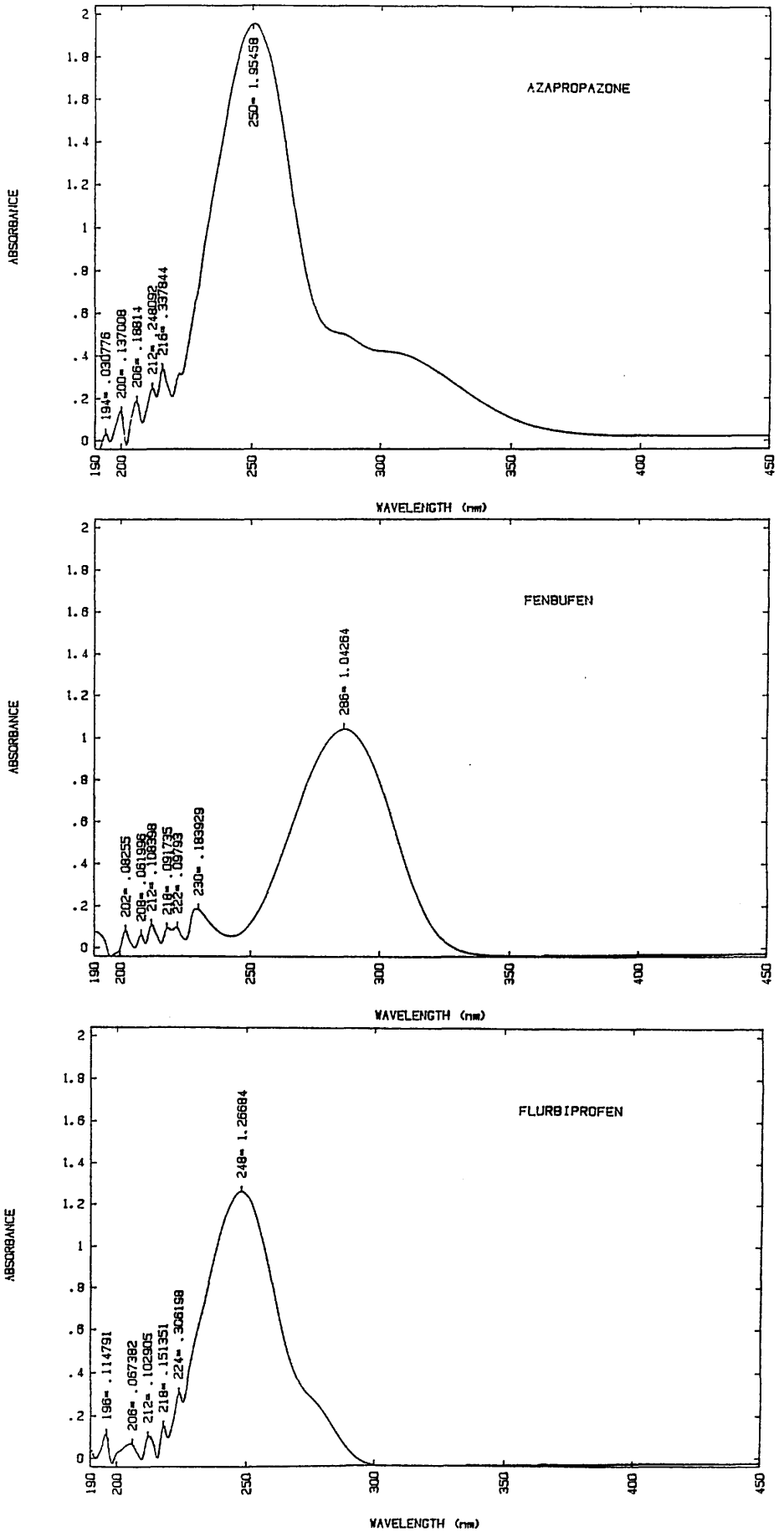


FIGURE 2.3.3: UV spectra of ibuprofen, indomethacin and ketoprofen.

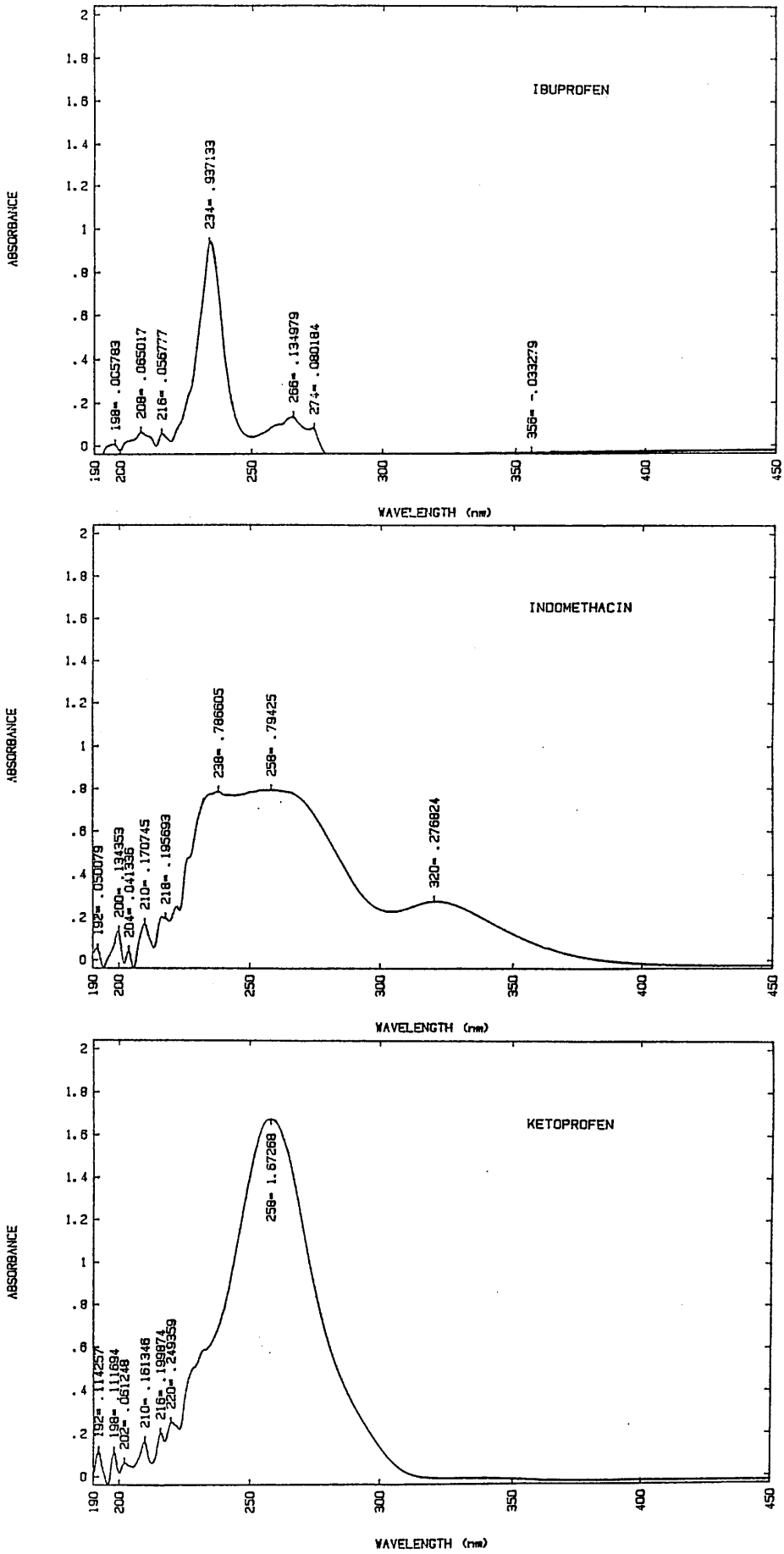


FIGURE 2.3.4: UV spectra of mefenamic acid, naproxen and phenylbutazone.

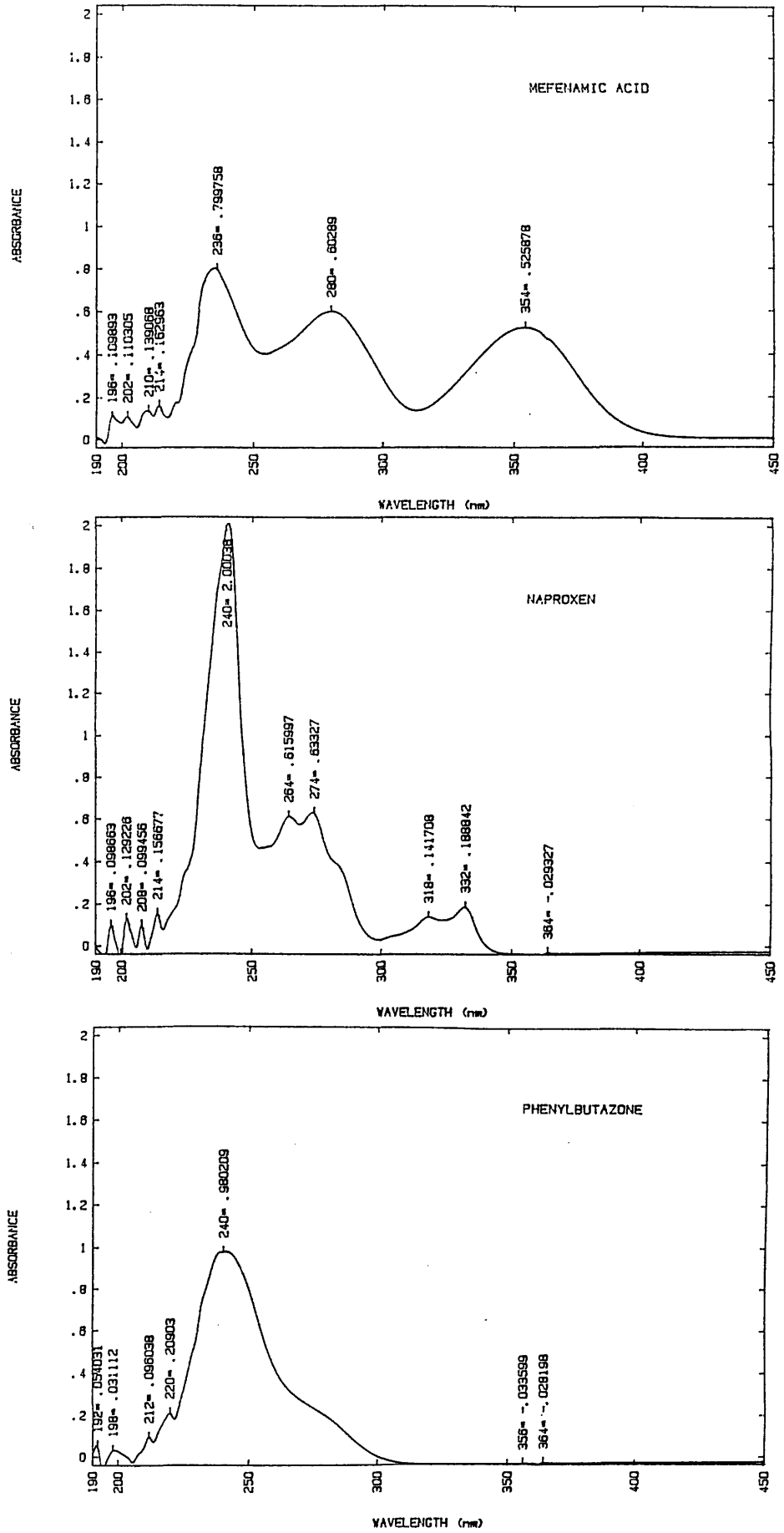
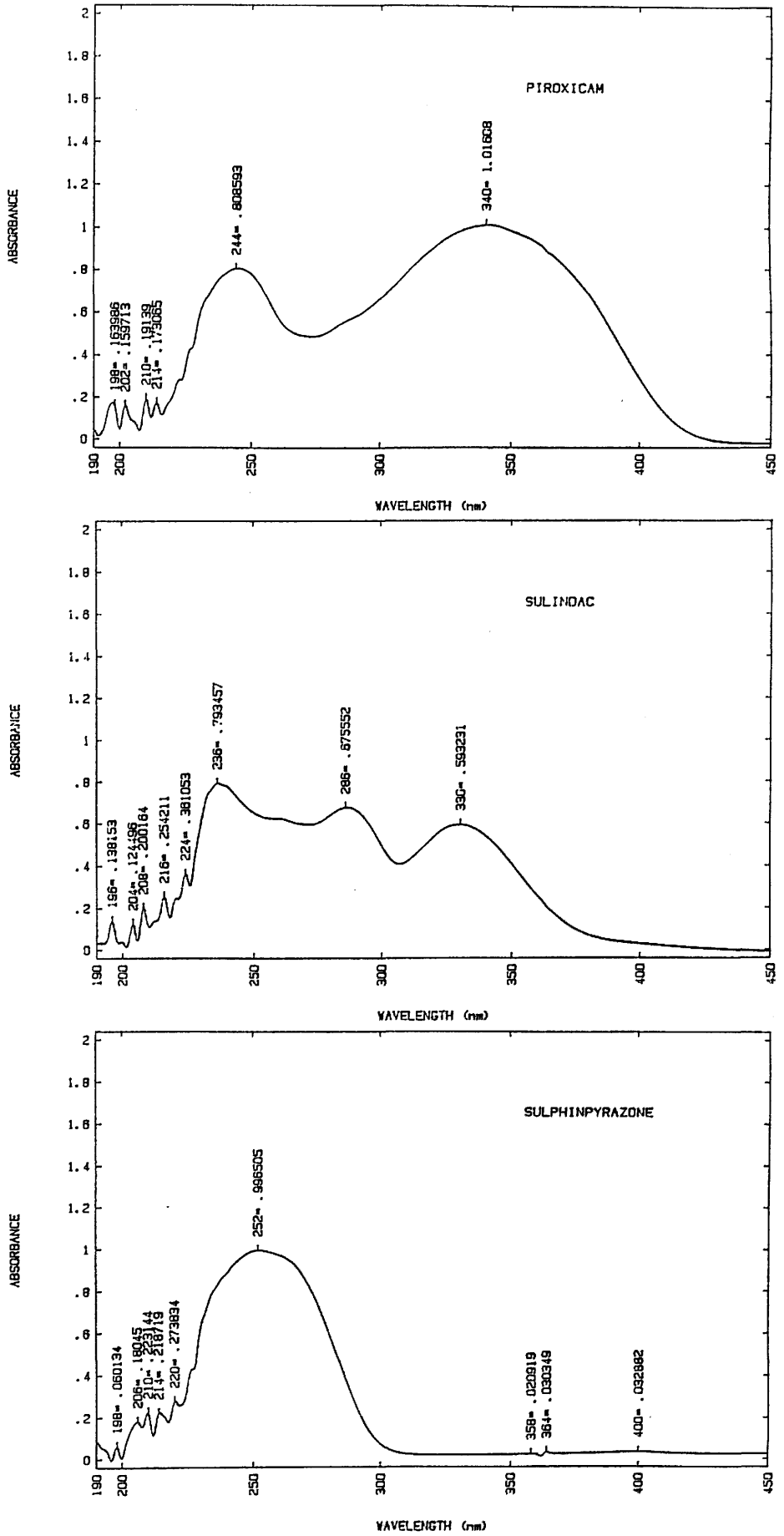


FIGURE 2.3.5: UV spectra of piroxicam, sulindac and sulphinpyrazone.



2.3.4 DISCUSSION

Reversed-phase HPLC was examined as a method of analysis since the NSAIDs in this study are all polar in nature. The drugs being considered are all acidic (see Table 2.3.7 for pka values) therefore the mobile phases tried were all acidic in order to suppress ion formation. If the drugs were ionised they would elute quickly and give poor peak shapes. Hence the use of ion pair chromatography for amines (basic drugs).

The ultimate aim of the HPLC analysis was to resolve completely the NSAIDs selected. There is an optimum range of values for k' in terms of resolution, separation time and band detection [39]; that range is $1 \leqslant k \leqslant 10$. When k' is initially small (< 1) resolution increases rapidly with increases in k', but when k' is greater than 5 resolution increases very little with increases in k'. Separations which involve k' > 10 result in long separation times and excessive band broadening to the point where detection of small samples is impossible. The capacity factors for phenylbutazone ranged from 0.83 to 3.55 for the seven mobile phases chosen.

Capacity factors vary with the stationary and mobile phases, but in practice k' is controlled with solvent strength. When k' values require to be increased a weaker solvent is used, and if k' values need to be reduced a stronger solvent is tried. Solvent strength can be defined quantitatively by the solvent strength

TABLE 2.3.7: Dissociation constants for selected NSAIDs (ref 50)

D R U G	pKa
Azapropazone	not available
Fenbufen	not available
Flurbiprofen	not available
Ibuprofen	4.4, 5.2
Indomethacin	4.5
Ketoprofen	not available
Mefenamic Acid	4.2
Naproxen	4.2
Oxyphenbutazone	4.7
Phenylbutazone	4.4
Piroxicam	6.3 (2:1 dioxane-water)
Sulindac	4.7
Sulphinpyrazone	2.8

parameter E^{o}. Solvent strength is listed for several solvents in Snyder and Kirkland's book [39]. Solvents with low polarities and low E^{o} values are weak solvents; for example, methyl acetate and acetone. Solvents with high polarities and high E^{o} values are strong solvents; for example, acetonitrile and methanol. Water has the highest polarity and E^{o} in the series. A binary or tertiary solvent has a combination of the solvent strength parameters of the solvents involved.

The changes in k' with various solvents can be seen in Table 2.3.3. Mobile phases 3, 4 and 5 have decreasing solvent strength, and the k' values for the three drugs increase as a result.

Methanol is a stronger solvent than acetonitrile and this is illustrated when mobile phases 1 and 2 are compared. The k' value for pheylbutazone with a methanol/buffer mobile phase is 1.83 and is 3.55 with an acetonitrile/buffer mobile phase. Finally, comparing mobile phase 6 and 7, we would expect mobile phase 6 to give smaller k' values since it is a stronger solvent; the k' value for mobile phase 6 for phenylbutazone is 0.97 and is 2.03 for mobile phase 7. Mobile phases 3 and 6 which resulted in $k' < 1$ were thought to be unsuitable in terms of their resolving potential.

Mobile phases 1 and 4 were used to examine a wider range of NSAIDs (see Table 2.3.4). Both systems were found to be satisfactory for the analysis of individual standard drug substances with respect to sensitivity, peak

shape and k' values. However, systems of these types did not provide sufficient resolution to permit unambiguous identification of unknown NSAIDs. It would most likely be necessary to use a gradient system in order to separate this range of compounds within a reasonable time. The problem of resolution would become more complex when urine samples containing metabolised drugs were analysed. The selectivity of the system could be improved by employing various detector systems in series, for example UV with fluorescence detection or UV with electrochemical detection.

Fluorescence was found to be suitable for flurbiprofen, naproxen, mefenamic acid and indomethacin in agreement with previous work described in the literature [87-90]. Ibuprofen was also found to fluoresce.

The most intense fluorescence is generally found in structures with aromatic character [91]. Most unsubstituted aromatic hydrocarbons fluoresce in solution and the quantum efficiency usually increases with the number of rings and their degree of condensation.

Substitution on the aromatic ring also causes changes in the fluorescence efficiency. The addition of an electron withdrawing group such as $-NO_2$, $-CO_2H$, -CHO or an halogen, dramatically reduces the fluorescence, while the introduction of an electron releasing group, such as $-NH_2$, -OH, $-OCH_3$ or $-CH_3$ increases the fluorescence.

Molecules that possess rigid structures also favour fluorescence. In this case the planarity inhibits the

loss of absorbed energy by other radiationless pathways.

It is, however, difficult to formulate absolute rules to say whether a substance will fluoresce or not. In the case of the drugs examined in Table 2.3.6 we may have expected none of them to fluoresce since they all contain both electron withdrawing and releasing groups. It is often best to use an empirical approach rather than a predictive one when assessing fluorescence detection for HPLC.

Electrochemical detection coupled to HPLC is now used to monitor a wide range of organic molecules [92]. Both electro-oxidation and electro-reduction are possible although electro-reduction is technically more difficult due to high background currents caused by dissolved oxygen and other reducible trace impurities [93].

Organic oxidations can be performed on most electron-rich compounds. Common reactions include phenol oxidative couplings, oxidation of primary and secondary alcohols to aldehydes and ketones, respectively, Kolbe de-carboxylation of carboxylic acids, oxidation of primary and secondary thiols, hydroquinones, indoles and xanthines [94-96].

The electrochemical analysis was performed using mobile phase 1 which contained 0.1M phosphate buffer since it is essential for electrochemical detection that the solvent is electrically conductive.

Electrochemical oxidation was found to be particularly suitable for mefenamic acid, sulphinpyrazone,

azapropazone and oxyphenbutazone. Indomethacin, sulindac and phenylbutazone also give a response but the detector was less sensitive for these substances under the conditions used. These seven drugs contain functional groups with an oxidation potential within the range of the electrode used. None of the other drugs examined in Table 2.3.6 gave a response under the conditions used. A change in mobile phase or electrode potential could facilitate a response but this was not investigated further.

2.3.5 CONCLUSION

HPLC was found unsuitable as a screening procedure for NSAIDs. An isocratic system such as the one used in the evaluation is suitable for the analysis of specific or known groups of compounds but does not provide sufficient resolution to analyse the twelve test compounds. It should also be noted that there are many other NSAIDs on the market which, in the presence of their metabolites and other urinary components, poses an insurmountable problem with respect to specificity. This problem could be reduced by the use of a gradient system to improve resolution, although these systems have in the past been unreliable and non-reproducible.

Alternatively, increased specificity could be achieved by using different detectors in series thereby increasing the amount of information avaiable on the compounds eluted from the column. Although the use of electrochemical and fluorescence detectors, for instance, does go some way to improving the selectivity of the system, they would not give an unambiguous identification of the NSAIDs and metabolites in authentic samples.

2.4. GAS LIQUID CHROMATOGRAPHY

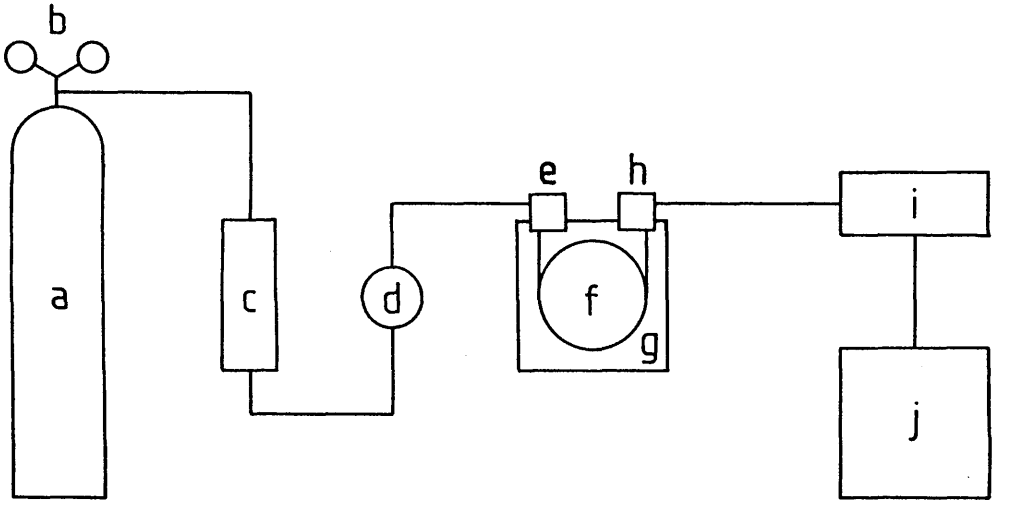
2.4.1 INTRODUCTION

Gas liquid chromatography (GC) has already been described as an example of partition chromatography in Section 2.2 and an outline of the chromatographic process has been given.

A schematic diagram of a typical gas chromatograph is shown in Figure 2.4.1. The mobile phase or carrier gas flows through the gas purifier, flow controller, injector, column and detector. The sample is introduced by micro-syringe through a rubber septum into the heated injector where it is immediately vaporised and transported onto the column. The various components travel through the column at different rates dependent on their vapour pressures and become separated. The partitioning behaviour of the sample between the mobile and stationary phases is very temperature-dependent, therefore the column is housed in an oven and the temperature is carefully controlled. When the sample components pass the detector (of which there are many types) at the end of the column they generate electrical signals which, after amplification, are fed to a recorder which presents the signals versus time as a chromatogram. This plot is useful for both qualitative and quantitative analysis.

Analysis can be performed isothermally or using a temperature programme. In the isothermal mode the column temperature remains constant throughout the analysis, whereas a temperature programme increases the column

Figure 2.4.1: Basic components of a gas chromatograph



Key

a – Carrier gas supply
b – Pressure gauge and regulator
c – Gas purifier
d – Flow controller
e – Injector
f – Column
g – Oven
h – Detector
i – Signal amplifier
j – Chart recorder

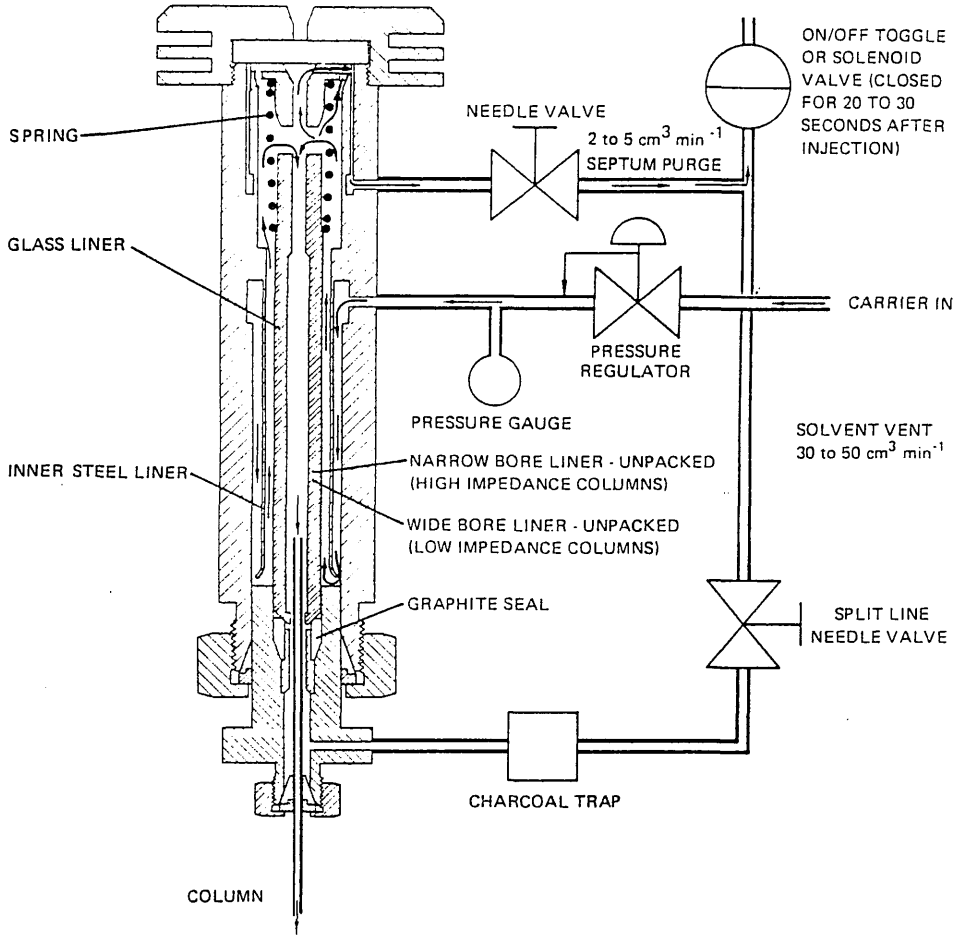
temperature in a uniform manner at a few degrees per minute. For the analysis of simple mixtures an isothermal run will suffice, but for the analysis of more complex mixtures a temperature programmed run may be necessary.

(a) SAMPLE INLET SYSTEMS

The introduction of a sample into a GC system is important as it affects the overall efficiency of the separation procedure and the accuracy and precision of the results. Liquid sample introduction has always been a problem in gas chromatography for a number of reasons. The sample must be introduced instantaneously into the system and then be evaporated rapidly, without decomposition, into the smallest possible gas volume. This "gas plug" must then be transferred into the column without loss. Also, the total amount introduced should not exceed the sample capacity of the column. In packed columns the sample capacity is high therefore the problems are less than for open-tubular (or capillary) columns.

There are a number of injection systems available for capillary column GC [97] and these include split systems, splitless systems and on-column sample introduction. The Groß splitless injector was the type of injector used in this study and is illustrated in Figure 2.4.2. In this type of injector the injector housing contains a glass liner. The column is placed inside the injector housing and projects into the glass liner. The column is no more than 10mm from the syringe needle, providing an all-glass system with minimum dead

FIGURE 2.4.2: The Groß splitless injector.



volume. When the syringe plunger is depressed the sample is transported onto the column with the carrier gas. Small sample volumes of no more than 1µl are used. If a larger volume was injected the sample would diffuse in the injector and lead to inefficient sample application and band broadening. This type of injector may be used to take advantage of the solvent effect (condensation of the solvent at the top of a cold column) to concentrate the solute at the top of the column. However, for high-boiling solutes, especially when temperature program GC is used, the solute zone remains sharp and minimal band broadening occurs. This is important as it may be very inconvenient to cool the column, say, to 40^{o} to condense methanol, or require subambient cooling for diethyl ether.

(b) TYPES OF COLUMN

There are two main types of column in GC: the packed column and the capillary column. Packed columns are generally made of glass or metal, are 1-5m in length and 3-6mm in diameter. The efficiency of the packed column is low, usually 500-2000 theoretical plates. The packing consists of an inert support coated with a thin film of liquid stationary phase. A table of some stationary phases available is given in Section 2.2 (Table 2.2.2). Capillary columns have been made of copper, stainless steel, glass and more recently vitreous silica. Capillary columns are usually 10-50m in length and 0.1-0.5mm in diameter, they have higher efficiencies than packed columns and are suitable for the analysis of

complex mixtures. Capillary columns can be classified into two groups: micropacked columns and open-tubular columns. Micropacked columns range in length from 5-25m have diameters of 0.6-1mm and have high efficiencies of about 50,000 theoretical plates. Open-tubular columns have an unrestricted flow through the bore of the column, they are 10-100m in length, have internal diameters of 0.1-0.5mm and have high efficiencies of 10,000 to 100,000 theoretical plates. They can be further subdivided according to the method of supporting the stationary phase:

(i) bonded phase columns (BPC)

(ii) wall coated open-tubular columns (WCOT)

(iii) support coated open-tubular columns (SCOT)

(iv) porous layer open-tubular columns (PLOT)

(i) BONDED PHASE COLUMNS (BPC)

In these columns, the stationary phase is formed by polymerisation of siloxane monomer on the inner surface of the capillary tube: covalent bonds form between the wall and the polymeric phase, thereby bonding it as a physically stable film on the surface.

BPC's are the most commonly used columns today. They are normally made of fused silica and have several advantages over the other open-tubular columns. Rearrangement of the bonded stationary phase film is virtually impossible so the column efficiency will be retained much longer than in other types of column. Contaminated columns can be washed with solvent to return them to their original performance. Pure vitreous silica

columns overcome many of the adsorption problems of glass or stainless steel columns once the surface has been deactivated. Vitreous silica columns also have much higher tensile strength and flexibility, although the exterior surface must be protected from oxidation and scratching, both of which weaken the column. Polyimide is the material usually used to coat the outer surface of the column. Fused silica capillary columns are 10-50m in length and have internal diameters of 0.1-0.5mm. A wide range of chemically bonded phases are now available including non-polar, medium polar and polar phases.

(ii) WCOT COLUMNS

WCOT columns have a thin film (0.1-0.5μm) of stationary phase coated on the inside wall of the capillary tubing. Glass rather than stainless steel capillary tubing is used because of its relative inertness. Wide-bore columns (0.5mm i.d.) have about half the efficiency of narrow bore columns (0.25mm i.d.) but usually they are easier to install and are less critical with respect to the effects of dead volume as the flow rate is higher. Both 0.25 and 0.5mm i.d. columns can be used with on-column injectors. Wide bore columns have more stationary phase and therefore a higher capacity than narrow bore columns. Wide bore columns also have the advantage that direct injection techniques without splitting may be used.

(iii) SCOT COLUMNS

SCOT columns are 0.5mm i.d. glass capillary

columns. The inner wall is coated with a layer of fine particle support material which in turn is coated with a thin film of stationary phase. The thicker effective film of stationary phase on a SCOT column permits a higher sample capacity than that of the WCOT columns, but SCOT columns are less efficient. Smaller diameter (0.3mm i.d.) SCOT columns are also available which have efficiencies approaching that of the WCOT columns.

(iv) PLOT COLUMNS

PLOT are similar to the SCOT columns, but have a porous layer on the inner wall of the glass capillary tubing, which is then coated with stationary phase.

(c) DETECTORS

There are various detection principles used for GC analysis [98] and these include:

(i) the flame ionisation detector (FID)

(ii) the nitrogen-phosphorus detector (NPD)

(iii) the electron capture detector (ECD)

(iv) the flame photometric detector

(v) the thermal conductivity detector (TCD)

(vi) the mass spectrometer (GC-MS)

(i) FLAME IONISATION DETECTOR (FID)

The FID is the most widely used GC detection system. The detector consists of a small hydrogen flame in an electrode gap. Organic compounds eluting from the column burn and form ions. The ion formation is a complex process, in which direct ionisation plays only a small part. The ions then travel to the collector electrode and thus a current is observed.

(ii) NITROGEN-PHOSPHORUS DETECTOR (NPD)

The NPD is used specifically to detect nitrogen and phosphorus-containing compounds. The detector is a modified FID containing a bead of an alkali metal salt. When heated the bead gives off alkali metal vapours. Decomposition products of N or P-containing compounds can accept electrons from alkali atoms causing ionisation and a large current increase. Precise control of the hydrogen and air flow rates allows the detector to respond selectively to N or P-containing compounds. Halogen and sulphur-containing compounds can also be detected under the correct flame conditions. Alkali metal salts used are caesium bromide, rubidium silicate, chloride or sulphate and potassium chloride or carbonate.

When compared with FID the NPD is approximately fifty times more sensitive to nitrogen compounds and five hundred times more sensitive to phosphorus compounds.

(iii) ELECTRON CAPTURE DETECTOR (ECD)

This detector contains a radioactive ß-emitter (^{3}H or ^{63}Ni) which ionises the carrier gas (usually argon/methane) forming slow electrons. When a sample capable of capturing electrons enters the detector it reacts with the electrons to produce negative ions. These negative ions combine with the positive gas ions. The number of free ions and electrons is therefore depleted in the presence of the sample and the current is reduced. ECD's are highly sensitive to molecules with electrophores, for example, halogen-containing compounds, conjugated carbonyls, nitrate esters and organometallics.

(iv) FLAME PHOTOMETRIC DETECTOR

The flame photometric detector is another type of flame detector and is specific for phosphorus and sulphur-containing compounds. The principle of operation is the measurement of radiation emitted by excited species in the flame. In a hydrogen flame, sulphur and phosphorus form excited species which emit radiation at 394nm and 526nm respectively.

(v) THERMAL CONDUCTIVITY DETECTOR (TCD)

The TCD is based on the principle that the filament will lose heat at a rate which depends on the composition of the surrounding gas. The change in temperature of the filament will give rise to a change in its electrical resistance. The detector contains two filaments, one in the gas flow from the column (the detector cell) and the other in the carrier gas flow (the reference cell). The two filaments form the arms in a Wheatstone bridge circuit. When no sample is eluted carrier gas passes through both cells, therefore, the bridge is balanced. When sample is eluted the resistance in the sample cell changes, an imbalance of the bridge occurs and is registered. The carrier gas used is either helium or hydrogen which both have high conductivities. The detector responds to any compound having a different thermal conductivity from the carrier.

(vi) MASS SPECTROMETRY (GC-MS)

The mass spectrometric detector is the most specific and sensitive detector available and will be discussed in detail in Section 2.5.

(d) DERIVATISATION

For a compound to be suitable for GC analysis the sample must be easily vaporised without decomposition. There are many compounds which do not fulfil this criterion but a chemical derivative of the compound may be suitable for GC analysis. The main reasons for derivatisation are as follows:-

1. to increase volatility of the sample;
2. to increase thermal stability of a compound;
3. to introduce functional groups which increase the sensitivity in selective detectors; and,
4. to improve separation and reduce tailing by masking polar groups.

The derivatisation reaction used should be rapid and quantitative and it should be possible to chromatograph the reaction mixture directly without pre-isolation of the derivative.

An extensive literature is available for chemical derivatisation [99-102], but some examples will be given here. Derivatisation methods can be classified into groups according to the reagents used and the reactions achieved, for example, silylation, acylation and alkylation. In the present study, silylation and alkylation reactions were used and these will be considered in more detail.

(i) SILYLATION

Silylation involves the replacement of an acidic hyrogen in the sample molecule with an alkylsilyl group such as the trimethylsilyl (TMS) group. The derivatives

are generally less polar, more volatile and more thermally stable than the parent compound. Water decomposes both TMS reagents and derivatives and solvents such as water and alcohols with active hydrogens should be avoided. Non-polar solvents such as hexane produce slow reactions, however. Pyridine is the commonly used solvent. It is an acid scavenger and basic catalyst. Dimethylformamide, dimethylsulfoxide, tetrahyrofuran and acetonitrile are also used. Examples of some reagents are N,O-bis (trimethylsilyl)acetamide (BSA), N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), N-trimethylsilylimidazole (TMSI), trimethylchlorosilane (TMCS) (also used as a catalyst with BSTFA) and hexamethylsilazane (HMDS). Using these reagents esters are formed from carboxylic acids, ethers from alcohols and N-TMS derivatives from amines. Some examples of reactions are given in Figure 2.4.3.

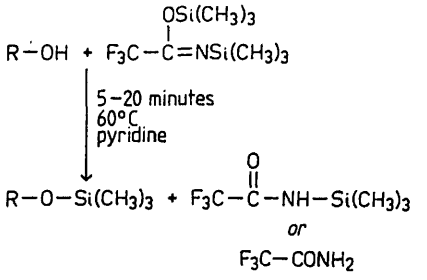
N- Methyl -N- (tert - butyldimethylsilyl) trifluoro-acetamide (MTBSTFA) is another silylation reagent. It donates a tert-butyldimethylsilyl moiety instead of the trimethylsilyl group. The advantage of this reagent is that both it and the derivatives it produces are less prone to hydrolysis than the other silylation reagents and derivatives already mentioned. An example of a reaction is given in Figure 2.4.3. Additional advantages of this derivative occur in GC-MS.

(ii) ALKYLATION

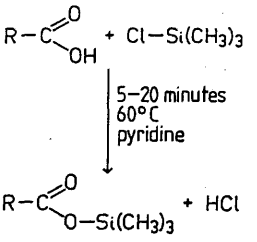
Alkylation is the addition of an alkyl group to an active functional group. Esterification of carboxylic

FIGURE 2.4.3: Silylation reactions using various reagents.

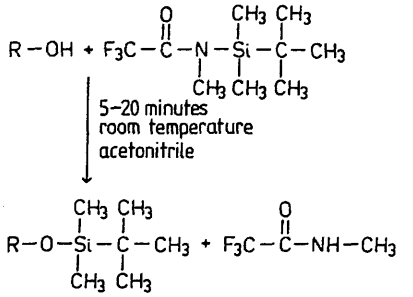
(a) Silylation using BSTFA.



(b) Silylation using TMCS



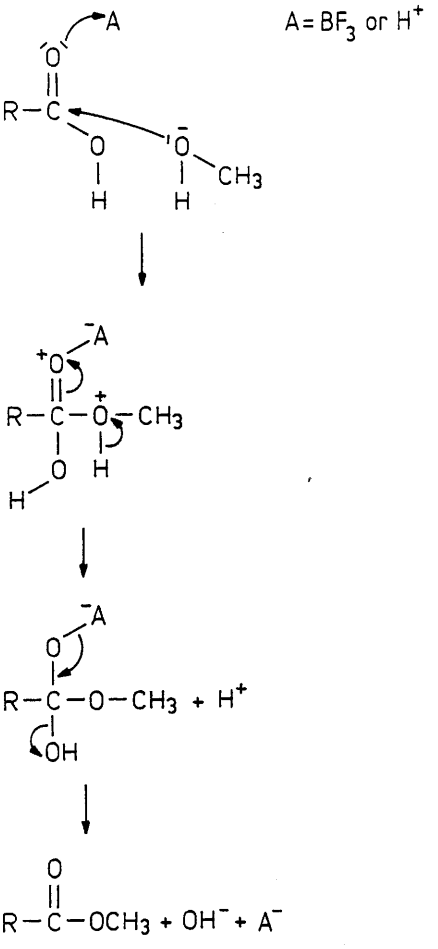
(c) Silylation using MTBSTFA



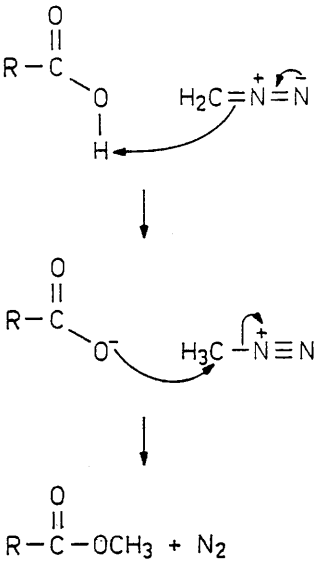
acids to form methyl esters is one of the most useful reactions. The ester is less polar, more thermally stable and more volatile than the acid therefore facilitating GC analysis. A number of methods are available for methylating compounds and these are discussed in detail in Blau and King's book [99]. Examples of some methylating reagents are an ethereal solution of diazomethane, a methanolic solution of boron trifluoride in ether and methanolic HCl. Figure 2.4.4 illustrates mechanisms for the reaction of these three reagents with a carboxylic acid.

FIGURE 2.4.4: Methylation reactions using various reagents.

Acid Catalysed Methylation



Methylation with Diazomethane



2.4.2 EXPERIMENTAL

(a) REAGENTS

1. Diethyl ether, Pronalys AR grade (May & Baker, Dagenham, England).
2. Methanol, HPLC grade (Rathburn Chemicals Limited, Walkerburn, Scotland).
3. Ethyl acetate (Ratherburn Chemicals Limited, Walkerburn, Scotland).
4. Digol, Analar grade (BDH Chemicals Limited, Poole, England).
5. Potassium hydroxide, Analar grade (BDH Chemicals Limited, Poole, England).
6. Diazald, N-methyl-N-nitroso-p-toluenesulfonamide (Aldrich Chemical Co.Ltd Limited, Gillingham, England).
7. Boron trifluoride etherate, redistilled (Sigma Chemical Company Limited, Poole, England).
8. Acetyl chloride (Sigma Chemical Company Limited, Poole, England).
9. BSTFA + 1% TMCS, Tri-Sil-Z, MTBSTFA, HMDS, Pyridine (Pierce, Life Sciences Laboratory Limited, Luton England).

(b) DRUG STANDARDS

As in Section 2.3.2 (b)

(c) APPARATUS

1. A Pye Unicam model 204 gas chromatograph fitted with an all-glass Groß splitless injector and a flame ionisation detector.

2. A wall-coated open-tubular column (24m, 0.5mm i.d., 1.10mm o.d.) with a liquid stationary phase of CP-SIL5 at a film thickness of 0.81μm.

(d) CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions employed were as follows:-

Column:	WCOT, 24m, 0.5mm i.d., 1.1mm o.d. with a liquid stationary phase of CP-SIL 5 at a film thickness of 0.81μm.
Carrier Gas:	Nitrogen (2mL/min).
Make-up Gas:	Nitrogen (30mL/min)
Injector Temperature:	275°C.
Detector Temperature:	300°C
Column Temperature:	Programmed from 80° to 280°C at a ramp rate of 4°/minute with initial and final isothermal periods of 2 minutes.

(e) PREPARATION OF DERIVATISING REAGENTS

(i) DIAZOMETHANE

Diazald* (1.8g) was placed in a dry flask. Diethyl ether (30ml), digol (4.5ml) and potassium hydroxide solution (6ml; 30% v/v) were then added to the flask consecutively. The flask was then placed in a water bath at 60°C. The diazomethane produced was collected via a side arm in the flask which was inserted into a 10ml

*This reagent is toxic and a potent carcinogen. Solutions of the gas are unstable and potentially explosive. The preparation and handling of the reagent were therefore carried out with care in a fume cupboard.

volumetric flask, containing diethyl ether (1ml), submersed in an ice-bath. The diazomethane solution remained stable for about one week when kept at 4°C.

(ii) BORON TRIFLUORIDE ETHERATE/METHANOL

Boron trifluoride etherate (3ml) was added to methanol (15ml, HPLC grade) and kept at 4°C until used.

(iii) ACETYL CHLORIDE/METHANOL

Acetyl chloride (3ml) was added dropwise to methanol (15ml, HPLC grade) in a round-bottom flask.

(iv) HMDS, PYRIDINE, TMCS

HMDS, dry pyridine and TMCS were pre-mixed in the ratio of 3:2:1, v/v/v. The solution was centrifuged before use to remove precipitated silica.

(f) PREPARATION OF DERIVATIVES OF NSAIDs

(i) METHYLATION WITH DIAZOMETHANE

Methyl esters of the drug standard were prepared by adding a few drops of a solution of diazomethane, in diethyl ether, to about 1mg of drug standard in methanol (1ml). Diazomethane was added dropwise until the solution remained yellow. The reaction was found to be complete on addition, but was allowed to stand at room temperature for 15 minutes before evaporating to dryness under a stream of nitrogen. The residue was redissolved in 5ml of ethyl acetate and about 0.5µl was used for GC analysis.

(ii) BORON TRIFLUORIDE ETHERATE/METHANOL

Boron trifluoride etherate/methanol 1:5 v/v (500µl) was added to about 1mg of drug standard in a reaction vial. The vial was sealed and the reaction mixture heated

at 60°C. The reaction was found to be complete after 1 hour. The solution was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 5ml of ethyl acetate and about 0.5µl was used for GC analysis.

(iii) METHYLATION WITH ACETYL CHLORIDE/METHANOL

Acetyl chloride/methanol 1:5 v/v (500µl) was added to about 1mg of drug standard in a reaction vial. The vial was sealed and the reaction mixture heated at 60°C. The reaction was found to be complete after 1 hour. The solution was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 5ml of ethyl acetate and about 0.5µl was used for GC analysis.

(iv) SILYLATION WITH BSTFA + 1% TMCS

BSTFA + 1% TMCS (100µl) and acetonitrile (100µl) were added to a vial containing about 1mg of drug standard. The vial was sealed and the reaction mixture heated at 60°C for 20 minutes. After this time acetonitrile (5ml) was added to the reaction vial and about 0.5µl was used for GC analysis.

(v) SILYLATION WITH TRI-SIL-Z

Tri-Sil-Z (100µl) was added to a vial containing about 1mg of drug standard. The vial was sealed and the reaction mixture heated at 60°C for 20 minutes. After this time acetonitrile (5ml) was added to the reaction vial and about 0.5µl was used for GC analysis.

(vi) SILYLATION WITH HMDS, PYRIDINE, TMCS

An aliquot of the HMDS, pyridine, TMCS mixture (100µl) was added to a vial containing about 1mg of drug

standard. The vial was sealed and heated at 60°C for 10 minutes. After this time acetonitrile (5ml) was added to the reaction vial and about 0.5µl was used for GC analysis.

(vii) tert-BUTYLDIMETHYLSILYLATION WITH MTBSTFA

MTBSTFA (100µl) and acetonitrile (100µl) were added to a vial containing about 50µg of drug standard. The vial was sealed and the reaction mixture heated at 60°C for 30 minutes. After cooling about 0.5µl was used for GC analysis.

2.4.3 RESULTS

(a) GC ANALYSIS OF SELECTED NSAIDs

Ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen and mefanamic acid did not chromatograph as their free acids. These six drugs were therefore derivatised and chromatographed successfully as their methyl esters, silyl esters and tert-butyldimethylsilyl esters. Figure 2.4.5 illustrates the resolution obtained with the three derivatives and Table 2.4.1 compares their retention indices. Methylation was performed using diazomethane, silylation with BSTFA + 1% TMCS and tert-butyldimethyl-silylation with MTBSTFA.

Piroxicam, indomethacin and sulindac did not chromatograph at all under the conditions used.

Phenylbutazone, sulphinpyrazone and azapropazone are discussed in Section 2.4.3(b).

(b) GC ANALYSIS OF PHENYLBUTAZONE, SULPHINPYRAZONE AND AZAPROPAZONE

Phenylbutazone, sulphinpyrazone and azapropazone chromatographed successfully as the free compounds and had retention indices of 2381, 2252 and 2427, respectively. Figure 2.4.6 illustrates a chromatogram obtained with a mixture of the three compounds.

Using ibuprofen as a model compound, ibuprofen, phenylbutazone and sulphinpyrazone were reacted with diazomethane, boron trifluoride/methanol and acetyl chloride/methanol. The products were analysed by GC and the results are given in Table 2.4.2.

Ibuprofen gave one peak with a retention index of

1529 with all four methylation procedures. This corresponded to the methyl ester of ibuprofen.

Phenylbutazone was not derivatised using boron trifluoride/methanol or acetyl chloride/methanol. The peak observed at 2381 corresponds to phenylbutazone itself. Phenylbutazone, however, formed several peaks when reacted with diazomethane. Three peaks were present after methylating for only 10 seconds in diazomethane, one peak corresponding to the free phenylbutazone. After reacting for 15 minutes, the phenylbutazone peak was no longer present. Figure 2.4.7 illustrates the progress of the reaction.

Sulphinpyrazone did not react with boron trifluoride/methanol, the peak observed at 2252 corresponding to sulphinpyrazone. Two peaks were observed after reacting sulphinpyrazone with diazomethane, one corresponding to sulphinpyrazone. Several peaks were observed when sulphinpyrazone was reacted with acetyl chloride/methanol. The reactions of phenylbutazone, sulphinpyrazone and azapropazone with diazomethane were studied using GC-MS. The results are given in Section 2.5.3(b).

Ibuprofen, phenylbutazone and sulphinpyrazone were reacted with BSTFA + 1% TMCS; Tri-Sil-Z; HMDS, pyridine, TMCS (3:2:1) and MTBSTFA. The reactions products were analysed by GC and the results are given in Table 2.4.3.

Ibuprofen gave one peak at 1615 with BSTFA + 1% TMCS and HMDS, pyridine, TMCS (3:2:1) corresponding to its

silyl ester. Ibuprofen did not react with Tri-Sil-Z and gave one peak at 1845 with MTBSTFA corresponding to its tert-butyldimethylsilyl ester.

Phenylbutazone and sulphinpyrazone were found not to react with any of the silylating reagents under the conditions used.

TABLE 2.4.1: Retention indices of selected NSAIDs after methylation, silylation and tert-butyldimethylsilylation.

DRUG	RETENTION INDICES		
	COMPOUND AFTER METHYLATION[1]	COMPOUND AFTER SILYLATION[2]	COMPOUND AFTER t-BUTYLDIMETHYL-SILYLATION[3]
Ibuprofen	1529	1615	1842
Flurbiprofen	1893	1971	2225
Naproxen	1981	2051	2302
Ketoprofen	2092	2161	2437
Fenbufen	2315	2440	2748
Mefenamic Acid	2072	2164	2435

1 Methylation was carried out with diazomethane (room temperature, 15 minutes).
2 Silylation was carried out with BSTFA + 1% TMCS (60°C, 20 minutes).
3 tert-Butyldimethylsilylation was carried out with MTBSTFA (60°C, 30 minutes).

Table 2.4.2: Retention indices of reaction products obtained by reacting selected NSAIDs with different methylating agents.

DRUG	RETENTION INDICES				
	Free Compound	Diazomethane R.T., 10 secs.	Diazomethane R.T., 15 mins.	BF_3/Methanol 60^oC, 1 hour	AcCl/Methanol 60^oC, 1 hour
IBUPROFEN	N D	1529	1529	1529	1529
PHENYLBUTAZONE	2381	2245, 2381, 2535	2245, 2535	2381	2381
SULPHINPYRAZONE	2252	1824, 2252	2252, 2445	2252	2252, 2319, 2460, 2609 2748, 2832

N D = not detected

Table 2.4.3: Retention indices of reaction products obtained by reacting selected NSAIDs with different silylating reagents.

DRUG	RETENTION INDICES				
	Free Compound	BSTFA + 1% TMCS $60^{o}C$, 20 mins.	Tri-Sil-z $60^{o}C$, 20 mins	HMDS, Pyridine TMCS (3:2:1) $60^{o}C$, 10 mins	MTBSTFA $60^{o}C$, 10 mins
IBUPROFEN	N D	1615	N D	1615	1845
PHENYLBUTAZONE	2381	2381	2381	2381	2381
SULPHINPYRAZONE	2252	2252	2252	2252	2252

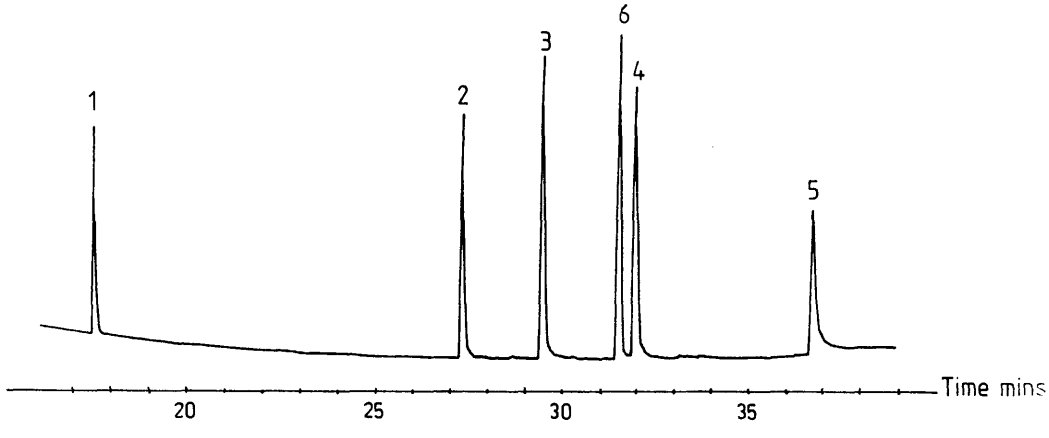
N D = not detected

FIGURE 2.4.5: GC analysis of products obtained after reacting a mixture of NSAIDs with (a) diazomethane (b) BSTFA + 1% TMCS and (c) MTBSTFA.

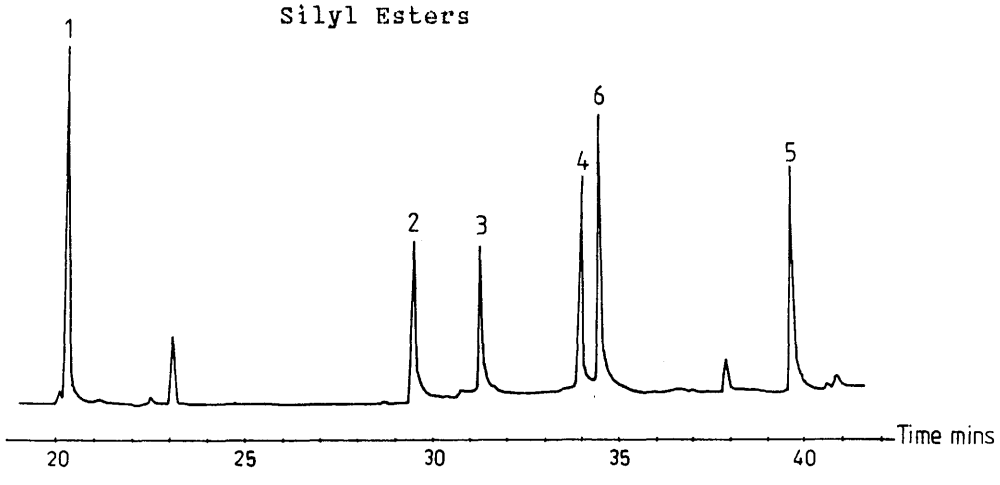
Peak identifications are: 1, ibuprofen; 2, flurbiprofen; 3, naproxen; 4, ketoprofen; 5, fenbufen; and 6, mefenamic acid.

Chromatographic conditions used are described in the text.

(a) Methyl Esters.



(b) Silyl Esters



(c) tert-Butyldimethylsilyl Esters.

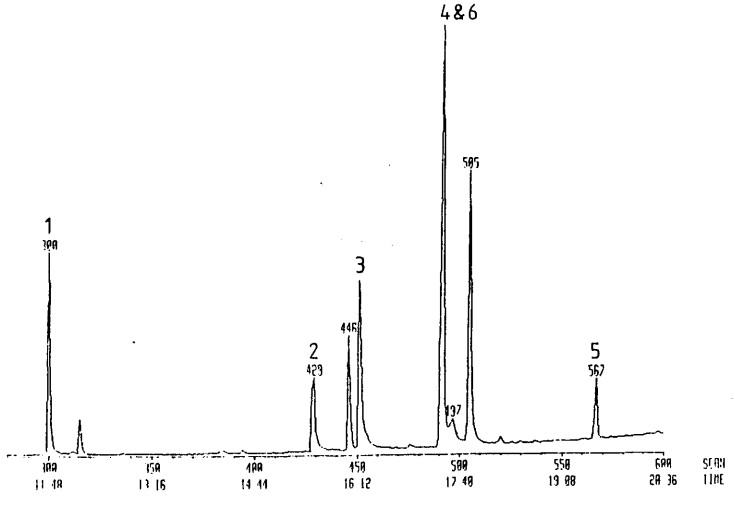


FIGURE 2.4.6: Typical chromatogram obtained with a mixture of phenylbutazone sulpinpyrazone and azapropazone.

Chromatographic conditions used are described in the text.

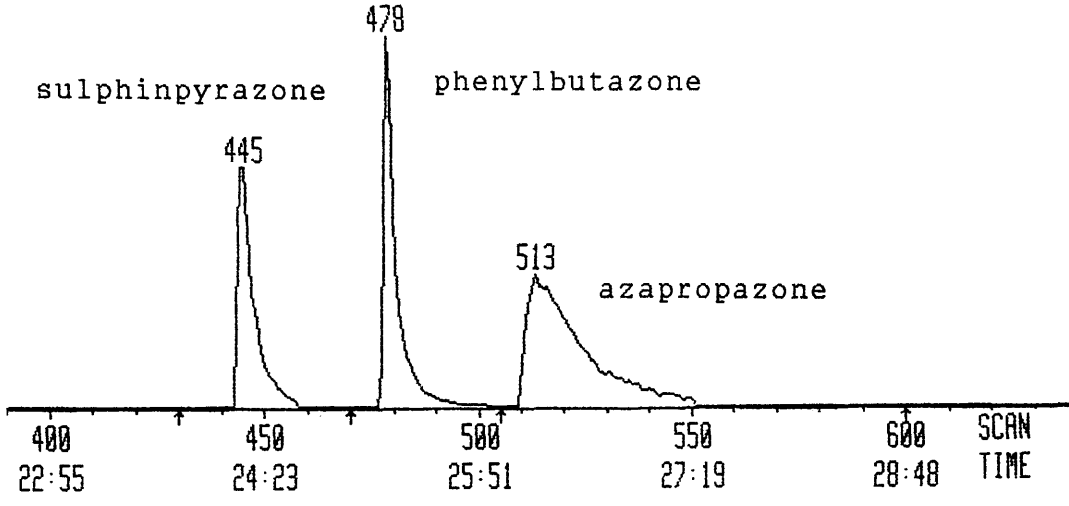
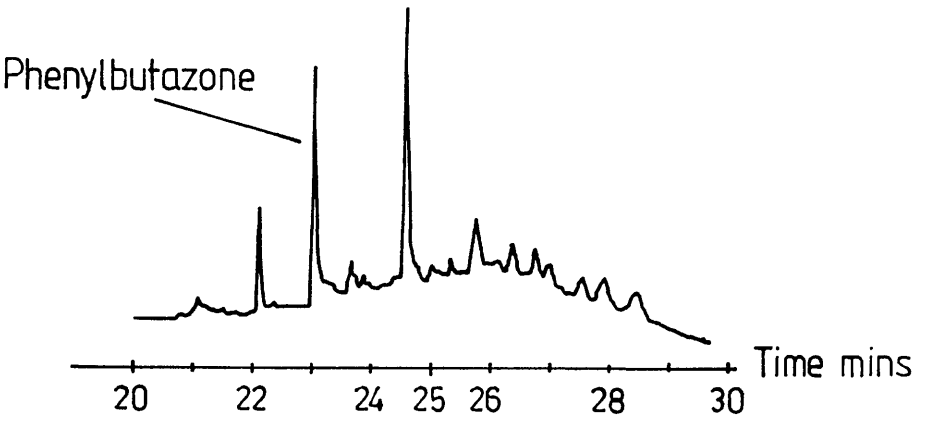


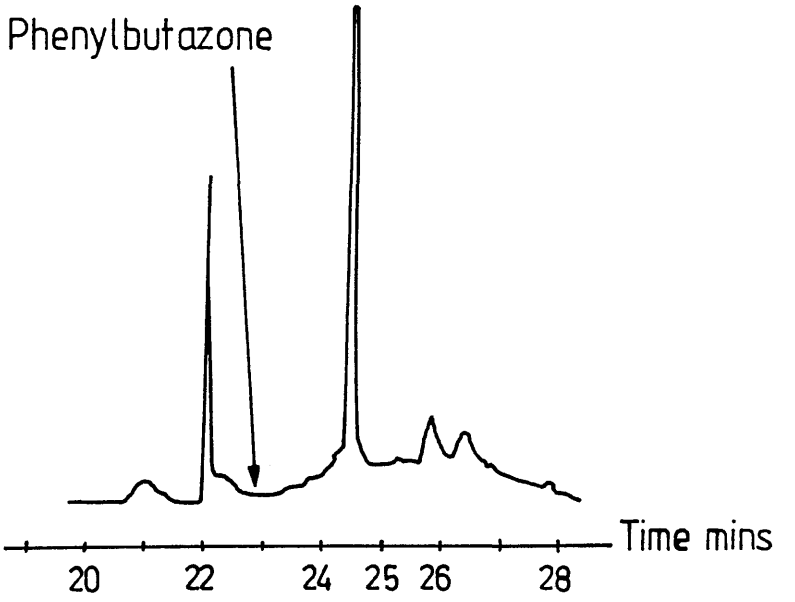
FIGURE 2.4.7: GC analysis of products obtained after reacting phenylbutazone with diazomethane for (a) 10 seconds, and (b) 15 minutes.

Chromatographic conditions used are described in the text.

(a) Reaction of phenylbutazone with diazomethane for 10 seconds.



(b) Reaction of phenylbutazone with diazomethane for 15 minutes.



2.4.4 DISCUSSION

In order to have sufficient resolving power to analyse NSAIDs in urinary extracts it was thought that a capillary column would be necessary. A fused silica column was not available so a wide bore (0.5mm i.d.) wall coated open-tubular column was chosen. This type of column had a high capacity and was resistant to water; it gave good resolving power and also allowed direct splitless injection to be used. If the samples to be analysed contained small amounts of drugs and/or metabolites it would be preferable to have as much of the sample on the column as possible, rather than losing a large percentage of it as would happen with a split injector system. The stationary phase chosen was CP-Sil 5 which is 100% dimethyl polysiloxane, a non-polar phase.

A temperature programmed run was chosen in order to give reasonable analysis times for a group of drugs with a wide range of retention indices.

A flame ionisation detector was used since it would be able to detect all of the drugs that emerged from the column. As with HPLC the analysis specificity could be improved by using different detectors, such as an NPD or ECD. These detectors were not tried, but reports have appeared in the literature which claim to detect the methyl ester of ketoprofen by ECD [103] and several authors report methods for indomethacin, also by ECD [104-106]. Phenylbutazone and indomethacin have also been reported to have been detected by NPD [7].

Out of the twelve test drugs which were examined only phenylbutazone, sulphinpyrazone and azapropazone were successfully chromatographed without derivatisation. Many literature methods are available for the GC analysis of phenylbutazone and sulphinpyrazone. However, Leach [107] noted that azapropazone oxidises at room temperaure within 1 hour. Samples containing azapropazone must, therefore, be analysed immediately or stored at $-20^{o}C$. The chromatogram in Figure 2.4.6 illustrates that phenylbutazone, sulphinpyrazone and azapropazone all gave tailing peaks. This is most likely due to deterioration of the GC column. Since sulphinpyrazone is the heaviest of these three molecules it would be expected to elute last, but it has the shortest retention time. This is probably due to thermal decomposition of the compound (this was confirmed by GC-MS analysis).

In order to chromatograph the other nine drugs as part of a drug screening procedure derivatisation by methylation, silylation and tert-butyldimethylsilylation were evaluated. Methylation, with ethereal diazomethane, silylation, with BSTFA + 1% TMCS and tert-butyldimethyl-silylation, with MTBSTFA were all successful for six of the drugs. These drugs all contain carboxylic acid functional groups, namely: ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen and mefanamic acid. In all cases the methyl esters eluted first followed by the silyl esters and lastly the tert-butyldimethylsilyl esters. This was to be expected as a result of the relative

increases in molecular weight provided by the derivatives. The necessity to derivatise these carboxylic acids agrees with many other authors [7,8,108]. However, ibuprofen, naproxen, ketoprofen, mefanamic acid and indomethacin have been chromatographed on fused-silica capillaries as their free acids [8,109]. Only ibuprofen chromatographed well and the other four compounds were said to have poor chromatographic properties.

Piroxicam, indomethacin and sulindac were not chromatographed by GC at all in this study. Sharp [8] reported piroxicam to have poor gas chromatographic properties and most literature methods of this drug have been by HPLC [110,111]. Several papers have agreed that indomethacin either forms several products on GC analysis [6,7] or has poor gas chromatographic properties [8,108]. Hunt and co-workers [6] identified the structures of the products produced by indomethacin under GC conditions. Sharp [8] also agrees with this present work in not successfully chromatographing sulindac by GC.

The six drugs containing carboxylic acid functional groups reacted quantitatively with diazomethane to form their methyl esters. When phenylbutazone and sulphinpyrazone were reacted with diazomethane multiple products were formed. Since this would be an undesirable complication to interpreting a GC chromatogram other methylating reagents were examined. Neither phenylbutazone or sulphinpyrazone reacted with boron

trifluoride/methanol. Phenylbutazone did not react with acetyl chloride/methanol but sulphinpyrazone formed several products with this reagent.

Silyl derivatives of phenylbutazone and sulphinpyrazone were not formed under the conditions used. This was perhaps not surprising because neither of these drugs have functional groups obviously amenable to silylation. However, the proton at C-4 is acidic and C-methylation has been noted at this position during the metabolism of phenylbutazone [112]. It was therefore necessary to establish the chemical form in which the drug and/or metabolites would be observed in the course of a routine drug screen.

2.4.5 CONCLUSION

Since only three out of the twelve NSAIDs examined could be chromatographed by GC as the free compounds, a GC screening procedure would have to involve a derivatisation step. If silylation or tert-butyldimethylsilylation were used nine out of the twelve compounds would be detected. Ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen and mefenamic acid would be detected as their silyl or tert-butyldimethylsilyl derivatives. Phenylbutazone, sulphinpyrazone and azapropazone would not react and would be detected as the free compounds.

If methylation was used as the derivatisation procedure, ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen and mefenamic acid would be detected as their methyl esters. It has been shown that phenylbutazone and sulphinpyrazone form multiple products when reacted with diazomethane or acetyl chloride/methanol. These reagents could therefore cause problems in quantitation of these compounds in a screening procedure. Although, on this occasion, it was shown that boron trifluoride/methanol did not react with phenylbutazone or sulphinpyrazone there is the possibility that these compounds may, to some extent, form derivatives with this reagent.

Piroxicam, indomethacin and sulindac did not chromatograph under the conditions used and would, therefore, not be detected in a GC screening procedure.

2.5 GAS CHROMATOGRAPHY/MASS SPECTROMETRY

2.5.1 INTRODUCTION AND THEORY

Mass spectrometry is the basis of the most specific and sensitive detector available for gas chromatography. It is possible to give an unambiguous indentification of a compound with 1ng or less of material.

The technique relies upon the ionisation and subsequent fragmentation of molecules and results in a mass spectrum, a graph of mass to charge ratios of the fragments plotted against relative abundance. Functional groups in the molecule direct the fragmentation. It is possible to deduce a possible structure from the fragmentation pattern and in many cases the molecular weight of the compound can be determined.

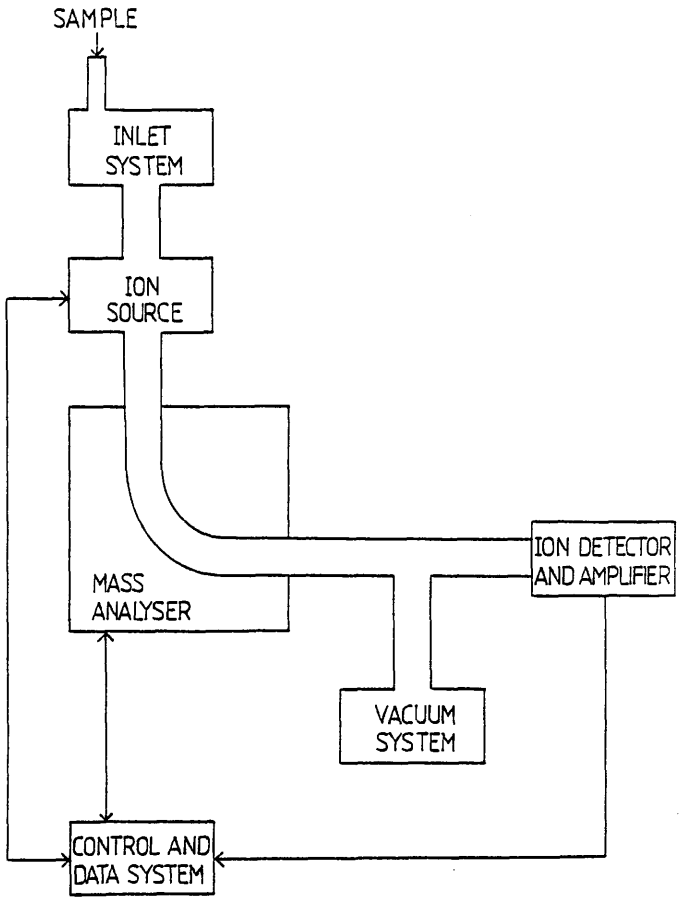
The basic components of a mass spectrometer are shown in Figure 2.5.1.

Throughout this study the mass spectrometer used was the VG 16F single focussing magnetic sector instrument. The technique and basic components of the mass spectrometer described here will be those of the VG 16F.

(a) SAMPLE INLET SYSTEMS

In order to minimise the number of collisions undergone by ions and thus maximise the number of ions reaching the detector the interior of a mass spectrometer is under high vacuum (10^{-6} torr). Sample inlet systems must therefore allow the introduction of a sample without significant loss of vacuum, especially in the mass analyser and detector regions of the spectrometer.

FIGURE 2.5.1: Schematic diagram of a mass spectrometer system.



There are four inlet systems available on the VG 16F mass spectrometer; the septum inlet, the direct probe and two gas chromatographic inlets.

(i) THE SEPTUM INLET

The septum inlet is for the introduction of volatile liquids or gases. This inlet consists of a 100ml reservoir and a molecular leak which meters the sample to the ion chamber at a constant volume rate. This inlet is particularly useful for the introduction of perfluorokerosene to calibrate the instrument. The reservoir is normally heated to about 150°C.

(ii) THE DIRECT PROBE

The direct insertion probe is used for the introduction of solid samples (~30µg or less). The solid sample is placed in a small quartz capillary tube and inserted through a vacuum lock close to the ion source. Due to the low pressure in the chamber and heating by contact with the source (250°C) the sample vaporises. The probe can also be heated directly to aid vaporisation.

(iii) GAS CHROMATOGRAPHIC INLETS

There are two gas chromatographic inlets on the VG 16F. One is used for a capillary column and the other for a packed column. A capillary column has a gas flow rate of about 1 to 2 ml/min which the vacuum pumps can easily pump away. The capillary column can therefore be inserted directly into the ion source. Packed columns have gas flow rates of about 20 to 30ml/min and they require a

molecular separator to prevent all the effluent entering the ion source, thus maintaining the vacuum. Only the jet separator will be discussed here as it is the one installed in the VG 16F.

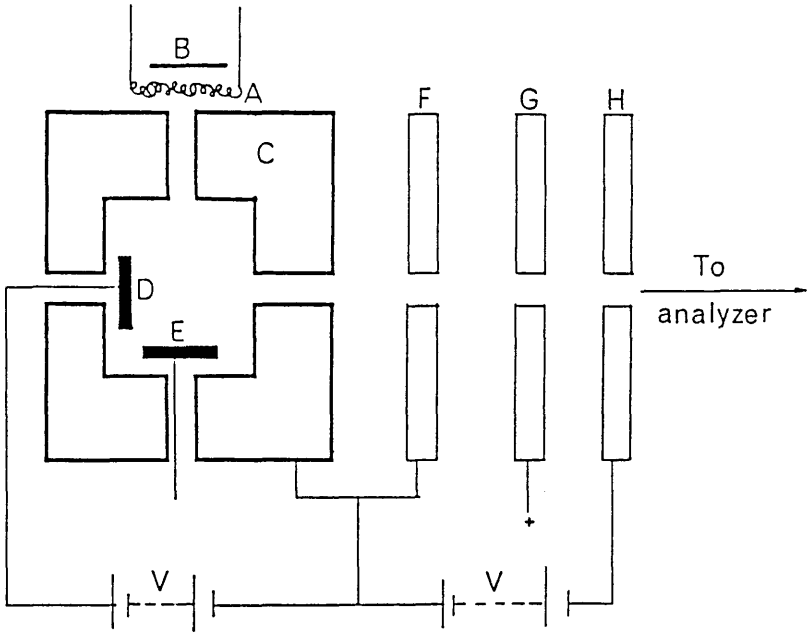
The single-stage jet separator consists of two aligned orifices, the gap between the orifices being evacuated by a rotary pump. As the effluent from the chromatographic column passes through the first orifice the lighter molecules of carrier gas (usually helium) have less momentum and tend to diffuse: these are then pumped away. Those heavier sample molecules have more momentum and so continue to the second orifice.

The two-stage jet separator consists of two single-stage separators in series.

(b) IONISATION METHODS

Various methods exist to produce ions in mass spectrometry. One of the most popular techniques is electron impact (EI). In the ion source (see Figure 2.5.2) electrons are emitted from a hot filament and are accelerated across the ion chamber to the collector anode. The vaporised sample molecules are bombarded by this stream of high energy electrons (70eV) and are consequently ionised. Since only about 10eV is required for ionisation of organic molecules some of the remaining energy absorbed by the molecules is used to break bonds, thus forming fragment ions. Both positive and negative ions are produced. Positive ion mass spectrometry is the most popular as these ions are produced in much larger

FIGURE 2.5.2: Ion source



Key

A.	Filament	E.	Trap
B.	Filament shield	F.	Source slit
C.	Source block	G.	Focussing plates
D.	Repeller	H.	Accelerating slit

numbers than are negative ions (by a factor of about 10^3). Only positive ion mass spectrometry will be considered here.

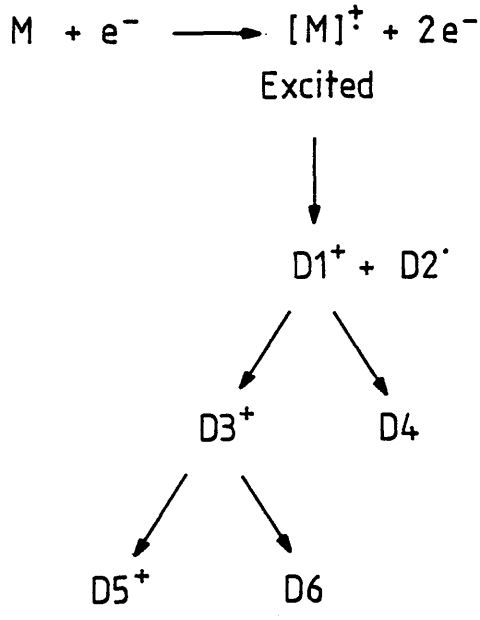
The simplest ionisation reaction is the loss of an an electron resulting in the molecular ion (M^+). The abundance of the molecular ion in the mass spectrum varies with the structure; depending on the inherent stability of the molecule, it can be a dominant ion, a weak ion or completely absent. The molecular ion can then rearrange or fragment into smaller ions which subsequently fragment themselves (see Figure 2.5.3). The resulting positive ions move out of the ionisation chamber through the slits and an ion lens system into the mass analyser, under the influence of a small positive repelling potential in the source.

The most important piece of information which may be obtained from a mass spectrum is the molecular weight. Certain classes of compounds do not show molecular ions and in other cases it is not always possible to identify the molecular ion. In order to resolve these problems a group of 'soft ionisation' techniques have been developed. These techniques generate a quasi-molecular ion and fragmentation is minimal. 'Soft ionisation' methods include chemical ionisation, secondary ion mass spectrometry (SIMS), fast atom bombardment (liquid SIMS), field ionisation, field desorption and atmospheric pressure ionisation.

Chemical ionisation is the most popular "soft

FIGURE 2.5.3: Ionisation by electron impact.

The source pressure is kept sufficiently low that ion decompositions are unimolecular. M represents the gaseous molecules and D1–D6 represent the daughter ions formed.



ionisation" method. Chemical ionisation utilises a reagent gas such as methane or isobutane which is present in a large excess. Electron impact causes mainly ionisation of the reagent gas and primary ions are produced. These primary ions then react with more reagent gas to produce a number of chemically reactive species such as CH_5^+ and $C_2H_5^+$, in the case of the reagent gas being methane (See Figure 2.5.4). These secondary ions can then act as Bronsted Acids and donate protons to the sample molecules, resulting in $(M + 1)^+$ quasi-molecular ions. These quasi-molecular ions may have sufficient energy to fragment, but in practice few fragmentation and rearrangement ions are observed. Chemical ionisation results in mass spectra with the quasi-molecular ion as the most intense ion in the spectrum.

Chemical ionisation then is a complementary technique to electron impact ionisation. Figure 2.5.5 illustrates the mass spectra of proline obtained by both electron impact and chemical ionisation techniques.

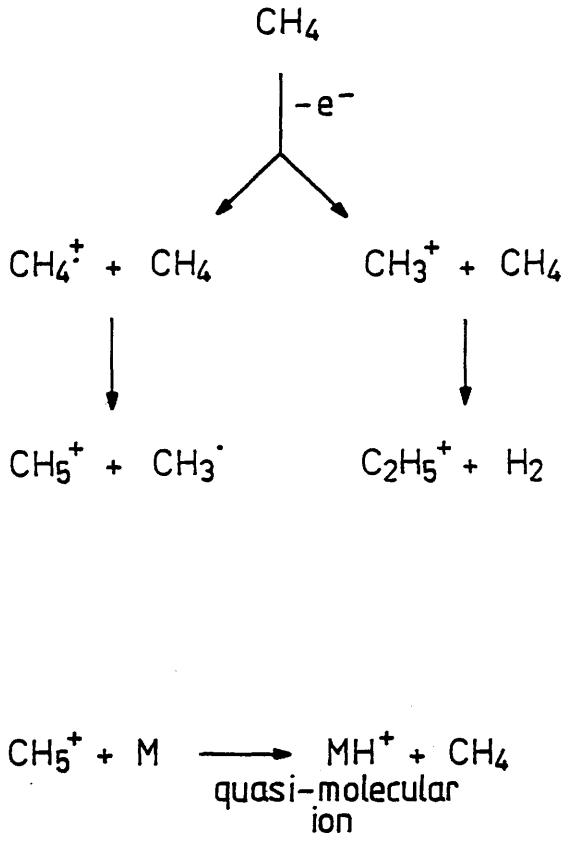
(c) MASS ANALYSER

The ions formed in the source are repelled through the source slit by a small potential difference between the slit and the repeller. These ions are then accelerated towards the mass analyser by a high potential difference (about 4 Kv) between the source block and the accelerating slit.

The linear ion beam leaving the ion source is separated into groups of ions of different mass to charge

FIGURE 2.5.4: Chemical ionisation with methane.

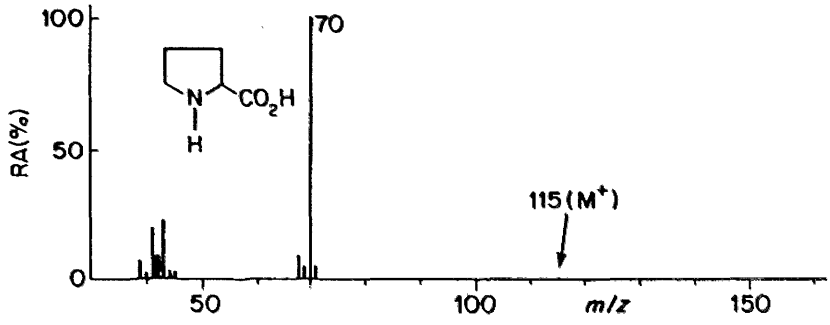
M represents the gaseous molecule and MH^+ is the quasi-molecular ion which is detected.



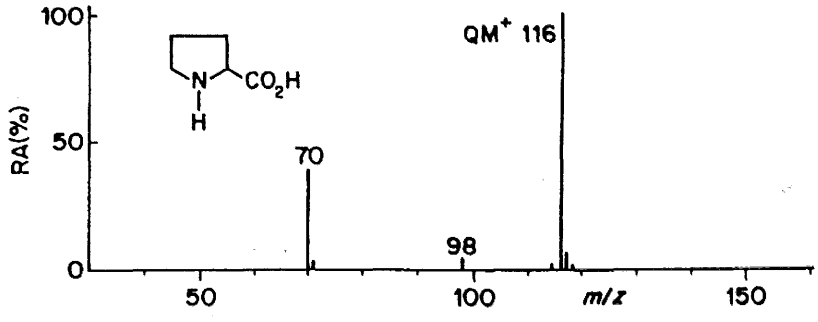
$$CH_5^+ + M \longrightarrow \underset{\text{quasi-molecular ion}}{MH^+} + CH_4$$

FIGURE 2.5.5: Mass spectrum of proline (a) by electron impact and (b) by chemical ionisation.

(a) electron impact mass spectrum



(b) chemical ionisation mass spectrum



ratios by the mass analyser. There are four different types of mass analysers commonly used in organic chemistry; the single focussing magnetic deflection mass analyser, the double focussing mass analyser (which contains a magnetic sector and an electrostatic sector), the quadrupole analyser and the time of flight analyser. Only the single focussing magnetic sector instrument will be discussed here as this is the type of mass analyser present in the VG 16F.

The most important parameter determined by the analyser is the mass resolution (R) which is defined as:

$$R = \frac{m}{\Delta m}$$

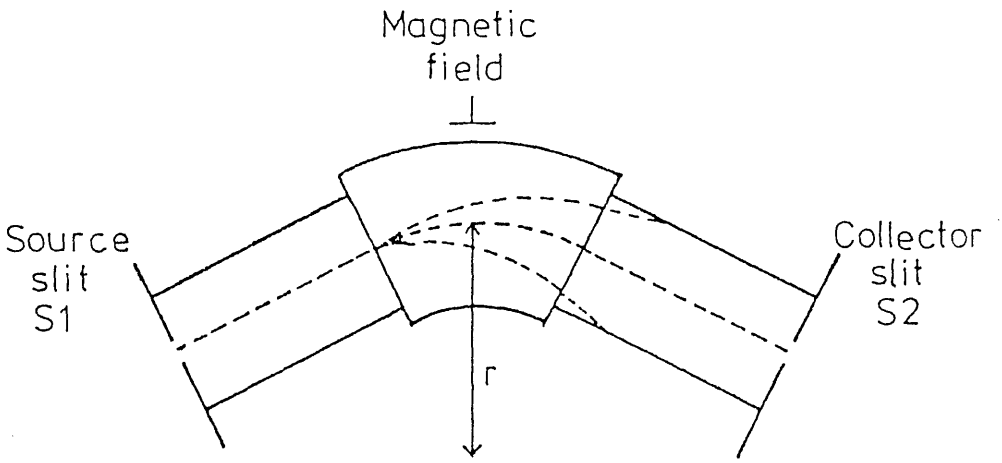
where m is the mass of the first peak in a consecutive pair of ions and Δ m the difference in the masses of the two peaks. The peaks are considered to be resolved if they are separated by a valley of 10% the peak height. Mass spectrometers may be of low, medium or high resolution.

Low resolution instruments have a resolution of the order of 1000, that is, they can distinguish ions of 1000 and 1001 amu or 100.0 and 100.1 amu. Single focussing magnetic sector and quadrupole mass spectrometers are low resolution instruments. Medium to high resolution instruments range from 10,000 to 150,000 and have double-focussing mass analysers.

The principle of the single-focussing magnetic sector instrument is shown in Figure 2.5.6. Ions formed in the source are accelerated through a voltage V

FIGURE 2.5.6: Schematic diagram of single-focussing magnetic deflection mass analyser.

'r' is the radius of curvature of the magnetic sector.



(V = 2000-8000V) toward the source slit which is at earth potential. The fall in potential energy for the ions is equal to their gain in kinetic energy, summarised in Equation 1, where e is the charge on an electron, Z is the number of such charges on an ion, V is the accelerating voltage, m is the mass of the ion and v is the velocity of the ion.

For an ion to reach the collector slit and be recorded it must traverse a path of radius of curvature r through the magnetic field of strength B. The equation of motion of the ion (Equation 2) expresses the balance between the centripetal force (or angular momentum) and the centrifugal force caused by this field.

$$ZeV = \tfrac{1}{2}mv \qquad \text{.....Equation 1}$$

$$mv^2/_r = B\ z\ v\ e \qquad \text{.....Equation 2}$$

Combining Equations 1 & 2 gives the basic mass spectrometer Equation 3,

$$m/_z = \frac{B^2r^2e}{2V} \qquad \text{.....Equation 3}$$

Thus by keeping r constant and by varying either the field strength or the applied voltage (as in th VG 16F) ions of different m/z ratios separated by the magnetic field can be made to reach the collector. The resolution of a magnetic sector mass spectrometer is determined principally by the radius of curvature and by the width of the source and collector slits.

$$R = \frac{kr}{S_1 + S_2}$$

where k is a constant and S_1 and S_2 are the widths of the source and collector slits. Decreasing the slit width, therefore, increases the resolution but also decreases the instrument's sensitivity.

Typical ion currents recorded in mass spectrometry are in the range 10^{-10} to 10^{-19} amps. These low currents are amplified by an electron multiplier before they reach the electronic amplifier.

Due to the amount of data generated by a mass spectrometer and the need for fast data acquisition and processing a computer is generally interfaced to the mass spectrometer. Chromatograms can be obtained from the output of the mass spectrometer by either recording the total ion current (TIC) or by recording a selected ion (known as Selected Ion Recording, SIR, or Selected Ion Detection, SID).

2.5.2 EXPERIMENTAL

(a) REAGENTS

1. Diethyl ether, Pronalys AR grade (May and Baker, Dagenham, England).

2. Methanol, HPLC grade (Rathburn Chemicals LImited, Walkerburn, Scotland).

3. Ethyl acetate (Rathburn Chemicals LImited, Walkerburn, Scotland).

4. Digol, Analar grade (BDH Chemicals Limited, Poole, England).

5. Potassium hydroxide, Analar grade (BDH Chemicals Limited, Poole, England).

6. Diazald, N-methyl-N-nitroso-p-toluenesulfonamide (Aldrich Chemical Company Limited, Gillingham, England).

7. BSTFA, + 1% TMCS, MTBSTFA and Pyridine (Pierce, Life Sciences, Laboratory Limited, Luton, England).

(b) DRUG STANDARDS

As in Section 2.3.2(b).

(c) Apparatus

1. A Perkin-Elmer Sigma 3B gas chromatograph fitted with a Groß split/splitless injector interfaced via a direct inlet to a VG 16F single-focussing magnetic sector mass spectrometer and a VG Series 2000 data system. Electron impact mass spectra were recorded repetitively (cycle time 2.5 seconds).

2. A wall coated open tubular column (24m, 0.5mm i.d., 1.1mm o.d.) with a liquid stationary phase of CP-SIL5 at a film thickness of 0.81 µm.

(d) GC-MS CONDITIONS

The GC-MS conditions employed were was follows:-

Column:	WCOT, 24m, 0.5mm i.d., 1.1mm o.d. with a liquid stationary phase of CP-SIL5 at a film thickness of 0.81μm.
Carrier Gas:	Helium (2ml/min)
Injector Temperature:	275°C
Column Temperature:	Programmed from 80°C to 280°C at a ramp rate of 4°/minute with initial and final isothermal periods of 2 minutes.
Ionising Energy:	70eV
Source Temperature:	240°C

(e) PREPARATION OF DIAZOMETHANE

As in Section 2.4.2 (e)(i).

(f) PREPARATION OF DERIVATIVES OF NSAIDs

(i) METHYLATION WITH DIAZOMETHANE

As in Section 2.4.2 (f)(i) except that the reaction products produced with phenylbutazone, sulphinpyrazone and azapropazone were diluted in methanol instead of ethyl acetate before injection.

(ii) SILYLATION WITH BSTFA + 1% TMCS

As in Section 2.4.2 (f)(iv).

(iii) tert-BUTYLDIMETHYLSILYLATION WITH MTBSTFA

As in Section 2.4.2 (f)(vii).

2.5.3 RESULTS

(a) MASS SPECTRAL ANALYSIS OF SELECTED NSAIDs

Ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen and mefenamic acid were derivatised and analysed by GC-MS as their methyl esters, trimethylsilyl esters and tert-butyldimethylsilyl esters. The mass spectral data obtained are listed in Table 2.5.1 and the full spectra are given in Figures 2.5.7-2.5.12.

(b) MASS SPECTRAL ANALYSIS OF PHENYLBUTAZONE, SULPHINPYRAZONE AND AZAPROPAZONE

Phenylbutazone, sulphinpyrazone and azapropazone were analysed by GC-MS. The mass spectral data obtained is listed in Table 2.5.2 and the full spectra are given in Figure 2.5.13.

(c) MASS SPECTRAL ANALYSIS OF THE REACTION PRODUCTS OF PHENYLBUTAZONE, SULPHINPYRAZONE AND AZAPROPAZONE WITH DIAZOMETHANE

Phenylbutazone, sulphinpyrazone and azapropazone were reacted with diazomethane as described in Section 2.5.2(f)(i) and the reaction products analysed by GC-MS.

The chromatograms obtained for the reaction products of phenylbutazone and azapropazone with diazomethane are given in Figure 2.5.14.

The retention indices and the mass spectrometric data of the reaction products obtained for the three drugs are listed in Table 2.5.3 and the full spectra are given in Figures 2.5.15-2.5.19.

TABLE 2.5.1 Mass spectral data for pure drug substances

COMPOUND	MASS SPECTRAL CHARACTERISTICS												
	$M^{+\cdot}$	Intensity	Other Ions % Abundance										
Ibuprofen	220	46	161	177	119	220	117	118	91	162	121	105	43	41
Methyl Ester			100	91	51	46	46	45	40	36	26	20	20	19
Ibuprofen	278	4	73	75	160	74	161	117	263	234	118	45	43	91
TMS# Ester			100	89	77	57	52	47	33	28	24	21	21	20
Ibuprofen	320	Not	263	75	73	264	117	161	265	118	119	115	305	105
BDMS* Ester		Present	100	63	56	41	24	20	16	15	14	14	5	5
Flurbiprofen	258	42	199	258	158	200	178	184	179	183	259	170	196	177
Methyl Ester			100	42	42	23	10	8	8	7	5	4	3	3
Flurbiprofen	316	12	180	73	165	75	179	301	74	178	181	316	199	45
TMS Ester			100	99	46	38	32	27	22	19	18	12	11	11
Flurbiprofen	358	Not	301	75	302	73	198	179	199	178	207	303	183	343
BDMS Ester		Present	100	38	34	32	20	18	14	11	11	9	8	3
Naproxen	244	84	185	244	186	141	170	154	115	245	142	153	155	152
Methyl Ester			100	84	44	28	24	18	18	11	11	7	6	5
Naproxen	302	100	287	186	244	303	184	243	141	75	170	154	153	74
TMS Ester			68	49	47	42	40	37	35	35	34	31	30	27
Naproxen	344	13	287	185	73	288	75	141	344	115	289	184	170	153
BDMS Ester			100	49	41	35	32	22	13	12	11	11	9	8

TABLE 2.5.1 Continued

COMPOUND	MASS SPECTRAL CHARACTERISTICS												
	M^{+}	Intensity	Other Ions % Abundance										
Ketoprofen	268	31	209	105	77	268	191	210	103	32	51	269	131	104
Methyl Ester			100	73	51	31	19	12	9	5	4	3	3	3
Ketoprofen	326	Not	73	282	75	77	263	281	311	105	74	284	45	312
TMS Ester		Present	100	62	24	20	18	11	9	9	5	3	3	2
Ketoprofen	368	Not	311	312	75	73	77	105	295	283	267	341	324	353
BDMS Ester		Present	100	37	31	28	23	22	18	8	8	7	5	4
Fenbufen	268	7	181	152	153	182	268	76	32	151	237	90	154	127
Methyl Ester			100	28	21	12	7	6	6	5	4	4	2	2
Fenbufen	326	24	181	152	75	182	311	153	73	326	151	312	237	76
TMS Ester			100	70	70	66	53	53	39	24	23	11	11	8
Fenbufen	368	Not	311	312	181	75	237	152	153	313	283	191	73	353
BDMS Ester		Present	100	39	33	30	24	24	16	14	11	9	8	7
Mefenamic Acid	255	94	223	255	208	222	224	194	180	256	195	209	77	97
Methyl Ester			100	94	46	34	29	27	21	18	10	7	6	4
Mefenamic Acid	313	52	223	313	224	208	73	222	194	180	314	75	298	209
TMS Ester			100	52	32	29	27	20	12	12	11	8	7	7
Mefenamic Acid	355	54	298	224	207	355	223	208	299	180	356	194	96	340
BDMS Ester			100	89	89	54	33	33	24	17	14	13	10	4

#TMS = trimethylsilyl
*BDMS = tert-butyldimethylsilyl

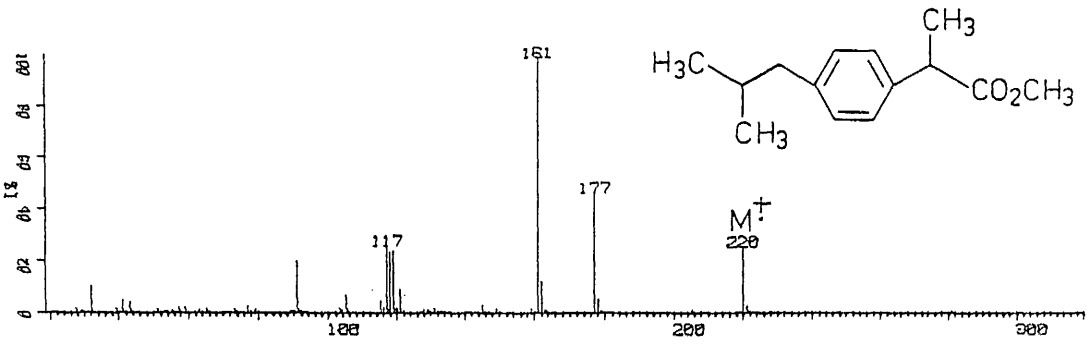
TABLE 2.5.2: Mass spectral data for pure drug substances

COMPOUND	MASS SPECTRAL CHARACTERISTICS												
	$M^{\pm}$	Intensity	Other Ions % Abundance										
Phenylbutazone	308	39	183	77	308	184	105	93	91	309	252	51	41	55
			100	77	39	22	14	11	11	9	7	7	6	5
Sulphinpyrazone	404	Not Present	278	77	279	105	130	131	119	249	152	78	51	280
			100	42	12	10	4	3	3	2	2	2	2	1
Azapropazone	300	58.18	160	300	189	145	161	188	42	190	146	104	77	55
			100	60	54	33	8	6	6	4	3	3	3	3

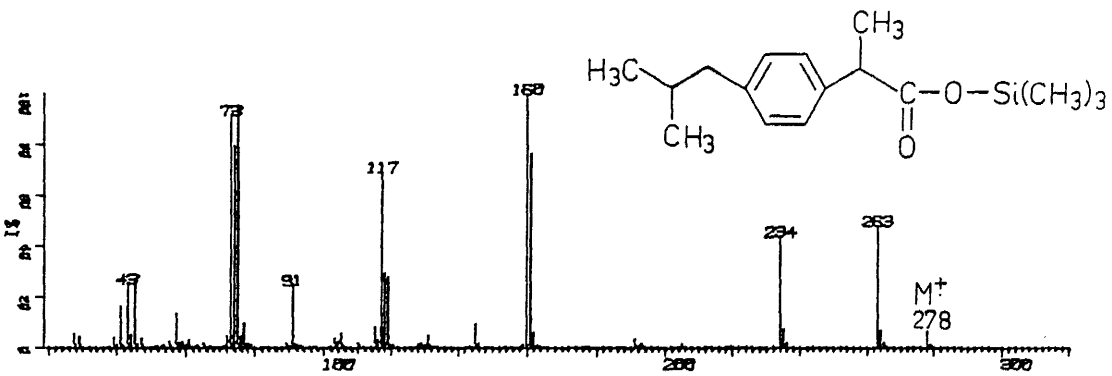
TABLE 2.5.3: Gas chromatographic and mass spectrometric data obtained for the reaction products of phenylbutazone, sulphinpyrazone and azapropazone with diazomethane

COMPOUND	Retention Index	Molecular Weight	Ions % Abundance Below											
Phenylbutazone	2276	322	183	77	322	266	105	118	83	41	184	323	119	91
Reaction Product 1			100	79	44	17	16	13	13	12	9	8	5	5
Phenylbutazone	2336	336	183	336	77	280	105	337	97	55	184	265	91	41
Reaction Product 2			100	50	44	17	12	11	11	11	9	6	5	6
Unreacted	2337	308	183	77	308	184	105	93	252	41	309	119	91	55
Phenylbutazone			100	84	37	26	16	15	10	10	9	9	9	8
Phenylbutazone	2527	322	77	188	322	160	279	129	146	91	219	280	225	119
Reaction Product 3			100	93	73	71	70	29	27	27	26	22	22	19
Phenylbutazone	2560	336	77	202	336	93	174	283	232	146	105	265	190	125
Reaction Product 4			100	58	51	41	33	28	26	24	24	19	19	19
Sulphinpyrazone	2143	418	292	77	105	144	119	293	182	64	145	130		
Reaction Produce			100	80	63	28	28	23	21	17	16	15		
Unreacted	2272	404	278	77	105	279	51	130	131	182				
Sulphinpyrazone			100	91	31	19	17	16	15	12				
Azapropazone	1765	314	145	160	188	104	77	89	146	161	314			
Reaction Product 1			100	71	41	24	19	18	12	9	3			
Azapropazone	2371	314	160	314	145	189	161	315	69	41	188	83	207	104
Reaction Product 2			100	46	26	17	16	10	10	10	9	9	4	4
Azapropazone	2448	314	174	314	72	175	315	285	242	241				
Reaction Product 3			100	36	27	8	7	7	3	3				
Azapropazone	2555	314	314	111	43	174	160	272	315	145	299	242	228	227
Reaction Product 4			100	53	47	39	17	14	12	11	8	8	7	4
Azapropazone	2627	314	174	314	72	285	175	315	242	241				
Reaction Product 5			100	61	40	7	6	5	3	2				

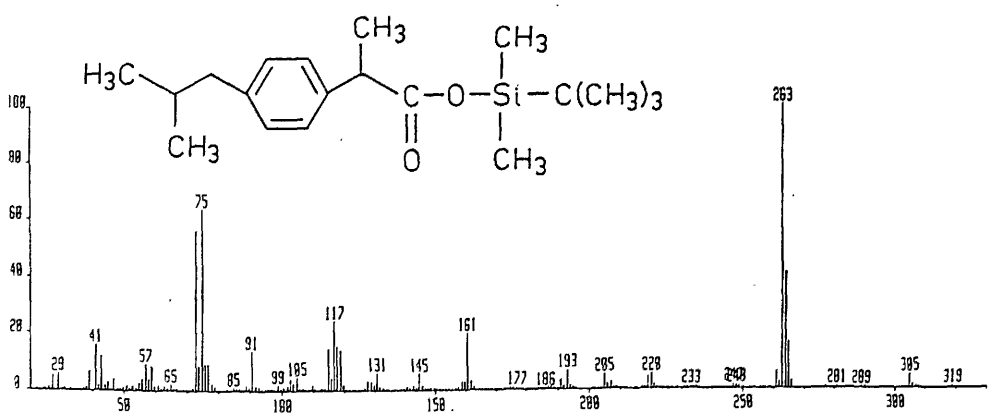
FIGURE 2.5.7: Mass spectra of the methyl, trimethylsilyl and tert-butyldimethylsilyl esters of ibuprofen.



Ibuprofen Methyl Ester — MW 220·31

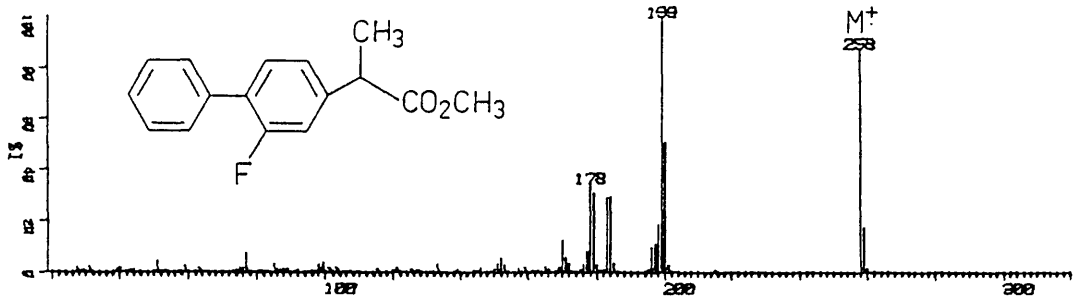


Ibuprofen TMS Ester — MW 278·47

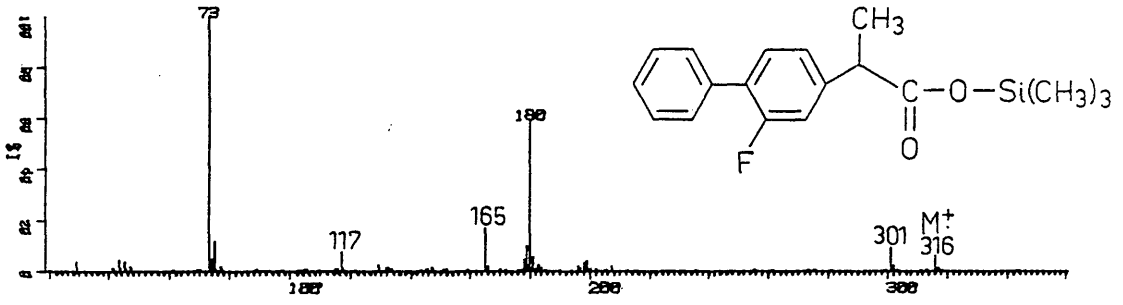


Ibuprofen BDMS Ester — MW 320·55

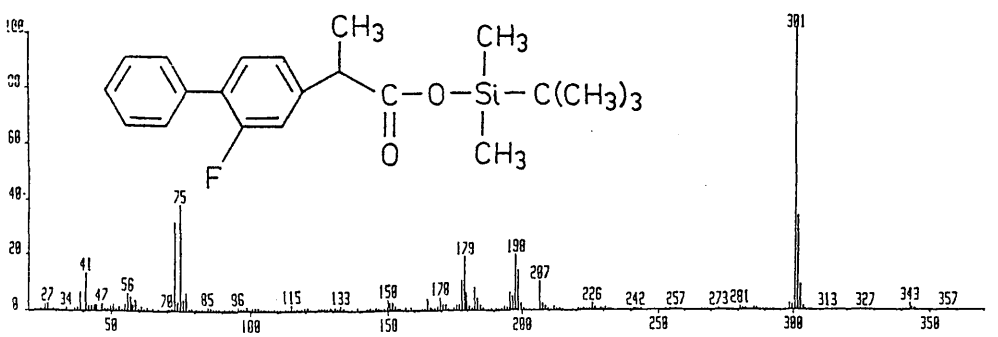
FIGURE 2.5.8: Mass spectra of the methyl, trimethylsilyl and tert-butyldimethylsilyl esters of flurbiprofen.



Flurbiprofen Methyl Ester — MW 258·29

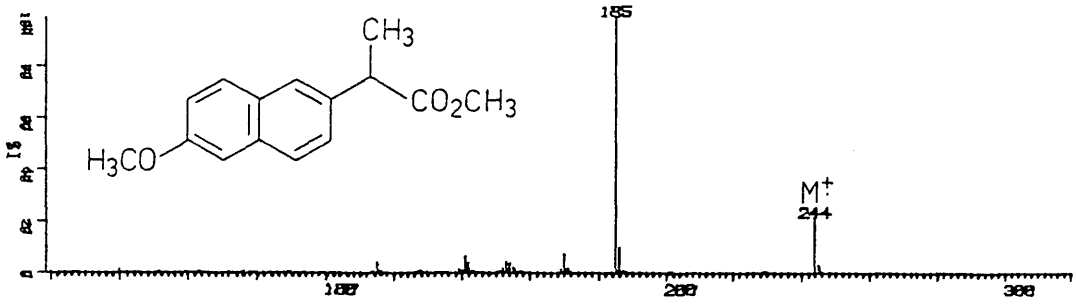


Flurbiprofen TMS Ester — MW 316·45

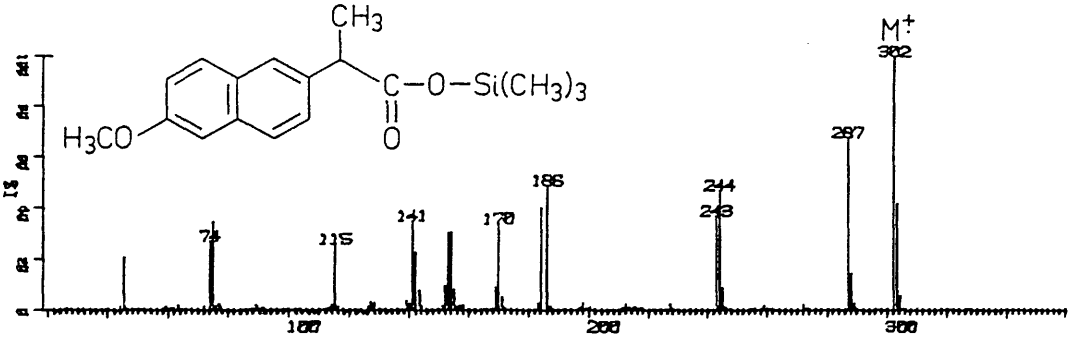


Flurbiprofen BDMS Ester — MW 358·53

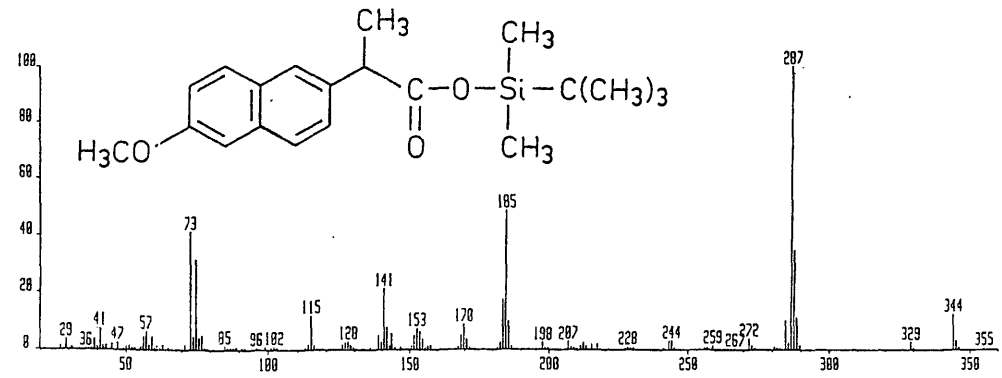
FIGURE 2.5.9: Mass spectra of the methyl, trimethylsilyl and tert-butyldimethylsilyl esters of naproxen.



Naproxen Methyl Ester — MW 244·29

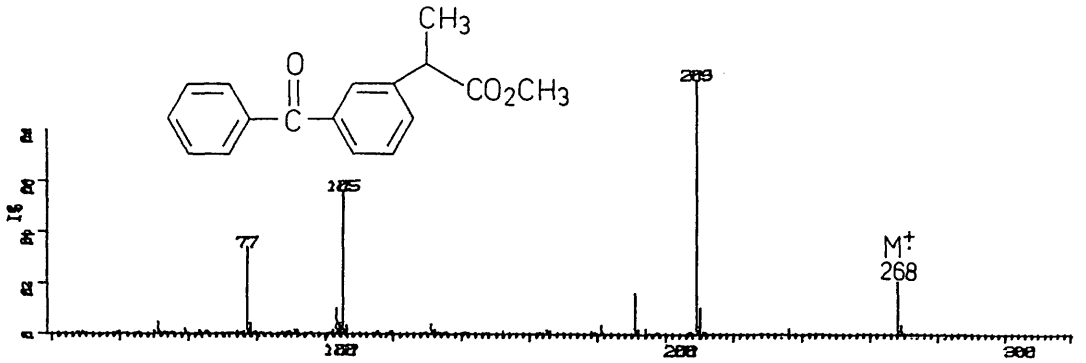


Naproxen TMS Ester — MW 302·45

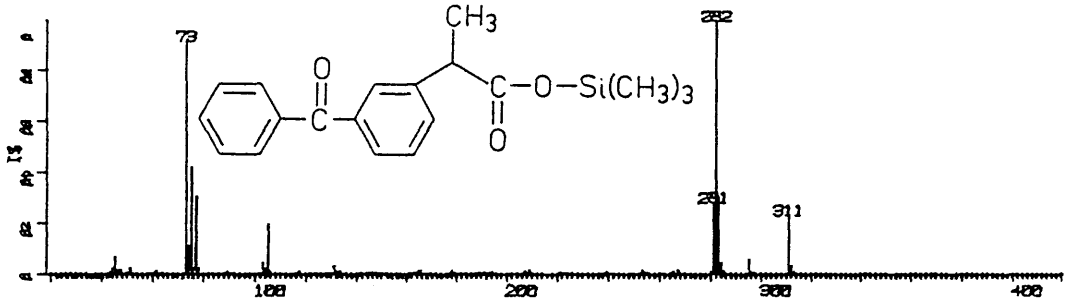


Naproxen BDMS Ester — MW 344·53

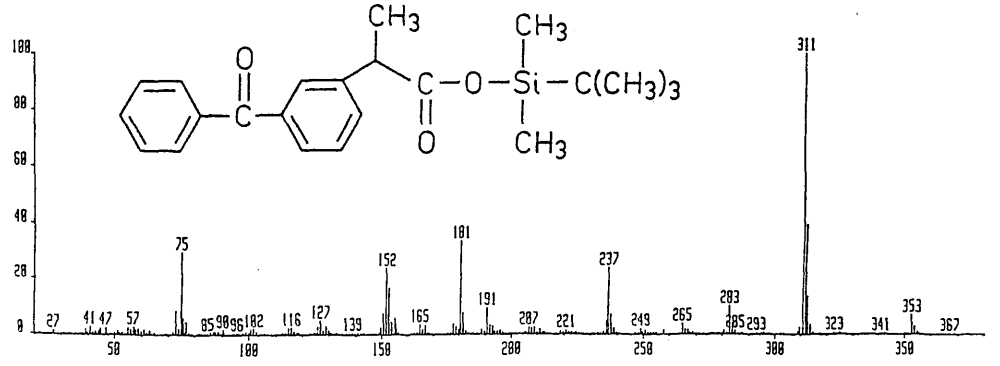
FIGURE 2.5.10: Mass spectra of the methyl, trimethylsilyl and tert-butyldimethylsilyl esters of ketoprofen.



Ketoprofen Methyl Ester — MW 268·31

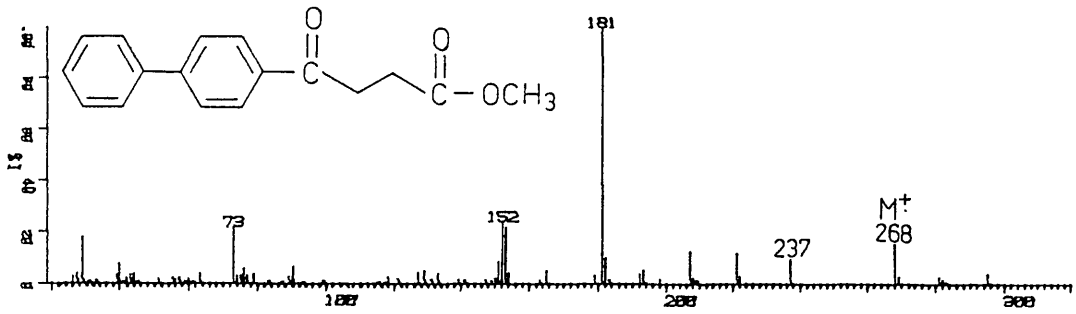


Ketoprofen TMS Ester — MW 326·47

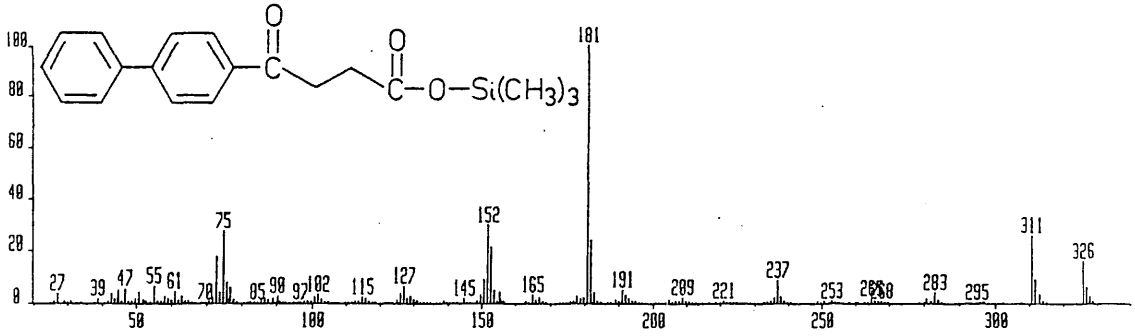


Ketoprofen BDMS Ester — MW 368·55

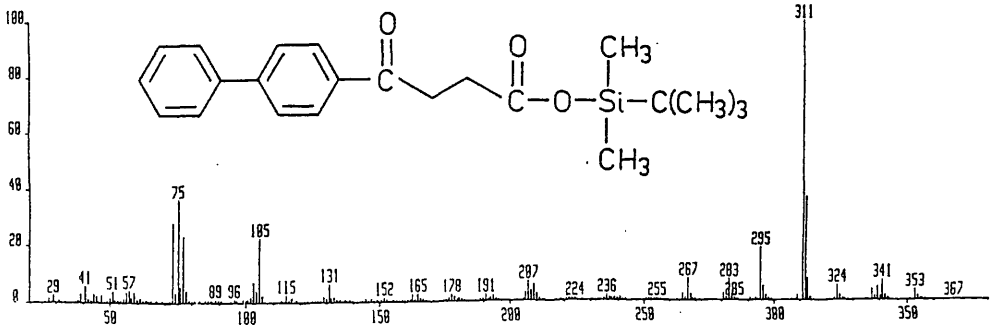
FIGURE 2.5.11: Mass spectra of the methyl, trimethylsilyl and tert-butyldimethylilyl esters of fenbufen.



Fenbufen Methyl Ester — MW 268·31

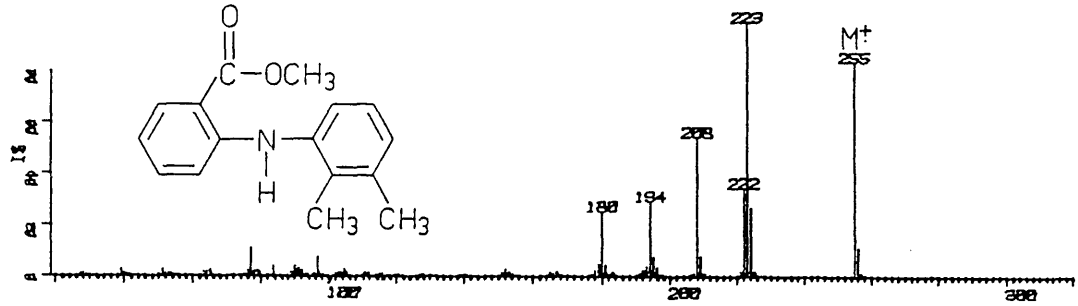


Fenbufen TMS Ester — MW 326·47

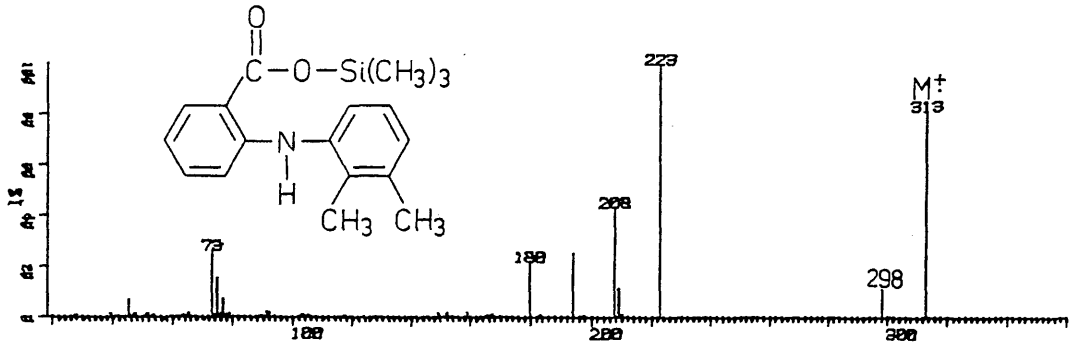


Fenbufen BDMS Ester — MW 368·55

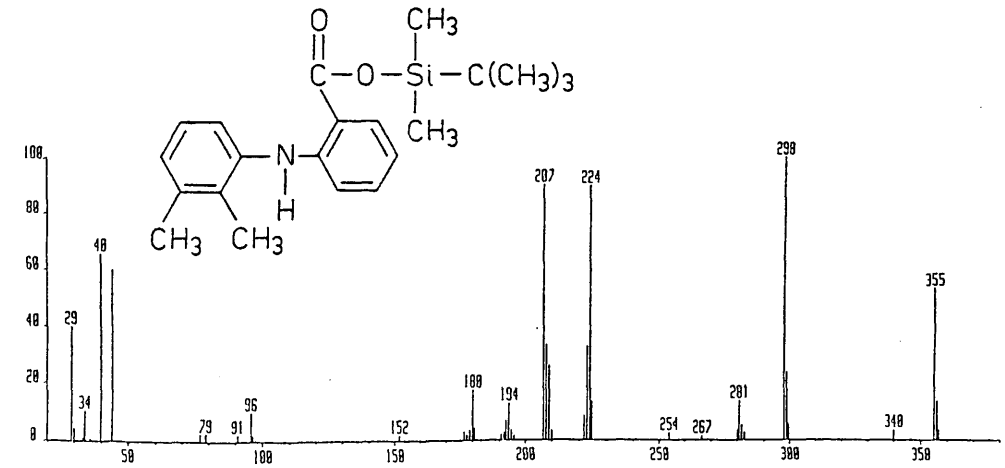
FIGURE 2.5.12: Mass spectra of the methyl, trimethylsilyl and tert-butyldimethylsilyl esters of mefenamic acid.



Mefenamic Acid Methyl Ester — MW 255·32

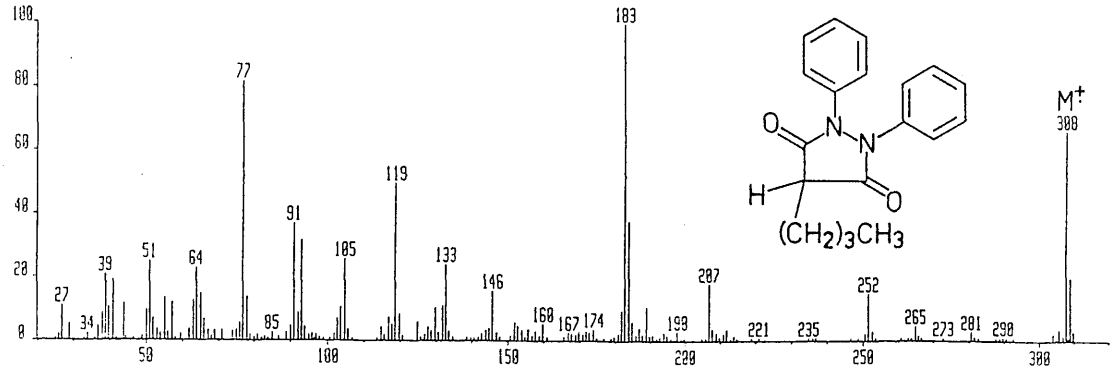


Mefenamic Acid TMS Ester — MW 313·47

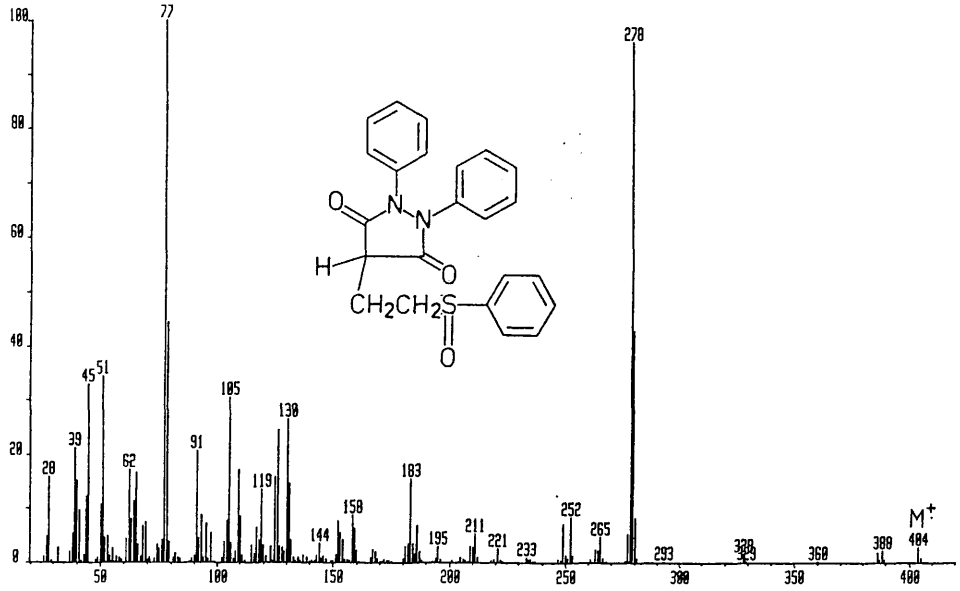


Mefenamic Acid BDMS Ester — MW 355·55

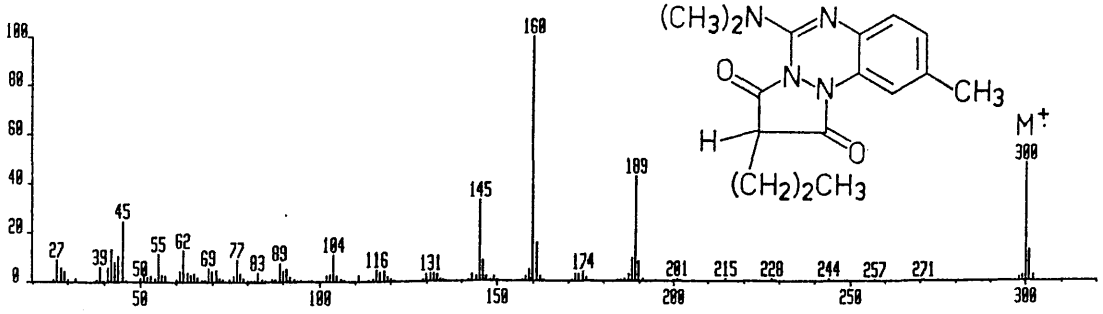
FIGURE 2.5.13: Mass spectra of phenylbutazone, sulphinpyrazone and azapropazone.



Phenylbutazone — MW 308·39

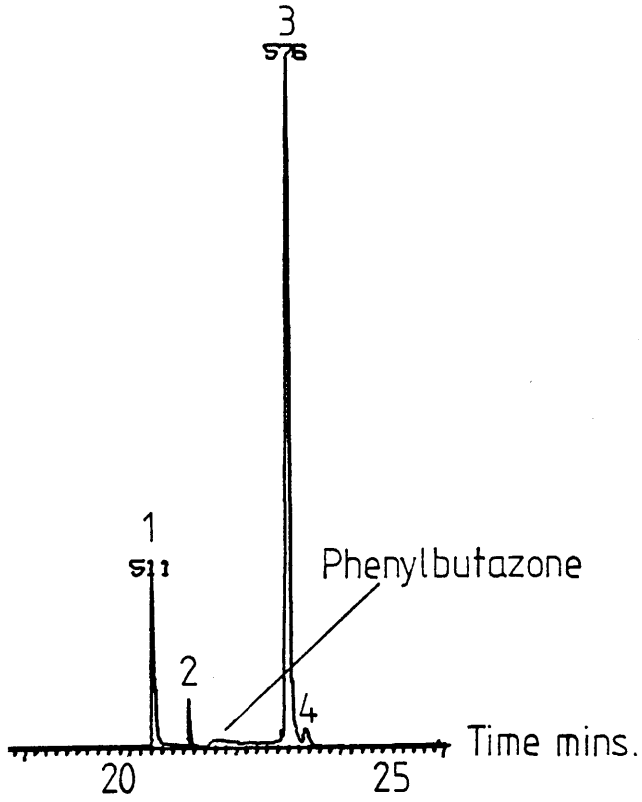


Sulphinpyrazone — MW 404·49

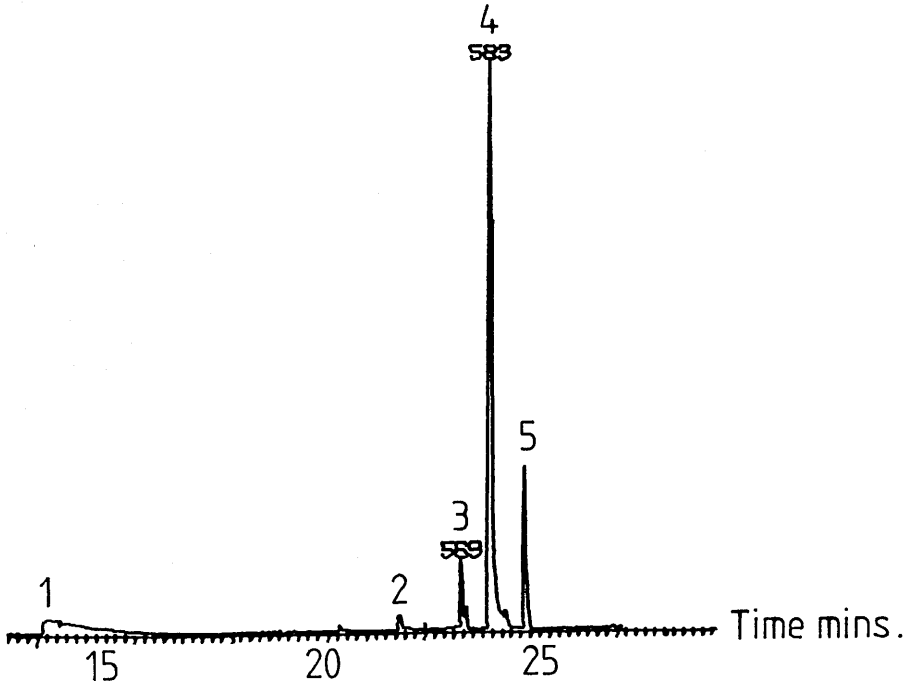


Azapropazone — MW 300·36

FIGURE 2.5.14: Chromatograms obtained for the reaction products of phenylbutazone and azapropazone with diazomethane.

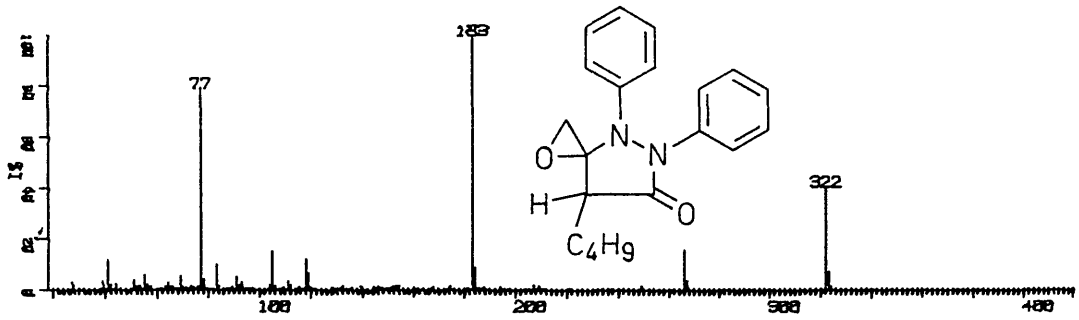


Phenylbutazone

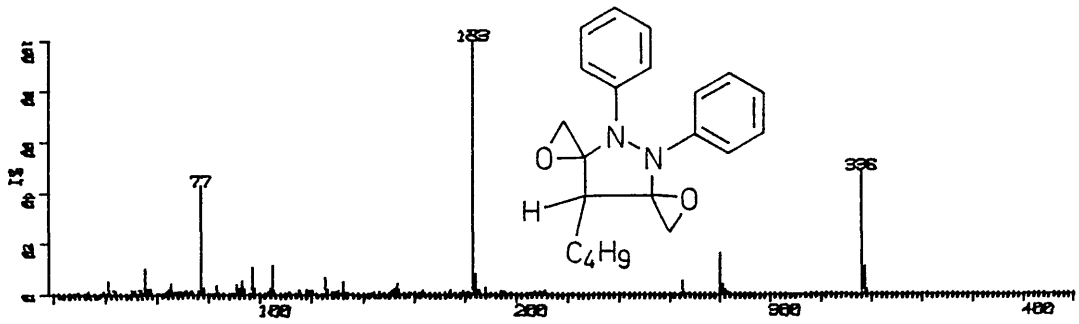


Azapropazone

FIGURE 2.5.15: Mass spectra obtained for the reaction products 1 and 2 of phenylbutazone with diazomethane.

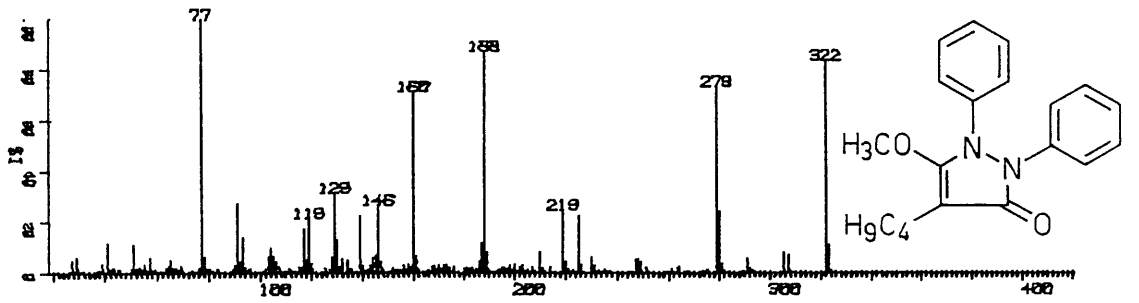


Phenylbutazone Reaction Product 1

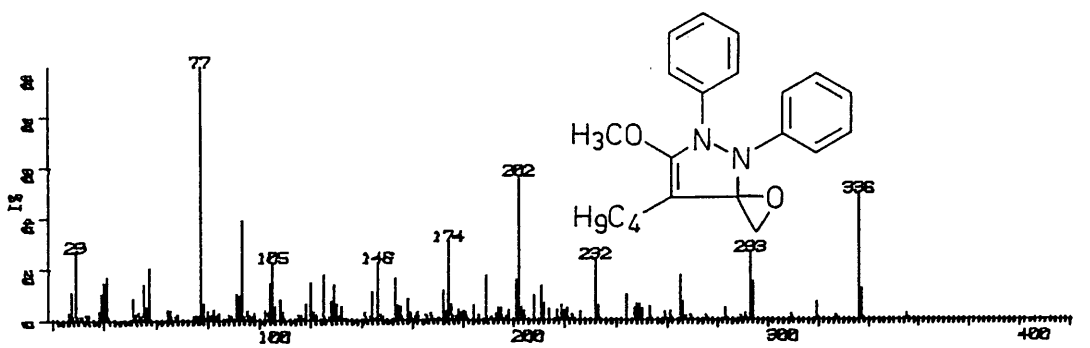


Phenylbutazone Reaction Product 2

FIGURE 2.5.16: Mass spectra obtained for the reaction products 3 and 4 of phenylbutazone with diazomethane.

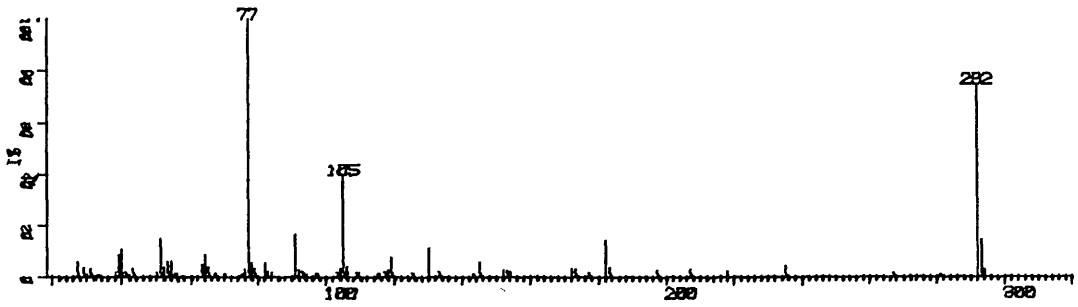


Phenylbutazone Reaction Product 3



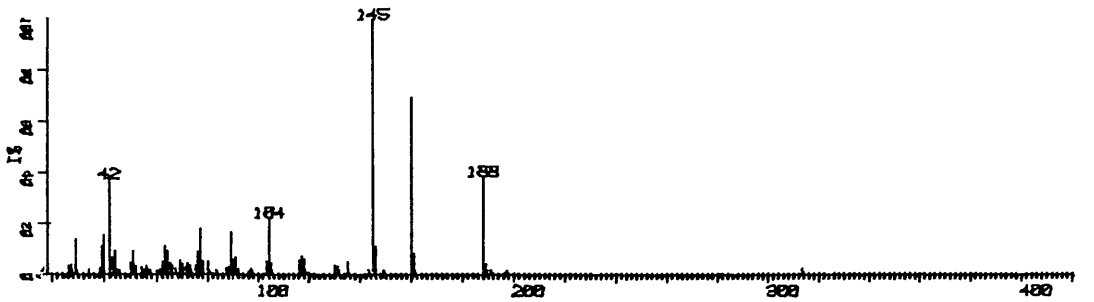
Phenylbutazone Reaction Product 4

FIGURE 2.5.17: Mass spectrum obtained for the reaction product of sulphinpyrazone with diazomethane.

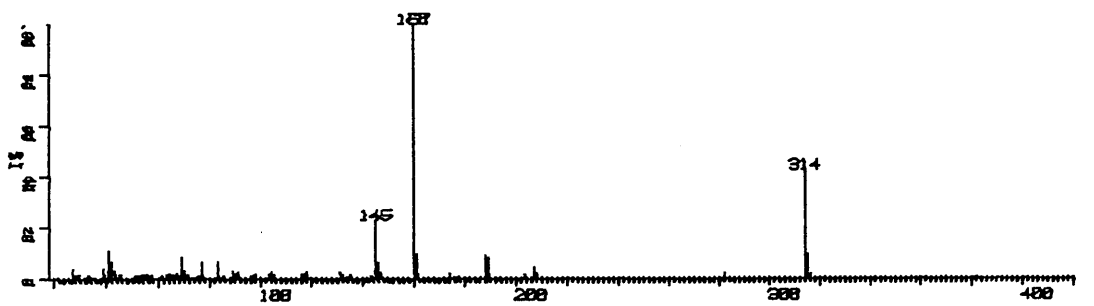


Sulphinpyrazone Reaction Product

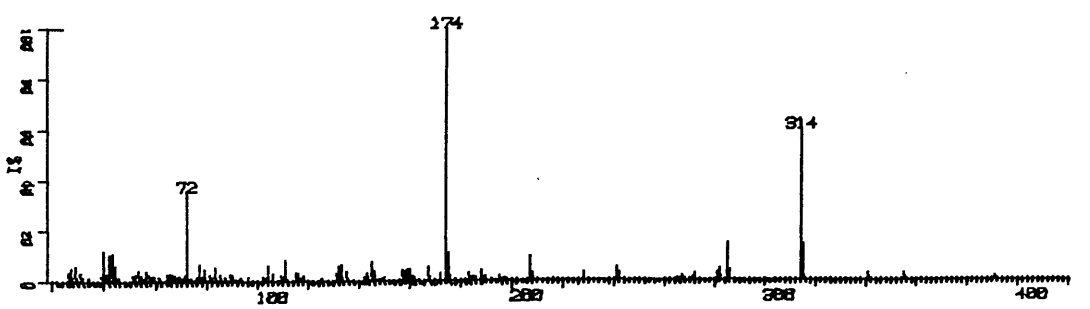
FIGURE 2.5.18: Mass spectra obtained for the reaction products 1, 2 and 3 of azapropazone with diazomethane.



Azapropazone Reaction Product 1

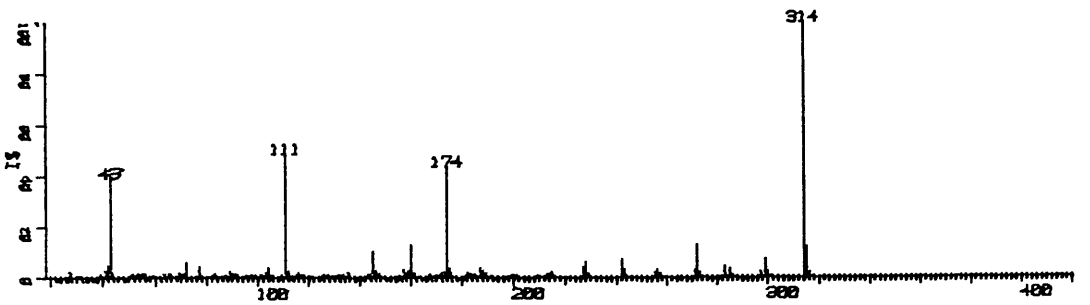


Azapropazone Reaction Product 2

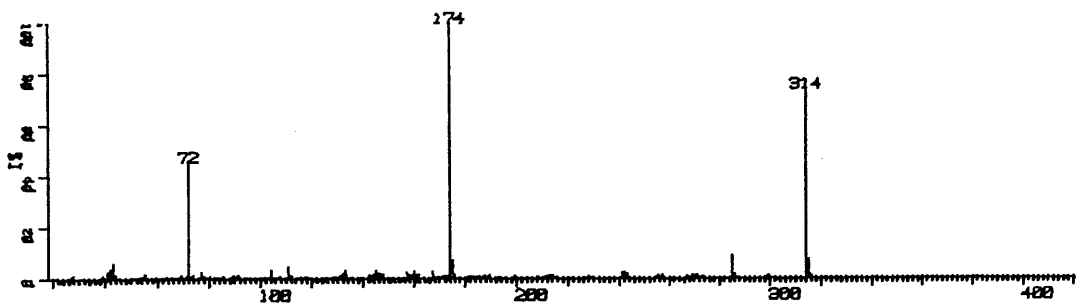


Azapropazone Reaction Product 3

FIGURE 2.5.19: Mass spectra obtained for the reaction products 4 and 5 of azapropazone with diazomethane.



Azapropazone Reaction Product 4



Azapropazone Reaction Product 5

2.5.4 DISCUSSION

(a) MASS SPECTRAL ANALYSIS OF SELECTED NSAIDs

The mass spectra of the methyl esters of ibuprofen, flurbiprofen, naproxen, ketoprofen, fenbufen and mefenamic acid showed several similar characteristics. All of the esters had a prominent molecular ion except fenbufen which had a molecular ion of 7% relative intensity.

Two common fragmentations seen in the spectrum of methyl esters were M-31 and M-59. These were due to loss of a methoxy group and loss of a carbomethoxy group. The six NSAIDs discussed here all exhibited an $[M-59]^+$ peak, although $[M-61]^+$ was of a higher relative abundance in both the fenbufen and mefenamic acid methyl ester spectra. This ion would arise from a proton transfer along with loss of the carbomethoxy group. The loss of 31 amu was seen in both the fenbufen and mefenamic acid methyl ester spectra.

The mass spectra of the silyl esters of five of the NSAIDs discussed here had a molecular ion. Ketoprofen did not exhibit a molecular ion. All of the six NSAIDs had a $[M-15]^+$ ion. This is usual for silyl esters and corresponds to the loss of a methyl moiety from the silyl group. All spectra of silylated

compounds had ions at m/z 73 and 75. These ions often formed the base peaks of the spectra, but were of low diagnostic significance. They have been shown [113] to have the structures illustrated in Figure 2.5.20.

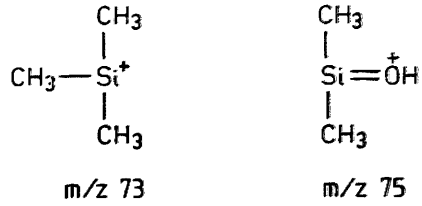
Only the tert-butyldimethylsilyl (BDMS) esters of naproxen and mefenamic acid exhibited molecular ions, the other four NSAIDs having $[M-1]^+$ peaks. In all cases the base peak in the BDMS ester spectrum was the $[M-57]^+$ ion, formed by loss of the tertiary butyl group.

The spectra of aromatic compounds often contained some or all of the following characteristic ions: m/z 77, 91 and 105. The formation of these ions is shown in Figure 3.4.1 and 3.4.2. These three ions can be found in many of the spectra of the methyl, silyl and BDMS esters of the NSAIDs discussed here.

IBUPROFEN

Cleavage of the 2-methyl-propyl side chain was evident in two of the ibuprofen spectra: m/z 177, $[M-43]^+$ in the methyl spectrum and m/z 234, $[M-44]^+$ in the silyl spectrum. The ion at m/z 161 was present in all three spectra and was due to loss of the carbomethoxy group, $[M-59]^+$ in the methylated compound, loss of the carboxy-silyl-ether group, $[M-117]^+$ in the

FIGURE 2.5.20: Ions found in the mass spectra of silylated compounds.



silylated compound and loss of the carboxy-tert-butyl-dimethylsilyl ether group, $[M-159]^+$ in the BDMS ester. The base peak in the silyl ester spectrum was in fact m/z 160, formed by loss of the 117 group mentioned above along with a proton transfer.

The cluster of ions present in all three spectra at m/z 117/118/119 was due to cleavage of the 2-methyl-propyl side chain along with loss of the carboxy ester group. The ion at m/z 117 was most abundant in the silyl spectrum because it was also due to the presence of the $[CO_2Si(CH_3)_3]^+$ ion.

FLURBIPROFEN

The base peak in the spectrum of the methyl ester of flurbiprofen was at m/z 199 and was due to loss of the carbomethoxy group. The BDMS ester spectrum also contained ions at m/z 198/199 but neither of these ions were of high relative abundance in the silyl ester spectrum. Loss of the carboxy-silyl-ether group was, therefore, not a favoured fragmentation of the silyl ester of flurbiprofen.

Peaks found at m/z 183/184 in the methyl and the BDMS spectra were due to losses of the carbomethoxy and the carboxy-tert-butyldimethylsilyl-ether groups respectively.

A dominant ion at m/z 180 which appeared in the spectrum of the silyl ester of flurbiprofen could be due to loss of the carboxy-silyl-ether group and a fluorine atom. The 180 ion was of low relative intensity in the methyl ester and BDMS ester spectra, but ions at m/z 178/179 were of high relative intensity. These ions could have been formed by loss of a fluorine atom along with a carbomethoxy group (or a carboxy-tert-butyldimethyl-silyl-ether group in the case of the BDMS ester) and a one or two proton transfer.

NAPROXEN

The ions at m/z 185/186, 184/186 and 184/185/186 appeared in the methyl, silyl and BDMS ester spectra respectively. These ions arose due to losses of the carboxy-ester groups in all three cases. An ion at m/z 170 also appeared in the three spectra and this was most likely due to loss of the carboxy-ester group and a methyl group. Loss of the carboxy-ester group and a methoxy group was shown by the presence of peaks at m/z 153/154 in the three spectra.

KETOPROFEN

The base peak in the methyl spectrum of ketoprofen at m/z 209 was due to loss of the

carbomethoxy group. The base peak in the silyl ester spectrum appears at m/z 282 (M-44) and was presumably due to loss of carbon dioxide. The mass spectrum of the BDMS ester of ketoprofen exhibitied a base peak at 311 which was the $[M-57]^+$ ion. The peak at m/z 191 in the methyl spectrum could have been due to the loss of a benzyl group. Apart from the usual aromatic ions at m/z 77 and 105 the other ions in the three ketoprofen ester spectra were of low relative intensity.

FENBUFEN

The peak at m/z 181 appeared in the three fenbufen spectra and was due to the $[C_6H_5-C_6H_4-CO]^+$ ion. The peak at m/z 237 in the methyl spectra was due to loss of the methoxy group. This peak was also present in the silyl and the BDMS ester spectra. The cluster of ions at m/z 151/152/153 present in all three spectra were due to the $[C_6H_5-C_6H_5]^+$ ion.

MEFENAMIC ACID

Nitrogen containing compounds such as mefenamic acid must follow the nitrogen rule: the molecular weights of compounds containing only C, H, N and O are odd when the number of nitrogens is odd and are even when the number

of nitrogens is even. The molecular ions in the three spectra of mefenamic acid all appeared at odd numbers, since mefenamic acid contains one nitrogen atom.

The ions present in the remainder of the three spectra were very similar to each other. Peaks at m/z 223 in the methyl and silyl spectra and m/z 224 in the BDMS spectra were of high relative intensity and were due to loss of the ether groups. The peak at m/z 208 present in the methyl and silyl spectra and that at m/z 207 in the BDMS spectra was due to further loss of a methyl group from the 223/224 ion.

The peak at m/z 194 also appeared in all three spectra and could have been due to the loss of the carboxy-ether groups along with proton transfers. Finally the peak at m/z 180 again appeared in the three spectra and was due to loss of the carboxy-ether group and a methyl group.

(b) MASS SPECTRAL ANALYSIS OF PHENYLBUTAZONE, SULPHINPYRAZONE AND AZAPROPAZONE

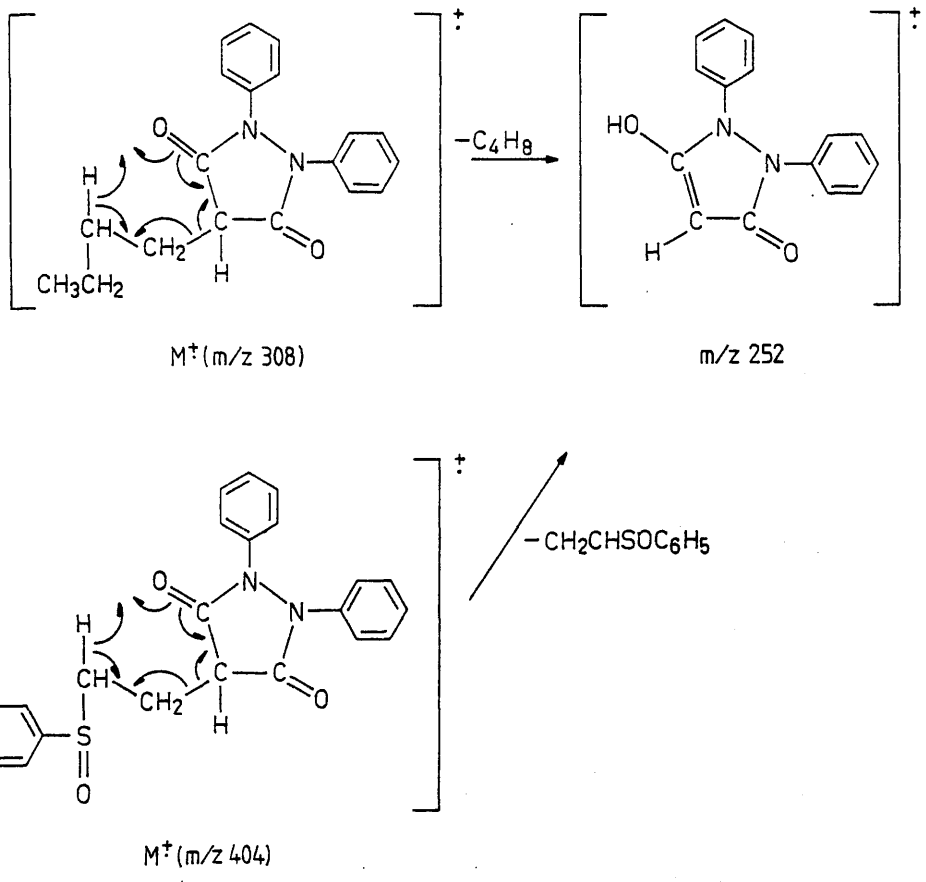
The mass spectra of some medicinal pyrazolidinediones have been studied. Yen and co-workers [114] include phenylbutazone in their analysis, while Unterhalt [115] and Locock and his colleagues [116] discuss phenylbutazone, oxyphenbutazone and sulphinpyrazone.

In the spectra obtained in this study molecular ions were observed at m/z 308, 404 and 300 for phenylbutazone, sulphinpyrazone and azapropazone respectively.

Unterhalt observed [115] that phenylbutazone, oxyphenbutazone and sulphinpyrazone undergo the McLafferty rearrangement to give a radical ion at m/z 252 (m/z 268 for oxyphenbutazone). Locock and co-workers studied the metastable ions present in all three spectra and concluded that this is a direct fragmentation from the molecular ion with the loss of the elements of butene or $C_6H_5SOCHCH_2$ in the case of sulphinpyrazone. These fragmentations are presented in Figure 2.5.21. The McLafferty rearrangement product (m/z 252) is also present in the spectra of sulphinpyrazone obtained during this study (Figure 2.5.13).

A minor fragmentation pathway for the molecular ion of phenylbutazone (also substantiated by the presence of metastable ions) is the loss of a propyl radical from the butyl side chain of the molecular ion to give an ion at m/z 265. In the spectrum of sulphinpyrazone, strong peaks at m/z 278 (100%) and at m/z 279 (127) represent the loss of C_6H_5SOH and C_6H_5SO from the side chain of the

FIGURE 2.5.21: The McLafferty rearrangement reactions of phenylbutazone and sulphinpyrazone.



sulphinpyrazone molecular ion.

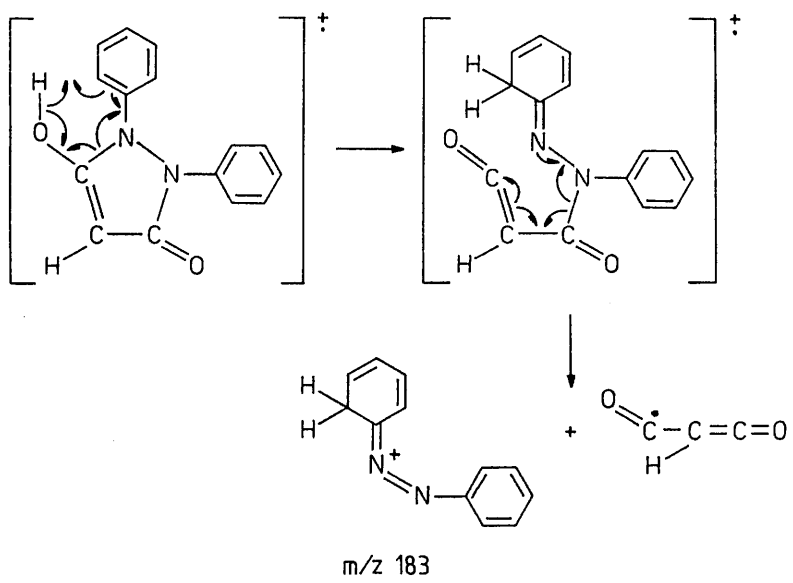
If azapropazone were to undergo a McLafferty rearrangement, an ion would be expected at m/z 258, and in fact an ion at m/z 257 is present in low abundance (1%).

Locock and co-workers have commented that the most characteristic fragments in the mass spectra of all the pyrazolidinediones which they studied were a series of peaks at m/z 182, 183 and 184 (198, 199 and 200 in oxyphenbutazone). The ions m/z 182, 183 and 184 are present in the mass spectra of phenylbutazone and sulphinpyrazone (Figure 2.5.13). The ion at m/z 183 is the base peak in the phenylbutazone spectrum. Locock and co-workers have shown that this ion arises from the radical ion (m/z 252) by hydrogen transfer as depicted in Figure 2.5.22. The peak at m/z 182 can be attributed to the formation of the azobenzene radical ion $(C_6H_5N_2C_6H_5)^+$.

Other ions present in the spectra of phenylbutazone and sulphinpyrazone, which may arise from the azobenzene radical are the result of the loss of a phenyl radical C_6H_5, to give $C_6H_5N_2^+$ (m/z 105) which subsequently may lose nitrogen to give $C_6H_5^+$ (m/z 77).

The base peak in the spectrum of

FIGURE 2.5.22: The formation of the m/z 183 ion in phenylbutazone.



azapropazone at m/z 160 and the collection of ions at m/z 188/189/190 may be formed as indicated in Figure 2.5.23. The ion at m/z 145 could be formed from the ion at m/z 160 by loss of a methyl group.

(c) MASS SPECTRAL ANALYSIS OF THE REACTION PRODUCTS OF PHENYLBUTAZONE, SULPHINPYRAZONE AND AZAPROPAZONE WITH DIAZOMETHANE

Phenylbutazone was reacted with diazomethane in methanol and the reaction products were examined by GC-MS. Four reaction products, whose spectra are indicated in Figures 2.5.15 and 2.5.16, were observed. Two products (1 and 3) have molecular ions at m/z 322 (i.e. phenylbutazone + 14) and the other two products (2 and 4) have molecular ions at m/z 336 (i.e. phenylbutazone + 28). Structures have been tentatively assigned to the four reaction products as indicated in Figure 2.5.24.

Reaction products 1 and 2 both show the loss of the butyl side chain (M-56) similar to phenylbutazone itself, while 3 and 4 have M-43 ions, corresponding to loss of a propyl group (part of the butyl side chain). The change in the fragmentation of the side chain in products 3 and 4 suggests that the reaction with diazomethane has altered the structure of the molecule at the carbon atom holding the side chain, which would be true if an enol ether

FIGURE 2.5.23: Possible cleavages of azapropazone to form the ions at m/z 160 and 189.

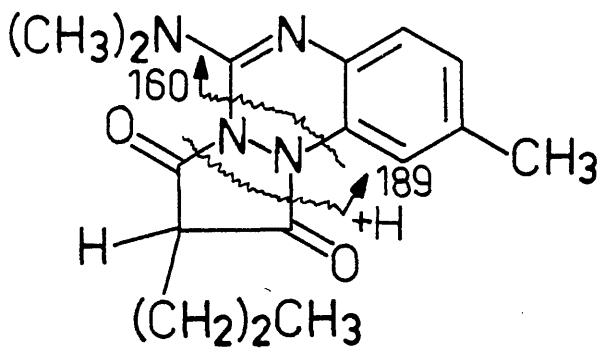
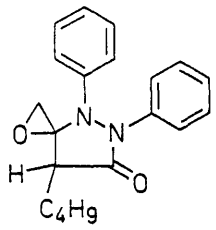
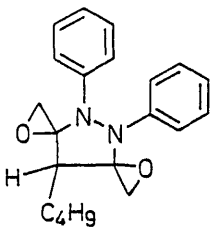


FIGURE 2.5.24: Suggested structures of the four reaction products of phenylbutazone with diazomethane.

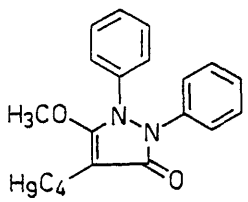
Reaction Product 1



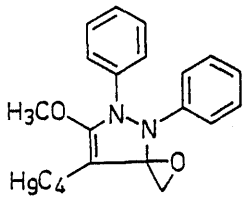
Reaction Product 2



Reaction Product 3



Reaction Product 4



had been formed. Since a vinyl bond would be more difficult to break than an alkyl bond, the loss of the butyl side chain would be less likely to occur when a vinyl bond is present. The presence of the vinyl bond would therefore explain the loss of a propyl group from the butyl side chain.

Bauer and co-workers [117] discuss the formation of oxiranes by the addition of CH_2N_2 to the carbonyl function of alpha-oxo acids when reacted with diazomethane. Bauer suggests that this occurs via a Zwitterion which is able to stabilise itself by elimination of N_2 and ring closure to form the oxirane ring as shown in Figure 2.5.25. It is possible that oxiranes could be formed when pyrazolidinediones are reacted with diazomethane to give the structures indicated in Figure 2.5.24.

The favoured reaction product is number 3. Figure 2.5.26 suggests possible fragmentations of the structure to give the m/z 188, 160 and 146 ions.

Sulphinpyrazone produced one reaction product when reacted with diazomethane in methanol. The mass spectrum of the reaction product does not contain a molecular ion, but has a base peak at m/z 292. By comparison with the mass spectrum of sulphinpyrazone which has a base peak at m/z 278, [M-126] due to loss of the C_6H_5SOH group it

FIGURE 2.5.25: The formation of an oxirane, when an alpha-oxo acid is reacted with diazomethane.

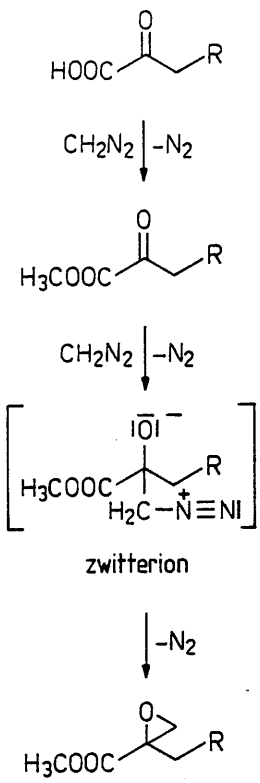
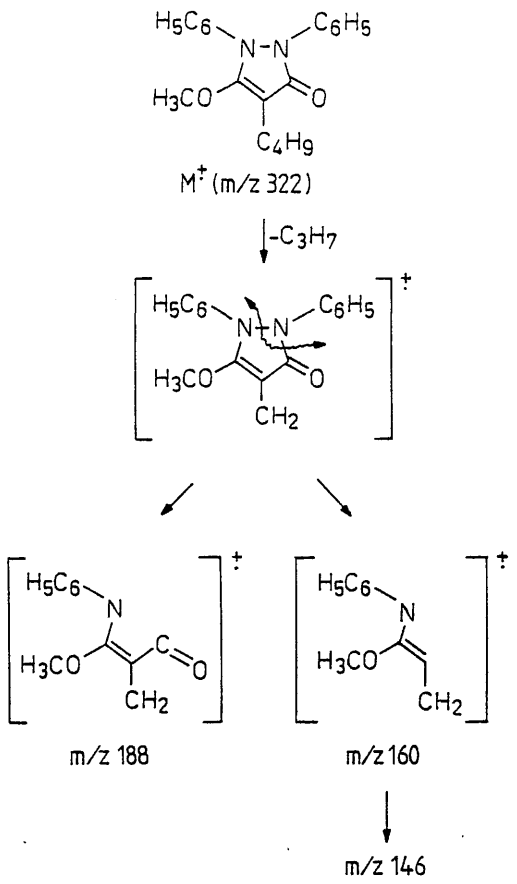


FIGURE 2.5.26: Predicted fragmentation of reaction product 3 (formed from phenylbutazone).

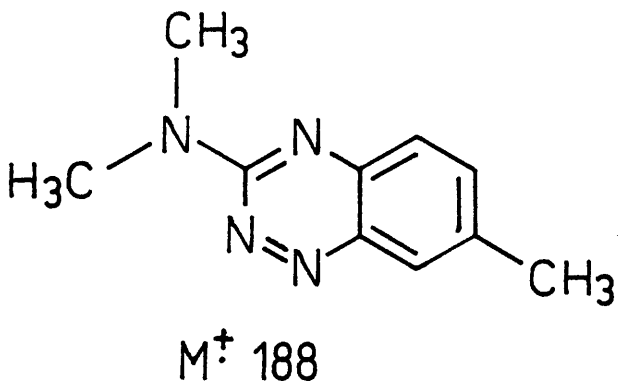


can be inferred that the molecular weight of the reaction products is 418. The reaction product is therefore a mono-methyl derivative of sulphinpyrazone. It is, however, difficult to predict its structure since there are very few diagnostic ions in the spectrum.

Azapropazone was also reacted with diazomethane in methanol; the chromatogram obtained from the reaction mixture (Figure 2.5.14) contained five major peaks. The early eluting, broad peak may be due to a pyrolysis product whose structure is given in Figure 2.5.27.

The other four peaks all have molecular ions at m/z 314 in their mass spectra suggesting that there are four different reaction products which all have one extra methyl group. These products could be similar to those suggested for phenylbutazone and tentative structures are given in Figure 2.5.28. Unlike phenylbutazone, azapropazone is not symmetrical about the pyrazolidine ring and each mono-derivative is a different geometric isomer. While it is difficult to assign structures to each product in the absence of further spectral data, the presence of (M-42) ions in products 2 and 4 suggests that these are oxirane derivatives while the presence of an (M-29) ion in products 3 and 5 suggests that these are enol ethers, following the same argument used for the products obtained from phenylbutazone.

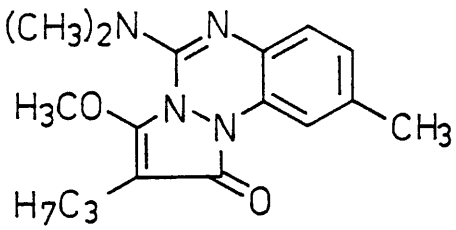
FIGURE 2.5.27: Pyrolisis product of azapropazone.

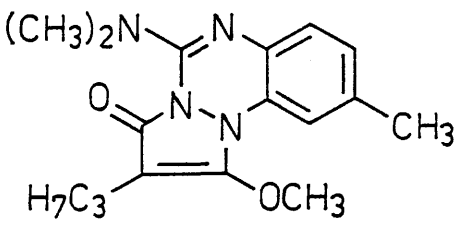


$M^{+\cdot}$ 188

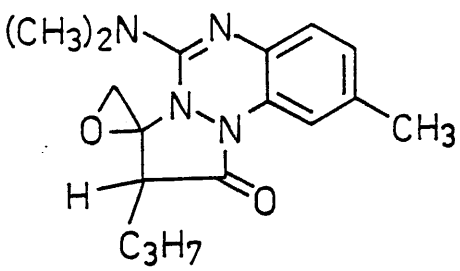
FIGURE 2.5.28: Suggested structures of the four reaction products of azapropazone with diazomethane.

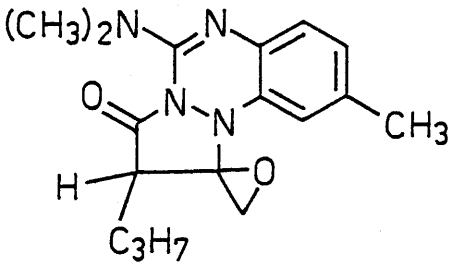
Reaction Products 3 and 5





Reaction Products 2 and 4





2.5.5 CONCLUSION

Ibuprofen, flurbirpofen, naproxen, ketoprofen, fenbufen and mefenamic acid could be uniquely identified from each other as the methyl, trimethylsilyl or tert-butyldimethylsilyl derivatives. Phenylbutazone, sulphinpyrazone and azapropazone could be analysed and identified. However, when these three drugs were reacted with diazomethane, as they could be in a screening procedure, they produced multiple reaction products which would complicate the analysis. These drugs would also be difficult to quantify if reacted with diazomethane.

Phenylbutazone, sulphinpyrazone and azapropazone did not react with the silylation reagents used in Section 2.4. If a derivatisation reaction was used in a screening procedure silylation would not affect the pyrazolidinediones and they could be identified as the free drug.

The proposed structures for the reaction products of the pyrazolidinediones with diazomethane need to be confirmed by further work. Sufficient quantities of the various products would have to be synthesised and identified using elemental analysis, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy.

Another approach which may help to identify the reaction products would be to examine the mass spectra

of the reaction products produced using [$^2H_2$]-diazomethane, although it should be noted that the fragmentations of the pyrazolidinedione reaction products are not simple reactions. Many appear to involve rearrangements and hydrogen transfers and the interpretation of deuteromethyl derivative spectra may be complex.

CHAPTER THREE

URINARY ACID PROFILE IN THE RACING GREYHOUND

3.1 INTRODUCTION

Dope testing in the racing greyhound is carried out using urine and since the anti-inflammatory drugs being examined were all acidic in nature it was essential to establish the normal urinary acid profile of the greyhound before attempting to assay the NSAIDs. The human urinary acid profile is already well documented [Ref. 118 and the references cited therein] since the identification and quantification of organic acids in human urine is used as a method of diagnosis for a group of hereditary diseases known as organic acidurias. These diseases, resulting from inborn errors of metabolism, are caused by the diminished activity or complete absence of a specific enzyme or enzyme co-factor which leads to the accumulation and increased excretion of acidic metabolites. Today some sixty different organic acidurias are known, involving a total of more than two hundred different acidic metabolites [119]. Phenylketonuria and maple syrup urine disease are two examples of organic acidurias occurring as inborn errors of metabolism [120]. If left untreated both of these diseases result in mental retardation. Phenylketonuria is caused by a deficiency in the activity of phenylalanine-4-hydroxylase. Since the normal conversion of phenylalanine to tyrosine is prevented,

the alternative pathway to phenylpyruvic acid and its metabolites is utilised and they are excreted in abnormally high concentrations in the urine (Figure 3.1.1). Maple syrup urine disease is revealed by high concentrations of 2-oxocarboxylic acids in the urine caused by a defect in the metabolism of the amino acids valine, leucine and isoleucine (Figure 3.1.2).

Urinary organic acids are the water-soluble end products or intermediates of the metabolism of amino acids, carbohydrates, biogenic amines, steroids, lipids and many other endogenous compounds. They may also be derived from exogenous sources such as food additives and drugs. They are a complex mixture of polar and moderately polar hydroxy-, keto-, mono-, di- and tri-carboxylic acids, aromatic and heterocyclic compounds as well as glycine and other conjugates which differ widely in their chemical properties and concentration in normal urine. Liebich has summarised the acids which have been identified in human urine and has classified them into seven major groups [120]. Table 3.1.1 lists the seven groups and gives some examples of urinary acids.

GC-MS has been the dominant technique used in the field of urinary organic acid analysis [121-124], though some workers have used GC alone and utilised retention indices from two columns of different polarities to establish the identity of the acids [119,125,126]. Lefevere and co-workers, however, report that it is impossible to make an unambiguous identification based on

FIGURE 3.1.1: Metabolism of phenylalanine in man, showing the normal pathway to acetoacetic acid and the alternative pathway to phenylpyruvic acid and its metabolites.

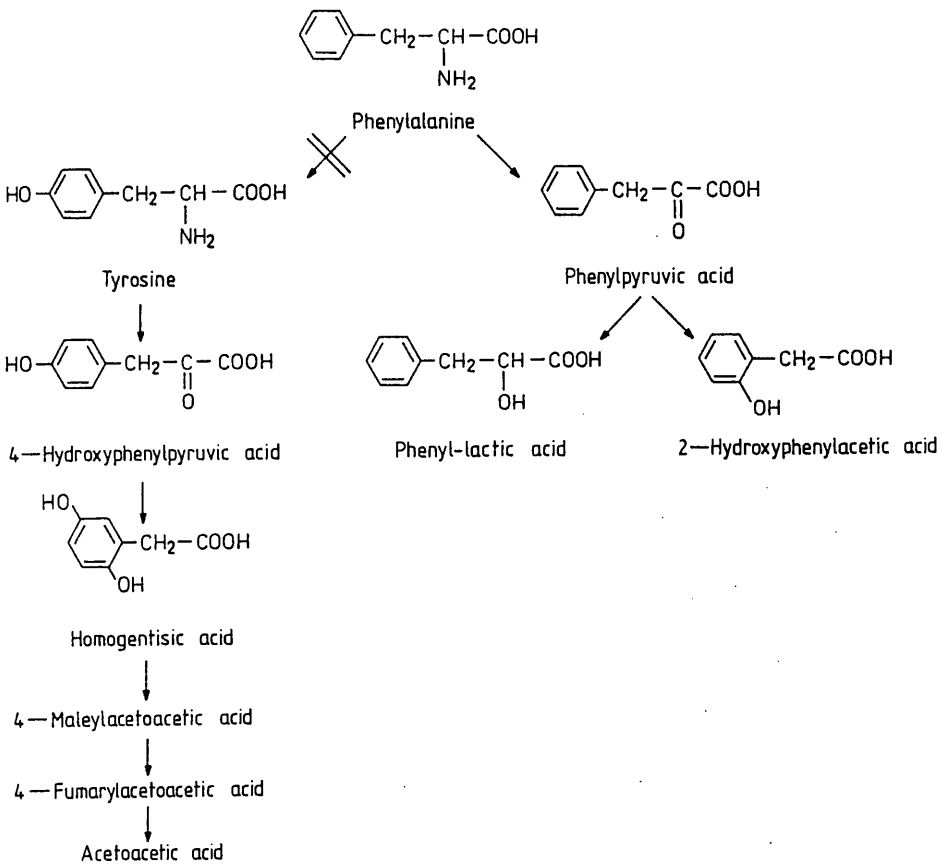


FIGURE 3.1.2: Metabolism of valine, leucine and isoleucine in man showing the normal pathways and the alternative pathways to the 2-oxocarboxylic acids observed in patients with Maple Syrup Urine disease.

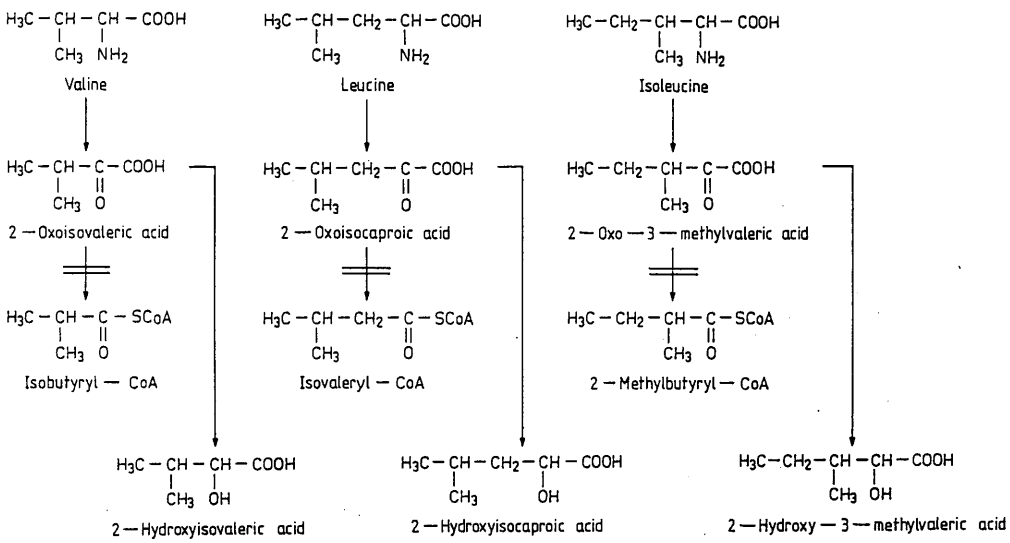


TABLE 3.1.1: Organic acids found in urine and serum.

DICARBOXYLIC ACIDS
Oxalic acid
Methylmalonic acid
Succinic acid
Glutaric acid
Adipic acid
Pimelic
OXOCARBOXYLIC ACIDS
Glyoxylic acid
Pyruvic acid
2-Oxobutyric acid
4-Oxovaleric acid
2-Oxoglutaric
2-Oxoadipic acid
HYDROXYCARBOXYLIC ACIDS AND LACTONES
Hydroxyacetic acid
Lactic acid
3-Hydroxyvaleric acid
Malic acid
D-Glucono-1,5-Lactone
Tartaric Acid
AROMATIC ACIDS AND PHENOLS
Phenol
Benzoic Acid
Vanillic acid
Phenylacetic acid
Mandelic acid
Phenyllactic acid
FURANCARBOXYLIC ACIDS
5-Methylfuran carboxylic acid
Furan-2,5-dicarboxylic acid
5-Hydroxymethyl furan carboxylic acid
3-Carboxy-4-methyl-5-(4-oxo-pentyl)-2-furanpropionic acid
NITROGEN-CONTAINING ACIDS
N-Methylleucine
Pyroglutamic acid
Nicotinic acid
3-Indoleacetic acid
Anthranilic acid
3-Indolelactic acid
ACID CONJUGATES
Propionylglycine
Isovalerylglycine
Hippuric acid
3-Hydroxyhippuric acid
N-Isovalerylglutamic acid
N-Phenylacetylglutamic acid

retention indices alone and that a final confirmation by GC-MS is necessary [119].

There have been some reports on the applications of HPLC to the study of organic acids in particular disease states [127,128], but Chalmers and Lawson [118] report that these methods have no particular advantage over the conventional GC procedure.

3.1.1 ISOLATION OF URINARY ORGANIC ACIDS

Various methods have been used to isolate organic acids from urine and these have been reviewed by several workers [120,122,123]. Solvent extraction and the use of anion-exchange procedures (an example of solid phase extraction) have been the most popular. Solvent extraction of acidified salt-saturated urine with ethyl or methyl acetate and/or diethyl ether has been preferred for routine clinical use [125,126,129,130]. These procedures, however, are not quantitative and polar compounds, such as di- and tricarboxylic acids and polyhydroxy acids are poorly extracted [131]. Solvent extraction also co-extracts interfering neutral compounds, such as urea and glycerol.

Anion-exchange using, for instance, DEAE-Sephadex has also been used by many workers [131,132,133]. This method is more quantitative than solvent extraction, gives better reproducibiltiy and eliminates interfering neutral compounds. Anion-exchange, however, requires the time-consuming lyophilisation of the aqueous pyridinium acetate solution which is used as the eluent. Large

amounts of sulphate and phosphate are also eluted in anion-exhange and these interfere with subsequent gas chromatography.

Most of the current applications of solid phase extraction use chemically modified silica and alumina which resemble chemically bonded phases for HPLC in their mode of action (or retention mechanism). Sorbents of different types are available to emulate straight phase, reversed phase, ion-exchange and affinity chromatography (not yet commercially available), depending on their substituents [134,135]. Appropriate choices of sorbent and solvents used in sample loading, sorbent washing and analyte elution permit solid phase extraction to be highly selective and often to provide extracts of sufficient purity for direct analysis by chromatographic methods following suitable derivatisation if necessary. Dalsh and Leonard reported [136] a solid phase extraction method for the extraction of organic acids from plasma. Recently Rumsby and co-workers reported [137] the use of a similar solid phase extraction method for the extraction of organic acids from various biological fluids including urine.

Another example of a solid phase extraction technique is the use of cross-linked polystyrene/divinyl benzene (XAD-2) resin. Its application is well recognised in analytical toxicology and it has been used in the isolation of drugs from various body fluids including urine [138-147]. These resins have also been utilised to

extract urinary organic acids [124,148].

For urine analysis the resin can be introduced directly into the urine or the sample may be passed through a column of the resin. Dieterle and co-workers [149] have also used micronised XAD-2 resin to form the support for preparative high-resolution liquid chromatography. This method was used to isolate the urinary glucuronides of sulphinpyrazone and also achieved a separation of a standard mixture of phenylbutazone and four of its metabolites.

In this project Amberlite XAD-2 resin was chosen as the solid phase for the extraction of the NSAIDs, organic acids and their metabolites from urine. Amberlite XAD-2 retains organic substances by virtue of hydrophobic/ lipophilic interactions on the resin surface. Aromatic substances are particularly well retained. Elution of adsorbed species requires the use of an organic solvent to break the lipophilic interactions, usually ethanol or methanol. Unlike other adsorbents based on silica or alumina there are no secondary ion exchange interactions present.

The use of XAD-2 resin for the isolation of drugs and metabolites from urine has several advantages over conventional solvent-solvent extraction; XAD-2 resin yields extracts which are free from emulsions and eluates do not require to be dried prior to evaporation. Eluates from XAD-2 resins are also free from inorganic phosphates and sulphates which could interfere with any subsequent enzyme hyrdrolysis step, for example, using glucuronidase.

3.1.2 DERIVATISATION OF URINARY ORGANIC ACIDS

Urinary organic acids are frequently highly polar, have low volatilities and are thermally unstable; they are therefore not suitable for direct analysis by GC and are normally derivatised first. The relative merits of various derivatisation procedures have been reviewed [118,122,123] and those commonly consist of the formation of esters of the carboxyl functions and ethers of free hydroxyl groups. Trimethylsilylation with BSTFA or BSA [119,124,125,131-133] and methylation with diazomethane [130,150-153] are the most commonly followed derivatisation procedures and are discussed briefly below. Keto-acids may require additional modification, normally by preparing an oxime derivative, to avoid formation of isomeric enol ethers and subsequent complications in the analysis.

Trimethylsilyl (TMS) derivatives are easy to prepare and most of them have good chromatographic properties. In addition, useful diagnostic ions are frequently obtained in the mass spectra of these derivatives. However, they are not stable during storage and the mass fragmentation pattern in some instances can be difficult to interpret especially in the case of unidentified substances. TMS reagents form derivatives with carboxyl groups, hydroxyl groups and phenolic groups of organic acids. Keto-acids have a tendency to yield multiple derivatives (enol-trimethylsilyl ethers) with silylation and are more easily determined if double

derivatives, such as oxime-TMS [154], methoxime-TMS [155] and ethoxime-TMS [156] are prepared.

Methyl esters of organic acids can also be readily prepared and have excellent chromatographic properties. They are not prone to hydrolysis like the corresponding TMS derivatives. The mass spectra of methyl esters are often easier to interpret and display ions which are more characteristic of the parent substance than those of the TMS derivatives. Diazomethane reacts with carboxyl groups, phenolic hydroxyls and the sulphydryl group of thiols. Diazomethane, though, has been found to produce a mixture of products when reacted with alpha-, beta-unsaturated acids and alpha-keto acids [157] and this complicates the interpretation of the spectra.

In this study four different derivatisation procedures were evaluated for the analysis of urinary acids in the racing greyhound by GC-MS. Having established the normal urinary composition and the derivatisation procedure which gave the simplest chromatographic profile, it would then be possible to establish screening procedures for NSAIDs and their metabolites in urine.

3.2 EXPERIMENTAL

3.2.1 REAGENTS

1. Amberlite XAD-2 resin, 80-100 mesh (BDH Chemicals Limited, Poole, England).
2. Acetone, reagent grade (BDH Chemicals Limited, Poole, England).
3. Ethanol, glass distilled (J. Burrough, London).
4. Methanol, HPLC grade (Rathburn Chemicals Limited, Walkerburn, Scotland).
5. Hydrochloric acid (BDH Chemicals Limited, Poole, England).
6. Ethyl acetate (Rathburn Chemicals Limited, Walkerburn, Scotland).
7. Diethyl ether, Pronalys AR grade (May and Baker, Dagenham, England).
8. Digol, Analar grade ((BDH Chemicals Limited, Poole, England).
9. Diazald, N-methyl-N-nitroso-p-toluenesulfonamide (Aldrich Chemical Company Limited, Gillingham, England).
10. Potassium hydroxide, Analar grade (BDH Chemicals Limited, Poole, England).
11. BSTFA + 1% TMCS (Pierce, Life Sciences Laboratory Limited, Luton, England).

3.2.2 ORGANIC ACID STANDARDS

Organic acids, known from previous work to occur in human urine, were obtained from BDH Chemicals Limited, Poole, England.

1.	o-Anisic	2.	cis-Aconitic
3.	Adipic	4.	Ascorbic
5.	Azelaic	6.	Benzoic
7.	iso-Butyric	8.	n-Butyric
9.	iso-Citric (tri-sodium salt)	10.	Fumaric
11.	2-Furoic	12.	Glucuronic
13.	Glutaric.	14.	Glycollic
15.	n-Hexanoic.	16.	Hippuric
17.	3-Hydroxybenzoic.	18.	4-Hydroxybenzoic
19.	Indole-3-yl-acetic.	20.	Lactic
21.	DL-Malic.	22.	Oxalic
23.	Pimelic.	24.	Succinic
25.	Tartaric.	26.	Vanillic
27.	iso-Valeric		

Salts of acids were treated with hydrochloric acid (1M) and the free acid was extracted into an organic solvent. The organic layer was removed and evaporated to dryness before derivatisation as described in Section (c).

3.2.3 APPARATUS

1. Perkin-Elmer Sigma 3B gas chromatograph fitted with a Groß split/splitless injector interfaced via a direct inlet to a VG 16F single-focussing magnetic sector mass spectrometer and a VG series 2000 data system. Electron impact mass spectra were recorded repetitively (cycle time 2.5 seconds).

2. A wall coated open tubular column (24m, 0.5mm i.d., 1.1mm o.d.) with a liquid stationary phase of CP-SIL5 at a film thickness of 0.81µm.

3. A Bucchi rotary evaporator.

3.2.4 GC AND GC-MS CONDITIONS

Gas chromatography and gas chromatography-mass spectrometry were carried out on systems similar to those described in Sections 2.4.2 and 2.5.2, respectively.

3.2.5 PREPARATION OF DERIVATIVES OF STANDARD ACIDS

(a) METHYLATION WITH DIAZOMETHANE

An ethereal solution of diazomethane (see Section 2.4.2(e)) was added dropwise to methanol (1ml) containing about 1mg of acid standard until the solution remained yellow. The solution was allowed to stand at room temperature for 15 minutes before evaporating to dryness under a stream of nitrogen. The residue was redissolved in 5ml of ethyl acetate and about 0.5µl was used for analysis.

(b) SILYLATION WITH BSTFA + 1% TMCS

BSTFA + 1% TMCS (100µl) and pyridine (20µl) were added to a vial containing about 1mg of acid standard. The vial was sealed and the reaction mixture heated at 60°C for 20 minutes. After this time hexane (5ml) was added to the reaction vial and about 0.5µl was used for analysis.

(c) tert-BUTYLDIMETHYLSILYLATION WITH MTBSTFA

MTBSTFA (100µl) and acetonitrile (100µl) were added to a vial containing about 50µg of acid standard. The vial was sealed and the reaction mixture heated at 60°C for 30 minutes. After cooling about 0.5µl was used for analysis.

3.2.6 COLLECTION OF URINE SAMPLES

A metabolic unit was set up in order to carry out this study, to administer therapeutic doses of drugs to

greyhounds and to collect urine samples in a controlled manner. The study was licenced by the Home Office and the metabolic unit was situated at the University of Glasgow Veterinary School. The upkeep of the unit was funded by the National Greyhound Racing Club of Great Britain.

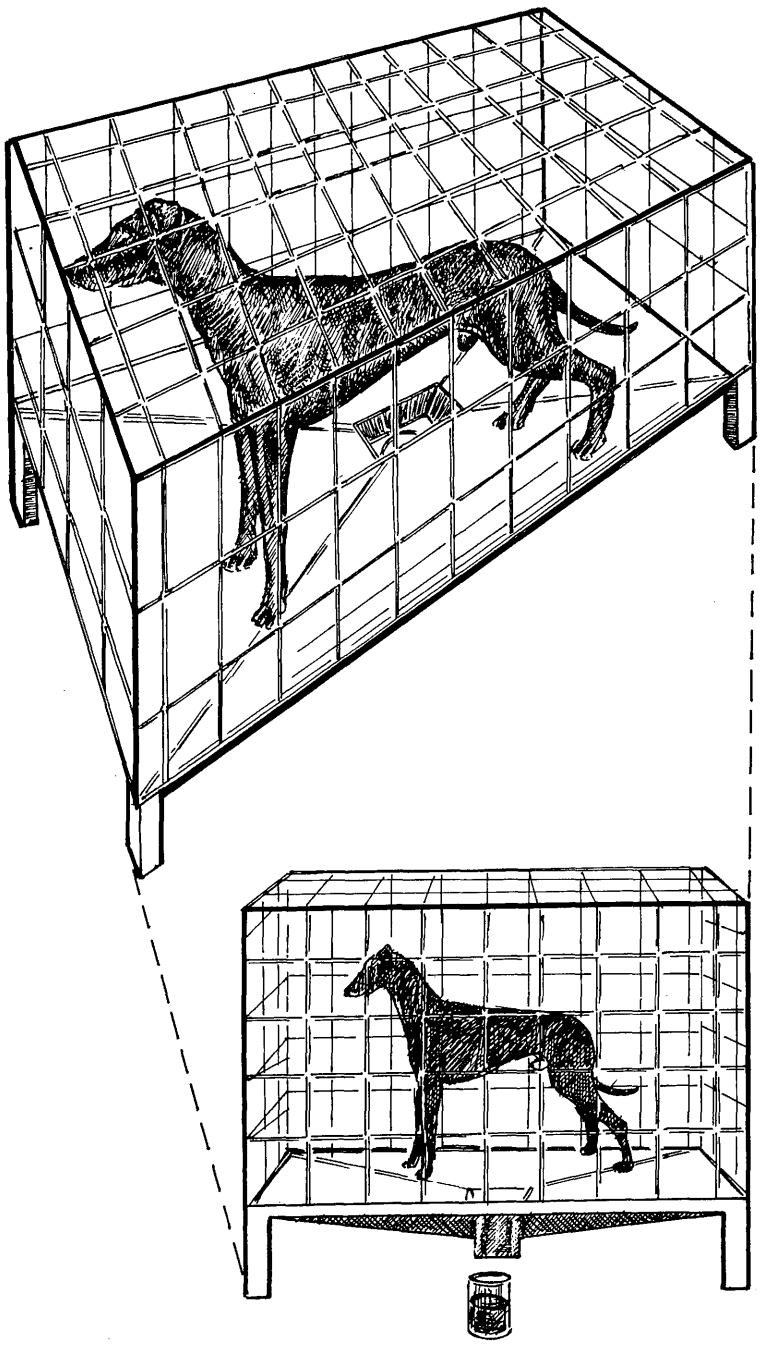
Four greyhounds (3 male and 1 female) were housed at the unit. The animals were cared for and blood and urine samples were taken by kennel nurses. The greyhounds were fed a normal diet of tinned dog food and biscuits.

Urine samples were collected by two procedures, either in a metabolic cage or by using a collection vessel during normal urination. The metabolic cage was about 2 x 2 x 1 metres in size and consisted of stainless steel supports covered in wire mesh. The floor of the cage, which was raised about 20cm off the ground, was also made of wire mesh (Figure 3.2.1). Under the flooring was a funnel made of stainless steel which collected urine passed by the animal in the cage. Urine samples were collected in a stainless steel jug, then transferred to polypropylene containers for carriage. Samples were stored at -20°C until analysed.

The second method of urine collection, outside the cage, was much simpler and involved the animal nurse collecting urine in a stainless steel jug while the animal was urinating.

When an animal had been administered a drug and the required number of urine samples had been taken, a washout period of about three weeks was allowed before dosing again.

FIGURE 3.2.1: Illustration of a metabolic cage.



COLLECTION OF BLANK URINE SAMPLES

Normal 24 hour urine samples were collected from the four greyhounds which were medication-free and were kept in individual metabolic cages for 24 hours. Twenty-four hour urine collections were analysed for individual dogs or as a pooled urine sample. Urine samples were collected separately from male and female dogs in the morning and evening on three successive days to observe variations.

3.2.7 PREPARATION OF AMBERLITE XAD-2 RESIN COLUMN

Amberlite XAD-2 resin (ca 20g) was slurry packed in water into a glass column (bed volume 30ml) and washed several times with acetone (total volume approximately 500ml) to remove any styrene present. The column was then washed with distilled water (approximately 200ml), ethanol (approximately 200ml) and distilled water (approximately 200ml) before use.

3.2.8 ISOLATION OF URINARY ORGANIC ACIDS

An aliquot of urine (20ml) was adjusted to pH 1 with 1M hydrochloric acid and passed through a freshly prepared column of XAD-2 resin (20g, bed volume 30ml) at a flow rate of 4ml/minute. The column was allowed to drain and was washed with distilled water (200ml) at the same flow rate. The column was allowed to drain once more and organic material retained on the resin was eluted with ethanol (100ml) at a flow rate of 2ml/minute. The extract was evaporated to near dryness on a rotary evaporator. The residue was then transferred quantitatively to a vial

and evaporated to dryness under nitrogen.

3.2.9 PREPARATION OF DERIVATIVES OF URINARY ACID EXTRACTS

(a) METHYLATION WITH DIAZOMETHANE

Urine extracts were dissolved in methanol (500µl) and treated with a freshly prepared ethereal solution of diazomethane (500µl). The solution was left at room temperature for 15 minutes. After this time the solution was evaporated to dryness under a stream of oxygen-free nitrogen. The sample was then redissolved in methanol (250µl) before analysis.

(b) SILYLATION WITH BSTFA + 1% TMCS

Dried extracts of urine were treated with BSTFA + 1% TMCS (200µl) and dry pyridine (50µl) in a vial. The vial was sealed and heated at 60°C for 1 hour. This solution was then injected directly into the GC.

(c) METHYLATION/SILYLATION

Dried extracts of urine were dissolved in methanol (500µl) and treated with an ethereal solution of diazomethane (500µl). The solution was left at room temperature for 15 minutes then evaporated to dryness under a stream of oxygen-free nitrogen. The sample was then treated with BSTFA + 1% TMCS (200µl) and dry pyridine (50µl). The vial was sealed and heated at 60°C for 1 hour. The solution was then injected directly into the GC.

(d) tert-BUTYL-DIMETHYLSILYLATION WITH MTBSTFA

Dried extracts of urine were treated with MTBSTFA (200µl) and acetonitrile (50µl) in a vial. The

vial was sealed and the reaction mixture heated at 60°C for 10 minutes. This solution was then injected directly into the GC.

3.2.10 IDENTIFICATION OF ORGANIC ACIDS IN URINE EXTRACTS

Organic acids in urine extracts were identified by comparison of their retention indices and mass spectral data with those of authentic acid standards run under the same conditions. When standards were not available identification was on the basis of the mass spectrum alone or from published gas chromatographic retention time and mass spectral data [118,152,158,159].

3.3 RESULTS

3.3.1 URINARY ACID STANDARDS

The retention indices and mass spectral data obtained after methylation, silylation and tert-butyldimethylsilylation of several standard acids, which are known to occur in human urine, are given in Tables 3.3.1, 3.3.2 and 3.3.3, respectively. Seventeen of the acids were successfully methylated and twenty-three successfully trimethylsilylated. A selected group of fourteen acids was also converted to tert-butyldimethylsilyl ethers (Figure 3.3.1, p225).

3.3.2 NORMAL URINARY ACID PROFILE OF THE RACING GREYHOUND

The average normal urinary acid profile of the racing greyhound was determined by GC analysis of a methylated extract of a pooled urine sample (Figure 3.3.2, p226). The chromatogram revealed a complex pattern of about seventy components present in varying quantities.

Samples of urine taken from two animals (1 male and 1 female) at different times of day and on different days were similarly analysed and showed variations between animals, according to the time of day and from day to day. An overall pattern, however, could be recognised as seen in the various chromatograms illustrated in Figures 3.3.3 to 3.3.5, pp227-230.

3.3.3 COMPARISON OF DERIVATIVES AND IDENTIFICATION OF GREYHOUND URINARY ACIDS

A pooled urine sample was extracted by the method described in Section 3.2.8. The final extract was

divided into four aliquots and was derivatised by four methods; methylation, trimethylsilylation, combined methylation/trimethylsilylation and tert-butyldimethylsilylation. The chromatograms obtained are compared in Figures 3.3.6 to 3.3.9, pp231-234.

The gas chromatographic and mass spectral data obtained for the four derivatives examined are listed in Tables 3.3.4 to 3.3.7. The peak numbers given in these tables correspond, as indicated, to the peak numbers in Figures 3.3.6 to 3.3.9. Compounds have been indentified by comparison with an authentic standard where available and the remaining peaks have been tentatively identified on the basis of their mass spectra or from published gas chromatographic retention times and mass spectral data [118,152,158,159].

A summary of the acids identified in the methylated and silylated extracts of greyhound urine is given in Table 3.3.8. Compounds which have been identified by comparison with an authentic standard are marked with *. This table groups acids of similar types together, and illustrates the structures of the acids identified.

TABLE 3.3.1: Gas chromatographic and mass spectrometric data for the methylated derivatives of standard organic acids known to be present in normal human urine.

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS														
		M^+	Intensity	OTHER IONS (% ABUNDANCE BELOW)												
cis-Aconitic Acid Methyl Ester	ND															
Adipic Acid di-Methyl Ester	1202	174	ND	59	55	114	111	101	74	43	143	73	41	83	42	
				100	72	58	53	53	40	34	31	27	27	19	18	
Ascorbic Acid Methyl Ester	ND															
Azelaic Acid di-Methyl Ester	1516	216	ND	55	74	152	83	59	43	111	41	69	143	185	87	
				100	92	68	66	55	55	52	49	41	38	36	34	
Benzoic Acid Methyl Ester	1066	136	52.2	105	77	136	51	106	50	78	137	76	74	135	92	
				100	78	52	24	9	6	3	2	2	2	1	1	
iso-Butyric Acid Methyl Ester	ND															
n-Butyric Acid Methyl Ester	ND															
iso-Citric Acid tri-Methyl Ester	1414	234	ND	143	101	59	43	69	42	57	29	175	144	31	153	
				100	83	37	28	10	9	8	8	5	4	4	3	
Fumaric Acid Methyl Ester	ND															
2-Furoic Acid Methyl Ester	955	126	82.3	95	126	39	96	38	127	68	67	37	29	97	8	
				100	83	28	21	7	3	3	3	2	2	1	1	

TABLE 3.3.1: Continuation (a)

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS													
		$M^{+\cdot}$	Intensity	OTHER IONS (% ABUNDANCE BELOW)											
Glucuronic Acid Methyl Ester	ND														
Glutaric Acid di-Methyl Ester	1104	160	ND	59	100	129	101	55	128	87	42	41	97	74	58
				100	77	50	31	30	15	11	10	9	4	3	3
Glycollic Acid Methyl Ester	ND														
n-Hexanoic Acid Methyl Ester	917	130	ND	74	87	43	59	41	29	27	99	55	42	39	71
				100	63	62	56	51	44	39	35	35	30	23	21
Hippuric Acid Methyl Ester	1602	193	3.2	105	77	134	51	193	137	106	50				
				100	40	17	7	3	3	3	1				
3-Hydroxy-Benzoic Acid Methyl Ester	1403	152	35.4	121	152	93	65	39	122	153	63	64	53	92	66
				100	35	26	13	9	5	4	4	3	3	2	2
4-Hydroxy-Benzoic Acid Methyl Ester	1431	152	33.5	121	152	95	65	39	122	63	153	92	64	53	38
				100	33	13	9	6	4	2	1	1	1	1	1
Indole-3-Acetic Acid Methyl Ester	1745	189	81.3	130	189	131	77	103	129	102	65	190	51	128	76
				100	81	35	27	20	17	11	9	7	7	5	3
Lactic Acid Methyl Ester	<900	104	ND	43	29	61	45	70	27	42	73	88	44	26	60
				100	54	53	50	27	24	17	10	8	6	2	1
DL-Malic Acid Methyl Ester	ND														

TABLE 3.3.1: Continuation (b)

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS													
		$M^{+\cdot}$	Intensity	OTHER IONS (% ABUNDANCE BELOW)											
Oxalic Acid	<900	118	8.3	59	29	60	31	45	44	30	118	56	46	41	33
Methyl Ester				100	47	20	19	18	10	10	9	5	2	1	1
Pimelic Acid	1311	188	ND	115	74	55	59	69	43	83	41	125	73	157	128
di-Methyl Ester				100	97	89	71	68	57	46	46	39	39	36	34
Succinic Acid	990	146	ND	115	55	59	114	87	45	27	116	56	31	29	57
di-Methyl Ester				100	40	26	14	5	3	3	2	2	2	2	1
Tartaric Acid	1179	178	ND	90	33	29	31	119	59	91	73	60	101	89	58
di-Methyl Ester				100	61	24	23	19	19	8	7	7	6	6	6
Vanillic Acid	1446	182	43.2	151	182	123	152	108	52	183	167	67	51	136	65
Methyl Ester				100	43	10	5	5	4	4	4	4	4	3	3
iso-Valeric Acid	ND														
Methyl Ester															

ND = not detected

TABLE 3.3.2: Gas chromatographic and mass spectrometric data for the silylated derivatives of standard organic acids known to be present in human urine.

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS													
		M^+	Intensity	OTHER IONS (% ABUNDANCE BELOW)											
cis-Aconitic Acid tri-TMS	1747	390	ND	29	73	147	75	375	148	285	149	43	229	133	116
				100	54	28	20	11	8	7	7	7	6	6	6
Adipic Acid di-TMS	1513	290	0.1	73	111	75	147	55	275	141	45	83	172	159	117
				100	60	57	35	35	13	13	10	9	7	6	6
Ascorbic Acid tetra-TMS	1952	464	ND	73	332	147	205	75	161	333	334	74	345	374	359
				100	64	62	36	33	30	20	10	9	8	7	7
Azelaic Acid di-TMS	1804	332	ND	73	75	55	317	201	147	117	129	152	149	45	204
				100	67	34	26	14	12	12	10	8	6	6	5
Benzoic Acid-TMS	1231	194	2.7	179	105	77	135	180	194	51	181	136	106	78	193
				100	43	19	16	7	3	3	2	2	2	2	1
iso-Butyric Acid-TMS	ND														
n-Butyric Acid-TMS	ND														
iso-Citric Acid tetra-TMS	1839	480	ND	73	273	147	75	274	375	74	45	363	347	148	149
				100	83	82	28	21	18	18	17	12	11	10	9
Fumaric Acid di-TMS	1345	260	ND	245	73	147	246	75	247	179	143	194	151	45	180
				100	23	14	11	9	5	5	5	3	3	3	2
2-Furoic Acid-TMS	1114	184	4.2	125	169	95	184	126	39	170	127	97	81	75	73
				100	38	12	4	4	4	3	2	1	1	1	1

TABLE 3.3.2: Continuation (a)

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS												
		M^+	Intensity	OTHER IONS (% ABUNDANCE BELOW)										
Glucuronic Acid-TMS-5 (Peak 1)	2017	554	ND	73	204	217	147	191	205	218	305	292	75	206	74
				100	82	78	28	16	11	10	9	8	7	5	5
Glucuronic Acid-TMS-5 (Peak 2)	2074	554	ND	73	204	217	147	191	75	74	205	218	292	305	143
				100	64	62	36	18	16	11	10	9	6	5	5
Glutaric Acid di-TMS	1396	276	ND	147	73	261	75	55	57	158	43	148	204	71	149
				100	69	45	31	28	19	17	16	13	10	10	10
Glycollic Acid di-TMS	1083	220	ND	73	147	66	45	148	74	59	43	205	177	149	133
				100	47	10	9	5	5	3	3	2	2	2	2
n-Hexanoic Acid-TMS	1062	188	0.4	173	75	73	117	174	132	131	129	74	145	175	145
				100	96	96	23	7	7	6	4	4	3	2	2
Hippuric Acid-TMS	1800	251	ND	105	206	73	77	207	75	236	51	45	106	74	208
				100	79	71	50	22	17	10	9	7	6	6	3
3-Hydroxy-Benzoic Acid di-TMS	1557	282	33.6	267	193	73	282	223	268	75	40	195	135	283	91
				100	44	44	34	30	21	18	12	11	11	9	6
4-Hydroxy-Benzoic Acid di-TMS	1620	282	18.7	267	193	223	73	268	282	269	224	194	126	195	283
				100	45	35	30	20	19	7	7	7	7	5	4
Indole-3-Acetic Acid-TMS	1917	319	18.1	202	73	203	319	130	75	304	204	129	320	321	
				100	28	19	18	16	12	6	6	6	5	4	
Lactic Acid di-TMS	1060	234	ND	73	117	147	75	148	66	191	190	173	118	45	219
				100	22	20	8	3	3	2	2	2	2	2	1
DL-Malic Acid tri-TMS	1499	350	1.8	73	147	233	75	245	190	74	189	149	148	133	101
				100	52	26	24	10	11	10	9	9	9	9	9

TABLE 3.3.2: Continuation (b)

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS													
		$M^{+\cdot}$	Intensity	OTHER IONS (% ABUNDANCE BELOW)											
Oxalic Acid di-TMS	1130	234	ND	73	147	45	148	74	72	66	75	196	149	59	43
				100	96	21	11	11	8	8	7	6	5	4	4
Pimelic Acid di-TMS	1610	304	ND	73	75	147	125	155	289	55	173	69	117	97	129
				100	65	39	37	33	30	19	17	17	10	7	6
Succinic Acid di-TMS	1307	262	ND	73	147	75	148	55	45	247	149	74	56	47	129
				100	93	18	8	7	7	5	5	4	3	3	2
Tartartic Acid tetra-TMS	1656	438	ND	73	147	292	219	423	293	189	75	305	221	294	277
				100	38	34	20	9	9	7	7	6	5	4	4
iso-Valeric Acid-TMS	ND														
Vanillic Acid di-TMS	1740	312	86.5	297	312	223	267	73	253	44	282	298	193	313	126
				100	87	69	62	61	52	37	32	23	22	21	21

ND = not detected

TABLE 3.3.3: Gas chromatographic and mass spectrometric data for the tert-butyldimethylsilylated derivatives of standard organic acids known to be present in human urine.

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS													
		M^+	Intensity	OTHER IONS (% ABUNDANCE BELOW)											
Adipic Acid di-BDMS	1947	374	ND	73	317	75	111	318	147	141	74	130	129	319	59
				100	67	55	28	21	14	13	13	12	12	11	11
o-Anisic Acid di-BDMS	1778	266	ND	135	209	77	210	92	136	194	41	179	251	165	105
				100	98	31	24	18	17	8	7	5	4	4	4
Azelaic Acid di-BDMS	2379	416	ND	359	73	75	360	313	129	361	55	77	74	314	115
				100	76	61	31	10	17	11	7	6	6	5	5
Benzoic Acid-BDMS	1567	236	ND	179	105	180	135	77	181	221	106	51	136	73	78
				100	27	20	17	16	8	5	4	4	3	3	2
iso-Citric Acid tetra-BDMS	>2800	648	ND	73	459	591	147	592	460	357	75	431	74	593	461
				100	32	25	24	14	14	14	14	13	11	9	8
Glutaric Acid di-BDMS	1949	360	ND	73	303	75	147	304	74	129	305	123	116	59	133
				100	70	37	29	20	12	11	10	10	9	9	8
n-Hexanoic Acid-BDMS	1380	230	ND	173	75	73	174	131	76	175	115	117	116	77	215
				100	80	18	17	13	9	7	7	5	5	5	4
Hippuric Acid-BDMS	2179	293	ND	105	236	77	73	75	237	106	248	238	193	59	278
				100	59	31	25	20	12	10	6	5	5	5	3
3-Hydroxy-Benzoic Acid di-BDMS	2141	366	0.06	309	310	73	311	267	235	193	135	75	253	209	149
				100	34	20	13	11	10	6	4	4	3	3	3

TABLE 3.3.3: Continuation

COMPOUND NAME	Retention Index	MASS SPECTRAL CHARACTERISTICS												
		$M^{+\cdot}$	Intensity	OTHER IONS (% ABUNDANCE BELOW)										
4-Hydroxy-Benzoic Acid di-BDMS	2221	366	0.04	309	73	310	267	235	193	135	126	265	91	209	351
				100	37	30	13	13	11	9	9	7	7	6	4
Lactic Acid di-BDMS	1576	318	ND	73	147	261	189	133	75	233	148	159	149	262	74
				100	97	43	35	22	22	21	19	18	15	13	12
Oxalic Acid di-BDMS	1612	318	ND	73	147	261	74	115	59	75	148	133	149	58	262
				100	70	30	18	17	16	15	14	12	10	10	9
Pimelic Acid di-BDMS	2169	388	ND	73	331	75	332	115	125	129	74	147	333	59	137
				100	73	62	22	14	14	12	11	10	9	8	7
Succinic di-BDMS	1846	346	ND	73	289	147	75	290	74	116	59	55	148	133	291
				100	46	34	29	14	12	11	10	9	8	8	7

ND = not detected

TABLE 3.3.4: Gas chromatographic and mass spectrometric data for compounds in a methylated extract of greyhound urine.

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
1	Benzoic Acid Methyl Ester	1073	136	105 100	77 56	136 34	51 19	106 4	50 4						
2	Phenylacetic Acid Methyl Ester	1139	150	91 100	150 89	65 33	92 24	59 22	39 17	63 8	89 7	151 5	90 5	51 5	
3	Phenylpropanoic Acid Methyl Ester	1234	164	104 100	91 68	105 31	164 17	133 9	78 8	51 8	79 7	103 6	77 6	92 5	65 5
4	3-Methyl-Quinoline	1269	143	143 100	128 8	115 7	144 6	142 5	116 3	101 2	51 2				
5	2-Amino-Benzoic Acid Methyl Ester	1294	151	119 100	151 42	92 41	120 26	40 15	43 11	128 10	86 10	65 10	39 10	99 9	91 7
6	Methyl Cinnamate	1321	162	91 100	162 22	92 17	103 15	121 14	65 13	135 10	77 8	44 8	119 6	63 5	31 4
7	3- or 4-Methoxy-Phenyl-acetic Acid Methyl Ester	1371	180	121 100	40 33	180 24	77 22	29 18	107 17	106 16	105 16	29 12	51 12	91 11	122 10
8	Suberic Acid di-Methyl Ester	1385	202	74 100	69 86	40 84	55 80	179 73	97 64	138 62	41 56	83 48	59 48	171 46	87 42
9	3-Hydroxy-Phenylacetic Acid Methyl Ester	1436	166	107 100	166 19	95 17	121 12	124 7	108 7						
10	Unidentified	1439		179 100	95 78	105 54	77 39	124 31	135 21	180 16	183 9	181 1	106 1		
11	4-Hydroxy-Phenylacetic Acid Methyl Ester	1448	166	107 100	166 9	108 4	77 4	95 3							
12	N-(2-Furanylcarbonyl)-Glycine Methyl Ester	1453	183	95 100	124 21	183 4	96 3	125 1							

TABLE 3.3.4: Continuation (a)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
13 & 14	A Methoxy-Phenylacetic Acid Methyl Ester + Unidentified	1477		121 100	40 50	159 25	180 17	103 15	91 10	75 9	116 9	193 8	122 8	129 5	58 5
15	Azelaic Acid di-Methyl Ester	1485	216	55 100	74 97	152 67	43 64	137 63	83 63	59 52	111 51	41 49	69 38	143 36	135 27
16	5-(3- or 4-Methoxy-Phenyl)-4-Hydroxy-Valeric Acid Methyl Ester	1492	238	179 100	137 73	73 47	165 38	238 33	135 33	163 30	180 24	151 22	77 20	223 17	94 17
17	4-Hydroxy-3-Methoxy-Phenylacetic Acid Methyl Ester	1515	196	137 100	196 24	138 10	122 9	94 6	197 4	193 4	127 4				
18	3,4-Dimethoxy-Benzoic Acid Methyl Ester	1521	196	196 100	165 83	46 26	181 18	120 16	107 14	79 14	197 13	59 13	127 12	77 12	55 12
19	4-Hydroxy-Phenyl-Propanoic Acid Methyl Ester	1535	180	107 100	120 99	180 52	121 46	77 26	119 23	91 18	65 15	60 12	151 11	108 10	
20	2-Keto,3-Hydroxy-3(4-Methoxyphenyl)-Propanoic Acid Methyl Ester	1541	224	165 100	137 59	151 31	66 30	166 28	138 28	67 27	153 17	109 13	123 12	95 10	94 10
21	3,4-Dimethoxy-Phenyl-Acetic Acid Methyl Ester	1552	210	151 100	210 32	165 19	156 10	123 10	152 8	153 6	167 4	138 4			
22	Tributyl Carbinol	1558	200	143 100	69 44	115 40	113 24	144 14	205 13	252 10	165 10	151 10	153 9		
23	Phenylpropanoyl-Glycine Methyl Ester	1561	221	91 100	121 64	162 62	102 41	221 24	65 21	132 18	90 13	45 13	220 11	39 10	

TABLE 3.3.4: Continuation (b)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
24	A C_{11}-Hydroxy-Triene Aliphatic Acid Methyl Ester	1570	210	88 100	95 62	81 53	109 48	178 39	154 38	59 32	150 30	55 27	297 7	282 6	267 5
25	Unidentified-probably a mixture	1604		123 100	105 36	77 36	195 25	135 21	129 21	95 21	161 19	106 19	51 16	192 13	78 2
26	Hippuric Acid Methyl Ester	1616	193	105 100	77 40	134 17	51 7	193 3	137 3	106 3	50 1				
27	N-Phenylacetyl-Glycine Methyl Ester	1666	207	91 100	92 89	88 64	30 61	56 39	118 33	65 30	207 26	116 25	39 9	107 5	90 5
28	Indole-3-Acetic Acid Methyl Ester	1755	189	130 100	189 30	103 4	102 4	129 3	131 1						
29	Indole-3-Carboxylic Acid Methyl Ester	1790	175	114 100	175 36	116 15	140 15	70 14	89 10	115 8					
30	Phthalic Acid Methyl Ester	1793	194	149 100	57 30	164 13	42 13	104 12	150 10	165 9	91 9	133 7	56 7	79 6	
31	3,4-Dimethoxy-Cinnamic Acid Methyl Ester	1802	222	222 100	191 43	163 34	164 30	87 27	115 20	207 15	147 12	223 10	105 10	119 9	148 8
32	2-Carboxy-4-Methoxy-Quinoline Methyl Ester	1853	217	159 100	158 23	115 17	160 12	102 12	217 11	143 10	130 5	129 4	89 3	88 3	
33	Palmitic Acid Methyl Ester	1870	270	74 100	87 64	43 32	75 14	41 13	55 12	57 9	69 6	143 4	83 4	88 3	270 2
34	Unidentified	1876	192?	121 100	192 50	43 18	161 12	122 11	135 8	73 8	150 7	134 6			
35	Phthalate	1883		149 100	150 8	135 8	133 7	239 5	285 4						

TABLE 3.3.4: Continuation (c)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
36	4-Methoxy-Phenyl-Propanoyl-Glycine Methyl Ester	1891	237	135	235	122	237	121	148	236	224	223	177	150	107
				100	82	57	51	25	15	12	13	11	11	10	10
37	4-Methoxy-Hippuric Acid Methyl Ester	1905	223	135	77	223	92	136	107	164	121	75	64	76	55
				100	11	9	6	5	4	3	3	3	3	2	2
38	4-Methoxy-Phenylacetyl-Glycine Methyl Ester	1920	237	121	122	148	237	107	91	88	78	56	178	238	150
				100	15	10	9	3	2	2	2	2	1	1	1
39	Unidentified	1930	296?	195	135	223	149	296	130	109	107	136	265	236	207
				100	91	61	53	37	24	18	18	16	15	15	15
40	Indole-3-Lactic Acid Methyl Ester	1937	219	130	219	131	77	160	129	103	102	88	128	118	116
				100	6	6	3	2	2	2	2	2	1	1	1
41	Unidentified Nitrogen Containing Aliphatic	1949	235?	175	176	235	51	69	75	107	56	73	83	74	133
				100	47	33	31	30	28	19	19	16	15	13	13
42	4-Hydroxy-Phenylacetyl-Alanine Methyl Ester	1964	237	107	43	178	160	147	74	44	40	75	237	136	108
				100	59	52	23	23	23	20	18	15	14	13	13
43	Unidentified Nitrogen Containing Aromatic	1972	235	107	179	75	235	136	91	147	132	205	88	207	108
				100	56	34	30	28	28	22	21	19	16	15	14
44	Background	1989		69	143	40	75	107	108	44	179	41			
				100	81	80	96	39	19	19	17	17			
45	2-,3- or 4-Methoxy-Phenylacetyl-Glycine Methyl Ester	2012	237	121	107	108	122	77	89	237	193	103	91	120	
				100	48	20	13	11	7	4	4	4	4	3	
46	2-,3- or 4-Hydroxy-Phenylacetyl-Glycine Methyl Ester	2024	223	107	108	134	88	67	77	81	55	121	223	109	95
				100	32	28	9	8	7	6	6	5	4	4	5
47	Unidentified Nitrogen Containing Compound	2029	285?	75	285	286	143	73	131	129	203				
				100	76	29	15	11	10	10	7				

TABLE 3.3.4: Continuation (d)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
48	Isomer of Oleic Acid Methyl Ester	2033	296	55	41	69	74	43	83	97	87	84	57	143	98
				100	91	51	49	40	35	30	27	27	26	24	23
49	Oleic Acid Methyl Ester	2040	296	55	43	69	41	74	83	87	84	97	222	264	296
				100	48	43	40	32	24	17	17	16	3	6	1
50	Unidentified	2056		146	221	205	58	91	118						
				100	36	20	20	15	10						
51	Stearic Acid Methyl Ester	2063	298	74	87	43	75	41	55	57	69	143	199	255	298
				100	66	35	26	14	12	10	7	4	2	3	3
52	Phenylacetyl-Cysteine Methyl Ester	2066	253	162	91	115	131	118	142	120	253	119	174	237	
				100	78	77	46	36	33	29	25	25	24	23	
53	3,4-Dimethoxy Hippuric Acid Methyl Ester	2081	253	165	253	166	77	79	254	137	122	73			
				100	14	8	5	4	3	3	3	3			
54	Either: a substituted benzoic acid; a phenyl-acetyl conjugate; or a benzimidazole type compound	2095	246?	118	91	92	113	155	112	43	246	65	55	119	
				100	67	44	23	18	14	13	10	10	7	6	
55	Quinoline-Glycine Methyl Ester	2113	244	128	185	129	156	244	212	186	102	101			
				100	59	48	21	14	8	8	8	8			
56	Unindentified	2124		217	158	189	75	89	130	131	218	174	129	159	43
				100	34	30	23	18	13	12	11	11	11	8	7
57	A C_{19} Monounsaturated Acid Methyl Ester	2145	310	55	69	41	43	74	83	70	97	56	84	87	57
				100	68	66	59	52	51	49	37	36	32	29	28
58	Heterocyclic Diacid Methyl Ester	2154	264	120	146	92	43	65	102	264	295	144	221	121	44
				100	53	25	21	19	13	12	9	9	8	7	7

TABLE 3.3.4: Continuation (e)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
59	Unidentified	2182	246?	91	79	80	67	92	41	93	118	55	155	83	106
	Similar to 54			100	74	62	58	52	49	48	45	37	35	34	31
60	Unidentified -	2200		75	73	70	130	214	221	311	61	55	235	41	292
	not an acid			100	87	74	52	48	47	44	41	38	21	21	17
61	Background	2231		313	75	314	73	129	117	131	55				
				100	80	14	13	8	8	6	5				
62	Unidentified	2268	337?	195	73	163	337	30	280	221	224	89	165	196	
				100	43	34	32	31	30	25	16	15	14	13	
63	N-Acetyl-Tryptophan	2287	260	130	201	43	131	29	77	260	170	159	103		
	Methyl Ester			100	10	6	5	4	3	2	2	2	2		
64	Unidentified	2293	323?	266	130	209	151	105	201	235	267	177	323	89	179
				100	84	51	37	32	27	25	23	23	21	21	19
65	Indole-Acetyl-Glycine	2363	246	130	246	131	77	129	103	247	102	187	157	128	65
	Methyl Ester			102	13	7	4	3	3	2	2	1	1	1	1
66	Background	2404		73	244	75	245	207	57	121	403	271	58		
				100	89	45	20	19	19	15	14	13	11		
67	Background	2426		244	73	461	245	403	75						
				100	86	11	11	10	7						
68	Background	2450		75	339	40	340	73	341						
				100	57	17	16	10	4						
69	Background	2477		75	341	73	342	129	117	207	131	343			
				100	42	13	11	7	6	5	4	4			
70	Diethylhexyl-	2507	390	149	57	71	70	167	55	113	69	150	112	83	279
	Phthalate			100	86	33	33	27	20	13	13	11	9	9	7

* Peak Number corresponds to Figure 3.3.6

TABLE 3.3.5: Gas chromatographic and mass spectrometric data for compounds in a silylated extract of greyhound urine.

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
1	Unidentified	1072		73	147	174	148	75	117	262	149	100	66	266	189
				100	89	19	13	10	6	5	4	4	4	3	3
2	Lactic Acid di-TMS or Oxalic Acid di-TMS	1079	234	73	147	117	207	66	74	148	191	190	118	88	219
				100	59	29	10	8	6	4	3	3	3	3	1
3	Lactic Acid di-TMS or Oxalic Acid di-TMS	1081	234	117	147	73	190	191	148	219	118	149	133		
				100	53	41	10	8	6	5	5	4	4		
4	Background	1089		207	295	208	133	209	178	77	296	186	179	150	194
				100	14	14	14	10	7	5	4	4	4	4	3
5	Background	1093		221	222	103	223	146	130	265	99	106	236	174	165
				100	30	24	13	11	10	9	8	7	6	6	6
6	Unidentified	1101		147	129	318	226	217	150	111	202	117	93	369	148
				100	21	16	11	11	11	11	10	10	10	9	9
7	Glycine di-TMS	1125	219	73	102	147	75	103	125	74	204	149	104	219	
				100	77	44	29	14	7	7	6	6	5	3	
8	Unidentified	1132	203?	73	152	142	167	130	188	149	174	74	153	144	193
				100	59	30	26	24	23	23	20	20	11	11	5
9	Unidentified	1135	260?	147	73	131	245	205	133	115	159	103	66	219	218
				100	58	53	33	10	10	10	9	9	9	6	6
10	Unidentified - no TMS peaks	1147		168	154	152	112	169	59	80	165	155	180	201	215
				100	90	74	49	48	41	33	18	15	7	3	2
11	4-Hydroxy-Butanoic Acid di-TMS	1161	248	73	147	75	117	233	189	68	74	103	177		
				100	98	23	13	7	5	5	5	4	3		

TABLE 3.3.5: Continuation (a)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
12	2-Hydroxy-2-Methyl-Butanoic Acid di-TMS	1169	262	73	145	147	75	146	148	74	219	133	247	149	59
				100	77	52	7	6	4	4	3	3	1	1	1
13	Unidentified	1181		75	156	159	147	157	116	100	104	160	90	187	
				100	66	19	19	8	8	8	7	6	6	5	
14	3-Hydroxy-But-2-eonic Acid di-TMS	1189	231?	73	147	75	216	188	148	144	131	231	114		
				100	69	26	12	11	7	6	6	4	4		
15	Dodecamethyl-Pentasiloxane	1195	384	281	147	73	369	282	370	371	283	148	149		
				100	90	56	21	15	13	12	9	7	3		
16	3-Hydroxy-iso-Valeric Acid di-TMS	1208	262	73	147	131	75	149	74	247	148	115	195		
				100	75	34	17	5	5	4	4	4	4		
17	Benzoic Acid TMS Ester	1228	194	179	105	147	77	135	73	180	74	194	148	106	90
				100	79	46	45	37	24	11	7	4	4	3	2
18	Caprylic Acid TMS Ester	1247	216	73	75	43	201	117	47	111	55	144	132	131	130
				100	68	41	20	19	13	10	9	7	5	4	4
19	Unidentified	1252		43	73	75	144	111	159	42	201	116	181	186	
				100	59	52	41	37	14	13	12	12	9	7	
20 - 25	Data not available														
26	Glutaric Acid di-TMS Ester	1307	176	147	73	75	261	148	217	149	55	69	45	232	143
				100	48	10	8	7	5	5	5	3	3	2	2
27	Unidentified - similar to 29	1329	260?	195	73	75	93	151	245	196	66	45	84	43	39
				100	82	14	13	12	10	8	6	6	4	4	4
28	Unidentified	1340		73	117	207	72	147	292	149	111	75	74		
				100	12	8	6	4	3	3	3	3	3		

TABLE 3.3.5: Continuation (b)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
29	Unidentified - similar to 27	1356	270?	195	73	255	151	196	93	75	62	270	197	149	66
				100	41	16	16	15	14	12	8	5	5	5	5
30	Unidentified	1371		73	75	147	117	243	100	149	148	59	55	45	74
				100	95	42	22	10	10	6	6	6	6	6	5
31	Unidentified	1376		73	75	147	55	261	43	259	184	149	148	117	158
				100	51	36	9	8	5	4	4	4	4	4	3
32	Unidentified	1381		73	393	239	254	117	40	394	219	218	171	75	
				100	10	8	6	5	5	4	4	4	4	3	
33	A Hydroxy-Heptanoic Acid di-TMS	1390	290	73	131	147	117	75	149	129	292	209	172	134	132
				100	35	21	14	10	9	9	7	6	5	5	5
34	2-Methyl-Glutaric Acid di-TMS Ester	1397	290	73	147	69	75	275	172	45	148	117	133	143	207
				100	72	58	21	11	6	5	5	4	4	4	4
35	3-Methyl-Glutaconic Acid di-TMS (Peak 1)	1410	288	73	147	82	75	198	109	273	45	183	148	55	170
				100	70	16	16	9	8	7	7	6	6	6	4
36	iso-Leucine-N-Acetyl-TMS	1421	245	73	86	128	43	75	45	230	133				
				100	75	57	36	24	10	8	8				
37	2-Hydroxy-Benzoic Acid di-TMS	1431	208?	75	193	73	43	208	117	194	151	57	89		
				100	90	48	42	21	21	14	13	13	11		
38	3-Methyl-Glutaconic Acid di-TMS (Peak 2)	1450	288	73	147	75	82	198	179	74	45	231	109	273	229
				100	40	26	12	7	6	5	5	4	4	3	3
39	Adipic Acid di-TMS Ester	1475	290	73	75	111	147	55	275	141	117	172	159	83	74
				100	53	33	23	12	9	8	8	6	5	5	4

TABLE 3.3.5: Continuation (c)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
40	Pyroglutamic	1489	273	73	131	156	75	147	37	157	231	228	69	55	40
	Acid di-TMS			100	51	43	40	18	12	10	9	9	7	7	7
41	Unidentified	1501		73	75	117	161	119	115	43	69	87	45	41	
				100	77	50	46	32	27	19	18	9	7	6	
42	Unidentified	1510	298?	73	195	147	298	103	74	196	75	45	283	208	
				100	39	21	9	6	5	4	4	4	3	3	
43	Unidentified	1515		73	147	271	254	284	169	197	123	117	45	255	272
				100	75	40	36	26	18	13	13	11	11	8	7
44	Unidentified	1524	310?	73	295	147	195	310	296	75	45	74			
				100	22	10	8	7	6	6	4	3			
45	3-Hydroxy-Phenylacetic	1536	296	73	149	147	75	45	267	253	164	74	296	177	
	Acid di-TMS			100	14	9	6	5	4	4	4	4	3	3	
46	3-Phenyl-Lactic	1558	310	73	193	147	75	45	194	74	220	148	142	219	91
	Acid di-TMS			100	82	61	18	12	9	9	7	6	6	5	3
47	Pimelic Acid	1571	304	73	147	75	125	155	55	289	69	173	129	97	45
	di-TMS Ester			100	52	52	31	26	24	18	17	14	9	9	9
48	2-Amino-Benzoic	1581	281	266	73	267	45	268	147	192	75	281	134	74	149
	Acid di-TMS			100	58	18	7	5	5	4	4	3	3	3	2
49	4-Hydroxy-Benzoic	1598	282	267	73	193	223	282	268	126	45	224	194	75	269
	Acid di-TMS			100	94	68	53	28	17	15	11	6	6	6	5
50	4-Hydroxy-Phenylacetic	1606	296	179	95	75	164	296	252	74	45	281	147	173	180
	Acid di-TMS			100	84	51	50	49	47	47	46	45	15	14	10

TABLE 3.3.5: Continuation (d)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
51	4-Hydroxy-Phenyl-propanoic Acid di-TMS	1613	310	193	73	194	295	310	192	178	75	45			
				100	54	12	10	5	5	5	4	4			
52	Lauric Acid di-TMS Ester	1620	272	73	75	117	40	257	43	55	132	57	45		
				100	44	21	17	13	13	12	9	9	5		
53	Unidentified	1625		73	175	173	75	147	205	103	117	55			
				100	35	19	16	15	8	8	5	5			
54	Suberic Acid di-TMS Ester	1666	318	73	75	55	303	187	147	83	169	117	139	129	149
				100	77	42	20	18	13	13	12	11	10	10	7
55	3-Hydroxy-Propanoic Acid di-TMS	1695	310	73	205	192	75	310	193	177	45	179	206	131	295
				100	84	84	82	37	26	18	11	10	8	7	3
56	2,5-di-Hydroxy-Benzoic Acid tri-TMS (Gentisic Acid)	1718	370	73	355	45	356	193	357	147	74	262	137	75	358
				100	55	10	9	7	5	5	5	2	2	2	1
57	cis-Aconitic Acid tri-TMS	1727	390	73	147	179	75	192	229	375	45	285	310	211	149
				100	62	47	27	18	17	9	9	6	5	5	5
58	4-Hydroxy-3-Methoxy-Phenylacetic Acid di-TMS	1736	326	73	209	326	75	311	327	267	210	74	45	21	
				100	35	26	19	12	6	6	4	4	3	3	
59	Unidentified	1753	327?	312	73	75	237	194	147	313	209	238	314	195	100
				100	87	54	37	21	21	14	10	8	6	5	4
60	3- or 4-Hydroxy-Mandelic Acid tri-TMS	1762	384	267	73	268	147	269	75	45	341	74	193	369	342
				100	63	48	19	13	9	9	6	4	3	3	3
61	Azelaic Acid di-TMS Ester	1766	332	73	75	55	317	201	147	117	129	152	217	74	45
				100	58	43	27	18	15	15	11	9	8	8	7

TABLE 3.3.5: Continuation (e)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
62	Unidentified	1771		40	265	281	34	44	280	208	193	58	223	210	168
				100	11	5	5	4	3	3	3	3	2	2	2
63	Hippuric Acid TMS Ester	1800	251	105	206	73	77	207	75	236	51	45	106	74	208
				100	79	71	50	22	17	10	9	7	6	4	3
64	N-(Phenylacetyl)-Glycine-TMS Ester	1832	265	91	73	30	221	250	118	75	92	102	265	65	174
				100	47	33	27	25	24	24	21	15	9	8	7
65	Unidentified-not a TMS derivative	1871	342?	327	342	312	253	70	343	328	270	283	223	43	313
				100	73	62	26	26	17	17	13	13	12	12	11
66	4-Hydroxy-Phenyl-Lactic Acid tri-TMS	1888	398	179	73	147	308	180	45	74	309	181	293	148	149
				100	55	33	25	25	8	6	4	4	3	3	2
67	Unidentified	1896		173	73	75	103	174	83	201	55	129	74	231	263
				100	72	18	13	9	7	6	5	4	4	3	2
68	3-(4-Hydroxy-Phenyl)-2-Propenoic Acid di-TMS	1908	308	73	75	219	293	308	147	249	216	174	309	294	220
				100	41	22	16	14	8	7	6	5	4	4	4
69	Unidentified	1925		73	103	217	147	75	307	268	196	74	195	104	218
				100	73	19	14	22	12	9	5	5	4	4	3
70	Unidentified	1931		73	103	75	217	147	307						
				100	91	20	15	14	11						
71	Unidentified	1948	398?	281	73	398	293	282	75	399	280	45	400	294	283
				100	60	58	39	27	16	15	10	7	5	5	5
72	4-Hydroxy-3-Methoxy-Phenyl-Lactic Acid tri-TMS	1998	428	209	73	210	147	338	204	179	117	211	413		
				100	43	11	8	6	4	4	4	3	2		

TABLE 3.3.5: Continuation (f)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
73	Palmitic Acid	2015	328	73	117	75	313	132	43	145	55	129	41	314	74
	TMS Ester			100	73	55	42	39	29	20	16	15	13	6	6
74	Unidentified	2031	333?	318	231	73	45	319	304	288	232	333	290	320	289
				100	79	62	30	27	19	11	10	7	7	6	6
75	4-Hydroxy-3-Methoxy-	2052	338	103	73	75	159	143	69	338	147	308	118	43	323
	Cinnamic Acid di-TMS			100	67	48	15	12	11	10	10	8	8	7	6
76	Unidentified	2083		179	73	75	308	43	180	293	309	74			
				100	76	48	20	13	7	6	5	4			
77	Heptadecanoic Acid	2111	342	73	75	117	327	341	132	145	45	43	129	74	41
	TMS Ester			100	60	13	10	7	7	6	6	6	5	5	5
78	Indole-3-Lactic	2148	421	202	73	203	193	75	179	218	206	45	204	74	421
	Acid tri-TMS			100	61	19	8	7	6	4	4	4	3	3	2
79	Unidentified	2167	353?	179	206	180	73	338	207	75	165	45	181	102	74
				100	99	33	30	23	20	7	6	6	5	5	4
80	4-Hydroxy-Hippuric	2181	339	193	294	75	73	117	339	55	194	129	295	145	43
	Acid di-TMS			100	36	21	19	15	14	10	9	9	7	6	6
81	Stearic Acid	2209	356	73	117	75	132	341	43	145	55	129	69	41	57
	TMS Ester			100	73	69	49	45	37	36	22	18	15	15	10
82	Unidentified	2264		73	118	91	245	103	92	132	75	147	74	217	98
				100	24	23	17	16	16	14	11	7	7	6	6
83	Unidentified	2533		130	149	73	157	57	131	202	167	304			
				100	36	19	17	10	9	7	7	4			

*Peak Number corresponds to Figure 3.3.7

TABLE 3.3.6: Gas chromatographic and mass spectrometric data for compounds in a tert-butyldimethylsilylated extract of greyhound urine.

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
1	Unidentified	1122		73	75	145	102	189	159	74	131	77	149	146	103
				100	58	36	12	6	6	5	4	4	3	3	3
2	Butanoic Acid BDMS Ester	1124	202	145	73	75	159	115	130	103	187	147	131	129	101
				100	95	50	23	9	8	3	2	2	2	2	2
3	Unidentified	1130		75	116	113	117	137	76	112	159	118	100	99	
				100	64	8	6	4	3	2	2	2	2	1	
4	3-Methoxy-n-Valeric Acid BDMS Ester	1159	246	147	73	189	148	117	149	133	190	131	72	74	191
				100	84	82	64	26	25	23	12	12	8	4	3
5	Unidentified	1195		149	75	133	57	142	41	150	147	73	151	59	134
				100	39	24	17	14	7	6	6	6	4	4	3
6	Methyl-Thio-Acetic Acid BDMS Ester	1205	220	163	205	133	164	89	164	206	173	119	47	134	73
				100	27	15	9	9	5	4	3	3	3	2	2
7	Propanoyl Glycine BDMS Ester	1226	245	146	188	73	130	147	189	75	74	148	132	116	190
				100	91	23	23	22	17	8	7	6	6	6	4
8	Acetyl Glycine BDMS Ester	1265	231	174	73	175	100	130	75	72	59	216	176	44	
				100	12	8	8	7	7	7	5	2	2	2	
9	Unidentified	1298	291?	73	115	77	284	234	74	59	75				
				100	23	8	5	5	5	4	3				
10	C_7 Aliphatic Acid Glycine Conjugate BDMS Ester	1302	301?	244	151	57	149	245	133	152	286	246			
				100	57	26	14	11	10	5	4	4			
11	Thioglycollic Acid di-BDMS	1314	320?	221	73	263	222	223	264	147	103	205	191	265	74
				100	78	54	33	18	11	6	6	5	5	4	4
12	Valeryl Glycine BDMS Ester	1330	273	147	73	75	154	216	189	148	59	149	57	45	74
				100	65	46	19	12	9	10	7	5	4	5	3

TABLE 3.3.6: Continuation (a)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
13	Unidentified	1350	290?	147	73	233	148	59	149	75	234	117	45	133	74
				100	99	81	31	17	16	14	13	8	8	6	6
14	C_9 Acid (+ double bond or ring) BDMS Ester	1358	270?	213	171	73	214	99	215	172	59				
				100	39	38	15	7	5	4	4				
15	Benzoic Acid BDMS Ester	1444	236	179	105	77	135	180	75	51					
				100	55	38	21	11	6	4					
16	Unidentified	1452	289?	73	147	232	75	183	233	148	168	100	124	259	274
				100	92	84	24	21	14	12	9	9	7	6	4
17	Unidentified	1461	295?	147	73	186	253	148	133	254	142	59	41	187	149
				100	42	26	26	8	5	4	4	4	4	3	3
18	Unidentified	1466		43	75	144	186	314	145	181	234	115	77	45	116
				100	80	50	16	5	5	4	3	3	3	3	2
19	Unidentified	1468		186	144	43	75	187	145	73	42	188	112	72	47
				100	96	65	54	8	6	5	5	3	3	3	3
20	Phenylacetic Acid BDMS Ester	1478		75	193	73	115	91	194	58	195	180	177	137	76
				100	43	18	9	7	6	4	3	3	3	3	3
21	Background	1488		73	207	115	75	147	208	74	209	249	193	180	173
				100	30	7	6	5	4	4	3	2	2	2	2
22	Unidentified	1499	303?	183	147	73	246	75	184	59	153	148	247	225	185
				100	48	26	16	11	8	5	4	4	3	3	3
23	Unidentified	1529	252?	195	93	196	149	151	75	73	66	58	197	41	39
				100	13	9	6	5	5	5	5	4	3	3	3
24	Unidentified	1550		75	58	77	79	69	149	134	184	76	73	52	45
				100	24	10	6	6	5	5	4	4	4	4	4
25	Unidentified	1558	252?	195	75	73	93	196	143	151	58	149	77	197	69
				100	33	11	10	8	8	6	6	4	4	3	3

TABLE 3.3.6: Continuation (b)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
26	Unidentified	1564	332?	73	147	75	275	179	189	187	149	133	59	289	276
				100	39	18	16	15	13	8	7	6	6	4	4
27	Unidentified	1572		73	147	75	289	189	187	149	261	209	148	74	133
				100	50	19	10	8	7	6	5	5	5	5	4
28	Unidentified	1581	303?	147	73	75	246	189	148	149	59	247	89	74	58
				100	81	30	23	9	8	6	6	4	4	4	4
29	Acetoacetic Acid BDMS Ester	1611	288	231	147	73	232	74	213	148	233	273	132	100	99
				100	72	61	16	15	7	7	5	4	4	4	4
30	Unidentified	1627		73	115	147	221	263	240	189	151	358	149	77	
				100	16	14	12	9	9	5	5	4	4	4	
31	2-Amino-Benzoic Acid BDMS Ester	1671	251	194	120	150	92	195	65	176	251	73			
				100	53	16	11	9	7	4	3	3			
32	Unidentifed	1677	360?	73	147	303	189	75	149	304	148	115	74		
				100	61	19	13	7	6	5	4	3	3		
33	Unidentified	1687		73	147	300	157	75	301	148	74	57	41		
				100	63	21	6	6	5	4	3	3	3		
34	Succinic Acid di-BDMS Ester	1712	346	73	289	147	290	116	115	133	148	75	291	129	331
				100	69	57	13	11	8	5	6	6	4	3	2
35	Unidentified	1723		73	147	303	75	123	74	304	59	41			
				100	38	17	11	4	4	3	3	3			
36	Unidentified	1797		73	147	263	115	275	74	77	59				
				100	8	7	7	6	3	3	2				
37	Unidentified	1802		73	303	223	193	208	147	91	289	102			
				100	18	15	10	9	9	7	6	5			
38	Unidentified	1822	374?	226	95	73	147	317	75	227	331	39			
				100	95	81	39	17	15	9	6	6			

TABLE 3.3.6: Continuation (c)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
39	3-Methyl-Glutaric Acid BDMS Ester	1824	372	73 100	315 74	147 36	316 11	187 9	109 9	74 5	82 4	317 2	241 2	225 2	148 2
40	Unidentified	1866	372?	73 100	315 36	147 35	273 10	211 7	316 6	183 5	74 5	59 5	129 4	109 4	
41	Unidentified	1878	400?	383 100	73 57	384 21	385 8	425 3	270 3	59 3	298 2	272 2	193 2	113 2	74 2
42	Adipic Acid di-BDMS Ester	1910	374	73 100	317 22	75 15	111 14	147 6	318 5	141 4	74 4	309 3	130 3	319 2	129 2
43	Palmitic Acid BDMS Ester	1939	370	313 100	147 61	73 39	314 13	182 6	128 6	315 5	148 4	123 4	239 3	355 2	330 2
44	Unidentified	1986		147 100	73 81	337 34	189 13	309 10	148 9	133 6	338 5	59 5	74 4		
45	Phenylpropanoyl-Glycine BDMS Ester	2003	321	105 100	264 78	73 64	44 59	91 45	236 24	77 13	265 8	75 6	146 5	237 4	192 4
46	Pimelic Acid di-BDMS Ester	2008	388	73 100	105 61	331 55	236 20	75 14	125 10	332 9	155 8	74 5	115 4	333 3	264 3
47	Hippuric Acid BDMS Ester	2022	293	236 100	105 51	77 46	192 25	73 20	237 15	106 15	51 5	248 4	193 4		
48	Unidentified	2037		73 100	75 80	285 56	91 48	250 44	286 11	251 7	129 7	132 6	313 6	115 6	74 6
49	N-Phenylacetyl-Glycine BDMS Ester	2059	307	250 100	91 91	73 34	251 20	30 10	132 9	116 5	92 5	252 4	104 4	65 4	115 3
50	Unidentified	2070		309 100	73 91	30 56	91 42	251 32	116 23	92 19	104 17	65 16	132 14	310 13	41 12
51	Indole-3-Acetic Acid BDMS Ester	2077	289	40 100	29 61	147 29	232 13	44 12	149 8	207 7	224 5	130 5	189 4	79 4	52 4

TABLE 3.3.6: Continuation (d)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
52	Suberic Acid di-BDMS Ester	2111	402	73	345	75	346	387	129	169	55	347	74	327	115
				100	87	25	17	9	7	6	6	5	4	3	3
53	Unidentified	2130		205	247	73	75	337	140	179	221	206	248	338	164
				100	55	53	45	27	15	10	9	9	8	7	5
54	Unidentified	2172		354	73	353	281	355	356	59	282	74	238	179	
				100	78	46	33	23	6	5	4	4	3	3	
55	Unidentified	2180		221	179	73	337	222	338	140	205	180	355	339	165
				100	54	53	30	12	9	9	7	7	5	5	5
56	Unidentified	2187		339	267	340	73	265	141	126	341	295	268	193	269
				100	24	14	13	6	6	6	5	5	5	5	3
57	Unidentified	2197		73	147	462	115	345	359	329	275	371	301	189	149
				100	39	12	10	8	5	5	4	3	3	3	3
58	Azelaic Acid di-BDMS Ester	2211	416	73	359	75	360	129	311	55	361	74	151	115	41
				100	80	32	15	7	6	6	5	5	3	3	3
59	Unidentified	2225		73	364	147	91	192	365	250	218	74	116	366	336
				100	27	23	18	7	6	5	5	5	4	3	3
60	Unidentified	2241		313	75	314	73	129	117	131	43	315	55	41	
				100	35	18	12	8	8	5	5	3	3	3	
61	Unidentified	2256		73	385	386	147	387	427	388	164	133	115	59	
				100	60	15	12	6	5	5	5	5	5	5	
62	Unidentified	2348		73	367	179	251	295	368	155	59	373	369	252	
				100	57	51	21	16	13	6	6	5	5	4	
63	Unidentified	2356		369	73	297	156	223	148	141	371	298			
				100	34	28	8	6	6	6	5	5			
64	Unidentified	2372		460	75	365	40	327	29	147	293				
				100	60	22	20	18	18	16	10				

TABLE 3.3.6: Continuation (e)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
65	Background	2408		73	439	74	59	193							
				100	41	5	3	2							
66	Background	2417		351	73	352	147	454	425	353	74	59	427		
				100	98	32	26	20	18	7	5	3	2		
67	Oleic Acid BDMS Ester or Isomer	2456	396	339	75	340	341	131	129	40					
				100	23	13	3	3	3	2					
68	Oleic Acid BDMS Ester or Isomer	2463	396	339	75	73	340	55	41	341	129	131	76	383	81
				100	51	41	14	9	6	5	5	4	4	3	3
69	Stearic Acid BDMS Ester	2487	398	341	73	147	75	342	129	117	131	57	55	148	43
				100	50	48	15	14	5	5	4	3	3	2	2
70	Background	2498		73	454	74	327	59	511						
				100	29	5	3	3	2						
71	Background	2509		73	370	57	71	59	394	339	262	134	55	211	147
				100	9	9	7	6	5	5	5	5	5	4	4
72	Unidentified	2530	417?	360	73	361	362	246	302	74	59				
				100	63	35	10	6	4	3	3				
73	Unidentified	2590		73	221	335	130	147	394	336	74	376	348	395	337
				100	81	64	20	17	12	10	6	4	4	3	3
74	Background	2612		355	377	73	336	378	337	184	379	74	279	59	
				100	79	71	18	15	6	6	3	4	3	3	
75	Background	2661		235	394	73	236	396	193	169	262	237	177	74	
				100	62	51	12	5	5	4	3	3	3	3	
76	Unidentified	2685		380	73	381	221	248	179	162	165	107	74		
				100	61	32	27	9	9	6	3	3	3		
77	Background	2697		73	147	498	179	251	221	74	148	59			
				100	53	13	8	7	5	5	3	3			

TABLE 3.3.7: Gas chromatographic and mass spectrometric data for compounds in a methylated/silylated extract of greyhound urine.

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
1	Urea di-TMS	1222	204	147	189	73	74	171	66	148	45				
				100	57	40	15	11	11	9	9				
2	Unidentified	1230	250	73	117	116	75	103	66	147	45	101	59	118	88
				100	48	19	14	10	10	6	6	4	4	3	3
3	Glycerol tri-TMS	1271	308	73	147	205	103	117	133	75	74	45			
				100	30	25	13	12	4	4	4	4			
4	Unidentified	1425		73	89	179	75	131	59	143	74	142	202	193	237
				100	45	23	14	10	10	9	9	8	7	7	6
5	Unidentified	1440		73	89	193	237	161	162	129	75	59	160	45	
				100	18	16	11	8	7	7	7	6	5	5	
6	Unidentified	1448		95	124	39	183	96							
				100	27	8	6	4							
7	Unidentified	1452		73	209	224	89	193	135	75	91	210	59	45	149
				100	96	47	22	20	16	16	15	12	12	12	10
8	Unidentified	1469		208	73	176	209	178	134	59	223	89	193	91	117
				100	30	21	13	13	13	13	11	8	7	7	6
9	Hydroxy-TMS Phenylacetic Acid Methyl Ester	1497	238	179	73	238	163	223	180	82	75	45			
				100	46	38	31	18	17	5	4	4			
10	Unidentified	1505		73	195	147	298	103	74	75	45	196			
				100	23	12	6	5	5	4	4	3			
11	Hydroxy-TMS Phenyl-propanoic Acid Methyl Ester	1521	252	193	73	194	252	177	237	195	45				
				100	38	10	7	6	3	3	3				
12	Unidentified	1560	252	205	252	73	89	206	177	193	192	179	253	221	75
				100	43	26	10	9	8	7	7	7	6	6	6

TABLE 3.3.7: Continuation (a)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
13	Unidentified	1585		73	179	252	89	72	91	131	180	163			
				100	67	18	17	15	12	11	10	9			
14	Unidentified	1593	268	209	73	210	89	135	74	45	253	225	211	97	75
				100	92	10	10	6	4	4	3	3	3	3	3
15	Hippuric Acid Methyl Ester	1626	193	105	77	134	51	193	106	161	78	209	50		
				100	40	24	11	5	5	3	3	2	2		
16	Unidentified	1632		134	77	51	106	179	238	268	161	73	193	50	253
				100	96	64	23	16	14	12	12	10	8	8	7
17	Unidentified	1648		105	73	91	206	264	45	265	178	250			
				100	79	42	30	19	19	13	13	10			
18	Unidentified	1665		121	73	199	192	122	223	207	129	92	89	267	116
				100	40	5	5	5	3	3	3	3	3	2	2
19	N-Phenylacetyl-Glycine Methyl Ester	1677	207	91	92	88	30	118	56	116	65	207	44	39	
				100	86	60	60	35	32	24	17	12	10	6	
20	Unidentified	1690		73	91	188	118	89	267	102	279	160	74	75	59
				100	35	28	21	21	19	19	14	13	11	10	7
21	Unidentified	1736		242	73	243	149	75	91	120					
				100	26	10	10	10	8	7					
22	Unidentified	1756		267	73	268	269	89	131	75	254	193	105	74	177
				100	70	14	7	7	4	4	3	3	3	3	2
23	Unidentified	1766		73	223	173	282	235	75	224	89	283	103	251	174
				100	83	72	67	42	12	9	8	7	7	6	6
24	Unidentified	1776		170	73	174	182	89	75	59	250	265	193	163	234
				100	91	35	6	6	6	6	4	2	2	2	1
25	Unidentified	1803		73	195	147	205	129	75	149	103	297	196	117	239
				100	20	11	7	7	6	5	5	4	4	4	3

TABLE 3.3.7: Continuation (b)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
26	Unidentified	1808		73	259	89	155	109	75	59	243	129	230	147	95
				100	16	15	10	10	8	7	5	4	3	3	3
27	Unidentified	1814		73	297	75	117	163	298	285	145	132	147	131	129
				100	23	17	9	8	6	6	6	6	5	5	5
28	Unidentified	1825		151	73	91	152	312	253	250	222	221	217	89	75
				100	48	7	5	3	2	2	2	2	2	2	2
29	Unidentified	1835	340?	340	73	281	293	341	282	294	75	309	342	283	265
				100	99	93	72	20	16	11	8	7	6	5	5
30	Unidentified	1851		73	209	159	147	217	210	205	103	75	311	158	133
				100	43	29	11	10	7	6	5	5	4	4	4
31	2-Carboxy-4-Methoxy-Quinoline Methyl Ester	1864	217	159	158	217	160	115	102	143	130	73	89	88	129
				100	29	20	20	20	19	11	9	9	5	5	4
32	Unidentified	1872		73	247	216	144	143	59	248	173	89	75	74	312
				100	18	13	6	6	5	4	4	4	4	4	3
33	Unidentified	1884		121	204	73	175	192	74	147	87	205	43	161	129
				100	88	78	51	50	40	36	21	18	17	11	10
34	Unidentified	1894		73	117	149	162	221	147	250	209	207	175	158	119
				100	30	29	12	8	7	4	4	3	3	3	3
35	Unidentified	1901		204	73	209	191	205	217	147	103	206	129	218	210
				100	79	58	16	10	9	8	6	5	5	3	3
36	4-Methoxy Hippuric Acid Methyl Ester	1919		73	103	135	268	117	269	217	173	147	75	74	281
				100	43	41	11	9	6	6	6	6	6	5	4
37	4-Methoxy-Phenylacetyl-Glycine Methyl Ester	1933		217	121	122	218	73	148	200	275	237	101	216	89
				100	98	19	12	11	9	6	5	5	5	4	4
38	Unidentified	1939		73	103	155	204	75	149	74	228	223	193	156	194
				100	29	15	8	6	4	3	3	3	3	3	2

TABLE 3.3.7: Continuation (c)

Peak No.*	COMPOUND NAME	Retention Index	Molecular Weight	Ions % Abundance Below											
39	Unidentified	1968		73	217	338	323	204	147	291	103	339	205	191	183
				100	11	9	8	8	7	4	4	3	3	3	3
40	Unidentified	1981		179	73	250	180	235	251	75	208	74	294	181	
				100	64	62	9	6	5	4	3	3	2	2	
41	Unidentified	1992		204	73	179	191	205	217	147	206	192	309	275	218
				100	44	18	17	11	8	7	5	4	3	3	3
42	Unidentified	2008		73	117	75	313	132	145	129	55	314	179		
				100	38	38	30	19	12	11	7	6	6		
43	Hydroxy-TMS-Phenylacetyl Glycine Methyl Ester	2030		179	73	180	295	206	45	165	163	193	181	82	89
				100	73	33	22	9	8	7	6	5	5	5	4
44	Unidentified	2059		202	73	203	363	204	304	218	130	277	200		
				100	45	13	3	3	2	2	2	1	1		
45	Stearic Acid Methyl Ester	2074		74	87	75	55	57	143	69	298	147	73	255	83
				100	68	18	12	10	7	6	5	5	5	4	4
46	Unidentified	2108		290	73	349	291	350	292	202	118	74	351	218	91
				100	95	50	17	7	6	5	4	4	3	3	3
47	Unidentified	2190		73	75	117	147	132	43	55	341	145	69	129	221
				100	72	45	26	22	18	16	14	14	13	11	7
48	Unidentified	2308		202	73	203	273	130	204	332	200	131	274	230	
				100	75	10	5	5	3	2	2	2	1	1	
49	Indole Acetyl Glycine Methyl Ester	2376		130	246	131	73	202	77	129	103	102	247	157	
				100	11	8	8	4	4	3	3	2	1	1	
50	Unidentified	2391		202	73	318	203	45	319	204	198	200	74	221	186
				100	37	19	19	11	3	3	3	2	2	1	1

* Peak Number corresponds to Figure 3.3.9.

TABLE 3.3.8: Organic acids identified by GC-MS in the urine of the racing greyhound.

NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
ALIPHATIC MONOCARBOXYLIC ACIDS				
Caprylic	$CH_3(CH_2)_6COOH$	144	-	1247(18)
Lauric	$CH_3(CH_2)_{10}COOH$	245	-	1620(52)
Palmitic	$CH_3(CH_2)_{14}COOH$	256	1870(33)	2015(73)
Oleic or Isomer	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	296	2033(48)	-
Oleic or Isomer			2040(49)	-
Heptadecanoic	$CH_3(CH_2)_{15}COOH$	284	-	2111(76)
Stearic	$CH_3(CH_2)_{16}COOH$	298	2063(51)	2533(83)
C_{19}Monounsaturated	$CH_3(CH_2)_7CH{=}CH(CH_2)_8COOH$	310	2145(57)	-
ALIPHATIC DICARBOXYLIC ACIDS				
Oxalic	$HOOC-COOH$	90	-	1079(2)* or 1081(3)*
Glutaric	$HOOC(CH_2)_3COOH$	132	-	1307(26)*
2-Methyl-Glutaric	$HOOC-CH_2-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-COOH$	146	-	1397(34)

TABLE 3.3.8: Continuation (a)

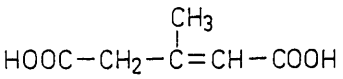
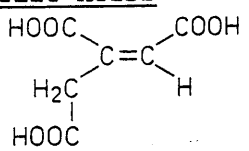
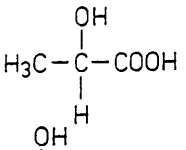
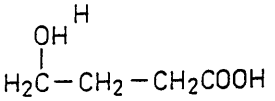
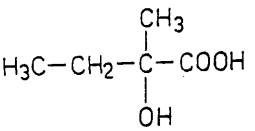
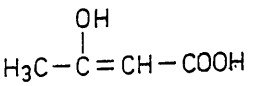
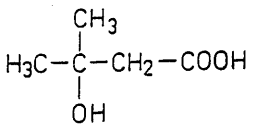
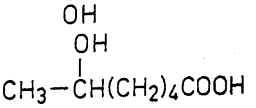
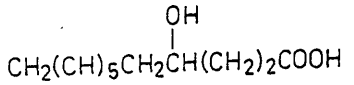
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
3-Methyl-Glutaconic		144	-	Peak 1 1410(35) Peak 2 1450(38)
Adipic	$HOOC(CH_2)_4COOH$	146	-	1475(39)*
Pimelic	$HOOC(CH_2)_5COOH$	160	-	1571(47)*
Suberic	$HOOC(CH_2)_6COOH$	174	1385(8)	1666(54)
Azelaic	$HOOC(CH_2)_7COOH$	188	1485(15)*	1766(61)*
ALIPHATIC TRICARBOXYLIC ACIDS				
cis-Aconitic		174	-	1727(57)*
HYDROXY-ALIPHATIC CARBOXYLIC ACIDS				
Lactic		90	-	1079(2)*
4-Hydroxy-Butanoic		104	-	1161(11)
2-Hydroxy-2-Methyl-Butanoic		118	-	1169(12)
3-Hydroxy-But-2-enoic		102	-	1189(14)
3-Hydroxy-iso-Valeric		120	-	1208(16)
Hydroxy-Heptanoic		146	-	1390(33)
C_{11}-Hydroxy-Triene		210	1570(24)	-

TABLE 3.3.8: Continuation (b)

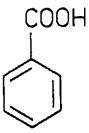
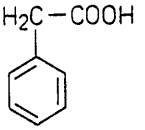
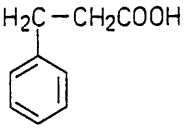
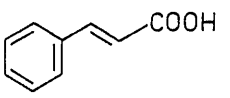
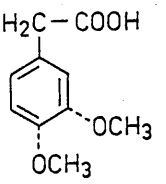
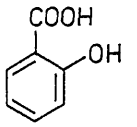
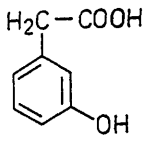
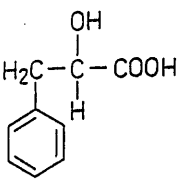
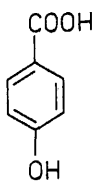
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
AROMATIC ACIDS AND PHENOLS				
Benzoic		122	1073(1)*	1228(17)*
Phenylacetic		136	1139(2)	-
Phenylpropanoic		150	1234(3)	-
Methyl Cinnamate		148	1321(6)	-
3- or 4-Methoxy-Phenylacetic		166	1371(7)	-
2-Hydroxy-Benzoic		138	-	1431(37)
3-Hydroxy-Phenyl-Acetic		152	1436(9)	1536(45)
3-Phenyl-Lactic		166	-	1558(46)
4-Hydroxy-Benzoic		138	-	1598(49)*

TABLE 3.3.8: Continuation (c)

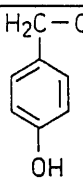
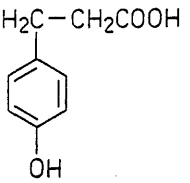
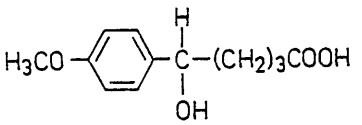
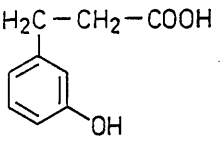
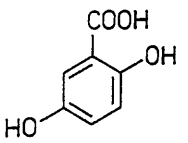
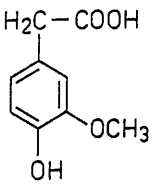
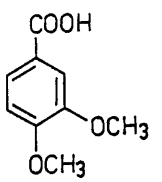
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
4-Hydroxy-Phenyl-Acetic		152	1448(11)	1606(50)
4-Hydroxy-Phenyl-Propanoic		166	-	1613(51)
5-(3- or 4-Methoxy-Phenyl)-4-Hydroxy-Valeric		224	1492(16)	-
3-Hydroxy-Phenyl-Propanoic		166	-	1695(55)
2,5-di-Hydroxy-Benzoic (Gentisic)		154	-	1718(56)
4-Hydroxy-3-Methoxy-Phenyl-acetic (Homovanillic)		182	1515(17)	1736(58)
3,4-di-Methoxy-Benzoic		182	1521(18)	-

TABLE 3.3.8: Continuation (d)

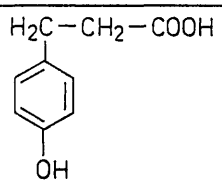
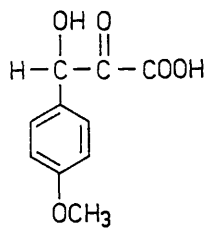
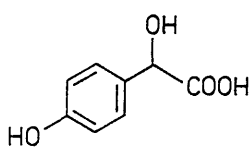
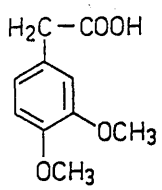
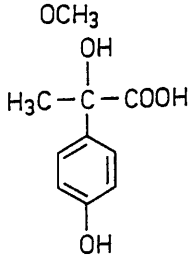
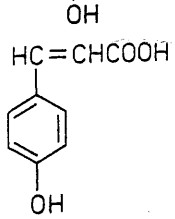
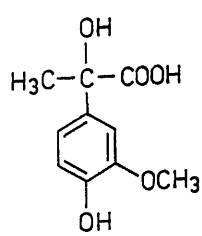
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
4-Hydroxy-Phenyl-Propanoic		166	1535(19)	-
2-Keto,3-Hydroxy-3(4-Methoxyphenyl)-Propanoic		210	1541(20)	-
3- or 4-Hydroxy-Mandelic		168	-	1762(60)
3,4-di-Methoxy-Phenylacetic		196	1552(21)	-
4-Hydroxy-Phenyl-Lactic		182	-	1888(66)
3-(4-Hydroxy-Phenyl)-2-Propenoic		164	-	1908(68)
4-Hydroxy-3-Methoxy-Phenyl-Lactic		212	-	1998(72)

TABLE 3.3.8: Continuation (e)

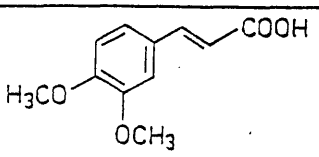
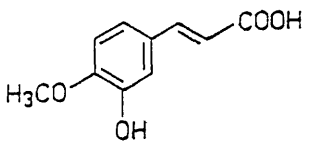
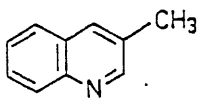
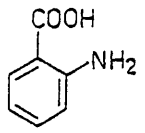
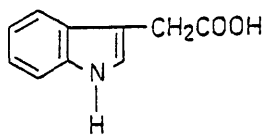
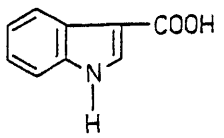
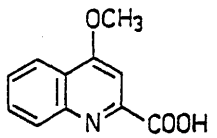
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
3,4-Dimethoxy-Cinnamic		208	1802(31)	-
4-Hydroxy-3-Methoxy-Cinnamic		194	-	2052(75)
NITROGEN-CONTAINING ACIDS				
Glycine	NH_2-CH_2-COOH	89	-	1125(7)
3-Methyl-Quinoline		143	1269(4)	-
2-Amino-Benzoic		137	1294(5)	1581(48)
Indole-3-Acetic		175	1755(28)*	-
Indole-3-Carboxylic		161	1790(29)	-
2-Carboxy-4-Methoxy-Quinoline		203	1853(32)	-

TABLE 3.3.8: Continuation (f)

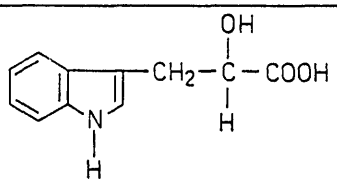
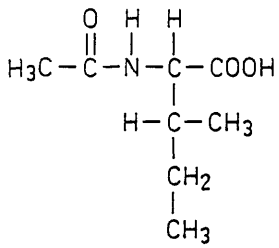
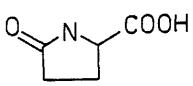
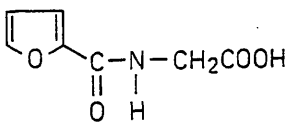
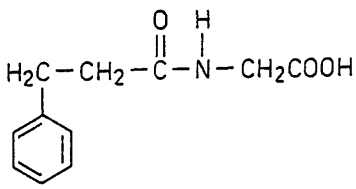
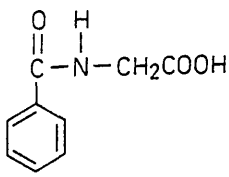
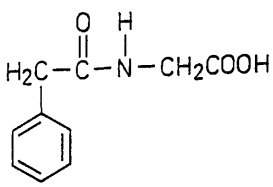
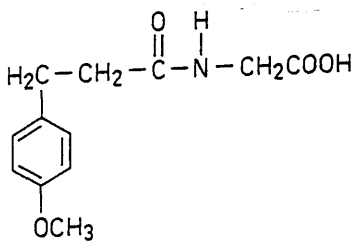
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
Indole-3-Lactic		205	1937(40)	2148(78)
ACID CONJUGATES				
Isoleucine-N-Acetyl		173	-	1421(36)
Pyroglutamic		127	-	1489(40)
N-(2-Furanyl-carbonyl)-Glycine		169	1453(12)	-
Phenylpropanoyl-Glycine		207	1561(23)	-
Hippuric		179	1616(26)*	1800(63)*
N-Phenylacetyl-Glycine		193	1666(27)	1832(64)
4-Methoxy-Phenyl-Propanoyl-Glycine		207	1891(36)	-

TABLE 3.3.8: Continuation (g)

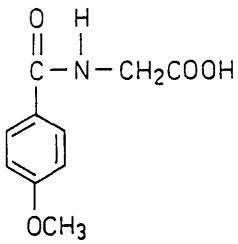
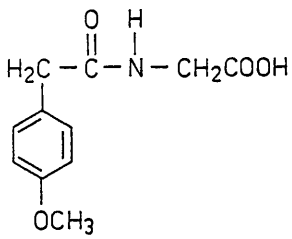
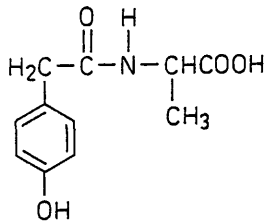
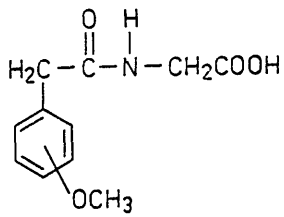
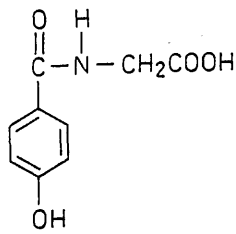
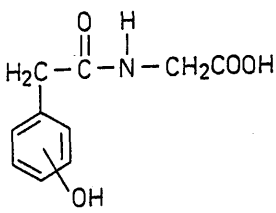
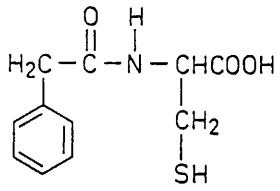
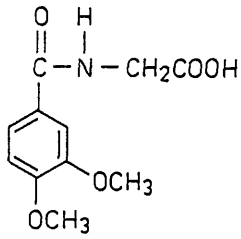
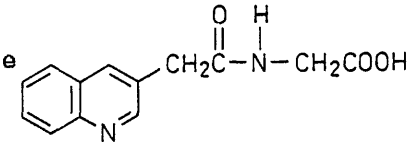
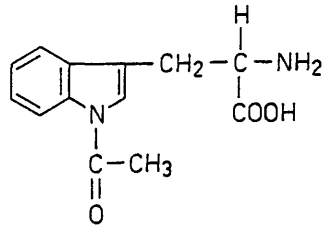
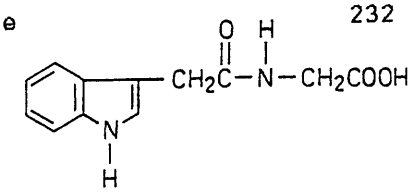
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
4-Methoxy-Hippuric		209	1905(37)	-
4-Methoxy-Phenyl-acetyl-Glycine		223	1920(38)	-
4-Hydroxy-Phenyl-acetyl-Alanine		223	1964(42)	-
2-,3- or 4-Methoxy-Phenylacetyl-Glycine		223	2012(45)	-
4-Hydroxy-Hippuric		195	-	2181(80)
2-,3- or 4-Hydroxy-Phenylacetyl-Glycine		209	2024(46)	-

TABLE 3.3.8: Continuation (h)

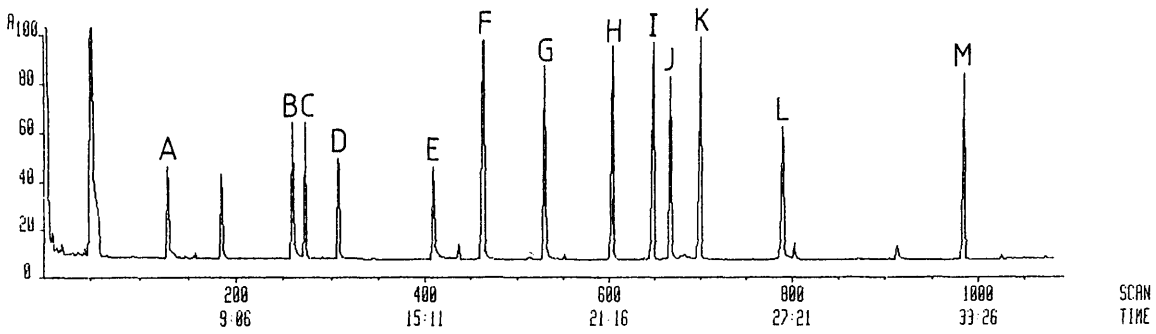
NAME OF ACID	STRUCTURE OF ACID	Molecular Weight of Free Acid	Retention Indice of Methylated Derivative[1]	Retention Indice of Silylated Derivative[2]
Phenylacetyl-Cysteine		239	2066(52)	-
3,4-di-Methoxy-Hippuric		239	2081(53)	-
3-Ethanoyl-Quinoline-Glycine		230	2113(55)	-
N-Acetyl-Tryptophan		246	2287(63)	-
Indole-Acetyl-Glycine		232	2363(65)	-

* Identified by comparison with an authentic standard

1 Number in parenthesis corresponds to the peak number in Figure 3.3.3(a) and Table 3.3.4.

2 Number in parenthesis corresponds to the peak number in Figure 3.3.3(b) and 3.3.5.

FIGURE 3.3.1: Chromatogram of standard acid mixture as their BDMS derivatives.



Identity of peaks;

A = n-Hexanoic acid
B = Benzoic acid
C = Lactic acid
D = Oxalic acid
E = o-Anisic acid
F = Succinic acid
G = Adipic + Glutaric acids
H = 3-Hydroxy-Benzoic acid
I = Pimelic acid
J = Hippuric
K = 4-Hydroxy-Benzoic acid
L = Azelaic acid
M = iso-Citric acid

FIGURE 3.3.2 : Chromatogram obtained for the analysis of a methylated extract of a pooled sample of greyhound urine.

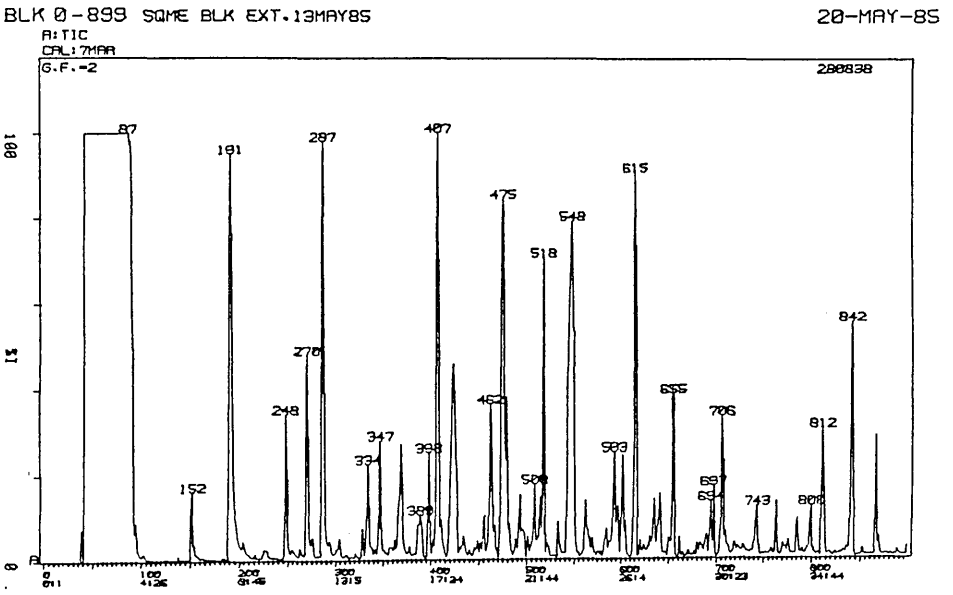
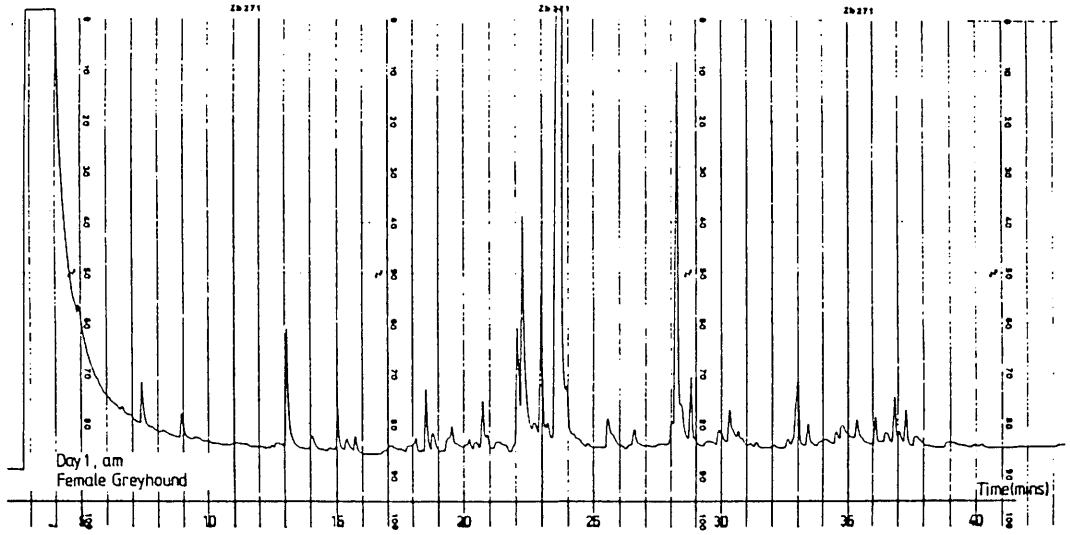
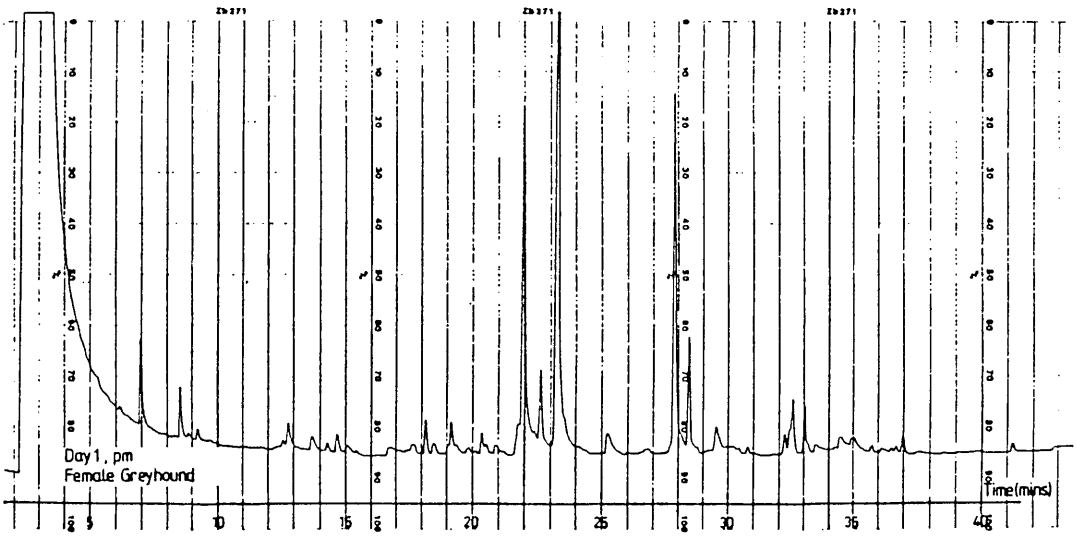


FIGURE 3.3.3a: Chromatograms obtained for the analysis of methylated extracts of urine samples taken from a female greyhound at different times.





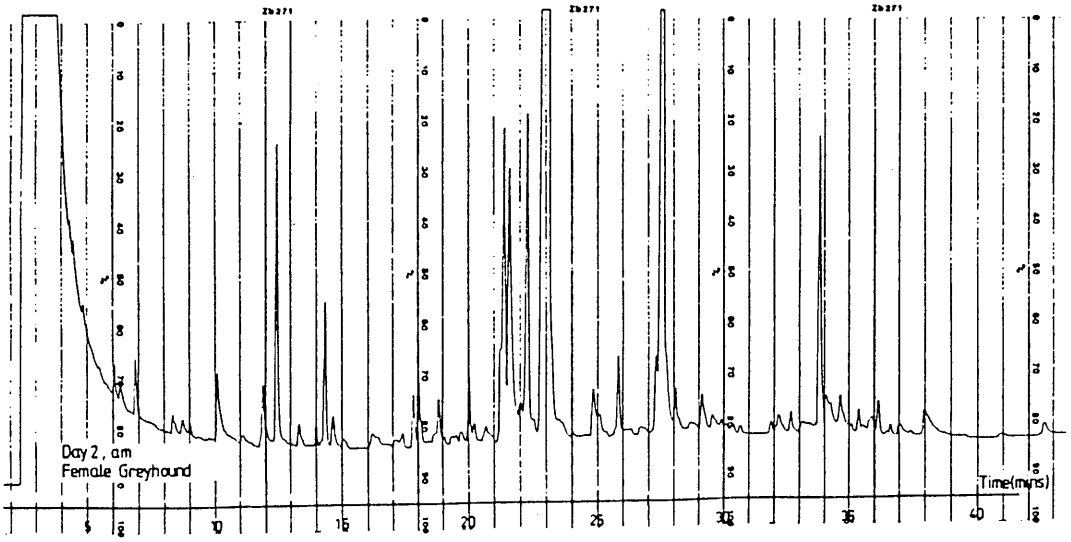
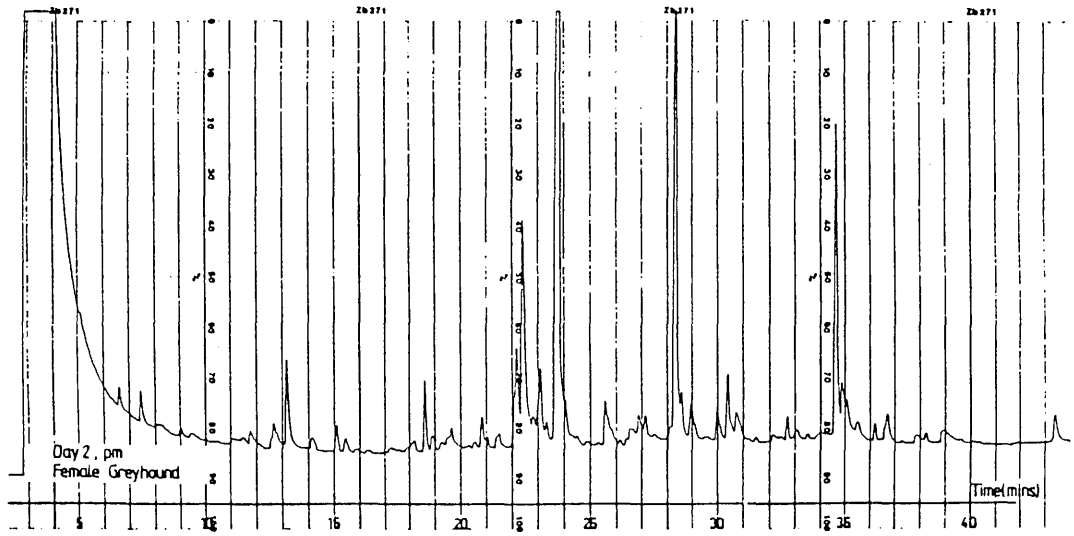
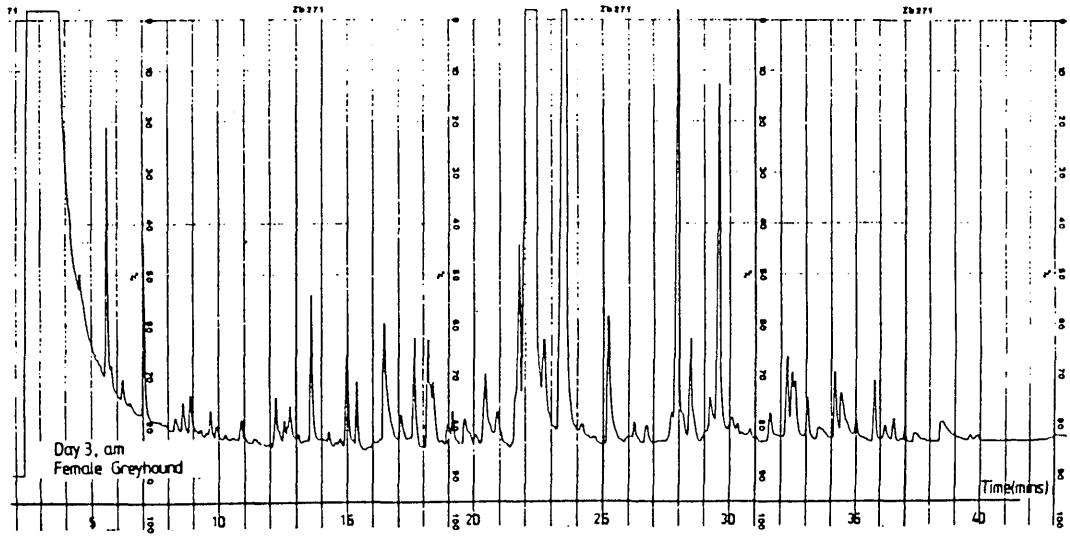


FIGURE 3.3.3b: Chromatograms obtained for the analysis of methylated extracts of urine samples taken from a female greyhound at different times.





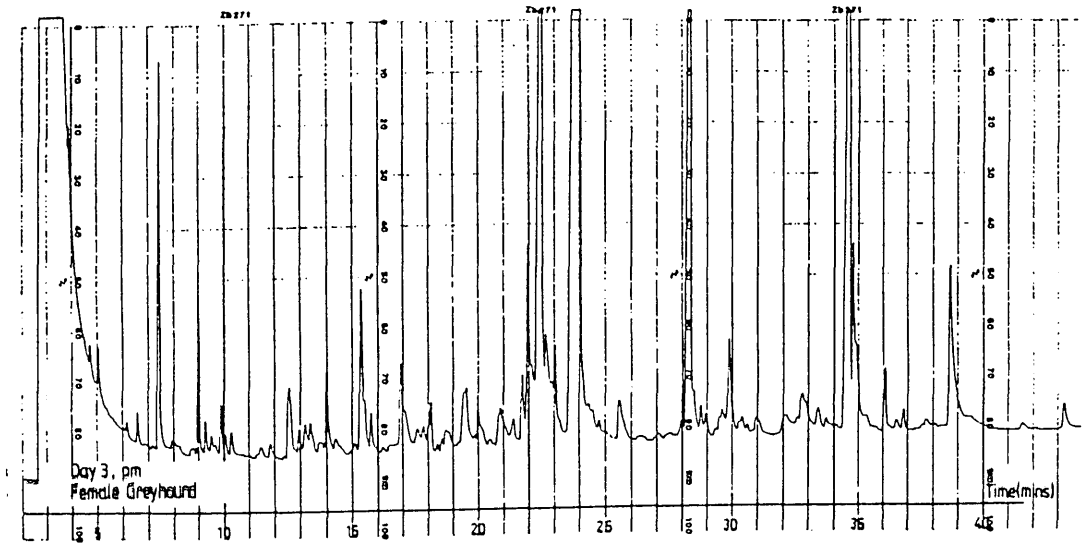


FIGURE 3.3.4: Chromatograms obtained for the analysis of methylated extracts of urine samples taken from a male greyhound at different times.

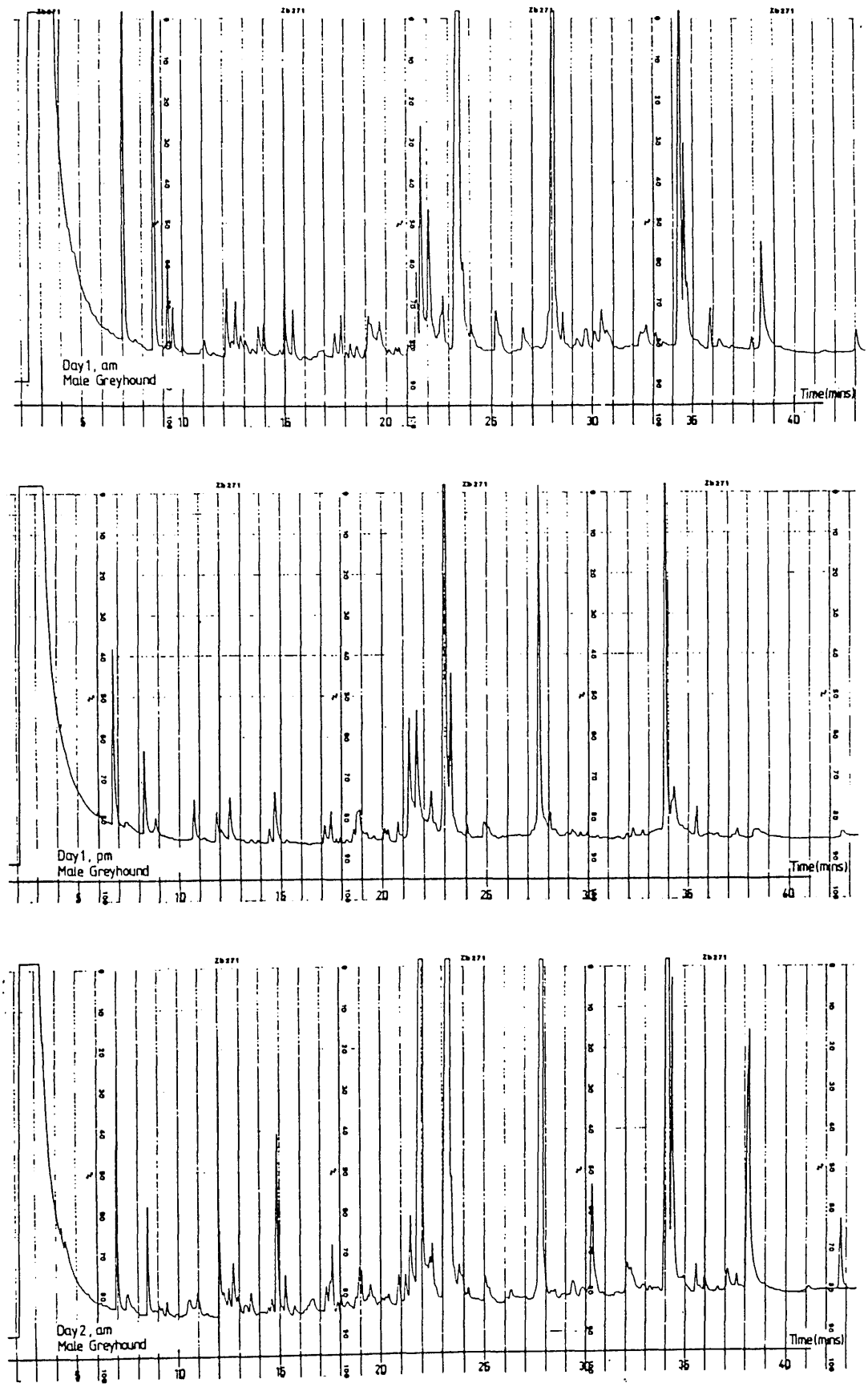


FIGURE 3.3.5: Chromatograms obtained for the analysis of methylated extracts of urine samples taken from a male greyhound at different times.

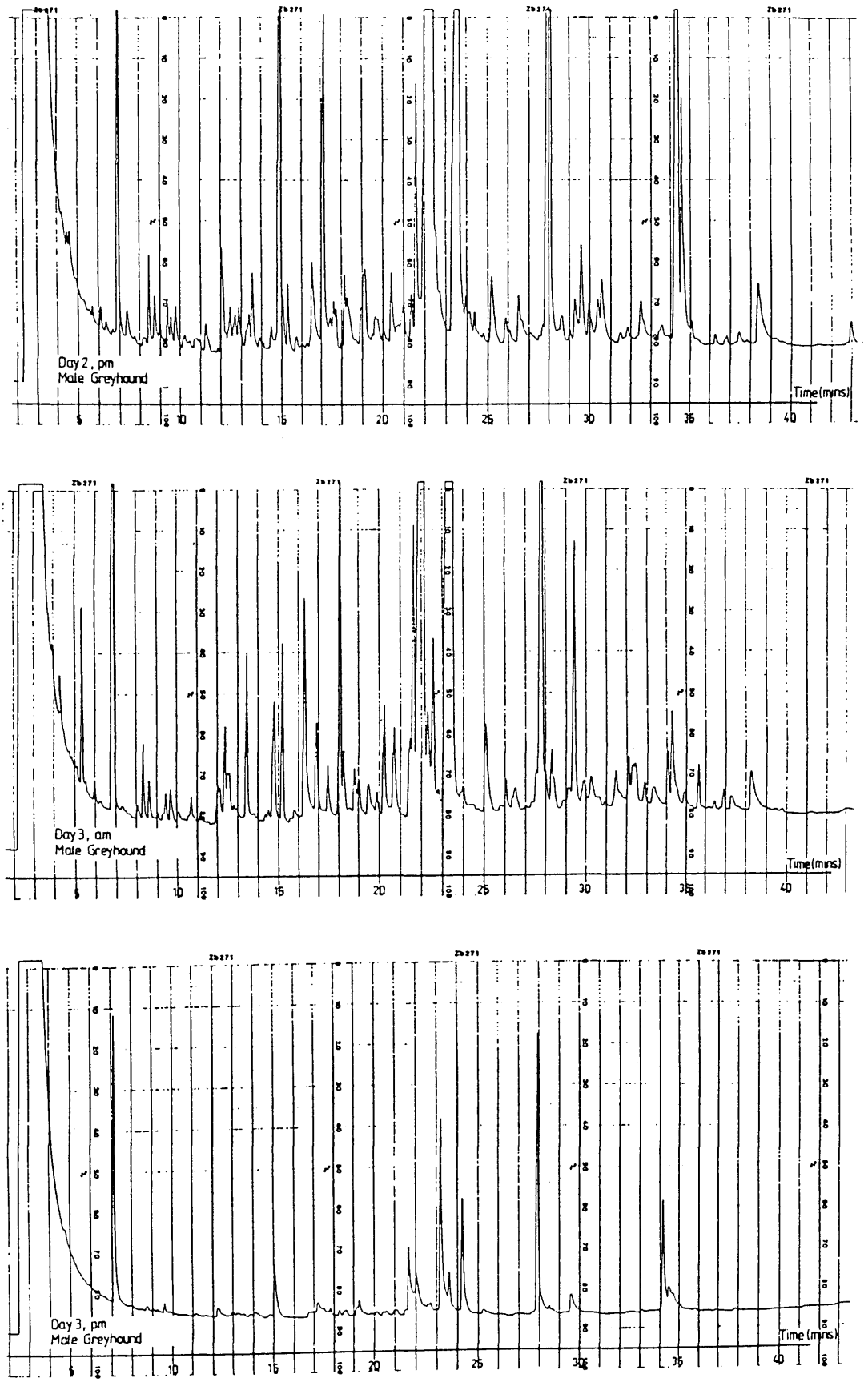
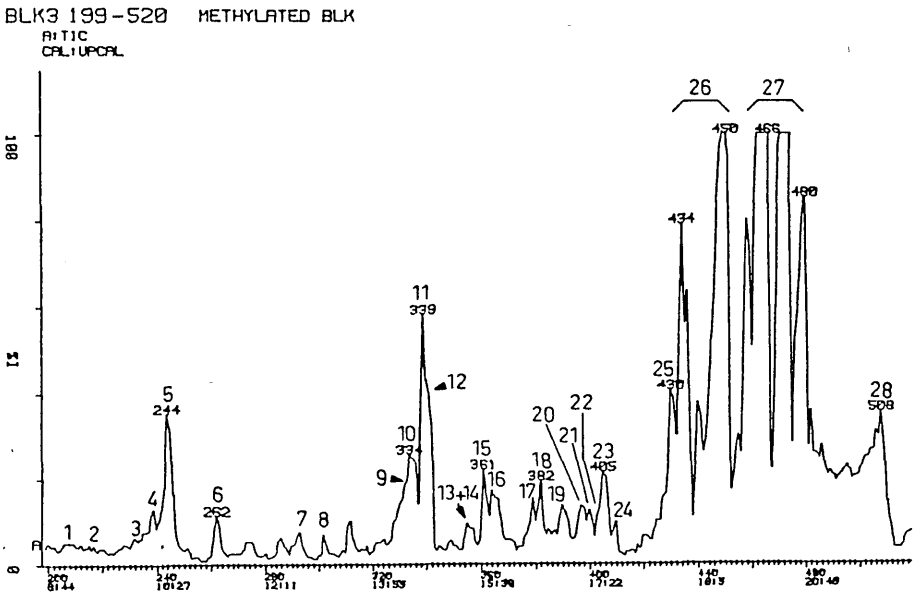


FIGURE 3.3.6: Chromatogram obtained for the analysis of a methylated extract of a pooled sample of greyhound urine. The peak numbers correspond to those in Table 3.3.4.



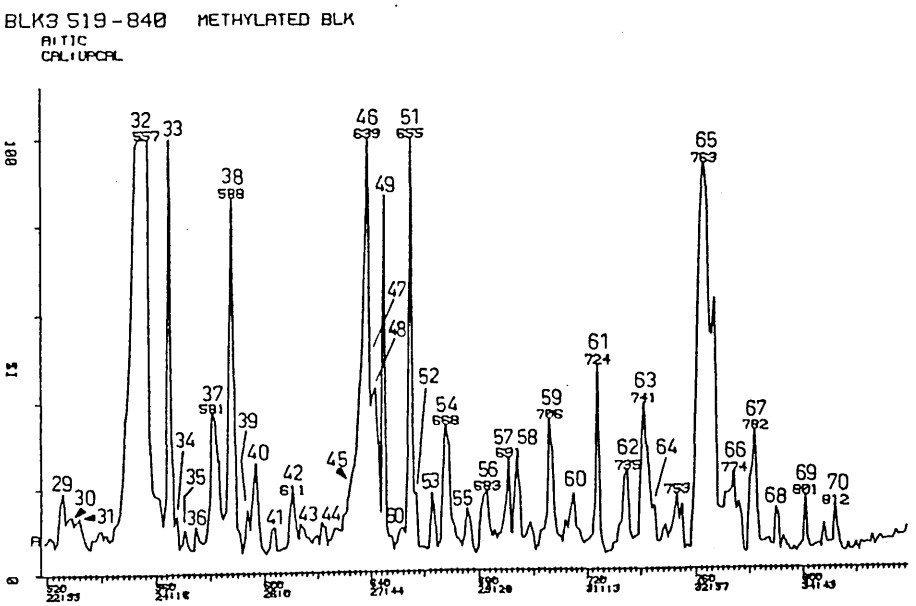


FIGURE 3.3.7: Chromatogram obtained for the analysis of a trimethylsilylated extract of a pooled sample of greyhound urine. The peak numbers correspond to those in Table 3.3.5.

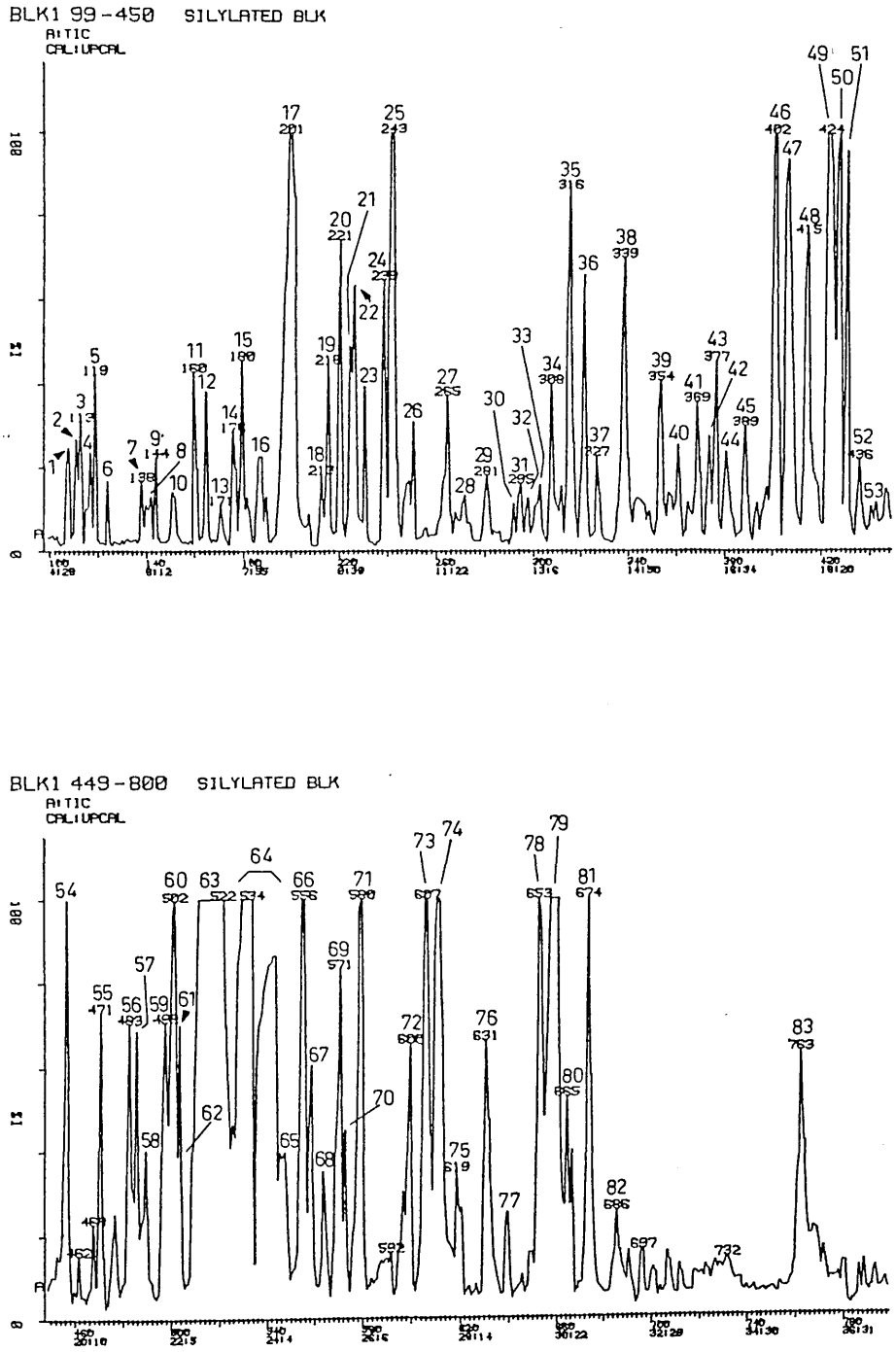


FIGURE 3.3.8: Chromatogram obtained for the analysis of a tert-butyldimethylsilylated extract of a pooled sample of greyhound urine. The peak numbers correspond to those in Table 3.3.6.

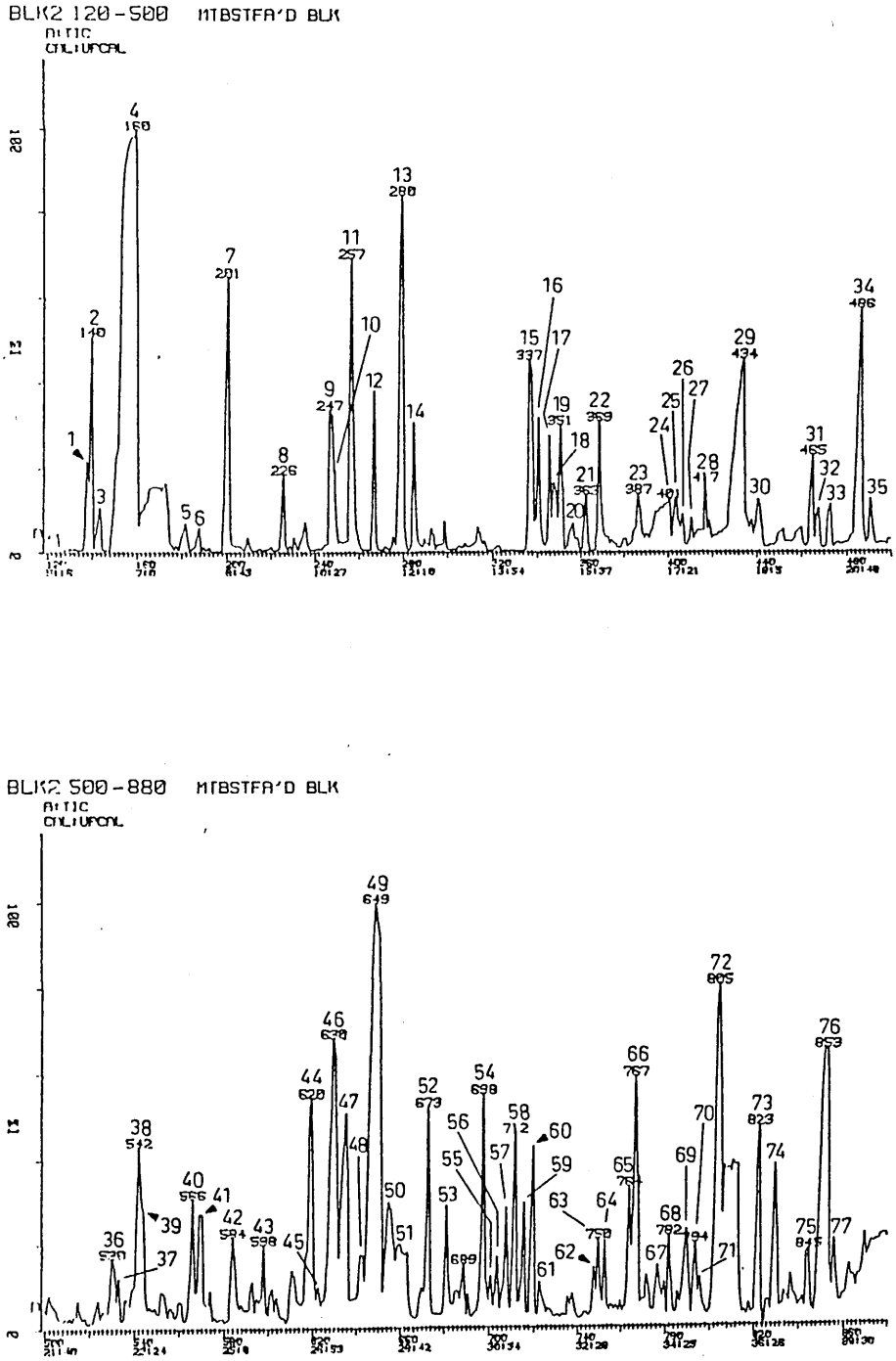
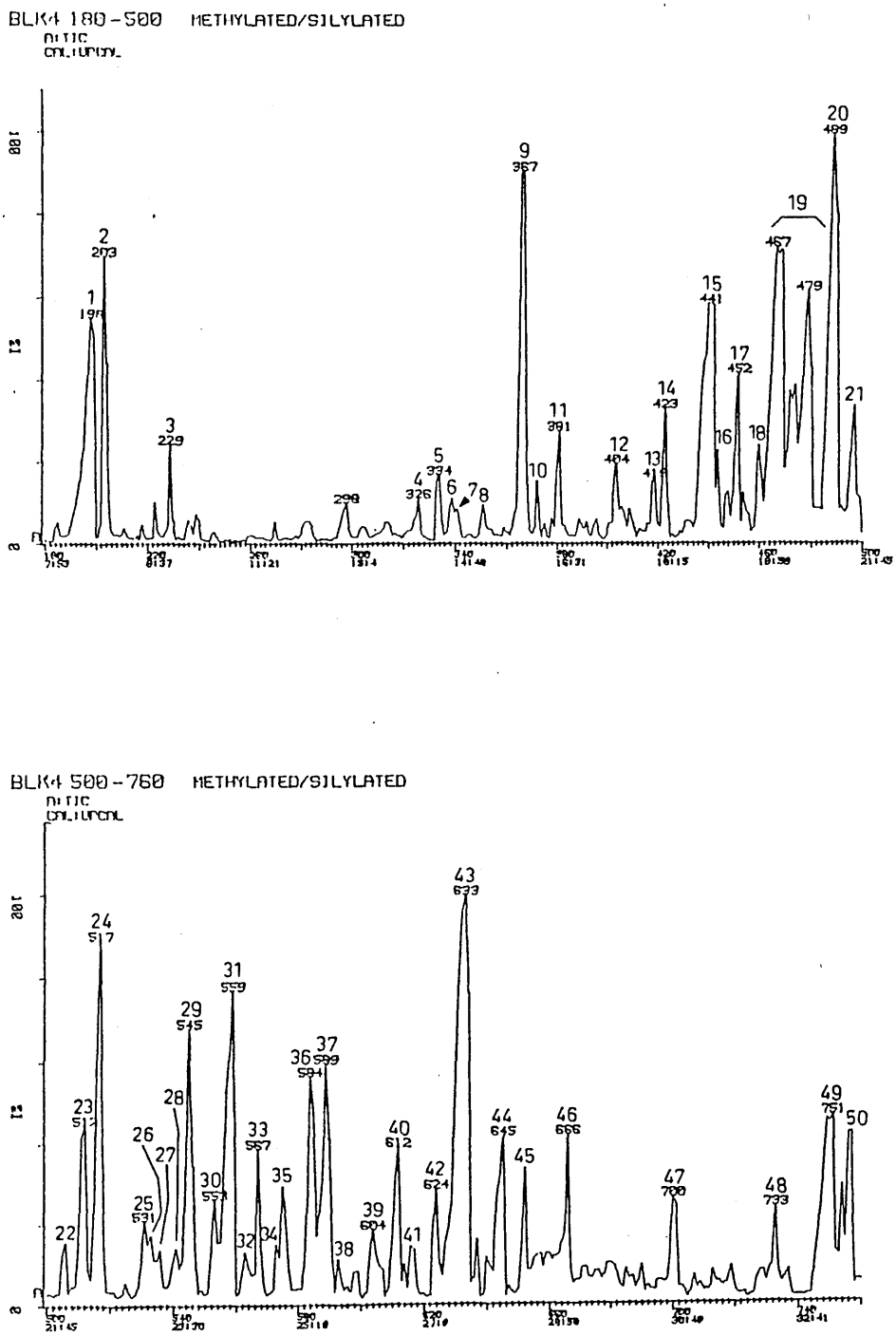


FIGURE 3.3.9: Chromatogram obtained for the analysis of a methylated/ silylated extract of a pooled sample of greyhound urine. The peak numbers correspond to those in Table 3.3.7.



3.4 DISCUSSION

3.4.1 URINARY ACID STANDARDS

A selection of organic acid standards known to occur in human urine [118] was reacted with three derivatisation reagents; seventeen acids were detected as their methyl derivatives, twenty-three as their trimethylsilyl (TMS) derivatives and fourteen as their tert-butyldimethylsilyl (BDMS) derivatives.

The methylated acids appeared to be stable but the silylated derivatives hydrolysed on evaporation of the reagents. When silylation of the acids was first examined in this project the reagents were evaporated and the residue redissolved in hexane before analysis. Since the evaporation step resulted in the hydrolysis of the silylated derivatives it was decided to omit this step and inject the standards directly in the reagent. The BDMS derivatives were also injected directly in the reagent.

A summary of the retention data obtained for the three derivatives is given in Table 3.4.1. This table illustrates that for each acid listed the methyl derivative elutes first, followed by the TMS derivative and lastly the BDMS derivative. The lengthening in retention time is due to an increase in molecular weight; methylation increases the molecular weight of a monocarboxylic acid by 14 units, silylation increases it by 72 units and tert-butyldimethylsilylation increases it by 114 units. This effect is exemplified in the increasing retention times of the various derivatives of

TABLE 3.4.1: Gas chromatographic retention indices for the methylated, silylated and tert-butyldimethyl silylated derivatives of standard organic acids.

NAME OF ACID	RETENTION INDICE		
	Methylated Derivative	Silylated Derivative	tert-Butyl-Dimethyl-Silylated Derivative
SATURATED MONOCARBOXYLIC ACIDS			
n-Butyric	ND	ND	-
iso-Butyric	ND	ND	-
iso-Valeric	ND	ND	-
n-Hexanoic	917	1062	1380
2-Furoic	955	1114	-
SATURATED DICARBOXYLIC ACIDS			
Oxalic	<900	1130	1612
Succinic	990	1307	1846
Glutaric	1104	1396	1949
Adipic	1202	1513	2063
Pimelic	1311	1610	2169
Azelaic	1516	1804	2379
UNSATURATED OXOCARBOXYLIC ACIDS			
cis-Aconitic	ND	1747	-
Fumaric	ND	1345	-
HYDROXY ALIPHATIC CARBOXYLIC ACIDS			
Glycollic	ND	1083	-
Lactic	<900	1060	1576
Malic	ND	1499	-
Tartaric	1179	1656	-
Citric	1414	1839	>2800
AROMATIC ACIDS			
Benzoic	1066	1231	1567
Anisic	-	-	1778
3-Hydroxy Benzoic	1403	1557	2141
4-Hydroxy Benzoic	1431	1620	2221
Vanillic	1446	1740	-
N-CONTAINING ACIDS			
Indole-3-Acetic	1745	1917	-
ACID CONJUGATES			
Hippuric	1602	1800	2179
'SUGAR ACIDS'			
Ascorbic	ND	1952	-
Glucuronic	ND	2017,2074	-

hexanoic acid. The methyl ester of hexanoic acid has a retention indice of 917, the TMS ester elutes at 1062 and the BDMS ester has a retention indice of 1380. The increase in retention is further emphasised in the dicarboxylic acids when there are two carboxylic groups available for derivatisation, as, for example, in the three derivatives of adipic acid.

This effect on retention time accounts for the fact that glycollic and malic acids were detected as their silyl esters but not as their methyl esters; the methylated acids eluted too close to the solvent front to be detected. Three of the acids, iso-butyric, n-butyric and iso-valeric were not detected as either the silyl or the methyl derivative as they were also masked by the solvent front.

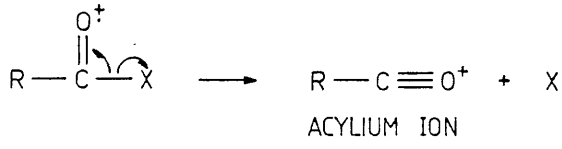
Two unsaturated acids, cis-aconitic and fumaric acids, were not detected as their methyl esters but were successfully silylated. It is known that acids of this type form non-volatile pyrazoline derivatives, when reacted with diazomethane, by substitution across the double bond [160-162].

The two 'sugar acids' which were examined, ascorbic and glucuronic acids, did not chromatograph as their methyl esters. These compounds contain several hydroxy groups, which would not be methylated and the compounds would therefore be too polar to chromatograph under the conditions used. Ascorbic acid chromatographed as a single peak corresponding to the silyl derivative of the

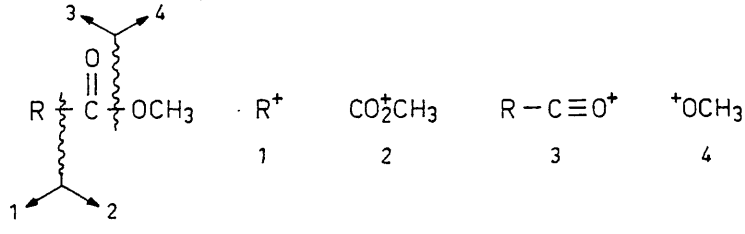
cyclic compound. Glucuronic acid gave two peaks with very similar mass spectra. These two peaks could be due to the silyl derivatives of two isomers of glucuronic acid.

(a) MASS SPECTRA OF METHYLATED DERIVATIVES OF ORGANIC ACID STANDARDS

The principal cleavage of carbonyl compounds is alpha to the C=O group;



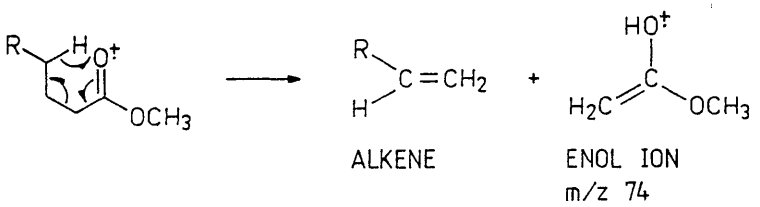
The formation of acylium ions by loss of $H^{\bullet}$, $R'^{\bullet}$, $R'O^{\bullet}$, $HO^{\bullet}$ and $H_2N^{\bullet}$ is an important diagnostic feature of the mass spectra of aldehydes, ketones, esters, acids and amides, respectively. In the fragmentation of methyl esters four possible ions can be formed by cleavage alpha to the carbonyl group:



The formation of the acylium ion by loss of a CH_3O radical (m/z 31) and the loss of a CO_2CH_3 radical (m/z 59) are two frequently observed fragmentations in the spectra of methyl esters and can be seen for many of the standard acids listed in Table 3.3.1.

Another useful feature in identifying carbonyl compounds is the loss of an alkene from the substituent group, with hydrogen transfer to the carbonyl oxygen.

This fragmentation is called the McLafferty rearrangement since F.W. McLafferty first observed this reaction when studying the mass spectra of a series of aliphatic methyl esters in the 1950's. The McLafferty rearrangement is illustrated below:



ALKENE

ENOL ION
m/z 74

The base peak in the mass spectrum of methyl hexanoate is the McLafferty rearrangement ion at m/z 74.

The aromatic methyl esters listed in Table 3.3.1 all contain a distinctive molecular ion and have an M-31 ion as the base peak. Another dominant fragmentation seen in the mass spectra of the aromatic esters is the M-59 peak, mentioned earlier.

Benzoyl compounds also give characteristic m/z 105 and 77 peaks in their mass spectra. These fragmentations are illustrated in Figure 3.4.1. Benzyl compounds generally afford $C_7H_7^+$ (m/z 91) and its decomposition product $C_5H_5^+$ (m/z 65), as shown in Figure 3.4.2.

Indole-3-acetic acid and hippuric acid both follow the Nitrogen Rule by having odd molecular weights. A discussion of the spectra of methyl esters of amino acid conjugates is given in Section 3.4.3.

(b) MASS SPECTRA OF SILYLATED DERIVATIVES OF ORGANIC ACID STANDARDS

The mass spectra of silylated derivatives often

FIGURE 3.4.1: The m/z 105 and its decomposition products are characteristic of benzoyl compounds.

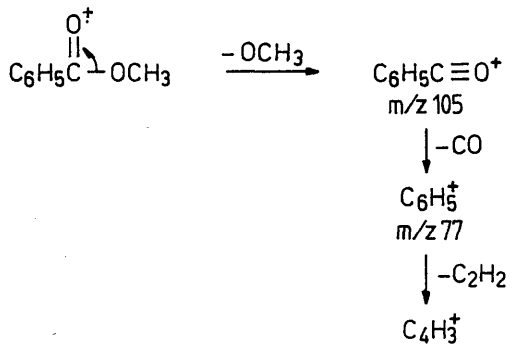
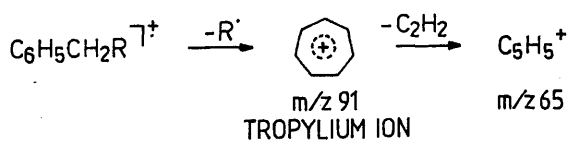
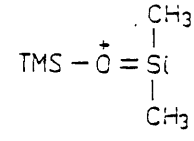


FIGURE 3.4.2: The m/z 91 ion and its decomposition products are characteristic of benzyl compounds.



contain intense ions at m/z 73, 74, 75, 76, 147 and 148, which have low diagnostic significance, the structures of the m/z 73 and 75 ions are illustrated in Figure 2.5.20. A ubiquitous ion found in compounds with two or more TMS groups occurs at m/z 147 and is formed by a rearrangement reaction [113]; its structure is depicted below:



m/z 147

Table 3.3.2 illustrates that the base peak in the mass spectra of silylated compounds is frequently at m/z 73 and the 147 ion is seen in many of the mass spectra listed including those of aconitic, azalaic, glutaric and pimelic acids.

It is evident from Table 3.3.2 that the molecular ions of silylated compounds are frequently not detected or are of low relative abundance. However, a fragment ion at M-15, arising by loss of a methyl radical from a TMS function, is always present and serves to indicate the molecular weight. A fragment equivalent to the McLafferty rearrangement ion at m/z 74 in methyl esters is at m/z 132 in the spectra of trimethylsilyl esters and is found in cases where the gamma hydrogen rearrangement is possible. As for the aromatic methyl esters, the aromatic silyl esters often contain ions at m/z 77, 91 and 105. The silyl esters of indole-3-acetic and hippuric acids also conform to the Nitrogen Rule and have molecular weights of 319 and 215, respectively.

(c) MASS SPECTRA OF tert-BUTYLDIMETHYLSILYLATED DERIVATIVES OF ORGANIC ACID STANDARDS

The BDMS derivative has not previously been used for the identification of urinary organic acids. Like the silylated derivatives, the spectra of BDMS derivatives also contain intense ions at m/z 73, 75 and 147 and aromatic BDMS esters exhibit ions at m/z 77, 91 and 105.

Table 3.3.3 illustrates that the BDMS esters do not normally exhibit a molecular ion. An M-1 ion of low intensity, however, is often present and indicates the molecular weight. A more useful diagnostic ion invariably seen at M-57 is due to loss of the tertiary butyl radical.

3.4.2 NORMAL URINARY ACID PROFILE OF THE RACING GREYHOUND

In order to obtain the average "normal" urinary acid profile of the racing greyhound, several attempts were made to collect twenty-four hour urine samples. It was not always possible to collect a complete twenty-four hour urine due to problems encountered with the animals. Although the animal was kept in the metabolic cage for a twenty-four hour period it frequently would not urinate in the cage. On release the animal would urinate and the animal nurses would attempt to collect a full sample, but this was not always possible. Catheterisation was tried on a few occasions, but this was thought to be too traumatic to the animals and to carry too high a risk of infection to carry out on a regular basis. In consequence the average profile was taken on pooled urine samples collected over an extended period of several days. Individual samples in this series were analysed separately as discussed in Section 3.3.2.

3.4.3 COMPARISON OF DERIVATIVES AND IDENTIFICATION OF GREYHOUND URINARY ACIDS

The chromatogram of the methylated extract was less complex than the other three chromatograms. This is most likely due to the fact that various hydroxylated compounds were not methylated on the hydroxyl functions and were too polar to chromatograph successfully. By contrast, the hydroxy groups can be silylated and also tert-butyl-dimethylsilylated, giving more complex profiles. When several urine extracts were chromatographed in sequence it was noted that the injection liner and the top of the column became contaminated. As a result, the injection liner was frequently cleaned and silylated to prevent polar material adsorbing on the inner glass surface. To maintain good chromatographic performance the front few centimetres of the column were removed when the chromatography was observed to have deteriorated. Current alternatives to this procedure are washing of the column (bonded phases) and incorporation of a retention gap to protect the analytical column.

The remainder of this section deals with the analysis of greyhound urinary acids as their methyl esters and as their trimethylsilyl derivatives.

(a) MASS SPECTRA OF ORGANIC ACIDS FOUND IN A METHYLATED EXTRACT OF GREYHOUND URINE

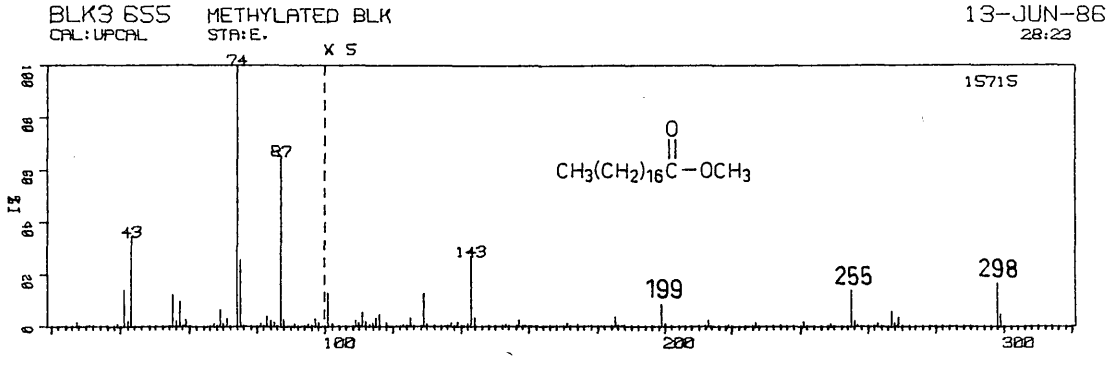
The mass spectra of long-chain aliphatic methyl esters have been described extensively by Ryhage and Stenhagen [163 and references therein]. The molecular ions are present and increase in intensity with increasing

chain length. The M-31 ion, conversely, decreases in intensity with increasing chain length. In addition to the m/z 74 base peak (already mentioned in Section 3.4.1), a dominant ion in methyl ester spectra is found at m/z 87. This is the most intense ion in a series which corresponds to m/z (59 + 14n), i.e. at 73, 87, 101, 115, 129, 143, 157, etc. The lowest member, m/z 73, is insignificant, whereas the abundance of m/z 87 is high and is favoured by resonance stabilisation. Ryhage and Stenhagen point out that in this ion series there is a periodic intensity enhancement every four methylene groups at m/z 143, 199, 255, etc. This can be clearly seen in the spectrum of methyl stearate (Figure 3.4.3). The other long-chain fatty acids identified also show evidence of these trends.

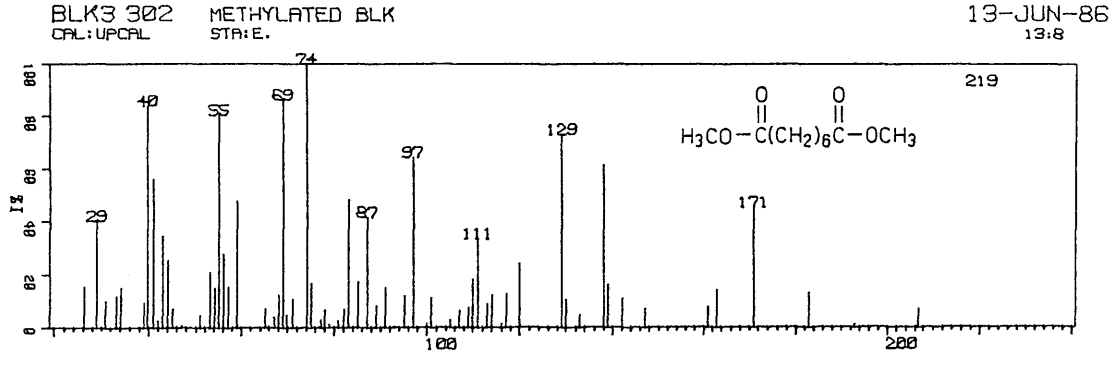
The spectra of the methyl esters of dibasic acids are more complex and differ considerably from the monobasic compounds. They have low-abundance molecular ions but usually M-31 peaks are evident. In addition to the (59 + 14n) series of ions characteristic of the monobasic esters two other series may be evident, i.e. (27 + 14n), present in all diesters, and (84 + 14n). Evidence of these three series is present in the spectra of suberic and azelaic acids (Figure 3.4.3) identified in the extract of greyhound urine.

A large number of aromatic compounds was identified in the urine extract, about fourteen of them as their methyl esters.

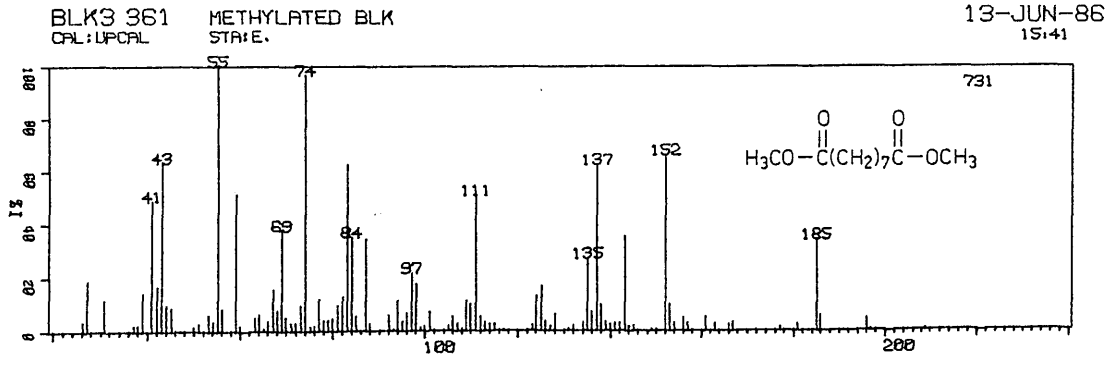
FIGURE 3.4.3: The mass spectra of the methyl esters of stearic, suberic and azelaic acids obtained from the GC-MS analysis of a methylated extract of greyhound urine.



Stearic Acid Methyl Ester



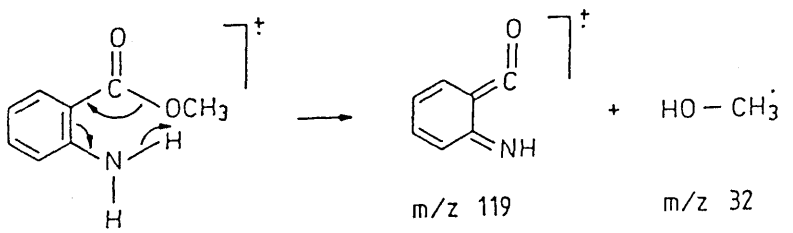
Suberic Acid di-Methyl Ester



Azelaic Acid di-Methyl Ester

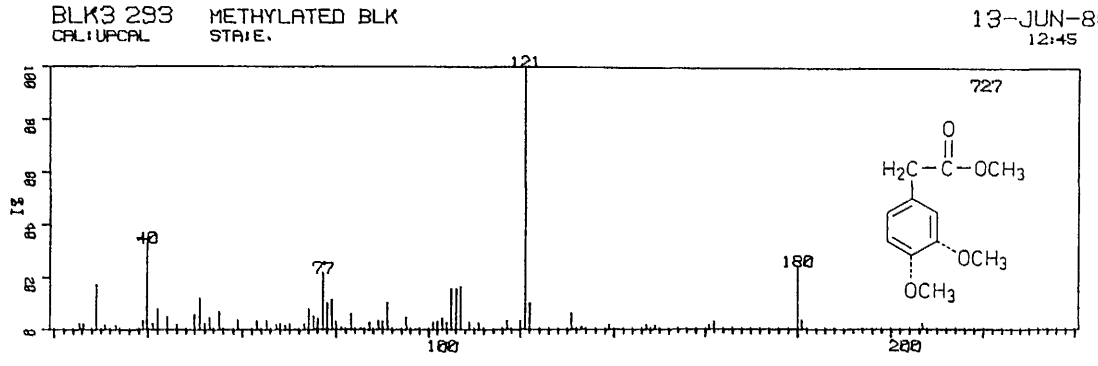
The presence of the aromatic ring appears to confer great stability on the molecular ions, which are much more prominent than for aliphatic compounds. Generally speaking all of the aromatic methyl esters identified did show prominent molecular ions. The other ions present in the spectra of aromatic compounds, m/z 77, 91 and 105, already mentioned in Section 3.4.1 are evident in the spectra listed in Table 3.3.4. Two examples of the spectra obtained for aromatic compounds are given in Figure 3.4.4.

The nitrogen-containing acids identified all had odd molecular weights. An unusual nitrogen-containing acid identified in an extract of the greyhound urine was 2-amino-benzoic acid (peak No.5 in Table 3.3.4). This compound illustrates a phenomena known as the '<u>ortho</u> effect'. When two substituents are <u>meta</u> or <u>para</u> they cannot interact through space, but when they are <u>ortho</u> they can exhibit the '<u>ortho</u> effect'. For instance, the base peak in the spectrum of the methyl ester of 2-amino-benzoic acid is at m/z 119 which is a loss of 32 (CH_3OH), instead of the usual loss of 31 ($-OCH_3$). This could be explained by the hydrogen transfer illustrated below:

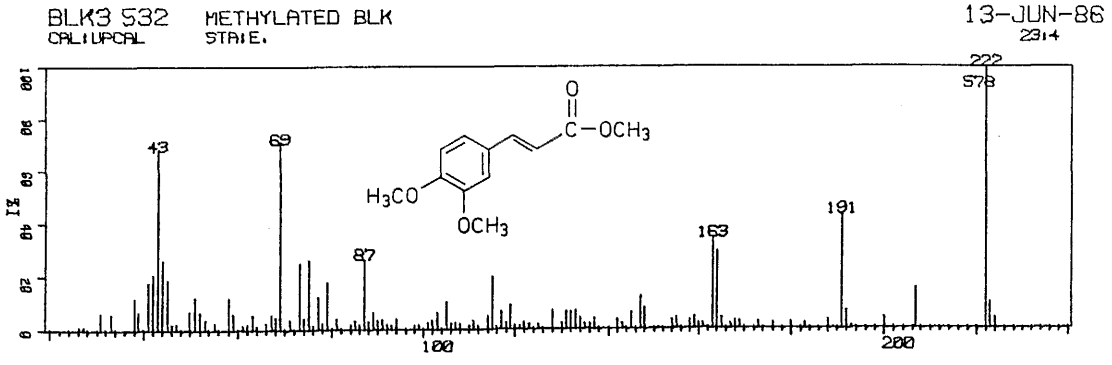


A similar ortho effect is seen for mefenamic acid (Figure 2.5.12, p135).

FIGURE 3.4.4: The mass spectra of the methyl esters of methoxy phenylacetic and 3,4-dimethoxy-cinnamic acids obtained from the GC-MS analysis of a methylated extract of greyhound urine.



Methoxy - Phenylacetic Acid Methyl Ester

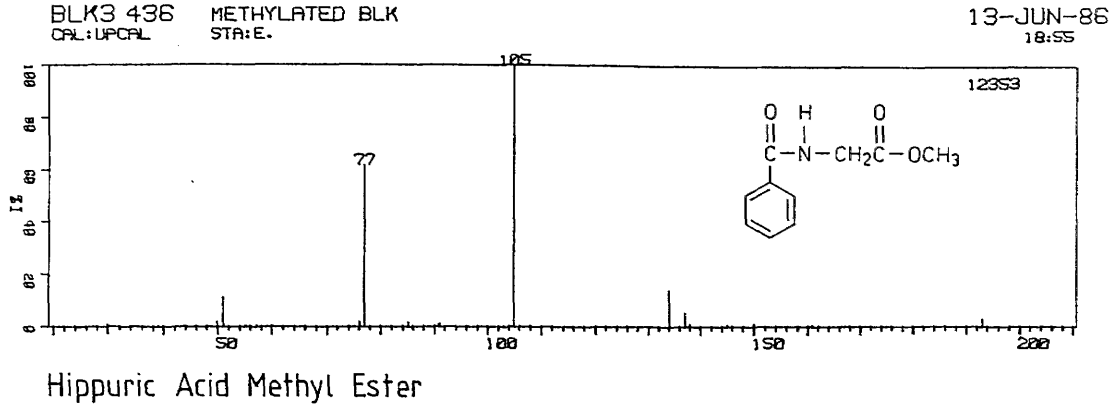


3,4 - Dimethoxy - Cinnamic Acid Methyl Ester

Another group of compounds which have been tentatively assigned structures are methyl esters of various amino acid conjugates. Ramsdell and co-workers [159] studied the spectra of twenty-one N-acylglycines as their methyl esters. All of the N-acylglycines identified in the greyhound urine extract were aromatic and all of their mass spectra contained molecular ions. Hippuric acid was available as an authentic standard therefore its mass spectrum was positively identified. The mass spectrum of hippuric acid is very simple and shows two main features associated with the glycine side chain; M-59 due to loss of the carbomethoxy group and M-88 which results from cleavage of the amide bond CO-NH with retention of charge on the acyl group (RCO^+) (Figure 3.4.5). The other acid conjugate structures were then tentatively identified from their molecular weights and from a knowledge of the fragmentation of the methyl ester of hippuric acid. The M-88 ion, seen in the spectrum of the methyl ester of hippuric acid, formed either the base peak or a major ion in almost all of the spectra, exceptions being phenylacetylglycine and its 4-hydroxy analogue. These molecules retain the charge on the glycyl group to give m/z 88 as a major ion.

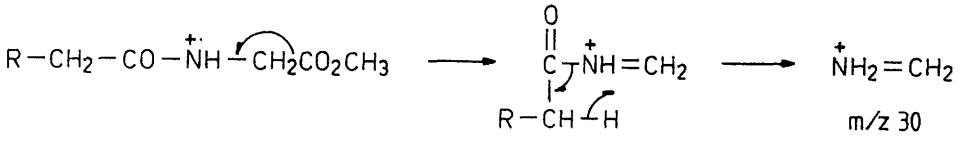
Two other fragmentations involving the amide group are also of significance, namely cleavage of the R-CO bond to yield M-116 and the formation of m/z 30. High-resolution mass measurement of the latter has shown

FIGURE 3.4.5: The mass spectrum of the methyl ester of hippuric acid obtained from the GC-MS analysis of a methylated extract of greyhound urine.



Hippuric Acid Methyl Ester

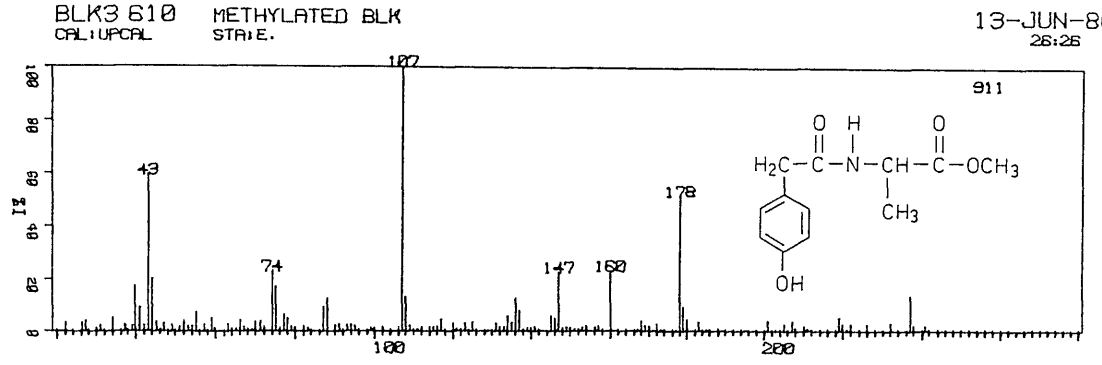
it to have the composition $CH_2 = {}^{+}NH_2$ [159] and it is probably formed as shown below:



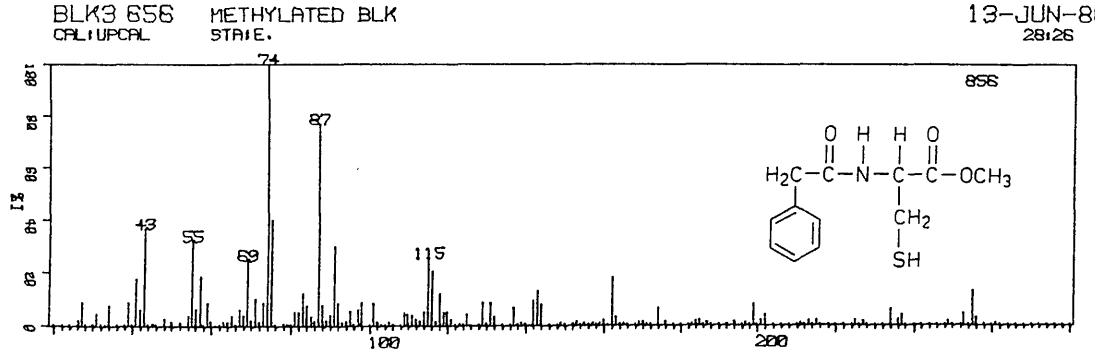
A significant ion in almost all spectra is $[M-116]^+$ and this is the base peak in cases where the ion is stabilised, for example, phenylacetylglycine.

In addition to glycine conjugates, three other conjugates were identified in the urine of the greyhound, namely, 4-hydroxy-phenylacetyl-alanine, phenylacetyl-cysteine and N-acetyl-tryptophan (Figure 3.4.6). The spectrum of 4-hydroxy-phenyl-acetyl-alanine has its base peak at m/z 107. This ion arises from M-130, a homologous equivalent to the M-116 ion in the glycine conjugate spectra. The presence of an ion at m/z 34 (H_2S) supports the fact that component number 52 in Figure 3.3.6 is a cysteine conjugate.

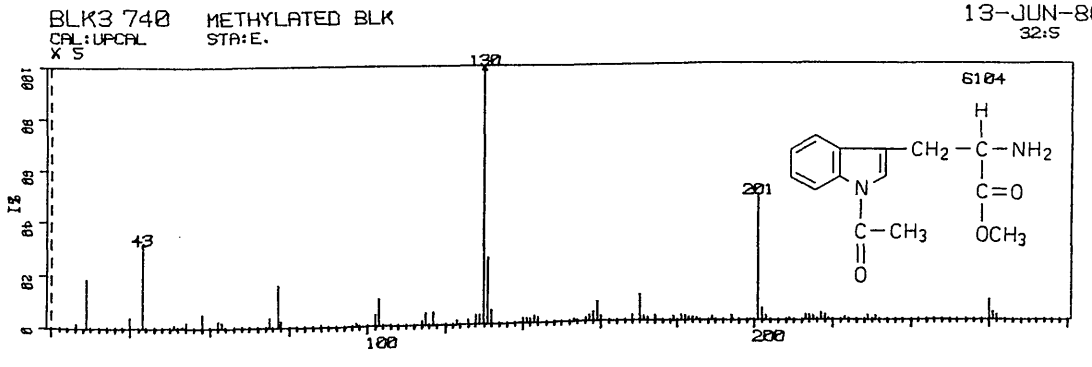
FIGURE 3.4.6: The mass spectra of the methyl esters of 4-hydroxy-phenylacetyl-alanine, phenylacetyl-cysteine and N-acetyl-tryptophan obtained from the GC-MS analysis of a methylated extract of greyhound urine.



4 - Hydroxy - Phenylacetyl - Alanine Methyl Ester



Phenylacetyl - Cysteine Methyl Ester



N - Acetyl - Tryptophan Methyl Ester

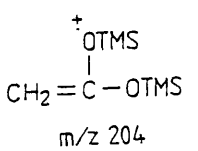
(b) MASS SPECTRA OF ORGANIC ACIDS FOUND IN A SILYLATED EXTRACT OF GREYHOUND URINE

As mentioned earlier, the aliphatic acid TMS esters give molecular ions of low intensity, although a fragment ion at M-15 is always present and serves to indicate the molecular weight of the compound. The spectrum of hydroxy-heptanoic acid TMS ester (Figure 3.4.7) illustrates many of the features common in spectra of short-chain hydroxy-acids. The presence of the silyl ether in the chain results in the alpha cleavage ion m/z 131, where the charge has been retained on the TMS-ether fragment. Retention of charge on the carboxy ether gives m/z 117. The fragmentation pattern indicates that the hydroxy group is either in the 3- or 5-position.

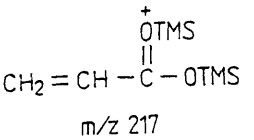
In long-chain acids, a series of carboxy ester-containing ions analogous to those in the mass spectra of methyl esters is observed at m/z 145, 159, 173, etc. This series also shows some periodicity, favouring ions at 145, 201, 257, etc. In addition, methane is lost from these to give a second lower-intensity ion series (m/z 129, 185, 269 ....).

The McLafferty rearrangement peak is prominent at m/z 132, as previously mentioned.

The mass spectra of diacids show the presence of additional fragmentation pathways resulting from the additional silyl group, notably the presence of rearrangement products such as the ions at m/z 204 and 217 which have the structures shown below [164]:-

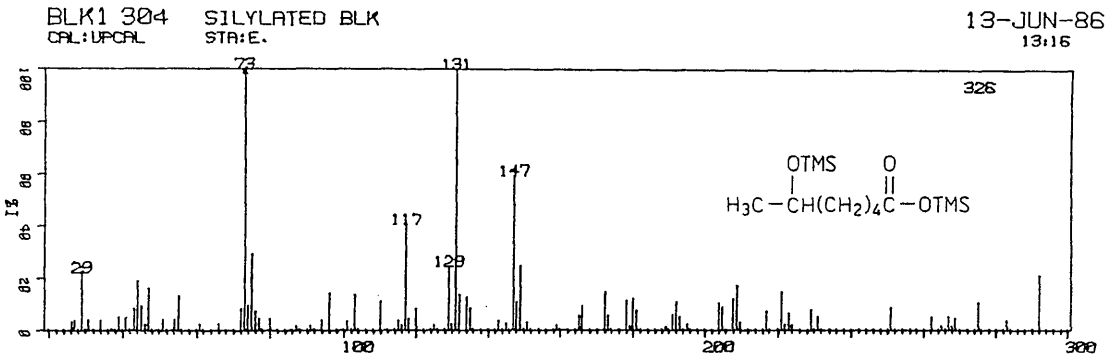


m/z 204



m/z 217

FIGURE 3.4.7: The mass spectrum of the silylated ester of hydroxy-heptanoic acid obtained from the GC-MS analysis of a silylated extract of greyhound urine.



Hydroxy - Heptanoic Acid TMS Ester

These ions are considered to be equivalent to the hydrogen rearrangement ions at m/z 74 and 87 respectively in the spectra of methyl esters. As noted above, the additional silyl group also gives rise to a commonly-observed ion at m/z 147, which is useful as an indicator that at least two silyl groups are present. Finally, the spectra of the dibasic acids show the presence of ions resulting from decarboxylation (M-44, for example m/z 245 in the spectrum of pimelic acid), although in the present study these did not reach the same degree of prominence as in previous work [164].

The fragmentation patterns of aromatic acid silyl derivatives are influenced by the presence of the aromatic ring which confers the molecule with some stability, resulting in visible, and in some cases prominent, M^{+} and $M-15^{+}$ ions. As expected, the fragmentation is also directed by the ester moiety, giving rise to characteristic ions according to the location of the carboxyl group - on the ring or isolated from it by one or more methylene carbon atoms. These features are shown by the three examples given in Figure 3.4.8. In the spectrum of 4-HO-benzoic acid, decarboxylation occurs from the M-15 ion by a cyclic mechanism [165]:

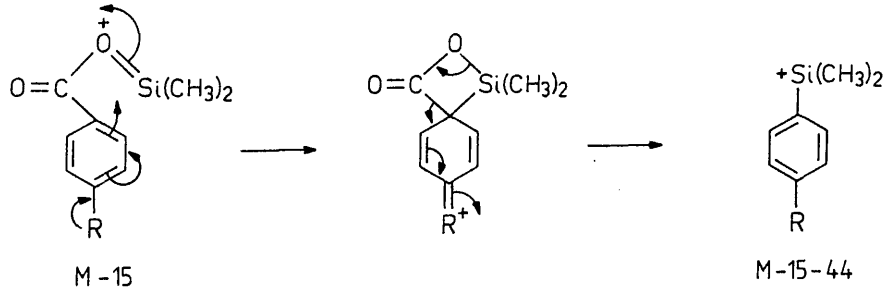
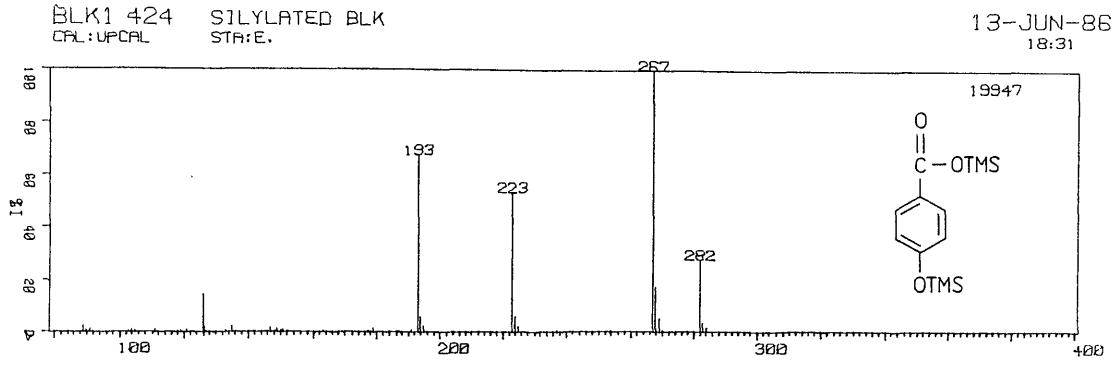
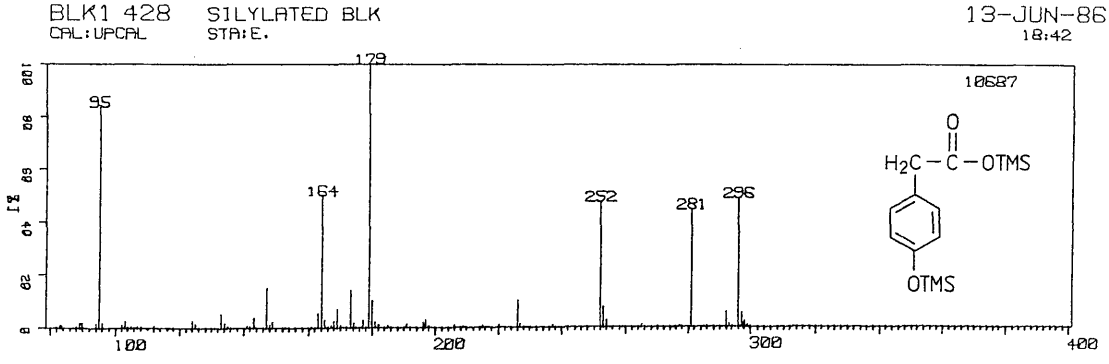


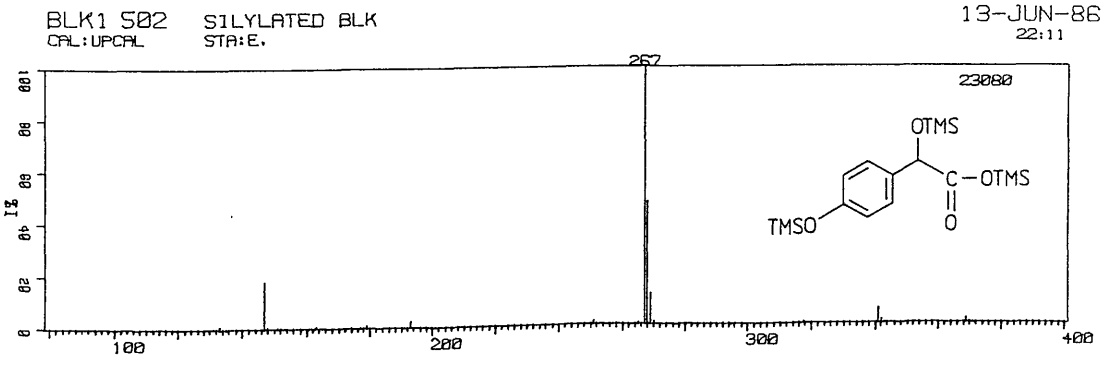
FIGURE 3.4.8: The mass spectra of the silylated esters of 4-hydroxy-benzoic, 4-hydroxy-phenylacetic and 4-hydroxy-mandelic acids obtained from the GC-MS analysis of a silylated extract of greyhound urine.



4-Hydroxy-Benzoic Acid di-TMS



4-Hydroxy-Phenylacetic Acid di-TMS



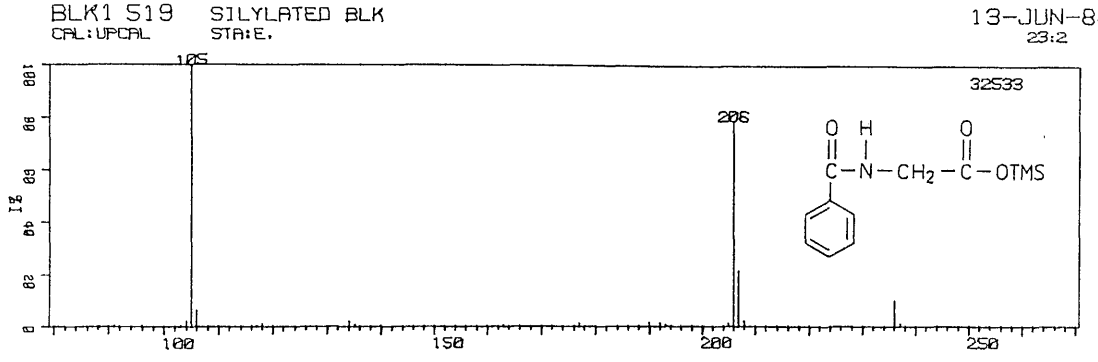
4-Hydroxy-Mandelic Acid tri-TMS

When the carboxy group is on an aliphatic carbon atom, as in phenyl acetic acid and its analogues, this mechanism is no longer possible and decarboxylation occurs directly from the molecular ion.

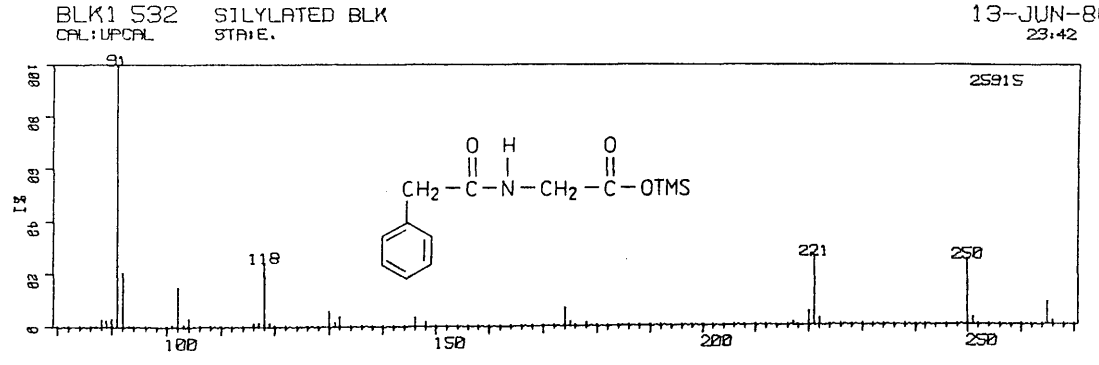
In 4-HO-benzoic acid, loss of the silyloxy group gives rise to an M-89 ion rather than the more familiar M-90 ion, because the carboxy group is directly substituted on the aromatic ring. By contrast, the structure of 4-HO-phenylacetic acid allows the possibility of loss of trimethylsilanol (M-90) and this ion is indeed visible at m/z 206, though at low intensity. The preferred mechanism is, instead, loss of the carboxysilyl group (M-117), giving rise to the base peak at m/z 179. The presence of an additional directing group on the side chain, such as the trimethylsilyloxy group in 4-HO-mandelic acid, further promotes this fragmentation and in this instance most of the ion current is carried by the M-117 ion at m/z 267.

Differences between aromatic and aliphatic acids also exist when these are conjugated with amino acids:- the spectra of hippuric acid and N-phenylacetyl-glycine are illustrated for comparison in Figure 3.4.9. Under the conditons used for silylation, the amide nitrogen was not derivatised. In the spectrum of hippuric acid, decarboxylation occurs together with loss of a proton, giving rise to a product ion which is stabilised by the aromatic ring. By the same token, the expected cleavage reaction through the amide carbon-nitrogen bond leaves the

FIGURE 3.4.9: The mass spectra of the silylated esters of hippuric acid and N-phenylacetyl-glycine obtained from the GC-MS analysis of a silylated extract of greyhound urine.



Hippuric Acid TMS Ester



N-(Phenylacetyl)-Glycine-TMS Ester

charge on the aromatic moiety and not on the aliphatic residue. In the spectrum of N-phenylacetyl glycine, decarboxylation gives rise to an M-44 ion but cleavage of the amide is accompanied by further loss of a hydrogen atom to give the ion at m/z 118, which is presumably also stabilised by delocalisation of the charge into the aromatic ring.

3.5 CONCLUSION

It is apparent from a consideration of Tables 3.3.4-7 that some components in the extracts remain unidentified and that the number of unidentified substances is greatest in the BDMS and methyl ester/silyl ether samples. This reflects the general problem of identifying unknown materials purely on the basis of their mass spectra, a difficult and skillful task which depends greatly on the availability of reference data. In the present instance, few data were available for those two derivatives and identification of acids was based on a knowledge of the types of acid present and on the presence of ions in the mass spectra consistent with the proposed structures.

It is concluded that, while novel derivatives such as the BDMS derivative yield useful mass spectra characterised by the presence of valuable diagnostic ions, a substantial amount of work requires to be done to enable them to be used effectively in screening for unknown substances, notably to establish a database of spectra of urinary acids derivatives.

Subsequent studies of the metabolism of NSAIDs were based on methylated extracts for this reason. Also, because the metabolic work was carried out in parallel with the urinary acid analysis, an early decision required to be taken concerning which derivative to use and the methyl derivatives were chosen because of the simpler nature of the chromatogram.

CHAPTER FOUR

DRUG METABOLISM

4.1 INTRODUCTION

In developing a screening procedure to comply, for example, with the International Olympic Committee's requirements, it is necessary for the analyst to know:

(a) The structures of the target analytes.

(b) The excretion rates and routes.

(c) The time period over which excretion takes place.

(d) The time period over which the methodology can detect the target analytes.

Having established the urinary acid profile for the greyhound, and selected derivatisation procedures and analytical methods, it was necessary to compile data on the metabolism and pharmacokinetics of individual NSAIDs. The development of a general screening procedure requires GC retention and mass spectral data for the metabolites as well as the parent drugs. While some information is available on the metabolism of these compounds in humans and other mammals, including rats, rabbits and beagle dogs, it was expected that both the metabolism and kinetics would differ in the greyhound, notably because of its lean physique and small fat depot.

An additional requirement of the N.G.R.C. is that drugs and their metabolites must be analysed quantitatively in urine to allow an interpretation to be

made of the significance of drugs detected.

Since the volume of urine excreted varies according to the condition of the dog the concentration of drugs present will also vary. A common method for compensating for these differences is to relate the urinary drug level to the creatinine concentration. Creatinine is excreted from the body at a constant rate [166]. This approach was used in the present study.

The following sections deal with the metabolism of five selected NSAIDs which are amongst the most commonly used drugs of this type and which were considered to be suitable model compounds. Ibuprofen is readily available as a non-proprietary drug and phenylbutazone has been previously detected in the course of routine race track testing.

4.2 GENERAL PHARMACOLOGY OF THE NSAIDs

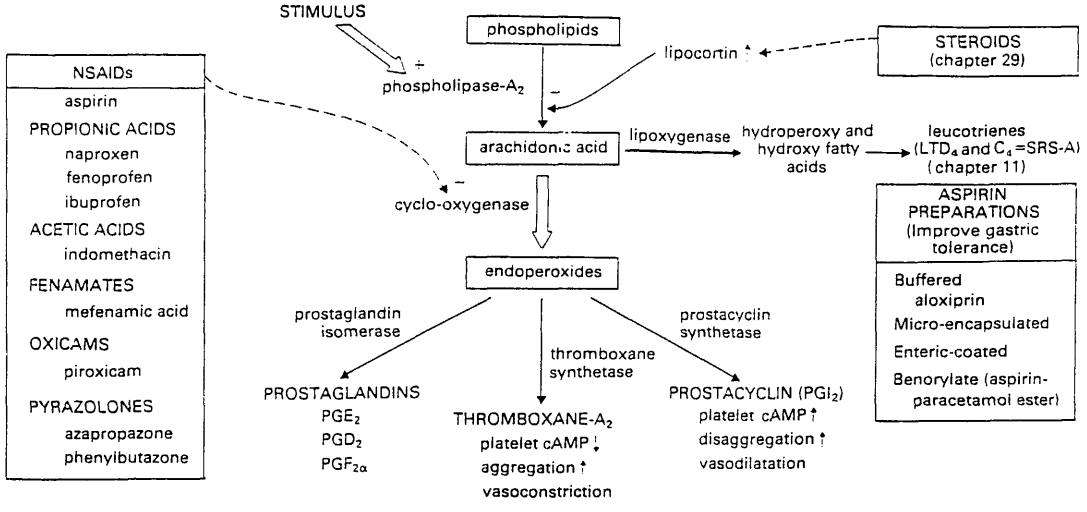
Non-steroidal anti-inflammatory drugs have been in use for more than a century. The active ingredient in the willow bark, salicin, was first discovered by Leroux in 1827 and in November of 1874, Thomas MacLagan [167], a Dundee physician first used salicin to treat a patient with rheumatic fever. Acetylsalicylic acid (aspirin) was synthesised in 1853, but the drug was not used until 1899. Since then it has remained one of the most widely employed remedies.

Due to the side effects of salicylic acid-based drugs, considerable effort has been expended to develop new compounds with similar pharmacological properties,

but with fewer side effects. In the 1950s the first successful alternative was mefenamic acid, followed in 1963 by indomethacin and in 1964 by ibuprofen. Many hundreds of analogues of these compounds have been synthesised but only about twelve are commonly prescribed in the U.K. (Table 1.3.1).

Although the NSAIDs have a wide range of chemical structures, they share a common set of pharmacological effects (anti-inflammatory, anti-pyretic and analgesic) which are best understood on the basis of their mechanism of action (Figure 4.2.1). This lies in the inhibition of prostaglandin biosynthesis at the step catalysed by cyclo-oxygenase [169]. While the detailed mechanism of this effect is not fully understood, available evidence suggests that the drugs bind to a site on the enzyme which is not the active site but is sufficiently close to it to inhibit the enzyme's catalytic activity. Prostaglandins are involved in the pathogenesis of inflammation and fever [5]. Prostaglandins of the E and F groups are involved in the mediation of the inflammatory response [170] and a rise in the concentration of prostaglandins in the brain is associated with disturbance of the thermoregulatory centre in the hypothalmus causing an increase in body temperature (PGE_1 is one of the most potent pyrogens known). The inhibition of prostaglandin synthesis in inflamed tissues also results in an analgesic effect. NSAIDs are effective against pain of low to

FIGURE 4.2.1: Mode of action of non-steroidal anti-inflammatory drugs.*



* Reproduced from Reference 168.

moderate intensity, especially where this is peripheral.

The commonest adverse effects of therapeutic doses of NSAIDs are dyspepsia and gastric and intestinal ulceration with consequent anaemia due to occult blood loss. These side effects may also be related to the inhibition of prostaglandin biosynthesis. For example, the gastric mucosa synthesises PGI_2 and the gastric erosion caused by indomethacin and other drugs in experimental animals can be prevented by administration of prostaglandins.

An additional side effect, which is used beneficially in the treatment of patients suffering from coronary heart disease, is the inhibition of the formation of blood clots because NSAIDs prevent the formation of thromboxane A_2, a potent aggregating agent, by the platelets.

4.3 EXPERIMENTAL

4.3.1 REAGENTS

In addition to the reagents listed in Section 3.2.1 the following were used in the drug metabolism work:

1. Anhydrous sodium acetate (BDH Chemicals Limited, Poole, England).
2. Glacial acetic acid (BDH Chemicals Limited, Poole, England).
3. Helix pomatia enzyme mixture (Uniscience Limited, London).
4. 2,3-Dicarboxy-naphthalene (Sigma Chemicals, Poole, England).

4.3.2 DRUG STANDARDS

The drug standards used were ibuprofen, ketoprofen, naproxen, mefenamic acid, phenylbutazone and flurbiprofen (as the internal standard). The suppliers of these drugs are listed in Section 2.3.2.

4.3.3 DRUG FORMULATIONS

The doses of drug administered to the greyhound were as follows:

Brufen (ibuprofen)	- 200mg
Orudis (ketoprofen)	- 50mg
Naproxyn (naproxen)	- 250mg
Ponstan (mefenamic acid)	- 250mg
Butazolidin (phenylbutazone)	- 100mg

4.3.4 PREPARATION OF SODIUM ACETATE BUFFER

REAGENTS:

0.5M Acetic Acid - 2.9ml of glacial acetic acid in 100ml of distilled water.

0.5M Sodium Acetate - 8.203g of anhydrous sodium acetate in 200ml of distilled water.

Acetic acid (0.5M,100ml) is mixed with sodium acetate (0.5M,150ml) and the pH was adjusted to 4.6 with concentrated hydrochloric acid.

4.3.5 PREPARATION OF STANDARD SOLUTIONS

About 5mg of drug standard was accurately weighed into a 50ml volumetric flask. The drug was dissolved in methanol and the solution made up to the mark with methanol and mixed well. An aliquot (250µl) of the appropriate standard solution (ibuprofen, ketoprofen, naproxen, mefenamic acid or phenylbutazone) and an aliquot (250µl) of the internal standard solution (flurbiprofen) were mixed and to this solution was added a freshly prepared solution of diazomethane until the solution remained yellow. The solution was then allowed to stand at room temperature for 15 minutes before evaporating to dryness under a stream of oxygen-free nitrogen. The residue was redissolved in methanol (250µl) and 0.1-0.5µl was used for GC-MS analysis.

4.3.6 CALCULATIONS

Drug concentrations were calculated using the formula:

$$\frac{A_2}{A_1} \times C = \text{Concentration of drug in unknown } (\mu g/\mu l)$$

where:

A_1 = peak area ratio of drug to internal standard for the standard solution.

A_2 = peak area ratio of drug to internal standard for the unknown solution.

C = concentration of drug present in the standard solution (about 100µg/ml).

4.3.7 PROTOCOL FOR THE ADMINISTRATION OF DRUGS AND THE COLLECTION OF BLOOD AND URINE SAMPLES

Greyhounds were administered with a single dose of an anti-inflammatory drug (quantity given in Section 4.3.3) and blood and urine samples were taken by licensed kennel nurses according to a specified protocol. An example of a protocol sheet is given in Table 4.3.1.

The timetable for the collection of samples was supplied to the kennel nurses along with the drug to be administered. The times given were to be used as a guide and could be changed to fit in with normal routine in the kennels. If, however, the sampling times were changed the nurse could make a note of the actual sampling time on the sheet.

Blood samples were collected from a vein in the neck in heparinised tubes and were gently mixed immediately after collection. They were then centrifuged (3500 rpm,15 minutes) and the plasma obtained transferred to polypropylene tubes. Urine samples were collected in a stainless steel jug, then transferred to polypropylene containers for carriage. All plasma and urine samples

TABLE 4.3.1: Timetable for collection of blood and urine.

DOG USED:

DRUG ADMINISTERED:

QUANTITY

TIME AND DATE OF DOSING

BLOOD		URINE	
Proposed time after dosing	Actual time after dosing	Proposed time after dosing	Actual time after dosing
Clear sample		Clear Sample	
1 hour(about 11 am)			
2 hours(about 12 noon)		2 hours(about 12 noon)	
3 hours(about 1 pm)			
4 hours(about 2 pm)			
6 hours(about 4 pm)		6 hours(about 4 pm)	
24 hours(about 10 am on day 2)		24 hours(about 10 am on day 2)	
		30 hours(about 4 pm on day 2)	
		48 hours(about 10 am on day 3)	

were stored at -20°C until analysed.

A washout period of about three weeks was allowed before an animal was administered with another drug.

4.3.8 EXTRACTION AND DERIVATISATION OF URINE SAMPLES

Urine samples were extracted and derivatised according to the following procedure:

1. Urine (5-20ml) was acidified with 1M HCl.
2. The acidified urine was loaded on a freshly prepared column of XAD-2 resin (preparation of column described in Section 3.2.5).
3. The column was washed with 200ml distilled water (flow rate 4ml/min).
4. The column was allowed to drain.
5. Organic material retained on the column was then eluted with 100ml ethanol (flow rate 2ml/min).
6. The eluate was evaporated to dryness on a rotary evaporator.
7. Sodium acetate buffer (pH 4.6, 20ml) was added and the tube sonicated for ca 30 seconds. Helix-pomatia enzyme mixture (300µl) was then added and incubated at 37°C for 24 hours to hydrolyse conjugates.
8. After incubatation was complete the mixture was allowed to cool to ambient laboratory temperature and then the total extract (including the enzymes and buffer) was loaded onto the XAD-2 column.
9. Steps 3-6 were repeated.
10. The residue was quantitatively transferred to a vial with methanol and evaporated to dryness with oxygen-free nitrogen.

11. The extract was redissolved in methanol containing the internal standard (flurbiprofen, 100µg/ml, 250µl) and freshly-prepared ethereal diazomethane (500µl) was added. The solution was allowed to stand at room temperature for 15 minutes, then evaporated to dryness under a stream of oxygen-free nitrogen.

12. The extract was redissolved in methanol (250µl) and 0.1-0.5µl was used for GC-MS analysis.

4.3.9 EXTRACTION AND DERIVATISATION OF PLASMA SAMPLES

Plasma samples were extracted and derivatised according to the following procedure:

1. A 1ml plasma sample was transferred to an extraction tube, then internal standard (flurbiprofen, 100µg/ml, 100µl) was added and vortex-mixed.

2. The mixture was acidified with 1M HCl (250µl), then extracted with heptane: ethyl acetate, 4:1 (6ml) by vortexing for ca 30 seconds.

3. The tube was then centrifuged (3500 rpm, 10 minutes).

4. An aliquot (4ml) of the organic layer was removed to a fresh tube and evaporated to dryness under a stream of oxygen-free nitrogen.

5. The extract was redissolved in methanol and a freshly prepared solution of diazomethane (500µl) was added. The solution was allowed to stand at room temperature for 15 minutes, and was then

evaporated to dryness under a stream of oxygen-free nitrogen.

6. The extract was redissolved in methanol (100µl) and 0.1-0.5µl was used for GC-MS analysis.

4.3.10 GAS CHROMATOGRAPHY/MASS SPECTROMETRY CONDITIONS

GC-MS was carried out on a system similar to that described in Section 2.5.2.

4.4 IBUPROFEN

4.4.1 INTRODUCTION

As mentioned earlier, ibuprofen was the first commercially successful drug in the series of substituted propionic acid derivatives. Naproxen and ketoprofen are also in this category and will be dealt with in Sections 4.5 and 4.6. Ibuprofen is readily available and can be purchased in Britain without a prescription. Basic information about ibuprofen is summarised in Table 4.4.1.

Ibuprofen is rapidly and almost completely absorbed from the gastro-intestinal tract following oral administration, giving rise to peak plasma levels after 1-2 hours. Therapeutic concentrations in plasma are usually in the range 20-30µg/ml. A review of the literature relating to toxicity of ibuprofen indicates that toxic effects in dogs might become apparent following single oral doses of 125mg/kg [171] and in humans following doses of approximately 200mg/kg [172]. In two reports of attempted suicide plasma concentrations of ibuprofen in the range 400-840µg/ml were measured. Although one subject was comatose all recovered within 24 hours [173 & 174].

Ibuprofen has an asymmetric carbon and exists as R(-) and S(+) optical isomers. The marketed preparation is racemic consisting of equal parts of both isomers. The S(+) isomer of ibuprofen is much more active than the R(-) isomer as an inhibitor of cyclo-oxygenase *in vitro*. However, *in vivo* the less potent R(-) isomer is converted

TABLE 4.4.1: Description and basic pharmacokinetic data for ibuprofen.

Chemical Name
α-Methyl-4-(2-methylpropyl)benzeneacetic acid

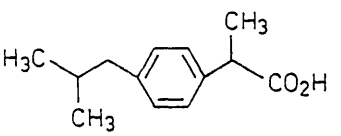
Chemical Abstracts Registry Number
15687-27-1

Non-Proprietary Name
Ibuprofen

Proprietary Names
Amersol, Apsifen, Brufen, Ebufac, Fenbid, Inabrin, Inflam, Librofen, Motin, Nurofen, Paxofen, Proflex, Relcofen, Seclodin and Uniprofen.

Empirical Formula
$C_{13}H_{18}O_2$

Structural Formula



Molecular Weight
206.27

Dissociation Constant
pKa = 4.4, 5.2

Physical Properties
A white crystalline stable solid. Melting point 75-77°C. Practically insoluble in water; soluble 1 in 1.5 of ethanol, 1 in 1 of chloroform and 1 in 2 of ether.

Dose
200, 400 and 600mg tablets.
Total daily dose up to 2400mg, although usually 1200-1800mg.

Plasma Half-Life
About 2 hours

Protein Binding
99%

Volume of Distribution
About 0.1 litre/kg

to the S(+) isomer; consequently, it is difficult to detect differences in the pharmacological activity of the two isomers in whole-animal studies [175]. The half-life of the active S(+) isomer is longer than that of the R(-) isomer [176].

4.4.2 METABOLISM AND EXCRETION

The metabolism of ibuprofen in mammals has been described by Mills and Co-workers [177]. In this and subsequent sections only humans and dogs will be considered.

Four metabolites have been identified in the urine of both species (Figure 4.4.1) although the relative proportions differ as shown in Table 4.4.2 [178-180]. Although ibuprofen disappears more slowly from plasma in dogs than in other species, the metabolites have not been detected in dog plasma suggesting that the metabolites are formed slowly and excreted rapidly. It has also been found that bile is an important route of excretion in the dog and about 25% of a dose is excreted in bile in the first three hours.

The literature data suggested the probable structures of ibuprofen metabolites in the greyhound but also that the relative proportions and excretion would be subject to species variation. In the present study published information concerning retention data and mass spectra of the known metabolites (Table 4.4.3) were used to search for the metabolites in greyhound urinary extracts.

FIGURE 4.4.1: The metabolism of ibuprofen.

Metabolites A and B have been characterised as the dextrorotatory (+) isomers (Reference 180).

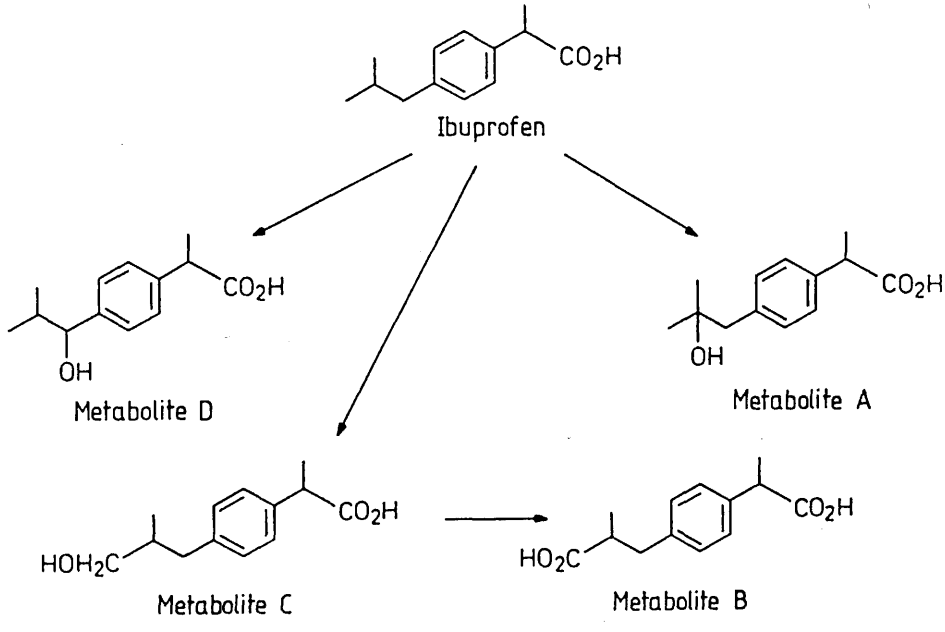


TABLE 4.4.2: Excretion of ibuprofen and its metabolites in urine.*

		Percentage of Dose Excreted	
		Man	Dog
Unconjugated	Ibuprofen	1	1
	Metabolite A	15	9
	Metabolite B	42	1
Conjugated	Ibuprofen	8	7
	Metabolite A	20	23
	Metabolite B	9	13
TOTAL		95	54

* taken from reference [172]

TABLE 4.4.3: Gas chromatographic and mass spectrometric data for the methylated derivatives of ibuprofen and three of its metabolites (from Reference 179).

COMPOUND NAME	Retention Index (OV-1,150°C)	MASS SPECTRAL CHARACTERISTICS									
		M^+	Intensity	OTHER IONS		(% ABUNDANCE BELOW)					
Ibuprofen	1515	220	19	161	177	117	119	19			
				100	35	22	22	21			
Metabolite A	1660	236	0.1	119	91	118	178	117	161		
				100	95	86	55	20	15		
Metabolite B	1775	264	23	205	145	177	117	91	118	121	204
				100	74	66	43	23	22	22	22
Metabolite D	1705	236	1	193	105	133	194	134			
				100	39	21	13	11			

4.4.3 RESULTS

Blood samples were collected from one greyhound and urine samples from two dogs according to the protocol described (Section 4.3.7). The samples were analysed according to the methods in Section 4.3. The results obtained from the analysis of ibuprofen in the plasma of animal 1 are given in Table 4.4.4 and are illustrated as concentration versus time in Figure 4.4.2 and as log concentration versus time in Figure 4.4.3. The results obtained for the analysis of ibuprofen in urine samples (expressed as µg/mL urine and µg/mg creatinine) are summarised in Table 4.4.5 and are illustrated in Figure 4.4.4.

Metabolites A and B were identified in samples of greyhound urine, their mass spectra are given in Figure 4.4.6. Metabolite A was detected in urine samples taken from animal 2 at 2, 6, 24 and 30 hours after administration. Metabolite B was detected in urine samples taken from animal 2 at 6 and 24 hours. Metabolites C and D were not detected. Relative concentrations of the metabolites in each sample were calculated from the peak areas of selected mass chromatograms for the metabolites and the internal standard. Estimated real concentrations were calculated by assuming that the total ion chromatogram (TIC) response ratios for the metabolites were the same as that for ibuprofen, which was quantified using an authentic standard (Table 4.4.6). Figure 4.4.5 illustrates the excretion of ibuprofen and its two metabolites.

The total ion current chromatograms obtained from extracts of greyhound urine taken at 2, 6, 24 and 30 hours after the administration of 200mg of ibuprofen are illustrated in Figure 4.4.7. These 4 chromatograms allow a comparison of the relative amounts of parent and metabolites present and also illustrates the compounds which may interfere in the analysis for ibuprofen and its metabolites.

Figure 4.4.8 illustrates the total ion chromatogram obtained from an extract of greyhound urine (6 hour sample) along with 4 mass chromatograms showing the positions of ibuprofen (m/z 205), metabolite A (m/z 178), metabolite B (m/z 205) and flurbiprofen, the internal standard (m/z 199).

4.4.4 DISCUSSION

In this study, a single 200mg dose of ibuprofen was administered to greyhounds of body weight 30kg, a dose of 6-7mg/kg and therefore higher than the normal adult dose in man of about 4mg/kg. The peak plasma level was observed 2 hours after dosage and was measured as 49µg/ml. This was consistent with the normal therapeutic range in man of 20-30µg/ml, considering the higher dosage level administered to the greyhounds. From the plasma data available for one greyhound (Figure 4.4.4, Table 4.4.3) a plasma half-life of about 1 hour can be estimated for the parent drug, using a single-exponential model to describe tbe fall in level as the small number of data points does not allow a more complex analysis to be

carried out. This half-life value indicates that ibuprofen may be cleared more rapidly in the greyhound than in man (half-life 2 hours) although a statistically significant number of trials would be necessary to make this conclusion valid. Nevertheless, if the drug is cleared rapidly, this may reflect the absence of a significant body fat depot, one of the expected features of greyhound metabolism.

In man, the literature suggests [172] that a total of about 8% of a dose of ibuprofen is excreted in the urine as either the parent drug or as its glucuronide conjugate. The present study indicates that a much lower fraction (0.1% of the dose) is excreted in urine as the parent drug or its conjugate in the first 24 hours. As mentioned below, it has not been possible to obtain a quantitative measurement of the total fraction of dose excreted in the urine as either the parent or metabolites: these measurements normally require the use of radiolabelled compounds which were not available in this study. The other major route of excretion *via* the bile and faeces was not examined.

Urinary concentrations are sometimes difficult to interpret because of variations in the urinary output and are often corrected using creatinine as an internal standard to allow for fluctuations in urinary output. The curves shown in Figure 4.4.4 for corrected and uncorrected concentrations of ibuprofen have the same shape, indicating that the urinary output was regular during the

course of the study. This was not true for all the metabolic experiments carried out (see below). In addition, a comparison of the curves for the two dogs used in the study indicates a significant variation between individuals during the absorption and excretion phases. These are influenced by many factors, including the concurrent diet, gastric pH and urinary pH. However, the latter was regularly monitored in the course of this work and was not found to vary sufficiently to affect urinary excretion to a significant extent. After about 30 hours, extremely small concentrations of ibuprofen (ca 10ng/mL urine) were present. The small levels were detected because the analysis was targeted for the drug using mass chromatograms i.e. for m/z 220 to locate methyl ibuprofen. However, in a general screening procedure, these peaks could have been easily missed or, if detected, the identification of the drug might remain uncertain.

Two known metabolites of ibuprofen were identified in greyhound urine by comparison of their mass spectra and gas chromatographic data with those published in the literature [176]. Their structures and mass spectra are shown in Figure 4.4.6. In the absence of authentic standards of the metabolites, accurate quantification of the metabolites was not possible. However, estimated concentrations were calculated using the (non-selective) TIC chromatogram by assuming that the metabolites had the same detector response as ibuprofen, which was independently quantified using a standard solution. On

this basis, metabolite A was present at a peak concentration 6 hours after dosage, of about 5 times that of ibuprofen and metabolite B was present at approximately half that of ibuprofen. Both metabolites were excreted over the same time interval as the parent drug. The implications of these results for a screening programme are:

(a) The time limit for detection of ibuprofen and its metabolites is 24-30 hours and this is not improved by using either metabolite as the target analyte instead of the parent drug.

(b) The detection of ibuprofen can be confirmed by including metabolite A, at least, in the list of target analytes in the screen. As both compounds are excreted together, both should be detected if the drug has been administered to the dog providing the sample.

The I.O.C. Medical Commission have ruled that, "the detection of a prohibited substance and/or one of its metabolites shall constitute an offence." This ruling is accepted by the N.G.R.C. provided it has been shown by the testing laboratory that the detected metabolite can be unequivocally related to the administration of the parent drug and that the concentration of the detected substance indicates that the performance of the dog would have been affected. The results indicate that if ibuprofen is detected it would have been administered in the 24 hours prior to the race meeting at which the sample was collected, and would constitute an offence under the rules of the N.G.R.C.

TABLE 4.4.4: Results obtained for the analysis of ibuprofen in plasma samples taken from greyhound 1 following the oral administration of ibuprofen 200mg.

T I M E (hours)	Concentration Ibuprofen In Plasma Sample (µg/ml)
0	0.00
1	9.08
2	49.32
3	22.54
4	21.39
6	4.38
24	0.00

TABLE 4.4.5: Results obtained for the analysis of ibuprofen in urine samples taken from two greyhounds following the oral administration of ibuprofen 200mg.

T I M E (hours)	Concentration Ibuprofen In Urine Sample (µg/ml)		Concentration Ibuprofen In Urine Sample (µg/ml)	
	Greyhound 1	Greyhound 2	Greyhound 1	Greyhound 2
0	0.00	0.00	0.00	0.00
2	1.55	0.00	1.25	0.00
6	0.70	1.65	0.44	0.65
24	0.03	0.15	0.02	0.06
30	0.01	0.05	0.01	0.03
48	0.00	0.05	0.00	0.03
54	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00

TABLE 4.4.6: Results obtained for the analysis of metabolites A and B of ibuprofen in urine samples taken from greyhound 1 following the oral administration of ibuprofen 200mg.

T I M E (hours)	Estimated Concentration of Metabolite A In Urine Sample (µg/ml)	Estimated Concentration of Metabolite B In Urine Sample (µg/ml)
0	0.00	0.00
2	0.10	0.00
6	7.14	0.71
24	1.80	0.26
30	0.22	0.00
48	0.00	0.00
54	0.00	0.00
72	0.00	0.00

FIGURE 4.4.2: Concentration versus time for the plasma ibuprofen levels obtained for greyhound 1 following the oral administration of ibuprofen 200mg.

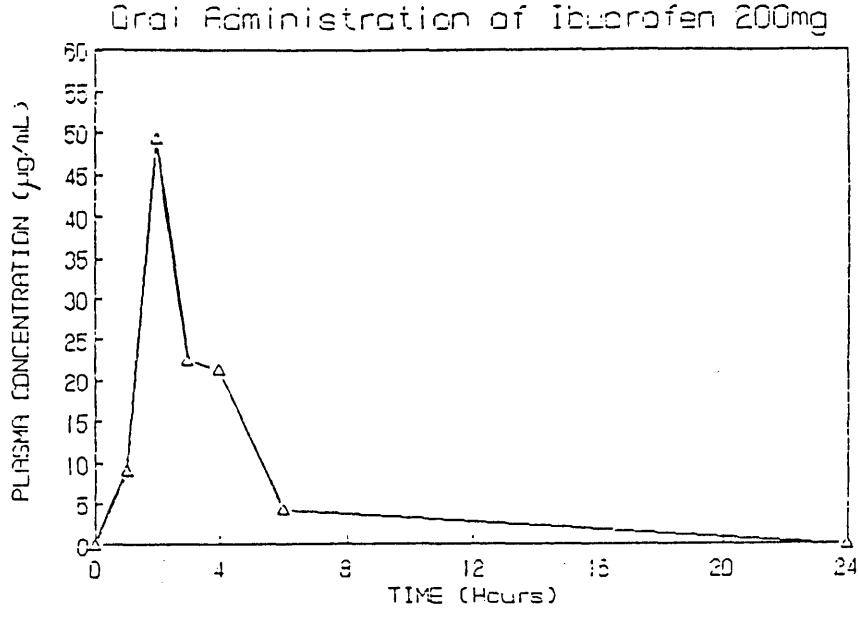


FIGURE 4.4.3: Log concentration versus time for the plasma ibuprofen levels obtained for greyhound 1 following the oral administration of ibuprofen 200mg.

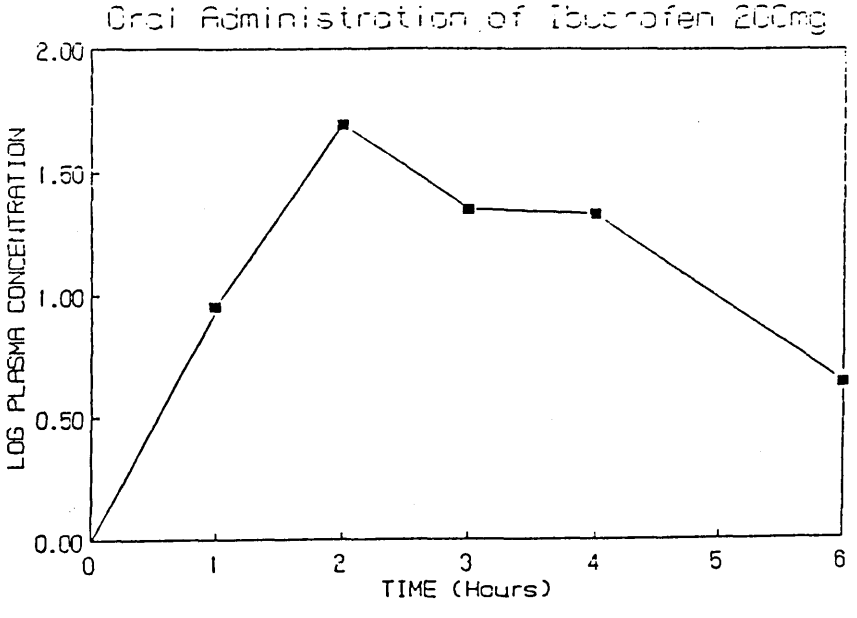


FIGURE 4.4.4: Excretion of ibuprofen in the urine of two greyhounds following the oral administration of ibuprofen 200mg.

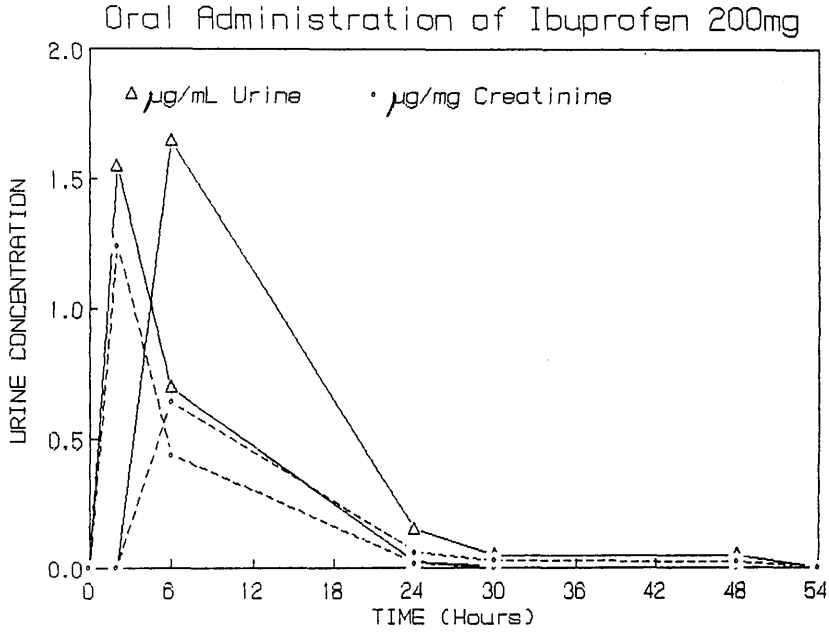


FIGURE 4.4.5: Excretion of ibuprofen and its two metabolites in the urine of greyhound 1 following the oral administration of ibuprofen 200mg.

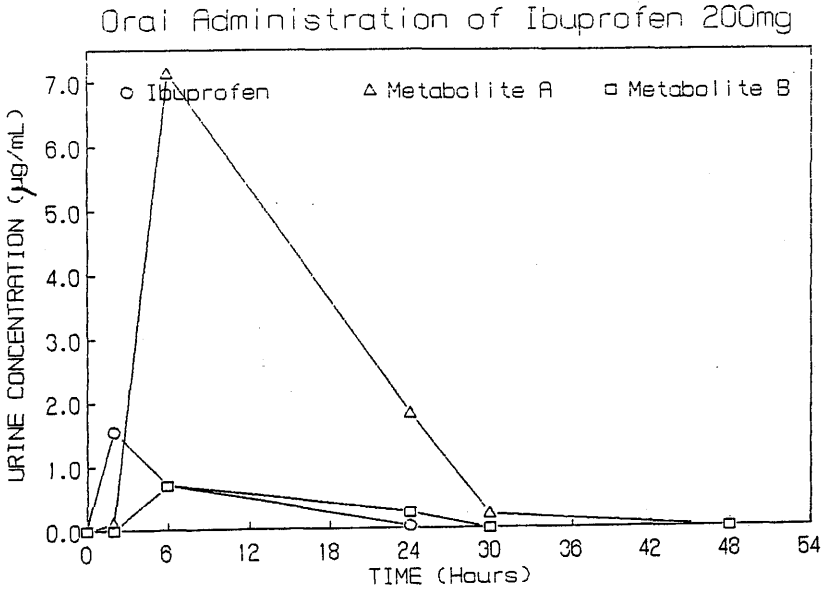


FIGURE 4.4.6: The mass spectra of the methylated esters of metabolite A and metabolite B obtained from the GC-MS analysis of a methylated extract of greyhound urine (6 hours after the administration of 200mg of ibuprofen).

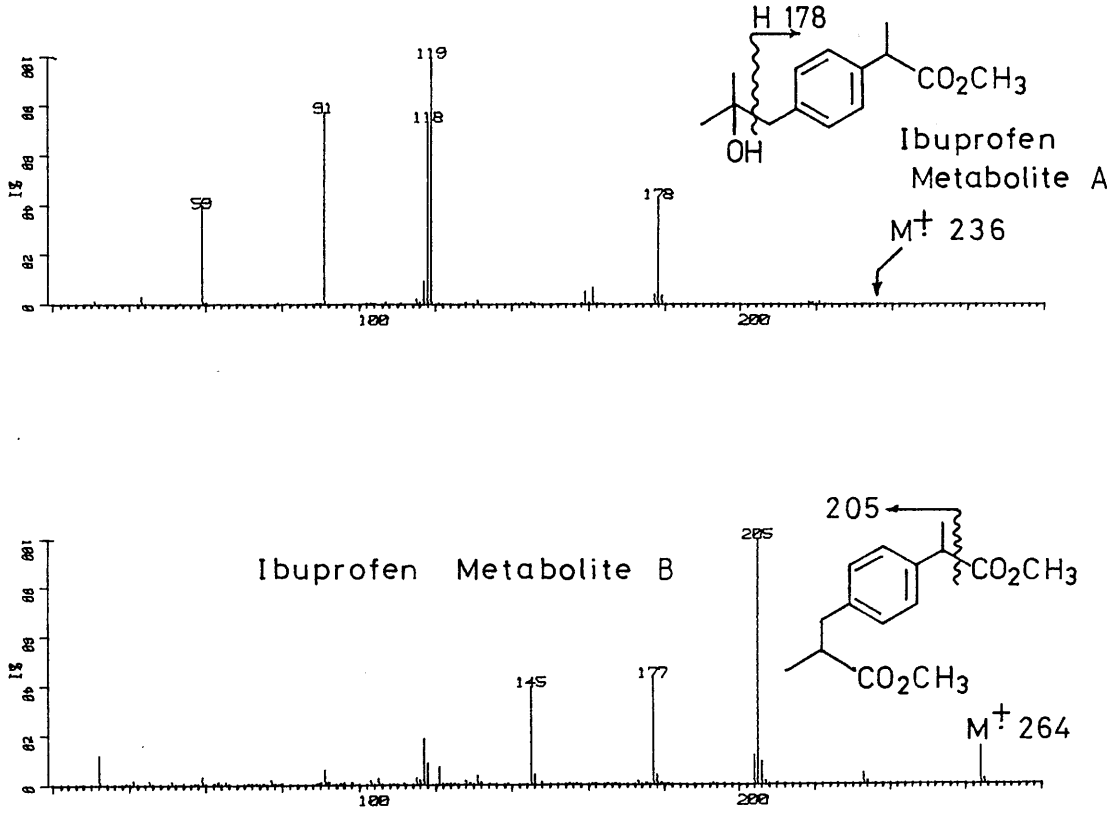
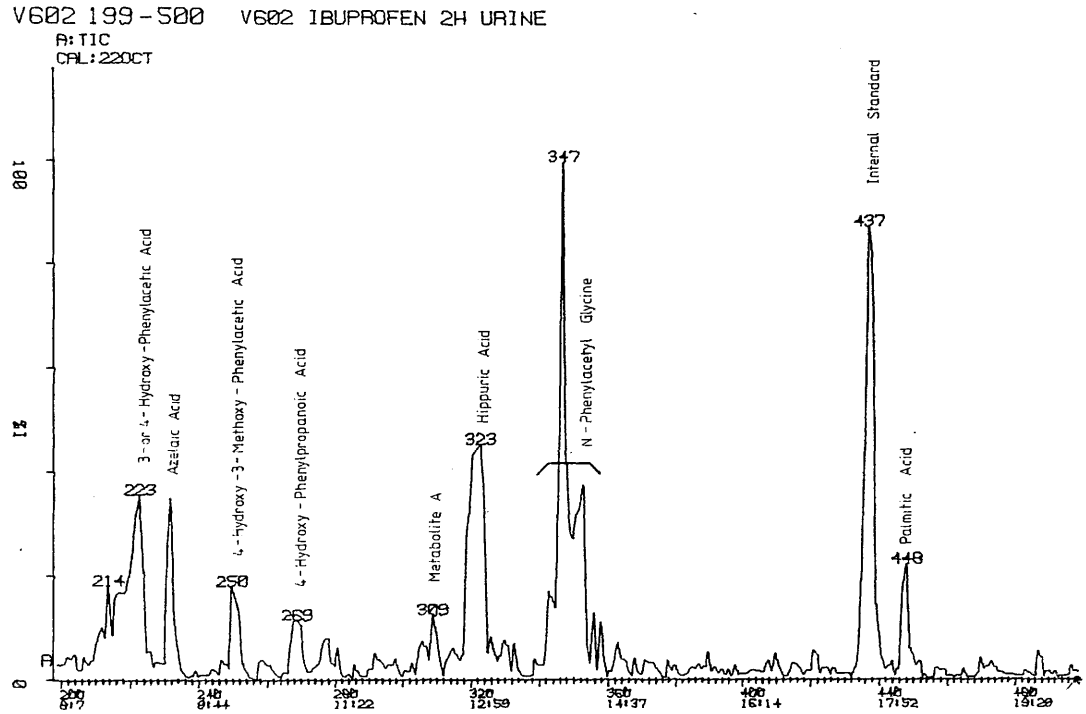


FIGURE 4.4.7: Chromatograms obtained from the GC-MS analysis of methylated extracts of greyhound urine (2 and 6 hours after the oral administration of 200mg ibuprofen).



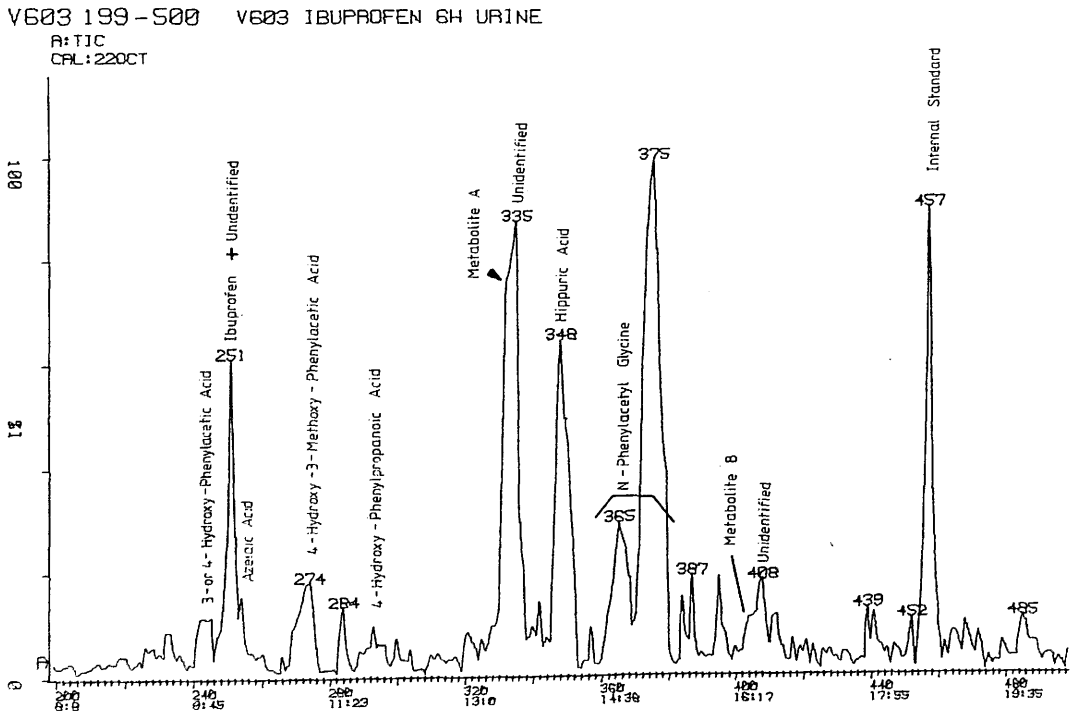
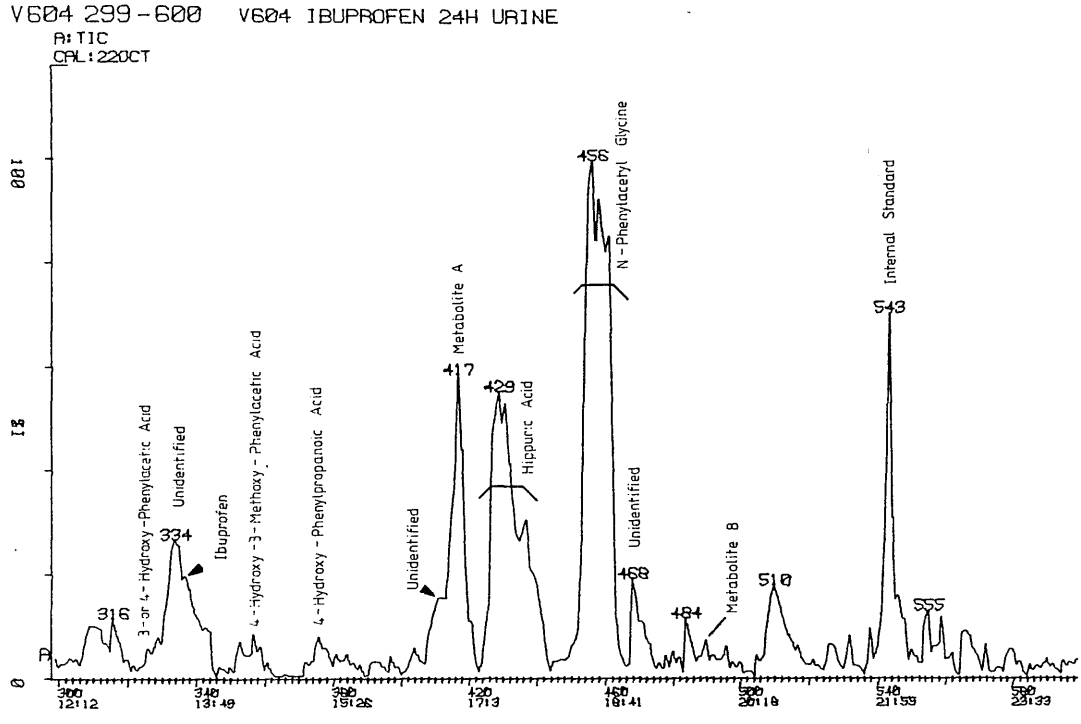


FIGURE 4.4.7 (Continuation a): Chromatograms obtained from the GC-MS analysis of methylated extracts of greyhound urine (24 and 30 hours after the oral administration of 200mg ibuprofen).



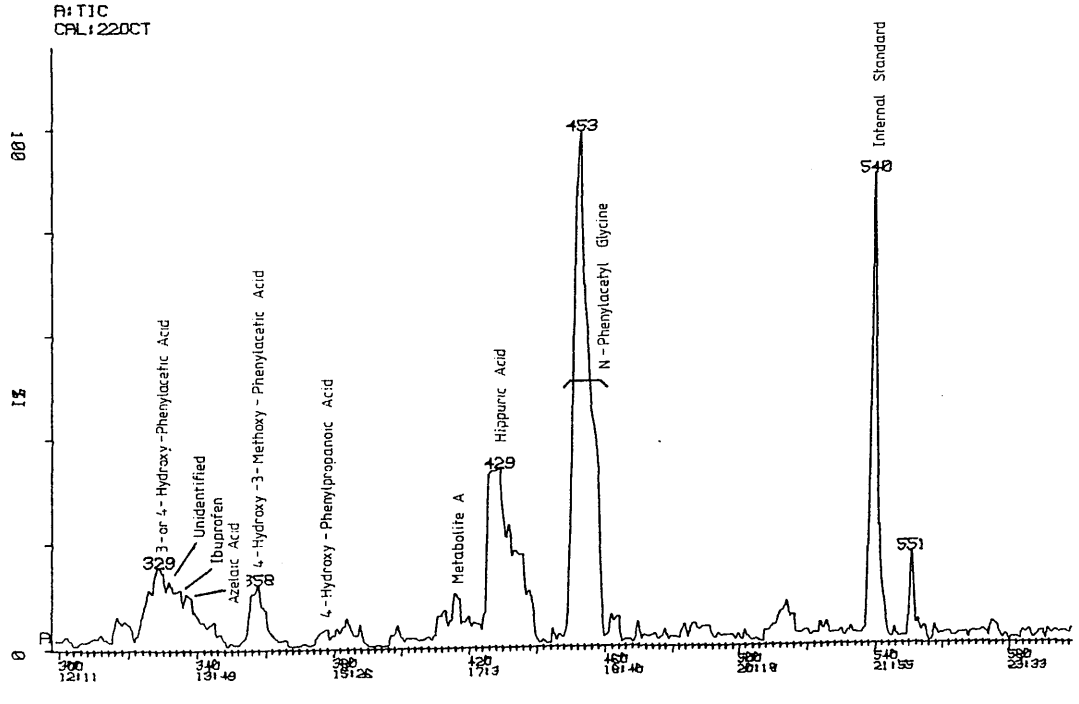
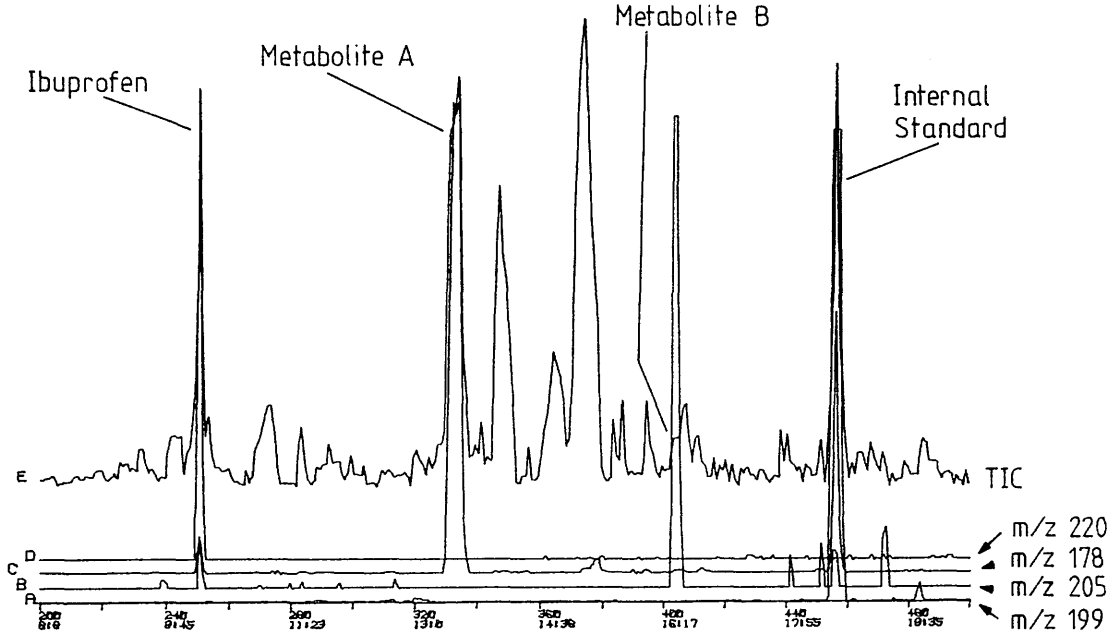


FIGURE 4.4.8: Total ion chromatogram and mass chromatograms (m/z 220, 178,205 and 199) obtained from the GC-MS analysis of a methylated extract of greyhound urine (6 hours after the oral administration of 200mg ibuprofen).



4.5 NAPROXEN

4.5.1 INTRODUCTION

Naproxen is the second member in the series of substituted propionic acid derivatisves which was administered to greyhounds. Basic information about naproxen is summarised in Table 4.5.1. Naproxen is the active D(+) isomer of 6-methoxy-∝-methyl-2-naphthalen-acetic acid. The inactive L(-) isomer is not contained in naproxen. Naproxen is completely absorbed from the gastro-intestinal tract, and peak plasma levels are reached in 2-4 hours. After a single oral dose of 250mg, peak plasma levels of 30-40μg/ml are achieved [181]. The biological plasma half-life, independent of dose, is 10-17 hours. The area under the plasma concentration versus time curve increases linearly with dosage up to 500mg twice a day, but with higher doses the plasma response is non-linear [182]. This non-linear response at high doses can be attributed to accelerated renal clearance as a result of a rise in levels in free naproxen when plasma protein binding capacity is exceeded. It has been suggested [181] that this self-regulating mechanism, which limits naproxen plasma levels in man, may well limit toxic effects should an overdose of naproxen be taken.

Frey and Rieh reported in 1981 [183] the pharmaco-kinetics of naproxen in mongrel dogs. After oral administration, of 5mg/kg, the drug was rapidly absorbed and maximal plasma concentrations were found at 0.5-3 hours after administration. The elimination

TABLE 4.5.1: Description and basic pharmacokinetic data for naproxen.

Chemical Name
(s)-6-methoxy-α-methyl-2-naphthaleneacetic acid

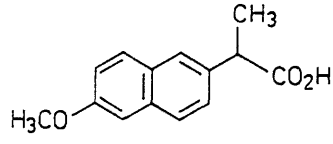
Chemical Abstracts Registry Number
22204-53-1

Non-proprietary Name
Naproxen

Proprietary Names
Equiproxen, Floginax, Laser, Naixon, Naprosyn(e), Naprix, Naxen, Proxen and Xenar

Empirical Formula
$C_{14}H_{14}O_3$

Structural Formula



Molecular Weight
230.26

Dissociation Constant
pKa = 4.2

Physical Properties
A white crystalline powder. Melting point about 156°C. Practically insoluble in water; soluble 1 in 25 of ethanol, 1 in 15 of chloroform and 1 in 40 of ether.

Dose
250 and 500mg tablets.
Total daily dose of about 500-1000mg

Plasma Half-Life
10 to 20 hours (mean 14)

Protein Binding
More than 99%

Volume of Distribution
About 0.1 litre/kg

half-life ranged from 45-92 hours.

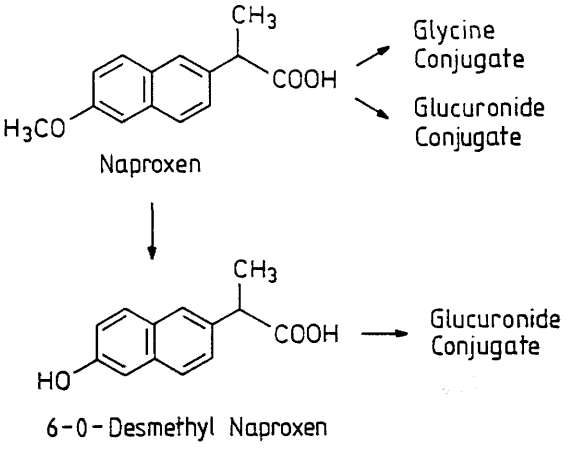
Fredell and Strand [184] described the clinical course of a patient who was thought to have ingested 25g of naproxen. The patient developed mild transient nausea and indigestion. The serum naproxen concentration, obtained approximately 15 hours after ingestion, was 414µg/ml. Waugh and Keatinge [185] have reported the development of hypoprothombinaemia in a patient who ingested 10g of naproxen. In addition to the coagulation abnormality, the patient also experienced nausea which persisted for several days.

4.5.2 METABOLISM AND EXCRETION

The main metabolite in human urine is 6-hydroxy-α-methyl-2-naphthylacetic acid (Figure 4.5.1) and is excreted, like naproxen, to a large extent as the glucuronide conjugate [186]. A glycine conjugate of naproxen has also been identified.

Runkel and his colleagues [187] studied the absorption, distribution, metabolism and excretion of naproxen in various laboratory animals and human subjects. He reported that with the exception of the dog, all of the species studied excreted naproxen and its metabolites predominantly in the urine. In the dog the major route was faecal excretion. In the human 94% of the dose administered appeared in the urine after 5 days, with only 1-2% in the faeces. Only 5-6% of the drug appeared as unchanged naproxen, 28% as the demethylated naproxen and the remainder was in the form of conjugates of the drug (predominantly the glucuronide ester).

FIGURE 4.5.1: The metabolism of naproxen.



4.5.3 RESULTS

A single 250mg dose of naproxen was administered to a greyhound and urine samples were collected according to the protocol described (Section 4.3.7). The samples were analysed according to the methods in Section 4.3. The results obtained for the analysis of naproxen in urine samples (expressed as µg/ml urine and µg/mg creatinine) are summarised in Table 4.5.2 and are illustrated in Figure 4.5.2.

The 6-O-des-methyl-naproxen metabolite was indentified in samples of greyhound urine: the mass spectrum obtained is given in Figure 4.5.3. The metabolite was detected in urine samples taken 24 and 48 hours after the administration of the drug but was not detected in the remaining urine samples. Relative concentrations of the metabolite in each sample were calculated as 1.04µg/ml in the 24 hour sample and 1.12µg/ml in the 48 hour sample from the peak areas of selected mass chromatograms for the metabolite and the internal standard. Estimated real concentrations were calculated by assuming that the TIC response ratio for the metabolite was the same as that for naproxen, which was quantified using an authentic standard.

The total ion current chromatograms obtained from extracts of grehound urine taken at 6, 24, 30 and 48 hours after the administration of 250mg of naproxen are illustrated in Figure 4.5.4. These 4 chromatograms illustrate the relative amounts of naproxen and its

metabolite present with time and also the compounds which may interfere in the analysis.

Figure 4.5.5 illustrates the TIC and 3 mass chromatograms obtained from an extract of greyhound urine (48 hour sample). The mass chromatograms show the positions of naproxen (m/z 185), the 6-o-des-methyl-naproxen metabolite (m/z 171) and flurbiprofen, the internal standard (m/z 199).

4.5.4 DISCUSSION

The limited data available from this study of naproxen in the greyhound are of value for the detection of the drug in future as part of a screening process. In several ways, naproxen differs from ibuprofen, in terms of its metabolism, excretion and the interpretation of observed levels in urine.

Naproxen is a more lipophilic drug than ibuprofen, due to the presence of the naphthalene ring in its structure, and in common with many other drugs with a lipophilic moiety, it persists in the circulation for a relatively long time. The biological half lives of naproxen and ibuprofen (10-17 hours and 2-4 hours respectively) illustrate this effect. This observation in humans appears to be upheld for the greyhound also, and the drug was readily observed in urine for the duration of the period of observation in this study, 72 hours, and would continue to be measurable for some time afterwards. The drug was absorbed well orally and could be observed in urine 2-6 hours after dosing, peaking during the period

6-24 hours after dosing. The importance of parallel measurements of urinary creatinine is well-illustrated in this study: examination of Figure 4.5.2 shows that the corrected and uncorrected graphs of urinary naproxen concentrations are different and that the concentrations of naproxen in the samples taken at 6, 24 and 72 hours are significantly different after correction.

The results indicate that naproxen is easily detected in greyhound urine. The urinary concentration rises to several microgrammes per millilitre (uncorrected) even after a single dose. Faecal excretion may be the predominant route in at least some species of dog [187] but appears not to pose a problem in this instance: as with ibuprofen, faecal excretion of naproxen was not examined in the study. The known metabolite of naproxen (O-desmethyl naproxen) was observed in the study at lower concentrations than naproxen, but not in all samples, suggesting that the parent drug is the target analyte of choice: the presence of the metabolite may be helpful in confirmation of the presence of naproxen but is not essential for a positive result in a urinary drug screen. The mass spectra of naproxen and its desmethyl metabolite show the presence of strong, characteristic ions, which also aids the detection and confirmation of their presence in urine. It is the presence of the naphthalene ring which imparts stability to the structures and influences the fragmentation reactions. In addition, there are no major interferences in the urinary acid profile to hinder

the detection and quantification of either the drug or its metabolite (Figure 4.5.5). Even the non-selective TIC trace appears to be free from significant interference, suggesting that naproxen might be amenable to analysis by GC with FID detection.

No other conjugates of naproxen were detected in this study, although the occurrence of a glycine conjugate of the parent has been reported in the literature [186]. In the absence of mass spectral and chromatographic data concerning this conjugate, the probable retention time and fragmentation pattern were predicted from those of components in the urinary acid profile (delta-I = 535, M^{+} 301, major fragments at m/z 242, 213 and 185 corresponding to loss of the carboxymethyl group and subsequent side-chain cleavages). However, examination of each of the urinary extracts failed to show the presence of this metabolite.

The creation in future of reliable criteria by which a urine sample will be considered to be positive for naproxen will clearly depend on the compilation of more data relating to a statistically significant number of dogs. Both plasma and urinary analysis will be required for this purpose. However, the present results have several implications for the screening process and interpretation of analytical results. Naproxen can be detected for several days following administration of even a single dose and it is likely that the drug will be detected long after it is significant to the performance

of the dog concerned in the race meeting at which the sample was collected. An interpretation will need to be made with respect to when the drug was administered and the intention of the dog's owner. Simple measurements of urinary concentration with creatinine correction may be sufficient if a threshold level is set, drawing from experience in dealing with caffeine, which poses a similar problem. For example, if the offence is defined as administration of naproxen less than three days prior to a race, then a suitable threshold level would be approximately 1 microgramme of naproxen per milligramme of creatinine.

TABLE 4.5.2: Results obtained for the analysis of naproxen in urine samples taken from a greyhound following the oral administration of naproxen 250mg.

T I M E (hours)	Concentration Naproxen In Urine sample (µg/ml)	Concentration Naproxen In Urine Sample (µg/mg creatinine)
	0.00	0.00
2	0.00	0.00
6	5.00	8.18
24	6.30	1.92
30	2.80	1.53
48	1.43	0.88
54	1.23	0.99
72	1.58	0.74

FIGURE 4.5.2: Excretion of naproxen in the urine of a greyhound following the oral administration of naproxen 250mg.

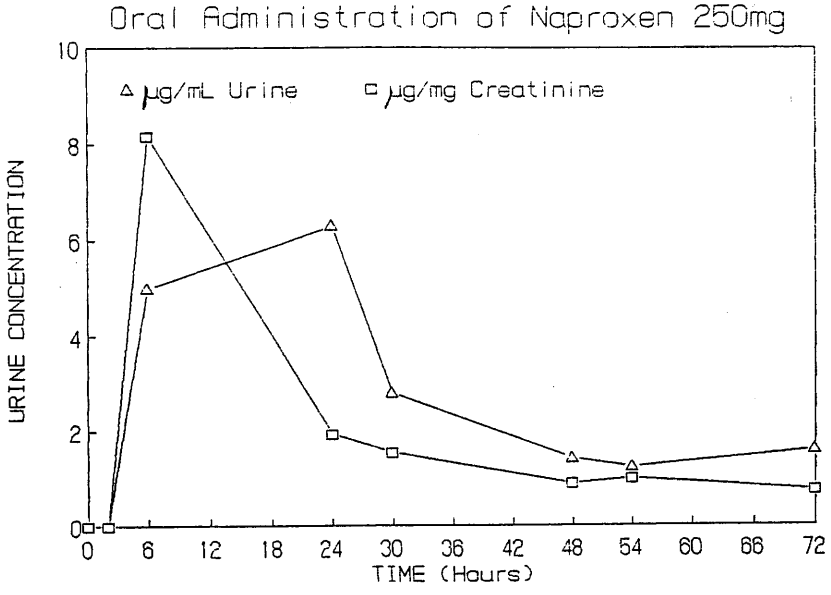
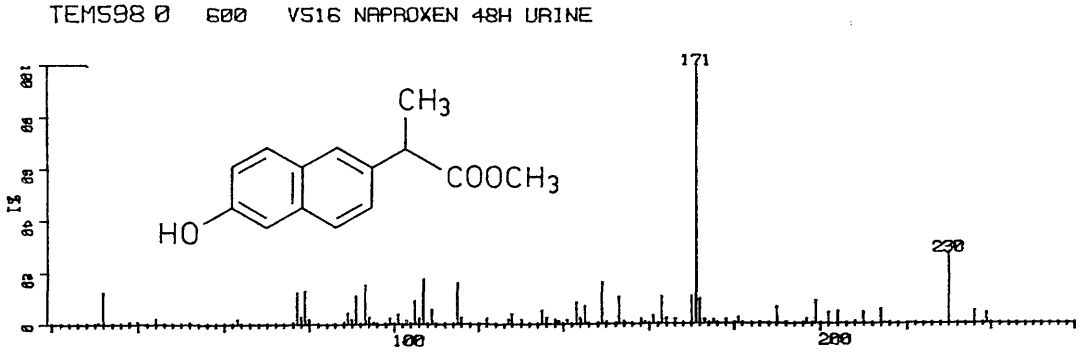
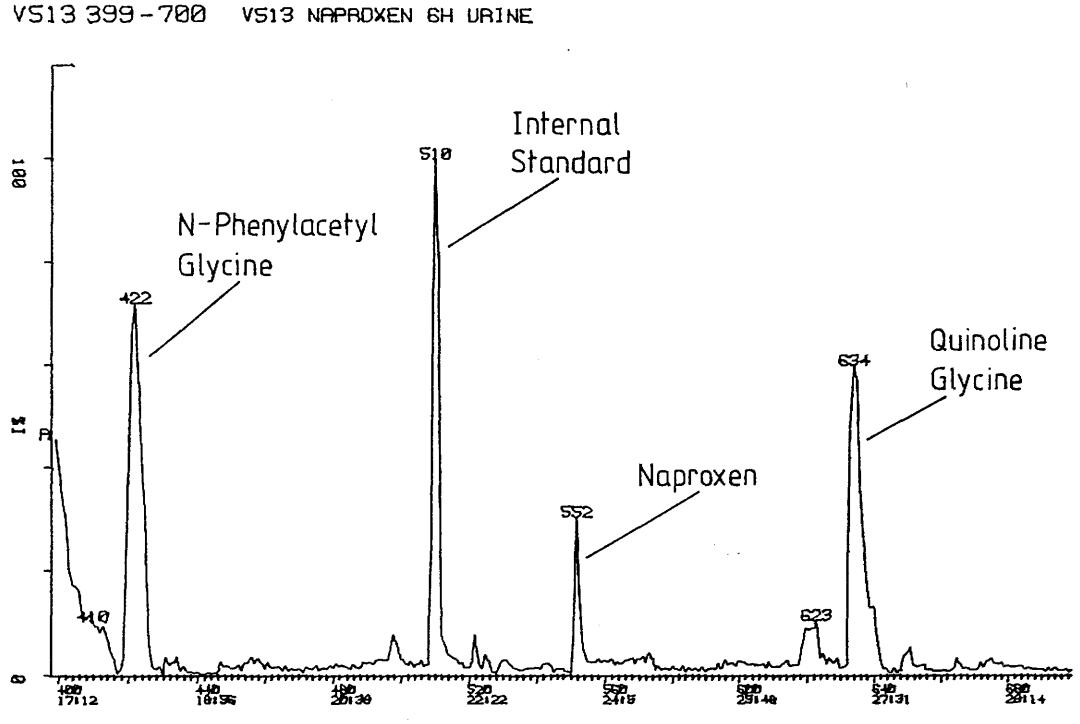


FIGURE 4.5.3: The mass spectra of the methyl ester of the 6-o-des-methyl-naproxen metabolite obtained from the GC-MS analysis of a methylated extract of greyhound urine (48 hours after the administration of 250mg of naproxen).



6-O-des-Methyl-Naproxen Methyl Ester

FIGURE 4.5.4: Chromatograms obtained from the GC-MS analysis of methylated extracts of greyhound urine (6 and 24 hours after the oral administration of 250mg naproxen).



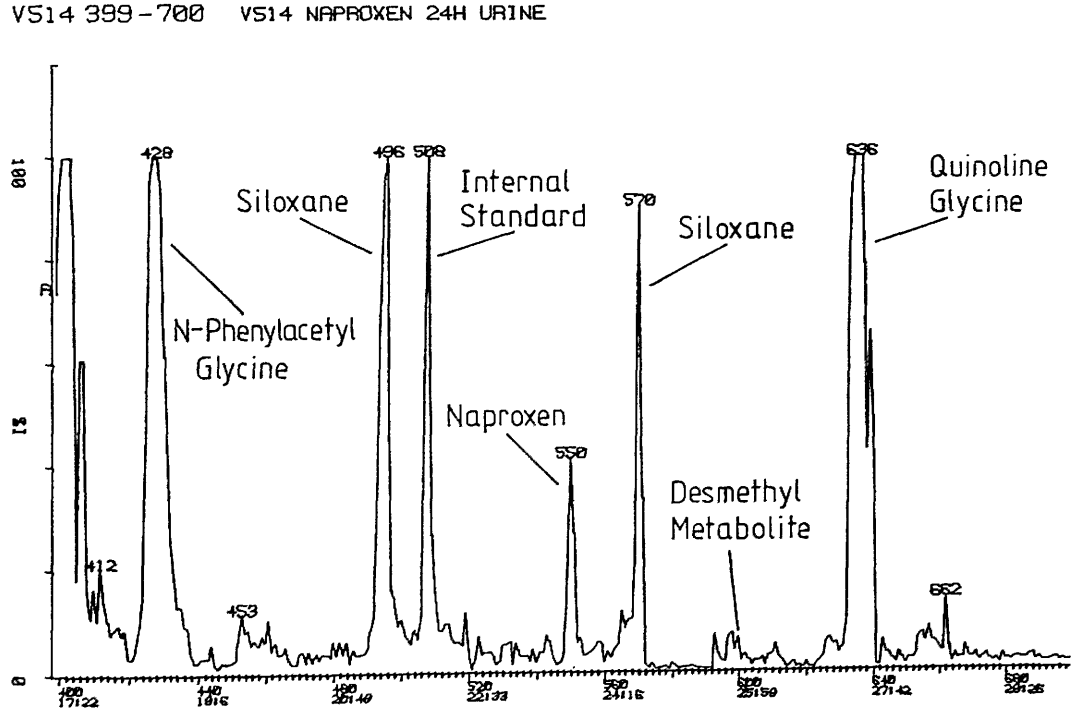
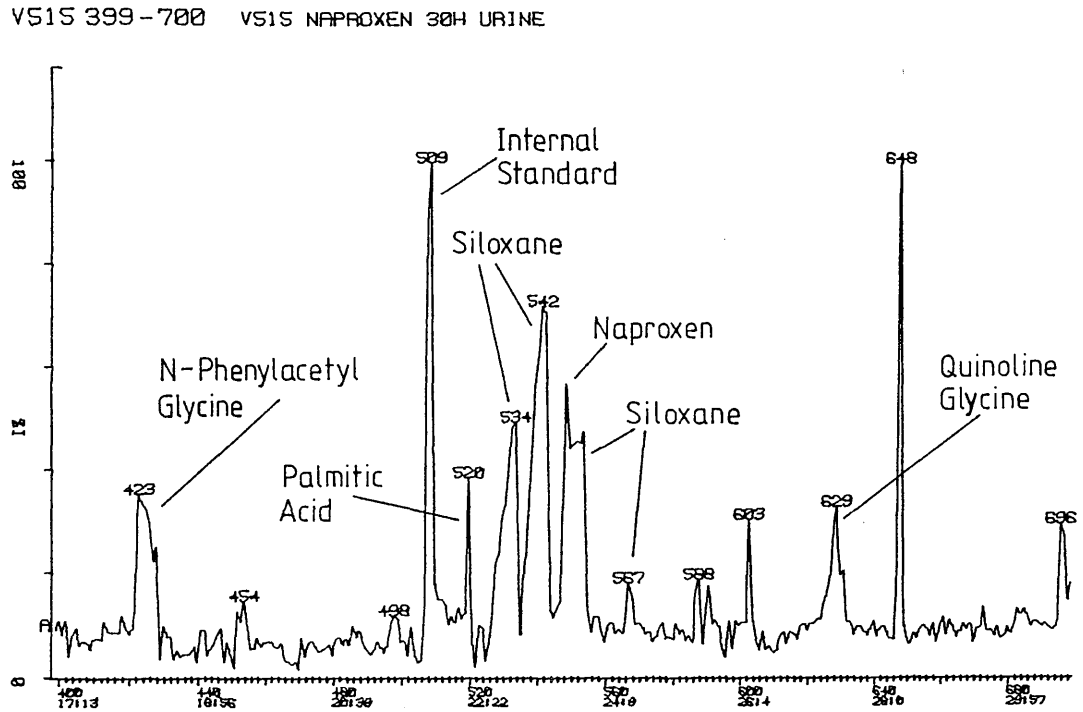


FIGURE 4.5.4 (Continuation a): Chromatograms obtained from the GC-MS analysis of methylated extracts of greyhound urine (30 and 48 hours after the oral administration of 250mg naproxen).



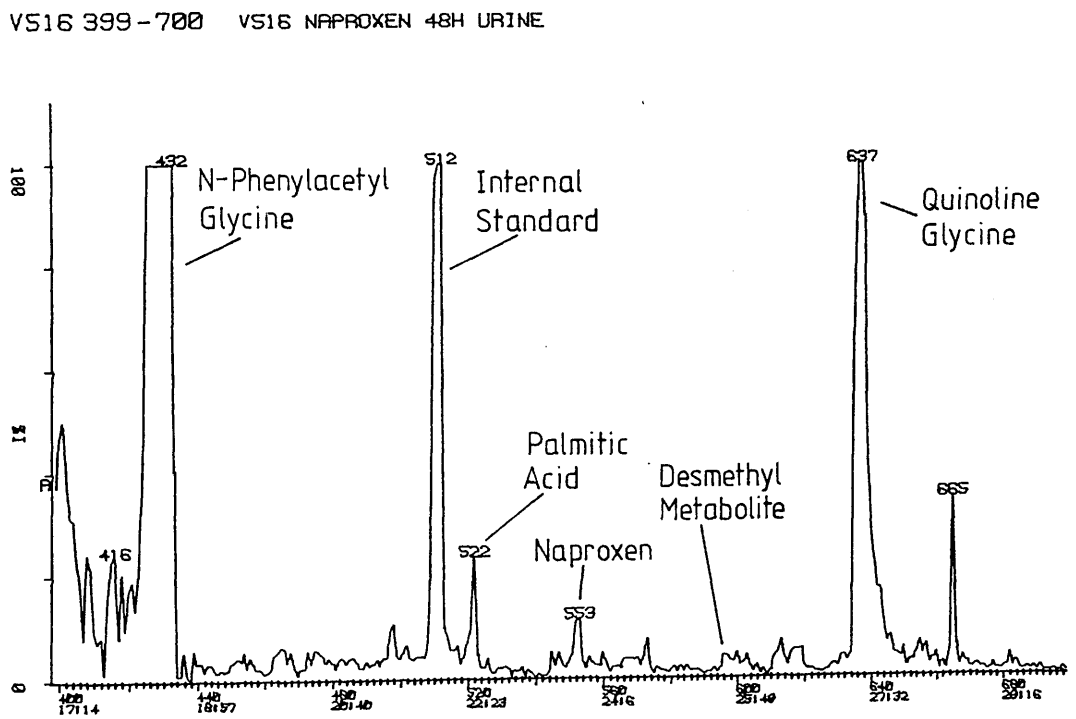
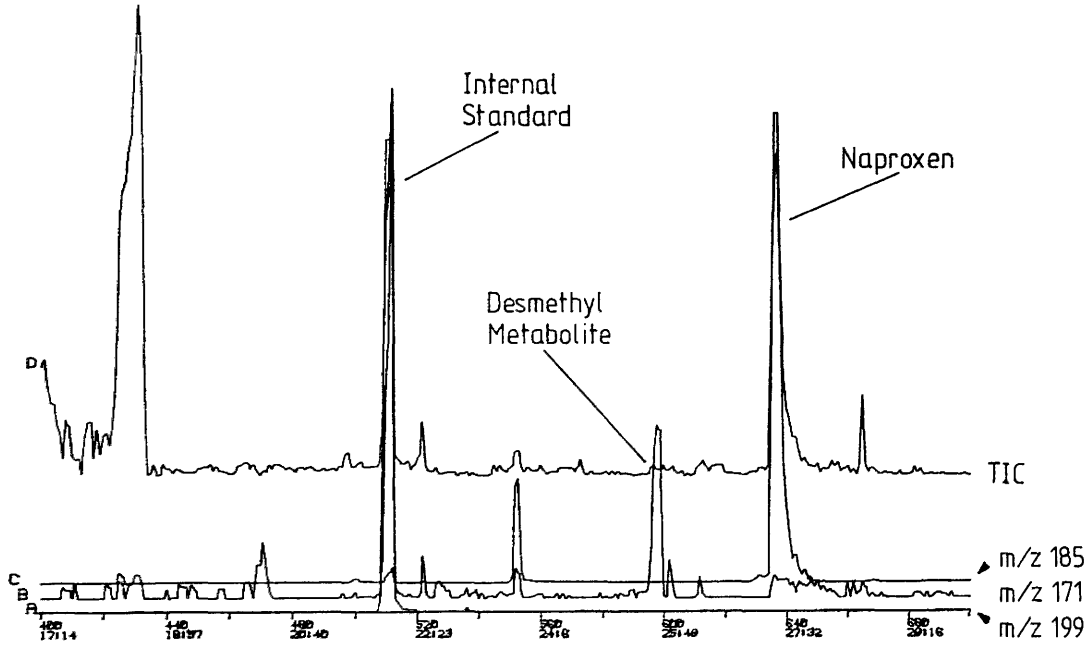


FIGURE 4.5.5: Total ion chromatogram and mass chromatograms (m/z 185, 171 and 199) obtained from the GC-MS analysis of a methylated extract of greyhound urine (48 hours after the oral administration of 250mg naproxen).



4.6 KETOPROFEN

4.6.1 INTRODUCTION

Basic information about ketoprofen, a propionic acid derivative, is summarised in Table 4.6.1.

Pharmacokinetic studies on ketoprofen [188] have shown that maximum concentrations of ketoprofen in plasma occur 30 minutes to two hours after oral dosing. In man, therapeutic concentrations in plasma are in the range 3-23μg/ml. The plasma disappearance curve of ketoprofen is multi-exponential, with a slower terminal phase of elimination [189]. Jolou and co-workers [190] have studied the acute toxicity of ketoprofen administered orally and subcutaneously in various animals, including the dog. The average lethal dose in the different animals ranged between 100 to 200mg/kg. Court and Volans [191] have summarised data on 20 suspected cases of overdose with ketoprofen where the only clinical features were drowsiness, abdominal pain and vomiting.

4.6.2 METABOLISM AND EXCRETION

After oral administration of ketoprofen, in man, excretion varied greatly amongst patients, 30-90% of the dose being excreted in 24 hours, but mostly during the first 6 hours [192]. In the dog, urinary excretion was relatively low, only 60% being excreted in 6 days [188]. Although ketoprofen is excreted in the bile, essentially all is reabsorbed, and little drug is present in the faeces [193]. Ketoprofen excretion in the faeces only accounted for about 1% of a dose in man.

TABLE 4.6.1: Description and basic pharmacokinetic data for ketoprofen.

Chemical Name
3-Benzoyl-α-methyl-benzeneacetic acid

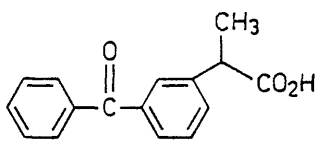
Chemical Abstracts Registry Number
22071-15-4

Non-Proprietary Name
Ketoprofen

Proprietary Names
Alrheumat, Capisten, Kefenid, Ketopron, Meprofin, Orudis, Oruvail and Profenid.

Empirical Formula
$C_{16}H_{14}O_3$

Structural Formula



Molecular Weight
254.29

Dissociation Constant
not known

Physical Properties
A white, crystalline powder. Melting point 93-96°C. Practically insoluble in water; freely soluble in ethanol, chloroform and ether.

Dose
50 and 100mg capsules.
Total daily dose of
100 to 250mg

Plasma Half-Life
1-4 hours.

Protein Binding
about 95%

Volume of Distribution
About 0.1 to 0.2 litre/kg.

Ketoprofen is largely excreted as its glucuronide conjugate in most species. A minor metabolic route involves hydroxylation to form phenolic derivatives. The detailed structures of the hydroxylated metabolites have not been reported in the literature.

4.6.3 RESULTS

A single 50mg dose of ketoprofen was administered to a greyhound and urine samples were collected according to the protocol described (Section 4.3.7). The samples were analysed according to the methods in Section 4.3. The results obtained for the analysis of ketoprofen in urine are summarised in Table 4.6.2. (Creatinine measurements were not available in this study).

Figure 4.6.1 illustrates the TIC and 3 mass chromatograms obtained from an extract of greyhound urine (6 hour sample). The mass chromatograms show the positions of ketoprofen (m/z 268 and 209) and flurbiprofen, the internal standard (m/z 199).

Figure 4.6.2 illustrates the mass spectra of an interfering peak, observed immediately before ketoprofen, and that of a large peak which elutes just after ketoprofen. These two peaks remain unidentified but, on the basis of the ions present in their mass spectra, they could be ketoprofen metabolites or diazomethane artefacts (see also Chapter 2).

4.6.4 DISCUSSION

In this study a single oral dose of 50mg of ketoprofen was administered to a greyhound (ca 1.5mg/kg).

Ketoprofen was detected at a high concentration in the 6-hour urine sample but thereafter relatively small amounts (2µg/ml) were detected up to 30 hours after administration. It has been reported [188] that urinary excretion of ketoprofen in the dog (most likely a beagle) is low but, in man, up to 90% of the dose could be excreted within 24 hours and mostly in the first 6 hours [192]. In this study about 46% of the dose administered to the greyhound was excreted in the first 6 hours (taking into account the total volume of urine collected in this period). The drug could not be detected after 48 hours.

The mass spectra in Figure 4.6.2 contain ions which are similar to the ketoprofen spectrum (Figure 2.5.10). The later-eluting peak has a molecular ion at m/z 282 (i.e. ketoprofen + 14) which suggests the addition of a CH_2 group. The identities of these peaks remain uncertain but could be due to a ketoprofen metabolite or a diazomethane artefact (see DISCUSSION, Section 2.5). If one or both of these peaks were due to a diazomethane artefact then the quantification of ketoprofen using diazomethane methylation would be difficult. It is undesirable to have such products which could further complicate a screening procedure. The limited data available in this study serve as a starting point for further investigation. However, it appears that ketoprofen is rapidly eliminated in the urine of the greyhound with peak levels at 6 hours after a single oral

dose. If ketoprofen was detected during a screening procedure in the urine of a greyhound at a race meeting then the animal must have been administered with the drug within the previous 24-30 hours. It is uncertain in this case whether or not metabolites were detected: further work would be necessary to ascertain these facts.

TABLE 4.6.2: Results obtained for the analysis of ketoprofren in urine samples taken from a greyhound following the oral administration of ketoprofen 50mg.

T I M E (hours)	Concentration Ketoprofen In Urine Sample (µg/ml)
0	0.0
2	1.0
6	291.0
24	6.0
30	2.0
48	0.0
54	0.0
72	0.0

FIGURE 4.6.1: Total ion chromatogram and mass chromatograms (m/z 268, 209 and 199) obtained from the GC-MS analysis of a methylated extract of greyhound urine (6 hours after the oral administration of 50mg ketoprofen). The mass spectra of the unidentified components A and B are given in Figure 4.6.2.

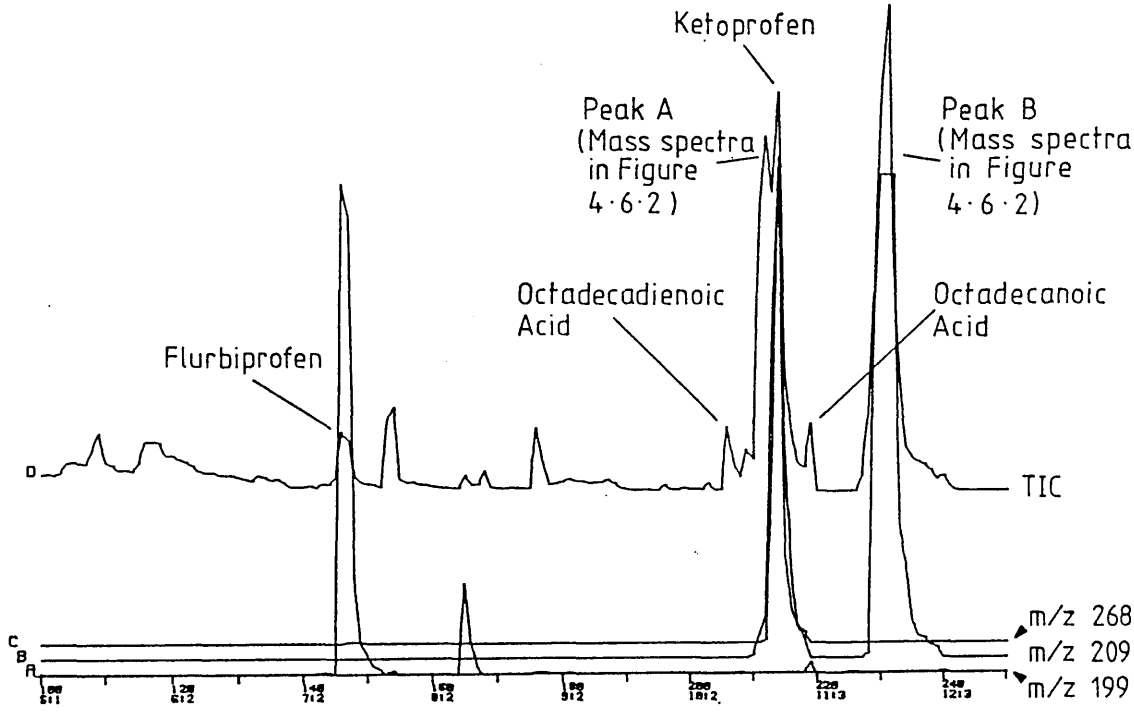
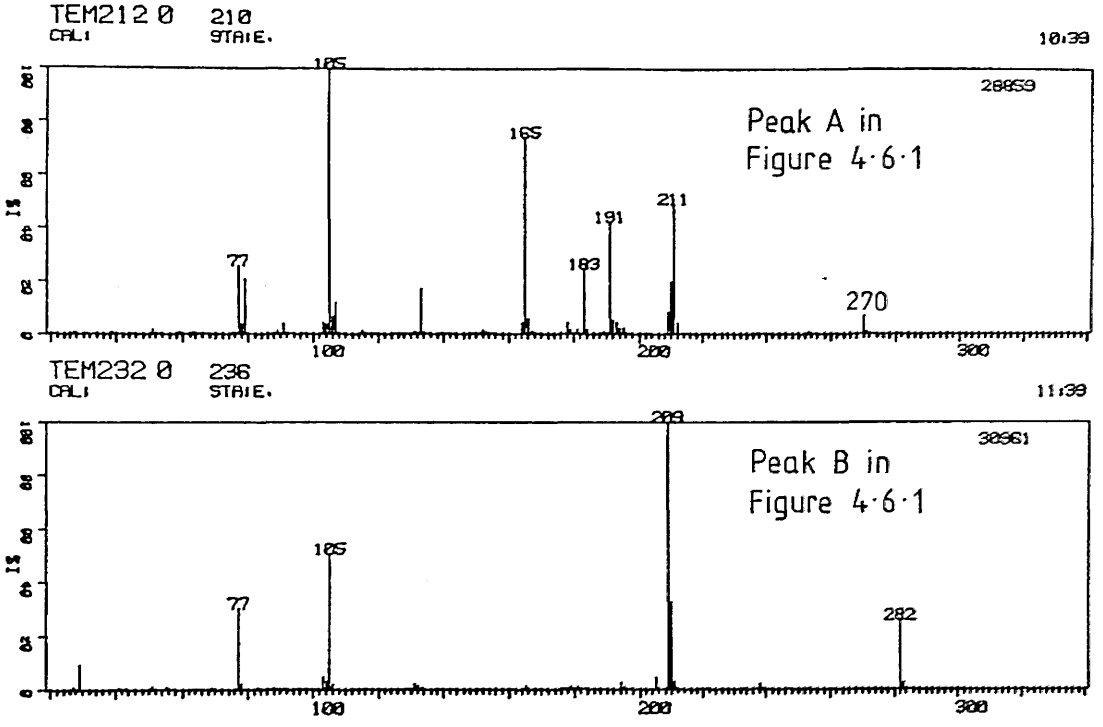


FIGURE 4.6.2: Mass spectra of two substances co-extracted with ketoprofen from greyhound urine (components A and B in Figure 4.6.1).



4.7 MEFENAMIC ACID

4.7.1 INTRODUCTION

Mefenamic acid is one of a series of substituted N-phenyl anthranilic acid derivatives (or fenamates) and has been marketed since the 1960s. Basic information about mefenamic acid is summarised in Table 4.7.1.

In a review of the metabolism of mefenamic acid by Glazko [194] it was shown that, after oral administration, mefenamic acid is rapidly absorbed and that blood levels reach a maximum in 2-4 hours. Following a single oral dose of 1g to 6 subjects a mean plasma concentration of 10μg/ml of free, unconjugated drug was attained which dropped to a 0.1μg/ml in 24 hours. Two metabolites have been isolated from plasma and identified as the 3'-hydroxymethyl derivative (Metabolite I), and 3'-carboxyl derivative (Metabolite II) (Figure 4.7.1). Metabolite I (present mainly as the glucuronide) attained peak plasma concentrations of about 10μg/ml after 3 hours, but disappeared from the plasma more slowly than the parent. Metabolite II formed more slowly, with peak plasma levels of about 10μg/ml occurring 6-8 hours after administration; approximately 50% of this metabolite was conjugated.

Court and Volans [191] have reported 73 mefenamic acid overdose cases and concluded that, unlike poisoning with other NSAIDs, mefenamic acid overdose appears to cause convulsions. Balali-Mood and co-workers [195] reported 54 mefenamic overdose cases over a period of 11

TABLE 4.7.1: Description and basic pharmacokinetic data for mefenamic acid.

Chemical Name
2-[(2,3-Dimethylpropyl)amino-benzoic acid

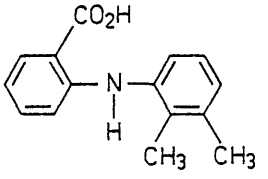
Chemical Abstracts Registry Number
61-68-7

Non-Proprietary Name
Mefenamic Acid

Proprietary Names
Coslan, Lysalgo, Parkemed, Ponstan, Ponstel, Ponsyl, Tanston and Vialidon.

Empirical Formula
$C_{15}H_{15}NO_2$

Structural Formula



Molecular Weight
241.28

Dissociation Constant
pKa = 4.2

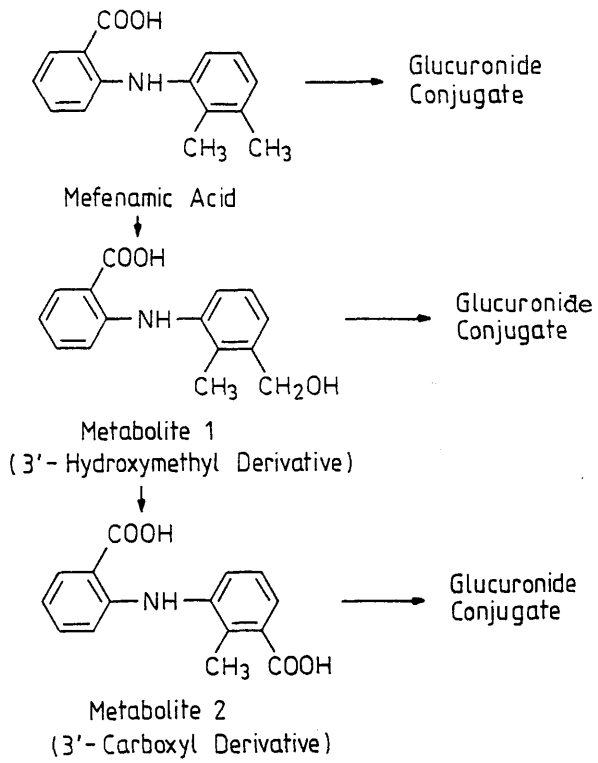
Physical Properties
A white to greyish-white microcrystalline powder. Melting point 230-231°C with effervescence. Practically insoluble in water; soluble in 1 in 185 of ethanol, 1 in 150 of chloroform and 1 in 80 of ether; soluble in solutions of alkali hydroxides.

Dose
250mg tablets.
750-1500mg daily

Plasma Half-Life
About 3-4 hours

Protein Binding
About 99%

FIGURE 4.7.1: Metabolic disposition of mefenamic acid in humans.



years. Twenty-nine patients had plasma concentrations above the therapeutic range (10µg/ml) on admission; 14 of these patients were asymptomatic (plasma concentration range 14-62µg/ml), 15 had muscle twitching and 11 progressed to grand mal convulsions (plasma concentration range 27-119µg/ml).

Metabolic studies [196] have shown that mefenamic acid is also rapidly absorbed in dogs and peak plasma levels are achieved in 2-4 hours after oral administration. When mefenamic acid was administered to dogs in long term studies at a daily dose of 50mg/kg, no adverse clinical or tissue reactions were observed, but at a daily dose of 100mg/kg slight hepatic damage was revealed on histological examination.

4.7.2 METABOLISM AND EXCRETION

In man about 52% of a dose is excreted in the urine in 48 hours (of this 6% is mefenamic acid, 25% is Metabolite I and 21% is Metabolite II). The maximum excretion rate for mefenamic acid was found in the 2-4 hour period after dosing, occurring mainly as the glucuronide. Metabolite I excretion peaked in the 4-8 hour period appearing almost entirely as the glucuronide and Metabolite II excretion peaked in the 6-8 hour period, with only about 30% conjugated. About 10-20% of a dose is excreted in the faeces over a 3 day period, mostly as unconjugated Metabolite II.

The major route of excretion of mefenamic acid in the dog is the faeces (appearing mainly as conjugated

mefenamic acid), with only small amounts appearing in the urine [194]. It appears that dog liver lacks the enzyme systems required for the oxidation of the 3'-methyl group to the hydroxymethyl.

In all species a high percentage of the administered dose is initially excreted in the bile but reabsorption from the intestine occurs (i.e. enterohepatic recycling) to varying extents in different species. The literature has suggested the possible structures of the mefenamic metabolites in the greyhound [194]. However, mass spectral data and GC retention times of the derivatives are not available.

4.7.3 RESULTS

A single oral dose of 250mg of mefenamic acid was administered to a greyhound. Blood and urine samples were collected and analysed according to the methods described in Section 4.3. The results obtained for the analysis of mefenamic acid in plasma are given in Table 4.7.2 and are illustrated as concentration versus time in Figure 4.7.2. The results obtained for the analysis of mefenamic acid in urine samples (expressed as µg/ml urine and µg/mg creatinine) are summarised in Table 4.7.3 and are illustrated in Figure 4.7.3.

Neither Metabolite I or II (Figure 4.7.1) were detected in any of the urinary extracts.

The TIC chromatogram obtained from an extract of greyhound urine (6 hour sample) along with 2 mass chromatograms showing the positions of mefenamic acid

(m/z 255) and 2,3-dicarboxy-naphthalene acetic acid, the internal standard (m/z 213) are illustrated in Figure 4.7.4.

4.7.4 DISCUSSION

Mefenamic acid appeared to be well absorbed orally in the greyhound, giving peak plasma concentrations of unconjugated mefenamic acid of about 3 µg/ml after 2-3 hours, following a dose of 250 mg (approximately 8 mg/kg). This compares well with clinical data in man which indicated an average plasma concentration of 10 µg/ml after a single oral dose of 1 gram (approximately 20 mg/kg). However, in the present study, the plasma level fluctuated markedly during the period up to 48 hours after dosing and a plasma half-life was not measured: in human subjects this was reported as 3-4 hours. The reason for the changes in concentration which took place was not established in this single experiment but is thought to be the result of enterohepatic circulation, which is known from the literature to be a prominent feature of the pharmacology of mefenamic acid.

Urinary concentrations of mefenamic acid were low throughout the course of the study, maximising at 0.5 µg/ml after 6 hours. Towards the end of the observation period, levels approaching the limit of detection were found. This is consistent with the major route of excretion being *via* the faeces. In this experiment, the creatinine concentrations in all samples were similar, and creatinine correction did not alter the

shape of the urinary excretion curve (Figure 4.7.3).

These results suggest that during a screening procedure mefenamic acid could be detected for 24-30 hours following a single dose, despite the fact that the drug is excreted in urine to a very small extent. The mass spectrum of the methyl ester of mefenamic provides salient diagnostic ions which permit the drug to be detected even in the presence of a large interference (methyl linoleate, Figure 4.7.4). No metabolites were observed in greyhound urine, leaving the parent drug as the target analyte. Interpretation of measured levels within a drug control programme will depend on the minimum period allowed between dosing an animal and a race: for example, a level above 0.1 µg/ml (uncorrected) would suggest that the dog had been given mefenamic acid within 24 hours of the race meeting at which the sample was collected.

TABLE 4.7.2: Results obtained for the analysis of mefenamic acid in plasma samples taken from a greyhound following the oral administration of mefenamic acid 250mg.

T I M E (hours)	Concentration Mefenamic Acid In Plasma Sample (µg/ml)
0	0.00
1	2.01
2	1.74
3	3.04
4	1.12
6	1.09
24	2.60
48	1.39

TABLE 4.7.3: Results obtained for the analysis of mefenamic acid in urine samples taken from a greyhound following the oral administration of mefenamic acid 250mg.

T I M E (hours)	Concentration Mefenamic Acid In Urine sample (ug/ml)	Concentration Mefenamic Acid In Urine Sample (µg/ml)
0	0.000	0.000
2	0.044	0.026
6	0.498	0.377
24	0.158	0.132
30	0.085	0.71
49.5	0.003	0.002
70.5	0.002	0.001

FIGURE 4.7.2: Concentration versus time for the plasma mefenamic acid levels obtained from a greyhound following the oral administration of mefenamic acid, 250mg.

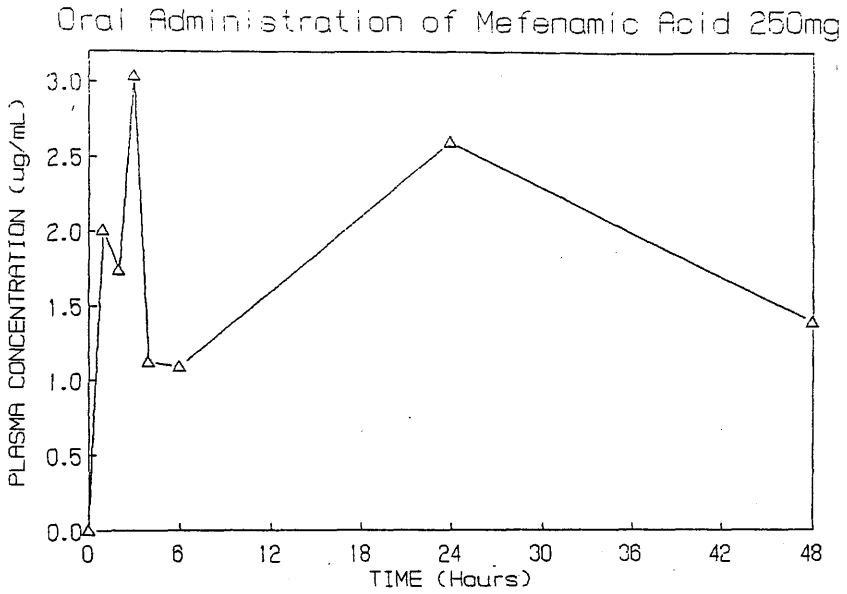


FIGURE 4.7.3: Excretion of mefenamic acid in the urine of a greyhound following the oral administration of mefenamic acid, 250mg.

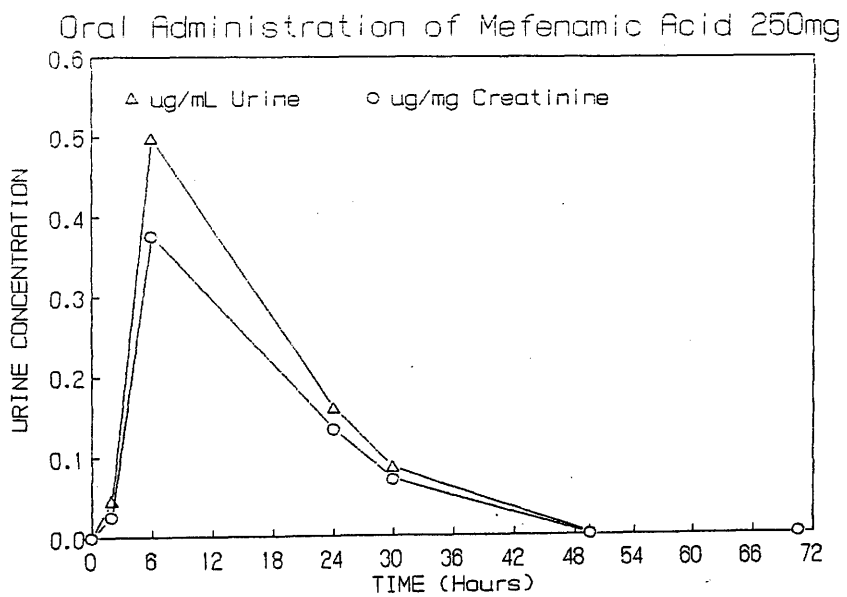
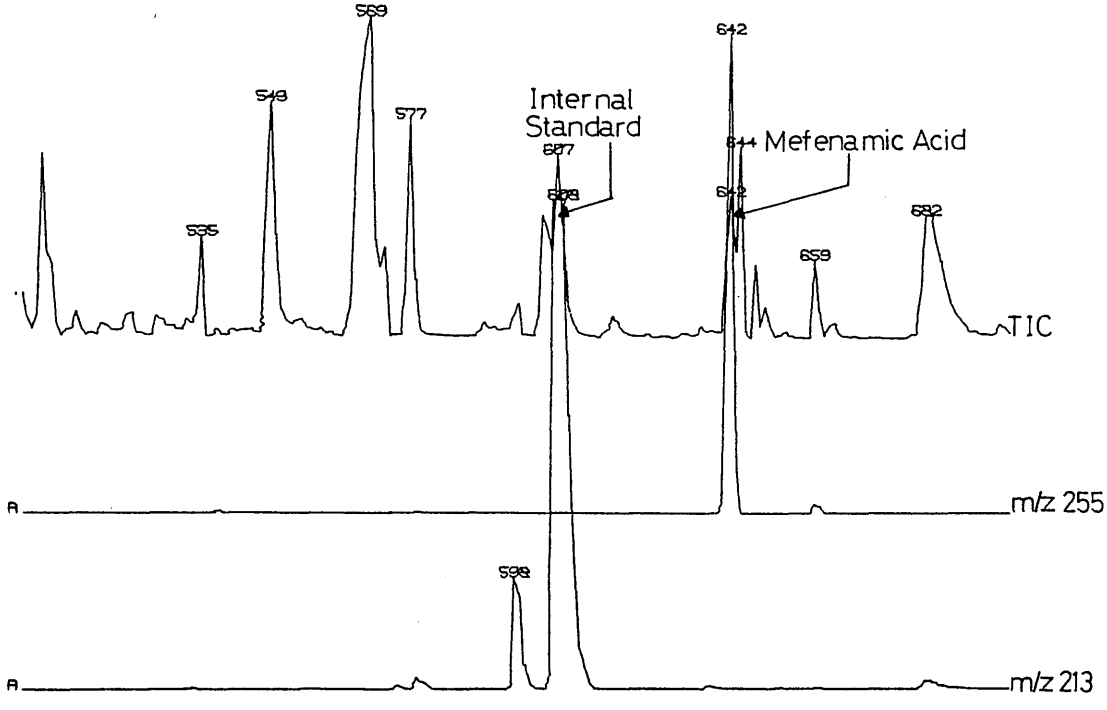


FIGURE 4.7.4: Total ion chromatogram and mass chromatograms (m/z 255 and 213) obtained from the GC-MS analysis of a methylated extract of greyhound urine (6 hours after the oral administration of 250mg mefenamic acid).



4.8 PHENYLBUTAZONE

4.8.1 INTRODUCTION

Phenylbutazone, the active ingredient of butazolidin, possesses anti-inflammatory, analgesic and anti-pyretic properties and has been used since 1949 in the treatment of rheumatic diseases. Due to serious toxicity (aplastic anaemia and agranulocytosis), mainly in the elderly, its use has been limited, since 1984 to the clinical treatment of ankylosing spondylitis. Phenylbutazone is one of a series of pyrazolone derivatives; basic information about this drug is summarised in Table 4.8.1.

Phenylbutazone is almost completely absorbed after oral administration. Following single oral doses of 100, 300 and 600mg to 6 subjects, peak plasma concentrations of about 14, 38 and 75μg/ml, respectively, were obtained in 1 to 7 hours (197]. Bruce and co-workers have reported [198] the pharmacokinetics of phenylbutazone in the dog. After oral administration of a single 150mg dose a maximum plasma concentration of 30.5μg/ml is achieved after 1 hour. The plasma half-life was 2.5 hours. However other workers have reported [199] that the plasma half-life increases with the size of the dose, for example, after 10 and 50mg doses the mean half-lives were 2.8 and 8.1 hours, respectively. Repeated dosing of phenylbutazone appears to cause induction of enzymes responsible for its metabolism in dogs but not in man, except to a moderate extent, resulting in a decrease in

TABLE 4.8.1: Description and basic pharmacokinetic data for phenylbutazone.

Chemical Name
4-Butyl-1,2-diphenyl-3,5-pyrazolidinedione

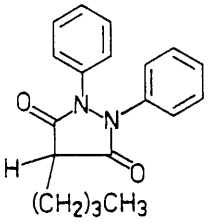
Chemical Abstracts Registry Number
50-33-9

Non-Proprietary Name
Phenylbutazone

Proprietary Names
Algoverine, Artrizin, Azolid, Butacote, Butagesic, Butazolidin(e), Butazone, Intrabutazone, Malgesic, Nadozone, Neo-Zoline, Phenbutazone and Tibutazone.

Empirical Formula
$C_{19}H_{20}N_2O_2$

Structural Formula



Molecular Weight
308.37

Dissociation Constant
pKa = 4.4

Physical Properties
A fine, white, crystalline powder. Melting point about 105°C. Practically insoluble in water; soluble 1 in 28 of ethanol, 1 in 1.25 of chloroform and 1 in 15 of ether.

Dose
100 and 200 mg tablets.
400-600mg daily.

Plasma Half-Life
About 2-5 days.

Protein Binding
about 99%

Volume of Distribution
0.18 litre/kg.

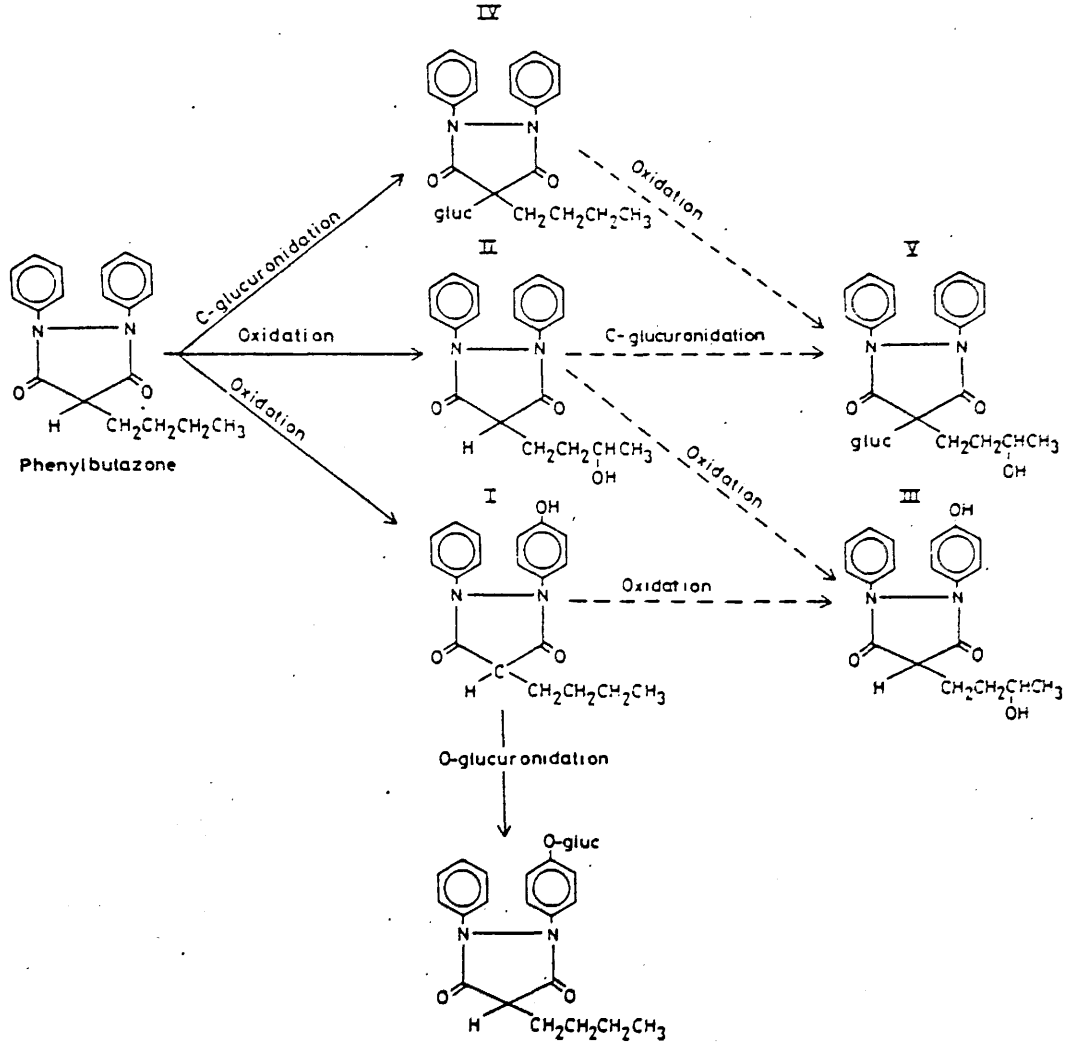
plasma concentration of the drug [200].

The estimated minimum lethal dose of phenylbutazone is 5g. Toxic effects during treatment are frequent and may occur even when the daily dose does not exceed 400mg. These side effects are usually associated with plasma concentrations above 100µg/ml [50]. Court and Volans have reported [191] a fatal dose of phenylbutazone as 2g in a 1-year-old, 2.9g in a child aged 15 and 5g in one of 4 years. Adults have suffered serious symptoms following the ingestion of between 4 and 40g of phenylbutazone and one adult who ingested between 14 and 28g of the drug developed pulmonary oedema 25 hours after the first dose and died 2 hours later.

4.8.2 METABOLISM AND EXCRETION

Phenylbutazone is extensively metabolised in man (Figure 4.8.1). The major routes involve side-chain oxidation, ring oxidation, combination of these and O- and C-glucuronidation [200,201]. Glucuronidation at a carbon atom, i.e. direct coupling of the pyrazolidine ring of phenylbutazone to glucuronic acid via a C-C bond, is an unusual metabolic transformation, first observed for phenylbutazone. Carbon glucuronidation has also been observed in sulphinpyrazone [112]. After a single dose, C-glucuronidation seems to be the dominant reaction, while oxidation becomes increasingly important after repeated administration. In plasma the pharmacologically active product of ring oxidation (p-hydroxy-phenylbutazone, oxyphenbutazone) is the major metabolite, whereas in

FIGURE 4.8.1 Major metabolites of phenylbutazone in man.
I = oxphenbutazone,
II = γ-hydroxyphenbutazone,
III = p,γ-dihydroxyphenbutazone,
IV = C-glucuronide of phenylbutazone,
V = C-glucuronide of metabolite II.
Broken lines indicated that the metabolic pathway is suggested by the structure of the metabolites, but that it has not been proven experimentally in man.
Gluc = glucuronic acid.*



* Reproduced from reference 201.

urine metabolites resulting from side-chain oxidation and C-glucuronidation predominate [202]. In both man and dog plasma decay of phenylbutazone is mostly due to metabolism as both urinary and biliary excretion are low [199]. Only about 1% of a dose is excreted unchanged in the urine.

4.8.3 RESULTS

A single oral dose of 100mg of phenylbutazone was administered to a greyhound. Urine samples were collected and analysed according to the methods described in Section 4.3. About 0.12 and 1.84µg/ml of phenylbutazone were detected in the 2- and 6-hour urine samples respectively, but not in any of the subsequent samples obtained. Figure 4.8.2 illustrates the total ion chromatogram along with 2 mass chromatograms showing the presence of phenylbutazone (m/z 308) and the internal standard, 2,3-dicarboxy-naphthalene (m/z 213). Neither oxphenylbutazone or any of the other oxidised metabolites were detected. In the absence of radio-labelled phenylbutazone it was not possible to monitor for the formation of C-glucuronides.

4.8.4 DISCUSSION

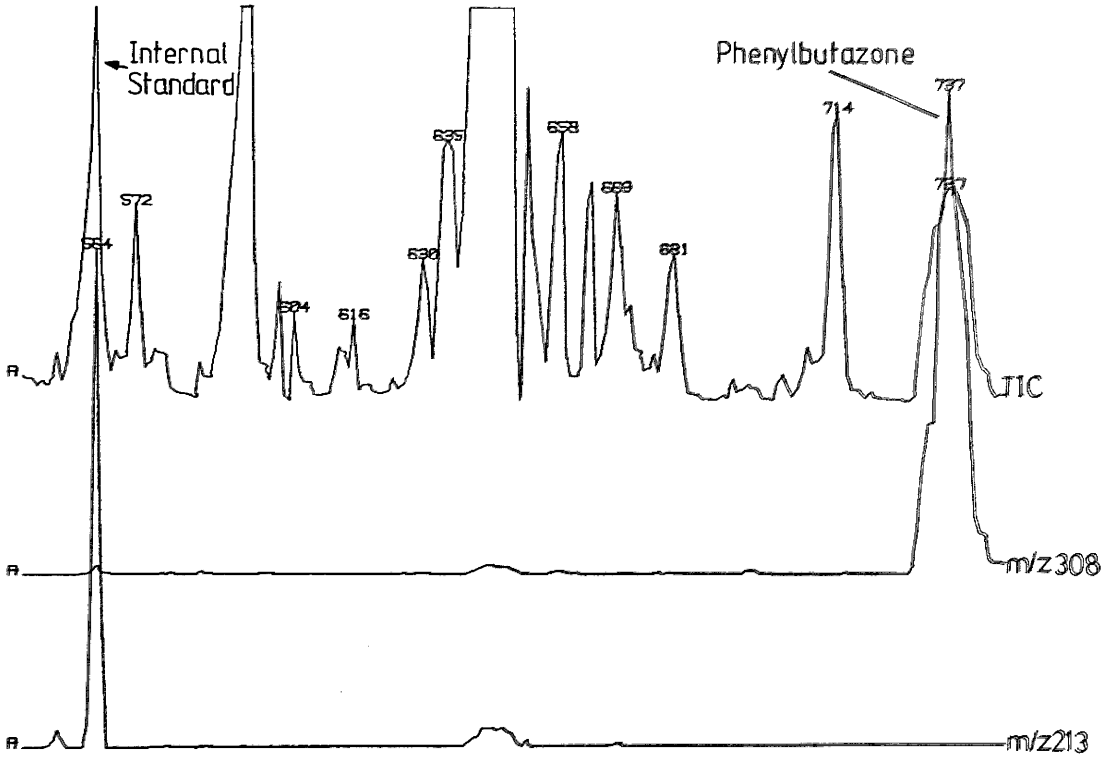
Following a single oral dose of 100mg of phenyl-butazone it was possible to detect the drug in urine up to 6 hours after administration. After this time the concentration of the drug fell below the detection limit in agreement with the literature reports that less than 1% of a dose is excreted in the urine. The dosage schedule used with the greyhounds allowed a sufficient interval

(4 weeks) to avoid significant induction of liver microsomal enzymes [198]. None of the known metabolites of phenylbutazone were observed, although it was recognised that C-glucuronides would not be sufficiently stable to be eluted through the GC column.

This study was carried out towards the end of the project. The difficulties in detecting phenylbutazone prompted the examination of possible reactions occurring between phenylbutazone and diazomethane. In this study the extracts had been methylated as it would be in a screening procedure. The results of this examination are given in Section 2.5 and showed that several products are in fact formed from phenylbutazone. Time did not permit the study to be repeated.

In conclusion phenylbutazone can be detected as the parent drug for short periods following administration in urine using the screening procedure developed during the course of this project. The detection of the drug in a race test sample would automatically imply that phenylbutazone had been administered shortly before the race. However, further investigation of the methylated products of phenylbutazone may extend the detection time although the drug appears to be excreted more rapidly in the greyhound than in man.

FIGURE 4.8.2: Total ion chromatogram and mass chromatograms (m/z 308 and 213) obtained from the GC-MS analysis of a methylated extract of greyhound urine (6 hours after the oral administration of 100mg phenylbutazone).



CHAPTER FIVE

CONCLUSIONS

The aim of this study was to develop suitable analytical methods for the detection and measurement of non-steroidal anti-inflammatory drugs in urine from the racing greyhound. The study was conducted in three parts, dealing with the evaluation of analytical methodologies, the investigation of the greyhound urinary acid profile and the metabolism of non-steroidal anti-inflammatory drugs in the greyhound, respectively.

The development of a screening procedure for non-steroidal anti-inflammatory drugs (NSAIDs) in urine is a complex problem because, although the drugs have similar pharmacological effects they encompass a wide range of chemical types which are extensively metabolised to form acidic products in urine and faeces. In urine especially the problem of detecting drugs and their metabolites is complicated by the presence of many other organic acids similar in structure to the compounds of interest. However, the administered dose of anti-inflammatories is relatively high and it is often possible to detect small quantities of the parent drug in urine. Screening procedures are therefore particularly required to overcome the problems of specificity rather than sensitivity.

In the present work only chromatographic procedures were considered. Another viable approach would be a

series of immuno-assays. These procedures, however, are time-consuming and expensive to develop for a diverse group of compounds and are unlikely to satisfy either the analytical or medico-legal requirements for specificity.

HPLC was found to be unsuitable as a screening procedure for NSAIDs. Isocratic systems such as the one used in this evaluation are suitable for the analysis of specific or known groups of compounds but are unable to resolve completely all of the twelve test drugs. It should also be noted that there are many other NSAIDs on the market which, in the presence of their metabolites and other urinary components, pose an insurmountable problem with respect to specificity. This problem could be reduced by the use of a gradient system to improve resolution, although these systems have in the past been unreliable and non-reproducible. Alternatively, increased specificity could be achieved by using different detectors in series thereby increasing the amount of information available on the compounds eluted from the column.

Careful use of pre- and post-column derivatisation reactions may also offer increased specificity and sensitivity, for example, hydroxylated metabolites might be distinguished from the parent drug or fluorescent derivatives might be prepared from compounds with no inherent fluorescence. GC is more suitable as the basis for a screening procedure because of the ready availability of high resolution capillary columns and reproducible temperature programming. The main drawback,

however, is the lack of thermal stability of some NSAIDs even following derivatisation of the acid function. As with HPLC, the problems of specificity can still arise and are amenable to the same approach of using multiple detectors or they can be largely overcome by the effective, though expensive, use of a mass spectrometer as the GC detector. From experience, a single capillary column is unlikely to be able to resolve the complex mixture of components present in acidic urinary extracts: an additional 'dimension' is required, supplied either by mass spectrometry or column switching techniques.

The screening procedure developed was based on capillary GC-MS. This is still the only readily available method with sufficient resolution, specificity and sensitivity to detect a diverse group of target analytes in a complex matrix which contains many endogenous masking agents. One possible alternative to GC-MS would be gradient HPLC-MS, but this technique has not yet been sufficienty developed to provide a robust procedure. Nevertheless a need remains for a preliminary screening procedure capable of dealing with large numbers of samples in which tentative positives could be confirmed by GC-MS. None of the common screening methods based on immunoassay or TLC are likely to provide a solution. Until an alternative is found, control of the abuse of non-steroidal anti-inflammatories must depend on the deterrent effect of random testing.

The greyhound urinary acid profile differed from

the human profile in both qualitative and quantitative composition. Despite the extensive research on urinary acids in the clinical context many of the compounds found in the urine of the greyhound remain unidentified. However, the mass spectra of many of the constituents have been tabulated and have proven to be a valuable reference collection during studies of drug metabolism. Further characterisation of the endogenous components and of factors affecting the profile will be required to allow more of the drugs and their metabolites to be detected.

The derivative of choice for the metabolic study was the methyl derivative because the mass spectra of many of the methyl derivatives of urinary acids have previously been recorded, reducing the number of unknown components in the analysis. However, the butyl-dimethylsilyl derivatives provided mass spectra with excellent diagnostic ions for the detection of target analytes. These derivatives might form a valuable alternative to the methyl derivatives or the trimethylsilyl derivatives used in clinical chemistry.

The analytical procedure developed during this project was able to detect five representative NSAIDs which had been administered to greyhounds and initial pharmacokinetic data was obtained with implications for the time intervals over which the drugs might be detected. Information was also obtained on metabolic routes of some of the drugs examined and possible cut-off levels were suggested. Future work would require a

statistically significant number of animals to be studied using different dosing regimens to establish a normal range of pharmacokinetic parameters of each drug of interest in the greyhound population.

Of the five drugs examined in this project phenylbutazone presented the most serious problems of detection due to formation of multiple products with diazomethane. This could be avoided with the use of the butyl-dimethylsilyl derivative.

The work carried out in this project has provided the N.G.R.C. with some of the information needed to control the abuse of drugs in its sport. Another aspect of control concerns the interpretation of the significance of drugs detected and their probable effects on performance. This problem will be addressed in future work by concurrent observations of the racing form of the dogs during the course of metabolic studies.

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