THE METABOLIC DISPOSITION OF PHENYLBUTAZONE AND OXYPHENBUTAZONE IN THE HORSE

by

Philip Barnaby Ward Smith

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University of London

Department of Pharmacology,
St. Mary's Hospital Medical School,
Paddington, London, W2 1PG

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ABSTRACT

Phenylbutazone has widespread application in equine medicine, and remains the drug of choice for treating horses with musculo-skeletal disorders, although recent concern over toxicity has led to calls for a re-evaluation of its veterinary use. Phenylbutazone and oxyphenbutazone are occasionally encountered as doping agents, and the most recent data suggests that they are responsible for over a third of all reported equine doping incidents. Despite the current interest in these compounds, and the potential utility of such knowledge, there is a paucity of information concerning their metabolic disposition in the horse. This thesis presents a study of the metabolic disposition of the two non-steroidal anti-inflammatory drugs (NSAID's), phenylbutazone and oxyphenbutazone, in the horse.

The following investigations were undertaken:-

^{14}C-Phenylbutazone and ^{14}C-oxyphenbutazone, the latter having been synthesised from diethyl [2-^{14}C] malonate, were administered to horses orally and intravenously on separate occasions. Urinary and faecal elimination of ^{14}C was monitored for up to 7 days after dosing, and plasma concentrations of phenylbutazone and oxyphenbutazone measured by HPLC. After oral administration phenylbutazone was rapidly and extensively absorbed, with an absolute bioavailability of 91%. Similarly, oxyphenbutazone was initially rapidly absorbed, after oral administration, but this was followed by a prolonged slow absorption phase, and consequently the plasma concentrations of oxyphenbutazone measured at 24 h after oral dosing, were double those achieved after intravenous administration. Oxyphenbutazone was not as extensively absorbed as phenylbutazone, with an absolute bioavailability of 78%. The plasma elimination half-lives of phenylbutazone and

oxyphenbutazone were 9.7 h and 6.3 h respectively, and these were independent of route of administration.

The pattern of elimination of phenylbutazone was independent of route of administration, with 55% of the dose found in the urine in 3 days and 40% in the faeces in 7 days. The incomplete absorption of oxyphenbutazone, however was reflected in the pattern of elimination of this compound. Urinary elimination accounted for 60% (i.v.) and 53% (p.o.) of the dose, with an additional 33% (i.v.) and 44% (p.o.) of the dose recovered in the faeces.

A full metabolic profile of the urinary elimination products of phenylbutazone and oxyphenbutazone was obtained. The parent drugs and metabolites were separated by HPLC, and characterised by GCMS. In addition to *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone, the two compounds previously identified as metabolites of phenylbutazone in the horse, three new metabolites were identified, namely ω-hydroxyphenylbutazone, γ-ketophenylbutazone and *p*,γ-dihydroxyphenylbutazone. >80% of the total urinary radioactivity was accounted for as these identified compounds. In contrast to phenylbutazone, oxyphenbutazone was excreted in the urine largely as the unchanged drug, accompanied by small amounts of *p*,γ-dihydroxyphenylbutazone. The kinetics of the urinary elimination of phenylbutazone and its major metabolites were also investigated. Up to 12 h after dosing, γ-hydroxyphenylbutazone was the major urinary metabolite, but in later samples oxyphenbutazone predominated.

The O-methyl and C-methyl derivatives of phenylbutazone were synthesised, and the characteristic mass spectral fragmentation patterns of these and additional derivatives established, enabling the structural elucidation of previously unidentified phenylbutazone metabolites.

In summary a comprehensive qualitative, quantitative and kinetic appraisal of the metabolic disposition of orally and intravenously administered phenylbutazone and oxyphenbutazone in the horse has been undertaken, and the findings are discussed in relation to the known toxicity of these compounds, and their implications for the detection of the illicit administration of these drugs to horses.

CONTENTS

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CHAPTER 1.

GENERAL INTRODUCTION

Doping:- an historical background

The term 'dope' first appeared in an English dictionary in 1899 and was defined as a mixture of opium and narcotics used for horses. The root of the word is South African where 'dop' means hard liquor and was thought to originate from the Dutch verb 'doopen' to swill. The Oxford English Dictionary now defines dope as a "drug etc. given to horse or greyhound to affect its performance or to athlete as stimulus."

For the purpose of implementing anti-doping regulations there are a variety of definitions for what constitutes doping. Some racing authorities such as those of Japan and Canada publish lists of prohibited substances, whilst in the U.K. the Horse Racing Anti-Doping Committee has defined doping as "the detection in its tissues, body fluids or excreta of any quantity of any substance which is either a prohibited substance or a substance the origin of which cannot be traced to normal and ordinary feeding and which by its nature could affect the performance of the horse." For the purpose of this definition a 'prohibited substance' is any substance originating externally, whether or not it was endogenous to the horse.

Doping in sport is not solely a recent phenomenon. In the 3rd Century B.C. athletes in the Olympic Games tried to improve their performance by eating mushrooms (Hanley, 1979) and Roman chariot horses were doped with *'hydromel'* a mixture of honey and water, an offence punishable by crucifixion. Since then, the doping of racehorses has become increasingly sophisticated, and the punishment less severe. Much of the historical background to doping in racehorses has been described by Tobin (1981): the first reported doping of racehorses occurred at Worksop, where an edict in 1666 banned the use of

exciting substances. Concern about stimulants at this date is odd, since most of the doping at this time was extremely crude and involved with stopping or 'nobbling' horses. The first reported trial involving doping was at Cambridge Assizes in 1812 where Donald Dawson was tried, and found guilty for poisoning horses at Newmarket with arsenic.

Stimulant doping appeared in England around the turn of the century, with the arrival of the American trainer Wishard. Wishard was accompanied by two American backers, who brought with them a substantial quantity of cocaine. It is estimated that in the four years in which they operated they made approximately two million pounds. Their usual routine was typified by their success with a horse called *Royal Flush.*

Royal Flush was purchased for a modest 450 guineas, and at six years of age was considered to be well over the hill. However, following the administration of a suitable quantity of cocaine *Royal Flush* finished first to win the Royal Hunt Cup at Ascot, and with a slightly increased dose, the Steward's Cup at Goodwood a couple of months later. This was the last coup that Wishard and his associates perpetrated as George Lambton, the leading English trainer of the time, had finally managed to convince the Jockey Club to take some action to prevent the administration of illegal stimulants.

The veterinary profession were worried about the effect of doping on the horse breeding industry, and brought the matter to the attention of the Jockey Club, who declined to take any action. George Lambton's solution was to quite openly dope his own horses, and after he achieved remarkable success with horses that had never won previously, the Jockey Club were finally convinced, and by the end of 1902 made doping an offence.

Other European racing authorities soon followed the English lead, but the embargo had little effect and doping became widespread throughout the continent. In 1910 the Austrian authorities employed the services of a Russian chemist, Bukowski. Bukowski claimed to be able to detect the presence of various drugs in the saliva of horses, but refused to disclose his methods. It seems unlikely that he was able to detect the secretion of drugs in saliva, but rather contamination from oral dosing. The tests were adequate for the small number of drugs (strychnine, morphine, cocaine, caffeine) in use at that time and the situation was partially resolved. History was made in 1912 when *Bourbon Rose* the winner of the Gold Cup in France yielded the first positive dope test, and was subsequently disqualified.

Meanwhile, the position in America was reaching ridiculous proportions, with tack rooms reportedly equipped like pharmacies, complete with scales, test-tubes, and an astonishing selection of drugs. Because the authorities made no attempt to enforce whatever anti-doping rules were in existence, an honest trainer had little choice but to adopt similar tactics. Some convictions were eventually obtained, but these were under the United States narcotic regulations, and not under any rules imposed by the racing authorities. Finally the Florida Racing Commission decided to tackle the problem, and after a visit to France to learn about saliva testing, routine drug testing was started. The situation was gradually brought under control as more and more States set up there own laboratories. In 1947 these men formed the Association of Official Racing Chemists (AORC).

The situation in England gradually worsened, partly as a result of the military use of amphetamines during the war, and after several notorious doping conspiracies a committee was appointed by the Jockey Club to investigate. As a result of their recommendations the Horse Racing Anti-Doping Committee

(HADC) was formed, and a decision taken to set up their own forensic laboratory. This laboratory, now known as the Horseracing Forensic Laboratory (formerly Racecourse Security Services) was originally set up under the auspices of the Animal Health Trust, but since then has become independent. Routine dope testing was introduced into Britain in 1963.

There are essentially two possible strategies for the detection of illegal medication; pre-race and post-race testing.

Pre-race testing

Pre-race testing is the only means which actually prevents the running of a horse that has received illegal medication, thereby removing a lot of the financial incentive to doping, since the horse is disqualified before the race; with post-race testing in the event of a positive, the prize money will be forfeited, but quite often the value of the purse is substantially less than the financial gains from betting, which are usually paid out immediately after the race. One of the first attempts at pre-race testing was carried out in Maryland in the 1930's. The basis of the test was the 'Straub Reaction', if a mouse is injected with morphine or a related opiate its tail will form an 'S' shaped curve. Samples of urine or saliva were injected into mice, and any horse that gave the positive reaction was automatically scratched. Specific for opiates, this test could not detect any other compounds and this procedure eventually lapsed. Despite many inherent advantages, pre-race testing is only carried out in a few States in America, because of the constraints imposed by various analytical and operational considerations: the number of samples is much greater and the time available for analysis much shorter. Blood or saliva is usually the only testing medium available, which imposes further limitations on analytical methods, as the sample volume is smaller and the concentration of drugs and/or drug

metabolites usually much lower than in urine. Since the testing equipment must be located at the local track, neither the amount nor the quality of analytical equipment is the same as that available at a central post-race laboratory. Pre-race testing is relatively expensive, and although a mobile laboratory would reduce the amount of personnel and analytical equipment needed, with race meetings taking place at up to ten different courses on the same day (in the U.K.), the amount of resources required would still be substantial.

Post-race testing

The usual method of post-race testing is to collect a urine sample (and in some instances a blood sample) as soon as possible after the race. Not all the horses are tested, usually the winners, beaten favourites, and any horses that have aroused the suspicions of the Stewards. Once selected the horses identity is confirmed by the trainer (or his representative), who witnesses the collection and labelling of the urine sample. In most systems the sample is split into two bottles labelled 'A' and 'B' and identified only by a code number. Initially only one sample is analysed, and in the event of a positive, the 'B' sample is made available for confirmatory analysis by an independent laboratory. While many chemists discard their negative samples, at least one racing authority keeps a portion of any negative sample in the freezer. If a new analytical technique then becomes available, or a particular pattern of drug abuse is suspected, the laboratory can reassay any previous samples. Although one positive sample is sufficient for action to be taken, a series of samples is much more compelling evidence. This was amply demonstrated by a case involving the Illinois Racing Board, who by the end of 1974 had accumulated 25,000 samples. During this time they suspected that a particular veterinarian was involved in the widespread doping of horses, but were unable to identify the compound involved. Eventually they obtained a sample of the 'dope' prior to its

administration to a horse, and it was subsequently identified as apomorphine. Armed with this knowledge, and after altering their analytical procedures, they reassayed the suspect samples, and found a string of positives for apomorphine.

The collection of urine samples is not without problems. The attendant has to wait for the horse to void a urine sample spontaneously which, in common with humans, horses are often reluctant to provide while being watched. In the early days of post-race testing there were several cases of positives for caffeine and nicotine, which had arisen because the attendants, having lost their patience, provided a personal sample in lieu of the equine sample; on one occasion a particularly frothy urine sample was on closer inspection found to be lager !

Types of doping

The late Professor E.G.C. Clarke of the Horse Racing Anti-Doping Committee divided doping under various categories which in a slightly modified form are illustrated in Table 1.1.

TABLE 1.1 Categories of medication in perfomance horses

1. **Medication to win**

   a) Acute:- either short acting stimulants e.g cocaine, or else small amounts of tranquillisers e.g. acetylpromazine to "take the edge off" an excitable horse.

   b) Chronic:- repeated dosing for weeks or months e.g. anabolic steroids.

2. **Medication to lose**

   Depressants: large doses of a tranquilliser/sedative e.g. acetylpromazine.

3. **Medication to restore normal performance**

   a) Non-steroidal anti-inflammatory drugs: e.g. phenylbutazone. Often permitted under controlled medication rules in certain States of America.

   b) Corticosteroids: e.g. betamethasone. Administered either systemically or intra-articularly to control joint pain; occasionally permissible in certain States of America.

   c) Local anaesthesia: nerve or joint blocks either temporarily with anaesthetics e.g. lignocaine, or more permanently by destruction of the nerve e.g. alcohol injections.

4. **Accidental**

   a) Dietary components: e.g. caffeine and theobromine from cocoa husks.

   b) Procaine from procaine penicillin.

5. **Medication to prevent the detection of other drugs**

   a) Administration of various compounds e.g. thiamine, thought by some to interfere with the detection of illegal medicaments.

   b) Administration of diuretics to dilute the concentration of other drugs in the urine: e.g. frusemide

   c) Administration of basic or acidic compounds to alter urinary pH e.g. bicarbonate

6. **Miscellaneous mechanisms**

   'Blood strategies'

Doping to win can conveniently be divided into chronic and acute doping. In acute stimulant doping the horse is given a rapidly acting stimulant such as amphetamine shortly before the race, this strategy is usually considered to be an 'inside job', and in order to be successful requires a good working knowledge of the pharmacology of the compound involved. Chronic doping is typified by the use of anabolic steroids, which are given for weeks or months before the event. Their use may be discontinued days before the race, as although the pharmacological effect will persist for some time, the compound may well be undetectable at the time of racing.

Paradoxically tranquillisers can also be used to improve a horse's performance, as some horses which demonstrate a sparkling ability on the home gallops, seem temperamentally unable to cope with the altered routine on a raceday. They become hyperexcitable, excessively nervous, and lose a lot of fluid by sweating. To overcome this problem small amounts of tranquillisers such as acetylpromazine are used. Obviously if the dose given is too large then, far from enhancing the horse's performance, the opposite will occur.

Doping to lose is typified by the administration of a large dose of tranquilliser or depressant. Generally speaking doping to lose is considered to be an 'outside job', since there are much easier and less detectable methods for a trainer to stop a horse e.g. overgalloping the horse the day before the race.

Medication to restore normal performance is usually concerned with the elimination of musculo-skeletal pain. It is illegal in Britain and most other countries, but the use of certain drugs is permitted in certain States of America. Once a horse is under controlled medication then the racing authorities must be notified, and in the case of phenylbutazone, once the horse has been given the drug it must continue to receive it throughout the season. This has led to a

'Catch 22' situation whereby analysts must verify that the horse has been given the medicament.

Accidental or inadvertent doping is often encountered, and is responsible for many of the positives reported. Procaine is a potent local anaesthetic, and as such can be used to 'block' a nerve or a joint: by preventing any discomfort it can allow a dangerously unsound horse to race. The use of any drug for this purpose is prohibited. Unfortunately procaine is also a product of the long acting antibiotic procaine penicillin, and may be excreted in urine for up to two weeks after a legitimate therapeutic dose. The pH of horse urine can vary from 4.5 to 10 (Tobin & Woods, 1979), and this can result in large variations in the urinary levels of some compounds. For a compound such as procaine, whose pK_a (8.7) is well within this range, this can theoretically result in a several thousand fold range in urinary levels, for the same urine volume and plasma concentration of the drug. These large variations can give rise to inadvertent positives (Tobin, 1981). Between 1947 and 1973 procaine was reported as a positive more times than any other drug, and it seems likely that a substantial proportion of these were inadvertent.

Another source of inadvertent positives is from compounds present in the diet. In the early 1970's there was a spate of positives for caffeine and theobromine, and this was eventually traced to the inclusion of cocoa husk in horse rations by a feedstuff manufacturer. In 1979 the horse *No Bombs* was disqualified after winning a £4000 race when both caffeine and theobromine were detected in the post race urine sample. This was traced to the horse having snatched and eaten a *Mars bar* from a stable boy on the way to the starting line. A similar event recently made front page news "Mars bars De Rigeur from £10,000 win" (*The Guardian* 7 Jan. 1987) after a *Mars bar* was given to a horse before it won a big race at Ascot.

Another cause of inadvertent doping may result from an increase in the sensitivity of the analysts methods. It is not uncommon for horses to receive medication in between races, and the drug or metabolites may be present in the urine long after the pharmacological effect has ceased. Experience will dictate for how long before the race the medication must be withdrawn, and this 'clearance time' is a measure of the sensitivity of the analysts testing procedure for the drug involved. An increase in the sensitivity of the test will result in an increased clearance time, and consequently a previously established 'safe' practice will now give rise to a positive. Recent advancements in analytical equipment and methodology (in particular the advent of combined gas chromatography-mass spectrometry) have increased the sensitivity of the testing procedures to such an extent that in some circumstances authorities may be compelled to act in situations where discretion would be more appropriate, or else to knowingly ignore their own regulations. The problem of calling positives on obviously non-effective levels of drugs, has led to proposals for quantification of drug levels, and the setting of a threshold level, below which no action would be taken.

Doping to prevent the detection of other drugs is occasionally attempted, and there are various strategies that have been tried for this. The administration of large quantities of a permitted substance is thought by some to mask the presence of an illegal medicant, but with modern analytical techniques this should not present a problem. By altering urinary pH one can enhance or reduce the elimination of some acidic or basic drugs. Amphetamine abusers sometimes co-administer bicarbonate to reduce its renal elimination (Hirom & Smith, 1975) thereby prolonging its effect, and decreasing the urinary concentration. In certain States of America where diuretics such as frusemide are permitted medication for 'bleeders' (horses suffering from epistaxis) the resultant dilution of the urine, which can be up to 50 fold, may interfere with

the detection of drugs and metabolites in the urine.

One of the more recent methods of enhancing athletic performance is by the manipulation of various blood strategies. By their nature these are extremely difficult to detect. Blood loading is reputedly both potent and widely used in athletics, and was used to great effect by the American cycling team in the Los Angeles Olympics (Beckett, 1986). A volume of blood is withdrawn weeks before the race, the red blood cells separated and stored, then a few hours before the race the blood cells are returned. This supposedly increases the oxygen carrying capacity of the blood, and thereby the performance. Although relatively straightforward with human athletes, the much greater circulating volume in the horse requires the transfusion of substantial amounts of blood.
By rendering the horse's blood alkaline by administration of bicarbonate, the onset of metabolic acidosis, which produces a shift to the left in the oxygen-haemoglobin dissociation curve and is therefore a contributory factor in the development of fatigue, can be delayed. This is thought to be of particular benefit in long races where exhaustion is a limiting factor in the performance.
The affinity for oxygen demonstrated by haemoglobin molecules is related to the concentration of intracorpuscular 2,3-diphosphoglyceraldehyde (2,3-DPG) (Benesch & Benesch, 1969). The avian equivalent of 2,3-DPG, inositol hexa-phosphate is found at elevated levels in migratory birds, and it is thought that by increasing 2,3-DPG levels athletic performance can be improved (Goodford *et al.*, 1978).

Future doping strategies and their detection

Advances in analytical techniques such as high performance liquid chromatography (HPLC), radioimmunoassay (RIA), capillary gas chromatography and combined gas chromatography-mass spectrometry (GCMS), have increased both

the scope and the sensitivity of the tests available, but there is some concern within the anti-doping laboratories about their ability to detect the possible doping agents of the future. As pharmaceutical chemists develop more and more specific and potent drugs, the dose required has decreased from grams to milligrams. The discovery of endogenous neuropeptides such as enkephalin and endorphin, and the subsequent development of synthetic analogues will present enormous problems for the analyst, since not only is the active amount minute (< 1*u*g) but also, present analytical techniques are not applicable for the separation and identification of these compounds. The picture is further complicated by the possible use of other endogenous compounds.

The amino acid L-tryptophan, the dietary precursor of the neurotransmitter 5-hydroxytryptamine (5-HT), is one of the main contenders in the search for an undetectable tranquilliser. Tryptophan loading not only has a tranquillising effect (Hartmann, 1982), but also increasing dietary tryptophan has been shown to produce analgesia (Lieberman *et al.*, 1982). One of the more esoteric techniques under consideration is laser therapy. Treatment with lasers has been demonstrated to elevate plasma 5HT levels by 400%, which may result in higher brain 5HT levels which have been shown to produce analgesia (Seltzer *et al.*, 1982).

A more immediate cause for concern is the increasing use of endogenous steroids, and the resultant problem of differentiating between natural variations in endogenous levels and the exogenous administration of these compounds, solely on the basis of urinary levels. As well as differences in urinary pH and volume, individual differences between horses will also give rise to variations. It seems unlikely therefore that one could establish that a high level of an endogenous compound was necessarily the result of the administration of that compound.

A measure of the variability of a normally distributed population is given by its standard deviation. Within ± 2.33 standard deviations of the mean one should find 99% of the population, therefore statistically the number of samples found with higher levels than this, entirely due to chance will be 0.5% or 1/200. Although this may seem negligible, it does mean that out of 10,000 horses tested there will be some 50 false positives. This was illustrated by the case involving *Hill House* (winner of the Schweppes Gold Cup at Newbury in 1967). The urine sample taken after his unexpected victory was found to contain abnormally high concentrations of cortisol; the horse was subsequently disqualified, and the owner and trainer suspended. The owner and trainer denied administering steroids, and proposed that the high levels of cortisol, although biochemically atypical, were normal for this individual horse and furthermore, they claimed that this was supported by independent scientific evidence. The horse was subsequently removed from the trainer, securely stabled at the Equine Research Station in Newmarket, and the urinary levels of cortisone periodically measured. The data obtained supported the trainer's case, the horse was re-awarded the race, and the suspension quashed. An essentially similar event concerning 19-nortestosterone and the horse *Vayran* occurred several years later; once again after further investigations the horse was re-awarded the race. As Tobin (1981) points out "the testing of some 10,000 horses a year is a good way of finding the biochemically atypical horses in the population."

The changing pattern of doping

For historical reasons that are not all that clear, the Association of Official Racing Chemists (AORC) have attempted to suppress the publication of the various methods used by their members for the detection of illegal medicaments and the whole of this area is surrounded by an air of secrecy. The only data

available on doping are those occasionally released by the AORC, which simply list the number of times various drugs have been detected by their members. It must be emphasised that the incidence of a reported positive does not necessarily reflect the incidence of doping with that compound, and that the absence of a compound does not mean that it is not being illicitly used. However some idea of the trends in the pattern of drug abuse can be obtained by comparing the statistics for the returns of the AORC for 1947-1973 and 1984-1985 (Tables 1.2 & 1.3). Unfortunately neither table is complete, and for various reasons the data are not strictly comparable. In the 26 years between 1947 and 1973, 77 compounds were reported as positives on at least two occasions, but in the 2 years 1984 and 1985, 128 compounds appeared more than once. The increase in the diversity of the drugs used reflects both the increasing number of compounds now available, and improvements in analytical techniques. Combined GCMS is now used in the majority of forensic laboratories, usually in combination with extensive data bases. The Horseracing Forensic Laboratory has a 'user defined' library of over 900 drugs, and with immediate access to the joint Environmental Protection Agency - National Institute of Health (EPA-NIH) library, a positive identification of over 33,000 compounds can be obtained within minutes of obtaining a mass spectrum. Although the number of drugs of abuse has increased, the incidence of finding a positive has remained fairly constant. The total number of positives in 1950, the first year for which data is available was 73 out of a sample of 37,518 or 0.20%, by 1970 the number of samples had risen to 126,643 and of these 191 or 0.15% were positive, the average incidence over this period was 0.12%. The total number of positives in the two years 1984 and 1985 was 3489 out of a total of 1,442,082 samples or 0.24%.

Trends in the type of drug used for doping can be seen In Table 1.4. where they are (rather crudely) classified on the basis of their pharmacological actions into

9 groups. The incidence in the detected abuse of local anaesthetics and CNS stimulants has decreased sharply, whilst the detection of diuretics, and non-steroidal anti-inflammatory drugs (NSAIDs) has increased. The increase in the appearance of NSAIDs was documented by Moss (1972), who listed the relative frequency for various years. In 1959 the incidence was 4%, in 1962 it had risen to 11% and four years later had doubled to 22%. In the last year reported (1970) the incidence had risen to 40% and the most recent data (1984-1985) show that NSAIDs accounted for over half of the total number of positives reported. Of these phenylbutazone and the related compound oxyphenbutazone (its active metabolite) were by far the most important, accounting for 64% and 8% of the NSAID positives, or 32% and 4% of all positives reported.

The dramatic increase in the incidence of NSAIDs is in part due to changes in the regulations, in particular the advent of controlled medication in America, and in part due to alterations in the sample reported on by the AORC. The data for 1984 and 1985 includes instances where the plasma or urinary concentration of a permitted substance exceeded an allowed maximum level, and drug positives from horse shows are now included in the AORC data.

TABLE 1.2 Drugs reported by members of the Association of Official Racing Chemists 1947-1973

Procaine	661	Mephenesin	6
Caffeine	482	Pemoline	6
Amphetamine	452	Pyrilamine	6
Phenylbutazone	267	Acetylsalicylic acid	5
Methylphenidate	193	Butacaine	5
Theobromine	127	Imipramine	5
Methamphetamine	121	Methoxamine	5
Dipyrone	116	Propiomaxine	5
Polyethylene Glycol	111	Tetracaine	5
Phenobarbitone	86	Chloroquine	4
Oxyphenbutazone	66	Hydrocortisone	4
Morphine	62	Levorphanol	4
Ephedrine	59	Prednisone	4
Strychnine	50	Mefenamic acid	4
Thiamine	50	Meperidine	4
Pentazocine	49	Cinchonidine	3
Nikethamide	44	Propoxyphene	3
Barbiturates	47	Sulphanilamide	3
Promazine	38	Sulphaphenazole	3
Methapyrilene	35	Thiabendazole	3
Nicotine	33	Acepromazine	2
Indomethacin	26	Antipyrine	2
Ethylaminobenzoate	23	Barbitone	2
Atropine	22	Codeine	2
Pipradol	21	Chloral hydrate	2
Phenothiazine	18	Dibucaine	2
Lignocaine	16	Doxapram	2
Chlorpromazine	15	Guaiacol	2
Prednisolone	13	Phemitone	2
Theophylline	12	Meprobamate	2
Mephentermine	11	Naphazoline	2
Leptazole	10	Pangamic acid	2
Acetophenitidin	9	Sulphonamide	2
Cocaine	9	Acetophanazine	1
Methocarbamol	8	Amydricaine	1
Phenylpropanolamine	8	Berberine	1
Salicylic acid	8	Bromide	1
Hyoscine	8	Camphor	1
Amylocaine	7	Capsaicine	1
Brucine	7	Chlorobutanol	1
Quinine	7	Cinchonine	1
Thozalinone	7	Cinchophen	1
Apomorphine	7	Danthron	1
Alcohol	7	Dapsone	1

TABLE 1.3 Drugs reported by members of the Association of Official Racing Chemists 1984 & 1985

Phenylbutazone	1107	Hydroxyethylpromazine sulfoxide	14
Procaine	208	Salicylic acid	14
Caffeine	186	Acetyl promazine	13
Flunixin	183	Pentazocine	13
Dimethyl Sulfoxide	172	Bumetanide	12
Frusemide	166	Bupivicaine	12
Oxyphenbutazone	136	Hydroxyethyl promazine	12
Apomorphine	68	Xanthines	12
Theobromine	66	3-Hydroxypromazine	11
T-61	54	Clenbuterol	11
Etorphine	47	Hordenine	11
Polyethylene Glycol	44	Hydroxypropyl promazine	11
Dipyrone	36	Nikethamide	11
Naproxen	35	Sulfaphenazole	11
Acetylpromazine Metabolites	31	2-(1-Hydroxypropyl) promazine	10
Dexamethasone	31	Nefopam	10
Prednisolone	28	Prednisone	10
Isoxsuprine	27	Primidone	10
Phenylpropanolamine	26	Guaiacol Glyceral Ether	9
Benzocaine	25	Mazidonol	9
Nalbuphine	24	Oxymorphone	9
Sulfamethoxazole	24	Pemoline	9
Theophylline	24	Dihydromorphone	8
Butorphanol	22	Lidocaine	8
Tetracaine	21	O-Desmethyl pyrilamine	8
Sulfadimethoxine	20	Trimethoprim	8
Trichloromethiazide	20	Arsenic	7
Guaifenesin	19	Meclofenamic acid	7
Methocarbamol	17	Mephentermine	7
Methylphenidate	17	Methadone	7
Hydrocortisone	16	Ephedrine	6
Sulindac	16	Estrane 3,17 diol	6
Chlorobutanol	15	Hydroxycamphor	6
Hydromorphone	14	Lignocaine	6

TABLE 1.4 Comparison of the types of positive samples found in 1947-1973 and in 1984-1985

	% incidence	
	1947-1973	1984-1985
1. Anaesthetics Procaine, lignocaine, amylocaine, tetracaine, butacaine etc.	21.0	8.3
2. Methylxanthines Caffeine, theobromine, theophylline etc.	18.9	8.5
3. CNS Stimulants Apomorphine, amphetamine, cocaine, methylphenidate etc.	27.0	4.0
4. CNS Sedatives Phenobarbitone, acetylpromazine, barbiturates, phenothiazine etc.	6.7	4.1
5. Nonsteroidal anti-inflammatory drugs		
a) **Pyrazolones** :- phenylbutazone, oxyphenbutazone, antipyrine etc.	10.2	36.2
b) **Others** :- indomethacin, flunixin, salicylic acid, ibuprofen etc.	5.1	13.7
6. Narcotic analgaesics Morphine, pentazocine, levorphanol, codeine, etorphine, methadone etc.	3.6	5.1
7. Steroids Prednisolone, hydrocortisone, estrane 3,17 diol etc.	0.6	2.6
8. Diuretics Frusemide, ethacrynic acid, bumetanide, thiazides etc.	-	6.0
9. Others Polyethylene glycol, thiamine, nicotine, strychnine etc.	6.9	11.5

Definition of non-steroidal anti-inflammatory drugs.

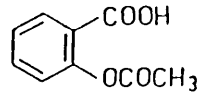
In their broadest sense, non-steroidal anti-inflammatory drugs (NSAIDs) can be defined as any substance other than a steroid that suppresses one or more components of the inflammatory response, and a variety of compounds, as diverse as dimethyl sulfoxide, gold salts, penicillamine, and enzyme preparations such as *orgotein* (superoxide dismutase) are included within this definition. However the term is more usually applied to compounds which inhibit the enzyme system which converts arachidonic acid to the various prostanoids which are involved in the production of the inflammatory response. Generally these compounds are weak organic acids, although there are some examples of neutral e.g. epirizole (*Mepirizole*) and basic compounds e.g. difenpiramide (*Difenax*). These non-steroidal anti-inflammatory drugs can conveniently be divided into various classes depending upon their chemical structure (Table 1.5.). In a review of the medicinal chemistry of acidic NSAIDs, Lombardino (1985) reported that 67 compounds were either on the market or had been assigned a generic name and were currently undergoing clinical trials in man.

TABLE 1.5 Chemical classification of acidic NSAIDs

CARBOXYLIC ACIDS

1. Salicylates

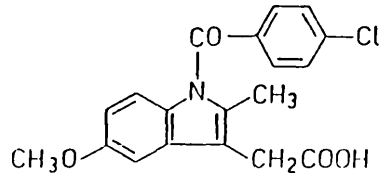
Acetylsalicylic acid (*Aspirin*)
Salsalate (*Disalsid*)



ASPIRIN

2. Acetic Acids

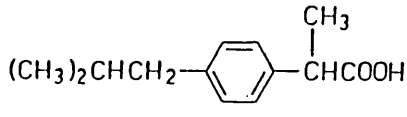
Indomethacin (*Indocin*)
Sulindac (*Clinoril*)
Diclofenac (*Voltarol*)
Zomeripac (*Zomax*)



INDOMETHACIN

3. Propionic Acids

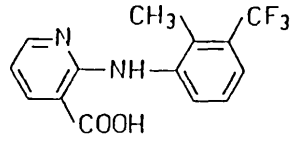
Ibuprofen (*Brufen*)
Naproxen (*Naprosyn*)
Ketoprofen (*Orudis*)
Benoxaprofen (*Opren*)



IBUPROFEN

4. Fenamates

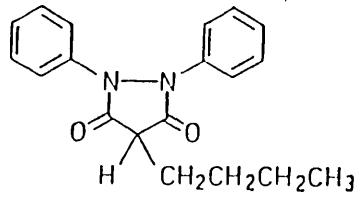
Mefenamic acid (*Ponstan*)
Flunixin (*Banamine*)



FLUNIXIN

PYRAZOLONES

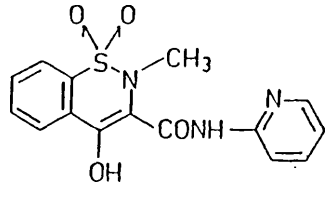
Azaproprazone (*Rheumox*)
Oxyphenbutazone (*Tanderil*)
Phenylbutazone (*Butazolidin*)
Feprazone (*Zepelin*)
Dipyrone (*Paralgin*)



PHENYLBUTAZONE

OXICAMS

Piroxicam (*Feldene*)
Isoxicam (*Maxicam*)



PIROXICAM

Mode of action of acidic NSAIDs

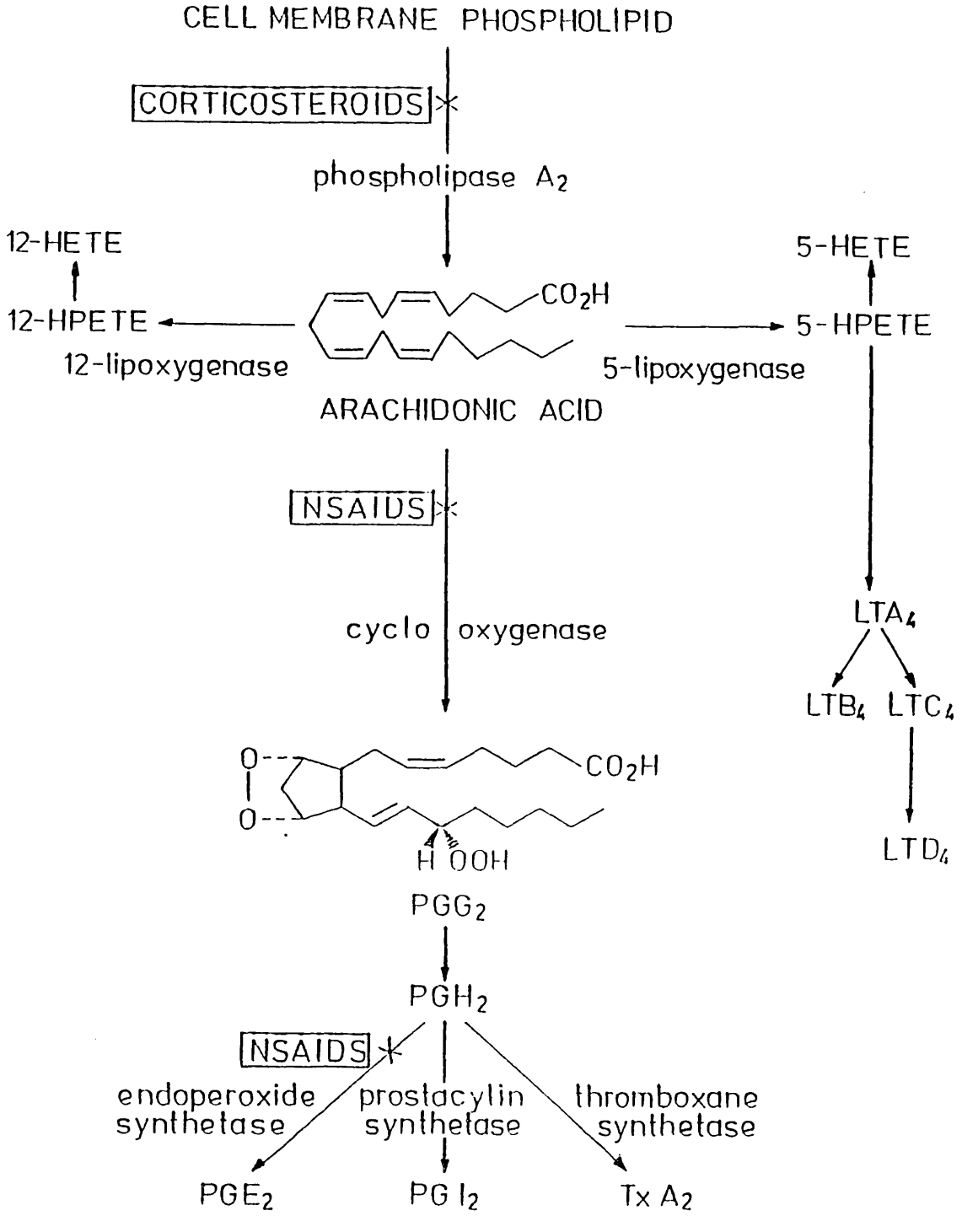
The classical signs of inflammation are invariably listed in the terms by which they were first observed. The first four are ascribed to Celsus (30 B.C.-A.D.38), and the fifth to Galen a century later. They are as follows; heat, redness, pain, swelling, and loss of function. The events associated with the development of these have been fairly well characterised, and it seems that a series of mediators is released sequentially in a response to the stimulus (Dawson & Willoughby, 1985). Immediately after the initial stimulus there is a transient vasoconstriction, followed by a prolonged vasodilation which results in the characteristic heat and redness. This is accompanied by increases in vascular permeability which result in the extravasation of plasma proteins and consequent oedema. Under normal circumstances, after the removal of the initial stimulus, the inflammatory response is then terminated by the migration of monocytes and polymorphonuclear leukocytes (PMN). If the response is not terminated then chronic inflammation develops, resulting in significant tissue damage, activation of the immune system and consequent loss of function. Among the mediators thought to be involved in the initial inflammatory response are histamine, 5-hydroxytryptamine, and the kinins. Originally the prostaglandins were postulated as mediators, although it now seems likely that their main role is as modulators of the inflammatory response (Willoughby *et al.*, 1973). The prostaglandins PGE_2 and PGI_2 directly produce vasodilation, and in addition act synergistically to amplify the changes in vascular permeability and pain produced by the kinins, while PGF_2 is a vasoconstrictor and acts as an anti-inflammatory. Although their exact role is not clear, the leukotrienes and thromboxane A_2, which are structurally related to prostaglandins, are also involved in the inflammatory process.

The biosynthesis of prostaglandins and related compounds from arachidonic acid is represented schematically in Fig 1.1. Phenylbutazone and other classical NSAIDs act by inhibiting the cyclo-oxygenase pathway, thereby preventing the formation of the prostaglandins. There is some evidence that they may also inhibit the endoperoxide isomerase pathway (Flower, 1974). Betamethasone and other corticosteroids are thought to inhibit phospholipase A_2, thereby preventing the release of arachidonic acid from cell membrane phospholipid (Flower & Blackwell, 1979), because they act at an earlier point in the cascade reaction this may explain their greater efficacy. The *in vivo* potency of a number of NSAIDs correlates well with their ability to inhibit the cyclo-oxygenase (or prostaglandin synthetase) enzyme *in vitro.*

In addition to their anti-inflammatory effects the classical NSAIDs generally possess both antipyretic and analgesic activity. The antipyretic activity is a result of the inhibition of the prostaglandin synthetase isoenzyme present in the hypothalamus, responsible for producing PGEs (especially PGE_1) in response to pyrogens, and the rise in body temperature in response to intracerebro-ventricular injections of PGEs is not prevented by these compounds. Although some NSAIDs (such as aspirin) have a direct analgesic effect, which may again be a result of inhibition of prostaglandin synthetase, in general they only relieve the pain of inflammation, and have no effect on other sorts of pain e.g. headaches. The inhibition of cyclo-oxygenase explains not only the antipyretic and anti-inflammatory effects of NSAIDs, but also the toxic effects which seem to be an inevitable accompaniment of these compounds. By inhibiting the formation of prostanoids, the biosynthesis of thromboxane A_2 a potent platelet aggregator is prevented, thereby prolonging clotting time. PGI_2 increases mucosal blood flow in the gastro-intestinal tract, and by preventing its formation NSAIDs may reduce the mucosal blood supply, resulting in hypoxia and subsequent ulceration. The combination of ulceration and inhibition of

platelet aggregation, may result in substantial blood loss into the gut, and gastro-intestinal disorders are by far the most frequently encountered adverse drug reaction associated with NSAID therapy. Because all NSAIDs act in a similar fashion it is not surprising that the majority of toxic effects are common to all of the compounds within this group, although there are differences in their relative efficacy/toxicity, their anti-inflammatory/ antipyretic/analgesic activity, and enormous variations in the individual patient response between drugs of this group.

FIGURE 1.1 **The biosynthesis of prostaglandins and related compounds formed from arachidonic acid.**



Code: PG, prostaglandin; TX, thromboxane; LT, leukotriene; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid.

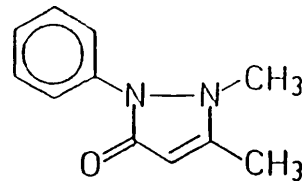
Phenylbutazone and other pyrazolones

The scarcity and consequent high price of quinine in the eighteenth century motivated a search for a cheaper substitute, and as a result a large number of compounds were synthesised some of which have proved to be valuable anti-inflammatory, antipyretic and analgesic agents. The anti-inflammatory activity of salicylates present in willow bark, previously used by ancient civilisations, was rediscovered by the Reverend Edward Stone in the Eighteenth Century (see Bowman & Rand, 1980). On hydrolysis the glycoside salicin present in willow bark liberates salicyl alcohol; this was first synthesised from phenol by Kolbe and Lautemann in 1860, and the related sodium salicylate first used therapeutically by Buss in 1875. Acetylsalicylic acid was introduced by Bayer under the tradename *aspirin* in 1899.

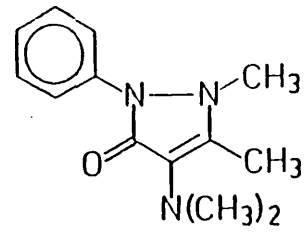
The first synthesis of a pyrazolone was reported by Knorr (1883), who prepared 3-methyl-1-phenyl-2-pyrazolin-5-one from ethylacetoacetate and phenyl-hydrazine. Methylation of this compound gave antipyrine, which was introduced into medicine in 1884. A few years later antipyrine was joined by the related compound aminopyrine. Both were originally employed for antipyresis (hence the name 'antipyrine'), but later they became used more for their analgesic action. The discovery of antipyrine stimulated research in this area, and by 1936 more than a thousand pyrazolone compounds had been synthesised and studied, although generally they had less desirable properties than antipyrine (Greenberg & Haggard, 1950). Phenylbutazone (1,2-diphenyl-3,5-dioxo-4-butyl-pyrazolidine) was one of a series of pyrazolone compounds first synthesised at Geigy Laboratories in the late 1940's. Phenylbutazone was originally marketed in 1949 as a co-solvent in an injectable formulation of aminopyrine *'Irgapyrin'*. It soon became clear that the dramatic relief obtained from this preparation, as compared to the oral preparation of aminopyrine was

due in part to the phenylbutazone content. The pharmacology of phenylbutazone was investigated by Wilhelmi (1949; 1950) and Pulver (1950) who demonstrated that it was a potent NSAID with analgesic and antipyretic activity. It was introduced into human medicine as '*Butazolidin*' in 1952, for the treatment of arthritis and similar musculo-skeletal disorders. The uricosuric actions of phenylbutazone made it particularly valuable for the treatment of gout, (and led to the development of sulphinpyrazone, a powerful uricosuric agent devoid of anti-inflammatory activity). The chemical structure of phenylbutazone, along with the structures of some other members of this group are illustrated in Figure 1.2.

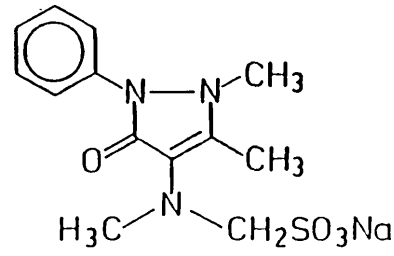
FIGURE 1.2 The chemical structures of some pyrazolone NSAIDs



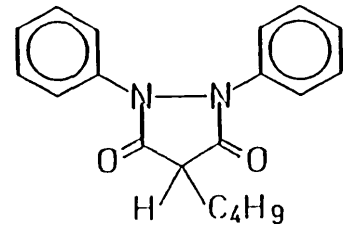
ANTIPYRINE



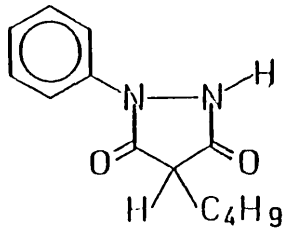
AMINOPYRINE



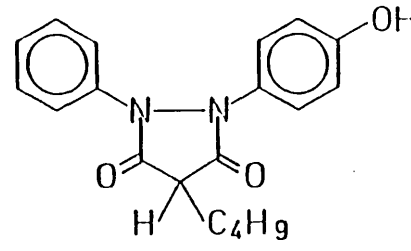
DIPYRONE



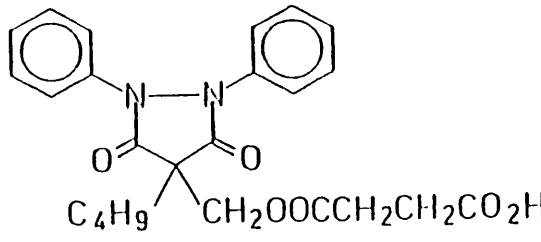
PHENYLBUTAZONE



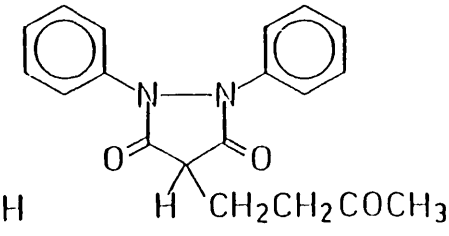
MOFEBUTAZONE



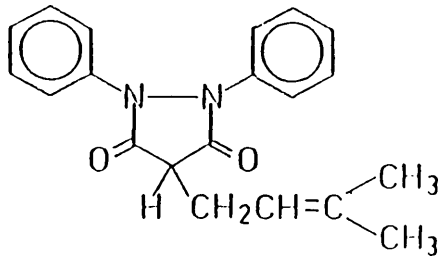
OXYPHENBUTAZONE



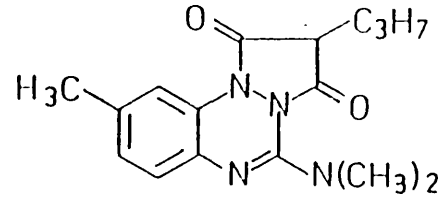
SUXIBUZONE



KEBUZONE



FEPRAZONE



APAZONE

The clinical use of phenylbutazone in the horse

Phenylbutazone was introduced into veterinary medicine in the 1950's and since then it has been the NSAID of choice for treating horses with musculo-skeletal disorders. Although a large number of similar drugs have been developed subsequently, phenylbutazone remains the NSAID most widely used in horses (Tobin, 1981; Lees & Higgins, 1985). Gabel considers phenylbutazone to be useful in the treatment of pedal osteitis (sore feet), cunean tendon bursitis (jacks), osselets, spavins, splints, navicular disease, ringbones, minor sprains and muscle soreness (Tobin, 1981). Phenylbutazone is also often given after the race to prevent the horse 'cooling out sore'. Considering its widespread use there are few reports on the therapeutic effects of phenylbutazone in the horse, but the dramatic improvements that may be expected are perhaps most clearly characterised by the *Dancer's Image* case. *Dancer's Image*, the champion Canadian two year old, finished first in the 1968 Kentucky Derby. The urine sample taken after the race proved positive for phenylbutazone, and the horse was subsequently disqualified. This precipitated a court battle which lasted for years and during which the Racing Commission produced a statement of their findings, which read as follows :-

> "*Dancer's Image* had prominent ankles before he began his career, that is ankles prone or predisposed to trouble under the strain and stress of racing. They were carefully watched and treated from the inception of his racing career by Louis C. Cavalaris, his trainer. The right ankle had begun swelling in early 1968 at Bowie Race Course in Maryland. It was X-rayed at the time of the Wood Memorial in New York by Dr. Girard and prior to and after the Kentucky Derby by Dr. Harthill. It was swollen on Saturday, April 27, 1968, and Sunday, April 28, 1968, and successfully treated by Dr. Harthill with the concurrence of Mr. Cavalaris upon the latter date with a four-gram dose of *Butazolidin* (the trade name for phenylbutazone). There was dramatic improvement on April 29, 1968, and the horse was in racing condition and worked out well. However, the condition of the right

ankle had again deteriorated by Thursday morning May 2, 1968, so much so that it was the worst it had been up to that time. The ankle was swollen and engorged, a condition known as a red-hot osselet. Dr. Scanlan, the track veterinarian at Churchill Downs, observed *Dancer's Image* that day and noticed the horse was so lame and sore that had his condition been the same on Saturday, May 4, 1968, Dr. Scanlan would have recommended to the Stewards that *Dancer's Image* be scratched. The ankle began dramatic improvement Thursday afternoon, was in good condition on Friday, May 3, 1968, and on Saturday morning and at the time of the race was in excellent condition and racing sound. The ankle remained sound after the race and through Sunday and Monday. However, by Tuesday, May 7, 1968, the ankle had reverted to its condition of the previous Thursday.

Effective use of phenylbutazone will dramatically improve the condition and health of a horse to the extent that it enables him to race when he would not otherwise be able to do so. It enables him to perform to his full potential when otherwise he would not be able to do so. Prohibition of the use of phenylbutazone is necessary in order to preserve honesty and integrity in racing because the indiscriminate use and withdrawal of the medication with many horses would vary their performance in racing. The Rules of Racing are designed to prevent use of medications and other devices for such a purpose.

Regardless of the amount of phenylbutazone and/or derivative thereof in the urine of *Dancer's Image* on Saturday, May 4, 1968, its presence in any amount shows that the administration affected the health and speed of the horse by enabling him to run racing sound."

Controversy over the use of phenylbutazone.

An aura of controversy surrounds the use of phenylbutazone in the performance horse. Along with all other NSAIDs, phenylbutazone is prohibited by the 'Rules of Racing' for flat, National Hunt, and point-to-point racing in Great Britain and in many other countries. The Federation Equestre Internationale (FEI) permit the limited use of phenylbutazone in horses competing in showjumping,

eventing and carriage driving, and it is doubtful whether showjumping and eventing could continue in their present format without the use of phenylbutazone. An attempt to ban the use of phenylbutazone in 1977 was overruled on a technical point by the then-president of the FEI, Prince Philip (Vogel, 1978). In 1978 it was decided in principle to impose a ban from 1981, but again this decision was set aside, and controversy continues regarding its use.

Not long after the introduction of phenylbutazone, it became clear that it would be of great benefit to horses racing and in training; the popularity and effectiveness of phenylbutazone soon led to pressure on the racing authorities to allow its use. In 1959 Colorado approved phenylbutazone for use in racing and the era of controlled medication was born. Since then under further pressure, an increasing number of States have permitted the use of phenylbutazone and it is estimated that in these States some 60% of the horses running are receiving phenylbutazone (Cannon, 1974).

The question of the effect of phenylbutazone on the performance of racehorses has often been raised. The Veterinary Chemists Advisory Committee to the National Association of State Racing Commissioners concluded that phenylbutazone did not change the innate ability of a horse to race but, by relieving inflammation, may enable him to race nearer to his maximum capacity. Studying the effect of phenylbutazone in time trials in horses, Sanford, (1978) found that the intramuscular administration of phenylbutazone increased the performance of their horses. These workers were surprised by their results, and concluded in a rather *post hoc* fashion, that phenylbutazone had acted to relieve subclinical lameness, rather than to stimulate the horses.

The racing authorities of Great Britain, Ireland and France issued a joint statement in 1971 reaffirming their intention not to relax the 'Rules of Racing' to permit the use of phenylbutazone and similar NSAIDs. This statement was subsequently endorsed by the Royal College of Veterinary Surgeons, but this was by no means the end of the matter, and the debate for and against controlled medication continues. Its proponents argue that it enables a slightly unsound horse to continue its racing career, the alternative being retirement (sometimes referred to as the 'bute or shoot' dilemma), and that the use of phenylbutazone does not allow a horse to perform above his innate ability, (a point refuted by Sanford's study) since horses running on phenylbutazone do not win a higher proportion of races than untreated horses (Tobin, 1981). Opponents of controlled medication have several concerns over its possible introduction. By manipulating the dosing regime a trainer could influence the performance of the horse, allowing it to be run 'hot and cold', another concern is that by permitting even one drug, they will have set a precedent, and that other drugs will follow (a controlled medication list for one State in the late 1970's included anabolic steroids, corticosteroids, diuretics, muscle relaxants, various NSAIDs, and the narcotic analgesic pentazocine). Their main concern however, is the possible detrimental effect of controlled medication on the quality of the breeding pool.

Vast amounts of the resources involved in horse racing are invested in the blood stock industry. Racehorses are selected for breeding primarily on the basis of their racing performance. If one adopts a classical view of evolution, then any medication (e.g. NSAIDs) that obscures the effect of undesirable traits, and thereby prevents their exclusion from the breeding pool, or (e.g. anabolic steroids) results in the acquisition of desired characteristics, thereby enhancing their inclusion into the breeding pool, will be detrimental to the breeding stock.

Problems associated with enforcing a controlled medication program

By the mid 1970's the majority of States in America had introduced controlled medication programs, and by far the most common permitted drug was phenylbutazone. In a survey of the restrictions governing the use of phenylbutazone in various States, Poppell (1979) reported that out of 18 States only 2 operated a total ban. The other 16 either allowed the use of phenylbutazone up to the day of racing, or else imposed a 'time rule' prohibiting its use within 24, 48 or 72 hours before the race. The simplest way to enforce a 'time rule' is to isolate the horse for the time period involved, unfortunately if the time period is long, and a large number of horses are involved, then this solution is not practical. Another method of enforcing the 'no race-day medication' rule is to set a maximum urinary concentration of phenylbutazone and metabolites. This limit is usually set at 165 *u*g/ml, and if higher levels than this are found this is taken to indicate that the rule has been infringed and the horse is disqualified. The scientific rationale behind this is not very clear, since at the moment there is no way of telling whether a given urine (or plasma) level of a drug has resulted from a small dose just before the sample was taken, or a large dose some time before. In addition the choice of urine as the testing medium was unwise, since as pointed out earlier the urinary concentration of a compound is dependent on many variables and this may result in a large range of concentrations for a single plasma level of the drug. Houston *et al.* (1983) demonstrated that urinary pH affects the urinary concentrations of phenylbutazone and its metabolites, as the pH increased from 4 to 8.5 the concentration of phenylbutazone increased 200 fold. In addition Soma *et al.* (1985) reported a complete lack of correlation ($r = 0.02$) between plasma and urinary levels of phenylbutazone.

A more rational approach is to use plasma levels, since this will remove two of the most important variables determining the urinary concentrations of phenylbutazone, namely changes in pH and volume. This is the solution adopted by the Federation Equestre Internationale (FEI), who set a maximum plasma concentration for phenylbutazone of 4 *u*g/ml, recently amended to a 5 *u*g/ml aggregate for phenylbutazone and its active metabolite oxyphenbutazone. Although plasma is vastly superior to urine as a forensic medium for the application of concentration rules, it is not without problems; there have been a number of studies to determine the range of plasma residues of phenylbutazone after various dosing schedules. Table 1.6. is a summary of these studies, and includes the mean plasma concentration at 24 h after the last dose, and a statistical projection of the expected maximum plasma level of one horse in 1000. In all the studies there was a wide intersubject variation in the plasma levels, and another important finding was that following repeated dosing the population distribution of the phenylbutazone plasma concentrations was log-normal. These two findings have important repercussions for the enforcement of any 'time rules' since as discussed earlier (in relation to steroids), if the upper limit is set too low then a significant number of people who stay within the rules will be judged guilty of infringing them, and if the level is too high then the rule will be ineffective. With a high coefficient of variation, and a positively skewed distribution it is virtually impossible to set a level that is an effective compromise.

TABLE 1.6 **A comparison and statistical projection of phenylbutazone plasma concentrations measured 24 h after various dosing regimes**

Study	Subjects	Dose regime	Phenylbutazone concentration Mean (*u*g/ml)	0.01% (*u*g/ml)
1.	49	8.8 mg/kg/day (p.o.) for 3 days then 4.4 mg/kg (i.v.)	4.1	24.0
2.	53	2 g (i.v.)	0.9	2.2
3.	43	2 g/day (i.v.) for 4 days	4.8	16.2
4.	62	4 g/day (p.o.) for 4 days then 2 g (i.v.)	5.3	23.5
5.	34	2 g/day (p.o.) for 2 days	4.1	23.2

All phenylbutazone plasma concentrations measured 24 h after the last dose. The statistical projection of the maximal plasma level of 1 horse in 1000 (0.01%) based on the arithmetic mean and population distribution of the sample.

Study 1. National Association of State Racing Commissioners (Tobin 1981).

Study 2. Gulfstream Race Track (Soma *et al.*, 1985).

Study 3. Keystone Race Track (Soma *et al.*, 1985).

Study 4. Keeneland Training Centre (Chay *et al.*, 1984).

Study 5. California (Houston *et al.*, 1985)

Plasma levels and therapeutic effect

Currie (1952) and Bruck *et al.* (1954) suggested an optimal therapeutic phenylbutazone plasma concentration in man, of between 50 and 100 ug/ml, with an increased incidence of toxic effects at higher levels (Bruck *et al.*, 1954). These findings have been refuted in later studies (Brooks *et al.*, 1975; Orme *et al.*, 1976) who could find no relationship between the plasma level and the clinical or toxic effects of phenylbutazone. The therapeutic levels in the horse have been reported as between 5 and 15 ug/ml (Jenny *et al.*, 1979; Gerring *et al.*, 1981), but there is some doubt that the relationship between the plasma levels and pharmacological effect is straightforward. Higgins *et al.* (1986) measured the levels of phenylbutazone both in plasma and in inflammatory exudate: 6 h after dosing the plasma levels of phenylbutazone exceeded those in the exudate (ratio 1.76), by 12 h the level of phenylbutazone in the exudate exceeded the plasma levels (0.56), and after 24 h the ratio was even lower (0.33). This alteration in the plasma/exudate level will further complicate any interpretation of plasma concentration data.

Toxicity of phenylbutazone in man

Phenylbutazone has a high margin of safety in man, with an estimated lethal dose of 500-1000 mg/kg: by the 1960's some 50 million people had been treated with phenylbutazone, with only one case of fatal overdose recorded (Rechenburg, 1962). However, soon after its introduction into human medicine there were reports of toxic side effects and as early as 1952 it was considered by some to be too dangerous for routine use (Granirer, 1952). The incidence of toxic side effects in man has been variously estimated at between 10 and 45% (Goodman & Gilman, 1980) and increases with the length of drug administration from 0.5% with short term (8-21 days) to 28% with long term therapy (5-54

months) (Rechenburg, 1962). In addition to the gastro-intestinal side effects common to all NSAIDs, phenylbutazone has two rare but dangerous side effects. It causes fluid retention, and in predisposed patients, this may precipitate cardiac failure, and there is a small but very significant incidence of life-threatening blood disorders including leucopenia, aplastic anaemia, thrombocytopenia, and erythema multiforme. Between 1964 and 1984 there was a total of 1693 adverse reactions associated with the use of phenylbutazone reported to the Committee on Safety of Medicines (CSM). Of these 445 were fatal (207 of these were attributed to aplastic anaemia, 63 to various white blood cell disorders and 19 to thrombocytopenia). The incidence of reports and fatalities for the related compound oxyphenbutazone was estimated to be twice that of phenylbutazone (Cuthbert, 1974). The indiscriminate use of phenylbutazone by some Medical Practitioners led the CSM to recommend restrictions on its use, and the total withdrawal of the more toxic derivative oxyphenbutazone. Although one of the oldest drugs in its class, phenylbutazone is still one of the most effective, and this is recognised in its continued use as a last resort for the treatment of ankylosing spondylitis.

Toxicity of phenylbutazone in the horse

The first reports of toxicity of phenylbutazone in the horses were reported by Gabriel and Martin (1962). When given as a single dose of up to 16g, phenylbutazone appeared to have no measurable toxic effects, but the use of repeated doses for prolonged periods (2-8 g/day for up to 32 days) was not so innocuous. Necrosing phlebitis of the portal veins was observed, and some ulcerative lesions were found in the intestines. One horse given 64 g over 4 days died but unfortunately because of tissue autolysis no histology was performed.

Between the appearance of this report and 1979 there were only a few reports of toxicity in the horse. There was some evidence for phenylbutazone induced hypoplastic anaemia (Dunavant & Murray, 1975), Hopes (1972) reported a fatal case of intestinal haemorrhage, a depressant or tranquillising effect was occasionally seen (Gabel *et al.*, 1977), and transient staggering was noted after an intravenous dose (Jeffcott & Colles, 1977). However, until 1979 phenylbutazone was widely considered to be non-toxic in the horse, with a wide safety margin. Phenylbutazone was sometimes prescribed at low doses for several years (Scott Dunn, 1972) apparently without any toxic effects, and in 1977 Gabel *et al.* wrote "toxicity to phenylbutazone is almost never seen in equine patients in spite of close observation and periodic blood counts done during thousands of courses of therapy."

In 1979 this position was questioned when Snow *et al.* (1979) reported the deaths of three Shetland ponies after the oral administration of phenylbutazone; at a dose of 10 mg/kg for 7 to 14 days, 7 out of the 8 horses treated developed signs of toxicity. These included anorexia, depression, abdominal oedema, oral ulceration and oedema of the large intestine. These findings were confirmed in a subsequent investigation (Snow *et al.*, 1981), decreases in plasma protein were apparent and two ponies died. At necropsy, massive intestinal ulceration was observed, mainly of the caecum and large intestine. Death was attributed to a protein-losing enteropathy, resulting in loss of blood volume and subsequent circulatory collapse.

Since the work by Snow and his colleagues, several other groups have undertaken similar studies, and the toxicity of phenylbutazone is now well established. Roberts (1981) described a case of hypoproteinaemia, and rupture of the large colon in an animal which had received phenylbutazone over 76 days. MacKay *et al.* (1983) administered phenylbutazone under 4 different

dosing regimes, and demonstrated a dose dependent toxicity. The horses receiving doses of 15 mg/kg/day and 30 mg/kg/day died between 4 to 7 days, and those on the lower dose (8 mg/kg/day) became anorexic and depressed after 2 to 4 days. In addition to the toxic effects previously described by Snow, a dose related renal papillary necrosis was observed, although it seems likely (Gunson & Soma, 1983) that concomitant dehydration is necessary for the development of this toxic manifestation. Alexander (1982) reported slight acidosis and decreases in urinary sodium and chloride and faecal chloride after the administration of 7.5 mg/kg for 4 days. Other toxic effects include elevated plasma liver enzyme levels (Lees *et al.*, 1983), and neutropenia (Snow & Douglas, 1983; Gerber, 1984). The results from the toxicity studies led several manufacturers to halve the recommended dose from 4.4 mg/kg twice daily to once daily (Taylor *et al.*, 1983).

Drug metabolism in the horse

The amount of published material on drug metabolism in the horse is very small, and there are several reasons for this. The development of mechanised power has relegated the horse from the important position it previously held, so that today, in the more prosperous countries of the world, the horse is used almost exclusively for sport and recreation. In financial terms the horse-racing industry is by far the most important activity within this area, and it is not surprising therefore that most studies into drugs and horses are financed by the racing authorities. This has two important repercussions, firstly the previously mentioned secrecy surrounding the work of the Association of Official Racing Chemists has meant that many of the studies performed have been published only in a form restricted to members, and secondly, the studies carried out are often incomplete, with the main emphasis on detection. In addition horses are far from ideal experimental animals for drug metabolism studies. They are

expensive to buy and maintain, need special facilities for the collection of biological samples, and consequently it is almost impossible to use them in large numbers. Their large size can also present problems of scale e.g. 10 *u*Ci is a fairly typical amount of a radiolabelled compound to administer to a 250g rat, but an equivalent pro rata amount in a 500 kg horse would be 20,000 *u*Ci.

Drug metabolism in relation to doping

Since metabolism is probably the major factor in determining the duration of action of most drugs, its relevance to doping is self evident. A clear understanding of metabolic transformations in the horse will have direct benefits for the design of drug-screening procedures, and may enable one to estimate certain parameters (e.g. the time, dose and route of administration) from the measurement of levels of a drug and metabolites in body fluids. Metabolites are often present in urine in higher concentrations than the parent compound, although they are often more water soluble than the parent compound and consequently difficult to extract with solvents. This is exemplified by apomorphine, which was notoriously difficult to detect in the urine of horses, but the identification of apomorphine glucuronide as a major urinary metabolite enabled many laboratories to incorporate a test for apomorphine into their drug-screening procedures, and resulted in several positives for this compound. The occurrence of certain metabolic pathways can also interfere with the detection of some compounds. Procaine has always been relatively easy to detect in horse urine, but for a long time it was impossible to detect in blood. The discovery that plasma esterases hydrolysed procaine (with an *in vitro* half-life of about 8 minutes), explained the difficulties in detecting procaine in plasma, and the addition of small amounts of an enzyme inhibitor, e.g. oxalate, to the blood sample permitted the estimation of plasma levels.

The need for establishing a pattern of metabolism, both quantitative as well as qualitative has recently become apparent with the growing use of endogenous steroids. As discussed earlier, it is very difficult to determine whether a high urinary level of an endogenous compound is due to the administration of that compound, or to the horse being biochemically atypical. An alternative strategy, which is possible with some compounds is to measure metabolite(s)/ parent drug ratios in the urine, and this approach has been used in human athletes to detect the administration of testosterone, by measuring the ratio of testosterone to epitestosterone (Donike *et al.*, 1983). Houghton *et al.* (1986) have recently developed a method for the detection of nandrolone (19-nortestosterone) in colts, based on measuring the oestranediol/oestrenediol ratio. Control values varied between 0.05 and 0.1, but following the administration of 400 mg of nandrolone they increased to up to 200, and remained high for up to fourteen days after the dose.

The metabolism of phenylbutazone

The first reports on the biotransformation of phenylbutazone appeared soon after its introduction into human medicine. Burns *et al.* (1953, 1955a) isolated two metabolites from human urine, and demonstrated that it underwent both aromatic and aliphatic oxidation, to yield γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone (oxyphenbutazone) respectively (metabolites I & II in Figure 1.3). In 1974 Bakke *et al.* investigated the metabolism of phenylbutazone in the rat and in addition to the two metabolites previously identified, reported the presence of a dihydroxylated metabolite, *p*,γ-dihydroxyphenylbutazone (III). Wagner *et al.* (1971) and McGilvery *et al.* (1974) reported the formation of three additional minor metabolites β-hydroxyphenylbutazone (IV), *p,p*-dihydroxyphenylbutazone (V) and γ-ketophenylbutazone (VI). In man, none of these metabolites were present as more than a few percent of the dose, and these

amounts were scarcely increased after incubation with ß-glucuronidase and sulfatase. Since the excretion of unmetabolised phenylbutazone accounted for no more than 1% of the dose, the fate of the vast majority of this compound remained unexplained. In 1975 Richter *et al.* (1975) reported the characterisation of a novel type of metabolite, namely the C-glucuronide of sulphinpyrazone, and in the following year they demonstrated the presence of the C_4-ß-D-glucuronides of phenylbutazone (VII), and γ-hydroxyphenylbutazone (VIII) in urine following the administration of ^{14}C-phenylbutazone to man (Dieterle *et al.*, 1976). These two compounds accounted for 40 % and 12 % of the total urinary radioactivity respectively. In addition, some 30% of the radiolabel was present in the faeces.

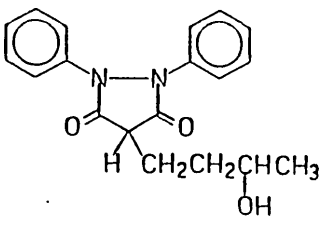
The metabolism of phenylbutazone and related pyrazolones in the horse

Phenylbutazone has been extensively used in equine medicine for some 30 years. Despite this its metabolism was not investigated in any detail until 1974 (Maylin), and a full study on the metabolic disposition of this drug in the horse has yet to be published. The findings of the previous investigations are summarised in Table 1.7. Schubert (1967) administered 10g p.o. and 6g i.m. to the same horse on separate occasions, and was able to demonstrate the presence of the parent compound in urine, and in addition tentatively identified *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone as phenylbutazone metabolites. The presence of oxyphenbutazone was confirmed in a subsequent investigation by Finnochio *et al.* (1970), but the identification was based solely on its U.V absorption spectrum. The first detailed metabolic investigation (Maylin, 1974) indicated that phenylbutazone was extensively metabolised, with less than 2% of an intravenous dose excreted in the urine as unchanged drug. Over 24 h a further 25% of the compound was excreted as the two previously identified metabolites, and these findings were later confirmed by Gerring *et al.*

(1981). One interesting finding was that after repeated dosing with phenylbutazone the proportion of these two metabolites varied, and the results obtained by Maylin (1974) are summarised in Table 1.8. Despite (a) widespread interest in the metabolism of phenylbutazone, (b) the publication of many papers dealing with the plasma kinetics of phenylbutazone, and (c) the renewed interest in the toxicity of this compound, to date 75% of the fate of phenylbutazone in the horse remains unexplained. There are no reports on the metabolism of oxyphenbutazone in the horse, and the only information concerning the disposition of this compound in the horse, is the report of a similar plasma elimination half-life to phenylbutazone (Gandal *et al.*, 1969).

The metabolism of antipyrine in the horse was investigated by Momose and Tsuji (1972a), who demonstrated that this compound was excreted as a mixture of 4-hydroxyantipyrine and 3-hydroxymethylantipyrine together with unchanged antipyrine, and essentially similar results were obtained with the related compounds dipyrone and aminopyrine (Momose & Tsuji, 1972b). Apart from these few studies, the fate of most pyrazolones, and pyrazolidiones (of which phenylbutazone and oxyphenbutazone are the prototypes), in the horse have yet to be determined.

FIGURE 1.3 Metabolites of phenylbutazone



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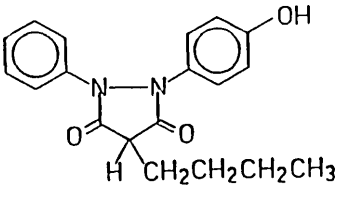

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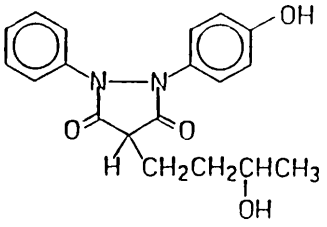

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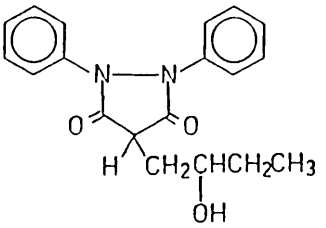

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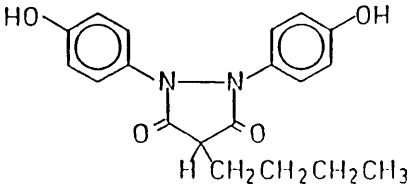

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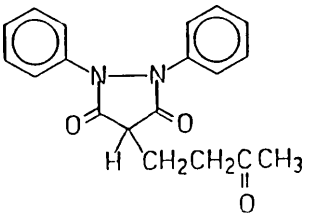

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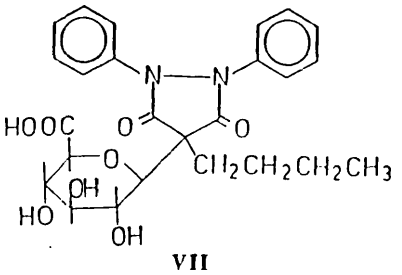
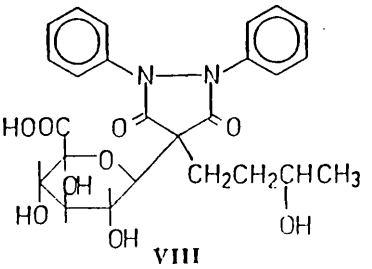


VII



VIII

I γ-hydroxyphenylbutazone. II *p*-hydroxyphenylbutazone. III *p*,γ dihydroxyphenylbutazone. IV β-hydroxyphenylbutazone. V *p*,*p*-dihydroxyphenylbutazone. VI γ-ketophenylbutazone. VII phenylbutazone-C-glucuronide. VIII -hydroxyphenylbutazone-C-glucuronide.

TABLE 1.7 The metabolism and elimination of phenylbutazone in the horse

Dose	Route	Finding	Reference
10g 6g	p.o. i.m.	Phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone found in the urine.	Schubert (1967).
4.4 mg/kg	i.v.	$t_{1/2}$ 3.5 h. 3.7% of dose recovered as unchanged drug in the 0-24 h urine.	Piperno *et al.* (1968).
2g	i.v.	Oxyphenbutazone detected as a urinary metabolite	Finocchio *et al.* (1970).
4.4 mg/kg for 4 days	i.v.	Phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone quantitated in urine.	Maylin (1974).

TABLE 1.8 The percentage recovery of dose as urinary metabolites following the daily intravenous administration of phenylbutazone (4.4 mg/kg)

From Maylin (1974).

	Phenylbutazone	Oxyphenbutazone	γ-hydroxyphenylbutazone	Total
Day 1	0.9	9.6	14.2	24.9
Day 2	1.7	14.0	10.1	25.8
Day 3	2.1	14.0	8.2	24.3
Day 4	1.6	10.5	6.4	18.5

Objectives of this study

Phenylbutazone is the most commonly encountered doping agent in racehorses, for the two years 1984/1985 phenylbutazone and oxyphenbutazone accounted for over a third of all doping positives reported to the AORC. The metabolic disposition of phenylbutazone in the horse has not been comprehensively investigated, although it is known for other non-equine species. There are few reports in the literature on the administration of oxyphenbutazone to horses, and the metabolic fate of this compound in the horse is unknown.

The metabolic disposition of a non-steroidal anti-inflammatory drug such as phenylbutazone, has important forensic implications for the detection of its illicit administration. In view of the absence of such information, the attainment of the following objectives was desirable.

1. To establish the systemic bioavailability of orally administered phenylbutazone and oxyphenbutazone in the horse.

2. To determine the principal routes of excretion, following the administration of radiolabelled phenylbutazone and oxyphenbutazone to the horse.

3. To establish the main metabolic pathways of phenylbutazone and oxyphenbutazone in the horse, by the isolation and identification of urinary metabolites.

4. To investigate the feasibility of using such metabolic and kinetic information for estimations of the time, amount, and route of administration in the context of detecting the illicit use of phenylbutazone in the horse.

CHAPTER 2.

THE BIOAVAILABILITY OF PHENYLBUTAZONE

INTRODUCTION

Phenylbutazone is extensively metabolised in the horse (Piperno *et al.*, 1968), with only 4% of the dose excreted unchanged in the 0-24 h urine. The principal metabolites so far identified arise from aromatic and aliphatic hydroxylation, yielding *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone respectively. In the horse, these three compounds together account for 25% of the dose (Gabel *et al.*, 1977), with the fate of the remaining 75% undetermined. Moss and Haywood, (1973) investigated the urinary concentrations of ^{14}C following the administration of ^{14}C-phenylbutazone to horses, but did not record the total urinary recovery, and the extent of urinary excretion is unknown.

Phenylbutazone is reported to be well absorbed by horses following oral administration (Gabel *et al.*, 1977) with peak plasma levels occurring some 2 h after dosing (Barragry, 1973). However, considerable variation was observed between animals, both in time to peak, and in peak concentrations obtained (Moss 1972; Gerring *et al.*, 1981; Sullivan & Snow, 1982). There is a paucity of information concerning the systemic availability of phenylbutazone in the horse, and the literature apparently contains no detailed studies of the excretion balance or bioavailability of the drug in this species. Previous bioavailability studies (Rose *et al.*, 1982; Snow *et al.*, 1983) have compared the absorption of various proprietary paste and powder formulations, and the influence of various feeding regimes, but were more concerned with pharmaceutical equivalence rather than absolute bioavailability. Because of the importance of such information for both the therapeutic use and the control of doping with this drug, the present study was undertaken.

MATERIALS AND METHODS

Study design and protocol

The study was conducted in 2 parts. In the initial feasibility study one horse received [4-^{14}C]-phenylbutazone by stomach tube, however an incomplete recovery of radioactivity was obtained, which necessitated alterations in the protocol. The second experiment was essentially a combined total balance and oral/i.v. cross-over study. Two horses received [*phenyl*-^{14}C]-phenylbutazone both orally and on a separate occasion intravenously, and in addition to urine, faeces was also collected. Details of the horses used and the samples taken are briefly described in Table 2.1 together with the rationale for the different protocols adopted in the various experiments. Experimental details common to both experiments are described under Study 1 and any different or additional details under Study 2.

Study 1. (Experiment 1)

Compounds

[4-^{14}C]-Phenylbutazone (1,2-diphenyl-3,5,-dioxo-4-[^{14}C]-butyl-pyrazolidine) sp. act. 0.3 uCi/mg, radiochemical purity by HPLC > 99% was provided by The Horseracing Forensic Laboratory. γ-, *m*- and *p*-hydroxyphenylbutazone were gifts from Ciba-Geigy (Basle, Switzerland). HPLC grade acetonitrile was purchased from Rathburn Chemicals Ltd. (Walkerburn, U.K). Phenylbutazone was purchased from Sigma Chemical Co. (Poole, U.K) , and all other chemicals used were purchased from BDH Chemicals Ltd. (Poole, U.K), and were of analytical grade.

TABLE 2.1 Details of the administration of ^{14}C-phenylbutazone and sampling procedures for experiments Nos. 1-5.

Experiment No.	1	2	3	4	5
Horse	Caspar	Caspar	Ginger	Ginger	Caspar
Position of ^{14}C Label	C_4*	*Phenyl*	*Phenyl*	*Phenyl*	*Phenyl*
Dose *u*Ci	30*	100	100	100	100
Dose mg/kg	8.33	7.1(1)	7.1	7.1	7.1
Route	p.o.	i.v.	p.o	i.v.	p.o
Saliva	+	+	+	-	-
Faeces 0-68 h	-	+	+	+	+
Faeces 68-150 h(2)	-	-	-	+	+

* Low plasma and urinary levels of ^{14}C were obtained after the administration of 30 *u*Ci of [4-^{14}C]-phenylbutazone and so the amount of radiolabelled material was increased to 100 *u*Ci in all further experiments. There was insufficient material labelled at C_4 for all of the intended experiments, and so [*phenyl*-^{14}C]-phenylbutazone was used instead.

(1). Because the bioavailabilty of phenylbutazone was unknown, the dose for the i.v. administration was reduced because of concern over the toxicity of this compound.

(2). In experiments 2 & 3 an incomplete faecal recovery was obtained and so the collection period was extended in experiments 4 & 5.

Animals

One gelded crossbred pony (body weight 360kg) received [4-^{14}C]-phenylbutazone (8.3 mg/kg, 30 *u*Ci) by stomach tube dissolved in 500ml of dilute aq. $NaHCO_3$ followed by 500ml water. The pony was given a small amount of hay 4 h prior to dosing at 11 am. The pony was supplied with small quantities of feed in order to obtain saliva samples, and was allowed free access to food 4 h after dosing.

Urine Collection

After administration of the drug, the horses were moved to a stable designed for the convenient collection of samples, in which the movement of the horse was restricted. Each morning the animals were removed and exercised for 30 min; horses generally do not urinate during exercise, but frequently do so soon after. Urine was collected using the methods described by Marsh *et al.* (1981). A harness over the horse's back holds in place a latex funnel, and its height and position are adjusted so it surrounds the penis. The urine runs from the funnel, via plastic tubing into the collection bottles containing the preservatives (see below) placed in a pit below the level of the animal. As far as possible urine samples were collected individually, and overnight this was achieved by using a large fraction collector. Working with large animals presents significant problems with sample collection and in the one experiment (No.5) an unavoidable sample loss occurred, and the method used to correct for this loss is discussed in the relevant section.

The volume and pH of the urine was measured, and duplicate 100 *u*l aliquots removed for scintillation counting. The urine samples were then immediately frozen and transported from Newmarket to London and stored at -20° until analysed.

Collection of saliva

Saliva samples were collected into a beaker immediately following blood collection. The ponies used had previously established explanted parotid papillae, parotid saliva being produced in copious amounts when food is given to the animal. Ponies are completely devoid of any Pavlovian response and will only produce saliva when actually chewing their food. The collection of the saliva was timed (usually for 1 min), and the volume measured. Duplicate aliquots (1ml) were taken from each sample and counted for ^{14}C.

Collection of Blood

An indwelling cannula was inserted into a jugular vein prior to drug administration, and samples collected at suitable intervals during the course of the experiment. Blood samples were collected into tubes containing lithium heparin (Monovettes, Sarstedt), centrifuged at 4000 rpm for 5 min, the plasma aspirated, and duplicate 1ml samples counted for ^{14}C.

Sample storage and preservation

To prevent the oxidative loss of phenylbutazone, urine was collected into bottles containing $Na_2S_2O_5$ (0.5g) and disodium EDTA (2.0g), and these compounds were added to plasma (final concn. approx. 5mM and 10 mM respectively) immediately after centrifugation. All samples were stored at -20° until analysed. Phenylbutazone and related pyrazolidinediones undergo facile oxidative degradation in solution, and these additives were shown in control experiments (see Chapter 3.) to prevent such breakdown.

Radiochemical techniques

The ^{14}C content of plasma, urine and other solutions was determined by liquid

scintillation spectrometry. Initial counting was carried out at Newmarket with a Packard TriCarb liquid scintillation spectrometer model 3320, with a Triton X-100-toluene based scintillant. Quench correction was by the channels ratio method. Subsequent counting was done using Scintran Cocktail T (BDH Chemicals, Poole, U.K.) a Triton X-100-toluene based scintillant, with a Packard TriCarb Model 4640 liquid scintillation spectrometer. Counting efficiency was assessed by reference to an external standard using a regularly determined quench correction curve.

Study 2. (Experiments 2,3,4,5)

Compounds

[*phenyl*-^{14}C]-Phenylbutazone (1,2-[(U)-^{14}C]-diphenyl-3,5-dioxo-4-butyl-pyrazolidine) sp. act. 19.9 *u*Ci/mg, radiochemical purity by T.L.C. >99%, was donated by Ciba-Geigy (Basle, Switzerland).

Animals

Two gelded crossbred ponies (body weights 360, 280 kg) were dosed on separate occasions with ^{14}C-phenylbutazone (7.1 mg/kg, 100 *u*Ci), either by stomach tube dissolved in 500ml of dilute aq. $NaHCO_3$ soln., followed by 500ml water, or by intravenous infusion over 4 min dissolved in 50ml of a solution containing 20% v/v NaOH (2.1ml), 10% v/v ethanol and 40% v/v propane-1,2-diol, in sterile water, final pH < 8.5. Food was withheld from the animals for 12 h prior to dosing which was at 11 am. As in the earlier study small amounts of food were given to enable the collection of saliva, and the ponies were allowed free access to hay after 4 h.

Faeces Collection

As far as possible each individual faecal void was collected, and the time recorded, but unfortunately this was not possible overnight because the horses were left unattended. After collection the samples (10-20 kg/day) were weighed and thoroughly mixed (without the addition of water) in an electric cement mixer. A sample of this crude homogenate (750g) was taken and then homogenised in a Moulinex 517 food processor and the liquidised faeces stored until analysed.

Faecal homogenates (0.3g) were combusted in duplicate with a Packard Tricarb Model 306 sample oxidiser, the $^{14}CO_2$ so produced trapped in Optisorb 1, and counted following the addition of Optisorb S scintillant (Fisons Ltd., Loughborough, U.K.).

High performance liquid chromatography (HPLC)

A M6000A pump was used with a WISP 710B autoinjector, an M440 absorbance detector equipped with a 254nm filter, an M720 Systems Controller and an M730 Data Module (all Waters Associates, Harrow, U.K.). The stainless steel column (250 x 5 mm i.d) was packed with 5*u* ODS-Hypersil (Shandon Southern Products Ltd., Runcorn, U.K.) and eluted at ambient temperature with a degassed mobile phase of acetonitrile-water (53:47 v/v) adjusted to pH 3.0 with glacial acetic acid, flow rate 2.0 ml/min. The identities of peaks were assigned by comparison of retention times with those of authentic standards. Under the conditions described, the elution times for γ-hydroxyphenylbutazone, *p*-hydroxyphenylbutazone, *m*-hydroxyphenylbutazone (internal standard) and phenylbutazone were 2.4, 2.9, 3.4, and 6.0 min respectively.

Treatment of plasma

To 0.5ml of plasma was added 0.5ml acetonitrile containing 10*u*g of m-hydroxy-phenylbutazone. Samples were vortexed for 30 s and then centrifuged for 10 min at 1000x*g* to separate precipitated protein. The supernatant was removed and 20 *u*l portions injected on to the HPLC column. Quantification of phenylbutazone was achieved using the peak area ratio method, with respect to the internal standard, *m*-hydroxyphenylbutazone. Plots of peak area ratio versus phenylbutazone concentration gave straight lines over the range 1 to 100 *u*g/ml with correlation coefficients always > 0.99.

Pharmacokinetic analysis

The decline in the plasma concentration of phenylbutazone with time following its i.v. administration was fitted to a two compartment open model, and pharmacokinetic parameters for both phenylbutazone and total ^{14}C were calculated by least square regression analysis and the method of residuals (Greenblatt & Koch-Weser, 1975) using the biexponential equation

$$C = C_1 e^{-\lambda 1 t} + C_z e^{-\lambda z t}$$

where C is the plasma concentration of phenylbutazone, C_1 and C_z are the ordinate intercepts of the two regression lines, λ_1 and λ_z are their associated hybrid rate constants and t is the time in h.

Following oral administration, pharmacokinetic parameters were similarly calculated. The total areas under the plasma level-time curves following oral and intravenous administration were measured by the trapezoidal method, extrapolation to infinite time being obtained from C_t/λ_z, where C_t is the last plasma concentration measured and λ_z the disposition rate constant. Following

intravenous administration total AUCs were also calculated from the equation

$$AUC = C_1/\lambda_1 + C_z/\lambda_z$$

There was good agreement between the values obtained following i.v. administration for both methods.

The systemic availability (f) of phenylbutazone was calculated from the equation

$$f = \frac{AUC\ (p.o)}{AUC\ (i.v)}$$

and all other parameters using standard pharmacokinetic equations (Allen *et al.*, 1982).

RESULTS

Study 1. (Experiment 1)

Excretion of ^{14}C

Urine samples were collected for up to 77 h after the oral administration of [4-^{14}C]-phenylbutazone, and by this time the radioactivity levels had returned to within twice background. Examination of the graph of cumulative excretion against time (Fig. 2.1) indicated that the urinary elimination of ^{14}C was essentially complete within this time but accounted for only 55% of the dose.

Radioactivity levels in plasma and saliva.

The log ^{14}C levels (μg equivalents) in plasma were plotted against time (Fig. 2.2). Peak plasma levels were obtained within 2 h and after a short plateau phase declined monoexponentially thereafter. The radioactivity levels in the saliva were monitored for 10 h after administration, and are illustrated in Fig. 2.3 together with the saliva flow rates and the ratio of ^{14}C in saliva/^{14}C in plasma. The initial rise in plasma ^{14}C following the oral administration of the drug is mirrored by a rise in saliva ^{14}C levels. The ratio of the ^{14}C levels in saliva and plasma was not constant (Fig. 2.4), and was dependent on the salivary flow rate, which varied from 8.5 ml/min to 68 ml/min.

FIGURE 2.1 **Cumulative urinary excretion of ^{14}C following the oral administration of ^{14}C-phenylbutazone**

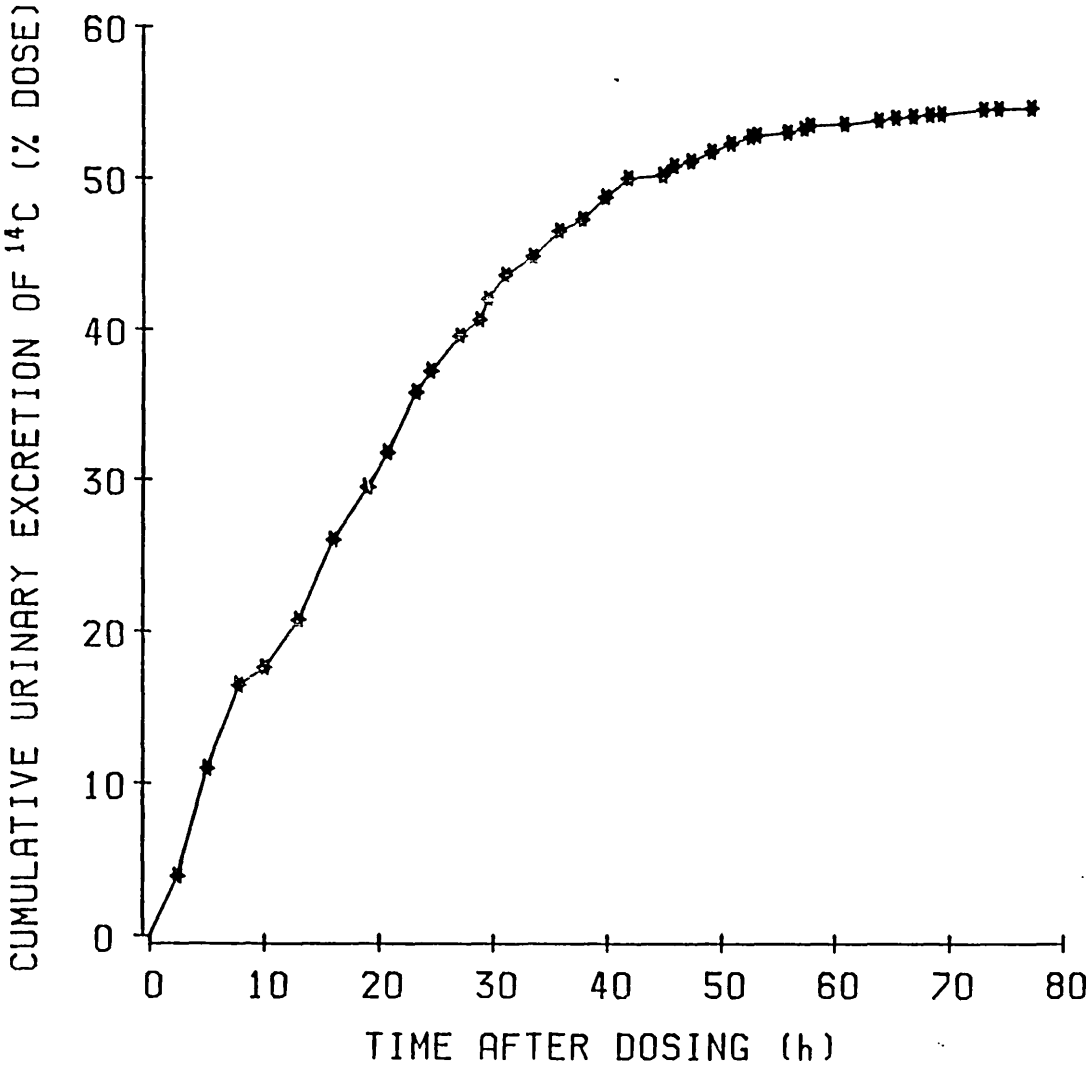


FIGURE 2.2 **Plasma level time curves for ^{14}C obtained in a horse following the oral administration of ^{14}C-phenylbutazone**

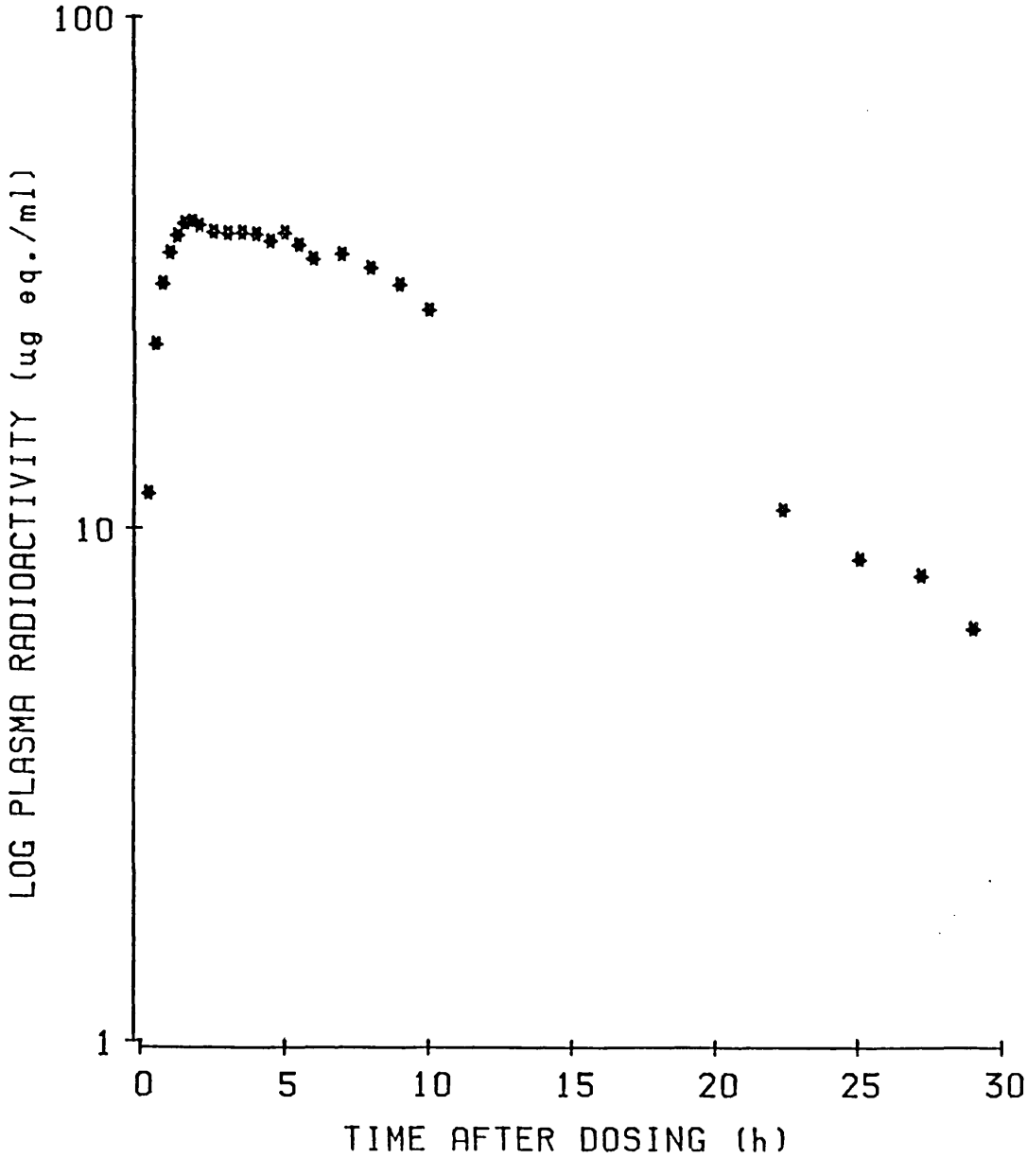


FIGURE 2.3 Salivary levels of ^{14}C obtained in a horse following the oral administration of ^{14}C-phenylbutazone

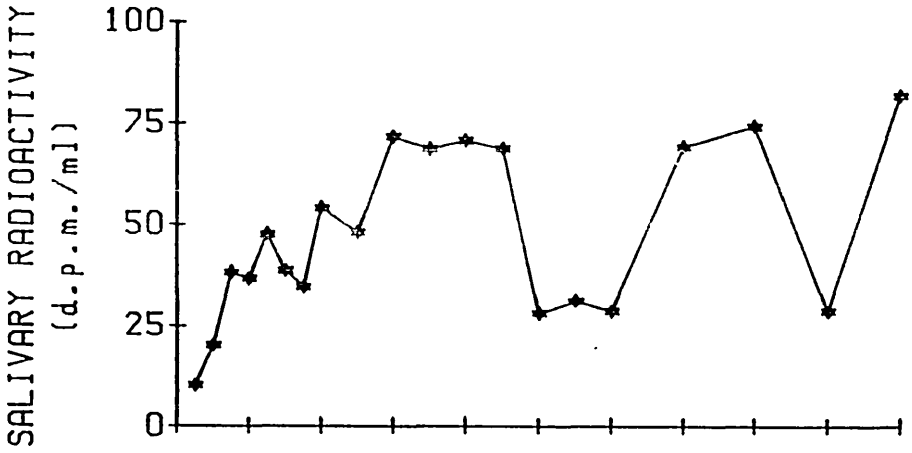
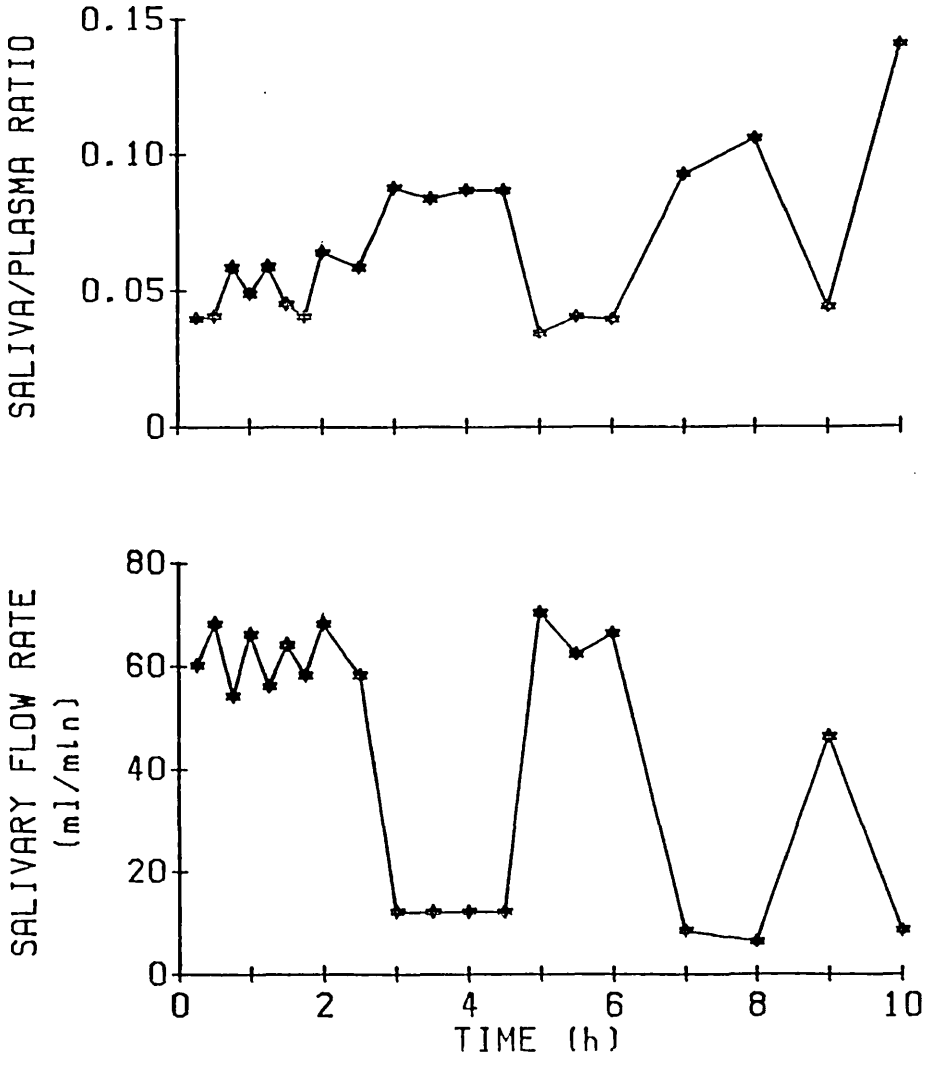


FIGURE 2.4 The influence of salivary flow rate on the ratio of ^{14}C levels in saliva and plasma following the oral administration of ^{14}C-phenylbutazone



Study 2. (Experiments 2,3,4,5).

Urinary excretion of ^{14}C

Urinary elimination of ^{14}C was essentially complete within 72 h, and accounted for some 55% of the dose. The recoveries were 57% and 44% (adjusted to 53%) after oral administration, and 54% after intravenous administration. The urinary recovery of ^{14}C following the oral dosing of ^{14}C phenylbutazone to one horse (Experiment 5), was significantly lower (44%), than that obtained in the rest of this study, and in the earlier feasibility study (Experiment 1). The percentage of the dose excreted in the urine in consecutive 16 h periods for all 5 experiments is given in Table 2.2. Examination of the appropriate rate plots (Figs. 2.5 & 2.6) indicate that the lower urinary recovery was the result of a lost sample. Estimation was made for the loss by calculating the log percentage dose excreted per hour, and plotting this against the midpoint for the sample. Extrapolation back to the missing time point gave the sample as 9% of the dose which increased the urinary recovery to 53%. Details of the calculations and tabulated data are included Appendix 1.

TABLE 2.2 **The urinary excretion of radioactivity for consecutive 16 h periods expressed as a percentage of dose following the oral and intravenous administration of ^{14}C-phenylbutazone**

Collection period	Experiment No. 1	2	3	4	Mean ± S.D. (1,2,3, & 4)	No. 5
0-16h	26.0	25.1	27.1	26.0	26.0 ± 0.8	16.0
16-32h	17.6	15.7	17.0	12.2	15.6 ± 2.4	14.9
32-48h	7.5	7.8	7.6	10.5	8.4 ± 1.4	7.8
48-64h	2.8	4.0	4.8	4.5	4.0 ± 0.9	2.4

FIGURE 2.5 **Ln excretion rate (% dose h^{-1}) plotted against the mid-point of consecutive 16 h sampling periods. Mean ± S.D. Experiments Nos. (1-4)**

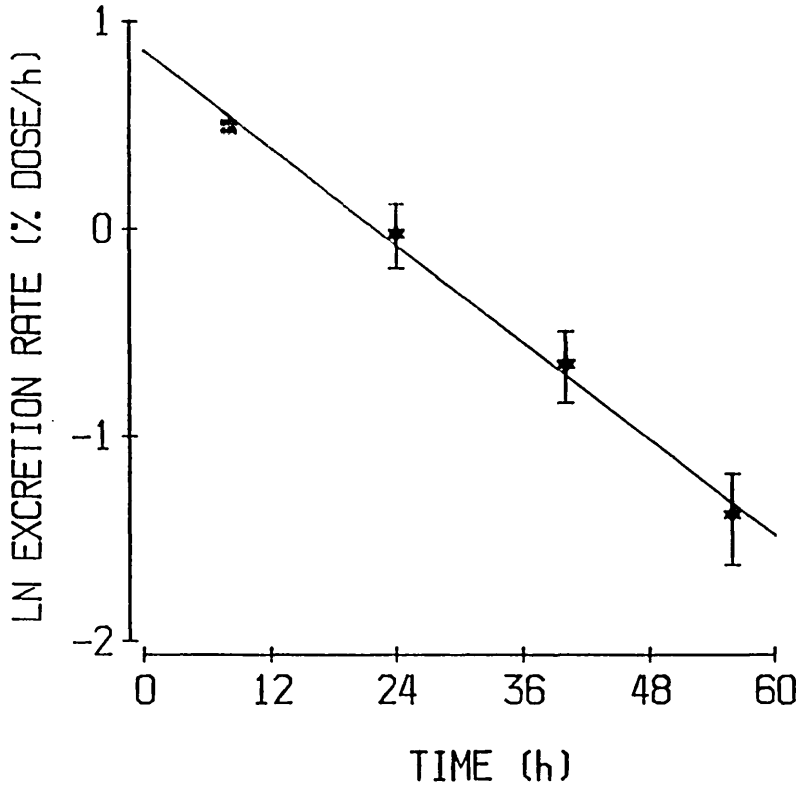
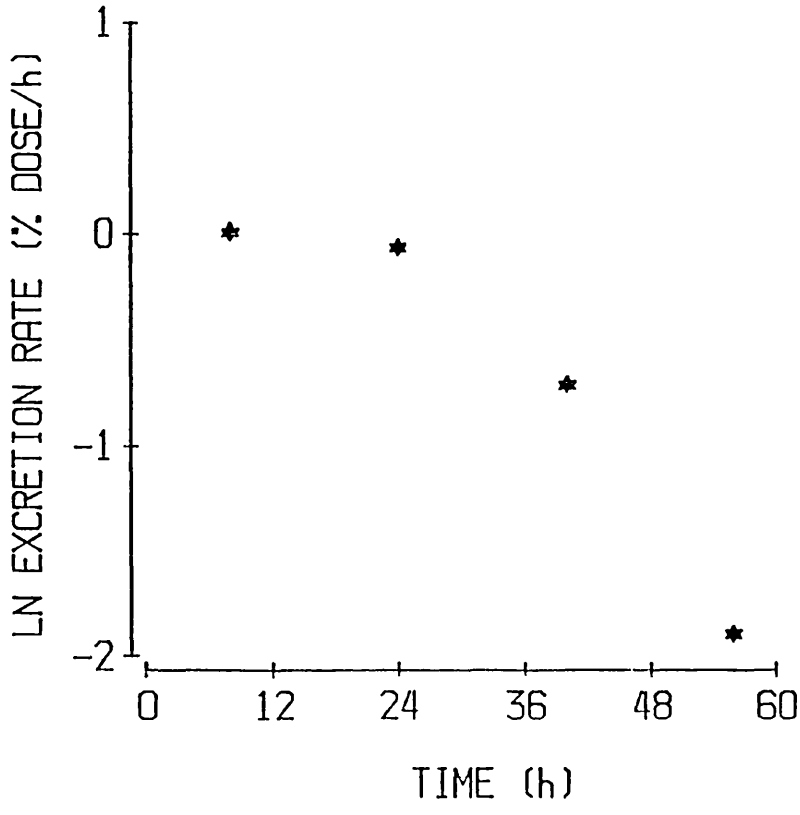


FIGURE 2.6 **Ln excretion rate (% dose h^{-1}) plotted against the mid-point of consecutive 16 h sampling periods. Experiment No. 5**



Faecal excretion of ^{14}C

After either oral or intravenous administration of ^{14}C-phenylbutazone no faecal elimination of ^{14}C was observed up to 20 h post dosing. In the first part of this study (Experiments 2 & 3), 20% (i.v) and 30% (p.o) of the dose was recovered in the faeces in the time period 20-68 h. Examination of the appropriate rate plots indicated that the faecal excretion of ^{14}C was not complete within this period and therefore in the remainder of the study (Experiments 4 & 5) the faecal collection period was extended to 150 h. Under these conditions 37% (i.v.) and 41% (p.o) of the dose was recovered in the faeces in this period (0-150 h), and at the end of which faecal elimination was essentially complete.

Total recovery of ^{14}C

An essentially quantitative recovery of administered ^{14}C was obtained within seven days. This was independent of the route of administration of ^{14}C-phenylbutazone, with 91% and 95% of the dose recovered after intravenous and oral administration respectively. The proportion of the dose excreted in urine and faeces was also independent of the route of administration. The results for both horses are illustrated in Fig 2.7 and Fig 2.8.

Plasma pharmacokinetics

A comparison between the phenylbutazone concentrations obtained from the HPLC analysis of plasma and the μg equivalents calculated from the plasma ^{14}C content revealed that at all times the major radioactive component was unchanged phenylbutazone. Fig. 2.9. illustrates the plasma concentration-time curves obtained for one of the horses following oral and intravenous administration.

Intravenous administration

Following intravenous administration the plasma concentrations of ^{14}C and of phenylbutazone declined biphasically with time. The experimental data were analysed according to a two compartment open model, and the various parameters describing this, corrected where appropriate for the weight of the horse, are listed in Table 2.3.

Oral administration

Following oral administration, phenylbutazone was rapidly absorbed. Peak plasma concentrations of phenylbutazone and of ^{14}C were obtained within 1.5 h, and fell monoexponentially thereafter. The data were fitted to a one compartment model. The pharmacokinetic parameters obtained for both horses are presented in Table 2.4, which includes estimates of the oral bioavailability of phenylbutazone.

FIGURE 2.7 **Cumulative urinary and faecal excretion of ^{14}C radioactivity by one horse (No. 1) given ^{14}C-phenylbutazone orally and intravenously on separate occasions**

Key: (✱——✱) Urine, (✱——✱) Faeces, (———) Total

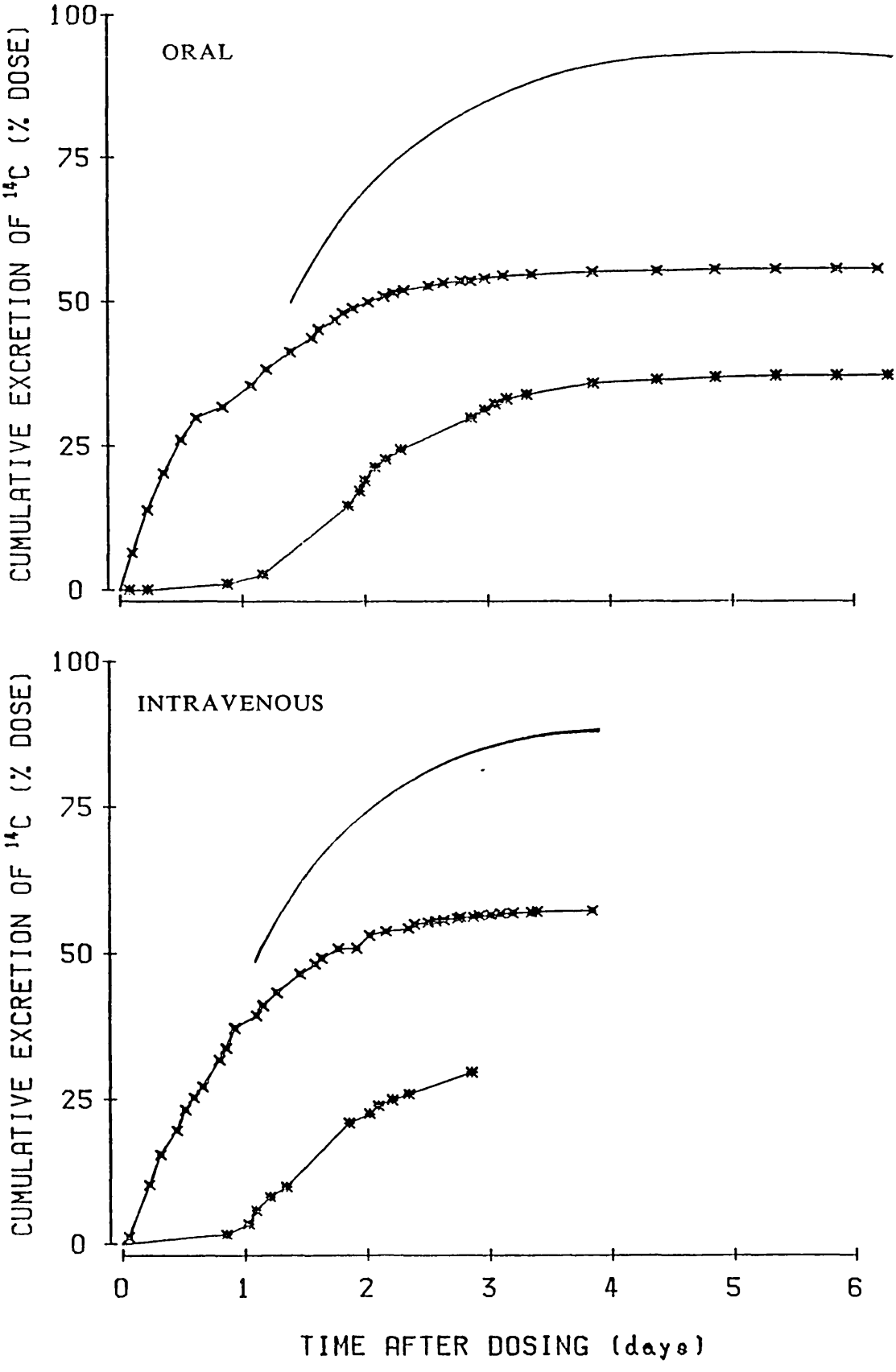


FIGURE 2.8 **Cumulative urinary and faecal excretion of ^{14}C radioactivity by one horse (No. 2) given ^{14}C-phenylbutazone orally and intravenously on separate occasions**

Key: (✱——✱) Urine, (✱——✱) Faeces, (———) Total

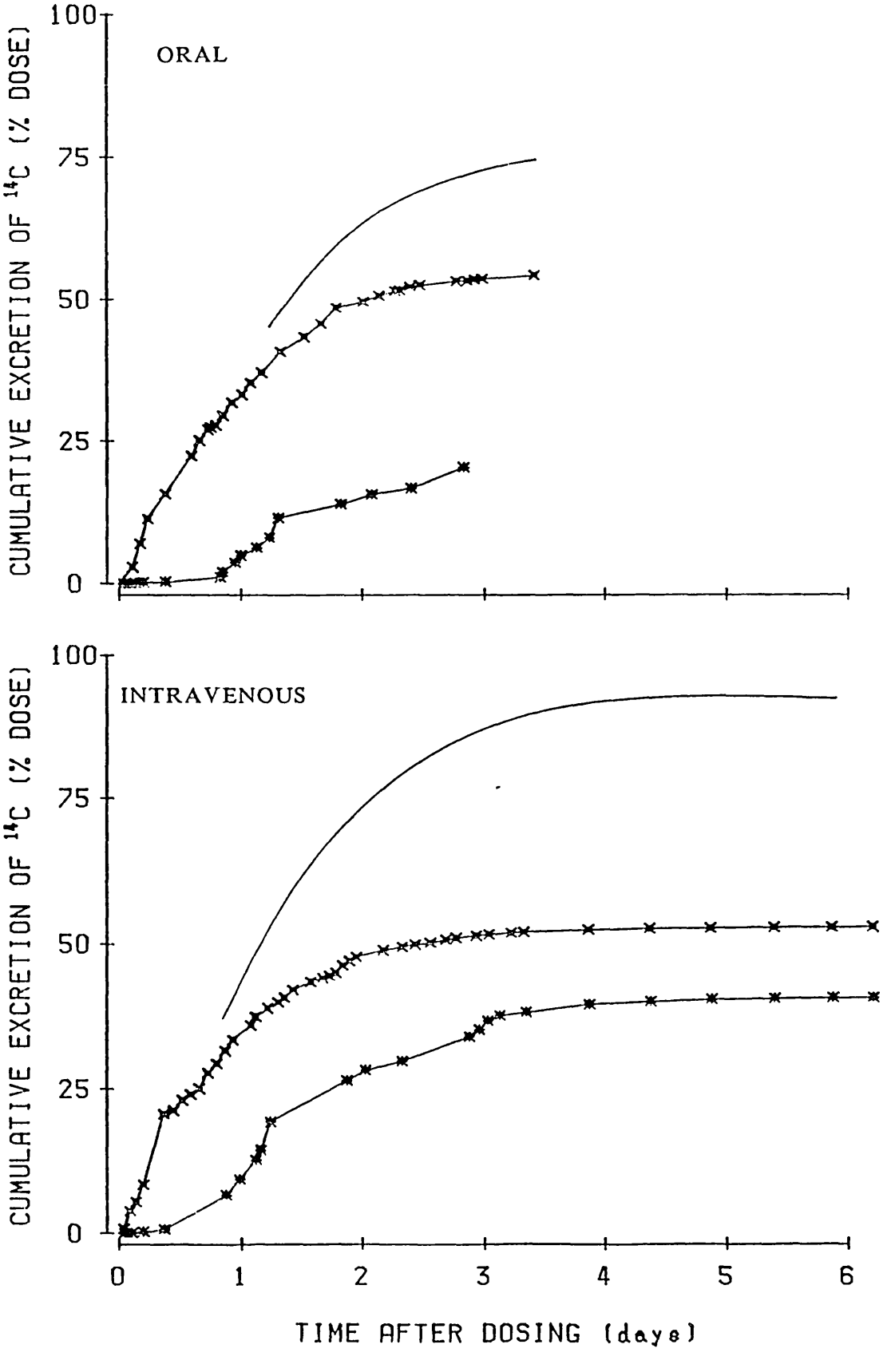


FIGURE 2.9 **Plasma level-time curves obtained in a horse given ^{14}C-phenylbutazone orally (•) and intravenously (o) on separate occasions**

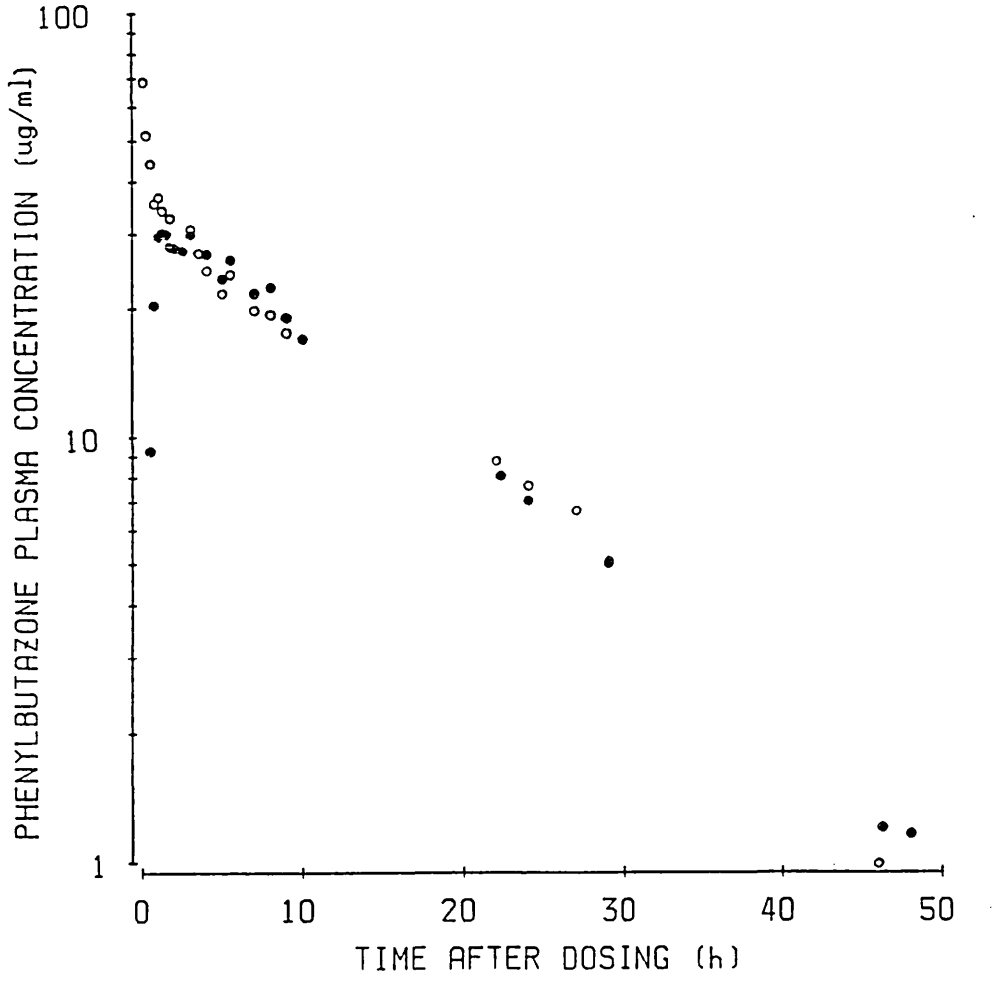


TABLE 2.3 **Pharmacokinetic parameters describing the plasma levels of ^{14}C radioactivity and phenylbutazone following the intravenous administration of ^{14}C-phenylbutazone to two horses**

	Horse 1	Horse 2
Phenylbutazone		
C^0 (μg ml^{-1})	73.9	72.2
C_1 (μg ml^{-1})	37.9	36.5
λ_1 (h^{-1})	3.58	2.71
C_z (μg ml^{-1})	36.0	35.7
λ_z (h^{-1})	0.07	0.07
$t_{0.5}$ (h)	9.7	9.7
App Vd ($mlkg^{-1}$)	192	193
App Vc ($mlkg^{-1}$)	95.6	97.8
Cl ($mlkg^{-1}h^{-1}$)	13.7	13.8
k_{12} (h^{-1})	1.73	1.26
k_{21} (h^{-1})	1.78	1.37
k_{10} (h^{-1})	0.14	0.14
AUC (μg ml^{-1}.h) (trapezoidal method)	520	520
AUC (μg ml^{-1}.h) (direct integration)	514	512
^{14}C equivalents		
$t_{0.5}$ (h)	9.9	10.4

TABLE 2.4 **Pharmacokinetic parameters describing the plasma levels of ^{14}C radioactivity and phenylbutazone following the oral administration of ^{14}C-phenylbutazone to two horses**

	Horse 1	Horse 2
Phenylbutazone		
C_{max}(μg ml^{-1})	36.2	30.1
t_{max} (h)	1.25	1.50
k_a(h^{-1})	4.56	2.97
λ (h^{-1})	0.07	0.07
$t_{0.5}$(h)	9.3	9.9
AUC (μg ml^{-1}.h) (trapezoidal method)	456	493
f % (Bioavailability of phenylbutazone)	87.5	95.3
^{14}C equivalents		
$t_{0.5}$(h)	9.7	10.1

DISCUSSION

The terminal plasma elimination half-life of phenylbutazone in this study was not dependent on route of administration, and there was good agreement for the two horses studied. The value obtained in this study (mean 9.7 h) was similar to that reported by Tobin *et al.* (1977) (7.9 h) at a slightly lower dose (6.6 mg/kg compared with 7.1 mg/kg in this study), but longer than that reported in Welsh mountain ponies (4.7 h) by Lees *et al.* (1985b) with a dose of 4.4 mg/kg, which is consistent with the known dose-dependent kinetics of phenylbutazone in the horse. Piperno *et al.* (1968) reported a half-life of 3.5h at 8.8 mg/kg increasing to 6 h at 17.6 mg/kg in thoroughbred and standardbred horses.

The renal elimination of phenylbutazone, a weak acid (pKa 4.6), might be expected to be dependent upon urinary pH. Low urinary pH (4.6-5.5) reduced the elimination rate of total ^{14}C following the administration of ^{14}C phenylbutazone (Moss & Haywood, 1973). An acidic urine does not explain the slower elimination observed in our study since the urine was alkaline (mean pH 7.5). A possible explanation for the longer half-life obtained in our study lies in the differences in the breed of horse used, since Argenzio and Hintz (1970) reported a longer antipyrine half-life in ponies than was reported for thoroughbreds (Powis & Snow, 1978). It is perhaps relevant to note that Vesell and Page (1968) suggested that the inter-individual variation of phenylbutazone metabolism in man is largely under genetic control.

The apparent volume of distribution of 190 ml/kg is similar to that obtained in Welsh mountain ponies (164 ml/kg) (Lees *et al.*, 1985b), cattle (100 ml/kg) (Eberhardson *et al.*, 1979), and man (172 ml/kg) (Triggs *et al.*, 1975), and consistent with that of an acidic drug highly bound to plasma protein (96%

Gandal *et al.*, 1969).

The ratio of radioactivity in saliva/plasma was inversely related to the salivary flow rate as previously observed with other compounds (see Moss, 1977), and varied between 0.05-0.10. The very low salivary radioactivity observed for ^{14}C-phenylbutazone is consistent with the concept that plasma and saliva are separated by a lipophilic membrane, permeable only to the unionised non-protein bound drug.

Following oral administration phenylbutazone was rapidly and well absorbed, with a bioavailability of 87% and 95% in the two ponies studied. The high systemic availability found in this study indicates that not only is phenylbutazone almost completely absorbed, but that it does not undergo any significant 'first-pass' metabolism.

There are few reports in the literature on the systemic availability of phenylbutazone in any species. De Backer *et al.* (1980) found a wide variation in the bioavailability of phenylbutazone in cows, results ranged from 42-95% with a mean of 67.5%. In a review of the clinical pharmacokinetics of phenylbutazone in man, Aarbakke (1978) could find no studies on the bioavailability of this compound in the literature. However he did suggest that phenylbutazone was completely absorbed in man, since Dieterle *et al.* (1976) recovered 90% of the radioactivity in the urine and faeces following the oral administration of ^{14}C-phenylbutazone, and Aarbakke *et al.* (1977) had demonstrated that the faecal elimination of radioactivity represented biliary excretion. Although this indicates a good absorption of drug related compound it is impossible to assess the systemic availability of phenylbutazone on this basis since it completely ignores any losses due to pre-systemic metabolism.

Since the inception of this study there have been several studies published on the bioavailability of phenylbutazone in the horse. Soma *et al.* (1983) reported a bioavailability of 91.8 $\pm$ 2.5% (n=6) in standard and thoroughbred horses. Lees *et al.* (1985b) used a proprietary formulation (*Equipalazone granules*; Arnold) which was administered as a 10 ml suspension to investigate the bioavailability in 3 young and 3 older ponies, results ranged from 50% to 98% with a mean of 77%.

It is of interest to compare the rate of faecal elimination of ^{14}C following phenylbutazone administration with the known kinetics of gastrointestinal transit in the horse. The absence of ^{14}C material in the faeces up to 20 h post-dosing was similar to the lag-time reported by Vander Noot *et al.* (1967) when they investigated the passage of feedstuffs through the digestive tract of the horse. In that study 95% of the marker used (Cr_2O_3) was recovered within 60 h, and similar results were obtained by Hintz and Loy (1966) who recovered 99% within 63 h. However, following the oral administration of ^{14}C phenylbutazone, faecal recovery of ^{14}C was by no means complete within 72 h of dosing. This strongly suggests that the continued elimination of ^{14}C in the faeces, after urinary elimination had ceased, was the result of faecal elimination of absorbed compound subsequent to biliary excretion, and not simply the consequence of incomplete absorption. Further evidence for this was that similar proportions of the dose were excreted in the faeces following intravenous and oral administration. These data are strongly indicative of biliary excretion. The extensive faecal elimination shown here accounts for the low recoveries of drug and metabolites obtained by other authors who collected only urine.

It should be noted that in species other than the horse phenylbutazone

ulceration is limited to the upper gastrointestinal tract (Rechenberg, 1962) but in the horse the majority of lesions occur in the large colon and caecum apart from some local ulcers of the oral cavity. Sullivan and Snow (1982), and Lees *et al.* (1985a) proposed that following oral administration a portion of the dose was adsorbed on to the fibre of the diet and subsequently released by fermentation in the caecum causing local ulceration. Snow *et al.* (1981) have suggested that the ulcerogenic activity of phenylbutazone is a local action, and arises from unabsorbed drug in the lumen of the gut. Support for this hypothesis was provided by Lees *et al.* (1985a) who recently demonstrated the *in vitro* binding of phenylbutazone to chopped hay. The delayed absorption of phenylbutazone following oral administration on a full stomach (Sullivan & Snow 1982; Lees *et al.*, 1983) may be explained by this hypothesis, but the observation by MacKay *et al.* (1983) that parenteral administration of phenylbutazone caused caecal rupture, and our finding of the high percentage of the dose excreted in the faeces following intravenous administration, suggests that the ulcerogenic effects of phenylbutazone may be a consequence of the exposure of the lower gastrointestinal tract to high levels of drug related material in the contents of the gut, presumably arising from the biliary clearance of the drug, thereby exposing the compound to an area it does not reach in other species.

CHAPTER 3.

THE METABOLISM OF PHENYLBUTAZONE

INTRODUCTION

Much of the background to the metabolism of phenylbutazone in the horse, was discussed in the Introductory Chapter. Despite widespread interest in the metabolism of phenylbutazone in the horse, oxyphenbutazone and γ-hydroxyphenylbutazone, the two metabolites originally identified by Schubert *et al.* (1967) 20 years ago, remain the only metabolites identified so far. Since the latter study, a number of different metabolites have been identified in other species, yet there are no reports in the literature, on the formation of these compounds by the horse. Prior to the inception of this study, the fate of some 75% of an administered dose of phenylbutazone remained undetermined. The discovery in the faeces of 40% of an intravenously administered dose of ^{14}C-phenylbutazone, goes some way to explaining the low recoveries previously reported, but knowledge of the fate of phenylbutazone in the horse is as yet incomplete.

Degradation of phenylbutazone

A problem frequently encountered in studies with phenylbutazone arises from the chemical instability of this compound. When stored as a solid, and under normal conditions, this is not a problem (Matsui *et al.*, 1977), but significant degradation occurs when the compound is stored in solution, even at low temperatures (Bellward *et al.*, 1972) Phenylbutazone is sensitive to both oxidative and hydrolytic conditions and several investigators have shown that when exposed to air e.g. on TLC plates, a large number of products arise spontaneously (Awang *et al.*, 1973; Fabre & Mandrou, 1981)

The degradation pattern of phenylbutazone has been discerned by Awang *et al.* (1973) (Fig 3.1), and is proposed to proceed through two main pathways, either

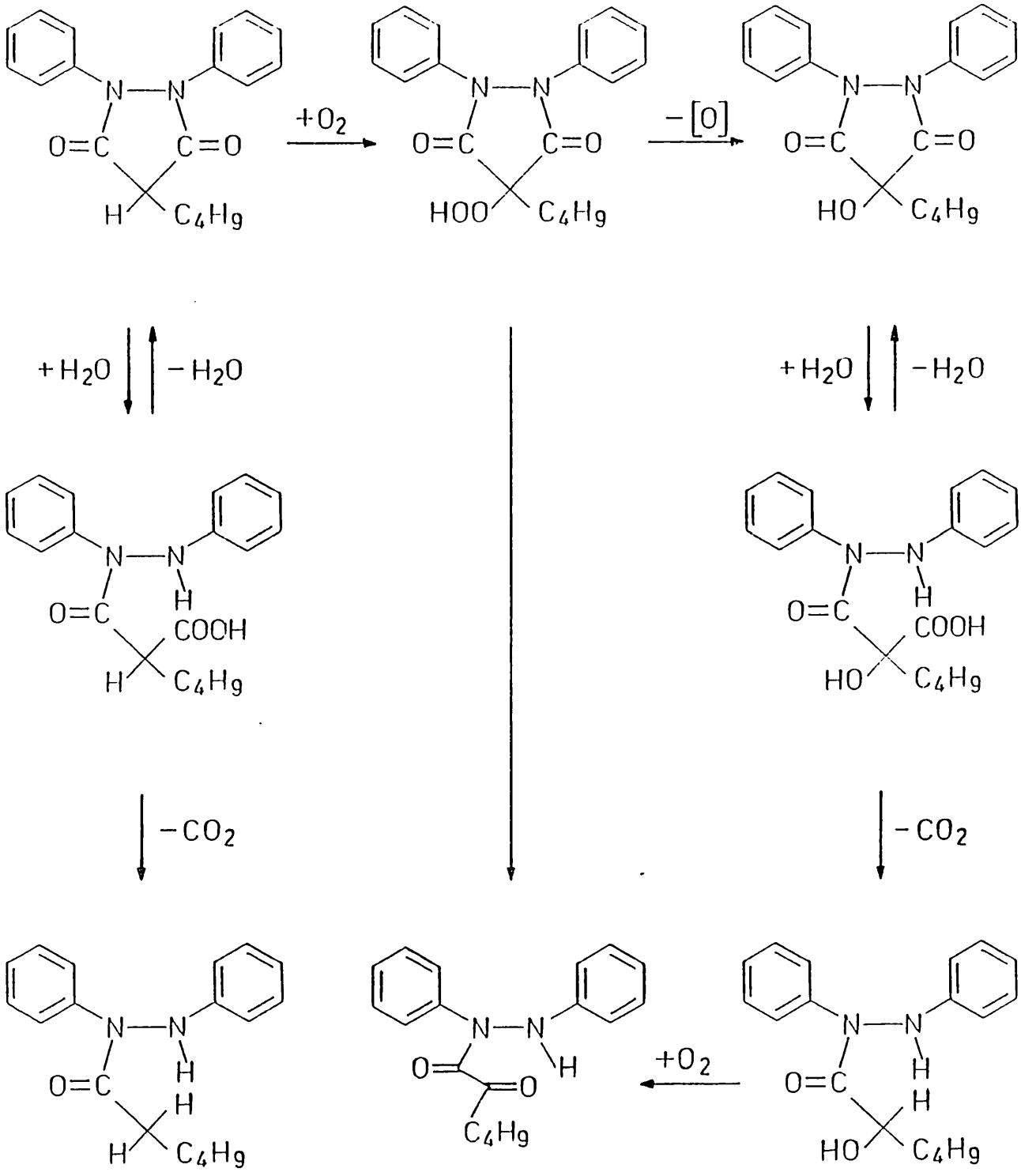
oxidation at the C_4 position via formation of a peroxide, or hydrolysis of the C-N bonds. The extent of each pathway is influenced by the reaction conditions.

The dissociable proton on the C_4 atom enables phenylbutazone to exist in solution in the three forms shown in Figure 3.2, a di-keto, an enol, and a mesomeric anion form. The interconversion between these forms probably contributes to the chemical instability of phenylbutazone since the degradative hydrolysis to butylmalonic acid mono(N,N'-diphenylhydrazide) is thought to proceed via the dissociated form (Schmidt, 1970).

The prevention of degradation is of importance in metabolic studies, especially when (as is the case with 4-hydroxyphenylbutazone) the degradation product is also a potential metabolite. Although the *in vivo* 4-hydroxylation of phenylbutazone has not been demonstrated in man or in animals, there are various reports of its production *in vitro*, and it is thought that the enzyme prostaglandin endoperoxide synthetase (PES) is responsible. Portoghese *et al.* (1975) demonstrated that the related compound oxyphenbutazone is metabolised to 4-hydroxyoxyphenbutazone by sheep vesicular gland microsomes, and recently Ichihara *et al.* (1986) showed that enzymes present in a leukocyte extract obtained from peritoneal exudate of rats metabolised phenylbutazone to 4-hydroxy- and 4-hydroperoxy- phenylbutazone. Favero & Winternitz (1980) investigated the microbial metabolism of phenylbutazone by the fungus *Rhizopus arrhizus*, and obtained 4-hydroxyphenylbutazone in an almost quantitative yield.

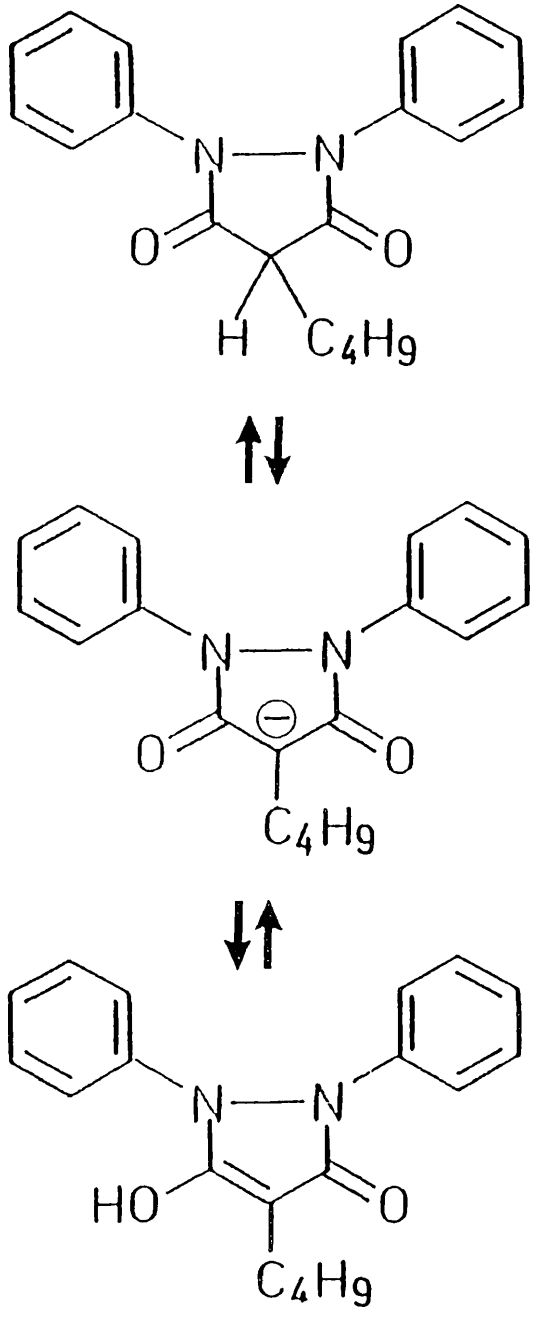
Because of the known chemical instability of phenylbutazone, special measures were taken to prevent the production of chromatographic artefacts, and to reduce the oxidative losses of phenylbutazone and metabolites on storage.

FIGURE 3.1. The degradation pathways of phenylbutazone
(From Awang *et al.*, 1973).



I, Phenylbutazone; II, 4-hydroperoxyphenylbutazone; III, 4-hydroxyphenylbutazone; IV, *n*-butyltartronic acid mono(N,N'-diphenyl)hydrazide; V, *n*-butylmalonic acid mono(N,N'-diphenyl)hydrazide; VI, N-(α-hydroxycaproyl)hydrazobenzene; VII, N-caproylhydrazobenzene; VIII, N-(α-ketocaproyl)hydrazobenzene.

FIGURE 3.2 **Interconversion of the three forms of phenylbutazone** (from Schmidt, 1970).



EXPERIMENTAL

Details of the experimental conditions are described in full in the preceding chapter; briefly two gelded crossbred ponies received ^{14}C-phenylbutazone (7.1 mg/kg, 100 *u*Ci) orally and intravenously on separate occasions. Urine and faeces were collected and the ^{14}C content determined. Any additional details are described below.

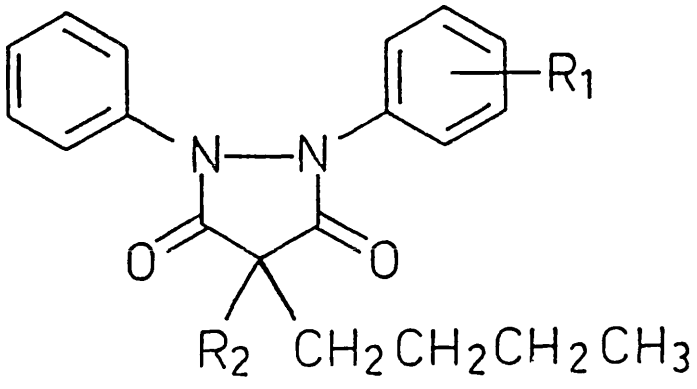
Compounds

Samples of possible phenylbutazone metabolites and degradation products were gifts from Ciba-Geigy (Basle, Switzerland). The chemical structures of the two ring opened compounds *n*-butyltartronic acid mono(N,N'-diphenyl)hydrazide and *n*-butylmalonic acid mono(N,N'-diphenyl)hydrazide are illustrated in Fig. 3.1 (IV and V), and the chemical structures of the other compounds in Table 3.1. HPLC grade methanol was purchased from Rathburn Chemicals Ltd., (Walkerburn, U.K.). Other materials were reagent-grade chemicals purchased from usual U.K. commercial sources and used without further purification.

Sample preparation

As described earlier, as far as possible each urinary voiding was collected separately (into bottles containing $Na_2S_2O_5$ and disodium EDTA to prevent degradation of phenylbutazone and metabolites); the volume, pH, and time of voiding were recorded. For the purpose of quantitative and qualitative analysis of the urinary metabolites, appropriate volumes of urine were pooled to provide representative 0-12, 12-18, 18-24, 24-48 and 48-72 h urine samples. The urine was clarified by centrifugation at 1500 x*g* for 10 min using an MSE Centaur 2 bench-top centrifuge.

TABLE 3.1 Structures of phenylbutazone and related compounds.



Compound.	R_1	R_2	Butyl group
Phenylbutazone	H	H	-
p-Hydroxyphenylbutazone	*p*-OH	H	-
m-Hydroxyphenylbutazone	*m*-OH	H	-
β-Hydroxyphenylbutazone	H	H	2-OH
γ-Hydroxyphenylbutazone	H	H	3-OH
ω-Hydroxyphenylbutazone	H	H	4-OH
γ-Ketophenylbutazone	H	H	3C=O
p,γ-Dihydroxyphenylbutazone	*p*-OH	H	3-OH
p-Hydroxy-γ-ketophenylbutazone	*p*-OH	H	3C=O
4-Hydroxyphenylbutazone	H	OH	-
4-Hydroperoxyphenylbutazone	H	OOH	-
4-Hydroxyoxyphenbutazone	*p*-OH	OH	-

For the GC analysis a portion of the urine (10ml) was evaporated to dryness at room temperature, initially under reduced pressure, and finally in a stream of nitrogen. The residue was redissolved in acetone (1ml) and methyl and methyl-trimethylsilyl derivatives formed (see below).

Enzyme treatments of urine

ß-*Glucuronidase.* Urine (2ml) was incubated at 37° overnight with ß-glucuronidase (1ml: Glucurase, 5000 units/ml, Sigma, Poole, Dorset, U.K.) and 0.2M acetate buffer, pH 5.0 (1ml). Control experiments were carried out either without enzyme, or in the presence of the specific inhibitor saccharo-1,4-lactone (2mg per tube): positive controls were carried out with phenolphthalein glucuronide (2mg per tube).

Sulfatase. Urine (2ml) was incubated at 37° overnight with sulfatase (2mg: type H-1 from *Helix pomatia*, Sigma; 18000 units/g) and 0.2 M acetate buffer, pH 5 (1ml). Incubations were carried out in the presence of saccharo-1,4-lactone (2mg per tube) to inhibit the ß-glucuronidase present in the enzyme preparation. A urine sample incubated without enzyme served as control.

Preparation of derivatives

Because of the low volatility and high polarity of most phenylbutazone analogues it was necessary to derivatise them before GC analysis: accordingly methyl (Me), and methyl-trimethylsilyl (Me-TMS) derivatives were prepared. Samples (1mg) were methylated by heating the sample at 60° for 1 h with methyl iodide (50*u*l) in acetone (100*u*l) over potassium carbonate. Methyl-trimethylsilyl (Me-TMS) derivatives were prepared by heating the methylated sample (1mg) at 80° for 2 h with a mixture of N,O-bis-trimethylsilylacetamide (100*u*l) and trimethylchlorosilane (50*u*l).

Radiochemical techniques

Fractions of HPLC eluate and all other solutions and extracts were assayed for ^{14}C using liquid scintillation spectrometry as detailed earlier. For radiochromatogram scanning of thin layer chromatographs, the plates were cut to a width of 5cm, and scanned using a Packard radiochromatogram scanner Model 7201.

Chromatography

Thin layer Chromatography

Thin layer chromatography (TLC) was carried out using silica gel F_{254} plates (Cat. No. 5554, E.Merck A.G., Darmstadt, FR Germany), layer thickness 0.2mm, 20 x 20 cm on aluminium support, developed to 15cm from the origin with cyclohexane-chloroform-methanol-acetic acid (60:30:5:5, by vol.), and the compounds located under UV light (254nm). The plates were pre-treated by soaking in a 1% alcoholic citric acid solution and dried for 1 h at 130°, as this had been demonstrated by Fabre & Mandrou (1981) to retard the *in situ* degradation of phenylbutazone. R_f values of phenylbutazone and potential metabolites are presented in Table 3.2. Samples for confirmatory GCMS were obtained by scraping the appropriate silica gel from the relevant area of the plate, and eluting the compound with acetone (10ml). The latter was then concentrated to 1ml, before further analysis.

High Performance Liquid Chromatography

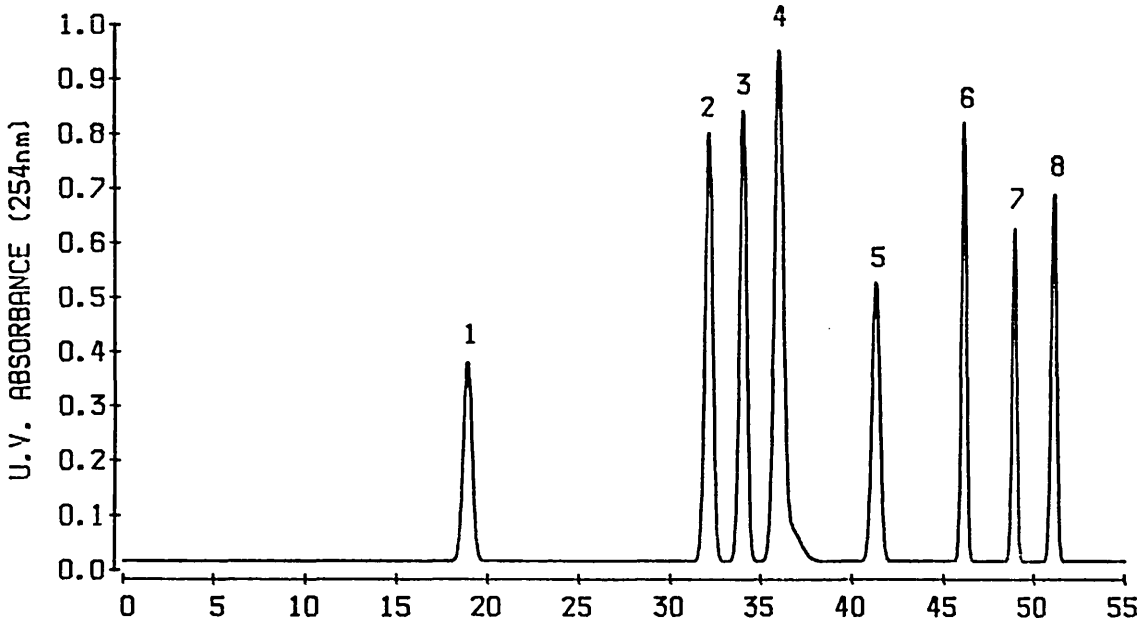
The analysis was accomplished by a reverse phase non-linear gradient elution technique. The apparatus consisted of two M6000A pumps, controlled by an M720 Systems Controller. UV detection utilised an M440 fixed wavelength detector equipped with a 254nm filter, and an M730 integrator. Samples were

introduced either manually with a U6K loop injector, or with an automatic injector WISP 710B, (all Waters Associates, Harrow, U.K.). The stainless steel column (250 x 5mm i.d.) was packed with 5*u* ODS-Hypersil (Shandon Southern Products Ltd., Runcorn, U.K.) and eluted at ambient temperature with a degassed mobile phase of methanol-water (25:75 v/v to 80:20 v/v) adjusted to pH 3.0 with glacial acetic acid, at a flow rate of 1.5 ml/min. Fractions of eluent (1.5ml) were collected into scintillation vials, using a LKB RediRac fraction collector. Details of the non-linear gradient, together with chromatographed standards are shown in Figure 3.3. Retention times of phenylbutazone and related compounds are presented in Table 3.2. For confirmatory GCMS analysis, the relevant fraction of HPLC eluent was collected into a glass vial, the mobile phase removed under reduced pressure using a Savant Speed Vac Concentrator, and the residue redissolved in acetone (1ml), before derivatisation if necessary.

Gas Chromatography

Gas chromatography was conducted with a Pye Unicam 304 instrument fitted with a capillary injector (SGE Unijector), a flame ionization detector, and a Pye Unicam CPD 4 integrator (Philips, Cambridge, U.K.). The gas chromatograph was equipped with a fused silica capillary column (25m x 0.32mm) coated with a chemically bonded phase of dimethyl silicone (Cat. No. 25QC3 SGE (U.K.) Ltd., Milton Keynes, U.K.). The conditions were set to give a split ratio of 50:1 with a column flow of about 1 ml/min of He, an injection port temperature of 200° and a detector temperature of 260°. The temperature program was held at 200° for 1 min after injection, and increased at 10°/min to 280°, and was held there for 5 min. Retention times for phenylbutazone and related compounds under these conditions are given in Table 3.3.

FIGURE 3.3 HPLC separation of authentic standards



1. *p*, γ -Dihydroxyphenylbutazone, 2. ω -hydroxyphenylbutazone, 3. γ-keto-phenylbutazone, 4. γ-hydroxyphenylbutazone, 5. *p*-hydroxyphenylbutazone, 6. *m*-hydroxyphenylbutazone, 7. 4-hydroxyphenylbutazone, 8. phenylbutazone.

Details of the non-linear gradient. Solvent A, 20% v/v methanol-water; solvent B 80% v/v methanol-water. Initial conditions 90% A, 15 min 82% A (Waters Assoc. curve No.4), 40 min 55% A (Waters Assoc. curve No.5) 45 min 65% A (linear).

TABLE 3.2 **Chromatographic characteristics of phenylbutazone and related compounds by TLC and HPLC**

Compound.	TLC R_f	HPLC R_t min
Phenylbutazone	0.48	51.1
p-hydroxyphenylbutazone	0.20	41.3
m-Hydroxyphenylbutazone		46.1
β-Hydroxyphenylbutazone	0.34	45.3 (45.9)
γ-Hydroxyphenylbutazone	0.23	36.0
ω-Hydroxyphenylbutazone		33.5
γ-Ketophenylbutazone		34.6
p,γ-Dihydroxyphenylbutazone	0.05	18.9
p-Hydroxy-γ-ketophenylbutazone		18.8
4-Hydroxyphenylbutazone	0.26	48.9
4-Hydroperoxyphenylbutazone		48.8 (51.1)
n-Butyltartronic acid mono(N,N'-diphenyl)hydrazide		48.2
n-Butylmalonic acid mono(N,N'-diphenyl)hydrazide		51.7

TABLE 3.3 **Chromatographic characteristics of phenylbutazone and authentic standards by capillary GC**

Compound (derivative).	R_t min
Phenylbutazone	8.16
Phenylbutazone (Me)	7.49
4-Hydroxyphenylbutazone	8.94
m-Hydroxyphenylbutazone (Me)	8.60
p-Hydroxyphenylbutazone (Me)	9.09
γ-Ketophenylbutazone (Me)	8.85
β-Hydroxyphenylbutazone (Me-TMS)	9.05 (8.79)
γ-Hydroxyphenylbutazone (Me-TMS)	9.29
ω-Hydroxyphenylbutazone (Me-TMS)	10.07
p,γ-Dihydroxyphenylbutazone (Me-TMS)	11.29

Gas Chromatography-Mass Spectrometry.

GCMS was performed in EI mode at the Horseracing Forensic Laboratory, Newmarket, with a Finnigan 1020 instrument. The capillary GC column was of fused silica (8m x 0.32mm i.d.) coated with a chemically bonded phase of dimethylsiloxane (Cat. No. 633225, Alltech U.K. Ltd., Carnforth, U.K.). The conditions were set to give a linear gas velocity of 40 cm/s of He, an injection port temperature of 240°, a detector oven temperature of 300° and a manifold temperature of 90°. There were slight differences in the temperature programs used for the GC analysis, but typically the oven temperature was initially set at 70°, programmed to rise at 15°/min to 290°.

Quantitative analysis of urinary metabolites

Aliquots (200*u*l and 500*u*l) of the 0-12, 12-18, 18-24, 24-48 and 48-72 h pooled urines were analysed by HPLC, and the separated metabolites quantitated by scintillation counting of serial fractions of the eluent. Radiochromatograms were constructed by plotting the ^{14}C content of each fraction against their elution time. Peaks for phenylbutazone and its metabolites in the radiochromatograms were assigned on the basis of identical chromatographic behaviour to that of the standards monitored by UV detection at 254nm.

RESULTS

Excretion of ^{14}C

These results are described in full in the preceding chapter. There was an essentially complete recovery of administered radiolabel within 6 days. The pattern of elimination was independent of the route of administration, with 55% of the dose found in the urine, and 40% in the faeces.

Prevention of phenylbutazone degradation

Pre-treating the silica TLC plates with citric acid, did not alter the chromatographic characteristics of phenylbutazone, or the major decomposition product (presumed to be 4-hydroxyphenylbutazone), but was effective at reducing the auto-oxidation of phenylbutazone, and this is illustrated in Figure 3.4. Macek (1968) suggested that the presence in the silica of small amounts of finely divided iron catalysed the oxidative degradation of phenylbutazone, and that by complexing the iron, citric acid protects phenylbutazone from oxidation.

Both $Na_2S_2O_5$ (5mM) and disodium EDTA (10mM) were effective in reducing the degradative losses on storage of phenylbutazone in urine. No breakdown occurred after storing a solution of phenylbutazone in urine (1 mg/ml) at room temperature for 4 days, and these results are illustrated in Figure 3.5. The exact mechanism of action of the two additives is unclear, but since $Na_2S_2O_5$ is usually classified as an antioxidant and disodium EDTA a chelating agent, it seems likely that they act by different mechanisms.

FIGURE 3.4 **Radiochromatograms demonstrating the effect of citric acid pre-treatment, on preventing the phenylbutazone decomposition on TLC plates**

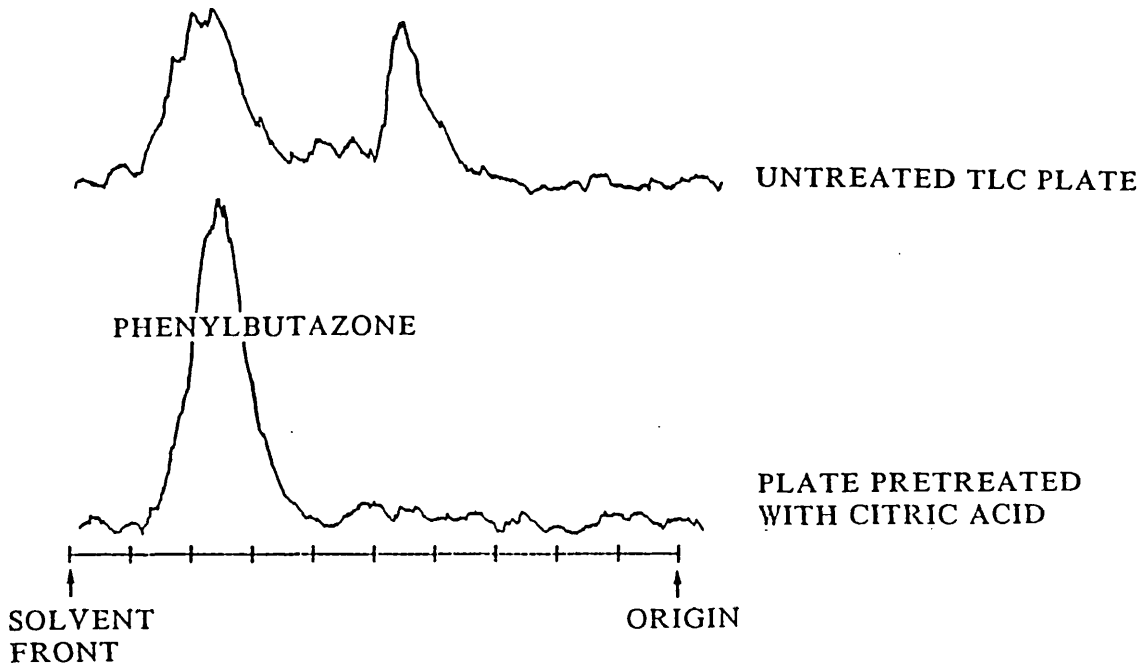


FIGURE 3.5 **Radiochromatograms of TLC plates demonstrating the effect of the preservatives EDTA and sodium metabisulfite on preventing phenylbutazone decomposition in urine**

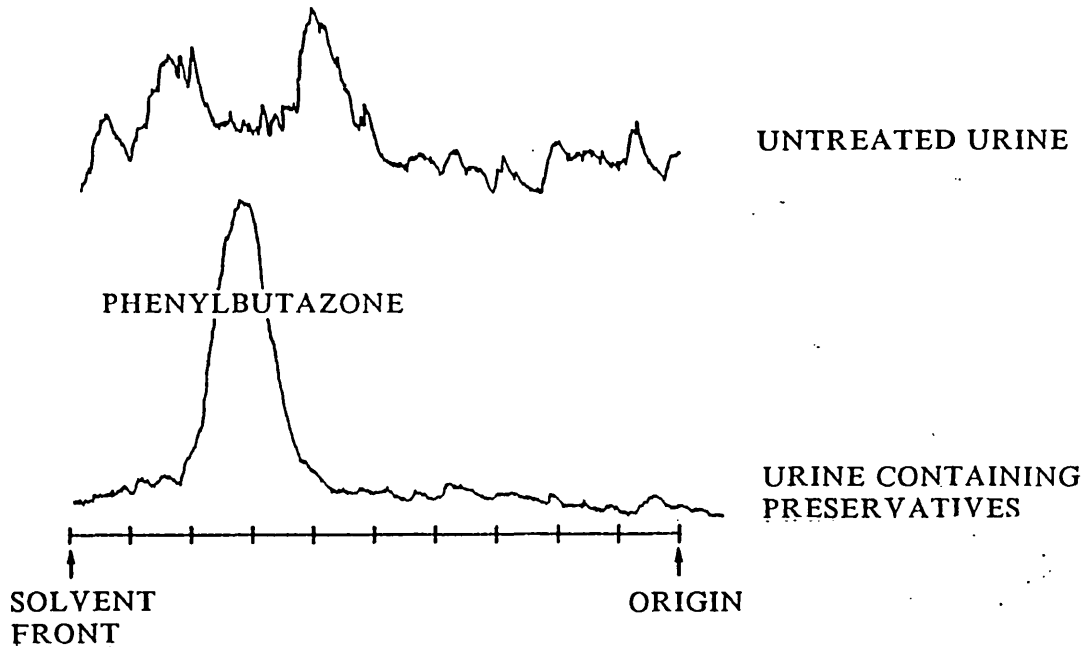
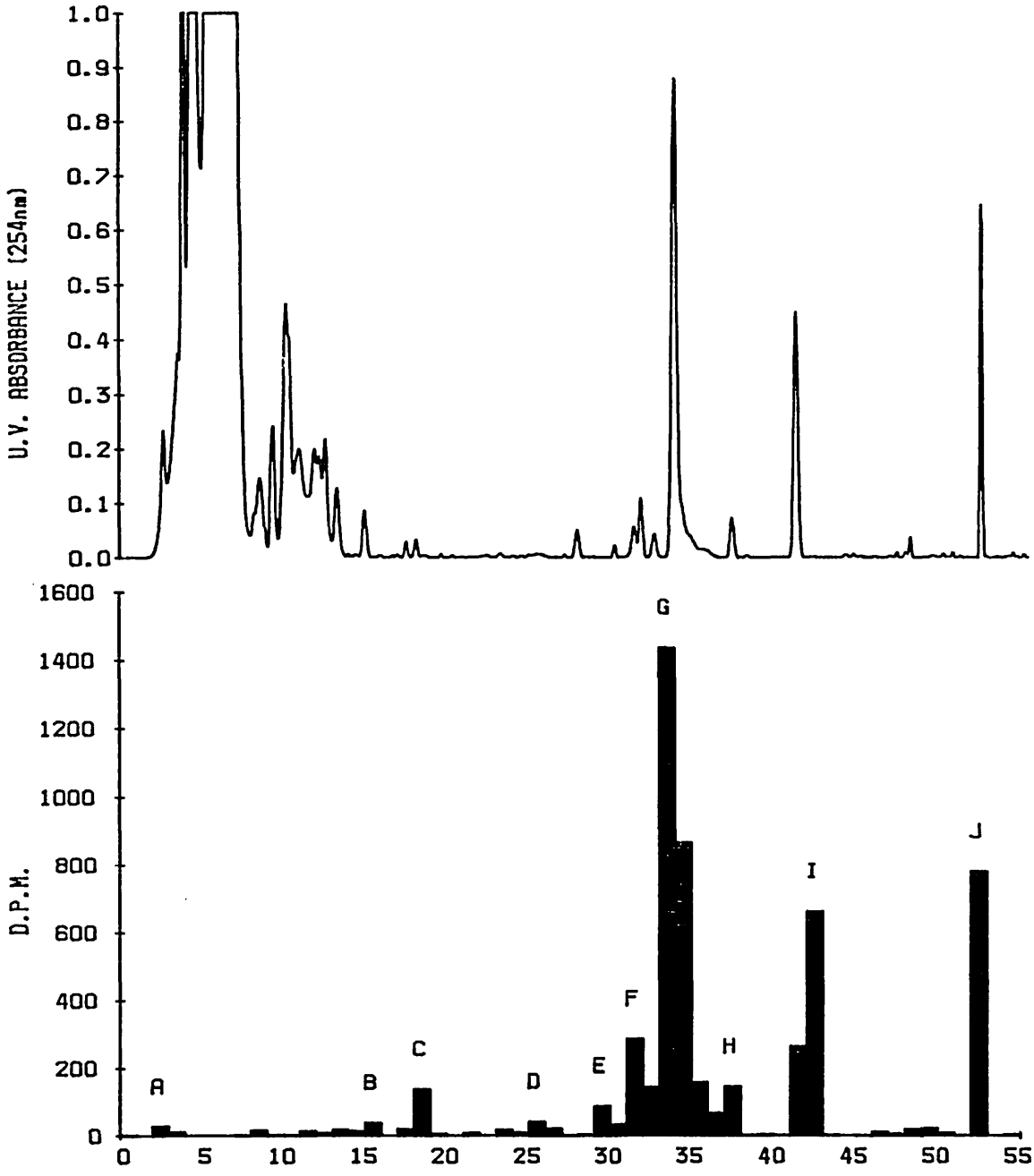


FIGURE 3.6 HPLC Radiochromatogram of 200 *u*l urine (0-12h) from a horse given ^{14}C-phenylbutazone



The identities of A, B, D, & H are unknown. C = *p*,γ-Dihydroxyphenylbutazone, E = ω-hydroxyphenylbutazone, F = γ-ketophenylbutazone, G = γ-hydroxyphenylbutazone, I = *p*-hydroxyphenylbutazone, J = phenylbutazone.

Derivatisation patterns

A full description of the sites and pattern of derivatisation, and their effect on the mass-spectral fragmentation processes is given in Chapter 7. Briefly, treatment with methyl iodide resulted in the formation of the C_4 methyl derivatives, the phenolic group present in compounds such as oxyphenbutazone was also methylated. Hydroxyl or keto functions in the butyl side chain were resistant to methylation, but successfully formed trimethylsilyl derivatives. A two stage derivatisation procedure (methylation followed by silylation) was adopted for compounds thought to contain an aliphatic hydroxyl group.

Chromatography of urinary metabolites

Preliminary chromatographic analysis of urine by TLC, and examination of the plates under UV light revealed the presence of many compounds, most of which were also present in drug-free urine. Three of the bands were tentatively identified by comparison of R_f values, as phenylbutazone, *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone. Confirmation of the identity of two of these, phenylbutazone and γ-hydroxyphenylbutazone, was obtained by GCMS. Because of the low levels of radioactivity, the remaining radiochromatographic analyses were carried out by HPLC.

A typical HPLC radiochromatogram is illustrated in Figure 3.6. The radiochromatogram was somewhat arbitrarily divided into 10 zones of radioactivity. Treatment of the urine with ß-glucuronidase and sulfatase, did not significantly alter this metabolic profile, although the interpretation was complicated by the presence of phenylbutazone degradation products, following the enzyme incubations. There was no evidence for the formation of 4-hydroxyphenylbutazone or any of the other known degradation products of phenylbutazone in the pre-incubation samples.

By comparison of retention times with authentic reference standards, 6 of the 10 radioactive zones were tentatively identified. These metabolites accounted for 85% of the total urinary radioactivity. Much of the unidentified urinary radioactivity was not associated with any discrete zone, and of the remaining 15%, no individual zone represented more than 2% of the total urinary radioactivity.

Confirmatory identification of urinary metabolites

Several techniques were used to separate and identify the urinary metabolites of phenylbutazone, and a summary of the criteria used for identifying each individual metabolite is included in Table 3.4. The proposed metabolic pathways of phenylbutazone in the horse are given in Fig. 3.13.

TABLE 3.4 Criteria used for the identification of urinary metabolites of phenylbutazone

Compound.	TLC	HPLC	GC	GCMS
Phenylbutazone	+	+	+	+[1]
p-Hydroxyphenylbutazone	+	+	+	+
γ-Hydroxyphenylbutazone	+	+	+	+[1]
ω-Hydroxyphenylbutazone		+	+	+
γ-Ketophenylbutazone		+		+
p,γ-Dihydroxyphenylbutazone		+		+

1. Samples obtained for confirmatory GCMS by TLC in addition to HPLC.

The identities of the urinary metabolites were assigned as follows :-

Phenylbutazone :- The compound tentatively identified as phenylbutazone had essentially identical chromatographic properties to an authentic standard in the following systems, TLC R_f 0.48, HPLC R_t 51.1 min, GC R_t 8.18 min, GCMS R_t 6.58 min. The mass spectrum obtained from this compound contained prominent ions at *m/z* 308 (M^+), 252 (M^+-C_4H_8), 183 ($C_6H_5N_2HC_6H_5$), 77 (C_6H_5), and was essentially identical to that obtained from an authentic phenylbutazone standard. The spectrum obtained from material isolated from urine is reproduced in Fig. 3.7 and that of authentic phenylbutazone in Fig. 7.2.

p-*Hydroxyphenylbutazone* :- The compound tentatively identified as *p*-hydroxyphenylbutazone had essentially identical chromatographic properties to an authentic standard in the following systems, TLC R_f 0.20, HPLC R_t 41.3 min, and as the dimethyl derivative GC R_t 9.09 min, GCMS R_t 8.41. The mass spectrum obtained from the dimethyl derivative of this compound contained prominent ions at *m/z* 352 (M^+), 296 (M^+-C_4H_8), 213 ($C_6H_5N_2HC_6H_4OCH_3$), 107 ($C_6H_4OCH_3$), 77 (C_6H_5) and was essentially identical to that obtained from an authentic oxyphenbutazone standard. The spectrum of the compound isolated from urine is reproduced in Fig. 3.8 and that of an authentic standard in Fig. 7.6.

γ-Hydroxyphenylbutazone :- The compound tentatively identified as γ-hydroxyphenylbutazone had essentially identical chromatographic properties to an authentic standard in the following systems, TLC R_f 0.23, HPLC R_t 36.0 min, and as the Me-TMS derivative GC R_t 9.30 min, GCMS R_t 7.56 min. The mass spectrum obtained from the Me-TMS derivative of this compound (reproduced in Fig. 3.9) contained prominent ions at *m/z* 410 (M^+), 395 (M^+-CH_3), 197, 183 ($C_6H_5N_2HC_6H_5$), 77 (C_6H_5), and was essentially identical to that obtained from

an authentic γ-hydroxyphenylbutazone standard (Fig. 7.10).

ω-Hydroxyphenylbutazone :- The mass-spectrum obtained from the Me-TMS derivative of the metabolite isolated from urine (HPLC R_t 33.5 min) is reproduced in Fig. 3.10. The spectrum contained prominent ions at *m/z* 410 (M^+), 395 (M^+-CH_3), 266 (M^+-C_4H_7OTMS), 227, 183 ($C_6H_5N_2HC_6H_5$), 77 (C_6H_5). Comparison of this spectrum with those of the Me-TMS derivatives of authentic standards of γ-hydroxyphenylbutazone and β-hydroxyphenylbutazone established the presence of a silylated hydroxyl group in the butyl side chain.. There were many similarities between the spectra of this metabolite and those of the other two aliphatic hydroxyl derivatives, but it was possible to distinguish between these compounds by the characteristic fragmentation patterns of their aliphatic side chains, and on this basis the compound was tentatively identified as ω-hydroxyphenylbutazone. At this stage a sample of authentic ω-hydroxyphenylbutazone was obtained. This compound had a similar HPLC R_t 33.5 min, and as the Me-TMS derivative had an essentially identical GC R_t 10.1 min, and mass spectrum as the metabolite. The mass spectrum of the authentic standard is reproduced in Fig. 7.11.

γ-Ketophenylbutazone :- The identity of the compound γ-ketophenylbutazone HPLC R_t 34.6 min, was confirmed by comparison of the mass spectrum obtained from material isolated from urine, with that of an authentic standard. The mass spectra of the methylated urine extract is reproduced in Fig. 3.11, and contained prominent ions at *m/z* 336 (M^+),266 (M^+-C_4H_6O), 183 ($C_6H_5N_2HC_6H_5$) 77 (C_6H_5), and was essentially identical to that of the authentic reference compound reproduced in Fig. 7.13.

p,γ-Dihydroxyphenylbutazone :- The compound with HPLC R_t 19.4 min was tentatively identified as *p*- γ -dihydroxyphenylbutazone, confirmation was

provided by GCMS. Material isolated by HPLC was derivatised (methylation followed by silylation), and subjected to GCMS. The spectra obtained from this compound did not contain the expected molecular ion at *m/z* 440 (340 + 2 methyl groups + 1 TMS group), but instead the molecular ion was at *m/z* 498, i.e. 58 a.m.u. higher than expected. This can be rationalised if one of the methyl groups (M.W. 15) was replaced with a TMS group (M.W. 73), later experiments with *p*-hydroxyphenylbutazone (Chapter 7.) confirmed that methylation of the phenol group does not always occur. The mass spectrum obtained is illustrated in Fig. 3.12, and contains prominent ions at *m/z*: 498 (M^+), 483 (M^+-CH_3), 271 ($C_6H_5N_2HC_6H_4OTMS$), 197, which is consistent with a monomethyl-disilyl derivative of *p*,γ-dihydroxyphenylbutazone.

FIGURE 3.7 The mass spectrum of material isolated from urine by TLC and identified as phenylbutazone

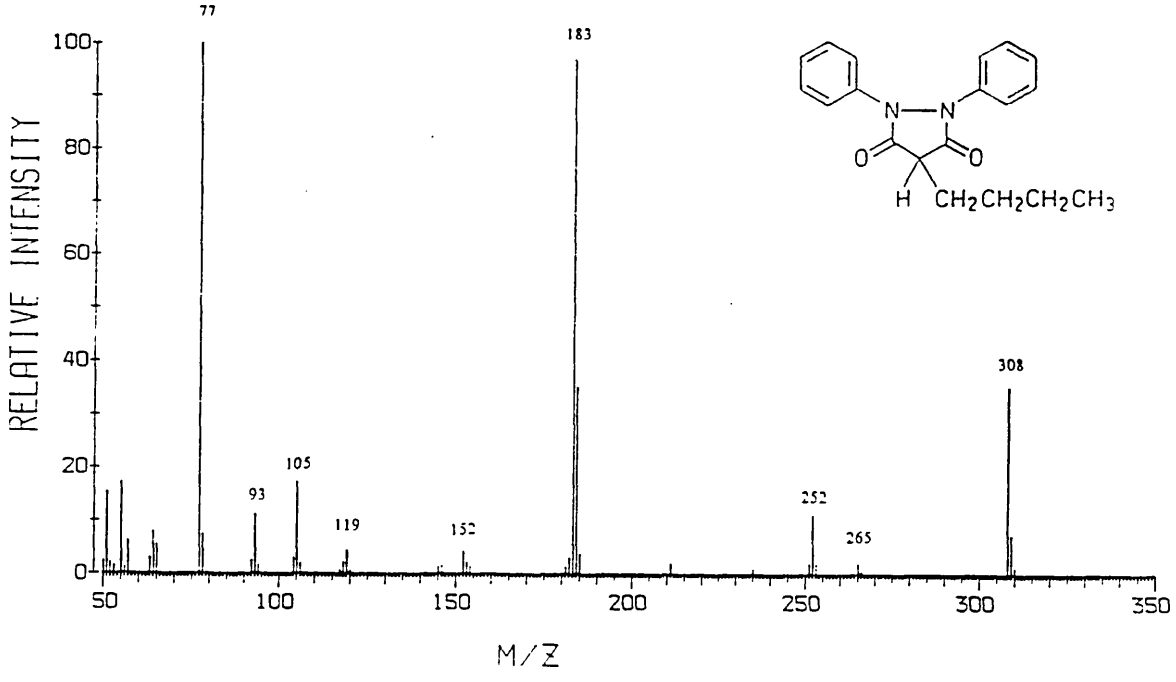


FIGURE 3.8 **The mass spectrum of material isolated from urine by HPLC and identified as the Me derivative of *p*-hydroxyphenylbutazone**

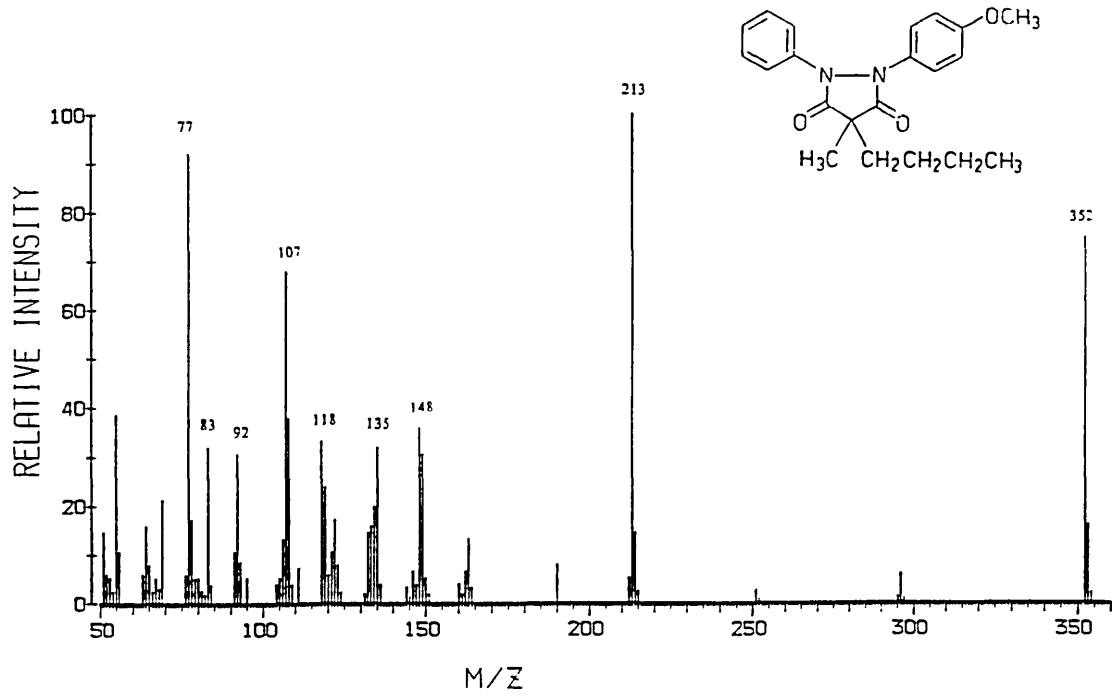


FIGURE 3.9 **The mass spectrum of material isolated from urine by HPLC and identified as the Me-TMS derivative of γ-hydroxyphenylbutazone**

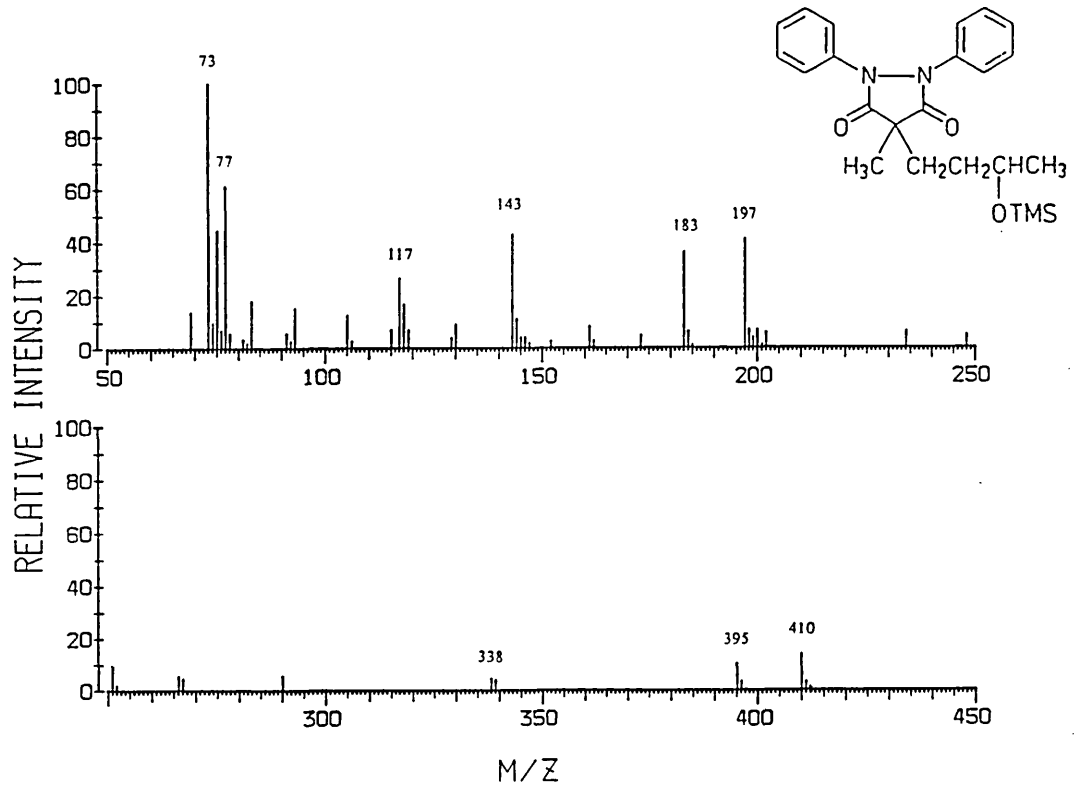


FIGURE 3.10 **The mass spectrum of material isolated from urine by HPLC and identified as the Me-TMS derivative of ω-hydroxyphenylbutazone**

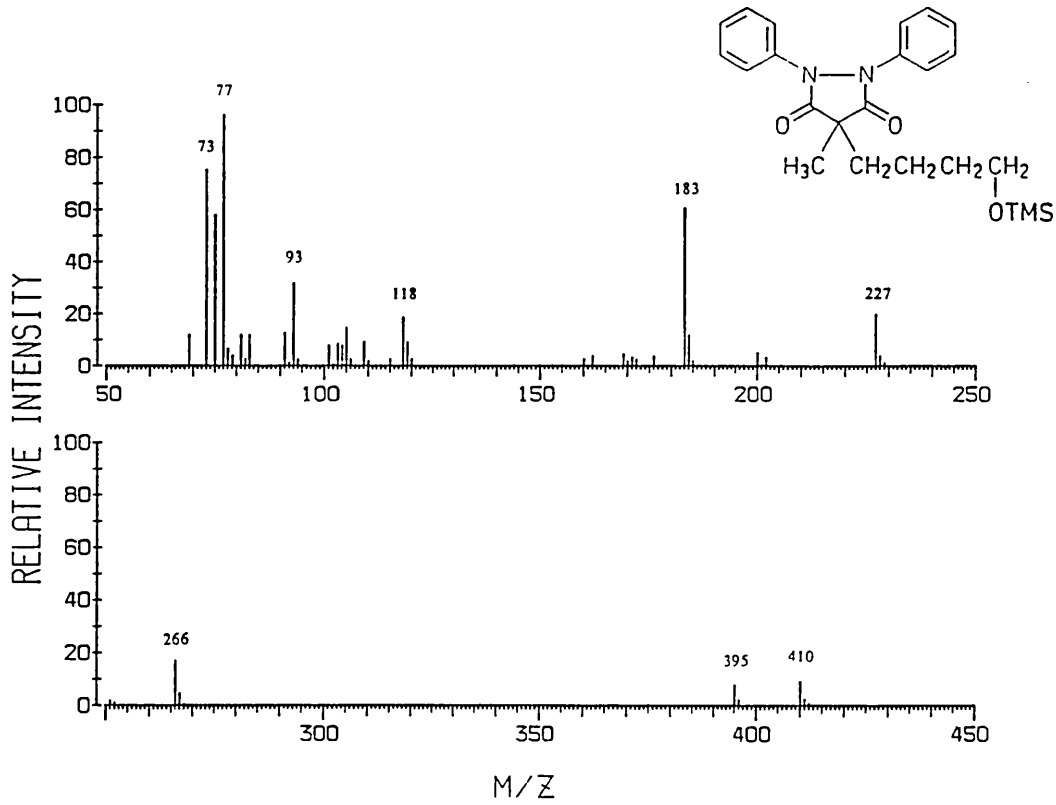


FIGURE 3.11 **The mass spectrum of material isolated from urine by HPLC and identified as the Me derivative of γ-ketophenylbutazone**

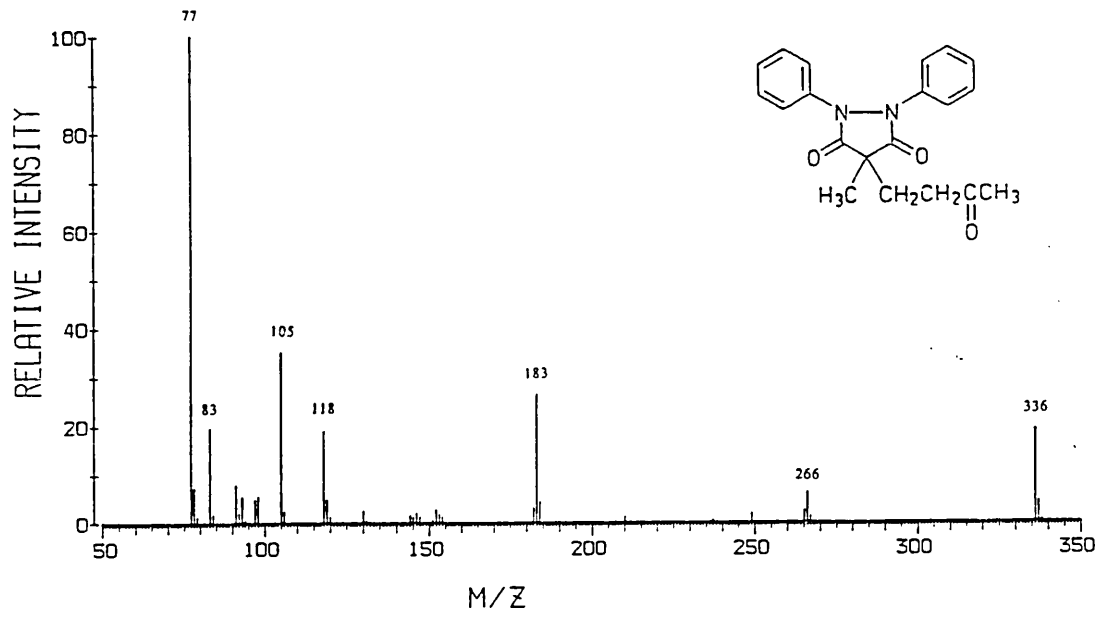


FIGURE 3.12 **The mass spectrum of material isolated from urine by HPLC and identified as the mono-methyl disilyl derivative of *p*,γ-dihydroxyphenylbutazone**

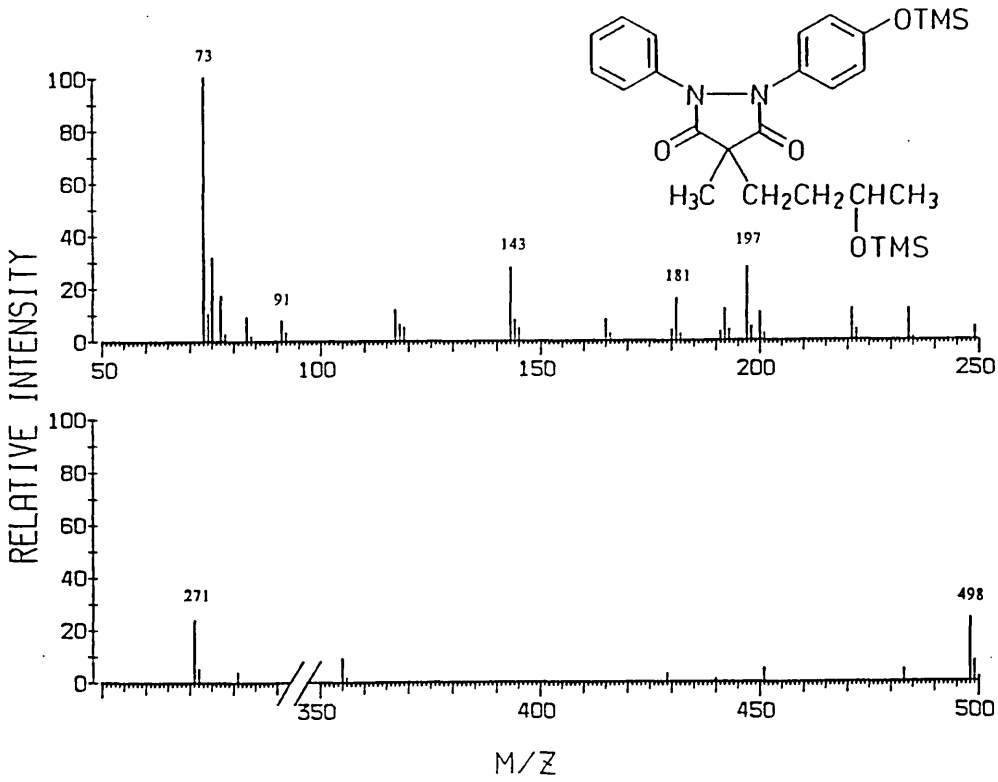
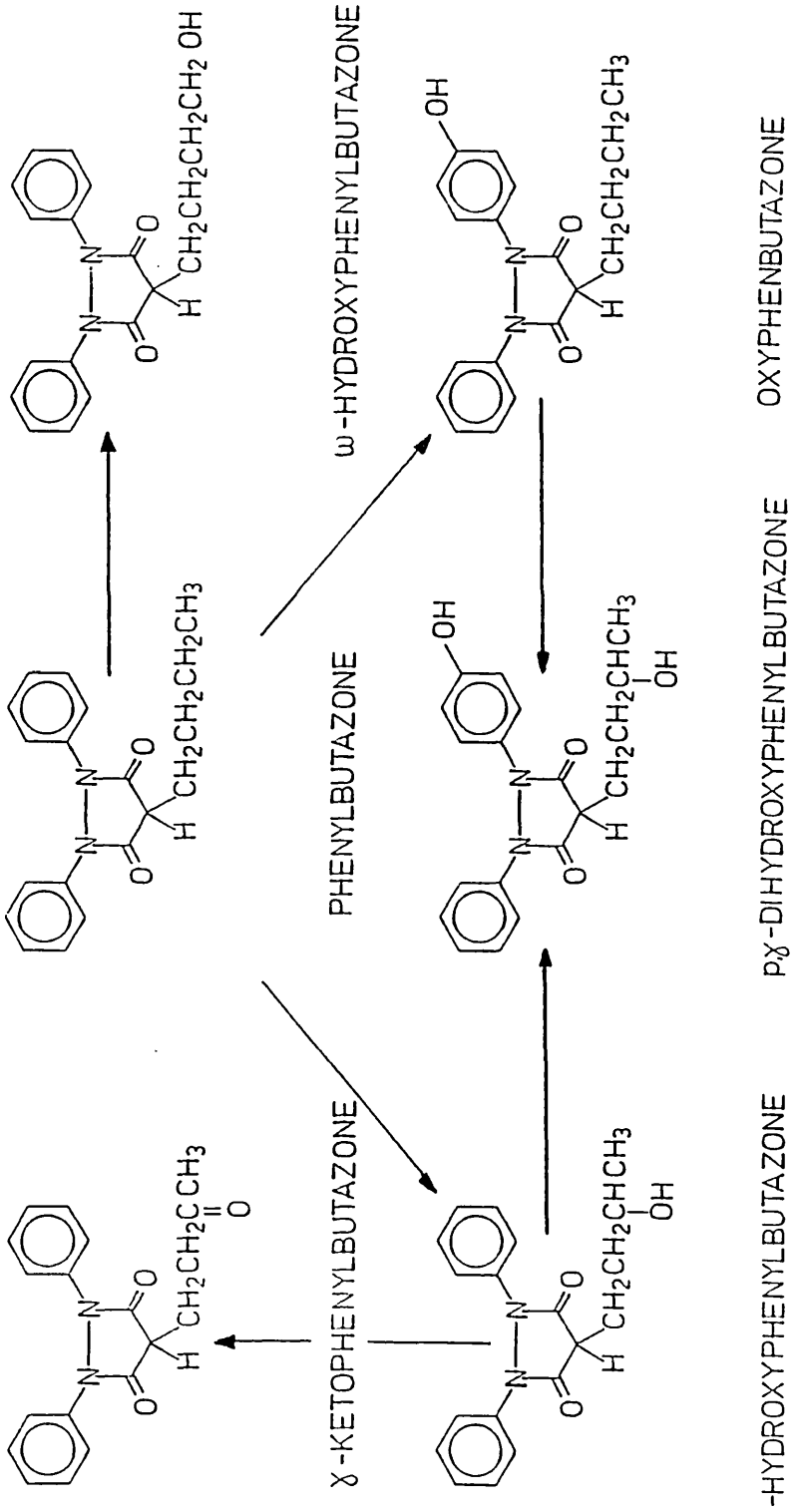


FIGURE 3.13 Proposed metabolic pathways of phenylbutazone in the horse



Quantitative aspects of the urinary excretion of phenylbutazone and its metabolites

The pattern of metabolism was independent of the route of administration, as illustrated in Tables 3.5 and 3.6. Because of the lost urine samples, the results from experiment No. 5 are recorded separately in Table 3.7. Quantitatively the most important metabolites are *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone, which account for 17.9% ± 2.0% and 16.5% ± 2.1% of the dose respectively (mean of experiments 1-4 ± s.d.). Unchanged phenylbutazone accounts for 5.3% ± 1.4% of the dose, and together with the minor metabolites, *p*,γ-dihydroxyphenylbutazone (2.1% ± 0.6%), ω-hydroxyphenylbutazone (3.1% ± 0.6%), and γ-ketophenylbutazone (1.7% ± 1.0%), a total of 46.6% ± 2.2 % of the dose or 85% of the urinary radioactivity has been accounted for.

Whilst there were slight variations between experiments in the relative proportions of the minor metabolites excreted, which was independent of the horse, there was a quantitative difference between the two horses in the excretion of the major urinary metabolites. Horse No. 1 (experiments 1 & 2) excreted 4.0% ± 0.1% of the dose as unchanged phenylbutazone, 19.9% ± 0.2% as *p*-hydroxyphenylbutazone, and 14.3% ± 0.1% as γ-hydroxyphenylbutazone, whilst Horse No. 2 (experiments 3 & 4) excreted 6.6% ± 0.7% of the dose as phenylbutazone 15.9% ± 0.3% as *p*-hydroxyphenylbutazone and 18.6 ± 0.4% as γ-hydroxyphenylbutazone.

TABLE 3.5 **The quantitative pattern of urinary metabolites following the oral administration of phenylbutazone**

Compound	Experiment No. 1		Experiment No. 3	
	% Dose	% Urinary ^{14}C	% Dose	% Urinary ^{14}C
p,γ-Dihydroxyphenylbutazone	2.83	5.20	1.88	3.29
ω-Hydroxyphenylbutazone	2.19	4.03	3.30	5.77
γ-Ketophenylbutazone	0.69	1.27	2.39	4.18
γ-Hydroxyphenylbutazone	14.43	26.53	18.99	33.21
p-Hydroxyphenylbutazone	19.74	36.28	16.25	28.42
Phenylbutazone	4.11	7.55	7.22	12.62
Total	43.98	80.85	50.03	87.49

TABLE 3.6 **The quantitative pattern of urinary metabolites following the intravenous administration of phenylbutazone**

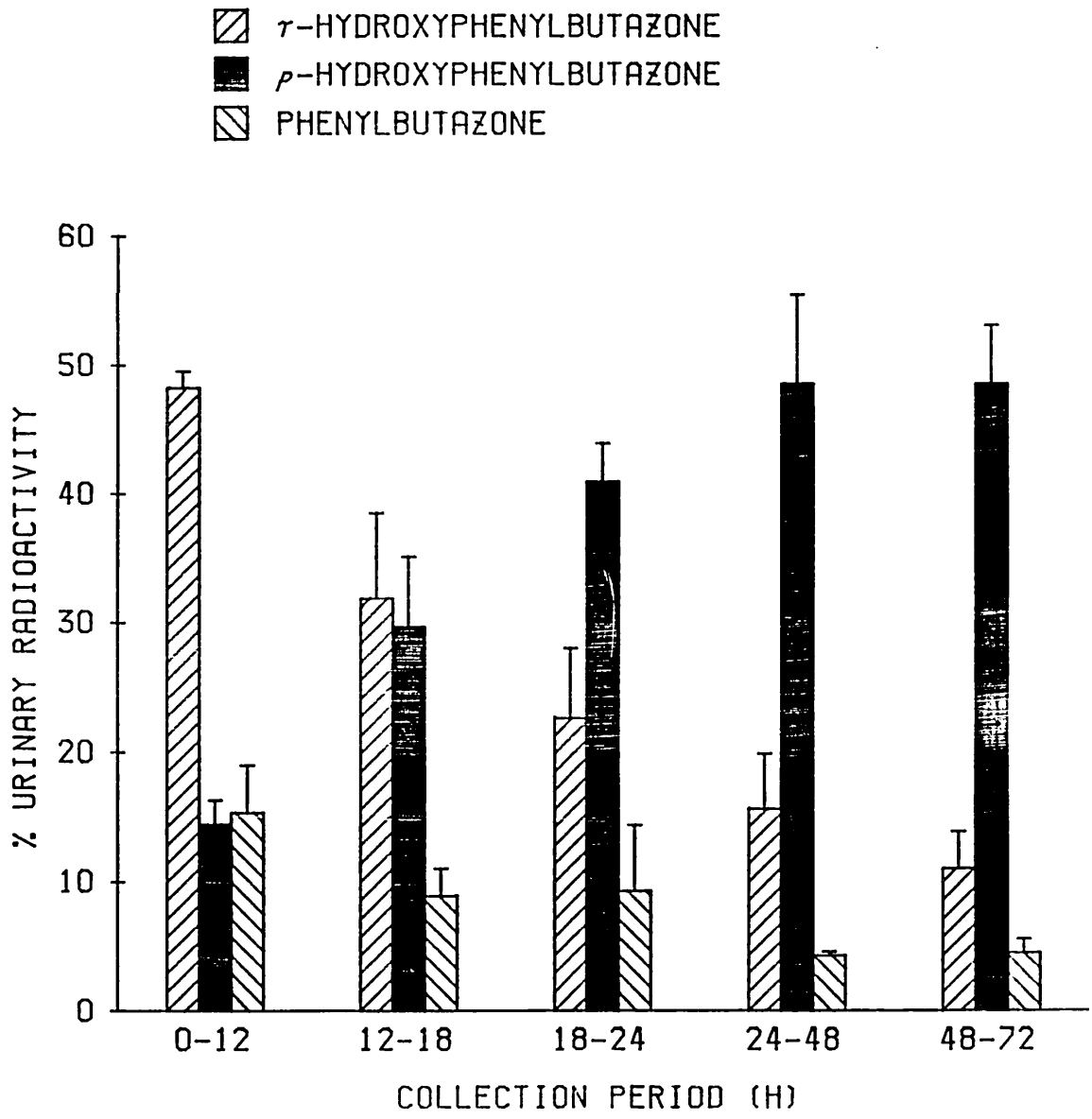
Compound	Experiment No. 2		Experiment No. 4	
	% Dose	% Urinary ^{14}C	% Dose	% Urinary ^{14}C
p,γ-Dihydroxyphenylbutazone	1.16	2.16	2.42	4.49
ω-Hydroxyphenylbutazone	3.35	6.25	3.65	6.77
γ-Ketophenylbutazone	3.13	5.84	0.76	1.41
γ-Hydroxyphenylbutazone	14.26	26.61	18.16	33.67
p-Hydroxyphenylbutazone	20.09	37.49	15.60	28.92
Phenylbutazone	3.89	7.26	5.90	10.94
Total	45.88	85.61	46.49	86.19

TABLE 3.7 The quantitative pattern of urinary metabolites following the oral administration of phenylbutazone

Compound	Experiment No. 5	
	% Dose	% Urinary ^{14}C
p,γ-Dihydroxyphenylbutazone	1.95	4.57
ω-Hydroxyphenylbutazone	2.38	5.58
γ-Ketophenylbutazone	1.03	2.41
γ-Hydroxyphenylbutazone	13.56	31.79
p-Hydroxyphenylbutazone	13.81	32.37
Phenylbutazone	2.40	5.63
Total	35.13	82.35

Analysis of the various pooled urine samples revealed a time dependent change in the pattern of the major urinary metabolites, and this is illustrated in Fig. 3.14. In the 0-12 h urine γ-hydroxyphenylbutazone and phenylbutazone were quantitatively the most important excretion products, and accounted for 48% and 15% of the total urinary radioactivity respectively. In the later urine samples these compounds were of decreasing importance in the overall elimination of phenylbutazone, and in the 48-72 h urine were responsible for only 11% and 4.5% of the total urinary radioactivity. Over the same time period *p*-hydroxyphenylbutazone accounted for an increasing proportion of the urinary radioactivity, from 14% in the 0-12 h urine to 49% in the 48-72 h urine.

FIGURE 3.14 **Time dependent alteration in the urinary metabolite profile following the administration of ^{14}C-phenylbutazone. Results expressed as a percentage of urinary radioactivity, mean ± s.d. (experiments Nos. 1-4)**



DISCUSSION

As might be expected for a lipophilic compound, the excretion rate of unchanged phenylbutazone is low, and the principal factor in determining its overall elimination is the rate of biotransformation. Phenylbutazone undergoes both aliphatic and aromatic carbon centre oxidations, and a summary of the metabolic pathways characterised for phenylbutazone in the horse is given in Fig. 3.13. The urinary metabolites identified confirm the importance of oxidative reactions in the metabolism of phenylbutazone in the horse, as suggested by earlier studies (Schubert *et al.*, 1967; Maylin, 1974).

Characteristically the metabolism of xenobiotics is a biphasic process, involving a plethora of reactions, which may be considered to be divided into two distinct types (Williams, 1959). Phase I reactions (oxidations, reductions, hydrolyses) introduce different functional groups into the molecule, and usually result in relatively minor changes in the structure of the compound. Phase II reactions are biosynthetic, whereby the exogenous compound is linked to an endogenous moiety. Formation of a conjugate results in large alterations in molecular structure, and these are almost invariably accompanied by a reduction in biological activity, and an increase in water solubility. If the parent compound possesses the necessary functional groups, then it may itself undergo Phase II biotransformations, although in most cases the ultimate excretion products are conjugates of Phase I metabolites.

At first glance one would not expect phenylbutazone to form conjugates directly, since it lacks the requisite functional groups (OH, COOH, NH_2, SH, etc.). However phenylbutazone and related pyrazolidinediones are unique in that they undergo a conjugation reaction in which the C_1' of glucuronic acid is directly attached to the C_4 of the heterocyclic ring. The formation of

C-glucuronides appears to be species dependent, and so far has only been demonstrated for humans and some primates; in all other species studied, the metabolism of phenylbutazone is dominated by Phase I reactions (Burns *et al.*, 1965; Bakke *et al.*, 1974; Faigle & Dieterle, 1977).

In terms of the known Phase I reactions there are a number of options available to phenylbutazone. These include oxidation in either the aliphatic side chain, or the aromatic rings. As mentioned earlier the pyrazolidinedione ring is known to undergo various non-enzymic oxidations (Awang *et al.*, 1973) and both the hydrolytic cleavage of the amide bonds in the pyrazolidinedione ring, and oxidation at the C_4 position must be considered as possible metabolic transformations.

The absence of 4-hydroxyphenylbutazone, butylmalonic acid mono(N,N' -diphenylhydrazide), or any of the other known degradation products of phenylbutazone, indicates that the pyrazolidinedione ring, although susceptible to non-enyzmic oxidations, is resistant to metabolic oxidation. These findings are consistent with the results obtained by Faigle and Dieterle (1977) who, using a specific and sensitive isotope dilution assay, concluded that the *in vivo* hydrolytic cleavage of phenylbutazone does not take place to any significant degree in man. In the only other detailed study published on the metabolism of phenylbutazone (Bakke *et al.*, 1974), 4-hydroxyphenylbutazone and 4-hydroxy-*p*-hydroxyphenylbutazone were identified in urine extracts. But these workers were unable to determine whether or not they were metabolites, since despite special precautions, such as developing the TLC plates in a nitrogen atmosphere, the artefactual formation of these compounds could not be completely abolished.

The aliphatic hydroxylation occurred predominantly at the 3 position in the butyl side chain resulting in the formation of γ-hydroxyphenylbutazone, and

this was accompanied by much smaller amounts of ω-hydroxyphenylbutazone, the 4-hydroxyl derivative. In contrast to the disposition of phenylbutazone in man (McGilvery *et al.*, 1974), there was no evidence for aliphatic hydroxylation at the 2-position. The preference for hydroxylation at the methylene carbon atom adjacent to the terminal carbon atom, termed '(ω-1) hydroxylation' is a well recognised phenomenon in drug metabolism (Trager, 1980) , and the formation of γ-hydroxyphenylbutazone might well be predicted from examination of the structure of phenylbutazone. Similarly the formation of ω-hydroxyphenylbutazone, resulting from the ω-hydroxylation of phenylbutazone, might have been predicted, and it is interesting that this is the first time that this compound has been described as a metabolite of phenylbutazone in the horse or any other species.

The formation of γ-ketophenylbutazone was previously demonstrated as a minor pathway in man (McGilvery *et al.*, 1974), but this is the first time it has been reported as a metabolite of phenylbutazone in the horse. γ-Ketophenylbutazone possesses anti-inflammatory actions, and under the name *Kebuzone* has itself been used as an antirheumatic agent (The Merck Index, 1983).

There are several metabolic pathways by which ketones can be formed, but the usual mechanism involves the alcohol dehydrogenase catalysed oxidation of a secondary alcohol. This is known to occur with barbiturates (Bush *et al.*, 1967), and the oxidation of γ-hydroxyphenylbutazone is the likely mechanism by which γ-ketophenylbutazone is formed. This is usually a minor pathway in the fate of xenobiotic secondary alcohols, as in general they are excreted (free or as a conjugate), or undergo further metabolism in another portion of the molecule. Consequently there are few examples in the literature of the *in vivo* conversion of a secondary alcohol to a ketone (McMahon, 1982).

The aromatic oxidation of phenylbutazone was apparently exclusively in the para position. Unfortunately an authentic sample of *o*-hydroxyphenylbutazone was not available, although from inspection of the mass spectra of both *m*-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone it would have been possible to identify *o*-hydroxyphenylbutazone had it been present in any of the samples submitted for GCMS. This regioselectivity of aromatic hydroxylation is not unexpected, although para hydroxylation may be accompanied by small amounts of the ortho hydroxylated product, and in addition there is evidence for a subgroup of P-450's which are product regioselective for meta-hydroxylation (Trager, 1980).

The identity of *p*,γ-dihydroxyphenylbutazone was established by GCMS, and co-chromatography, it had previously been positively identified as a major metabolite in the rat and as a minor metabolite in man, but this is the first time it has been reported as a metabolite of phenylbutazone in the horse. The HPLC system used was unable to adequately resolve *p*,γ-dihydroxyphenylbutazone and *p*-hydroxy- γ-ketophenylbutazone, consequently the figures quoted for the recovery of the dihydroxylated metabolite may include small amounts of the keto compound, although it is worth noting that there was no evidence from the GCMS results of the presence of this compound in any of the samples analysed.

From the experiments performed in this study, it is not possible to determine the sequence of the two hydroxylations responsible for the formation of the dihydroxylated metabolite, although it is worth noting that in the rat this compound is formed following the administration of both γ-hydroxyphenyl-butazone and *p*-hydroxyphenylbutazone, suggesting that either metabolite is capable of undergoing further hydroxylation.

The quantitative pattern of metabolites in urine revealed a slight preference for

aromatic over aliphatic hydroxylation, but since urinary excretion represented just over half of the total excretion of this compound, it is not possible to draw too many conclusions about the relative importance of these two pathways in the overall metabolism of phenylbutazone. The situation in the rat is slightly different; γ-hydroxyphenylbutazone was present in the urine in much larger amounts than oxyphenbutazone, although to some extent this was offset by the predominance of oxyphenbutazone in the bile (Bakke *et al.*, 1974).

Several groups have attempted to explain the low recoveries obtained, following the administration of phenylbutazone to the horse, by postulating the formation of conjugates. Gerring *et al.* (1981) suggested that phenylbutazone metabolites undergo conjugation with glucuronic acid prior to renal elimination, other workers (Davis *et al.*, 1973) reported that the formation of the ethereal sulfate of oxyphenbutazone was quantitatively important in the urinary elimination of phenylbutazone by ponies. Tobin *et al.* (1986) hypothesised that either 4-hydroxyphenylbutazone, or its glucuronide was a potential metabolite in the horse, but in support of this, misleadingly cited the finding of phenylbutazone-C-glucuronide in man (Dieterle *et al.*, 1976).

The results from the enzyme treatments of urine provide little evidence for the urinary elimination of either glucuronides or sulfates of phenylbutazone or its metabolites. For various reasons it is impossible to state categorically that conjugation does not take place, but since over 80% of the total urinary radioactivity could be accounted for as identified Phase I metabolites, if conjugates are produced, then quantitatively they are of little importance in the overall urinary elimination of phenylbutazone.

For the enzymic hydrolysis of glucuronides a positive control (phenolphthalein glucuronide) was available, but no such control was used for the sulfatase

treatment. Since the antioxidant $Na_2S_2O_5$ is known to inhibit the enzyme sulfatase at a concentration 10 times lower than that added as a preservative to the urine (Rose, 1986), the results from the sulfatase treatment are best treated with caution, although Perel *et al.* (1964) reported that the ethereal sulfate of *p*-hydroxyphenylbutazone was readily hydrolysed in aqueous solution. Similarly the presence of C-glucuronides cannot be completely excluded, as they are resistant to enzymic hydrolysis (Dieterle *et al.*, 1976), and the free compound would not be released.

The predominance of oxidative mechanisms in the formation of the urinary metabolites of phenylbutazone in the horse is essentially similar to the situation in the rat and the dog. In the rat oxyphenbutazone and p,γ-dihydroxy-phenylbutazone, which both contain phenolic hydroxyl groups, are conjugated with glucuronic acid to some extent; along with smaller amounts of γ-hydroxy phenylbutazone glucuronide and the very small amounts of sulfate conjugates, these compounds account for less than 5% of the total urinary radioactivity. In the dog oxyphenbutazone is excreted in part (18-28%) as a glucuronide conjugate (Perel *et al.*, 1964), although since oxyphenbutazone is only a minor urinary metabolite of phenylbutazone in the dog, and 70% of the urinary radioactivity has been identified as free compounds, conjugation is quantitatively unimportant in the metabolism of phenylbutazone in the dog.

The relative unimportance of conjugation reactions in the metabolism of phenylbutazone in these animal species is in stark contrast to the situation in man. Although phenylbutazone undergoes both aliphatic and aromatic hydroxylation in man, these metabolites are not excreted free to any appreciable extent, and C-glucuronidation dominates the metabolism of phenylbutazone in man.

In context of the therapeutic use of phenylbutazone in the horse, it is worth noting that most of the metabolites so far identified in the horse are pharmacologically active. Oxyphenbutazone is itself an established anti-inflammatory agent, γ-hydroxyphenylbutazone the other major urinary metabolite possesses uricosuric properties (Burns *et al.*, 1960) and, as previously mentioned, γ-ketophenylbutazone has been used as an antirheumatic agent. It is possible that rather than acting to terminate pharmacological activity, the metabolism of phenylbutazone may contribute to the overall pharmacological effect of a dose of phenylbutazone.

The time dependent change in the pattern of urinary metabolites is of some interest, both from a purely mechanistic viewpoint, and because of the possible implications in dope testing. The alteration with time in the urinary metabolic profile, may enable one to determine the time of drug administration, from a single urine sample; if so, then this will be of definite value in regulating controlled medication programmes, with particular importance for the enforcement of 'time rules'. The urinary kinetics of phenylbutazone and metabolites are investigated in Chapter 6.

CHAPTER 4.

THE SYNTHESIS OF ^{14}C-OXYPHENBUTAZONE

INTRODUCTION

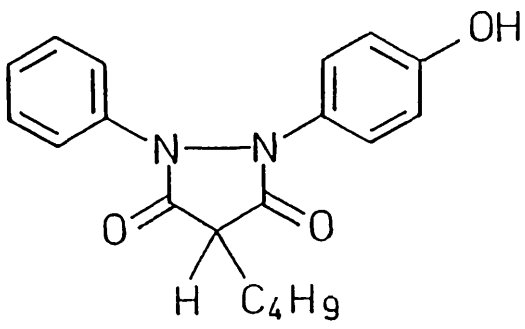
Once the metabolic disposition of phenylbutazone in the horse had been established, there were several reasons for investigating the disposition of the related compound oxyphenbutazone. Although much was made of the paucity of metabolic information on phenylbutazone, the situation with regard to oxyphenbutazone is far worse; to date there is no report in the literature of any detailed metabolic study on this compound in the horse.

Metabolism studies are most easily carried out with radiolabelled material, and the benefit of using these techniques was illustrated in the earlier experiments; in particular for the determination of the faecal elimination of drug and metabolites, following the administration of ^{14}C-phenylbutazone. Accordingly, a small amount (200 uCi) of radiolabelled oxyphenbutazone was required for investigations into the metabolic disposition of oxyphenbutazone in the horse, and since this compound was not commercially available it was decided to synthesise it.

Although it would have been relatively straightforward to prepare ^{3}H labelled oxyphenbutazone, ^{14}C was chosen in preference, because of the known difficulties of conducting metabolic and pharmacokinetic studies with some tritiated compounds (Di Carlo, 1979). Having decided upon the label, the position of the labelling was then considered. The previous experiments on the disposition of phenylbutazone, were carried out with the compound labelled in either the phenyl rings or at the C_4 position in the pyrazolidinedione ring; the butyl side chain, the easiest position to label, was thought unsuitable because it is known to undergo extensive metabolism and was considered to be possibly metabolically labile. For the intended experiments, oxyphenbutazone with a minimum specific activity of 15 uCi/mmol was required, with a radiochemical

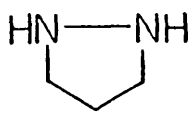
purity of >98% and a total activity of 200 μCi.

Because much of the literature on the synthesis of antipyrine, phenylbutazone and related compounds dates from the turn of the century, these compounds are usually described by the use of trivial and semi-trivial names, and both phenylbutazone and oxyphenbutazone are invariably named using non IUPAC nomenclature. The use of this non-systematic nomenclature may lead to misunderstanding, and it is worth reviewing some of the terms commonly used. Both phenylbutazone and oxyphenbutazone (I) can be considered as derivatives of pyrazolidine (II) (systematically named as tetrahydropyrazole), since they both contain an unsaturated five membered heterocyclic ring containing two adjacent nitrogen atoms. They are classified as pyrazolidiones, since at least one of the carbon atoms in the heterocyclic ring is part of a carbonyl group, and more specifically as pyrazolidinediones, as this indicates both the complete saturation of the heterocyclic ring, and the number of carbonyl functions. Phenylbutazone and oxyphenbutazone are often termed along with antipyrine as pyrazolones, although for the former compounds this is strictly incorrect, as the term pyrazolone (derived from pyrazoline) indicates one degree of unsaturation.



(I)

Oxyphenbutazone



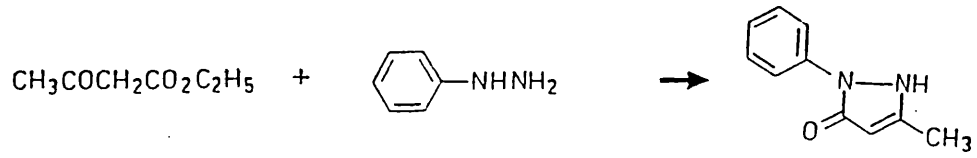
(II)

Pyrazolidine

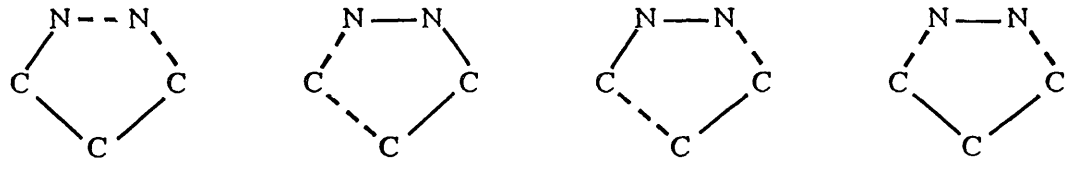
Synthesis of pyrazolones and pyrazolidinediones

The first synthesis of a pyrazolone was reported by Knorr in 1883, who prepared 3-methyl-1-phenyl-2-pyrazolin-5-one from ethylacetoacetate and phenylhydrazine (Eq.1). Subsequent methylation of this compound gave antipyrine, the first synthetic antipyretic and analgesic.

Eq. 1.



Following the successful production of antipyrine, several thousand related compounds have been synthesised (Greenberg & Haggard, 1950). The key to the synthesis of antipyrine, phenylbutazone, oxyphenbutazone and all other pyrazolones and pyrazolidinediones is the formation of the heterocyclic ring, and this can be approached from several different starting points. Khaletski and Moldaler (1963) classified the methods of synthesising the pyrazolidine nucleus, according to the order of bond formation, into four discrete classes.

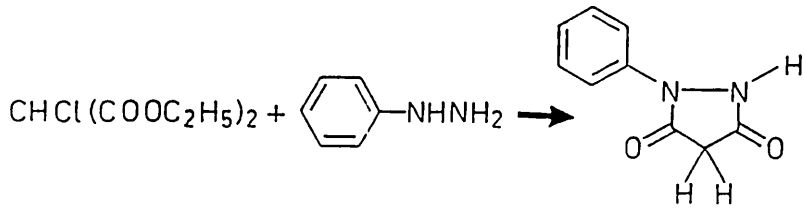


A B C D

Where the unbroken lines represent bonds present in the reactants, and the dotted lines indicate the bonds formed in the final synthesis.

There are examples in the literature for all of these, but for the synthesis of phenylbutazone and other 3,5-pyrazolidinediones, class 'D' is by far the most widely used, since the other methods usually require uncommon starting materials and/or several steps. The main type of reaction in class D, is the condensation of malonic acids (or esters, or acid chlorides), with a hydrazine derivative (Eq. 2.), and this was the method by which Burmeister and Michaelis (1891) synthesised the first 3,5-pyrazolidinedione (1-phenylpyrazolidine-3,5-dione). Since then many different pyrazolidinediones have been synthesised by this method. Ruhkopf (1940) obtained various compounds in this way and found that with increasing size and unsaturation of the substituents the tendency to ring formation decreases.

Eq. 2.



The original method of synthesising phenylbutazone (Geigy, 1948) was to attach the butyl side chain to preformed 1,2-diphenyl-3,5-pyrazolidinedione, but it was soon recognised that by using a butylmalonic acid derivative, for the synthesis of the heterocyclic nucleus, that this second step could be eliminated. The synthesis of oxyphenbutazone is achieved in a similar fashion, although the situation is complicated by the need to protect the phenolic hydroxyl group. Since the work by Geigy (1950) only minor improvements have been made to this basic method, the most notable of which was the discovery by Fucik and Koristek (1956) that sodium iodide catalysed the reaction.

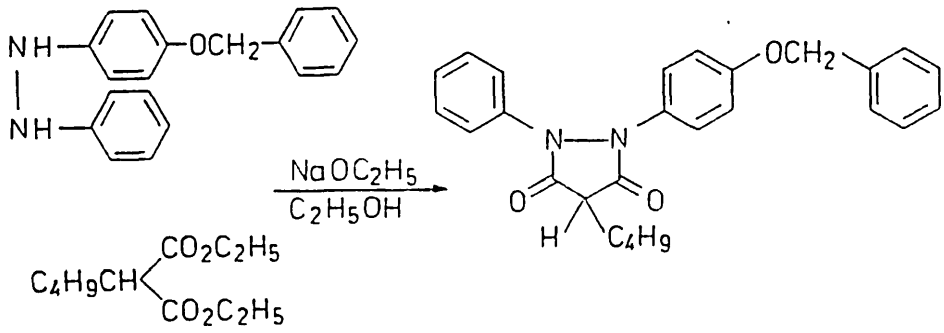
Choice of synthetic route

Comparison of the available synthetic routes to oxyphenbutazone showed that the method of Burmeister and Michaelis offered the easiest approach. All of the starting materials were either available, or comparatively easy to synthesise. The radiolabelled compound diethyl [2-^{14}C]-malonate was commercially available, allowing the synthesis of [4-^{14}C]-oxyphenbutazone.

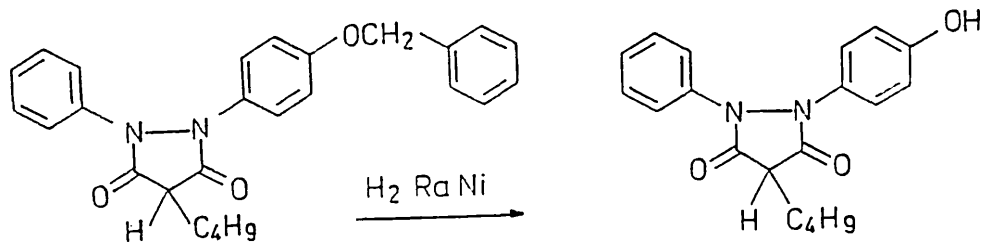
The reaction scheme is as follows :-

Eq. 3. $CH_2(CO_2C_2H_5)_2 + CH_3(CH_2)_3I \xrightarrow[C_2H_5OH]{NaOC_2H_5} CH_3(CH_2)_3CH(CO_2C_2H_5)_2$

Eq. 4.



Eq. 5.



The alkylation and condensation steps (Eq. 3. & Eq. 4.) have been carried out both as two completely separate processes, or else consecutively in the same reaction vessel, since the conditions required for both reactions are the same. Yeomans (1973) when investigating the synthesis of ^{14}C-phenylbutazone, obtained higher yields by performing these two steps concurrently, and it was decided to adopt this procedure for the synthesis of oxyphenbutazone.

EXPERIMENTAL

Compounds

Diethyl [2-^{14}C]-malonate sp. act. 58 *u*Ci/mg, radiochemical purity 97%, was purchased from Amersham International plc., (Amersham, U.K.). 1-Bromobutane, 1-iodobutane, benzyl chloride, *p*-hydroxyazobenzene, diethylmalonate, and *n*-butyldiethylmalonate were purchased from Aldrich Chemical Co Ltd., (Gillingham, U.K.), and used without further purification. Unlabelled oxyphenbutazone was obtained from Sigma Chemical Company Ltd., (Poole, U.K.). Raney nickel was prepared from aluminium-nickel powder by the standard method. A sample of *p*-benzyloxyhydrazobenzene was donated by Ciba-Geigy (Basle, Switzerland). All other chemicals used were of analytical grade, and purchased from usual U.K. commercial sources.

High performance liquid chromatography

The equipment used for the HPLC analysis was described in Chapter 3. The compounds were separated using a pre-packed C18 cartridge system (25 x 4 mm i.d. Hibar LiChroCART RP-18, Cat. No. 15539 BDH Ltd. Poole, U.K.) with UV detection at 254 nm. The column was eluted at ambient temperature with a degassed mobile phase of methanol-water (70:30 v/v) adjusted to pH 3.0 with glacial acetic acid, at a flow rate of 1.5 ml/min.

Radiochemical techniques

The ^{14}C content of solutions was determined by liquid scintillation spectrometry, as described earlier. Fractions of the HPLC eluent were collected into scintillation tubes using a LKB RediRac fraction collector, counted following the addition of scintillant, and radiochromatograms prepared by plotting the ^{14}C content against time.

Mass spectrometry

Direct insertion MS in the EI mode was performed at the Horseracing Forensic Laboratories, Newmarket, with a Finnigan 4000 instrument under the control of a Finnigan 6110 data system. The temperature of the probe was programmed to rise from 140° to 240° over 2 min, ionising voltage 70 eV.

Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectra were recorded at 250 MHz using a Bruker WM 250 spectrometer and ^{13}C-NMR spectra at 400 MHz with a Bruker WM 400 spectrometer (University of London Intercollegiate NMR Service, Kings College, London). All shifts are quoted (in ppm) relative to the internal standard TMS.

Melting point determination

Melting points were determined using an Electrothermal apparatus, in open glass capillary tubes. Uncorrected values are quoted.

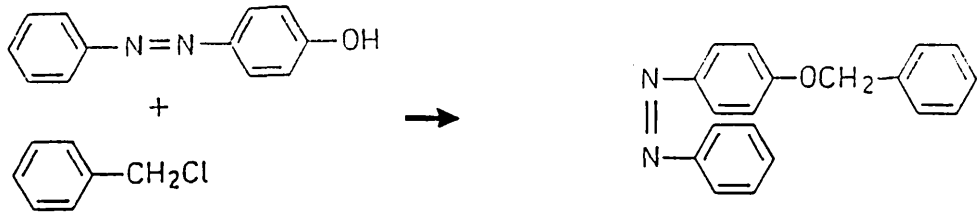
Infra red spectrometry

IR spectra were recorded as nujol mulls in KBr discs with a Perkin-Elmer grating spectrophotometer (Model 157G).

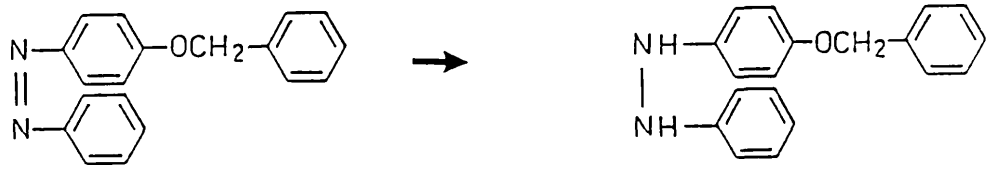
Synthesis of intermediates

A small amount (1g) of *p*-benzyloxyhydrazobenzene was available, but unfortunately the scale of the intended syntheses was such that further material was required. The method used to synthesise *p*-benzyloxyhydrazobenzene was essentially that of Pfister & Hafliger (1957). *p*-hydroxyazobenzene was benzylated with benzylchloride (Eq. 6.) and the resultant *p*-benzyloxy-azobenzene reduced with zinc and alkali (Eq. 7.).

Eq. 6.



Eq. 7.



Benzylation of p-*hydroxyazobenzene*

p-Hydroxyazobenzene (19.8g, 0.1mol) was added to a solution of sodium methoxide prepared from sodium metal (2.3g, 0.1mol) and dried methanol (50ml). The solution was warmed gently until the solid was completely dissolved, then benzylchloride (12ml, 0.12mol) added, and the mixture refluxed for 3 h. The solution was cooled in an icebath and the crystalline product (20.1g, 70% yield) removed by filtration, washed with water and ice-cold ethanol, and recrystallised from ethanol (m.p. 113-115°, lit. val. 114-116°).

Reduction of p-benzyloxyazobenzene

p-Benzyloxyazobenzene (14.4g, 0.05mol) was added to a solution of sodium hydroxide (6g) in ethoxyethanol (100ml) and water (1ml). The solution was maintained in a nitrogen atmosphere, heated to 95° and zinc powder (15g, 0.25mol) added over 3 h, after a further 3 h the solution became almost colourless. The hot solution was then filtered under nitrogen, the residue washed with hot ethoxyethanol, and the cooled filtrate added to 400ml of cold water. The resultant crystals (8.7g, 59% yield) were extracted by filtration under nitrogen, washed with water and recrystallised under nitrogen from ethanol. (m.p. 99-101°, lit. val. 100-103°). The mass spectrum obtained following the direct insertion of this compound is reproduced in Fig. 4.1 and

was consistent with *p*-benzyloxyhydrazobenzene: *m/z* (rel. intensity) 290 (M^+, 18), 288 (M^+-H_2, 30), 199 (M^+-$C_6H_5CH_2$, 38), 91 (100). *p*-Benzyloxyhydrazobenzene is easily oxidised and was stored under nitrogen until required.

Synthesis of p-*benzyloxyphenylbutazone*

p-Benzyloxyhydrazobenzene (1.45g, 5mmol) and *n*-butyldiethylmalonate (1.08g, 5mmol) were placed in a 25 ml flask together with a small amount (3mg) of sodium iodide. The flask was fitted with a reflux condenser, and the apparatus constructed as to maintain a nitrogen atmosphere. The reactants were heated to 70°, with continuous mixing using a magnetic stirrer. A solution of sodium ethoxide (5ml, 10mmol) was slowly added over 2 h, and the mixture was then left to reflux overnight. After the overnight reflux, the apparatus was converted for distillation under nitrogen, and the ethanol allowed to distill from the reaction mixture over a period of about 30 min. 10 ml of xylene was added and the mixture heated for 1 h until the remaining ethanol and xylene had completely evaporated. The solid residue was transferred using hot water (2 x 25ml) into a separating funnel, and extracted with chloroform (3 x 20ml) and diethyl ether (20ml). Acidification of the solution with conc. HCl to pH 1 gave a cream coloured precipitate, which was extracted into diethyl ether (3 x 25ml), and the extract evaporated to dryness under a stream of nitrogen. The crude product was recrystallised firstly from ethanol/water, then from hexane, and dried *in vacuo* to give a white solid (m.p. 125-128°, lit. val. 132-133°)

The purified product (1.4g, 68% yield) gave one peak R_t 6.50 min, when examined by HPLC, and the identity was confirmed as *p*-benzyloxyphenylbutazone by ^{1}H-NMR ($CdCl_3$) δ0.9 (t, 3H), 1.25-1.60 (m, 4H), 2.05-2.15 (m, 2H), 3.4 (t, 1H), 5.0 (s, 2H), 6.9 (m, 2H), 7.2-7.4 (m, 12H), and by direct insertion MS (Fig. 4.2); *m/z* (rel. intensity), 414 (M^+ ,100), 323 (M^+-$C_6H_5CH_2$, 86), 91 (92).

FIGURE 4.1 **Direct insertion mass spectrum of *p*-benzyloxyhydrazobenzene**

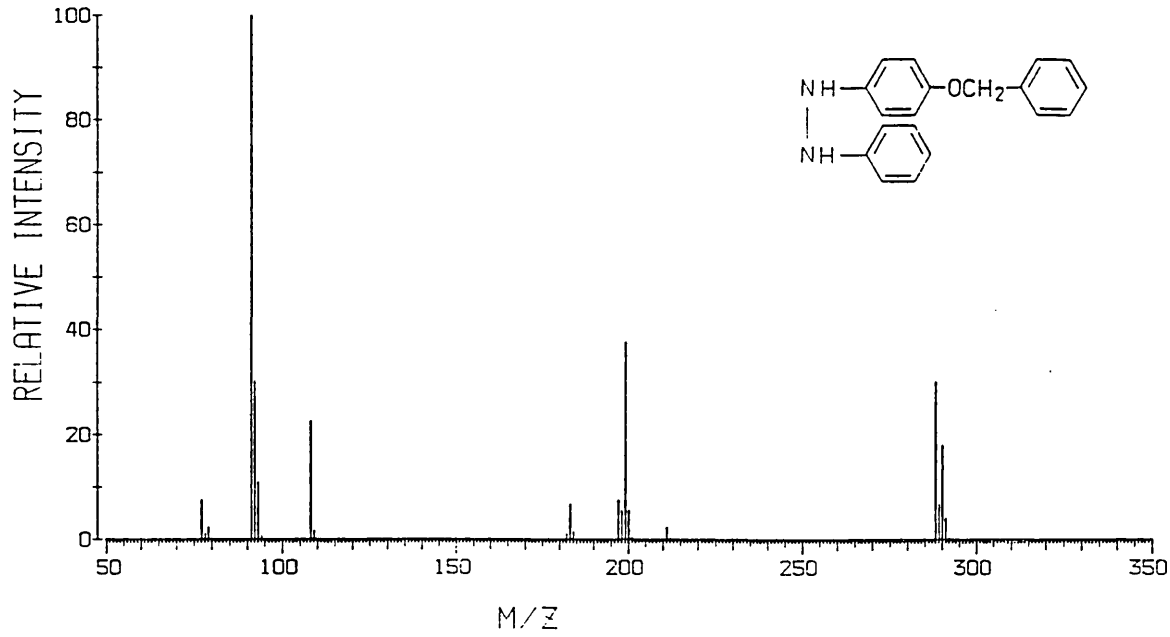
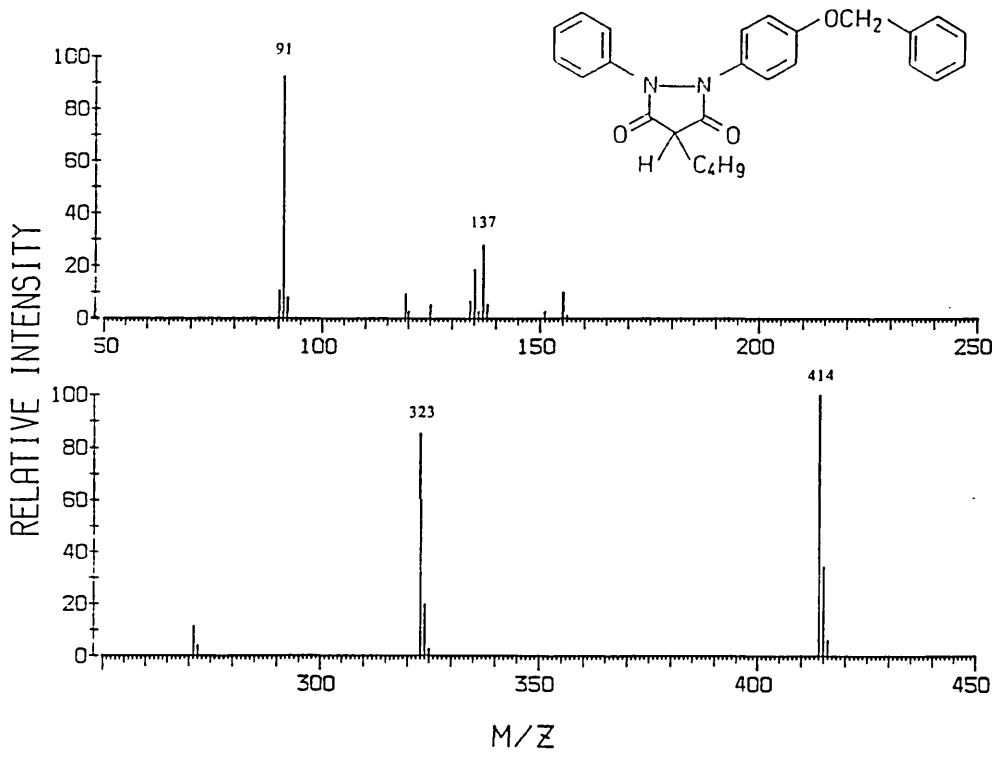


FIGURE 4.2 **Direct insertion mass spectrum of *p*-benzyloxyphenylbutazone**



Trial synthesis

Synthesis of p-*benzyloxyphenylbutazone*

Early attempts to synthesise *p*-benzyloxyphenylbutazone from *p*-benzyloxyhydrazobenzene, diethylmalonate, and 1-bromobutane, by carrying out the butylation and condensation steps consecutively in the same reaction vessel were unsuccessful. GC analysis of the products from the butylation step indicated that a mixture of diethylmalonate, butyldiethylmalonate and dibutyldiethylmalonate were obtained. By replacing 1-bromobutane with 1-iodobutane, and by adding the sodium ethoxide slowly the formation of the unwanted dibutyl product was eliminated, and *p*-benzyloxyphenylbutazone was obtained in yields of around 60%.

Hydrogenolysis of p-*benzyloxyphenylbutazone*

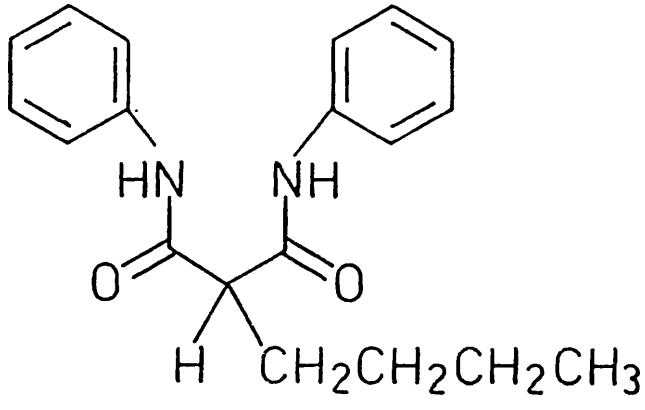
p-Benzyloxyphenylbutazone (1.04g, 2.5mmol) was dissolved in a solution of ethanol (20ml) and 1M NaOH soln. (10ml). A suspension of Raney nickel (approx. 1g) was added and the reactants stirred in a hydrogen atmosphere. Although it was possible to monitor the reaction by measuring the uptake of hydrogen, it was much easier to follow the reaction by HPLC. 100 *ul* portions of the reaction mixture were removed, neutralised with acetic acid, and subjected to HPLC; by this means it was possible to monitor both the disappearance of the starting material *p*-benzyloxyphenylbutazone, and the appearance of the hydrogenolysis product oxyphenbutazone.

HPLC analysis of the hydrogenolysis products of *p*-benzyloxyphenylbutazone R_t 6.50 min, revealed the successful formation of oxyphenbutazone R_t 2.47 min, but in addition the presence of a minor component R_t 3.00 min. The amount of this impurity was found to increase with reaction time, and was formed at the expense of oxyphenbutazone. 4-Hydroxyphenylbutazone was identified by

Avery & Yeomans (1974) as an impurity formed during the synthesis of phenylbutazone, and although the analogous oxidation product, 4-hydroxyoxyphenylbutazone might be formed during the preceding condensation step, it was thought unlikely that it would be formed under the reductive conditions of the hydrogenolysis step, and this was confirmed by HPLC analysis of an authentic standard of 4-hydroxyoxyphenbutazone R_t 2.10 min.

Because the impurity seemed to be formed at the expense of oxyphenbutazone, the hydrogenolysis reaction was repeated with oxyphenbutazone as the substrate, and good yields of the impurity were obtained. As a possible aid to the identification of this compound the reaction was repeated with phenylbutazone, and again a good yield of an analogous hydrogenolysis product was obtained.

The material from the hydrogenolysis of oxyphenbutazone was isolated, purified, and identified as 2-*n*-butyl(N-phenyl-N'-*p*-hydroxyphenyl)-malonamide (**III**) by mass, infra-red, ^{1}H, and ^{13}C magnetic resonance spectroscopy.



(III) 2-*n*-Butyl(N-phenyl-N'-*p*-hydroxyphenyl)-malonamide

Replacing the Raney nickel with another hydrogenation catalyst (10% palladium on charcoal) did not prevent this unwanted reaction, but by carrying out the reaction with the minimum amount of ethanol necessary to dissolve the *p*-benzyloxyphenylbutazone the second unwanted hydrogenolysis reaction was entirely eliminated.

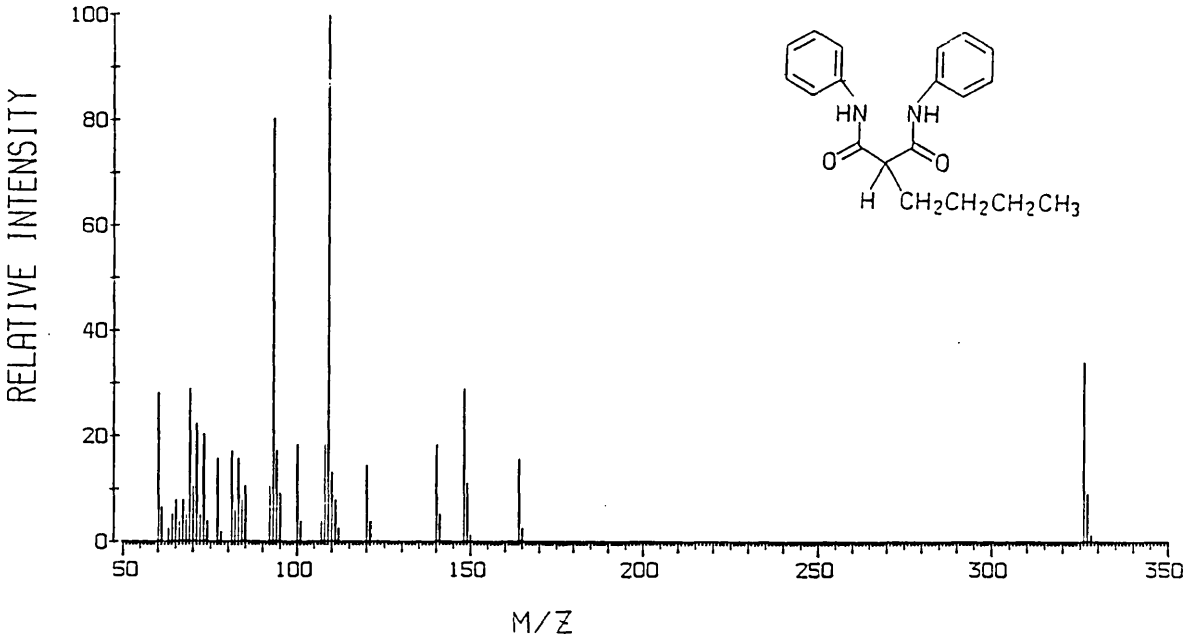
Proof of the structure of the malonamide

The mass spectrum of the material obtained from the hydrogenolysis of oxyphenbutazone is reproduced in Fig. 4.3. Comparison of this spectrum with that of oxyphenbutazone indicated an increase in the molecular weight of 2 mass units, from 324 to 326. The principal feature in the mass spectra of 1,2-diphenyl-3,5-pyrazolidinediones is the series of ions at *m/z* 182, 183, and 184 arising from the cleavage of the heterocyclic ring and the resultant formation of a stable azobenzene type ion. The corresponding ions for oxyphenbutazone *m/z* 198, 199, 200 appear 16 mass units higher because of the hydroxyl group in the phenyl ring. The complete absence of any similar series of ions in the spectra of the isolated material suggests prior cleavage of the azo bond, and the intense peaks at *m/z* 109, 93 which are thought to arise from hydroxyaniline ($C_6H_5NH_2OH$) and aniline ($C_6H_5NH_2$) ions respectively support this assumption.

The infra-red spectra of the two compounds were not dissimilar, the main differences were the absence of the band at 1288 cm^{-1} (C-N stretch), and alterations in the bands at 1700 and 1748 cm^{-1} (C=O stretch) and 1300 cm^{-1} (characteristic of pyrazolidiones). Supporting the mass spectral evidence of a disruption of the heterocyclic nucleus. Thus it now seemed likely that the impurity was formed by reductive cleavage of the pyrazolidione ring.

Further corroboration was obtained from the proton nuclear magnetic resonance spectra. The main difference between the spectra of oxyphenbutazone and the isolated material was the appearance of 2 broad singlets at δ 9.6 and 9.9, these signals disappeared after equilibration with deuterated water, suggesting they were arylamine protons. The conclusive evidence for the structure was obtained from ^{13}C nuclear magnetic resonance studies. Comparison of the spectra obtained for oxyphenbutazone and the isolated material, with the spectrometer operating in off-resonance mode, indicated that there was no change in the number of protons bound to any of the individual carbon atoms. Therefore the only position where the 2 additional protons could be added was on the two nitrogen atoms.

FIGURE 4.3 Direct Insertion mass spectrum of the material obtained from the hydrogenolysis of oxyphenbutazone



Synthesis of ^{14}C-oxyphenbutazone

*1. Synthesis of [4-^{14}C]-*p*-benzyloxyphenylbutazone.*

Diethyl[2-^{14}C]malonate 500 *u*Ci was diluted with unlabelled diethylmalonate to give a total of 0.40g (2.5mmol), to this was added iodobutane (0.46g 2.5mmol), and *p*-benzyloxyhydrazobenzene (0.80g 2.5mmol) together with a small amount of sodium iodide (3mg) and heated in a nitrogen atmosphere to 70° with constant stirring. A solution of sodium ethoxide prepared from clean sodium metal (0.40g) and dried ethanol (5ml) was added over 2 h. The mixture was then refluxed overnight, the apparatus converted for distillation under nitrogen, and most of the ethanol distilled off over 30 min. 10ml of xylene was then added and the mixture heated until the remaining ethanol and xylene had completely evaporated. The residue was then dissolved in hot water (2 x 25ml), extracted with chloroform (3 x 20ml), and diethylether (20ml). The solution was then acidified to pH 1 and a small amount of yellow precipitate obtained, this was extracted with diethylether (3 x 25ml), decanted and evaporated to dryness under a stream of nitrogen.

Radiochromatographic analysis (by HPLC) of the crude product revealed a disappointing yield (<10%) together with a low radiochemical purity. In order to minimise losses of the small amount of ^{14}C-*p*-benzyloxyphenylbutazone obtained, 90 mg of carrier *p*-benzyloxyphenylbutazone was added to the radiolabelled product, which was then recrystallised from ethanol/water. At this stage the recrystallised product was still not completely pure, but it was decided to continue to the next step without further purification.

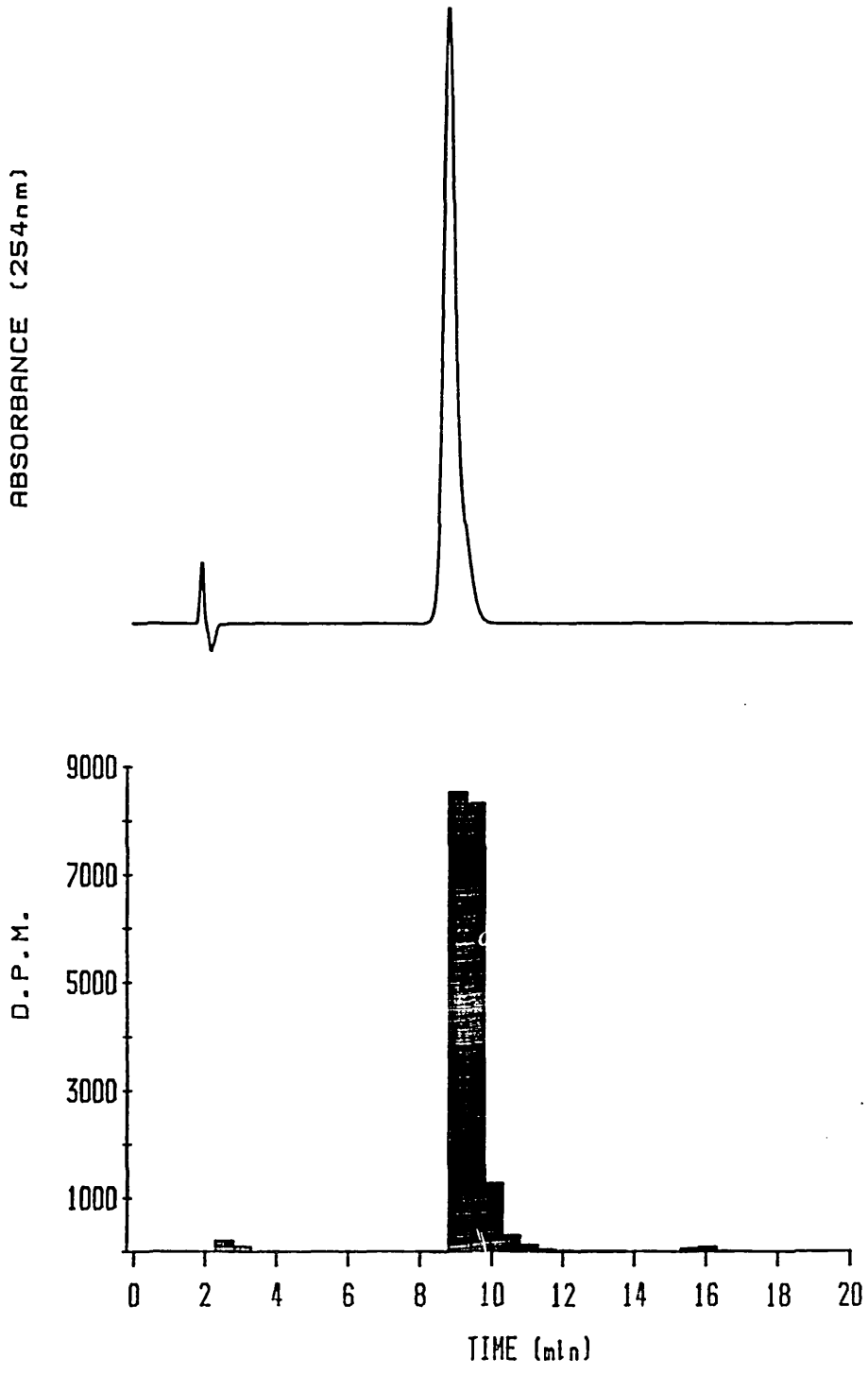
*2. Hydrogenolysis of ^{14}C-*p*-benzyloxyphenylbutazone.*

The radiolabelled *p*-benzyloxyphenylbutazone (200mg,0.5 mmol) was dissolved in a solution of ethanol (1ml) and 1M NaOH (10ml). A suspension of Raney

nickel (0.3g) was added and the reactants stirred in a hydrogen atmosphere. The progress of the hydrogenolysis reaction was monitored by HPLC as previously described. After 3h all but a trace of the starting material had disappeared, and the HPLC analysis indicated the successful formation of oxyphenbutazone. The solution was filtered to remove the Raney nickel, transferred to a separating funnel, extracted with diethyl ether (3 x 25ml), and the extract evaporated to dryness under a stream of nitrogen. The crude oxyphenbutazone was then recrystallised from ethanol/water following the addition of 100mg of unlabelled oxyphenbutazone. Radiochromatographic analysis revealed that the presence of a small impurity (10%), but this was removed after two further recrystallisations.

The radiochemical purity of the final product as determined by reverse phase HPLC was >97%, and the radiochromatogram obtained is reproduced in Fig. 4.4. A total of 27 *u*Ci of the purified oxyphenbutazone with a specific activity of 0.12 *u*Ci/mg (40 *u*Ci/mmol) was recovered; a radiochemical yield of 5-6%.

FIGURE 4.4 Radiochromatogram of [4-^{14}C]-oxyphenbutazone



DISCUSSION

The finding that phenylbutazone and oxyphenbutazone undergo catalytic reduction to form malonamides is of some interest from a chemical viewpoint. In an attempt to obtain phenylbutazone derivatives with reduced toxicity, Bavin *et al.*, (1955) investigated the *in vivo* anti-inflammatory activity of a number of phenylbutazone analogues, including the ring-opened analogue of phenylbutazone, *n*-butylmalondianilide. In the test system used (light induced erythema in the guinea-pig) *n*-butylmalondianilide, although less effective than phenylbutazone, was approximately equipotent with monophenylbutazone (*Mofebutazone*). Renewed concern over the toxicity of phenylbutazone has stimulated interest in this area, and recently Vennerstrom and Holmes (1987) evaluated the potential anti-inflammatory activity of a series of ring-opened analogues of phenylbutazone. Although less active than phenylbutazone, in the *in vitro* inhibition of microsomal prostaglandin-H (PGH) synthase, several of them showed promising activity.

The synthesis of these compounds was accomplished by the condensation of the appropriate aniline derivative with diethyl butylmalonate, and although generally successful, poor results were obtained for the synthesis of compounds containing a phenolic group, with yields as low as 3%. The catalytic reduction of pyrazolidiones offers an alternative route to the synthesis of malonamides. For the two compounds investigated, (phenylbutazone and oxyphenbutazone) the corresponding malonamides were obtained in quantitative yield, and an additional advantage of this method is that it is applicable to the synthesis of non-symmetric compounds, i.e. those with different substituents in the phenyl rings. Paradoxically the reverse of this reaction, i.e. the dehydrogenation of malonamides to give pyrazolidinediones, was used to obtain phenylbutazone from *n*-butyl diphenylmalonamide following treatment with a mixture of sulphur and aluminium chloride (Esteve, 1960).

CHAPTER 5.

THE METABOLIC DISPOSITION OF OXYPHENBUTAZONE

INTRODUCTION

Oxyphenbutazone (1-phenyl-2-(*p*-hydroxyphenyl)-3,5-dioxo-4*n*-butyl-pyrazolidine), was originally identified as a metabolite of phenylbutazone, following the administration of this compound to man (Burns *et al.*, 1955a; 1955b). With some foresight, the authors speculated that oxyphenbutazone was an 'active metabolite', contributing to the *in vivo* pharmacological effects of phenylbutazone. Preliminary studies confirmed this view, demonstrating that oxyphenbutazone had a similar spectrum of pharmacological activity to phenylbutazone (Brodie *et al.*, 1956) and in a clinical trial Yu *et al.* (1958) considered it to be as effective as phenylbutazone for the treatment of gouty arthritis. In a similar study, Hart and Burley (1959) reported that oxyphenbutazone, although slightly less potent than phenylbutazone, was superior, because of a lower incidence of gastric side-effects, consequently it was not long before oxyphenbutazone was introduced into human medicine in 1959 under the trade name *'Tanderil'*. However, since then it has become apparent that oxyphenbutazone is almost twice as toxic as phenylbutazone (Cuthbert 1974), resulting in the CSM recommending the total withdrawal of this compound.

The contribution made by oxyphenbutazone to the overall anti-inflammatory activity of phenylbutazone in the horse, received official recognition, when the FEI amended their regulations regarding the permitted limited use of phenylbutazone. Originally the FEI set a maximum plasma concentration of phenylbutazone of 4 *u*g/ml, but this was recently altered to a 5 *u*g/ml aggregate for phenylbutazone and oxyphenbutazone. The anti-inflammatory effect of oxyphenbutazone in the horse was evaluated by Gorman *et al.* (1968), and at a dose of 6 mg/kg p.o. oxyphenbutazone significantly reduced the inflammatory response, in and around artificially created surgical incisions.

Initial studies in man indicated that the metabolic disposition of oxyphenbutazone was similar to that of phenylbutazone. Following oral administration, oxyphenbutazone is well absorbed, slowly eliminated, with a plasma half-life of about 3 days, and extensively metabolised, with only 4% of a given dose recovered in the urine within 2 days (Burns *et al.*, 1955a; Yu *et al.*, 1958).

There are several reports in the literature of studies comparing the plasma elimination half-lives of phenylbutazone and oxyphenbutazone in various species, but few have addressed the specific aspects of the metabolic disposition. Perel *et al.* (1964) reported that following the intravenous administration of oxyphenbutazone (100 mg/kg) to two dogs, 10.5% and 15% of the unchanged drug and 18% and 28% respectively as the glucuronide were recovered in the 0-24h urine, with an additional 1-2% of the dose excreted as oxyphenbutazone in the bile. When human subjects were given oxyphenbutazone orally (800mg), between 1 and 5% of the dose was excreted as the glucuronide in the 0-24h urine. Wagner *et al.* (1971) investigated the metabolism of oxyphenbutazone in man and dog and in addition to confirming these earlier findings, reported that oxyphenbutazone was subject to both aromatic and aliphatic hydroxylations, with an additional hydroxyl group introduced in either the butyl side chain, or the benzene ring.

There are no reports in the literature on the metabolism of oxyphenbutazone in the horse. Gandal *et al.* (1969) administered oxyphenbutazone intravenously (5, 10 or 15mg/kg) to 3 ponies and 2 geldings; these workers concluded that it had a similar elimination half-life to that of phenylbutazone in the horse, but was less strongly bound to horse plasma (87%), than phenylbutazone (96%). In the only other report on the administration of oxyphenbutazone in the horse, Tobin *et al.* (1977) pretreated thoroughbred mares with oxyphenbutazone and demonstrated

a small, but statistically significant increase in the plasma elimination half-life of subsequently administered phenylbutazone.

Davis *et al.* (1973) reported that "following oral administration, oxyphenbutazone was absorbed to about the same extent and at the same rate as phenylbutazone in ponies" apart from this rather vague statement, there are no reports on the pharmacokinetics of orally administered oxyphenbutazone in the horse, and consequently the bioavailability of oxyphenbutazone in this species is unknown.

Our earlier investigations into the metabolism of phenylbutazone in the horse, indicate that the majority of the oxyphenbutazone produced from the metabolic hydroxylation of phenylbutazone is excreted without undergoing further metabolism. However, for a variety of reasons one cannot automatically assume that the metabolic disposition of exogenously administered oxyphenbutazone, will be the same as that of oxyphenbutazone formed *in vivo.*

Phenylbutazone and to a lesser extent oxyphenbutazone have been implicated in various dose-dependent effects (see Chapter 6.). It is to be expected, that the administration of oxyphenbutazone *per se* will result in much higher tissue concentrations of this compound compared to those produced from the *in vivo* hydroxylation of phenylbutazone, and this may result in a variety of pharmacokinetic and pharmacodynamic differences.

Alterations in the disposition of administered oxyphenbutazone and that produced *in vivo*, might also be expected from stereochemical considerations. Phenylbutazone is a prochiral compound, with several potential asymmetric centres. In man, hydroxylation at the C_3 position in the butyl side chain results in the production of optically active γ-hydroxyphenylbutazone (Burns *et al.*,

1955a) and since the C_4 position in the heterocyclic ring is also a prochiral centre, the formation of oxyphenbutazone is also potentially stereoselective. One might expect some differences in the metabolic disposition of the two oxyphenbutazone enantiomers, and consequently if one enantiomer is preferentially formed, one would expect differences between the disposition of the resultant non-racemic mixture and that of the racemate.

Oxyphenbutazone is one of the more frequently encountered doping agents, (Table 1.3), and although it is not clear how many of these positives resulted from the administration of phenylbutazone, if oxyphenbutazone itself is not a drug of abuse, then it is certainly a potential one. For the reasons outlined above, it is impossible to predict the metabolism of oxyphenbutazone in the horse with any great degree of certainty. Without this information, it is impossible to be sure that the screening procedures currently adopted for the detection of the illicit use of this compound are adequate.

Several aspects of the metabolic disposition of phenylbutazone in the horse remain unexplained; in particular, a rationale for the alterations in the urinary metabolite profile was sought. It was hoped that a knowledge of the metabolic disposition of oxyphenbutazone, would result in a greater understanding of the situation regarding phenylbutazone, which in turn might resolve some of these issues. Accordingly the present study was designed, in order to evaluate the excretion balance, bioavailability, and metabolism of oxyphenbutazone in the horse.

EXPERIMENTAL

Study design and protocol

The study design was similar to that of the previous experiments with phenylbutazone; that is essentially a combined, total balance and oral/i.v. cross over study. One gelded crossbred pony received ^{14}C-oxyphenbutazone (7.1 mg/kg, 10 *u*Ci) orally and intravenously on separate occasions. Serial blood samples were obtained, and the plasma concentrations of oxyphenbutazone measured. Urine and faeces were collected, the ^{14}C content determined, and urinary metabolites separated and quantitated by HPLC.

Compounds

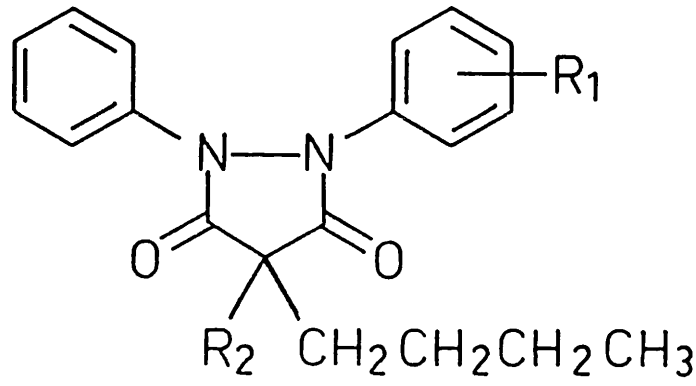
[4-^{14}C]-Oxyphenbutazone (1-(*p*-hydroxyphenyl)-2-phenyl-3,5-dioxo-4-[^{14}C]-butyl-pyrazolidine) sp. act. 0.12 uCi/mg, radiochemical purity by HPLC > 97% was synthesised as previously described. Unlabelled oxyphenbutazone was purchased from Sigma Chemical Co. (Poole, U.K.). Samples of possible metabolites and degradation products were gifts from Ciba-Geigy (Basle, Switzerland) and are illustrated in Fig. 5.1. HPLC grade methanol and acetonitrile were purchased from Rathburn Chemicals Ltd., (Walkerburn, U.K). Other materials were reagent-grade chemicals purchased from usual U.K. commercial sources and used without further purification.

Animals

One gelded crossbred pony (body weight 280 kg) received [4-^{14}C]-oxyphen-butazone (7.1 mg/kg, 10 *u*Ci), by stomach tube dissolved in 500ml of dilute aq. $NaHCO_3$ soln., followed by 500ml water, and on another occasion (two months later), by intravenous infusion over 4 min, dissolved in 50ml of a solution containing 20% v/v NaOH (2.1ml), 10% v/v ethanol and 40% v/v propane-1,2-

diol, in sterile water, final pH < 8.5. Food was witheld from the pony for 12 h prior to dosing which was at 11 am, and the pony was allowed free access to hay 2.5 h later.

TABLE 5.1 Structures of oxyphenbutazone and related compounds.



Compound.	R_1	R_2	Butyl group
Oxyphenbutazone	*p*-OH	H	-
p,γ-Dihydroxyphenylbutazone	*p*-OH	H	3-OH
p-Hydroxy-γ-ketophenylbutazone	*p*-OH	H	3 C=O
4-Hydroxy-*p*-hydroxyphenylbutazone	*p*-OH	OH	-

Sample collection and storage

The procedures adopted for the collection, preparation and storage of samples were identical with those used in the earlier experiment, although on this occasion saliva was not collected. Briefly; blood samples were collected into tubes containing lithium heparin, from an indwelling jugular cannula, and the plasma aspirated after centrifugation. Faeces and urine were collected as voided, the faecal samples were homogenised and combusted using the previously adopted procedures, and the ^{14}C content determined by liquid scintillation spectrometry. All samples were stored at -20° in the presence of

$Na_2S_2O_5$ (approx. 5mM) and disodium EDTA (approx. 10mM) to prevent degradative losses, until analysed.

As far as possible each individual urinary voiding was collected separately, and the volume, pH, and time of voiding recorded, appropriate volumes of urine were then pooled to provide representative 0-12, 12-24, 24-48 and 48-72 h urine samples. Urine was incubated with ß-glucuronidase and sulfatase and incorporated positive and negative controls as previously described.

High performance liquid chromatography

The apparatus consisted of two LC 6A solvent delivery systems, combined with a variable wavelength absorbance detector SPD 6A, operated at 254nm, a SCL 6A system controller, and a CR3A integrator-recorder. Samples were introduced with an SIL 6A automatic injector (all Shimadzu, Kyoto, Japan). Separation was achieved in reverse-phase mode using a pre-packed cartridge system (25 x 4 mm i.d. Hibar LiChroCART RP-18 Cat. No. 15539 BDH Ltd., Poole, U.K.).

Two solvent systems were used; for the analysis of oxyphenbutazone in plasma an isocratic mobile phase of acetonitrile-water (40:60 v/v) adjusted to pH 3.0 with glacial acetic acid, at a flow rate of 2 ml/min. The identities of the peaks were assigned by comparison of retention times with those of authentic standards. Under the conditions described, the elution times for *m*-hydroxy-phenylbutazone (internal standard) and oxyphenbutazone were 5.2 and 6.5 min respectively.

The analysis of urinary metabolites was accomplished by a linear gradient elution technique. The column was eluted at ambient temperature with a degassed mobile phase of methanol-water (30:70 v/v to 70:30 v/v over 40 min) adjusted to pH 3.0 with glacial acetic acid, at a flow rate of 1.5 ml/min. Under

these conditions the retention times for *p*-hydroxy- γ -ketophenylbutazone, *p*,γ-dihydroxyphenylbutazone, 4-hydroxyoxyphenbutazone and oxyphenbutazone were 13.5, 13.6, 25.8 and 30.1 min respectively.

Treatment of plasma

To 0.5ml of plasma was added 0.5ml acetonitrile containing 10*u*g of *m*-hydroxyphenylbutazone. Samples were vortexed for 30s and then centrifuged for 10 min at 1000 x *g* to separate precipitated protein. The supernatant was removed and 15*u*l portions injected on to the HPLC column. Quantification of oxyphenbutazone was achieved using the peak area ratio method, with respect to the internal standard, *m*-hydroxyphenylbutazone. Plots of peak area ratio versus oxyphenbutazone concentration gave straight lines over the range 1 to 100 *u*g/ml with correlation coefficients always > 0.99.

Pharmacokinetic analysis

The alteration in the plasma concentration of oxyphenbutazone with time, following the intravenous administration of oxyphenbutazone was fitted to a two compartment open model, and pharmacokinetic parameters calculated by least square regression analysis and the method of residuals, using the standard pharmacokinetic equations given in Chapter 2.

Quantitative analysis of urinary metabolites

Aliquots of the 0-12, 12-24, 24-48, and 48-72 h pooled urine samples were analysed by HPLC, serial fractions of the eluent collected and metabolites quantitated by scintillation counting of the relevant fractions.

Confirmatory identification of urinary metabolites

For the confirmatory GCMS analysis, a portion of urine (10ml) was evaporated to dryness, initially under reduced pressure, and finally in a stream of dry nitrogen. The residue was redissolved in acetone (1ml) and a portion of this (10*ul*) derivatised to form Me and Me-TMS derivatives before submission to GCMS at the Horseracing Forensic Laboratory, Newmarket as previously described in Chapter 3.

RESULTS

Excretion of ^{14}C

Urine and faeces samples were collected for up to 7 days after the administration of ^{14}C-oxyphenbutazone, and by this time the radioactivity levels had returned to background, the results obtained following both oral and intravenous administration are presented in Fig. 5.1. Urinary elimination was essentially complete within 72 h, and accounted for 60% (i.v.) and 53% (p.o.) of the dose. As in the earlier experiments with phenylbutazone, there was a lag period of about 20 h before the appearance of ^{14}C in the faeces, and again the continued faecal excretion of ^{14}C was observed after urinary excretion had ceased, 33% (i.v.) and 44% (p.o.) of the dose was recovered in the faeces. An essentially quantitative recovery (97% and 93%) was obtained after both oral and intravenous dosing, although the relative proportions of the dose excreted in the urine and faeces appeared to be dependent on the route of administration, with a greater proportion of the intravenous dose excreted in the urine.

Plasma pharmacokinetics

A comparison between the oxyphenbutazone plasma concentrations obtained from the HPLC analysis, and the μg equivalents calculated from the ^{14}C content, indicated that at all times the major (>95%) radioactive component present in the plasma was unchanged oxyphenbutazone. Fig. 5.2. illustrates the oxyphenbutazone plasma concentration time curves following the oral and intravenous administration of oxyphenbutazone.

Intravenous administration

Following the intravenous administration of oxyphenbutazone, the plasma

concentrations of both ^{14}C and oxyphenbutazone declined biphasically with time. The experimental data were analysed according to a two compartment open model, and the various parameters describing this, corrected where appropriate for the weight of the horse, are listed in Table 5.2.

Oral administration

Following oral administration, oxyphenbutazone was rapidly absorbed, with peak plasma levels of both ^{14}C and oxyphenbutazone obtained within 1 h, thereafter the levels declined, but not in a simple monoexponential fashion. The log oxyphenbutazone plasma concentration time curve is illustrated in Fig. 5.3. The experimental data were analysed by both one compartment and two compartment models, but neither model adequately fitted the data. Least squares regression analysis of the last five data points gave a straight line (slope 0.114) with a correlation coefficient > 0.998, and the value of the elimination half-life obtained from this (6.1h) was thought to be a better estimate than that obtained from either pharmacokinetic model.

FIGURE 5.1 **Cumulative urinary and faecal excretion of ^{14}C radioactivity by a horse given ^{14}C-oxyphenbutazone (7.1 mg/kg) orally and intravenously on separate occasions**

Key: (✱——✱) Urine, (✱——✱) Faeces, (———) Total

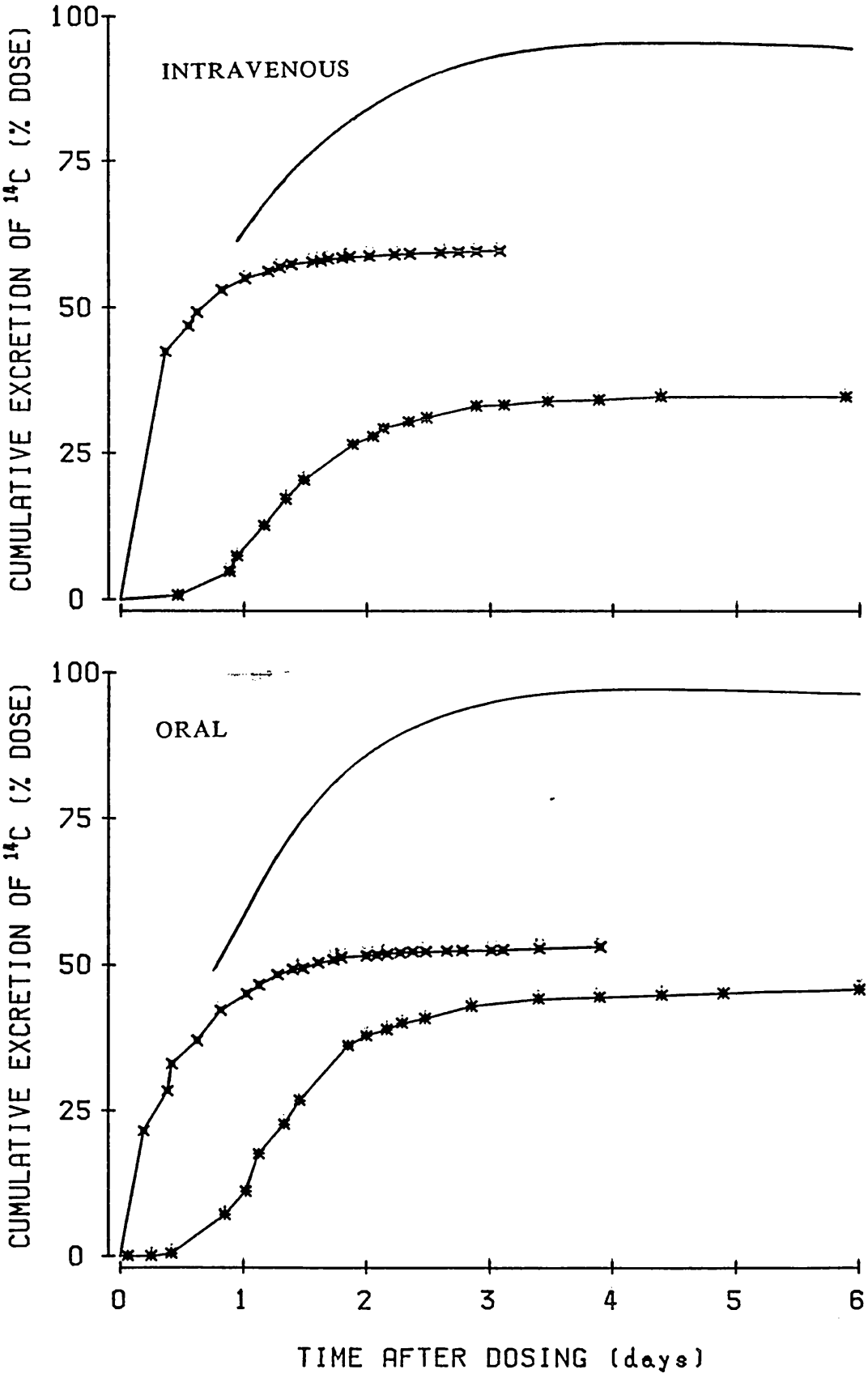


FIGURE 5.2 **Oxyphenbutazone plasma level-time curves obtained in a horse given ^{14}C-oxyphenbutazone (7.1 mg/kg) intravenously**

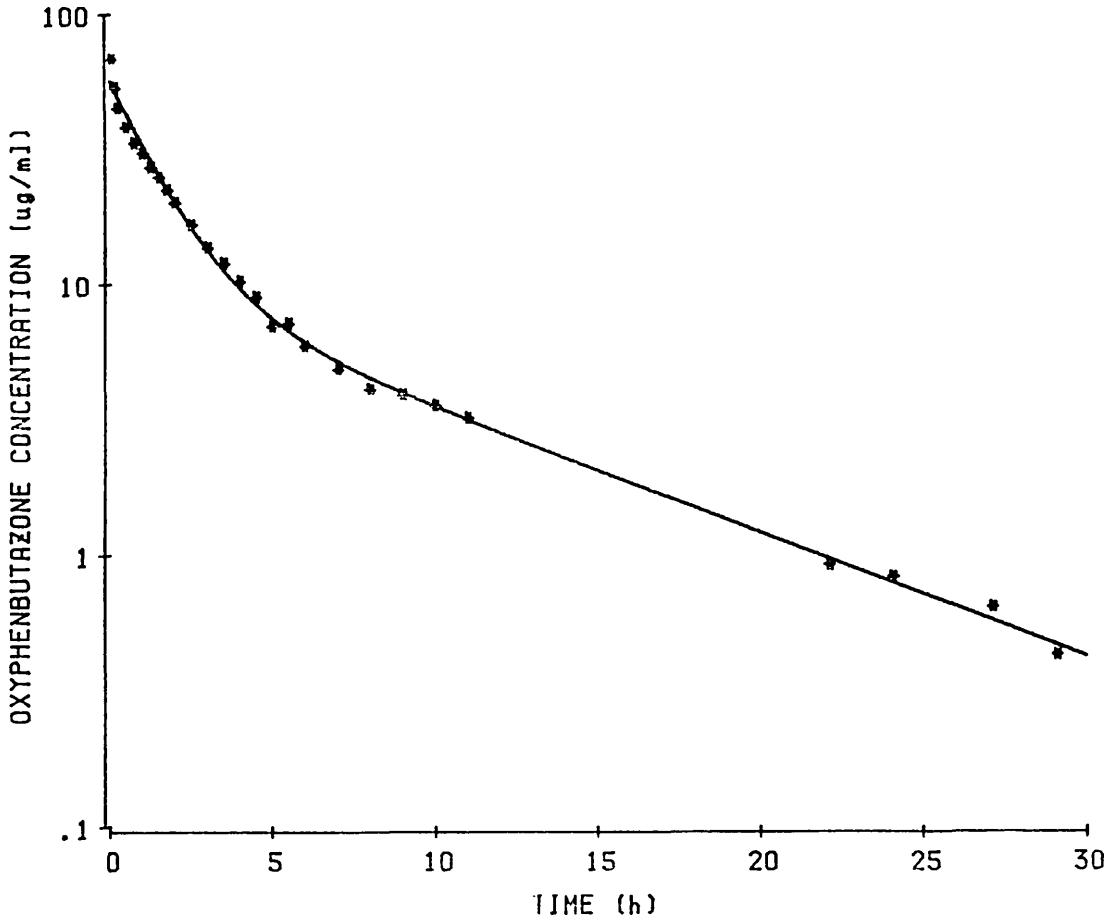


FIGURE 5.3 **Oxyphenbutazone plasma level-time curves obtained in a horse given ^{14}C-oxyphenbutazone (7.1 mg/kg) orally**

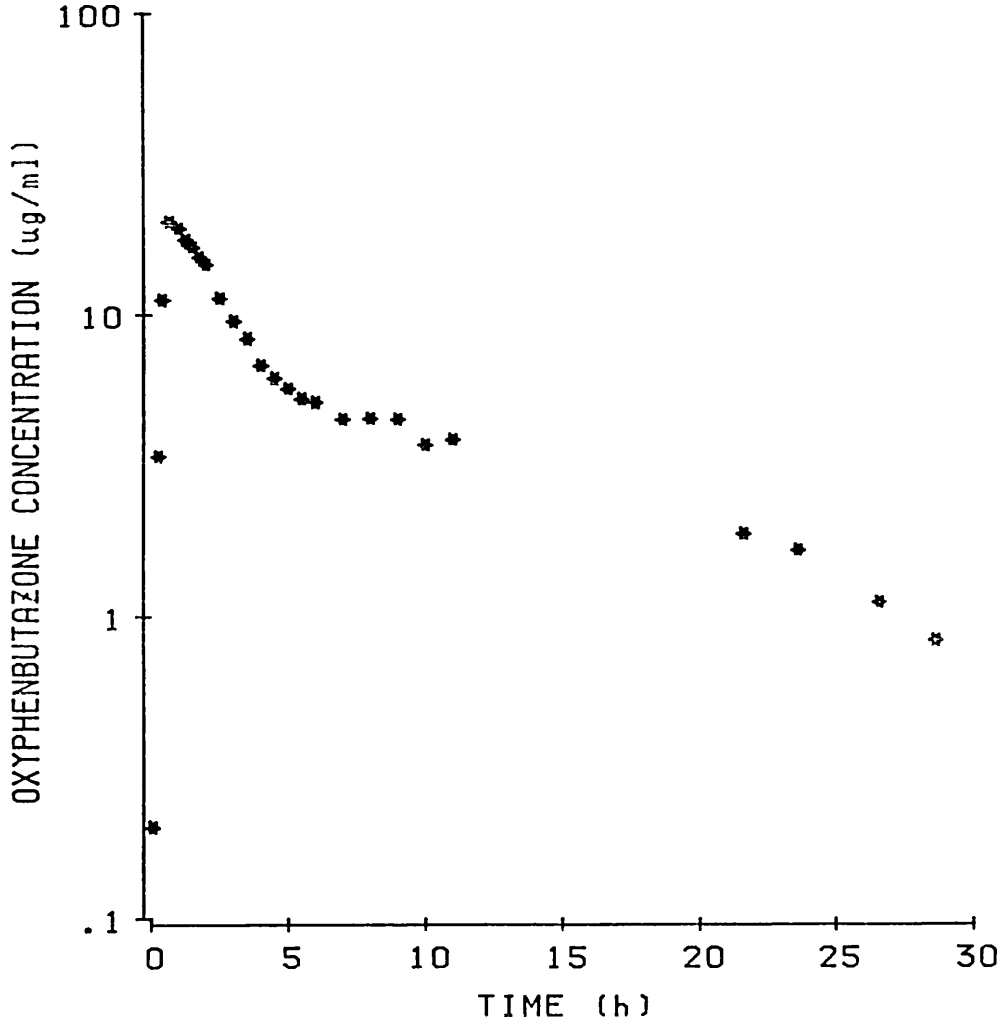


TABLE 5.2 **Pharmacokinetic parameters describing the plasma levels of oxyphenbutazone following the intravenous administration of ^{14}C-oxyphenbutazone (7.1 mg/kg) to one horse**

C^0 (ug ml^{-1})	56.6
C_1 (ug ml^{-1})	46.7
λ_1 (h^{-1})	0.67
C_z (ug ml^{-1})	9.88
λ_z (h^{-1})	0.10
$t_{0.5}$ (h)	6.6
Vz (mlkg^{-1})	412
Vc (mlkg^{-1})	125
Cl (mlkg^{-1}h^{-1})	43.3
k_{12} (h^{-1})	.2248
k_{21} (h^{-1})	.2062
k_{10} (h^{-1})	.3387
AUC (ug ml^{-1}.h) (trapezoidal method)	164
AUC (ug ml^{-1}.h) (direct integration)	164

TABLE 5.3 **Pharmacokinetic parameters describing the plasma levels of oxyphenbutazone following the oral administration of ^{14}C-oxyphenbutazone to one horse**

C_{max}(ug ml^{-1})	20.3
t_{max} (h)	0.67
λ (h^{-1})	0.114
$t_{0.5}$(h)	6.10
AUC (ug ml^{-1}.h) (trapezoidal method)	122
f % (Bioavailability of oxyphenbutazone)	78.4

Chromatography of urinary metabolites

Urinary metabolite profiles were obtained by radiochromatographic analysis of the pooled urine samples, with tentative identification based on co-chromatography. In all of the urine samples the vast majority of the radioactivity was associated with unchanged oxyphenbutazone, which accounted for 80% of the radioactivity in all samples. Of the remainder, some 2-3% was associated with the solvent front, and most of the rest was present in two discrete peaks, *p*,γ-dihydroxyphenylbutazone (3-5%), and an unknown metabolite HPLC R_t 22.9 min (4-8%). The pattern of urinary metabolites was independent of route of administration and there was no alteration in the metabolite profile with time.

Confirmatory identification of urinary metabolites

The presence of oxyphenbutazone and *p*,γ-dihydroxyphenylbutazone, indicated by co-chromatography with authentic standards was confirmed by GCMS.

Oxyphenbutazone :- The mass spectrum obtained following GCMS of material isolated from urine is reproduced in Fig. 5.4. This spectrum contained prominent ions at *m/z* 352 (M^+), 296 (M^+-C_4H_8), 213 ($C_6H_5N_2HC_6H_4OCH_3$), 107 ($C_6H_4OCH_3$) consistent with dimethylated oxyphenbutazone, and was essentially identical to that obtained from an authentic sample of oxyphenbutazone (Fig. 7.3).

pγ-Dihydroxyphenylbutazone :- The mass spectrum obtained following GCMS of material isolated from urine is reproduced in Fig. 5.5. This spectrum contained prominent ions at *m/z* 440 (M^+), 425 (M^+-CH_3), 213 ($C_6H_5N_2HC_6H_4OCH_3$), 197, and was essentially identical to the spectrum

obtained from the Me-TMS derivative of an authentic standard of *p*,γ-dihydroxyphenylbutazone (Fig. 7.15).

FIGURE 5.4 **The mass spectrum of material isolated from urine and identified as the dimethyl derivative of oxyphenbutazone**

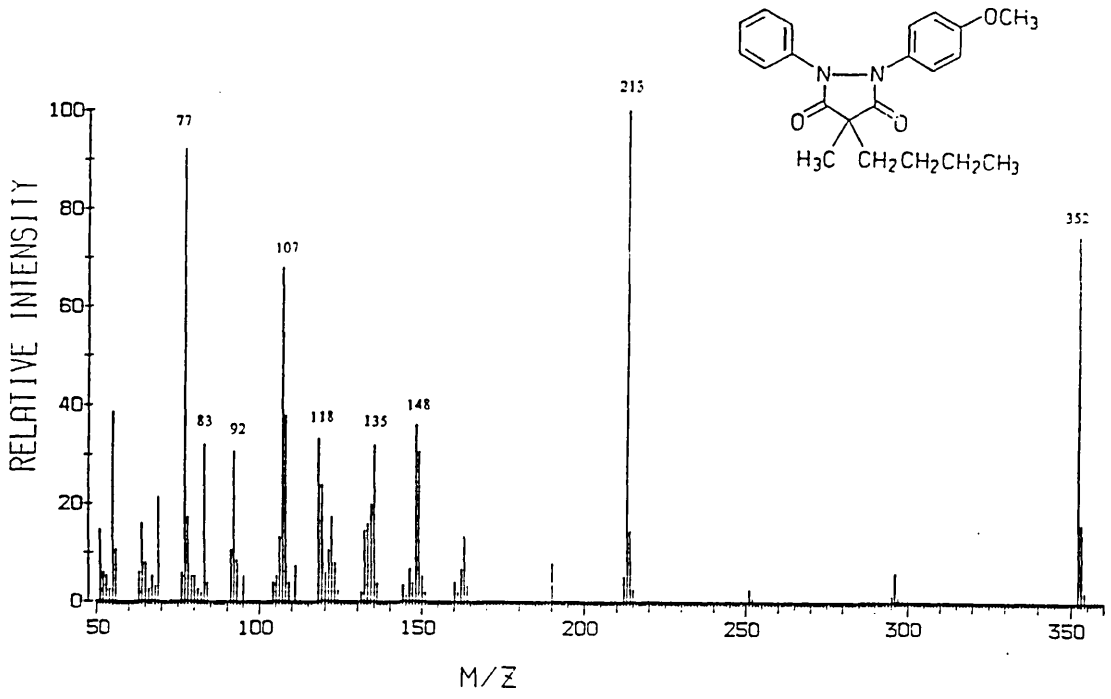


FIGURE 5.5 **The mass spectrum of material isolated from urine and identified as the Me-TMS derivative of *p*, γ-dihydroxyphenyl-butazone.**

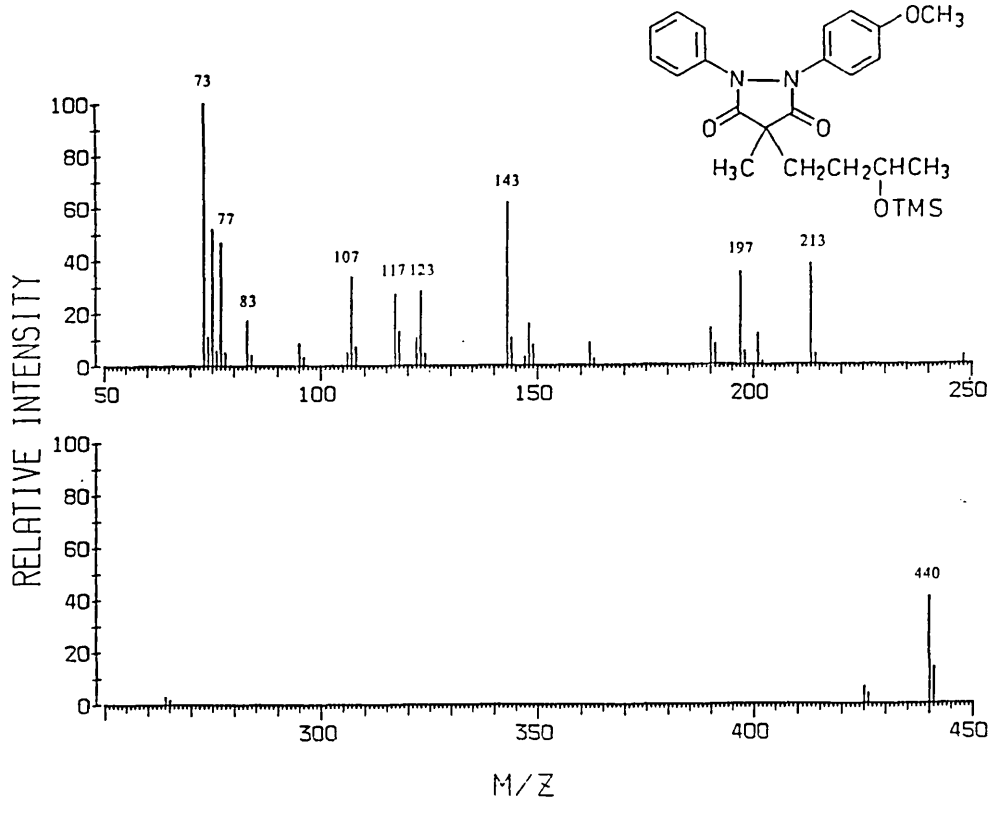


FIGURE 5.6 Plasma elimination half-lives of phenylbutazone and oxyphenbutazone in various species

Species	Phenylbutazone		Oxyphenbutazone		Reference
	$t_{1/2}$(h)	Dose	$t_{1/2}$(h)	Dose	
Man	72	15mg/kg	72	15mg/kg	Brodie *et al.*, 1955a.
Rhesus Monkey	8	15mg/kg	8	15mg/kg	Perel *et al.*, 1964.
Dog	6	50mg/kg	0.5	50mg/kg	Dayton *et al.*, 1967.
Rat	6	100mg/kg	6	50mg/kg	Perel *et al.*, 1964.
Cow	55[1]	5mg/kg	1.3[2]	5mg/kg	[1]De Backer *et al.*, 1980. [2]Gandal *et al.*, 1969.
Pig	3.7	20mg/kg	1.5	10mg/kg	Hvidberg & Ramussen, 1975.
Rabbit	3	100mg/kg	4	50mg/kg	Perel *et al.*, 1964.

DISCUSSION

Following oral administration oxyphenbutazone was incompletely absorbed, with an absolute bioavailability of 78%. The initial rate of absorption was rapid, with the peak plasma concentration obtained within 1 h. In the period immediately after this the plasma concentration of oxyphenbutazone declined rapidly with an apparent half-life of about 0.5 h. After 5 to 6 h, there was a prolonged plateau phase in the plasma concentration-time profile, and it was not until >15 h after dosing that the true elimination phase became apparent.

There are several potential explanations for the plateau in the plasma pharmacokinetics of oxyphenbutazone following its oral administration, but by comparison with the pharmacokinetics after intravenous administration, the most likely explanation is a continued slow absorption of oxyphenbutazone. Delayed absorption of phenylbutazone was observed after its administration following food (Rose *et al.*, 1982; Sullivan & Snow, 1982), which led to the proposal (Sullivan & Snow, 1982; Lees *et al.*, 1983; Bogan *et al.*, 1984) that phenylbutazone was adsorbed onto the fibrous component of the diet, and subsequently released by fermentation. This explanation may account for the results we obtained in this study, since although oxyphenbutazone was administered following an overnight fast, the horse was given hay 2.5 h after dosing. An alternative explanation is that the initial rapid absorption of oxyphenbutazone takes place when it is in the acidic environment of the stomach, and following gastric emptying, the prolonged slow absorption occurs from the less acidic regions of the gastrointestinal tract. The pH of the stomach will also be higher following feeding reducing the absorption of acidic drugs such as oxyphenbutazone.

The terminal plasma elimination half-life of oxyphenbutazone was independent

of the route of administration, with similar values obtained in both experiments 6.5 h (i.v.) and 6.1 h (p.o.). However, presumably because of the continued slow absorption of oxyphenbutazone, the plasma concentrations measured 24 h after oral administration, were double those achieved following the intravenous administration of this compound.

A good recovery of ^{14}C was obtained after both the oral and intravenous administration of ^{14}C-oxyphenbutazone. The slightly greater proportion of the dose recovered in the faeces after oral dosing (44%), compared with intravenous administration (33%) is suggestive of incomplete absorption, and consistent with the results obtained from the bioavailability calculations.

It is of some interest to compare the results obtained following the administration of phenylbutazone and of oxyphenbutazone at the same dose (7.1 mg/kg) and to the same horse. Oxyphenbutazone is eliminated at a faster rate than phenylbutazone, with terminal plasma half-lives of 6.3 h and 9.1 h respectively. The faster elimination of oxyphenbutazone was not a consequence of higher urinary pH, since the mean urinary pH (6.92 $\pm$ 0.59) in this study was similar to that in the experiments with phenylbutazone (7.54 $\pm$ 0.40).

The value for the apparent volume of distribution of oxyphenbutazone (412 ml/kg) was double that obtained for phenylbutazone in the earlier experiment (190 ml/kg). Similar differences were reported in the pig (Hvidberg & Ramussen, 1975) with values of 280 and 180 ml/kg for oxyphenbutazone and phenylbutazone respectively. Oxyphenbutazone is bound to plasma proteins to a lesser extent than phenylbutazone, in both the horse (87% and 96% Gandal *et al.*, 1969) and the pig (97% and 98%, Hvidberg & Ramussen, 1975), and the larger volume of distribution of oxyphenbutazone compared to phenylbutazone may be attributed to this.

There is good evidence that in the horse (Gerring *et al.*, 1981) oxyphenbutazone is actively secreted by the renal tubules. This process is usually assumed to be independent on the degree of protein binding, however since glomerular filtration is limited to free drug, the lower protein binding of oxyphenbutazone is likely to contribute to the greater renal elimination of this compound, resulting in the shorter elimination half-life.

Unlike phenylbutazone, oxyphenbutazone is eliminated in the urine largely as the unchanged compound, and consequently the elimination half-life is mainly determined by the rate of renal excretion. The differences in the renal excretion of these two compounds can be attributed to differences in their physico-chemical properties. Oxyphenbutazone is less lipophilic than phenylbutazone, with K_p values of 0.6 and 2.2 respectively (peanut oil-Sorenson buffer system; Perel *et al.*, 1964). The lower lipophilicity of oxyphenbutazone is likely to reduce the tubular reabsorption of this compound, thereby enhancing its renal excretion.

The relatively rapid renal elimination of oxyphenbutazone may explain why, unlike phenylbutazone which is extensively metabolised, oxyphenbutazone is eliminated largely as the unchanged compound. With the exception of the C-glucuronide, phenylbutazone is unable to form conjugates without first undergoing Phase I metabolism. Oxyphenbutazone however, contains a functional group that is a suitable site for the formation of glucuronides and sulfates, yet there was no evidence that conjugation occurred to any significant extent in the metabolism of this compound.

The absence of any significant metabolism of oxyphenbutazone in the horse is in contrast to the situation in man, where oxyphenbutazone like phenylbutazone

is extensively metabolised. The inability of man to either rapidly metabolise or excrete oxyphenbutazone results in a relatively long elimination half-life for this compound. The differences between species in the metabolic handling of phenylbutazone and oxyphenbutazone are reflected by the plasma pharmacokinetics, and it is of some interest to compare the plasma elimination half-lives of these two compounds in other species (Table 5.6). Although there are difficulties in comparing the half-lives reported by different authors, because of differences in the dosage, route of administration and method of pharmacokinetic analysis used.

As one might expect from the physico-chemical differences between these two compound, in general oxyphenbutazone had either a similar (man, rhesus monkey, rat, rabbit) or shorter (horse, pig, cow, dog) elimination half-life than phenylbutazone. One exception to this is the cat, which was reported by Davis *et al.* (1973) to have a much longer oxyphenbutazone elimination half-life compared to phenylbutazone. The inability of the cat to form phenolic glucuronides (Robinson *et al.*, 1958), may provide an explanation for the relatively longer half-life of oxyphenbutazone in this species. The short phenylbutazone half-life is a consequence of its rapid metabolism, mainly to oxyphenbutazone. Once produced the cat is unable to form glucuronides of oxyphenbutazone (or presumably to rapidly metabolise or excrete oxyphenbutazone by any other route), resulting in the long elimination half-life of this compound.

CHAPTER 6.

THE NON-LINEAR URINARY KINETICS OF PHENYLBUTAZONE METABOLITES

INTRODUCTION

The quantitative and qualitative aspects of the metabolism of phenylbutazone was described earlier in Chapter 3. The major urinary metabolites were γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone, which were of almost equal importance in the overall urinary elimination of phenylbutazone. The relative proportions of these two metabolites in urine, varied with the time after the administration of phenylbutazone. Up to 12 h after dosing γ-hydroxyphenylbutazone was the major urinary metabolite, but in the later urine samples the predominant metabolite was *p*-hydroxyphenylbutazone. This time dependent change in the urinary metabolite profile was observed in both horses, and after both intravenous and oral administration.

Many of the processes involved in the metabolic disposition of a given compound are of a capacity limited nature, and these can be subdivided into both passive and active processes. The rate(s) of the latter are usually governed by Michaelis-Menten kinetics, under these conditions the relationship between the rate of the process and the concentration of drug varies. If the drug concentration is much lower than the rate constant K_m, then the rate approximates to that of a first-order process (also termed linear kinetics because a semi-log plot of rate against concentration gives a straight line). As the concentration of the drug approaches K_m the rate is no longer proportional to the concentration, and once the concentration is much higher than K_m then the rate is essentially independent of concentration, and thus zero-order.

Zero-order kinetics are also described as non-linear kinetics, although the terms are not interchangeable, since 'non-linear' does not imply zero-order. In addition to non-linear kinetics resulting from the saturation of a particular pathway or process, they can also arise from interactions between compounds.

Desmethyldiazepam a metabolite of diazepam inhibits the metabolism of the parent compound (Klotz & Reimann, 1981), and as a result the kinetics describing the metabolism of diazepam are non-linear. Many acidic compounds are bound with a high affinity to plasma protein binding sites, and these can be displaced by other compounds with similar physicochemical properties. Other examples of non-linear kinetics can arise from time-dependent effects, such as the induction of a particular metabolic pathway, or as a consequence of the accumulation of a metabolite after chronic administration. The physiological actions of a drug may alter the metabolic disposition of the drug, and obviously at very high dose levels, toxicological effects will result in non-linear kinetics.

The metabolic disposition of a drug can usefully be divided into 4 discrete, but inter-related phases; absorption, distribution, metabolism and excretion. Non-linear kinetics have been demonstrated for absorption (riboflavin, Levy *et al.*, 1966), distribution (disopyramide, Meffin *et al.*, 1979), metabolism (salicylate, Levy *et al.*, 1972), and excretion (bethanidine, Chremos *et al.*, 1976). Following the oral administration of a given compound, the composition and rate of appearance of urinary excretion products, are a sequela of all of these processes, and consequently non-linear urinary kinetics can arise if any one of these individual phases is non-linear.

Phenylbutazone has been associated with several non-linear pharmacokinetic processes, and in addition a large number of drug interactions have been reported for this compound. Burns *et al.* (1953) reported a non-linear relationship between dose and plasma levels, with similar plasma levels obtained after 800 or 1600 mg/day, although at lower doses (100-400 mg/day) the plasma AUCs increased with dose in a linear fashion (Lukas *et al.*, 1974). Burns *et al.* (1953) postulated that since phenylbutazone is strongly bound to plasma proteins, at high dose levels the proportion of non-bound drug is increased, and

thus the elimination enhanced. Evidence for this was provided by Dayton *et al.* (1973) who reported an increase in the percentage of unbound phenylbutazone from 1-2% at a plasma concentration of 100 *u*g/ml to 12% at 250 *u*g/ml. Another consequence of the extensive plasma protein binding of phenylbutazone, is the displacement of other plasma protein bound drugs by phenylbutazone (Aggeler *et al.*, 1967; Shoeman & Azarnoff, 1975) which will tend to enhance their elimination, and increase their pharmacological effect.

Given acutely phenylbutazone decreases the elimination of tolbutamide (Christensen *et al.* 1963), and has been demonstrated both *in vitro* and *in vivo* to inhibit the enzyme responsible for demethylating ethylmorphine (Cho *et al.*, 1970). The chronic administration of phenylbutazone reportedly increases the metabolism of aminopyrine (Chen *et al.*, 1962), digitoxin, dihydrocortisone and phenylbutazone itself (Davies & Thorgeirsson, 1971).

There is good evidence that phenylbutazone and its metabolites *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone are actively secreted by the renal tubules (Gutman *et al.*, 1960; Perel *et al.*, 1964). This process is saturable, both by the compound itself, and in a competitive fashion by compounds such as probenicid that are excreted by the same mechanism (Weiner, 1967). Phenylbutazone has been demonstrated to inhibit the renal excretion of various compounds e.g. hydroxyhexamide (Field *et al.*, 1967).

Unfortunately, in many of the studies reported on the interactions of phenylbutazone with other drugs, the effects were assessed simply by comparison of elimination half-lives before and after the co-administration of phenylbutazone. Because phenylbutazone may influence several of the processes involved in the metabolic disposition of the drug, there are problems in determining which particular interaction is responsible for the altered half-life. The difficulties

of attributing a particular mechanism to a given effect, are illustrated by the interaction of phenylbutazone with warfarin.

Concomitant administration of phenylbutazone results in an enhancement of the anti-coagulant effect of warfarin, originally this was attributed solely to the displacement of warfarin from plasma proteins, however it is now recognised that the mechanism is more complex. In addition to its effects on protein binding, phenylbutazone alters the metabolism of warfarin in a stereospecific fashion. Phenylbutazone has little effect on the metabolism of the R-enantiomer, but markedly inhibits the metabolism of the much more potent S-enantiomer (Lewis *et al.*, 1974.)

A further complication is the contribution made by phenylbutazone metabolites to these drug interactions. Oxyphenbutazone is also strongly protein bound, and partially displaces phenylbutazone from plasma proteins (Hvidberg *et al.*, 1968). Oxyphenbutazone has been demonstrated to inhibit the oxidative metabolism of phenytoin (Soda *et al.*, 1975) and phenylbutazone (Jahnchen & Levy, 1972) in the rat, and dicoumarol and warfarin in man (Davies & Thorgeirsson, 1971).

A number of non-linear kinetic processes have been implicated in the metabolic disposition of phenylbutazone in the horse. Dose-dependent plasma kinetics were demonstrated by Piperno *et al.* (1968), doubling the dose from 4.4 to 8.8 mg/kg increased the plasma concentrations of phenylbutazone by 33%, a further doubling to 17.6 mg/kg resulted in plasma concentrations 300% higher. The elimination half-life was also dose dependent, increasing from 3.5 h at the lowest dose to 6 h at the highest. Concomitant administration of phenylbutazone and isopropylaminophenazone increased the half-lives of both of these compounds, and this was demonstrated to be a consequence of decreased plasma clearance, rather than an effect on the volume of distribution

of these compounds (Jenny *et al.*, 1979).

Maylin (1974) reported an increase in the elimination half-life from 5.1 to 6.1 h, and an alteration in the composition of urinary metabolites (Table 1.8), following the intravenous administration of phenylbutazone on 4 consecutive days. These findings were unconfirmed by a subsequent study (Soma *et al.*, 1983), although other time-dependent urinary kinetics were noted. In the latter study, phenylbutazone was administered orally for 4 days, and on the 5th day given intravenously, the urinary concentrations of oxyphenbutazone decreased immediately after the intravenous administration of phenylbutazone, and this was accompanied by an increase in the urinary excretion of phenylbutazone.

There are a number of possible explanations for the non-linear urinary kinetics observed in our study. As mentioned earlier, if any of the processes involved in the absorption, distribution, metabolism or excretion of a compound is of a non-linear nature, this is likely to result in non-linear urinary kinetics. Any number of non-linear processes can occur simultaneously, and with the additional complication of metabolite mediated effects, it may be impossible to determine the exact nature of the mechanism(s) involved. However, it was hoped that by the utilisation of various graphical methods, and by comparison of the results obtained following the administration of both phenylbutazone and oxyphenbutazone, that many of the possible candidates which could theoretically be responsible for the non-linear urinary kinetics could be excluded.

Apart from interest in the mechanism(s) underlying the non-linear urinary kinetics, the resultant time dependent alteration in the metabolite profile is of forensic relevance, since it may enable the time of administration to be estimated.

METHODS

Details of the radiochromatographic analysis of phenylbutazone and its major metabolites in the 0-12, 12-24, 24-48 and 48-72 h pooled urine samples were presented in Chapter 3, and a summary of the results obtained by this method is presented in Figure 6.1. In addition to the analysis of these collective samples, phenylbutazone γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone were quantitated in individual urine samples. Because of the large numbers of samples involved, it was inappropriate to use the radiochromatographic method used previously, instead these compounds were separated by an identical HPLC method to that used earlier, but quantitation was by UV absorbance, rather than radiochemical content.

Chromatography.

The results from the earlier radio-HPLC analyses were used to construct calibration curves for phenylbutazone,γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone. These were prepared by comparison of the integrated areas of each peak of interest, against the ^{14}C content of the corresponding fraction of HPLC eluent. Plots of peak area versus ^{14}C content gave straight lines over the for all three compounds with correlation coefficients >0.995, and a typical calibration curve is presented in Figure 6.2. Aliquots (100-500 *ul*) of the individual urine samples were injected directly onto the HPLC column using a WISP 710B autoinjector (Waters Associates, Harrow, U.K.) and the concentrations of phenylbutazone, γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone calculated from their integrated peak areas.

FIGURE 6.1 **The urinary excretion of phenylbutazone γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone expressed as a percentage of the dose. Mean $\pm$ s.d. of experiments 1-4.**

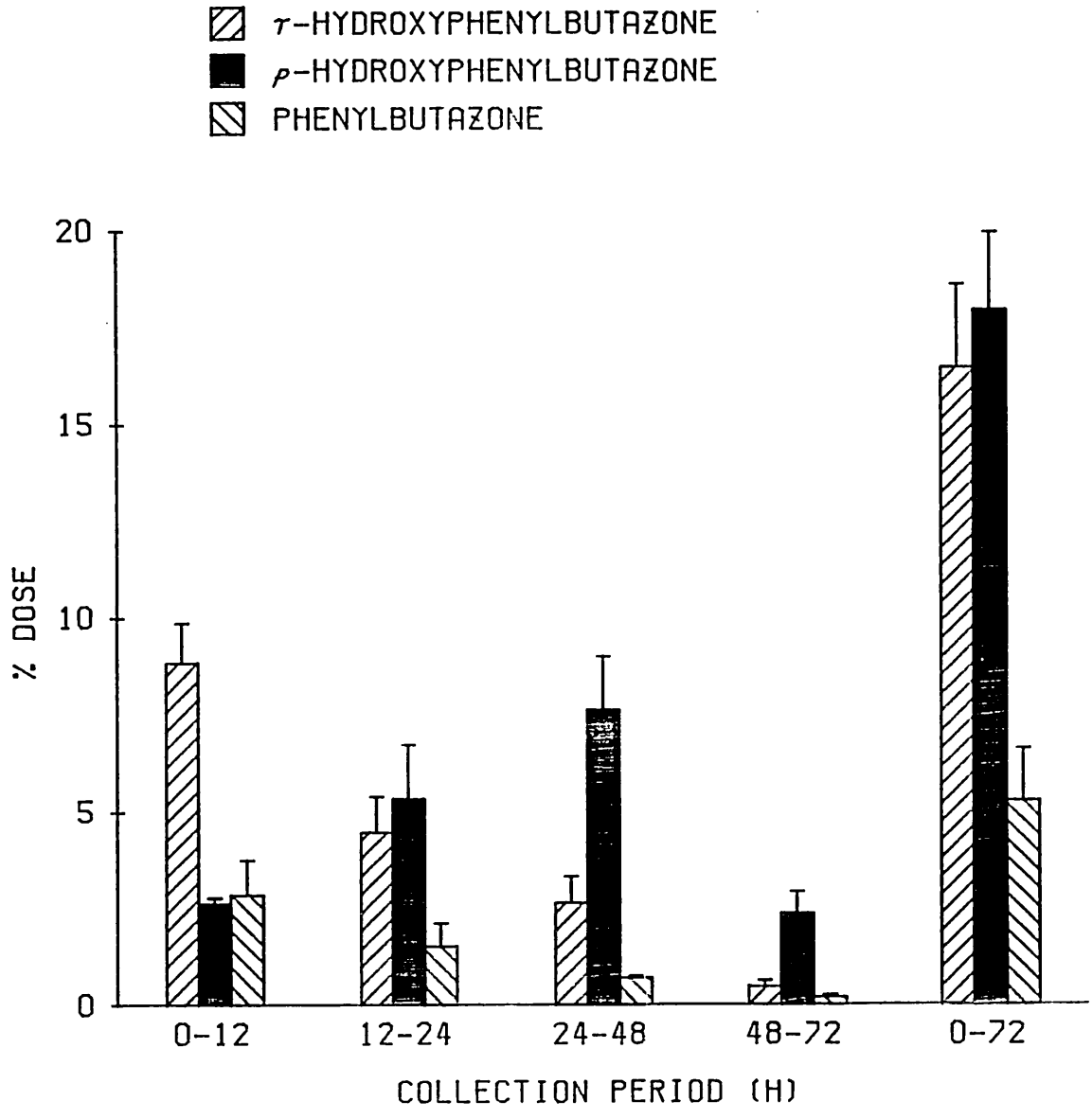
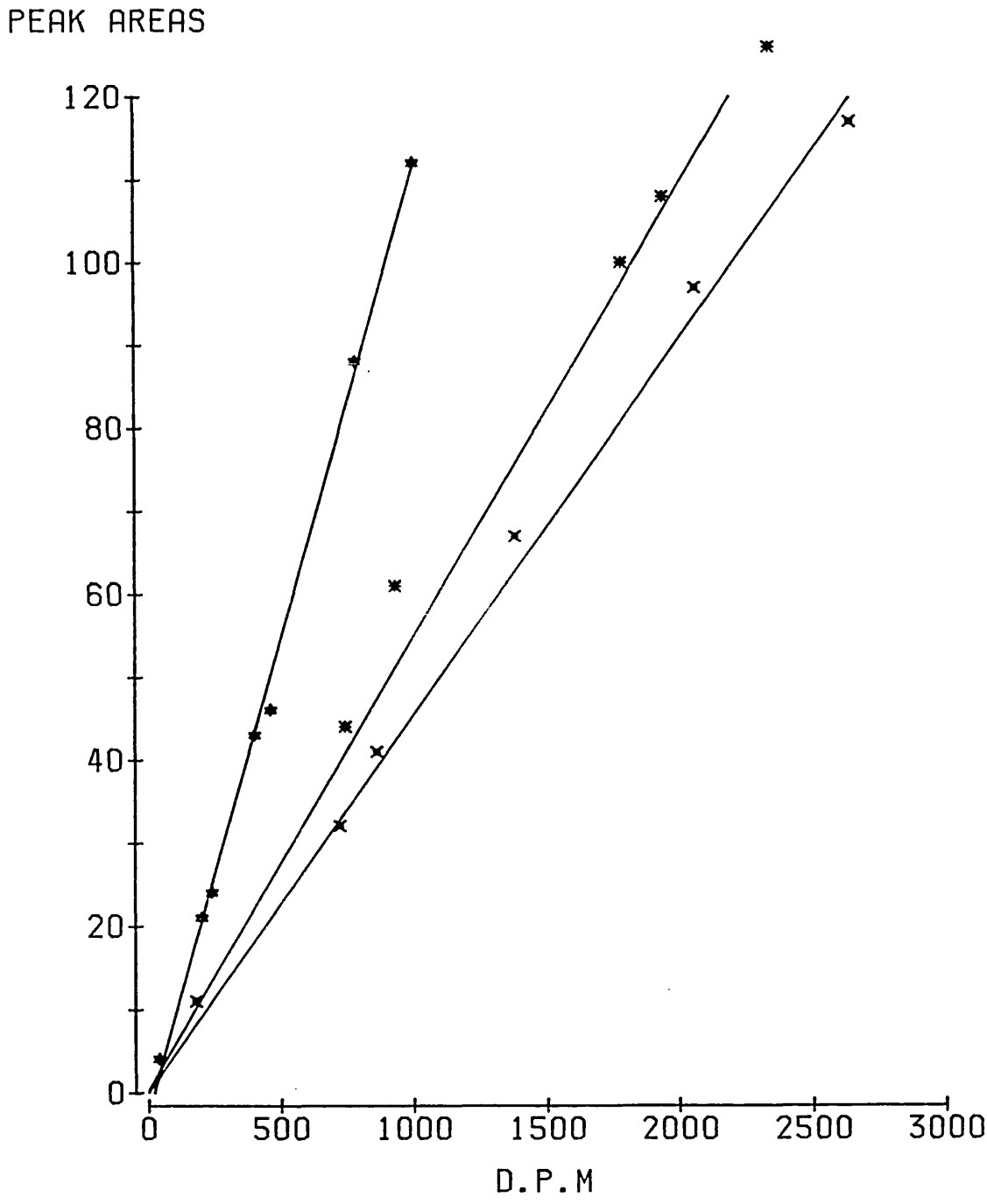


FIGURE 6.2 **Calibration curves for the assay of phenylbutazone and its major metabolites in urine**

Key: (✱) phenylbutazone, (✳) *p*-hydroxyphenylbutazone, (✕)γ-hydroxyphenylbutazone



RESULTS

Pooled urine samples

The ln excretion rates for urinary radioactivity, phenylbutazone, γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone were obtained from the analysis of the pooled urine samples, and plotted as a function of time (against the mid-point of the time interval). Similar results were obtained for both horses, and after both routes of administration, and the averaged results from experiments 1-4 are illustrated in Figures 6.3.

The semi-logarithmic plots of phenylbutazone excretion and γ-hydroxyphenylbutazone excretion declined linearly over the whole time period, illustrating the first-order urinary elimination of these compounds. Similar results were obtained for the excretion of total urinary radioactivity, although there was some evidence for an initial short plateau phase over the first 12h.

The results obtained for *p*-hydroxyphenylbutazone demonstrate the non-linear elimination of this compound. Over the time period 0-12 h the excretion rate of this compound was similar to that of phenylbutazone, but in the following 12h the excretion rate of *p*-hydroxyphenylbutazone almost doubled, and it was not until between 24-48 h after dosing that the excretion rate of this compound decreased. The results from one experiment (No.1) together with the equivalent plot obtained following the oral administration of *p*-hydroxyphenylbutazone are reproduced in Figure 6.4. In contrast to the situation resulting from the administration of phenylbutazone, the log excretion rate of *p*-hydroxyphenylbutazone following the administration of *p*-hydroxyphenylbutazone decreased in a linear fashion over the time period 0-72h.

FIGURE 6.3 **Semi-log plots of the urinary excretion of total radioactivity, phenylbutazone (PBZ),γ-hydroxyphenylbutazone (γ-OH) and *p*-hydroxyphenylbutazone (*p*-OH), following the administration of ^{14}C-phenylbutazone. Mean ± s.d. of experiments 1-4**

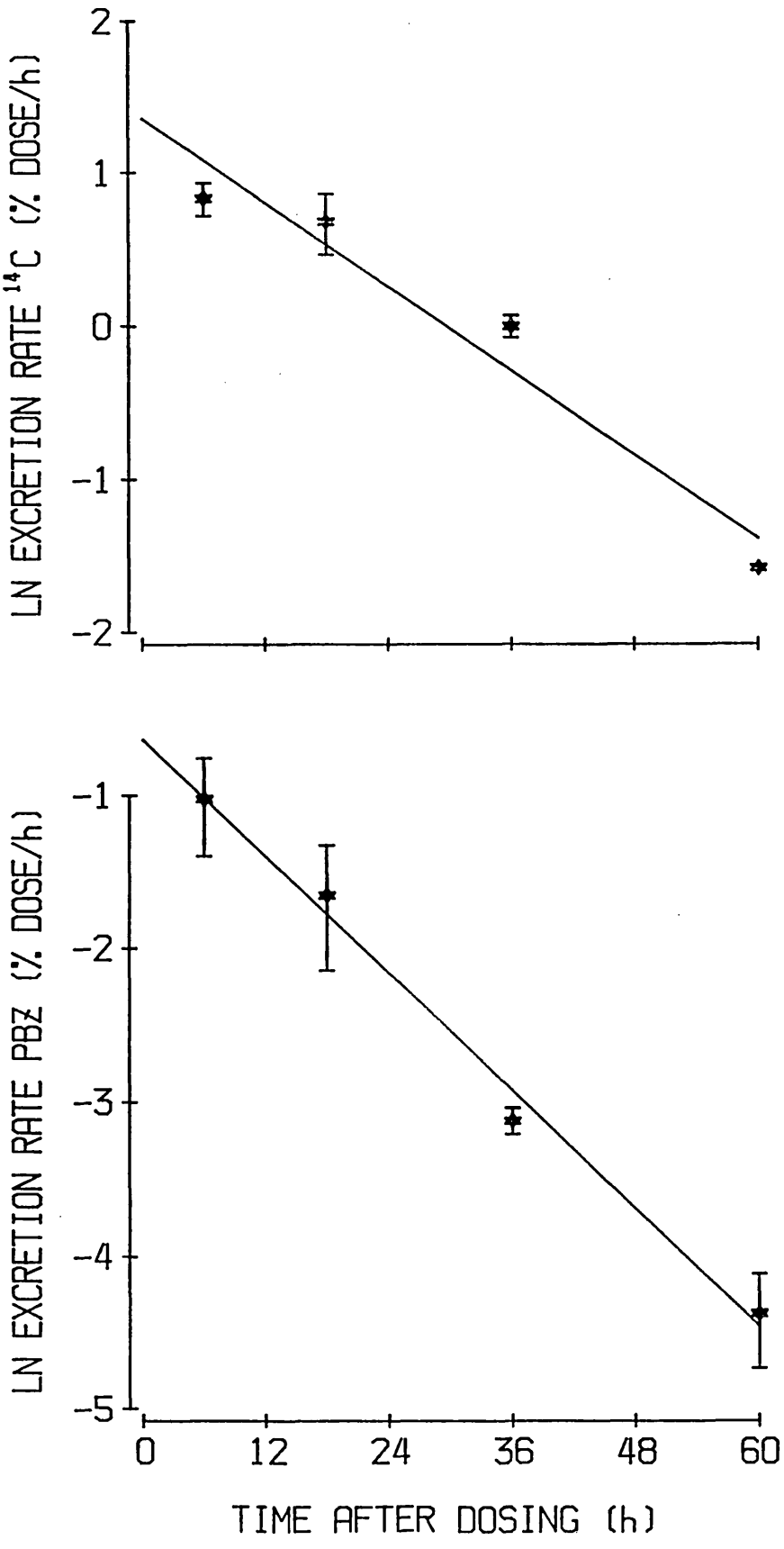


FIGURE 6.3 Continued

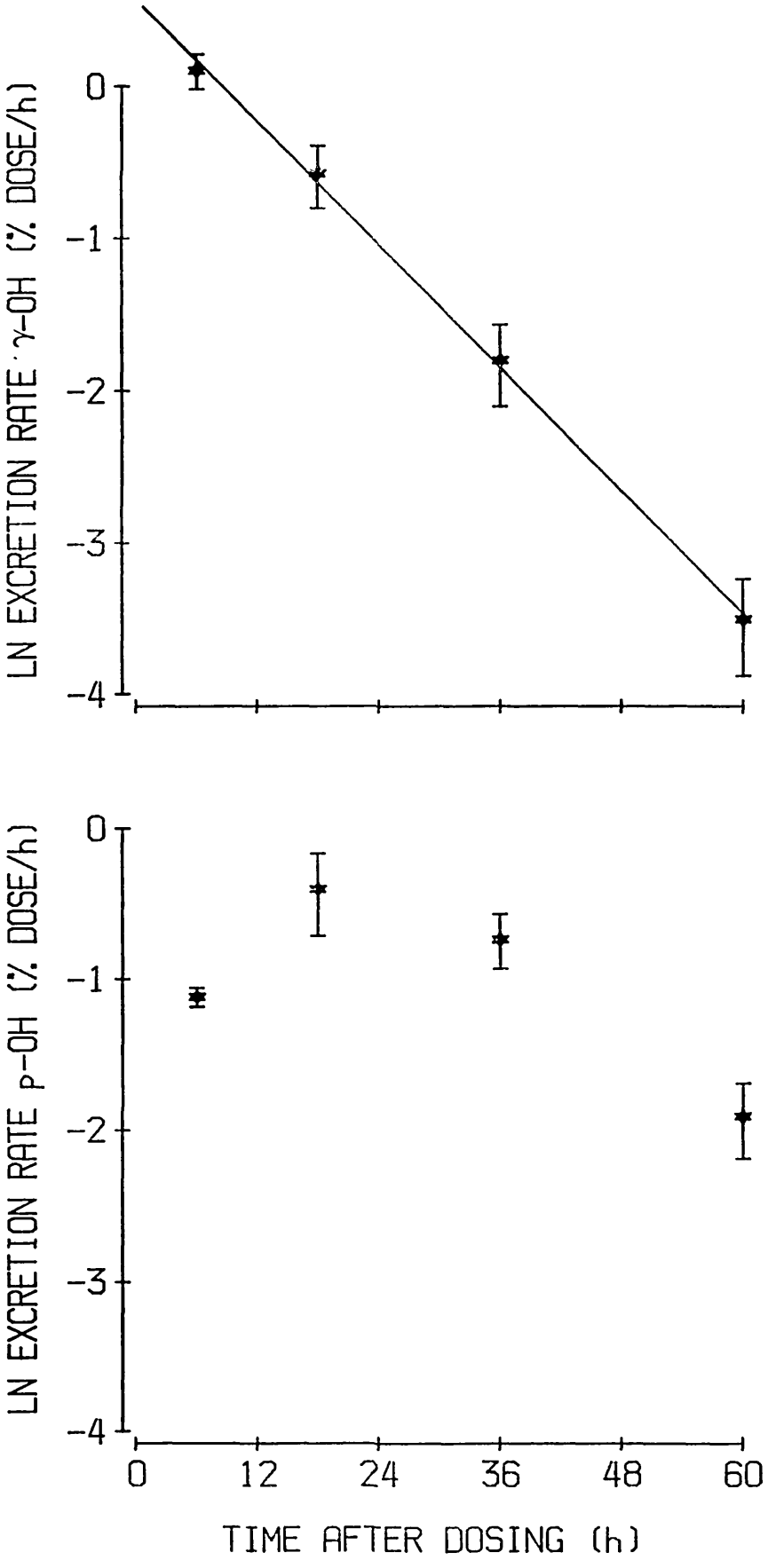
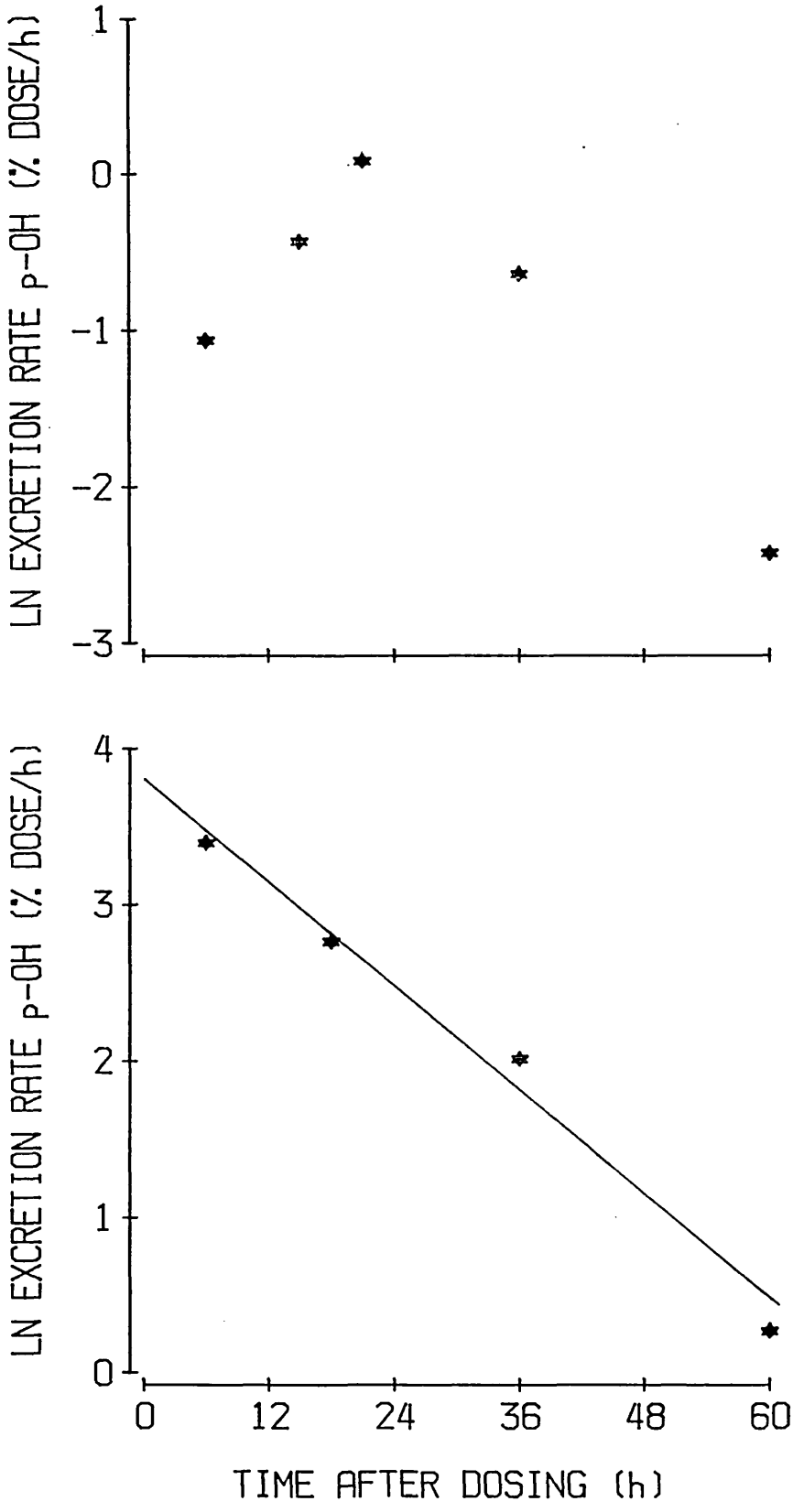


FIGURE 6.4 **Semi-log plots of the urinary excretion of *p*-hydroxyphenylbutazone following the oral administration of ^{14}C-phenylbutazone (upper graph) and ^{14}C-oxyphenbutazone (lower graph)**



Individual urine samples

Attempts to obtain a more accurate picture by plotting the log excretion rate of *p*-hydroxyphenylbutazone against time for individual urine samples, were frustrated because of the large variations in the urine flow-rates. The results for one experiment (No. 3.) are illustrated in Figure 6.5. The maximum value for the urinary excretion rate of *p*-hydroxyphenylbutazone was obtained some 30 h after dosing, however the excretion rates of γ-hydroxyphenylbutazone and phenylbutazone were also high for this urine sample, which was a consequence of the high urine flow rate.

Another way of representing the time dependent alteration in the urinary metabolite profile is to plot the ratios of the urinary concentrations of the various metabolites against the sample time. The results obtained by this method for experiments 1, 2, 3, 4, and 5 are illustrated in Figs. 6.6 to 6.10. The ratios of the urinary concentrations of *p*-hydroxyphenylbutazone/ γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone/phenylbutazone increased with time in an approximately linear fashion. The slopes of the lines were calculated by least squares regression analysis, and in all cases $r > 0.85$. There was considerable variation between experiments in the values of the slopes obtained for each metabolite ratio, and also within experiments in the relative gradients of the two lines.

FIGURE 6.5 **Effect of urinary flow rate on the log excretion rate of phenylbutazone, γ-hydroxyphenylbutazone and *p*-hydroxyphenylbutazone following the administration of phenylbutazone. Experiment No. 3**

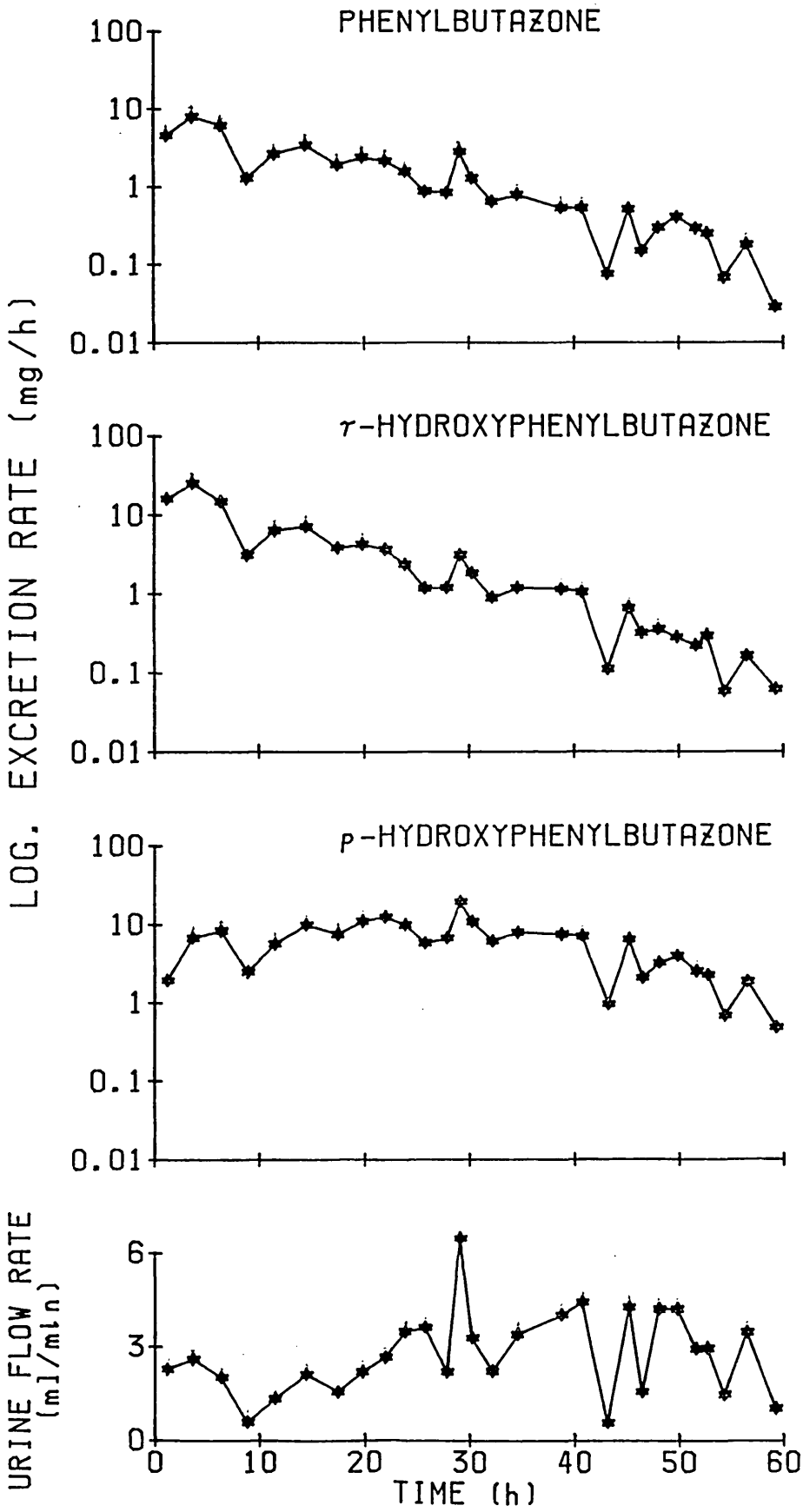


FIGURE 6.6 **The time dependent alteration in the relative urinary concentrations of *p*-hydroxyphenylbutazone (*p*-OH), phenylbutazone (PBZ), and γ-hydroxyphenylbutazone (γ-OH) following the oral administration of phenylbutazone to one horse. Experiment No. 1**

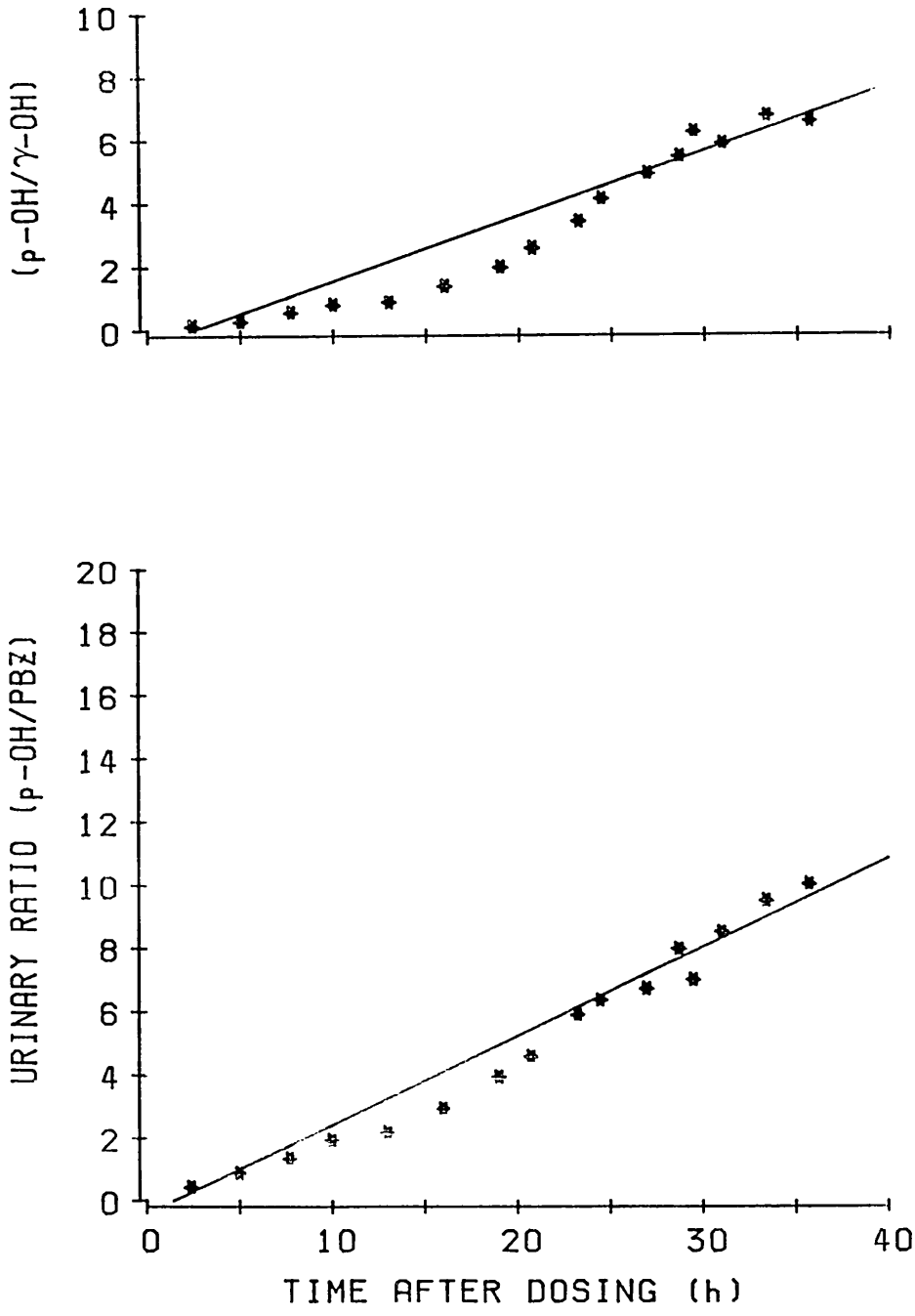


FIGURE 6.7 **The time dependent alteration in the relative urinary concentrations of *p*-hydroxyphenylbutazone (*p*-OH), phenylbutazone (PBZ), and γ-hydroxyphenylbutazone (γ-OH) following the oral administration of phenylbutazone to one horse. Experiment No. 2**

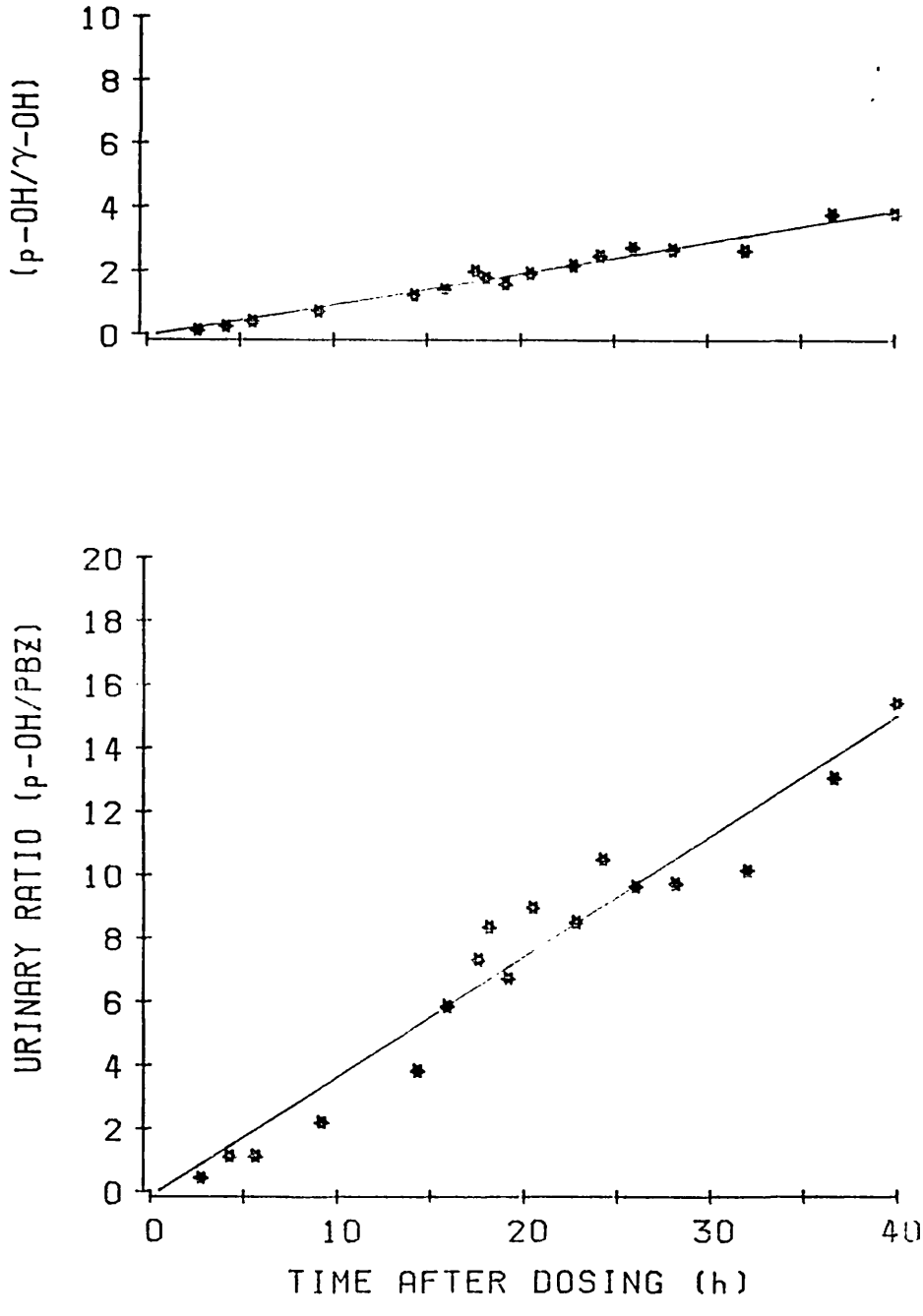


FIGURE 6.8 **The time dependent alteration in the relative urinary concentrations of *p*-hydroxyphenylbutazone (*p*-OH), phenylbutazone (PBZ), and γ-hydroxyphenylbutazone (γ-OH) following the intravenous administration of phenylbutazone to one horse. Experiment No. 3**

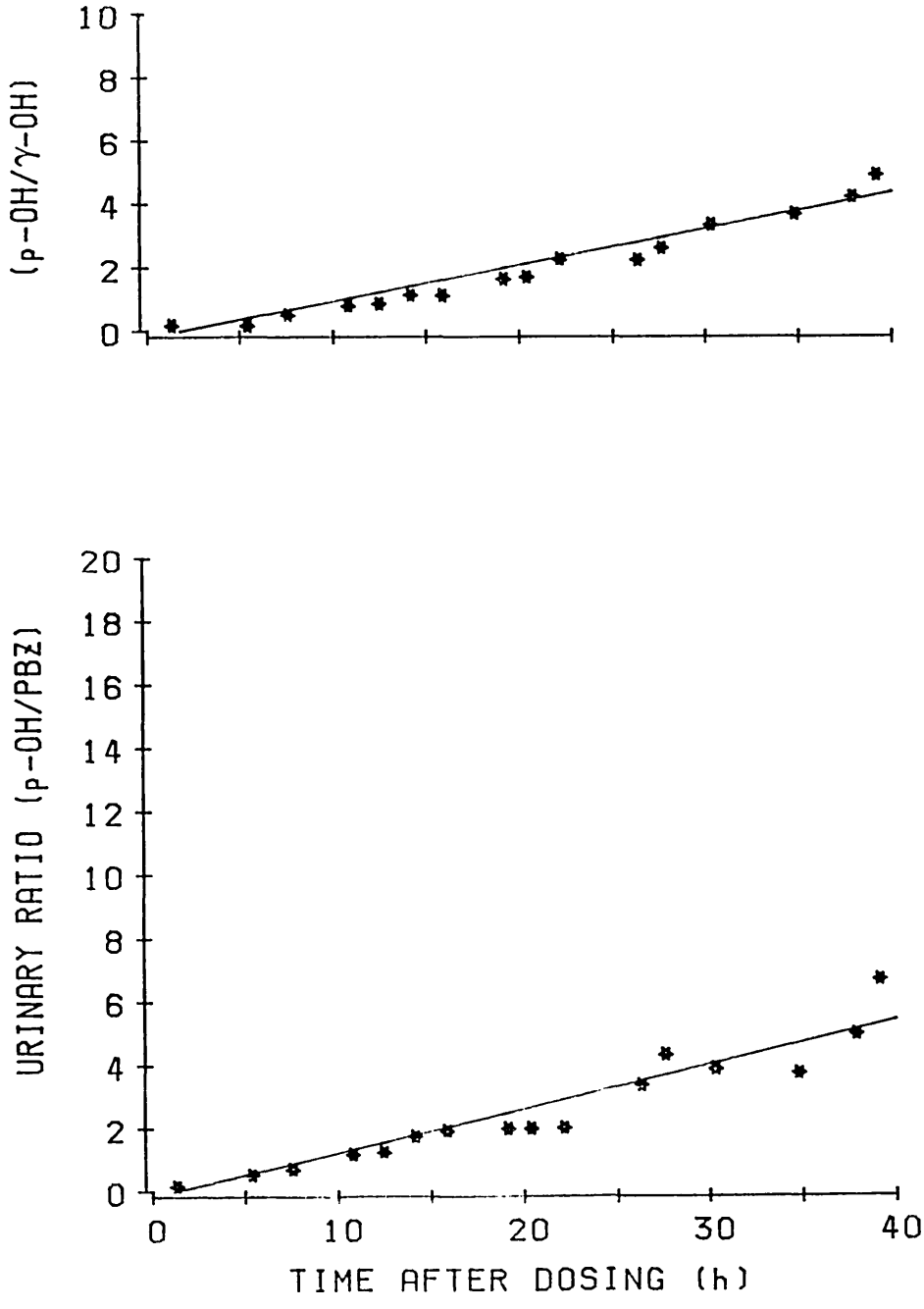


FIGURE 6.9 **The time dependent alteration in the relative urinary concentrations of *p*-hydroxyphenylbutazone (*p*-OH), phenylbutazone (PBZ), and γ-hydroxyphenylbutazone (γ-OH) following the intravenous administration of phenylbutazone to one horse. Experiment No. 4**

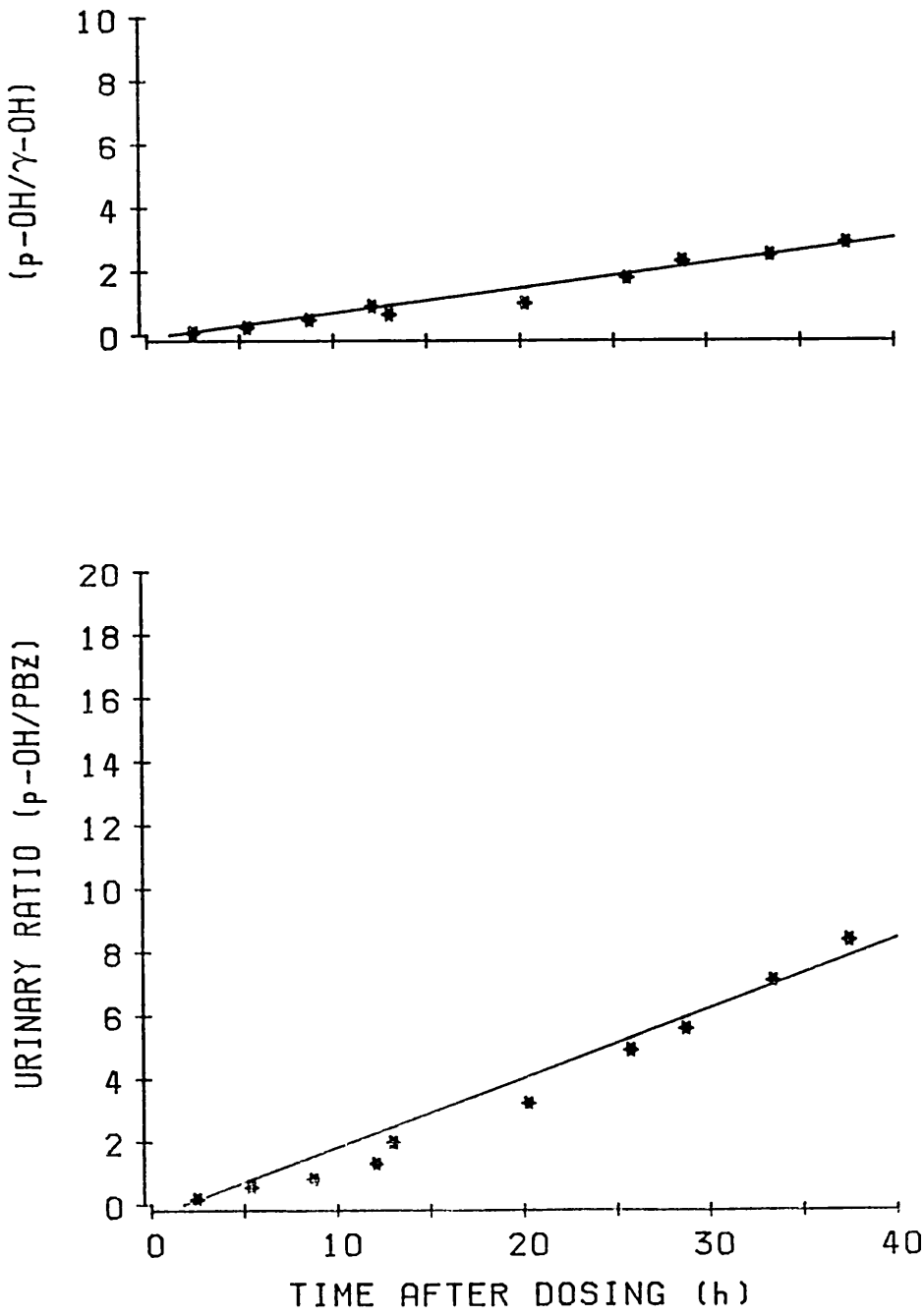
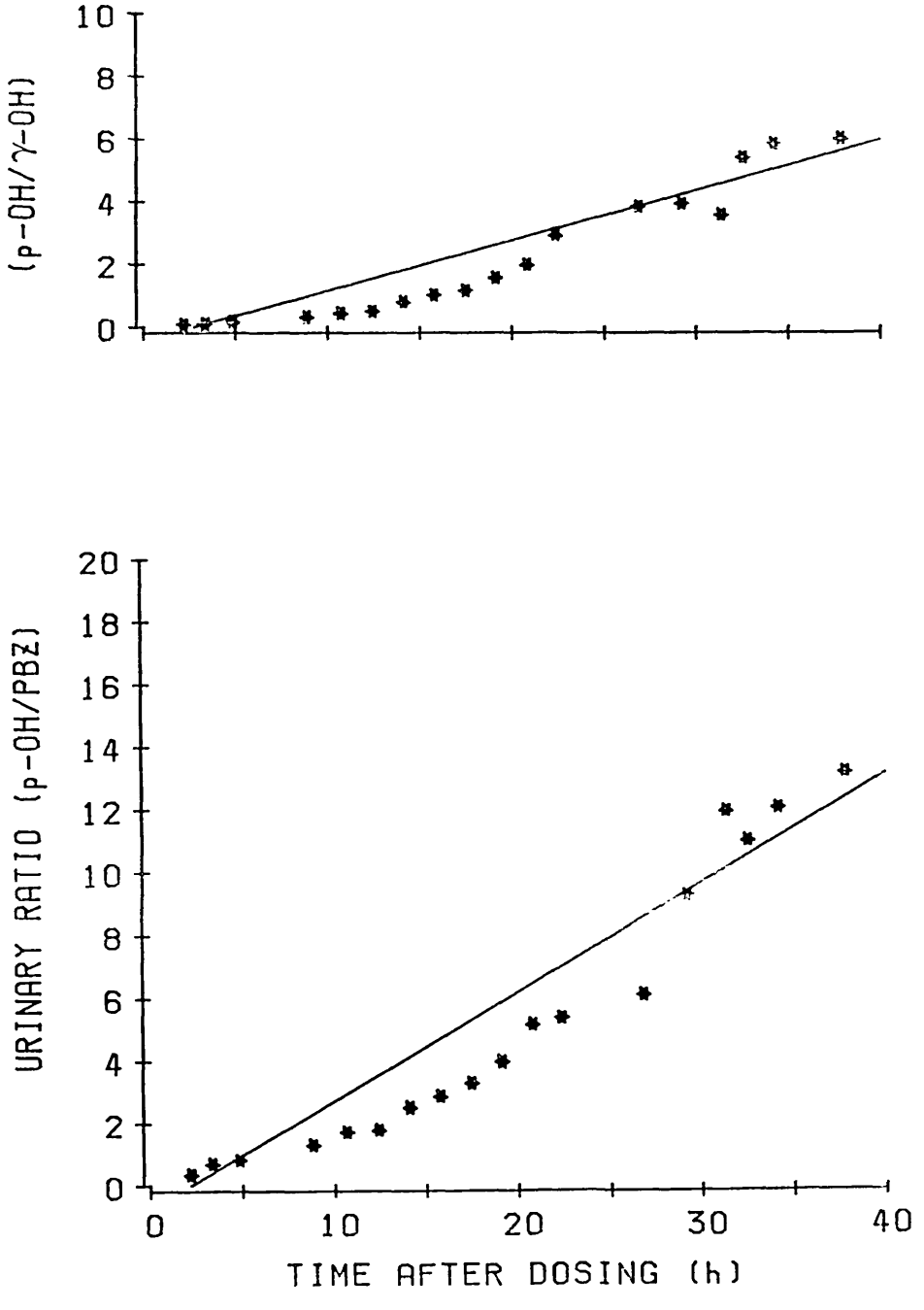


FIGURE 6.10 **The time dependent alteration in the relative urinary concentrations of *p*-hydroxyphenylbutazone (*p*-OH), phenylbutazone (PBZ), and γ-hydroxyphenylbutazone (γ-OH) following the oral administration of phenylbutazone to one horse. Experiment No. 5**



DISCUSSION

Because the urinary kinetics of both total radioactivity and of the individual compounds were essentially identical after both oral and intravenous administration of phenylbutazone, it seems reasonable to discount absorption in the production of the non-linear kinetics. Furthermore, since the plots of the ln excretion rates for the various metabolites, indicate that the alteration in the urinary metabolite profile with time is directly attributable to the non-linear excretion of *p*-hydroxyphenylbutazone, yet after the administration of this compound the ln excretion rate was linear, other potential explanations can be excluded. The enterohepatic recirculation of *p*-hydroxyphenylbutazone is a likely explanation for the anomalous urinary kinetics, but since there was no evidence for this after the administration of oxyphenbutazone this can be discounted, similarly the suggestion that the delayed excretion of *p*-hydroxyphenylbutazone is a consequence of a longer elimination half-life than phenylbutazone is contrary to the results obtained after the administration of oxyphenbutazone.

From this one can conclude that the non-linear urinary kinetics of *p*-hydroxyphenylbutazone do not result from the metabolic disposition of *p*-hydroxyphenylbutazone *per se*, but rather from either the non-linear metabolic formation of this compound, or else from some interaction between this compound and phenylbutazone and/or metabolites.

Non first order kinetics can result as a consequence of effects on the protein binding of drugs. In the horse oxyphenbutazone is bound to plasma proteins to a lesser degree than phenylbutazone (Gandal *et al.*, 1969), and although some mutual displacement has been reported in man (Hvidberg *et al.*, 1968), it seems extremely unlikely that any protein binding effects are responsible for this

effect, since one would expect the initial excretion rate of *p*-hydroxyphenylbutazone to be high (when plasma levels of phenylbutazone are high), rather than the opposite as is this case.

As discussed earlier (Chapter 5.) it seems likely that phenylbutazone and especially the two metabolites *p*-hydroxyphenylbutazone and γ-hydroxyphenylbutazone undergo active renal secretion. This is a saturable process, and as such, may account for the anomalous kinetics observed in our study, however since *p*-hydroxyphenylbutazone was the only compound whose elimination was inhibited, processes other than simple saturation of renal transport must be involved. Either one must assume that both phenylbutazone and γ-hydroxyphenylbutazone are preferentially transported, or else that phenylbutazone or a metabolite thereof inhibits the active transport of *p*-hydroxyphenylbutazone.

A likely candidate for the inhibition of active tubular secretion is γ-hydroxyphenylbutazone, Burns *et al.* (1958) demonstrated that this compound was considerably more potent than phenylbutazone as a uricosuric agent. Most uricosuric agents of this type have a paradoxical effect of inhibiting the tubular secretion of uric acid at low doses, whilst inhibiting its active reabsorption at higher doses. However there is no evidence that *p*-hydroxyphenylbutazone accumulates in plasma to any significant extent, and it seems unlikely that substantial amounts of *p*-hydroxyphenylbutazone accumulate elsewhere in the body.

The most likely explanation for the alteration in the urinary excretion of *p*-hydroxyphenylbutazone, involves the non-linear formation of this compound. Saturation of the enzyme(s) responsible for the *p*-hydroxylation of phenylbutazone is unlikely to account for the anomalous urinary kinetics of this compound, as the plot of the ln excretion rate of *p*-hydroxyphenylbutazone

against time does not have an initial plateau phase as would be expected with Michaelis-Menten kinetics, but rather an initial phase of depressed excretion, followed by a substantial increase, and therefore enzyme inhibition and/or enzyme induction must be involved. Enzyme inhibition can be by product (*p*-hydroxyphenylbutazone), substrate (phenylbutazone) or by some other metabolite, and can be of either a competitive or non-competitive nature.

Perrier *et al.* (1973) has postulated that the two compartment model usually associated with a biexponential concentration time curve, may result from product inhibition, where the apparent distribution phase represents the faster rate of metabolism prior to inhibition. The decline in the plasma concentrations of phenylbutazone is biexponential, but the data obtained from the ln excretion rate plots indicates that this is not a result of product inhibition, although this does not exclude product inhibition as the mechanism for the time dependent urinary kinetics.

Oxyphenbutazone has been demonstrated to inhibit the elimination of phenylbutazone, in rats the plasma half-life was increased from 2.95 to 4.91 h ($p<.001$) this was a consequence of both decreased plasma clearance and increased volume of distribution (Janchen & Levy, 1972). In the horse Tobin *et al.* (1977) demonstrated a statistically significant increase in the plasma elimination half-life of phenylbutazone (6.6mg/kg i.v.) from 7.6 to 9.4 h (f test $p<.01$) after pretreatment with oxyphenbutazone (11.0 mg/kg). In both the rat and the horse, the effects produced by oxyphenbutazone were relatively small for the doses used. Product inhibition alone, is unlikely to account for the anomalous urinary kinetics observed after the administration of phenylbutazone. Several groups (Gerring *et al.*, 1981; Soma *et al.*, 1985) have reported an increase with time in the plasma concentrations of oxyphenbutazone following the administration of phenylbutazone, which would not explain the increase in the

excretion of oxyphenbutazone observed after the initial phase.

Phenylbutazone inhibits the metabolism of various compounds, and there is some evidence that it can inhibit its own metabolism (see Aarbakke, 1978; Davies & Thorgeirsson, 1971). Substrate inhibition is an adequate explanation for the non-linear urinary kinetics of *p*-hydroxyphenylbutazone, although the dramatic rise in the excretion of this compound between 12 and 18h after the administration of phenylbutazone is strongly suggestive of the induction of this metabolic pathway.

Enzyme inhibition and enzyme induction involve entirely distinct mechanisms, and so there is no *a priori* reason why a compound should not be both, in fact the biphasic *in vivo* effect of a number of compounds considered as inhibitors, but which cause inhibition only in the acute phase, and thereafter act as enzyme inducers in a subchronic or chronic phase is well documented. And although classical enzyme induction as typified by phenobarbital pretreatment takes 3-4 days for significant effect (Conney *et al.* 1960) there is good evidence that other inducers acting by a different mechanism can enhance the microsomal oxidation of compounds immediately (Cinti, 1978). These compounds are able to enhance the *in vitro* activity of the drug metabolising enzymes, and this 'activation' is not mediated via increased protein synthesis, but possibly by some allosteric interaction with the enzyme system.

Serrone *et al.* (1961) noted a biphasic response in the effect of MPDC (N-methyl-3-piperidyl-(N',N')-diphenylcarbamate) on hexobarbital sleeping times. Up to 12 h after pretreatment with MPDC the sleeping times were increased, suggesting inhibition of the metabolism of hexobarbital, but after 24h the sleeping times were significantly reduced from control values suggesting enhanced hexobarbital metabolism. Several years later Cho *et al.* (1970)

demonstrated a similar effect with phenylbutazone. Pretreatment of rats with a single dose of phenylbutazone causes a non-competitive inhibition of ethylmorphine oxidation for a period of 6-12 hours, but thereafter the demethylation of ethylmorphine was enhanced.

The identity of the compound(s) responsible for this initial inhibition and subsequent enhancement of enzyme activity is unknown, although since Cho et al. (1970) reported a competitive inhibition *in vitro* and a non-competitive inhibition *in vivo*, the possibility that it is a metabolite of phenylbutazone, rather than phenylbutazone *per se* cannot be excluded. Kitagawa *et al.* (1971) observed a 40% increase in the rate of the *in vivo* microsomal hydroxylation of phenylbutazone on the addition of 0.06mM *p*-aminophenol, this effect was also observed with aniline, although this was limited to microsomes from male rats. Faigle *et al* (1977) detected minute amounts of aniline after the administration of phenylbutazone to man, although since the hydrolytic cleavage of the pyrazolidione nucleus does not take place *in vivo* to any significant degree it is doubtful whether this is involved in the increases in enzyme activity.

Further evidence for the initial phase of enzyme inhibition is provided by the demonstration of a dose-dependent half-life for phenylbutazone in the horse (Piperno *et al.*, 1968), rat (Varma, 1979) and dog (Dayton *et al.*, 1967; Kitagawa *et al.*, 1968). Although the 'apparent half-life' will increase at high doses, Michaelis-Menten type kinetics cannot produce a true dose-dependent increase in the terminal elimination half-life.

The time dependent alteration in the urinary metabolite profile can be explained by either the inhibition of the enzymes responsible for the *p*-hydroxylation of phenylbutazone, or by an induction or activation of this enzyme soon after the administration of phenylbutazone. Either one of these

mechanisms will adequately explain all of our observations, but for the reasons outlined above, both of these processes may be involved.

Maylin (1974) originally reported the time dependent alterations in the phenylbutazone urinary metabolite profile, which led to suggestions (Tobin, 1979) that reasonable estimates of the time of administration could be made by a comparison of the ratios of phenylbutazone to its metabolites. This approach was adopted by McBurney (1981) to estimate the time and dose since the ingestion of diazepam by the measurement of the urinary ratios of the three diazepam metabolites, oxazepam, desmethyldiazepam and temazepam.

The proposed underlying mechanism, responsible for the non-linear urinary kinetics of phenylbutazone has several implications for the use of this ratio in determining the time of administration. In addition to the numerous factors such as age, sex, diet, environment, illness etc. which can influence the metabolism of many compounds, there is the added complication of the inhibition/activation/induction effect. Thus one might expect the urinary kinetics to be influenced by factors such as the amount and rate of drug administered, by multiple or single dosing, the co-administration of other compounds, and because of differences in the pK_a values of the three compounds, urinary pH.

The variation in the results we obtained following the administration of phenylbutazone to two different horses on a total of five separate occasions, suggest that although under controlled conditions, reasonable estimates of the time of administration could be made from the measurement of the concentrations of phenylbutazone and its two major metabolites in a single urine sample this method is unlikely to be applicable for the implementation of 'time rules'.

However despite these reservations, the measurement of the ratios of the urinary metabolites of phenylbutazone might enable one to distinguish between a horse that has been given a small dose the day of the race, and one that had been treated with phenylbutazone several days before the raceday, but still had traces of phenylbutazone in the urine.

CHAPTER 7.

THE DERIVATISATION AND MASS SPECTRAL FRAGMENTATION PATHWAYS OF PHENYLBUTAZONE ANALOGUES

INTRODUCTION

Despite the interest in the analysis of pyrazolidinediones in general, and in phenylbutazone and oxyphenbutazone in particular, there is very little information available on the derivatisation, and mass spectra of these compounds. The usual purpose of derivatising functional groups is to modify the physicochemical properties of the compound to suit a particular analytical procedure. In the majority of forensic laboratories the final confirmatory identification of most doping agents is by GCMS, and many metabolic studies also now rely on GCMS for the identification of metabolites. Although phenylbutazone can be analysed directly by this method, it is necessary to derivatise all of the known metabolites of phenylbutazone prior to analysis by GCMS. There is a wide variety of derivatising agents available, but methylation and/or silylation are the usual methods employed for the types of functional groups present in phenylbutazone and its metabolites.

Midha *et al.* (1974a) reported a method for the GC analysis of phenylbutazone and oxyphenbutazone in which the compounds were derivatised by on-column 'flash methylation' with trimethylanilinium hydroxide. Under these conditions phenylbutazone gave two GC peaks (R_t 7.1 min and 14.9 min) with peak areas in the ratio 5:1. These two compounds were analysed by GCMS, and the mass spectra obtained for both of these compounds contained a molecular ion at *m/z* 322, indicating that the two compounds were isomeric monomethyl derivatives of phenylbutazone. Phenylbutazone contains two possible sites for attachment of the methyl group, either an enolised carbonyl group, or the C_4 position of the heterocyclic ring (I and II in Figure 7.1). The authors concluded that the major product was the O-methyl derivative of phenylbutazone (1,2-diphenyl-3-methoxy-4-*n*-butyl-5-oxo-pyrazoline).

Similar results were obtained with oxyphenbutazone, although here the situation is slightly complicated because oxyphenbutazone contains a phenolic group which can also be methylated. Like phenylbutazone, the major product from the flash methylation of oxyphenbutazone was assigned as an O-methyl derivative (1-phenyl-2-(*p*-methoxyphenyl)-3-methoxy-4-*n*-butyl-5-oxo-pyrazoline): however, the mass spectrum Midha *et al.* (1974a) attributed to this compound was essentially identical to that reported by Unterhalt (1972) as the C-methyl derivative (1-phenyl-2-(*p*-methoxyphenyl)-4-methyl-4-*n*-butyl-3,5-dioxo-pyrazoline).

Attempts to derivatise γ-hydroxyphenylbutazone by an identical flash methylation method (Midha *et al.*, 1974b) resulted in 4 GC peaks, 3 of which were identified as breakdown products. Interestingly, the authors concluded that the fourth compound was the C-methyl derivative of γ-hydroxyphenyl-butazone (1,2-diphenyl-4-methyl-4-(3-methoxy)-butyl-3,5-dioxopyrazoline), but gave no explanation as to why on this occasion, a C-methyl derivative should be formed, when previously they had reported the preferential formation of O-methyl derivatives by this method.

At about the same time, Locock *et al.* (1974) reported the main mass spectral fragmentation pathways of phenylbutazone and some other pyrazolidinediones. Although they did not specifically address the situation regarding the O-methyl and C-methyl derivatives of phenylbutazone or oxyphenbutazone, they did determine the fragmentation pathways of some simpler C-methyl derivatives, and these were in agreement with the general pathways previously outlined for the C-methyl derivative of oxyphenbutazone by Unterhalt (1972).

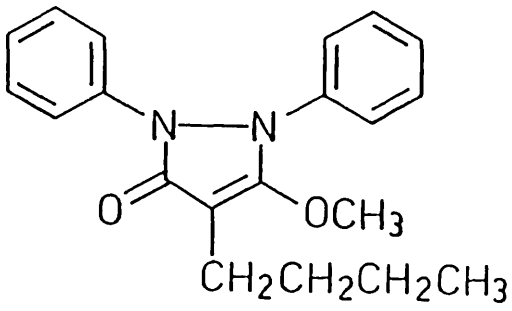
Several years later, Midha *et al.* (1978) reported a method for the GC analysis of γ-ketophenylbutazone, and again several products were obtained. In the article

the authors stated that the major product of the reaction of diazomethane with phenylbutazone was the C-methyl derivative, the characterisation being apparently by preparative TLC and ^{1}H-NMR, although no details are given. Based on this, and presumably also on the assumption that γ-ketophenylbutazone and phenylbutazone will form similar derivatives, they concluded that the major product obtained by treating γ-ketophenylbutazone with diazomethane was the C-methyl derivative.

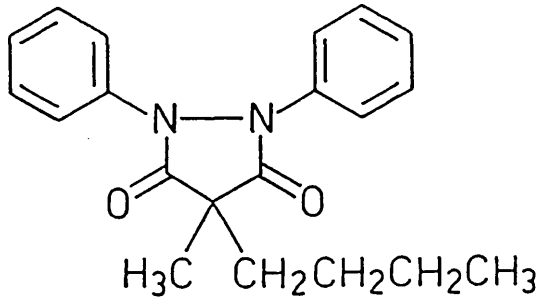
Although the basic fragmentation patterns of phenylbutazone and oxyphenbutazone have been determined previously (Unterhalt, 1972; Locock *et al.*, 1974; Sabih, 1974) the literature contains little information on the mass spectra of other phenylbutazone metabolites, and the derivatisation pathways of these compounds are by no means certain. Many of the known metabolites of phenylbutazone are isomeric, resulting from the introduction of a single hydroxyl group into the molecule, consequently molecular weight measurements are of little use for the identification of these compounds, and the determination of the position of hydroxylation by mass spectrometry must rely on the different fragmentation patterns of each isomer.

Because of the uncertainty concerning the regioselectivity of the methylation of phenylbutazone and related compounds, it was decided to synthesise and isolate sufficient quantities of both O- and C- methyl phenylbutazone, for definitive structural elucidation by IR and ^{1}H-NMR spectroscopy, before investigating their mass spectral characteristics.

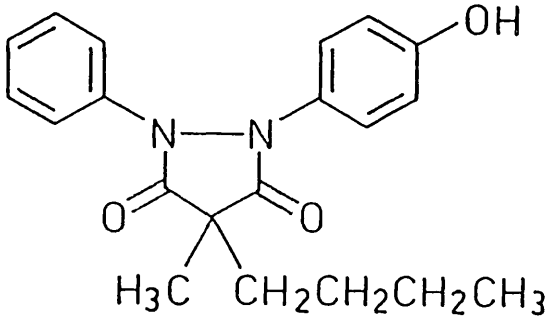
FIGURE 7.1 Structures of the C-methyl and O-methyl derivatives of phenylbutazone and oxyphenbutazone



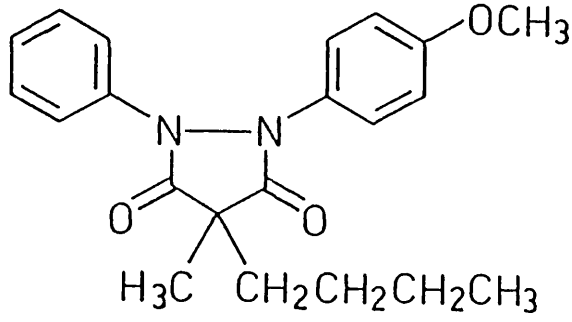
I



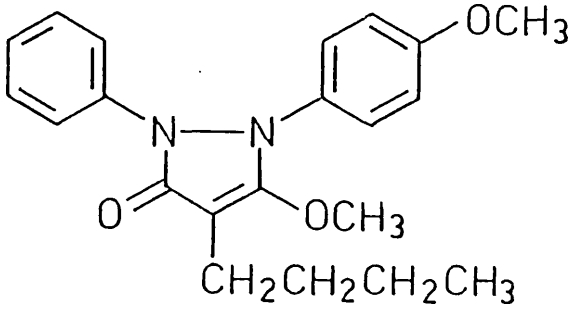
II



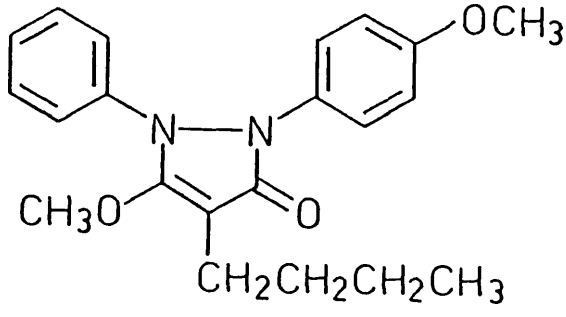
III



IV



Va



Vb

EXPERIMENTAL

Compounds

Authentic samples of the compounds listed in Table 7.1 were donated by Ciba-Geigy. Diazald (N-methyl-N-nitroso-*p*-toluenesulfonamide), iodomethane, iodomethane-d_3, Carbitol-d_1 (2-(2-ethoxyethoxy)ethan(ol-*d*)) and sodium deuteroxide (30% soln. in D_2O) were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). N,O-bis-trimethylsilylacetamide and trimethyl-chlorosilane were purchased from Sigma Ltd. (Poole, Dorset, U.K.). Other materials were reagent-grade chemicals purchased from usual U.K. sources, and used without further purification.

Chromatography

The equipment used for the HPLC analysis was described earlier in Chapter 2., compounds were separated using a stainless steel column (250 x 5 mm i.d.) packed with 5*u* ODS-Hypersil (Shandon Southern Products Ltd., Runcorn, U.K.), and eluted with a degassed mobile phase of methanol-water (60:40 v/v) adjusted to pH 3.0 with glacial acetic acid, at a flow rate of 1.5 ml/min.

Mass Spectrometry

Mass spectrometry was performed in EI mode (ionizing potential 70 eV) at the Horseracing Forensic Laboratory, Newmarket. Samples were introduced either by direct insertion using a Finnigan 4000 instrument under the control of a Finnigan 6110 data system, probe temperature 180°, or by GC-MS using a Finnigan 1020 instrument. For the GCMS a capillary column of fused silica (8m x .32mm i.d.) coated with a chemically bonded phase of dimethylsiloxane was used (Cat. No. 633225 Alltech U.K. Ltd.). The conditions were set to give a linear gas flow of 40 cm/sec. He, an injection port temperature of 240° a

detector oven temperature of 300° and a manifold temperature of 90.° There were slight variations in the temperature programs used for the GC analysis of individual compounds, but typically the temperature was initially set at 70° and programmed to rise at 15°/min to 290.°

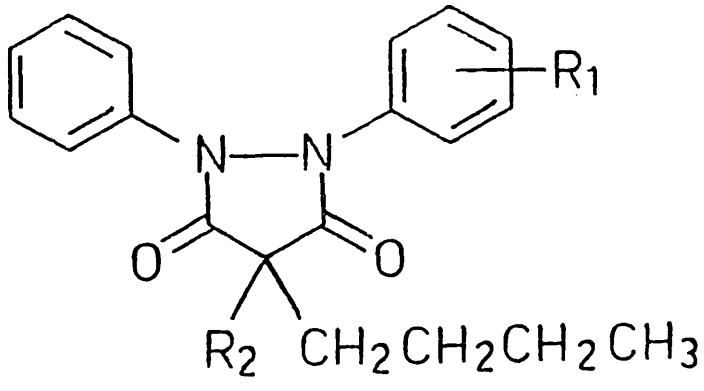
Preparation of deuterodiazomethane

Diazald (0.5g) in anhydrous ether (8ml) was cooled in ice and a mixture of carbitol-d_1 (5g) and 30% sodium deuteroxide (2g) in anhydrous ether (5ml) was added. After cooling for 10 min, the flask containing the mixture was attached to an apparatus comprising a cold finger condenser and collecting flask and the ethereal solution of deuterodiazomethane was gently distilled.

Preparation of derivatives

Samples of authentic standards (1mg) were methylated either by heating the sample at 60° for 1 h with iodomethane (50*u*l) in acetone (100*u*l) over K_2CO_3, or at room temperature with an ethereal solution of diazomethane. Diazomethane in ether was added to the sample until the yellow coloration persisted, and the sample allowed to react for 10 min, before the solvent was removed in a stream of nitrogen. Deuteromethylated derivatives were prepared by similar methods, using either deuterodiazomethane or iodomethane-d_3. Trimethylsilyl (TMS) derivatives were prepared by heating the sample (1mg) at 80° for 2 h with a mixture of N,O-*bis*-trimethylsilylacetamide (100*u*l), and trimethylchlorosilane (50*u*l), and methyl-trimethylsilyl (Me-TMS) derivatives similarly formed after prior methylation with iodomethane.

TABLE 7.1 Structures of phenylbutazone and related compounds



Compound.	R1	R2	Butyl group
Phenylbutazone	H	H	-
p-Hydroxyphenylbutazone	*p*-OH	H	-
m-Hydroxyphenylbutazone	*m*-OH	H	-
m,m-Dihydroxyphenylbutazone *	*m*-OH	H	-
β-Hydroxyphenylbutazone	H	H	2-OH
γ-Hydroxyphenylbutazone	H	H	3-OH
ω-Hydroxyphenylbutazone	H	H	4-OH
γ-Ketophenylbutazone	H	H	3 C=O
p,γ-Dihydroxyphenylbutazone	*p*-OH	H	3-OH
4-Hydroxyphenylbutazone	H	OH	-

* *m*-hydroxyl group in both phenyl rings.

Synthesis of O-methyl phenylbutazone (I)

Ethanolic KOH (10ml) was added to a solution of Diazald (N-methyl-N-nitroso-*p*-toluenesulphonamide, 2.0g) in diethyl ether (30ml). The solution was gently warmed, and the ethereal solution of diazomethane distilled over into a flask containing phenylbutazone (1.54g, 0.5mmol) in diethyl ether (5ml). The mixture was left at room temperature for 30 min, and then the excess diazomethane destroyed with 15ml glacial acetic acid. The solution was added to 250ml of aq. K_2CO_3, the organic phase removed and washed with aq. K_2CO_3 (4 x 250 ml). The extract was evaporated to dryness under reduced pressure, and the crude off-white product (0.81g, 50% yield) recrystallised from ethanol/water to give a white crystalline solid (m.p. 80-84°). HPLC analysis of the purified product revealed the successful formation of the O-methyl derivative, R_t 15.7 min, accompanied by small amounts (1-2%) of the C-methyl derivative of phenylbutazone R_t 13.0 min. There was no peak corresponding to phenylbutazone R_t 9.7 min.

Synthesis of C-methyl phenylbutazone (II)

Iodomethane (10ml, 0.16mol) was added to a solution of phenylbutazone (1.54g, 0.5mmol) in acetone (30ml) containing K_2CO_3 (0.5g). The solution was refluxed at 60-70° under a $CaCl_2$ drying tube for 30 min, allowed to cool and then added to 250ml of dilute aq. K_2CO_3. The white precipitate was extracted with diethyl ether (2 x 50ml), the organic phase washed with aq. K_2CO_3 (4 x 250ml), decanted and the solvent removed under reduced pressure. The crude product (0.76g, 47% yield) was recrystallised from ethanol to give a white crystalline solid (m.p. 110-112°). HPLC analysis of the final product resulted in one peak R_t 13.0 min.

Attempted synthesis of C-methyl-p-methoxyphenylbutazone (IV)

Iodomethane (20ml, 0.32mol) was added to a solution of oxyphenbutazone (13g, 0.04mol) in acetone (75ml) containing K_2CO_3 (1g). The solution was refluxed at 60-70° under a $CaCl_2$ drying tube for 30 min, allowed to cool and then added to 250ml of dilute aq. K_2CO_3, and the white precipitate extracted with diethyl ether (2 x 50ml). The ethereal solution was washed with aq. K_2CO_3 (4 x 250ml), separated and the solvent removed under reduced pressure. The crude product (8.25g, 62% yield) was recrystallised from ethanol/water, to give a white solid (m.p. 74-77°). HPLC analysis of the final product from the iodomethane-methylation of oxyphenbutazone revealed one compound R_t 8.2 min, with a longer retention time than the starting material (R_t 5.9 min), but no peak was obtained on GCMS, suggesting that the compound was incompletely methylated.

RESULTS

Proof of structures of O-methyl and C-methyl phenylbutazone

Comparison of the IR spectra of phenylbutazone and the two methyl derivatives supports the proposed structural assignments. The prominent bands at 1720 and 1755 cm^{-1} (C=O stretch) obtained with phenylbutazone were equally prominent in the spectrum of the C-methyl derivative (II) (1725 and 1760 cm^{-1}), but absent in the spectrum of the O-methyl derivative (I). Further confirmation was obtained from ^{1}H-NMR studies. The triplet at δ 3.35 produced by the proton at the C_4 position of phenylbutazone was absent in the spectra of both the methylated derivatives, and replaced by a singlet integrating for 3 protons, at δ 3.9 in the spectrum of the O-methyl derivative, and at δ 1.5 for the C-methyl derivative. The multiplet δ 2.0 arising from the methylene group adjacent to the heterocyclic ring of phenylbutazone, appeared as a triplet (δ 2.4) in the spectrum of the O-methyl derivative, and as a multiplet (δ 1.9) for the C-methyl derivative.

Identification of the product of the methylation of oxyphenbutazone.

Inspection of the ^{1}H-NMR spectra for this compound and comparison with the spectra obtained for oxyphenbutazone itself and C-methylphenylbutazone confirmed that this compound was the C-monomethyl derivative of oxyphenbutazone (1-phenyl-2-(*p*-hydroxyphenyl)-4-methyl-4-*n*-butyl-3,5-dioxopyrazoline, (III). The triplet arising from the C_4 proton δ 3.4 in oxyphenbutazone was replaced by three proton singlet at δ 1.5 and the multiplet arising from the methylene group (δ 2.0) was shifted downfield (δ 1.9). The broad singlet at δ 6.5 produced by the phenolic proton was still present in the spectra of the methylated derivative, indicating that the phenolic group was not methylated.

The mass spectra of phenylbutazone, O-methylphenylbutazone (I), and C-methylphenylbutazone (II), are reproduced in Figures 7.2, 7.3 and 7.4, and partial spectra of these compounds together with the deuteromethyl derivatives in Table 7.2. The mass spectrum obtained after the direct insertion of the C-methyl derivative of oxyphenbutazone (III) is reproduced in Figure 7.5.

FIGURE 7.2 Mass spectrum of phenylbutazone

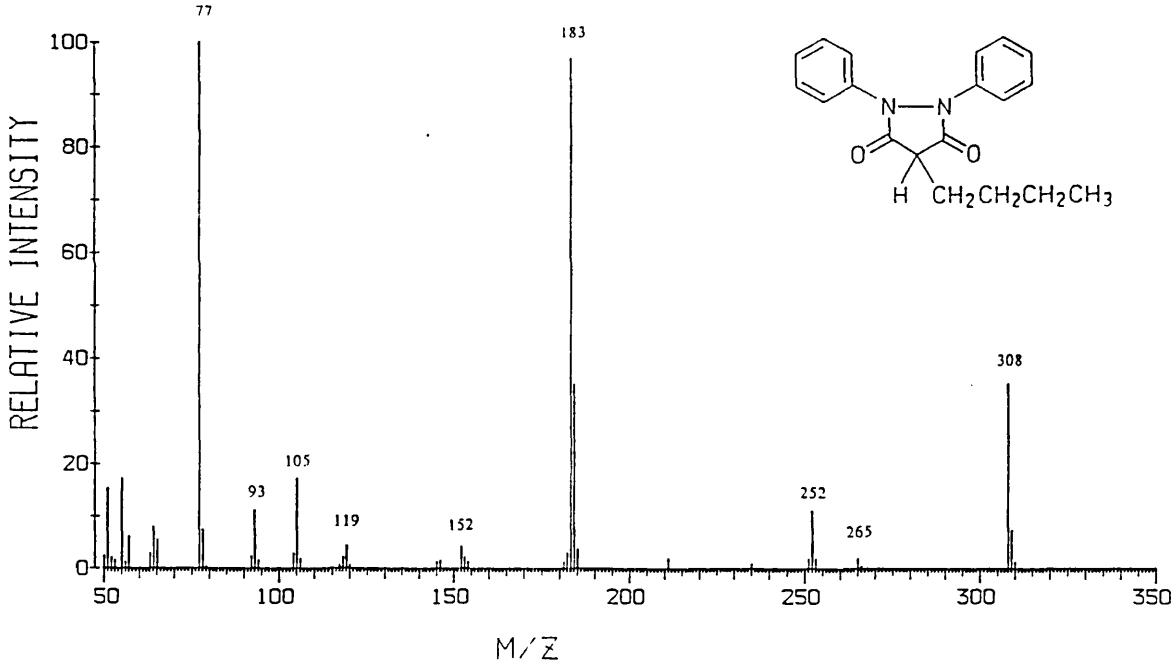


FIGURE 7.3 Mass spectrum of O-methylphenylbutazone (I)

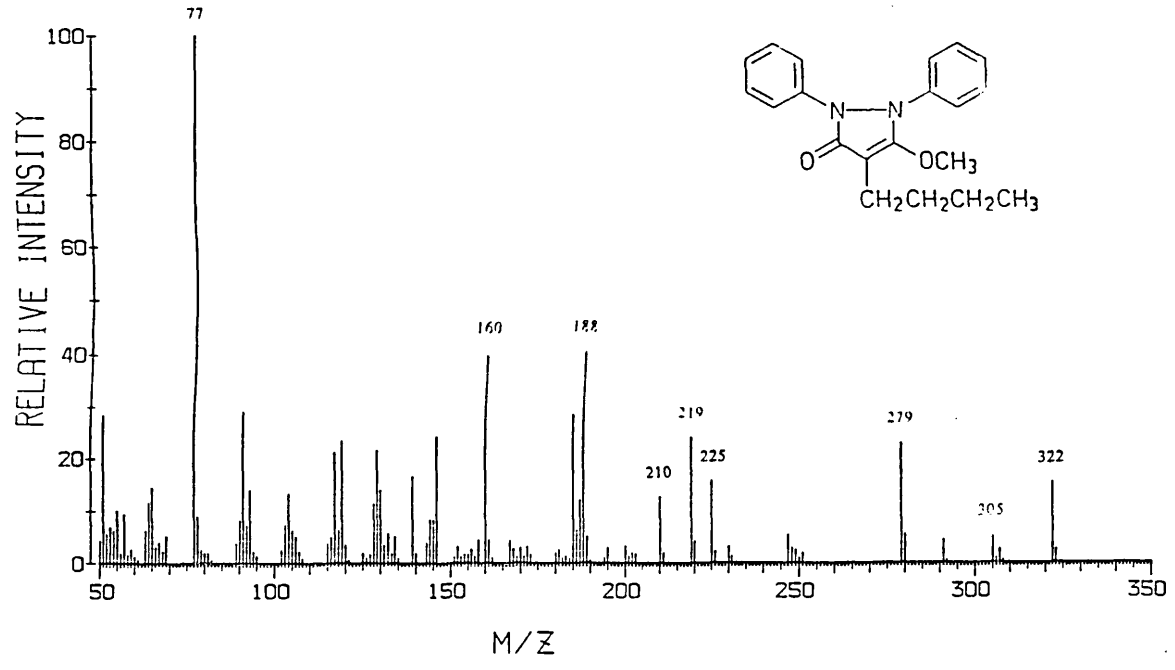


FIGURE 7.4 **Mass spectrum of C-methylphenylbutazone (II)**

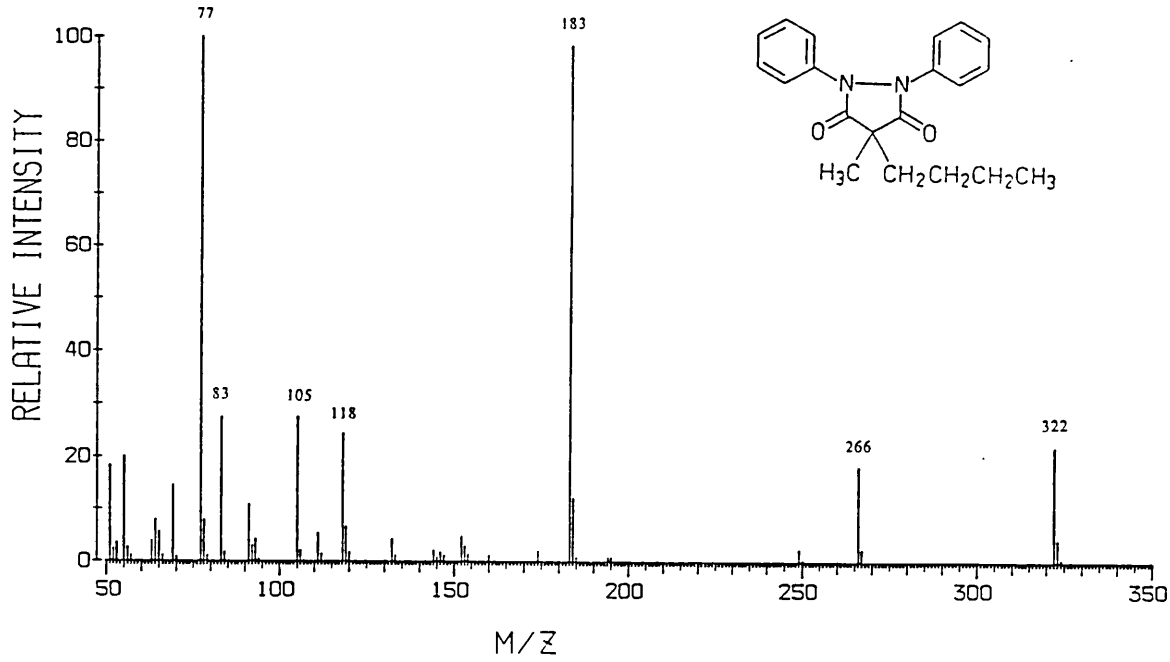


FIGURE 7.5 **Mass spectrum of C-methyl-*p*-hydroxyphenylbutazone (IV)**

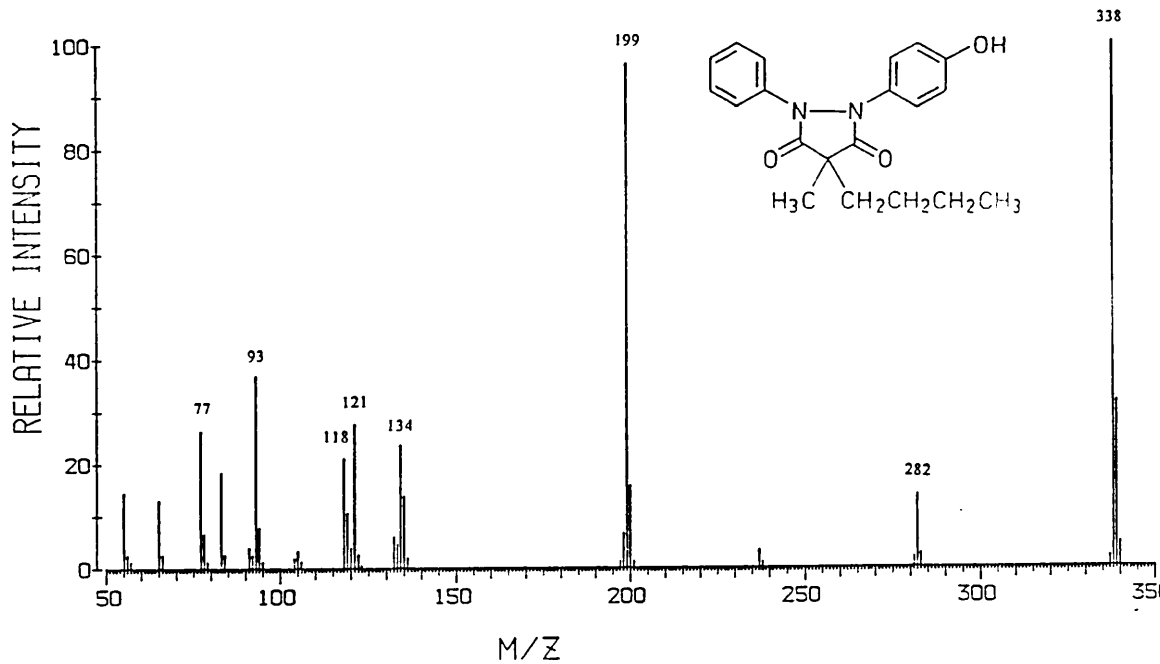


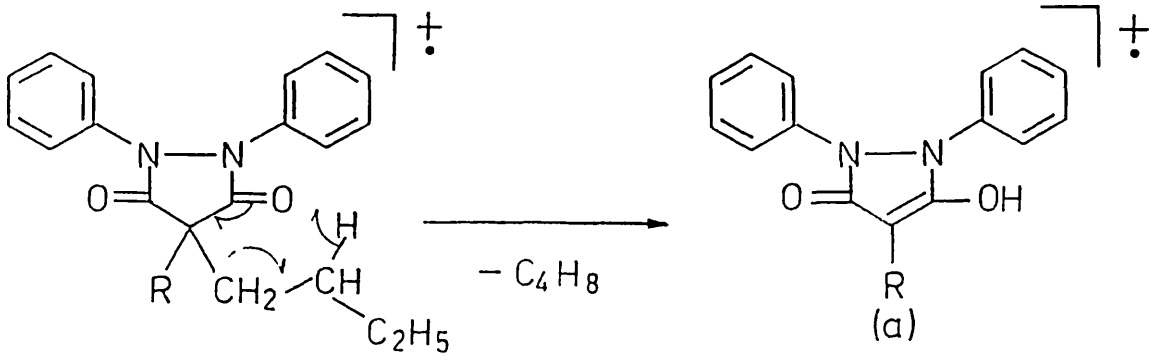
TABLE 7.2 Partial spectra of phenylbutazone and methylated derivatives

Phenylbutazone		C-Methyl-phenylbutazone		C-Methyl(d_3)-phenylbutazone		O-Methyl-phenylbutazone		O-Methyl(d_3)-phenylbutazone	
m/z	Rel. Int.	*m/z*	Rel. Int.	*m/z*	Rel. Int.	*m/z*	Rel. Int	*m/z*	Rel. Int.
308	(36.3)	322	(22.8)	325	(41.1)	322	(14.9)	325	(54.7)
						305	(5.1)	308	(17.7)
						291	(4.1)	291	(18.0)
265	(1.9)					279	(23.4)	282	(80.4)
252	(10.8)	266	(13.0)	269	(22.2)				
						247	(5.7)	247	(17.1)
						225	(15.8)	228	(34.2)
						219	(24.7)	219	(61.4)
						210	(13.0)	210	(75.3)
						188	(39.9)	191	(75.3)
183	(96.9)	183	(80.4)	183	(100)				
167	(1.2)	167	(1.3)	167	(1.3)	167	(4.1)	167	(7.6)
						160	(40.5)	163	(54.1)
152	(4.6)	152	(5.1)	152	(4.4)	152	(2.1)	152	(5.1)
						146	(24.0)	146	(40.5)
						139	(16.5)	142	(29.7)
						129	(21.5)	129	(33.5)
119	(4.6)	119	(7.9)	119	(4.1)	119	(23.7)	119	(41.8)
		118	(19.0)	121	(15.2)				
						117	(21.2)	117	(28.2)
105	(17.2)	105	(21.2)	105	(17.4)				
						104	(13.0)	104	(19.6)
91	(10.1)	91	(11.4)	91	(7.3)	91	(13.9)	91	(28.8)
77	(100)	77	(100)	77	(58.9)	77	(100)	77	(100)
69	(5.2)	83	(25.3)	86	(14.9)				

Fragmentation pathways of phenylbutazone and C-methyl derivatives.

Phenylbutazone and the C-methyl derivatives (II & III) undergo a McLafferty rearrangement, resulting in the loss of C_4H_8 from the molecular ion, and the formation of a radical ion (a) at *m/z* 252 for phenylbutazone, and at *m/z* 266 (C-methyl-phenylbutazone), *m/z* 269 (C-methyl-d_3-phenylbutazone), and *m/z* 282 (C-methyl-*p*-hydroxyphenylbutazone) for its derivatives.

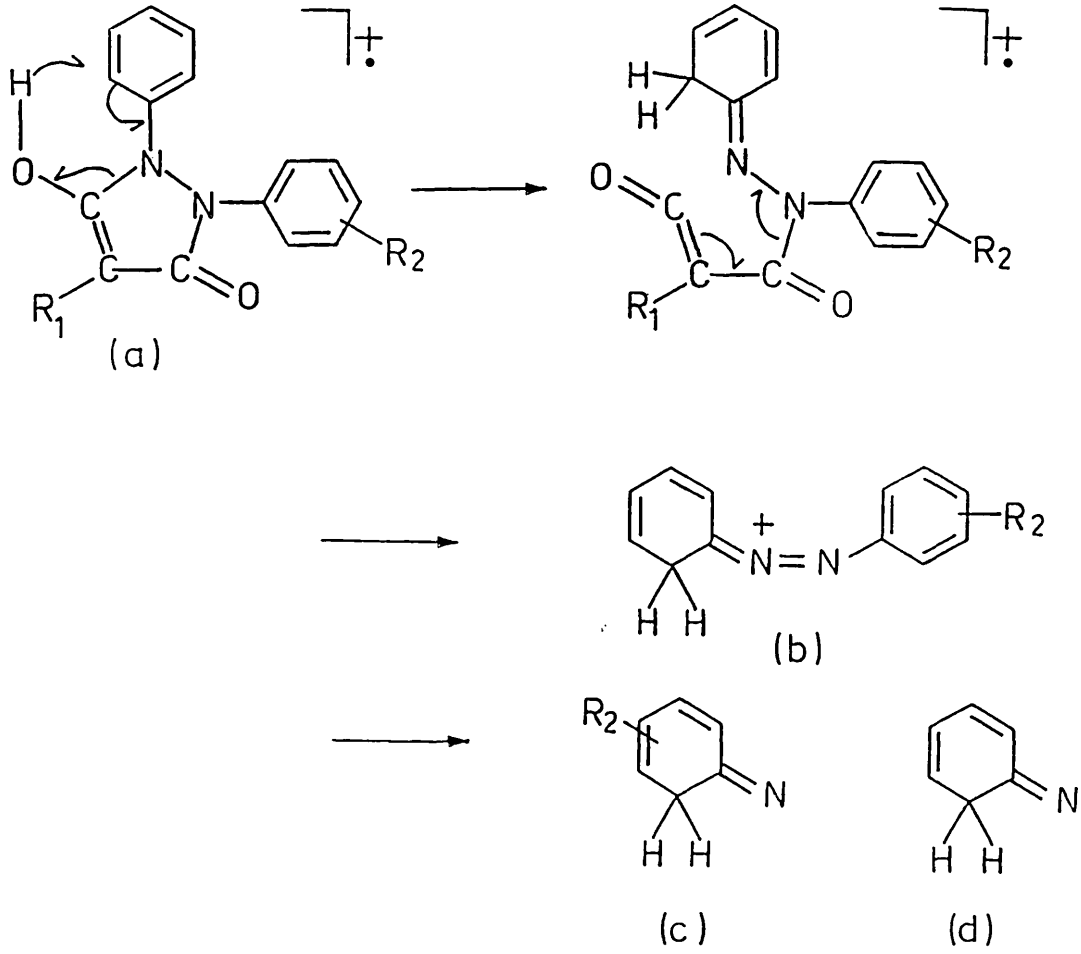
Scheme I



R=H or CH_3

The major fragmentation pathway of phenylbutazone and the two C-methyl derivatives (II & III) is cleavage at the carbonyl sites of the pyrazolidione ring, producing a series of ions at *m/z* 182, 183, 184 (198, 199, 200 in III). The ion (b) at *m/z* 183 and m/z 199 in the spectra of the C-methyl derivatives probably originates from the McLafferty rearrangement ion (a), since the molecular ion is unable to enolise (Scheme II). The ions (c) and (d) proposed by Locock *et al.* (1974) to result from the fragmentation of the hydrazobenzene ion (b) are also included in Scheme II.

Scheme II

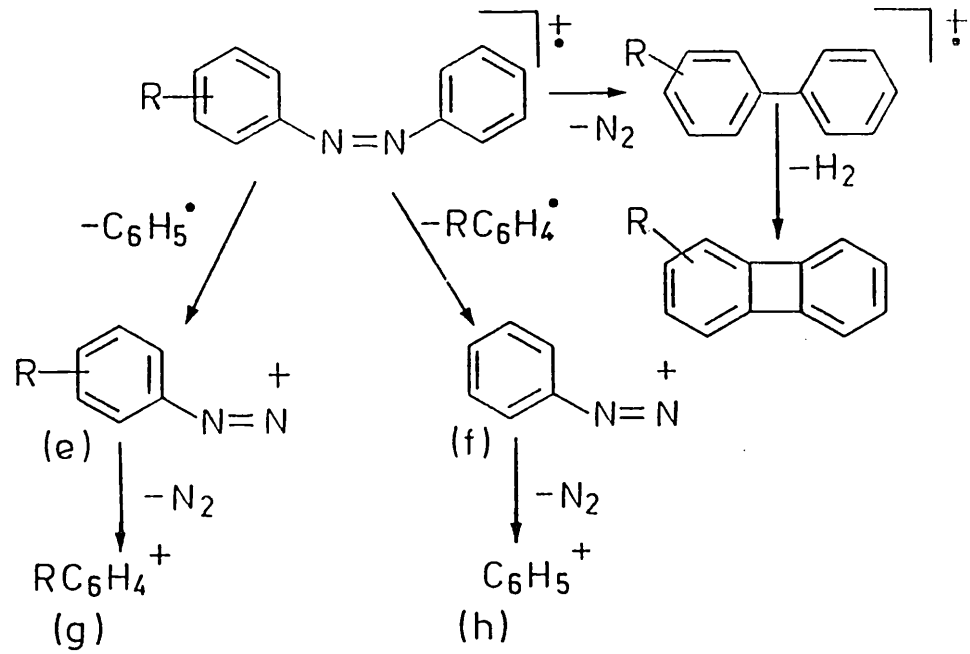


R_1 = H or CH_3,

R_2 = H, OH or OCH_3

Locock *et al.* (1974) attributed the ion at *m/z* 182 to the formation of an azobenzene radical ion, $(C_6H_5N_2C_6H_5)^+$, which then gives rise to a series of skeletal rearrangement ions at *m/z* 152, 153, 154. These rearrangement ions together with the ions (e-h) at *m/z* 105, 77, (121, 105, 93, 77, in the spectra of III) were originally described by Bowie *et al.* (1967) in a study of mass spectra of azobenzenes, and the mechanisms for their formation are given in Scheme III. The ions (e) to (h) for various compounds are recorded in Table 7.3.

Scheme III



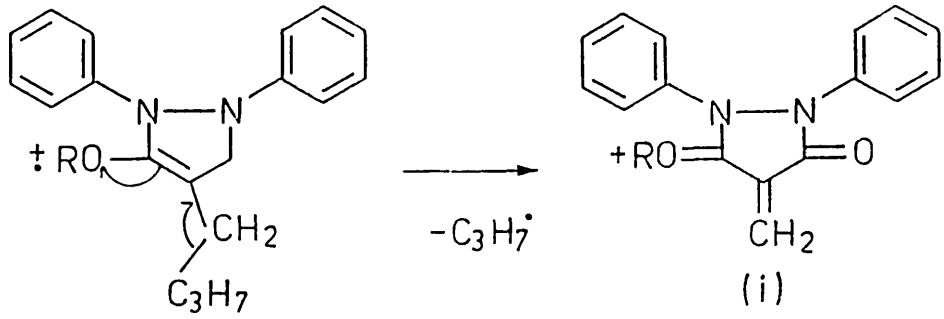
R_1= H, OH or OCH_3

R_2= H, OH or OCH_3

Fragmentation of O-methylphenylbutazone.

A minor fragmentation pathway for phenylbutazone is the loss of a propyl radical (C_3H_7)· from the butyl side-chain to give an ion (i) at *m/z* 265. There was no equivalent ion in the spectrum of C-methylphenylbutazone, but these were prominent *(m/z* 279 O-methylphenylbutazone, *m/z* 281 O-methyl-d_3 -phenylbutazone) in the spectra of the O-methyl derivatives. The mechanism for the formation of this ion (i) is suggested in Scheme IV. Minor pathways in the fragmentation of O-methylphenylbutazone are the loss of OH and OCH_3 from the molecular ion.

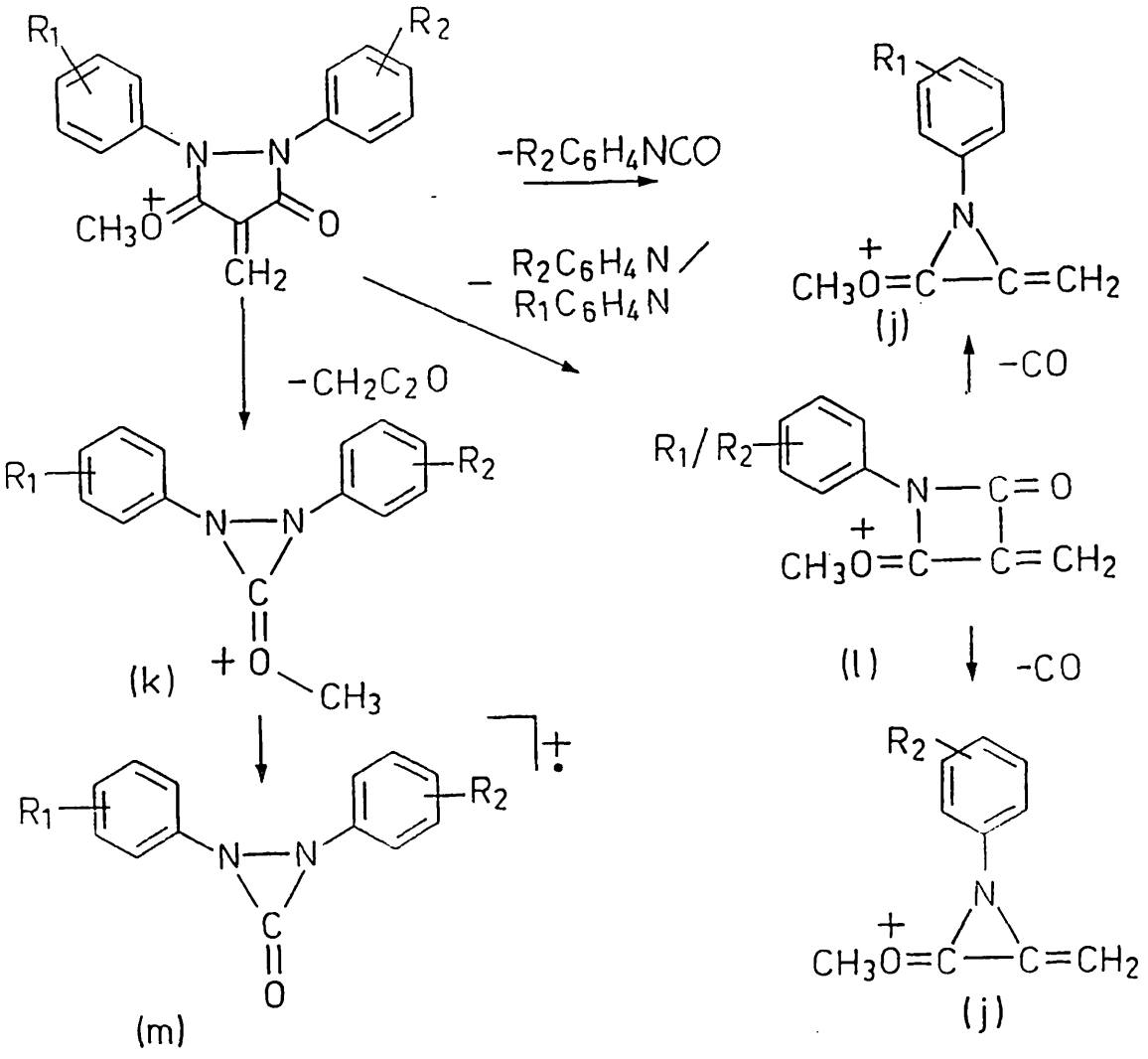
Scheme IV



R=H or CH_3

Unterhalt proposed the formation of two ions (j) and (k) from this ion (i), arising from the loss of C_6H_5NCO or CH_2C_2O respectively. This pathway is illustrated in Scheme V, and includes the possible structures of two other ions (l) and (m).

Scheme V



Phenylbutazone: $R_1 = R_2 = H$

O-methoxy-*p*-methoxyphenylbutazone (Va): $R_1 = p\text{-}OCH_3$ $R_2 = H$

O-methoxy-*p*-methoxyphenylbutazone (Vb): $R_1 = H$ $R_2 = p\text{-}OCH_3$

O-methoxy-*m*-methoxyphenylbutazone (VIa): $R_1 = m\text{-}OCH_3$ $R_2 = H$

O-methoxy-*m*-methoxyphenylbutazone (VIb): $R_1 = H$ $R_2 = m\text{-}OCH_3$

Mass spectra of p-hydroxyphenylbutazone derivatives.

GCMS analysis of the reaction products of diazomethane and oxyphenbutazone revealed the formation of three different dimethyl derivatives with R_t 4.41, 6.21, and 6.27 min). The mass spectrum of the compound with GC R_t 4.41 min, (Fig. 7.6) was consistent with that of C-methyl-*p*-methoxyphenylbutazone (IV): *m/z* 352 (M^+), (a) 296 (M^+-C_4H_8), (b) 213 ($C_6H_5N_2HC_6H_4OCH_3$), (c) 121 ($NC_6H_4OCH_3$), (d) 91 (C_6H_5N), (e) 135 ($C_6H_4N_2OCH_3$), (f) 105 ($C_6H_5N_2$), (g) 107 ($C_6H_4OCH_3$), (h) 77 (C_6H_5).

The mass spectra obtained for the two peaks R_t 6.21 and 6.27 min, (V_1 and V_2 in Fig. 7.8) were characteristic of O-methyl derivatives: *m/z* 352 (M^+), (i) 309 (M^+ -C_3H_7), (j) 190/160 (i-C_6H_5NCO)/(i-$CH_3OC_6H_5NCO$), (k) 255 (i-54), (l) 218/188 (i-C_6H_5N)/ (i-$C_6H_5NOCH_3$), (m) 240. However there were differences between the two spectra, notably in the intensities of the ions (j) and (l). The formation of two isomeric O-methyl derivatives is expected, since the methylated carbonyl function can be on either side of the heterocyclic nucleus (Va and Vb, Fig. 7.1).

Based on the fragmentation pathway proposed by Unterhalt (1972) (Scheme V) it should be possible to distinguish between the two O-methyl isomers (Va and Vb), by the mass of the ion (j). One would expect this ion to appear at *m/z* 190 for the isomer Va which has the *p*-methoxyphenol group and the methylated carbonyl function on the same side of the pyrazolidione ring, and at *m/z* 160 for Vb. However there were peaks at *m/z* 160 and 190 in the spectra of both isomers, possibly due to the ion (l) which can be formed with either phenyl ring, eliminating CO to form both ions (j). The results obtained with the deuteromethyl derivatives (Table 7.4) suggest that there must be an additional mechanism for the formation of the ion at *m/z* 190, since there was not only the expected equivalent ion at *m/z* 196 for one isomer, but also an ion at *m/z*

193 for the other. Consequently the exact identity of the two isomers remains uncertain.

Mass spectra of m-hydroxyphenylbutazone derivatives.

In a similar fashion to *p*-hydroxyphenylbutazone, diazomethane treatment of *m*-hydroxyphenylbutazone resulted in the formation of three isomeric dimethyl derivatives (R_t 4.14, 5.56, and 6.07 min). The mass spectrum of the C-methyl derivative is reproduced in Fig. 7.7, and selected ions for the two O-methyl derivatives ($\mathbf{VI}_1$ & $\mathbf{VI}_2$) are presented in Table 7.4. There was little difference between the spectra of the C-methyl derivatives of *m*- and *p*- hydroxy-phenyl-butazone apart from the low relative intensities of the ions at *m/z* 121 and 135 for the *m*-hydroxy compound (Figs. 7.6 & 7.7). There were considerable differences between the spectra of the O-methyl derivatives of these two compounds, and it was possible to distinguish between them by the difference in the relative intensities of the ions at *m/z* 160/218 for compounds $\mathbf{V}_1$ & $\mathbf{VI}_1$ and at *m/z* 190/77 for compounds $\mathbf{V}_2$ & $\mathbf{VI}_2$ (Table 7.4).

Unfortunately a sample of *o*-hydroxyphenylbutazone was not available for comparison, but one would expect to be able to distinguish between the C-methyl derivative of this compound and the C-methyl derivatives of *m*- and *p*-hydroxyphenylbutazone by the fragmentation pattern of the substituted azobenzene radical ion *m/z* 212, $(C_6H_5N_2C_6H_4OCH_3)^+$. Bowie *et al.* (1967) could not distinguish between the spectra of *m*- or *p*- substituted azobenzenes, but reported a significant difference in the fragmentation patterns of *o*-substituted compounds, particularly in the spectra of *o*-methoxy azobenzenes. The presence of the *o*-OMethyl substituent exerts a strong '*ortho effect*' and an additional ion (*m/z* 93) corresponding to an amine $(C_6H_5NH_2)^+$ is observed in the spectra of *o*-methoxyazobenzene, which is absent from the spectra of the other C-methyl derivatives (Table 7.3).

Mass spectrum of 4-hydroxyphenylbutazone

The spectrum obtained after the GCMS analysis of this compound is reproduced in Figure 7.9. The principal features of this mass spectrum are the molecular ion at *m/z* 324, an absence of a McLafferty rearrangement ion at *m/z* 268 (M^+ -C_4H_8), and a prominent peak at *m/z* 184.

FIGURE 7.6 **Mass spectrum of C-methyl-*p*-methoxyphenylbutazone**

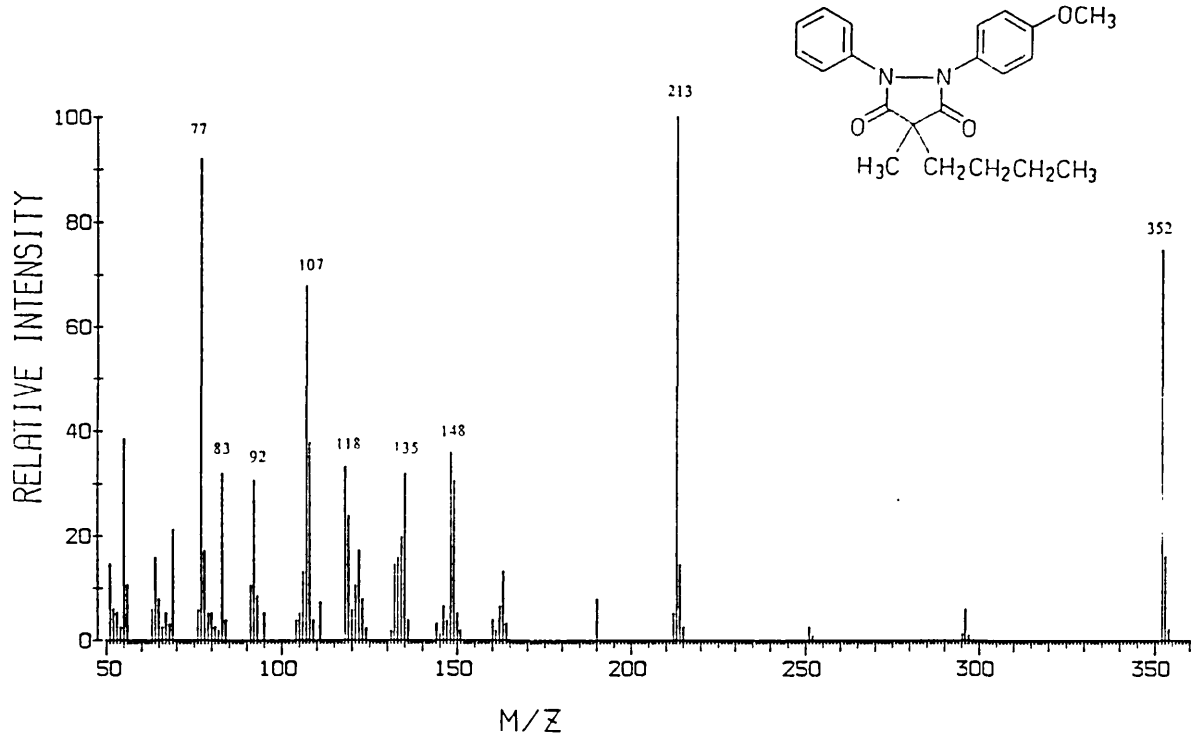


FIGURE 7.7 **Mass spectrum of C-methyl-*m*-methoxyphenylbutazone**

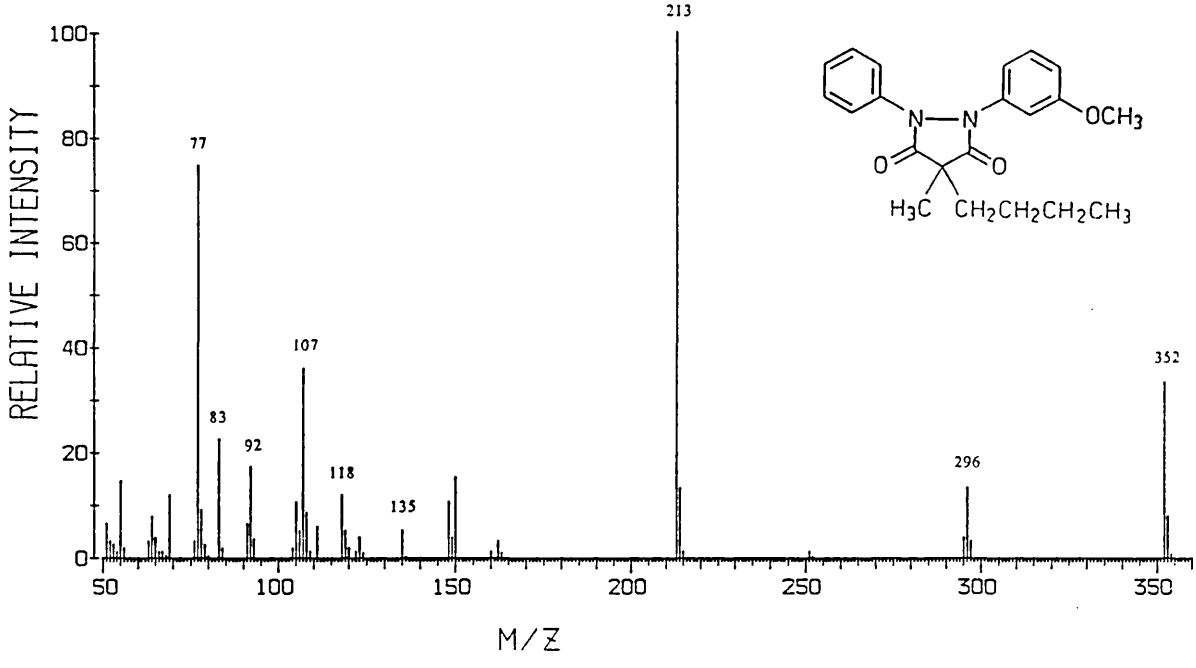
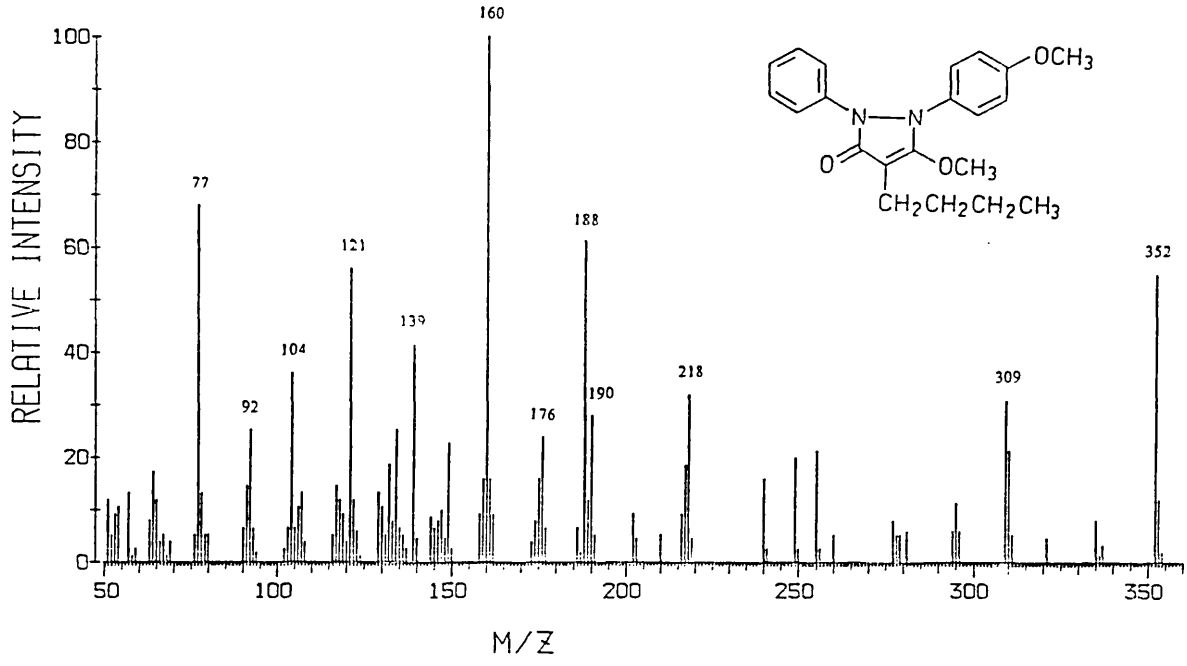


FIGURE 7.8 **Mass spectra of the two isomers of O-methyl-*p*-methoxyphenylbutazone**



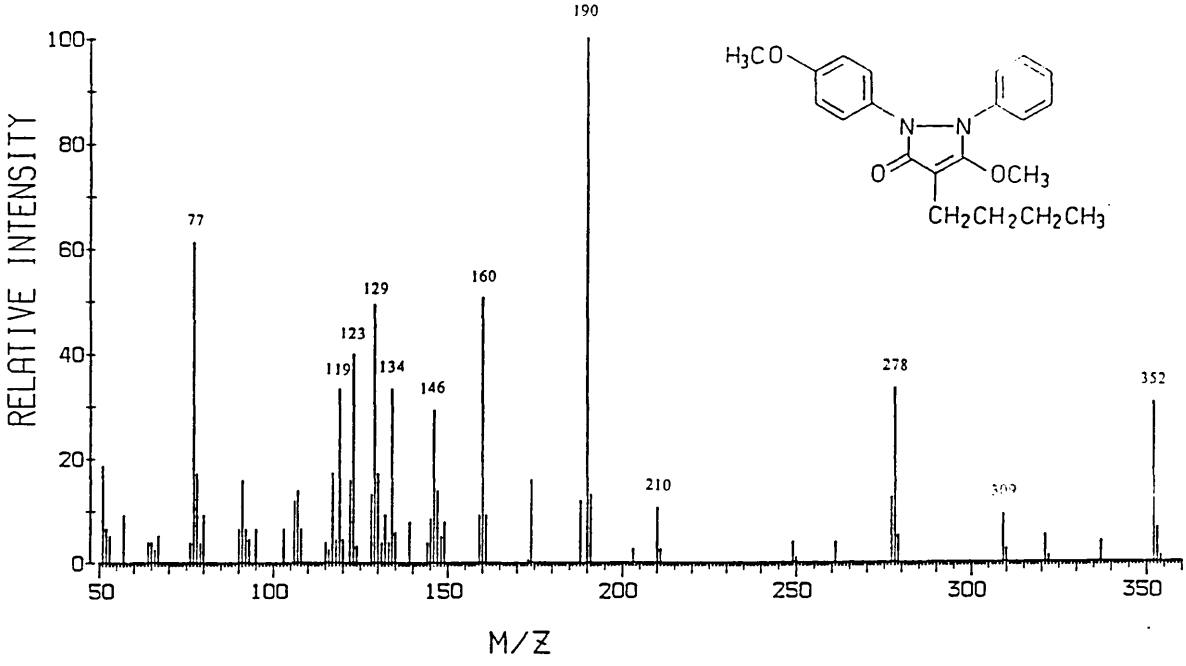


TABLE 7.3 **Relative abundance of selected ions in the mass spectra of various C-methylated phenylbutazone derivatives**

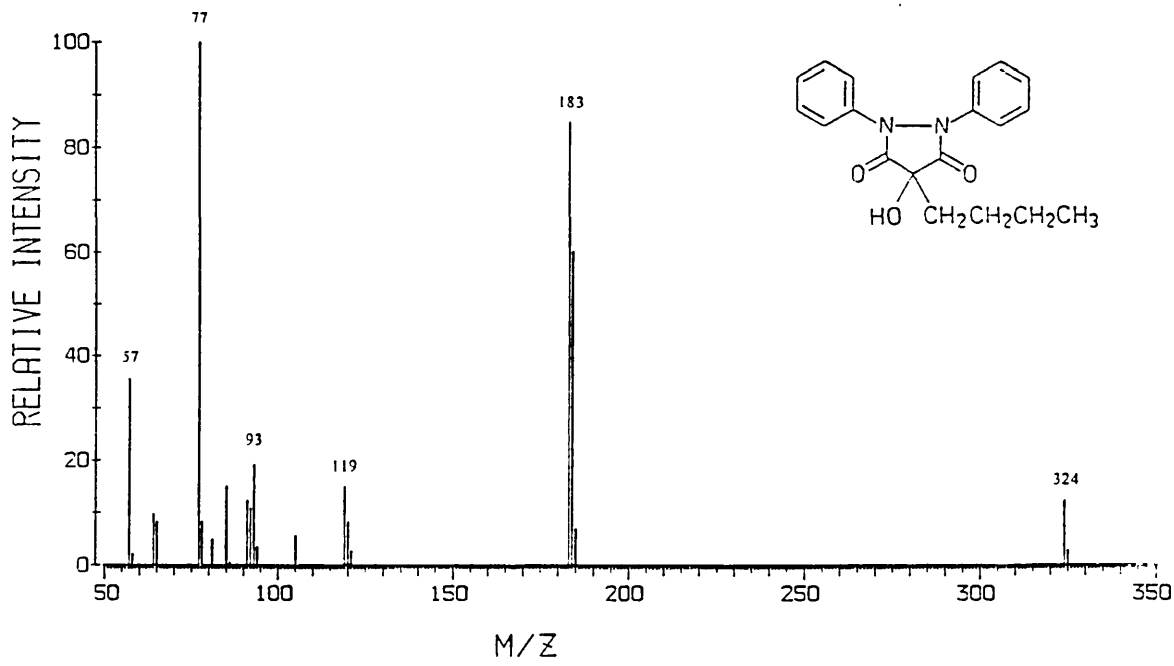
Compound		Ion						
		(b)	(c)	(d)	(e)	(f)	(g)	(h)
Phenylbutazone	*m/z*:-	183	91		105		77	
	(Int.)	80.4	11.4		21.2		100	
p-hydroxy-	*m/z*:-	199	107	91	121	105	93	77
phenylbutazone	(Int.)	95.7	5.8	4.3	27.3	3.4	37.3	25.6
p-methoxy-	*m/z*:-	213	121	91	135	105	107	77
phenylbutazone	(Int.)	100	10.9	9.2	25.0	5.1	44.2	69.0
m-methoxy-	*m/z*:-	213	121	91	135	105	107	77
phenylbutazone	(Int.)	100	n.d.	6.4	5.6	10.7	35.7	74.3
p-methoxy-	*m/z*:-	216	124	91	138	105	110	77
phenylbutazone-d_6	(Int.)	100	8.1	5.4	26.6	4.8	50.9	44.8
m,m-dimethoxy-	*m/z*:-	243	121		135		107	
phenylbutazone	(Int.)	100	N.D.		10.1		68.1	
m,m-dimethoxy-	*m/z*:-	249	124		138		110	
phenylbutazone-d_9	(Int.)	88.0	1.3		9.3		100	

TABLE 7.4 **Relative intensities of selected ions in the spectra of O-methyl derivatives**

Compound	Ion (i)	Ion (j)	Ion (j)	Ion (l)	Ion (l)
	m/z (Int.)	*m/z* (Int.)	*m/z* (Int.)	*m/z* (Int.)	*m/z* (Int.)
I	279 (23.4)	160 (40.5)		188 (39.9)	
I-d_6	282 (80.4)	163 (54.1)		191 (79.3)	
V_1	309 (30.7)	160 (100)	190 (28.0)	188 (61.3)	218 (32.0)
V_2	309 (8.7)	160 (50.7)	190 (100)	188 (12.0)	218 (--)
V_1-d_6	315 (33.3)	160 (100)	196 (27.3)	191 (52.7)	224 (22.0)
V_2-d_6	315 (4.0)	163 (58.7)	193 (100)	191 (8.3)	224 (--)
VI_1	309 (32.0)	160 (22.7)	190 (25.3)	188 (12.0)	218 (100)
VI_2	309 (12.7)	160 (52.0)	190 (9.3)	188 (30.7)	218 (10.7)
VI_1-d_6	315 (38.0)	160 (26.7)	196 (21.3)	191 (10.0)	224 (100)
VI_2-d_6	315 (24)	163 (70.0)	193 (12.0)	191 (45.3)	224 (14.0)

The base peak in the spectra of compounds VI_2 and VI_2-d_6 was at *m/z* 77.

FIGURE 7.9 Mass spectrum of 4-hydroxyphenylbutazone



Mass spectra of aliphatic hydroxyl derivatives.

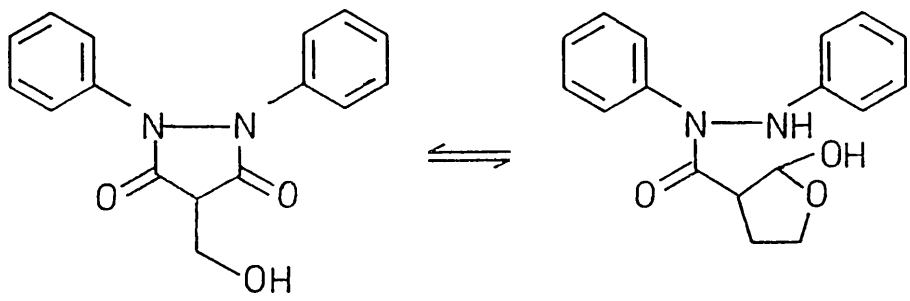
Me-TMS derivatives of the three aliphatic hydroxyl derivatives of phenylbutazone were analysed by GCMS and the mass spectra obtained reproduced in Figs. 7.10, 7.11, and 7.12. γ-Hydroxyphenylbutazone (R_t 12.45 min) and ω-hydroxyphenylbutazone (R_t 13.44 min) both gave single peaks on GC but β-hydroxyphenylbutazone gave two peaks A and B on GC, (R_t 13.28 and 14.22 min respectively).

The mass spectra of these compounds contained a molecular ion at *m/z* 410, (*m/z* 413 after deuteromethylation) consistent with monomethyl monosilyl derivatives. The methylation of these compounds was performed with iodomethane, and consequently one would expect the formation of C-methyl derivatives, this was confirmed by the presence of ions (a) at *m/z* 266 (269 for the deuteromethyl derivative), and (b) *m/z* 183 in the spectra of these compounds.

There were significant differences in the spectra of the three aliphatic hydroxyl derivatives, and it was possible to identify each compound by its characteristic fragmentation pattern. The spectrum of the methyl-TMS derivative of γ-hydroxyphenylbutazone contained a prominent ion at *m/z* 143 which may result from cleavage of the aliphatic side chain (Scheme VI), and the subsequent loss of a proton from the rearrangement ion. This ion was absent in the spectra of the other aliphatic derivatives. Other ions characteristic of γ -hydroxyphenylbutazone were at *m/z* 197 (200 for the CD_3-TMS derivative) and at 117. The mechanism for the formation of the ion at *m/z* 117 (and at *m/z* 131 for β-hydroxyphenylbutazone) is given in Scheme VII.

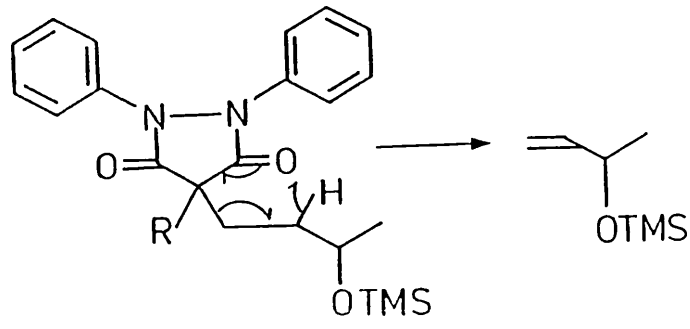
The spectrum of ω-hydroxyphenylbutazone was characterised by a prominent ion at *m/z* 227 (found at 230 for the CD_3-TMS derivative) consistent with the elimination of the hydrazobenzene from the molecular ion.

The spectra of the two compounds obtained from the derivatisation of the β-hydroxyphenylbutazone standard both contained a prominent ion at *m/z* 131, suggesting that they were isomeric forms of β-hydroxyphenylbutazone and not different compounds, furthermore the ions at *m/z* 266 and 183, common to both spectra indicated they were both C-methyl derivatives. Underivatised β-hydroxyphenylbutazone also gave two peaks on HPLC suggesting that the authentic standard was a mixture of two isomers, and that the two GC peaks were not different derivatives of one compound. γ-Hydroxyphenylbutazone can exist in two forms, either the straight chain form or as a lactone (Denss *et al.*, 1957; Girod *et al.*, 1957). The two forms of γ-hydroxyphenylbutazone have different chromatographic properties (Alexander *et al.*, 1985), but since the lactone form is thermodynamically favoured only one peak is usually obtained with this compound. It is likely that β-hydroxyphenylbutazone can form a lactone, since this has been demonstrated for the related compound 1,2-diphenyl-3,5-dioxo(2-hydroxyethyl)-pyrazoline (VII) (Denss *et al.*, 1957), and the existance of these two isomeric forms of β-hydroxyphenylbutazone, may explain the two HPLC peaks, and the two GCMS peaks obtained for this compound.



(VII)

Scheme VI



Scheme VII

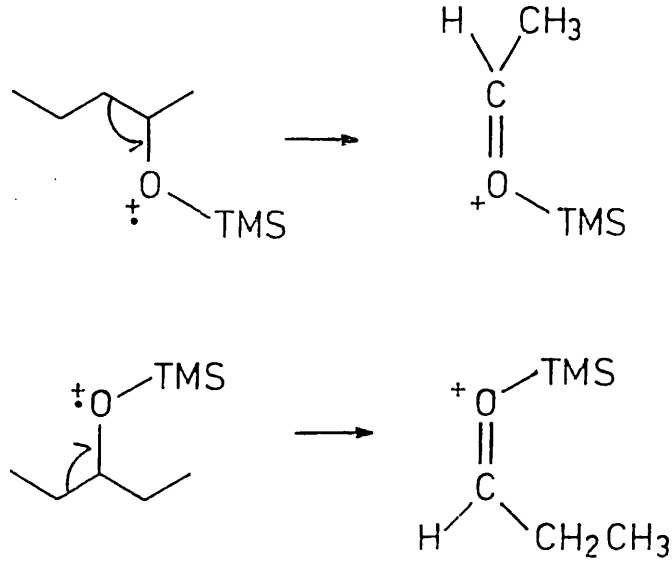


FIGURE 7.10 **Mass spectrum of the Me-TMS derivative of γ-hydroxy-phenylbutazone**

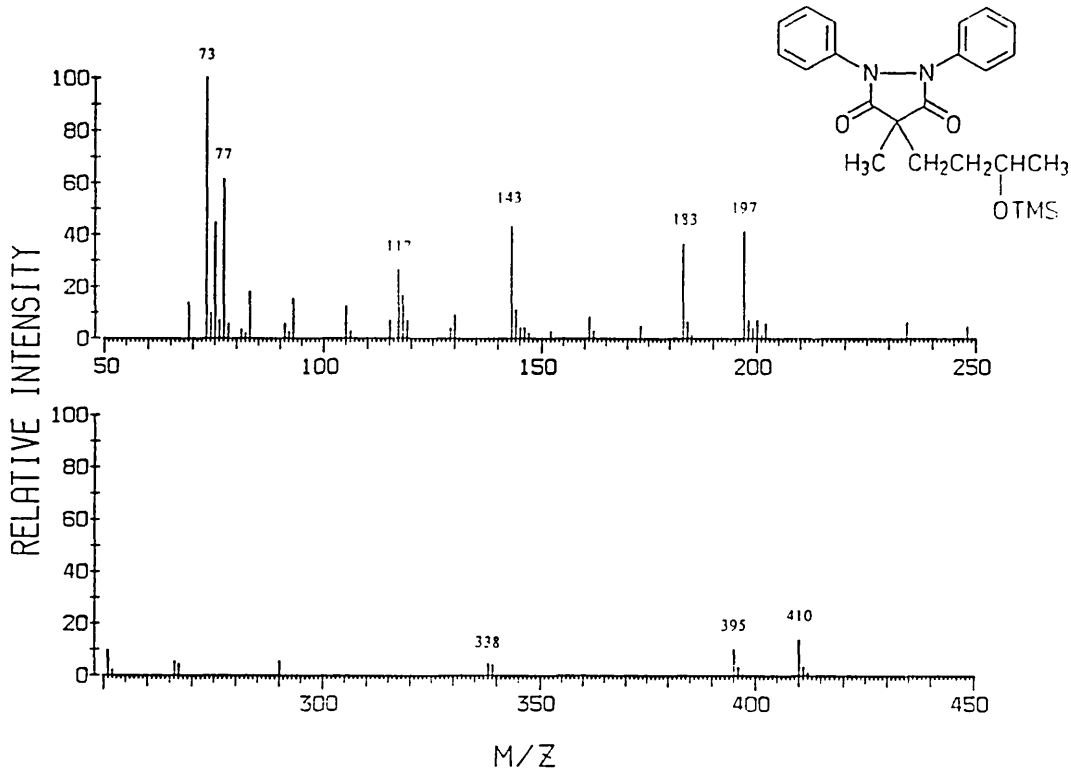


FIGURE 7.11 **Mass spectrum of the Me-TMS derivative of ω-hydroxy-phenylbutazone**

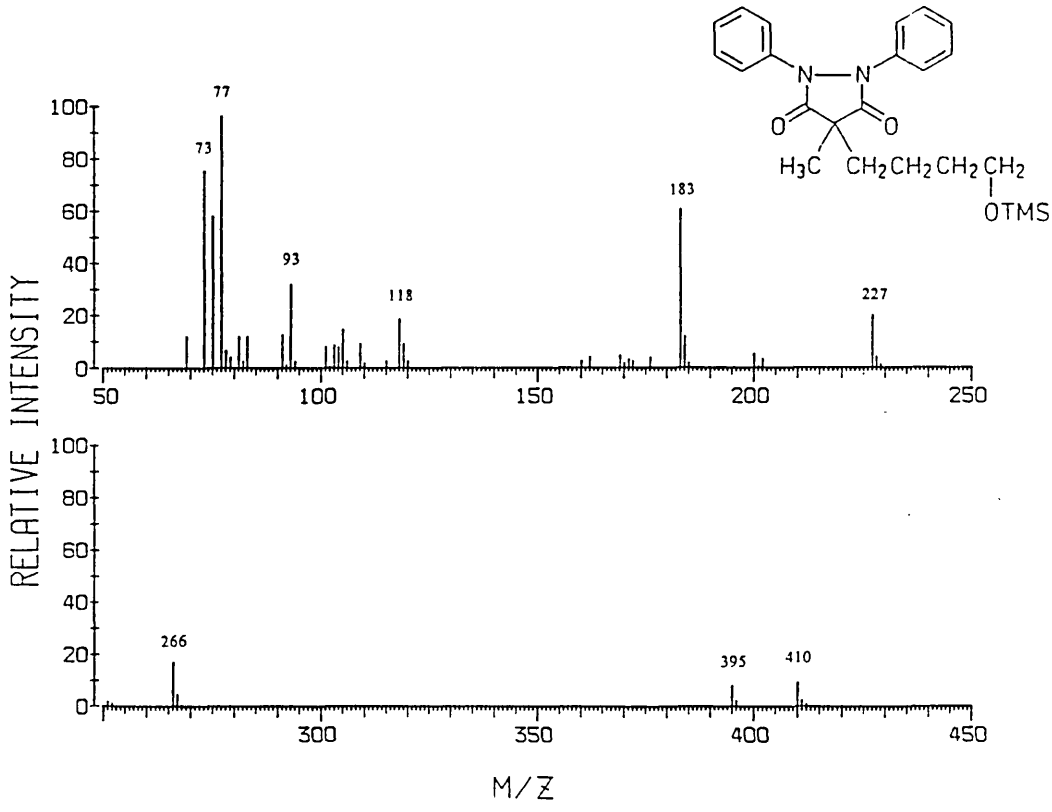
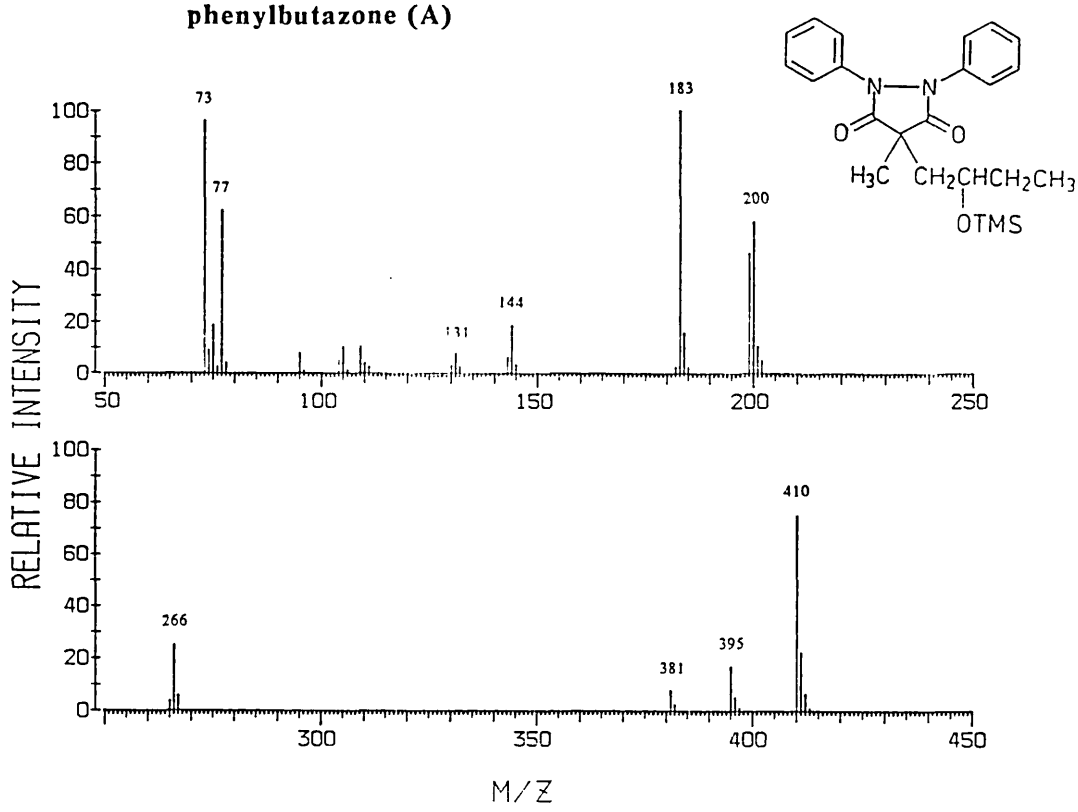
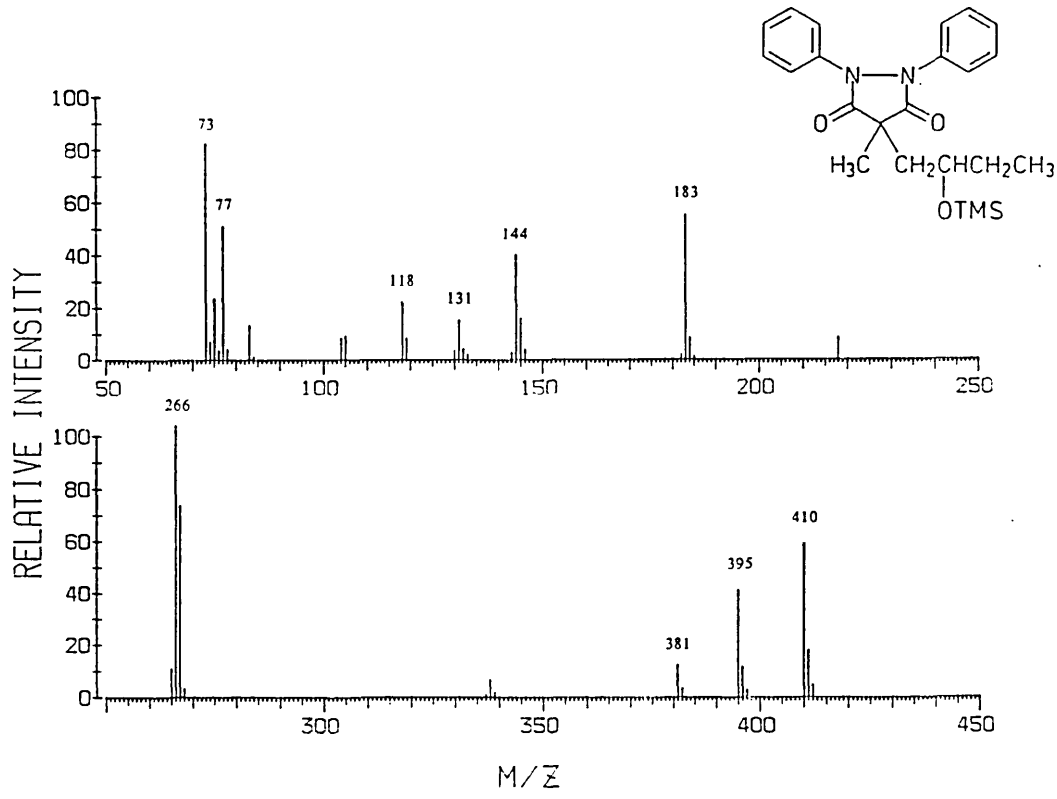


FIGURE 7.12 Mass spectrum of the Me-TMS derivative of β-hydroxy-phenylbutazone (A)



Mass spectrum of the Me-TMS derivative of β-hydroxy-phenylbutazone (B)



Mass spectra of γ-ketophenylbutazone.

The authentic standard of this compound gave one peak on HPLC, but three GC peaks were obtained following derivatisation (methylation and silylation). The mass spectra obtained for these compounds indicate that one of the compounds (R_t 11.09 min, Fig. 7.13) was a simple C-methyl derivative *m/z*: 336 (M+), 266 (M^+-C_4H_6O), 183 ($C_6H_5N_2HC_6H_5$) whilst the other two were isomeric C-Methyl-TMS derivatives (R_t 14.41 min and R_t 14.56 min, A & B in Fig. 7.13) *m/z*: 408 (M^+), 266 (M^+-C_4H_5OTMS), 183 ($C_6H_5N_2HC_6H_5$).

The mass spectra of both methyl-TMS derivatives contained an ion at *m/z* 338 representing transfer of the TMS group to the carbonyl function and a McLafferty rearrangement with loss of C_4H_6O, Scheme VIII. It was possible to assign structures to the two isomers by the prominence of the ion at *m/z* 143 (Scheme VI) and the very weak McLafferty rearrangement ion (*m/z* 266) in the spectra of A.

Scheme VIII

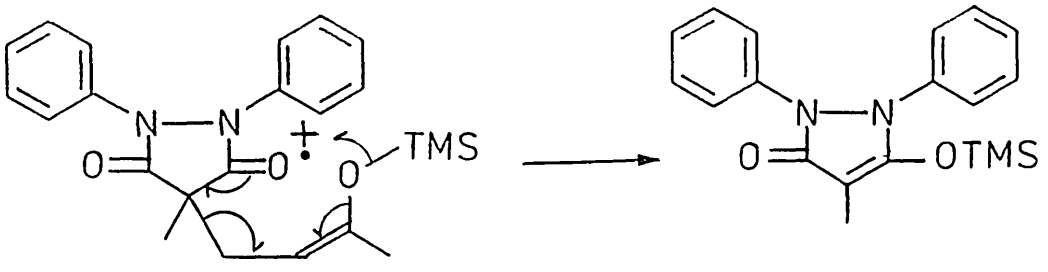


FIGURE 7.13 Mass spectrum of C-methyl-γ-ketophenylbutazone

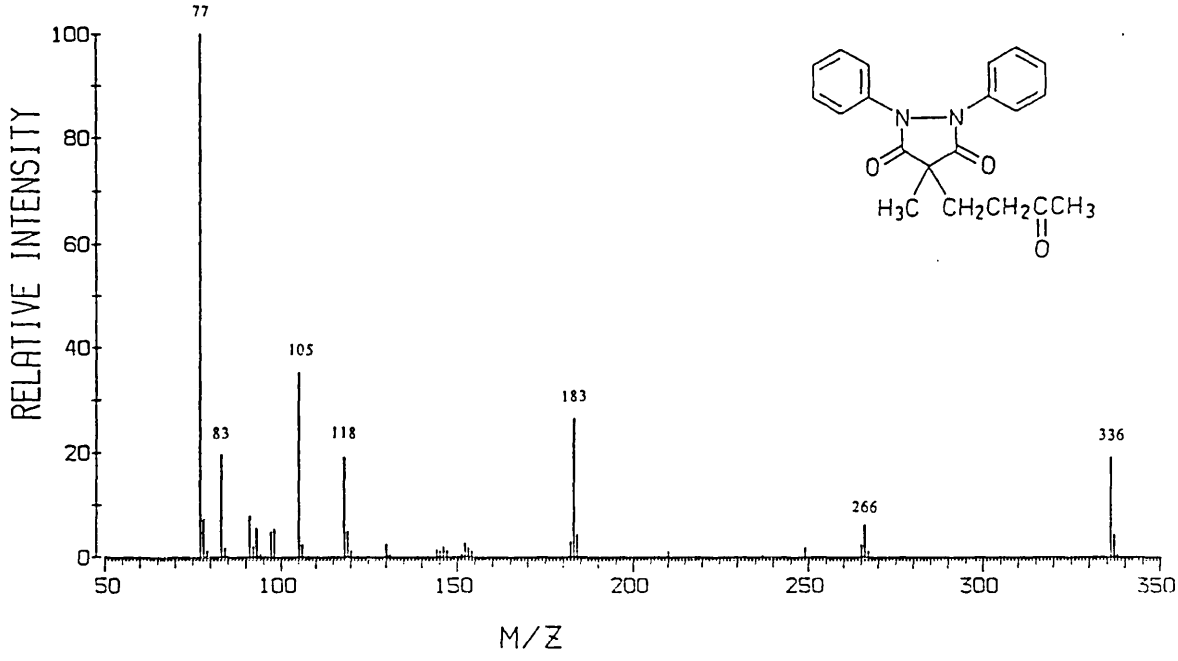
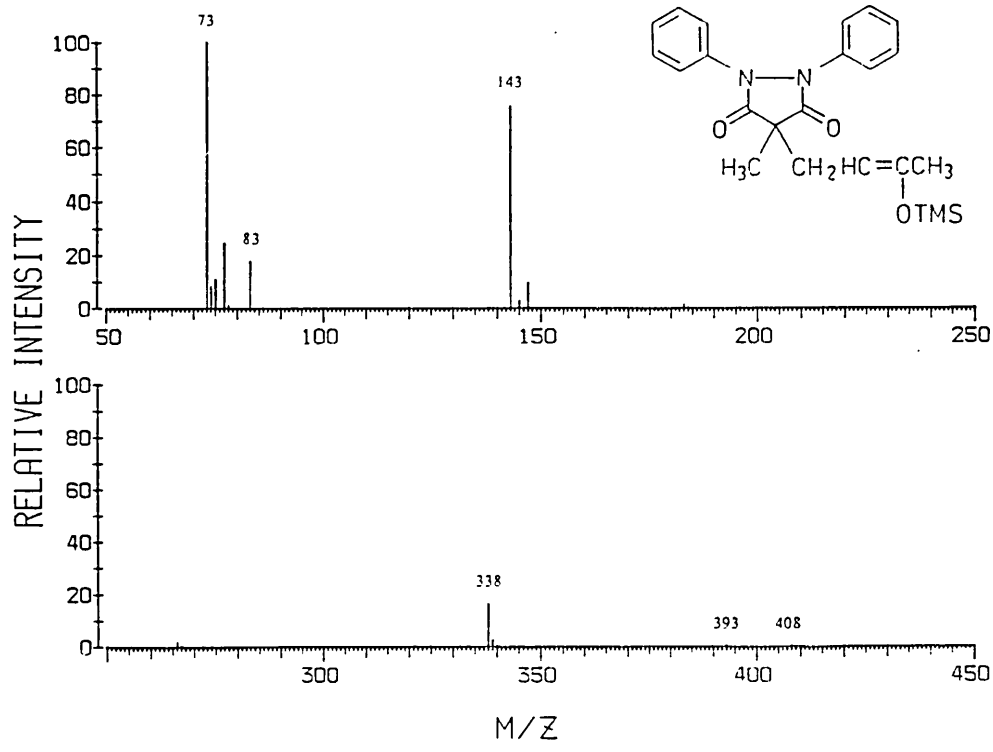
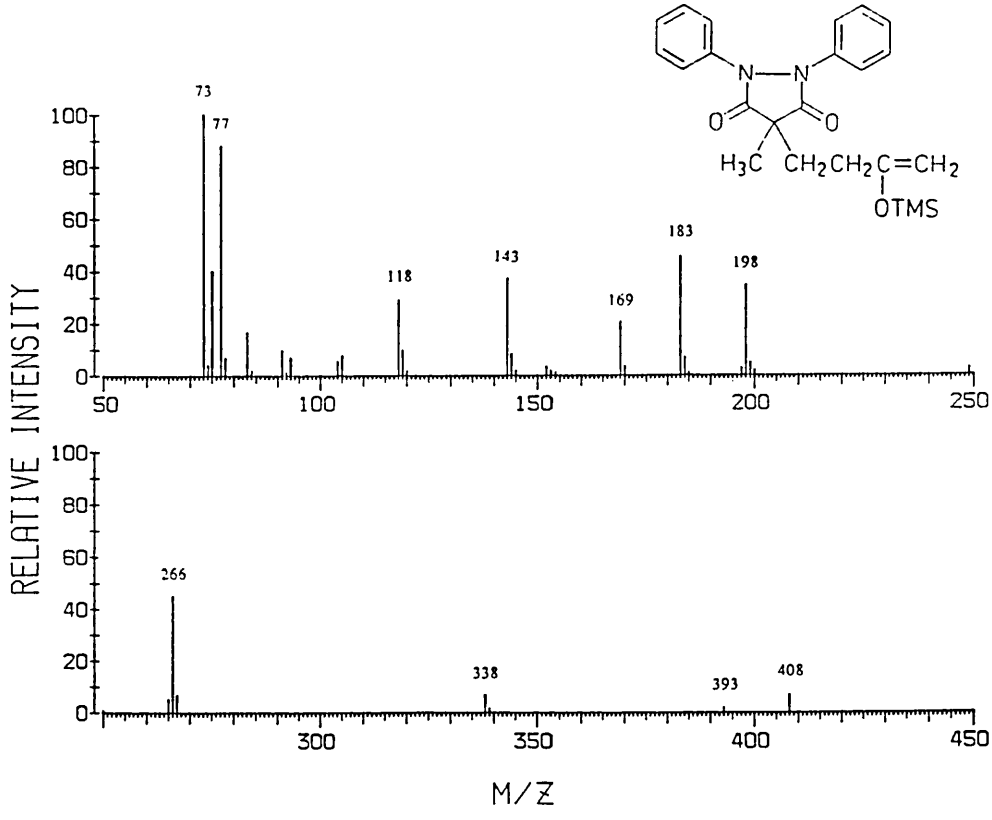


FIGURE 7.14 Mass spectrum of Me-TMS γ-ketophenylbutazone (A)



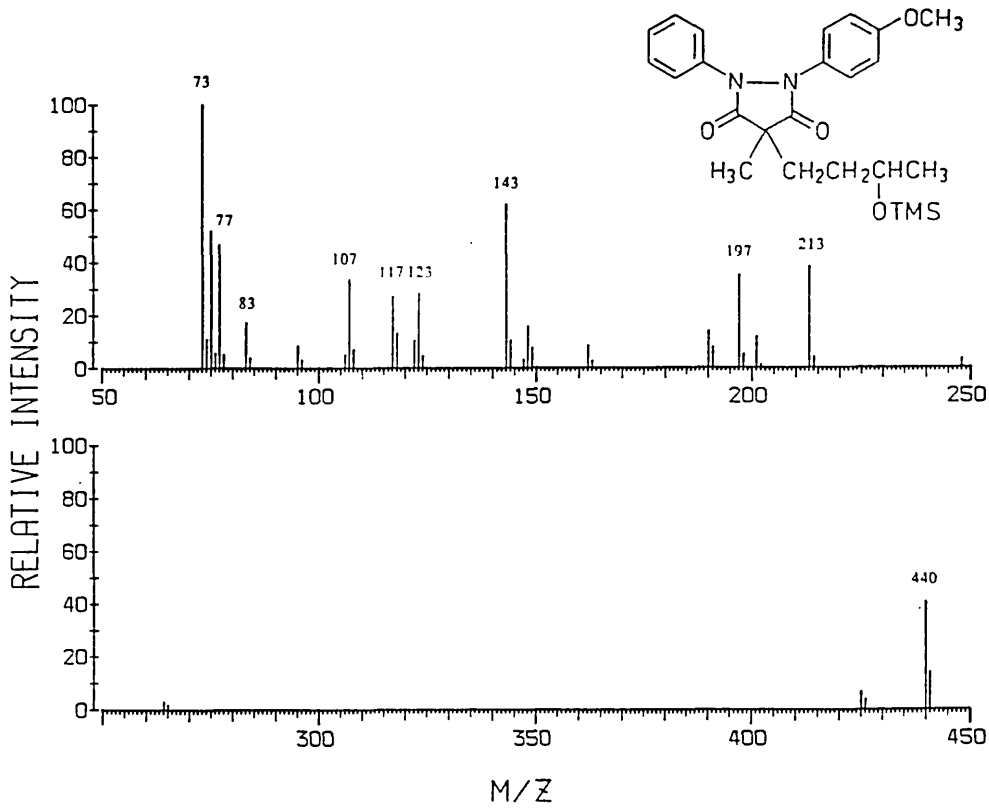
Mass spectrum of Me-TMS γ-ketophenylbutazone (B)



Mass spectrum of p, γ-dihydroxyphenylbutazone.

The mass spectrum of the Me-TMS derivative of this compound (Figure 7.15) contained a molecular ion at *m/z* 440 (*m/z* 446 after deuteromethylation) consistent with the formation of a dimethylmonosilyl derivative. As expected the mass spectrum of this compound showed ions in common with the spectra of both C-methyl-*p*-methoxyphenylbutazone (*m/z* 213, 123, 107) and the Me-TMS derivative of γ-hydroxyphenylbutazone (*m/z* 197, 143 and 117).

FIGURE 7.15 Mass spectrum of the dimethyl monosilyl derivative of *p*,γ-dihydroxyphenylbutazone.



DISCUSSION

It might seem surprising that the methylation of phenylbutazone with iodomethane takes place exclusively at carbon rather than oxygen. However phenylbutazone is a 'carbon acid' whereby the dissociating proton is attached to a carbon atom (Stella, 1975), and the C_4 position in the pyrazolidinedione ring is quite strongly acidic (pK_a 4.5): such systems are well known to undergo C-alkylation (Khaletski & Moldaler, 1963; Dieterle *et al.*, 1976). The acidic nature of the proton at C_4 explains why methylation proceeds more rapidly at this position, than at the less acidic phenolic group (pK_a 10) of oxyphenbutazone. The relative difficulty in methylating the phenol group explains why early attempts to derivatise the metabolite *p*, $\underline{\gamma}$ -dihydroxyphenylbutazone resulted in a monomethyl disilyl derivative, rather than a dimethyl monosilyl derivative (Chapter 3.)

Methylation with diazomethane resulted in a mixture of O-methyl and C-methyl derivatives. An advantage of O-methylation is that it is easier to distinguish between the mass spectra of the O-methyl derivatives of *m*-hydroxy and *p*-hydroxy phenylbutazone, although this was outweighed by the increased complexity of the chromatograms, since methylation with diazomethane gave three products from each of these compounds. For most of the compounds investigated, the derivatisation procedure (methylation with iodomethane and subsequent silylation) resulted in a single peak on GC, and the identities of all of the compounds could be assigned from mass spectral data alone.

The use of 'designer' drugs is of some concern to racing authorities, by slight alterations in their chemical structure, numerous analogues of proscribed drugs can be produced, many of which will have similar physiological effects to the original compound. Apart from the obvious problems for jurisdictions that

operate a list of proscribed compounds, designer drugs are of some concern for authorities that operate a total ban, since successful prosecution may depend on the unambiguous identification of the compound involved. A large number of analogues of phenylbutazone have been synthesised, and many have significant anti-inflammatory activity (Bavin *et al.*, 1955; Burns *et al.*, 1960; Khaletski & Moldaler, 1963). The detection of the illicit administration of these compounds will probably be based on GCMS, and consequently the general fragmentation patterns of 3,5-pyrazolidinediones are of forensic significance.

The following summary outlines the derivatisation patterns and diagnostic ions for hydroxylated phenylbutazone derivatives, and may also apply to a wider range of phenylbutazone analogues.

1. Treatment with methyl iodide results in the preferential formation of C-methyl derivatives, and the methylation of any phenolic groups present in the molecule. Aliphatic hydroxyl groups are resistant to methylation but are readily silylated. The γ-keto function in the butyl side chain is also silylated, although the compound will successfully chromatograph as a simple methyl derivative. The determination of the molecular weight enables one to distinguish between aliphatic and aromatic hydroxyl derivatives.

2. The presence of an ion at *m/z* 213 indicates monohydroxylation in the phenyl ring, an ion at *m/z* 243 indicates two aromatic hydroxyl groups and an ion at *m/z* 183 none. The characteristic fragmentation of these ions enables the position of hydroxylation to be determined, and should also distinguish between monohydroxylation in both rings, or dihydroxylation in one.

3. The position of aliphatic hydroxylation can be determined by the presence of ions at *m/z* 143, 197, 117 (γ-hydroxyl), *m/z* 381, 144, 131 (β-hydroxyl) *m/z* 227 (ω-hydroxyl).

4. The mass spectrum of *p*,γ-dihydroxyphenylbutazone demonstrates that these general rules can be applied for the structural elucidation of compounds containing both aromatic and aliphatic substituents.

SUMMARY

This thesis presents an investigation into the metabolic disposition of phenylbutazone and oxyphenbutazone in the horse. Phenylbutazone is the most widely used non-steroidal anti-inflammatory drug in equine medicine, and recently has become the most commonly encountered doping agent in racehorses. There is a paucity of information concerning the metabolic fate of these compounds in the horse.

A clear understanding of the metabolic disposition of non-steroidal drugs such as phenylbutazone and oxyphenbutazone is of direct relevance to the design of drug-screening procedures. Furthermore, this information may enable one to ascertain additional parameters such as the time of administration, which is of particular importance for the regulation of controlled medication programs.

In the course of the present work, a number of the processes concerning the metabolic disposition of phenylbutazone and oxyphenbutazone have been studied; evidence has been presented to support a number of conclusions, summarised briefly below.

1. After oral administration phenylbutazone was rapidly and extensively absorbed, with an absolute bioavailabilty of 91%. Oxyphenbutazone was not as extensively absorbed as phenylbutazone, with an absolute bioavailability of 78 %. The oral absorbtion of oxyphenbutazone appeared to be biphasic, with an initial rapid absorbtion followed by a prolonged slow absorbtion.

2. Oxyphenbutazone was eliminated at a faster rate than phenylbutazone with plasma elimination half-lives of 6.3 h and 9.7 h respectively. The

elimination half-lives of both of these compounds were independent of the route of administration, but a consequence of the prolonged absorption of oxyphenbutazone was that the plasma concentrations measured at 24 h after the oral administration of this compound were double those achieved after intravenous administration.

3. Both urinary and faecal routes of elimination are of importance in the overall elimination of phenylbutazone and oxyphenbutazone. The pattern of elimination of phenylbutazone was independent of the route of administration, whilst the pattern of elimination of oxyphenbutazone reflected the incomplete absorbtion of this compound.

4. Phenylbutazone was extensively metabolised by oxidation at both aliphatic and aromatic carbon centres. In contrast to phenylbutazone oxyphenbutazone was excreted in the urine largely as the unchanged drug. The pattern of the urinary elimination products for phenylbutazone and oxyphenbutazone was independent of the route of administration.

5. The pattern of the urinary elimination products of phenylbutazone altered with time; a consequence of either the initial inhibition or subsequent enhancement of the enzyme(s) responsible for the *p*-hydroxylation of phenylbutazone.

APPENDIX 1.

URINARY DATA FOLLOWING THE ADMINISTRATION OF PHENYLBUTAZONE

TABLE A1.1 Urinary elimination of ^{14}C following the administration of ^{14}C-Phenylbutazone. Experiment No. 4.

Sample No.	Time (h)	% Dose	Midpoint time (h)	Log_e (% dose excreted h^{-1})
1	2.50	6.44	1.25	0.948
2	5.42	7.39	3.97	0.928
3	8.75	6.43	7.08	0.657
4	12.08	5.75	10.42	0.542
5	16.66	3.86	14.36	-0.174
6	20.25	1.85	18.46	-0.654
7	25.75	3.77	23.00	-0.386
8	28.75	2.73	27.25	-0.094
9	33.42	3.07	31.08	-0.415
10	37.70	2.35	35.56	-0.598
11	39.06	1.46	38.38	0.068
12	42.26	1.65	40.66	-0.654
13	43.88	1.09	43.06	-0.386
14	45.83	0.85	44.85	-0.821
15	48.83	1.18	47.33	-0.942
16	51.75	0.92	50.28	-1.139
17	53.66	0.61	52.72	-1.155
18	55.75	0.50	54.72	-1.427
19	60.25	0.76	58.00	-1.772
20	63.42	0.51	61.83	-1.833
21	66.75	0.32	65.08	-2.333
22	68.75	0.05	67.75	-3.649
23	71.50	0.38	70.13	-1.980
24	75.00	0.50	73.25	-1.966
25	80.75	0.20	77.86	-3.352
26	92.75	0.49	86.75	-3.194
27	105.25	0.17	99.00	-4.343
28	116.75	0.11	111.00	-4.710
29	128.75	0.09	122.75	-4.962
30	140.75	0.06	134.75	-5.298
31	149.75	0.02	145.25	-6.214

TABLE A1.2 **Urinary elimination of ^{14}C following the administration of ^{14}C-Phenylbutazone. Experiment No. 5.**

Sample No.	Time (h)	% Dose	Midpoint time (h)	Log_e (% dose excreted h^{-1})
1	1.03	0.70	0.52	-0.387
2	2.20	4.15	1.62	1.260
3	3.36	1.66	2.78	0.352
4	4.86	1.90	4.12	0.235
5	8.86	3.26	6.86	-0.203
6	10.72	0.57	9.80	-1.167
7	12.40	1.81	11.56	0.074
8	14.08	0.97	13.20	-0.548
9	15.76	0.99	14.93	-0.527
10	17.48	2.73	16.63	0.462
11	19.15	1.61	18.32	-0.035
12	20.83	2.14	20.00	0.238
13	22.36	1.96	21.60	0.246
14	25.86	2.54	24.12	-0.321
15	26.86	1.14	26.36	0.343
16	29.20	1.53	28.03	-0.422
17	31.36	1.03	30.28	-0.749
18	32.53	0.74	31.95	-0.449
19	34.20	1.35	33.36	-0.212
20	37.86	1.42	36.03	-0.947
21	40.20	0.55	39.03	-1.440
22	41.53	0.44	40.86	-1.103
23	42.86	0.45	42.20	-1.088
24	44.20	1.31	43.53	-0.016
25	45.53	0.85	44.86	-0.454
26	46.86	0.68	46.20	-0.669
27	52.12	1.10	49.50	-1.565
28	55.86	0.54	54.00	-1.938
29	58.36	0.47	57.12	-1.671
30	61.36	0.27	59.86	-2.397

(Continued over page)

TABLE A1.2. (Continued)

31	64.36	0.51	62.86	-1.766
32	66.36	0.34	65.36	-1.760
33	Unknown	0.25		
34	70.36	0.15		
35	72.86	0.20	71.62	-2.526
36	77.36	0.22	75.12	-2.996
37	79.86	0.16	78.62	-2.733
38	92.86	0.33	86.36	-3.689
39	104.86	0.21	98.86	-4.074
40	116.86	0.10	110.86	-4.828
41	129.36	0.10	123.12	-4.828
42	140.86	0.05	135.12	-5.521
43	148.86	0.03	144.86	-5.521

Figure A1.1. **Elimination of urinary radioactivity. Ln % Dose h^{-1} plotted against sample midpoint time following the administration of ^{14}C-phenylbutazone (Experiment No. 4.)**

Equation of line Y= -0.050X + 1.136 r= -0.966

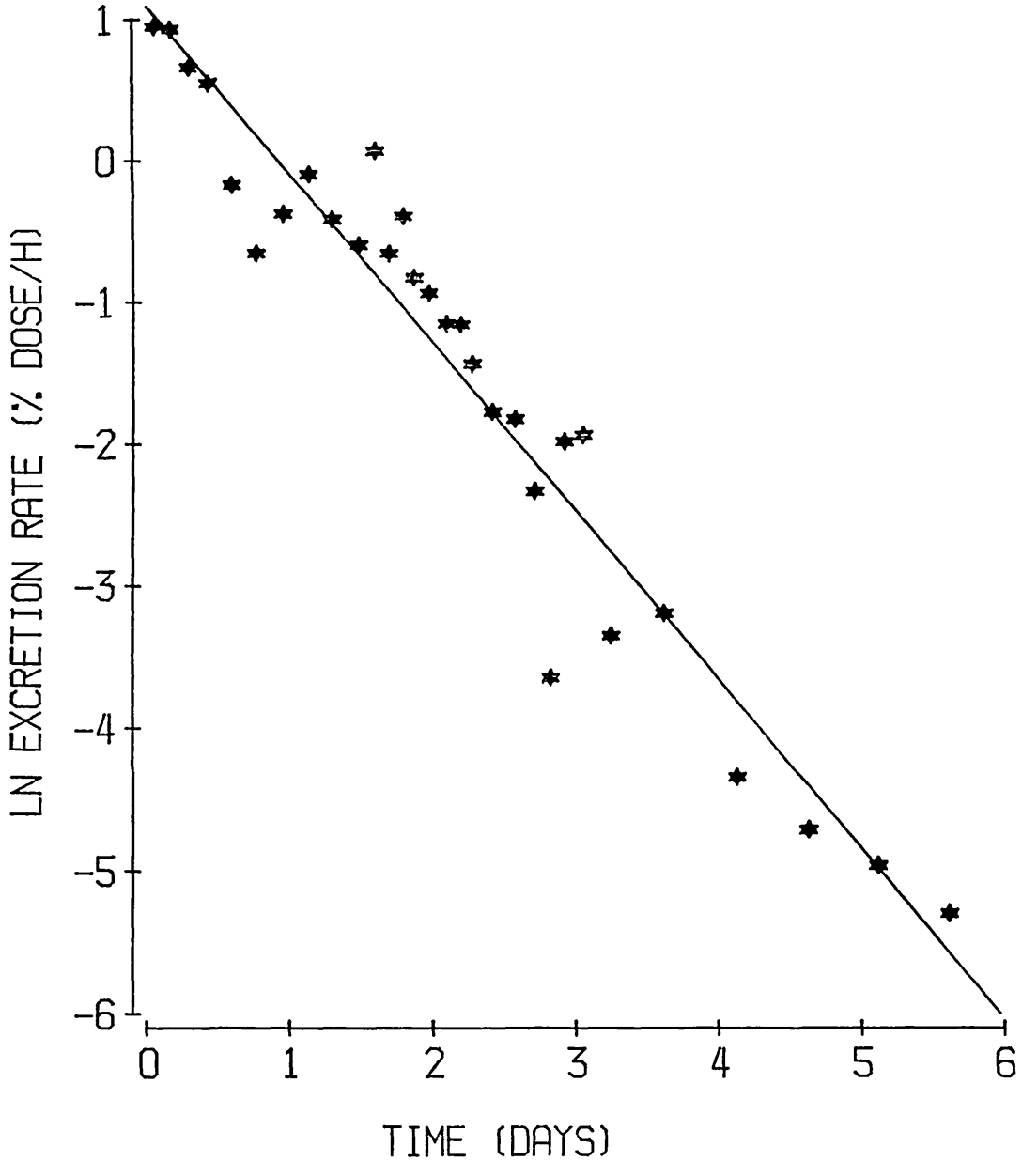
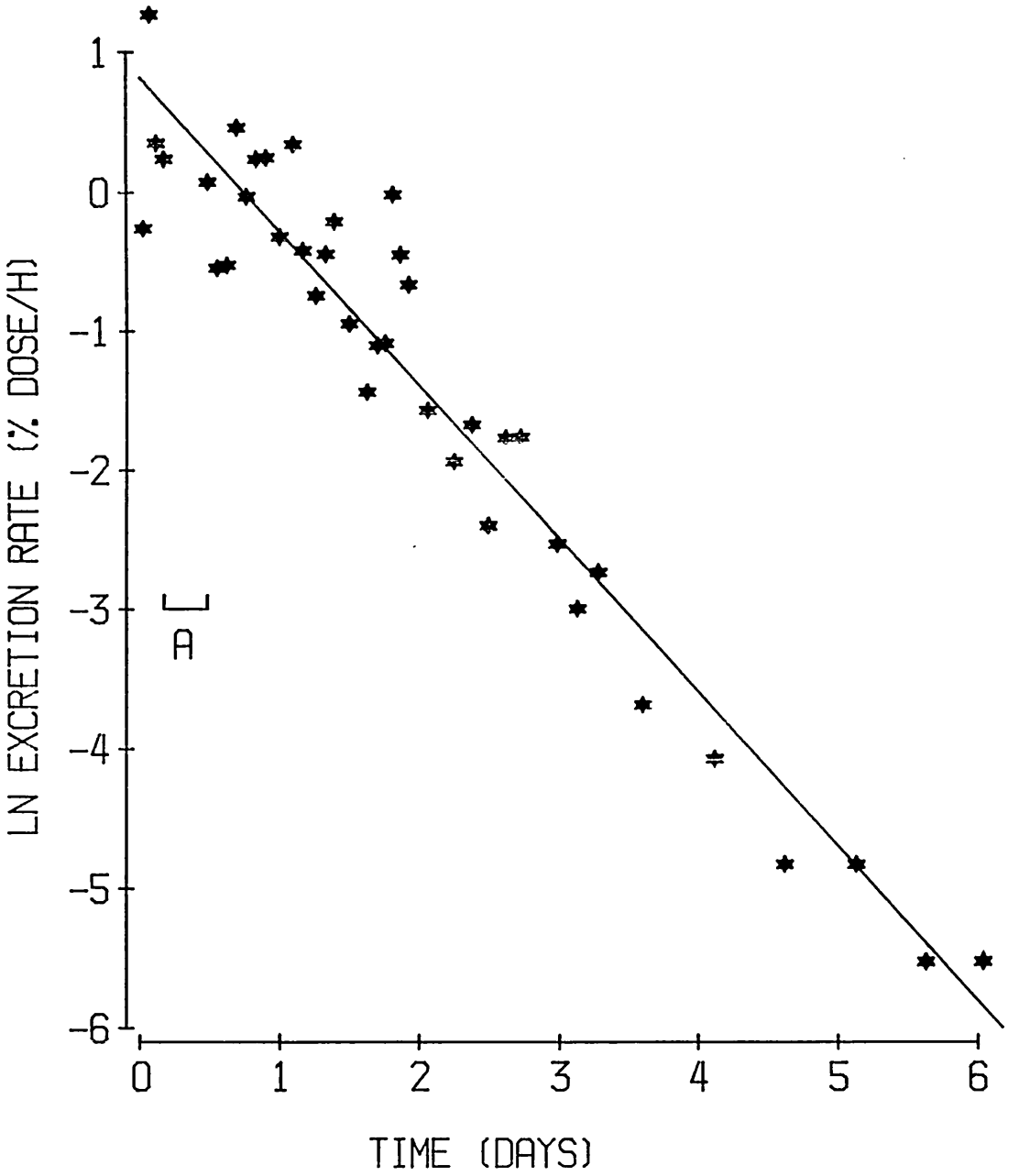


Figure A1.2. **Elimination of urinary radioactivity. Ln % Dose h^{-1} plotted against sample midpoint time following the administration of ^{14}C-phenylbutazone (Experiment No. 5.)**

Equation of line Y= -0.046X + 0.804 r= -0.964



Calculations to account for sample losses

Figure A1.2 which is the plot of the natural logarithm of the percentage dose excreted per hour against the midpoint time for each sample, is used to account for the lost samples. The sample known to be lost occured in the time period (4.12-11.57 h). Over this time period (7.45 h) 243 ml of urine were collected, compared to a mean urinary flow rate of 187 ml h^{-1} during the rest of the experiment or 1390 ml for an equivalent time period. The amount of the lost sample is calculated from the straight line obtained by regressional analysis using the least squares method.

For the time period 4.12-11.57 h the ln (% dose excreted h^{-1}) changes from 0.498 to 0.168, and at the midpoint of this time (7.84 h) the value is 0.333 or 1.395 % dose excreted h^{-1}. The collection time was 7.45 h and therefore the expected amount excreted over this time period is 10.39% of the dose. The amount actually recovered was 1.13%, and therefore the amount of sample lost is the difference between these two values, 9.27% of the dose.

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