THE STUDY OF THE METABOLISM OF PHENYLBUTAZONE

(4-BUTYL-1,2-DIPHENYLPYRAZOLIDINE-3,5-DIONE)

IN RATS

THESIS

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A B S T R A C T

In this study the metabolism of the anti-arthritic drug, phenylbutazone, was investigated in female Wistar rats, and the results compared with those of other workers in this field.

Two interrelated projects were undertaken. The first covered the pattern of excretion, isolation and characterisation of the metabolites and decomposition products of phenylbutazone in rats dosed post-orally with the drug. It was found that the major route of excretion was via the urine and over 50% of the administered dose was excreted in the first 24 hours by this route. A small percentage of the dose was excreted in the faeces. The following compounds were identified using chromatographic and autoradiographic techniques:

p-Hydroxy derivative of phenylbutazone
γ-Hydroxy derivative of phenylbutazone in both its molecular forms
(ring lactone and straight chain hydroxyl)
4-Hydroxy derivative of phenylbutazone
p-γ-Dihydroxy derivative of phenylbutazone
p-4-Dihydroxy derivative of phenylbutazone
Hydrolysable conjugates (possibly glucuronides)
Water soluble non-hydrolysable conjugates.

The second project dealt with the quantitation of the water insoluble

compounds isolated in the initial work. Using a unique technique, combining inverse isotope dilution assay and spectrophotometric analysis, it was found that the major metabolite was the γ-hydroxy derivative of phenylbutazone, present in both its molecular forms. Oxyphenbutazone was a minor metabolite and the p-γ-dihydroxy derivative of phenylbutazone was present only in very low concentration.

These results did not conform with those of previous workers in this field who reported the γ-hydroxy derivative of phenylbutazone, in one molecular form only, as the major metabolite and the dihydroxy derivative as the second metabolite with a higher concentration in the urine than oxyphenbutazone. This disparity could be due to the fact that these workers took no account of the presence of the two molecular forms of the γ-hydroxy derivative of phenylbutazone with their different polarities and different R_f values. The present study showed that the straight chain hydroxyl isomer was probably mistakenly identified as the p-γ-dihydroxy derivative of phenylbutazone. This theory is supported by the fact that the percentage dose recovered by the previous workers of the γ-hydroxy and p-γ-dihydroxy derivatives together equalled the percentage dose recovered in this study of the two molecular forms of the γ-hydroxy derivative.

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ABBREVIATIONS

ADP	Adenosine-5'-diphosphoric acid
APS	Adenosine-5'-phosphosulphate
ATP	Adenosine-5'-triphosphoric acid
Bq	Bequerel
cpm	Counts per minute
Cyt	Cytochrome
dpm	Disintegrations per minute
DSC	Differential Scanning Calorimetry
e	Electron
E	Efficiency
Fp	Flavoprotein
FpH_2	Flavoprotein (reduced)
GC	Gas chromatography
GC/MS	Gas chromatography/Mass Spectrometry
HSA	Human Serum Albumin
IM	Intramuscular
l.s.c.	Liquid Scintillation Counter
mfo	Mixed Function oxidase
MS	Mass Spectrometry
NAD	Nicotinamide-adenine-dinucleotide
$NADH_2$	Nicotinamide-adenine-dinucleotide (reduced)
NADP	Nicotinamide-adenine-dinucleotide phosphate

$NADPH_2$	Nicotinamide-adenine-dinucleotide phosphate (reduced)
NHI	Non-haem-iron protein
4-OH	4-Hydroxy
γ-OHPB	γ-Hydroxy Derivative of phenylbutazone
OPB	Oxyphenbutazone
PAP	3'-phospho-adenosine-5'-phosphate
PAPS	3'-phospho-adenosine-5'-phosphosulphate
p	Para
PB	Phenylbutazone
PBOH	Hydroxy Derivative of phenylbutazone
p-γ-DiOH	Para-γ-Dihydroxy
PP	Inorganic phosphate
r.p.m.	Revolutions per minute
s.a.	Specific activity
t	Tertiary
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
UDPG	Uridine-diphospho-glucose
UDPGA	Uridine-diphospho-glucuronic acid
UV	Ultraviolet light
K_p	Partition co-efficient for the system peanut oil / Sørensen buffer pH 7,4

NB When referring to the Boehringer Mannheim enzyme, -Glucuronidase/ arylsulfatase the form of spelling used is sulf...; at all other times the term sulph... is employed

INTRODUCTION

CHAPTER I

PYRAZOLIDINE 3,5-DIONES AND DERIVATIVES

Phenylbutazone is a derivative of pyrazolidine 3,5-dione (Figure 1) and has been the subject of extensive investigation as an antiarthritic drug.

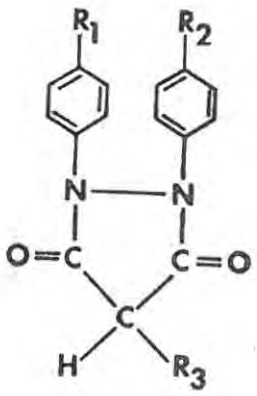


Fig. 1 Basic Structure of Phenyl Pyrazolidine 3,5-dione

Other related compounds have been investigated as uricosuric and anti-inflammatory agents. These are listed in Table 1.

Table 1 STRUCTURE OF PHENYLBUTAZONE AND RELATED DRUGS

Number	Compound	R_1	R_2	R_3
1	Phenylbutazone	H	H	$(CH_2)_3CH_3$
2	γ-Hydroxy derivative	H	H	$(CH_2)_2CHOHCH_3$
3	p-γ-Dihydroxy derivative	OH	H	$(CH_2)_2CHOHCH_3$
4	p-Hydroxy derivative (Oxyphenbutazone)	OH	H	$(CH_2)_3CH_3$
5	p-Nitro derivative	NO_2	H	$(CH_2)_3CH_3$
6	p-p'-Difluoro derivative	F	F	$(CH_2)_3CH_3$
7	p-p'-Dichloro derivative	Cl	Cl	$(CH_2)_3CH_3$
8	Dimethylsulphone derivative	CH_3SO_2	CH_3SO_2	$(CH_2)_3CH_3$
9	Isopropyl derivative	H	H	$CH(CH_3)_2$
10	Ethylsulphoxyphenyl derivative	H	H	$(CH_2)_2SOC_6H_5$
11	γ-Keto derivative	H	H	$(CH_2)_2COCH_3$
12	Ethylsulphonyphenyl derivative	H	H	$(CH_2)_2SO_2C_6H_5$
13	Prenazone	H	H	$CH_2CH=C(CH_3)_2$
14	p-Hydroxy-tertiary butyl derivative	OH	H	$C(CH_3)_3$
15	p-Hydroxy-isopropyl derivative	OH	H	$CH(CH_3)_2$
16	p-p'-Dimethyl derivative	CH_3	CH_3	$(CH_2)_3CH_3$
17	4-Phenylthioethyl derivative	H	H	$(CH_2)_3SC_6H_5$
18	p-Hydroxy-α-keto derivative	OH	H	$CO(CH_2)_2CH_3$

It has been shown that variations in the ring structure, R_1 and R_2, and in the side chain, R_3, cause marked changes in the physico-chemical properties of these derivatives. These changes, in turn, have a direct influence on their pharmacokinetic and therapeutic values (15, 18, 24, 44, 65).

The correlation between structure and some of the important pharma=cokinetic and therapeutic values is shown in Table 2. (15, 18, 19, 21, 24, 39, 44, 48, 55, 56).

Plasma binding studies of the derivatives of phenylbutazone with human plasma were undertaken (24, 44) using the method described by Burns (11).

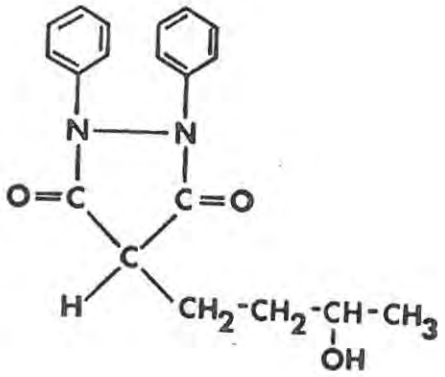
It was found that:

(i) the majority of the derivatives were highly bound, in the order of 98-99%;

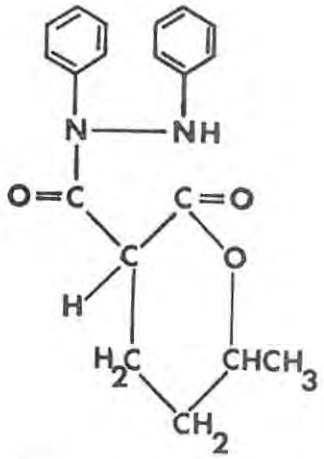
(ii) substitution of a hydroxyl group in the gamma position of the side chain reduced the binding capacity of the γ-hydroxy and p-γ-dihydroxy derivatives of phenylbutazone to 94% and 83% respectively. However, substitution with other polar groups, such as a keto group, in this position had no effect on binding (44). Therefore it appeared that the equilibrium reaction of the two molecular forms, one of which was a lactone, reduced the drug-protein interaction and thereby lowered the plasma binding capacity of the γ-hydroxy derivatives (Figure 3).

Table 2 PHARMACOKINETIC AND THERAPEUTIC VALUES OF DERIVATIVES OF PYRAZOLIDINE 3,5-DIONE

Derivative Number	Plasma Binding %		Half-life h		pKa	24 h urinary excretion % dose	Kp	Uricosuric dose mg
	Man	Dog	Man	Dog		Man		
1	99	92	72	7	4,5	< 1	2,2	800-1 000
2	94	85	10	2	4,0	8	0,6	150- 300
3	83	63	4	2	4,3	< 50	0,01	
4	99	89	72	0,5	4,7	< 2	0,6	800-1 000
5	98	98	20	12	3,2	< 2	1,0	30- 100
6	99	91	40	47	4,5	< 1	1,0	1 500
7	99	99	20	33	4,0	< 1	83	1 500
8	98	87	1	0,5	2,6	40	0,6	100- 150
9	98	96	72	5,0	5,5	< 1	3,4	1 200 - no effect
10	99	90	2,5	2	2,8	43	0,5	30- 70
11	98	97	-	-	2,0	42	-	-
12	99	90	3	2	2,7	35	0,5	100
13	-	91	20	4,8	5,1	< 0,1	-	-
14	99	87	1	1	7,1	-	8,0	-
15	96	79	12	1	5,8	-	0,4	-
16	-	-	-	-	4,9	24	-	1 200 - no effect
17	99	99	3	7	3,9	< 1	1,6	150- 300
18	99	91	8	3	2,3	41	0,4	100



Straight chain hydroxyl isomer



Ring lactone isomer

Fig. 2 Molecular Forms of the γ-Hydroxy Derivative of Phenylbutazone

The biological half-life of phenylbutazone in man has been reported as 72 hours (11). Substitution of the hydroxyl group in the R_1 position had no effect on the half life, but the introduction of the more acidic nitro group reduced the half life to twenty hours (56). Derivatives with halides in the R_1 and R_2 positions also reduced the half-life; that of the difluoro derivative to 40 hours and of the dichloro derivative to 20 hours (24). Substitution with a highly acidic group, such as methylsulphone, in these positions had an even more marked effect, reducing the half-life to one hour (24). It appeared that there was an inverse relationship between the acidity of the substituent and the half-life of the derivative.

Substitution of an isopropyl group in the R_3 position also had no effect on the half-life of phenylbutazone (24) but, as in the case of ring substitution, the introduction of more acidic groups reduced the half-life. For example, substitution of a γ-hydroxy group reduced the half-life to ten hours (55) and of ethylsulphoxyphenyl to 2½ hours (21).

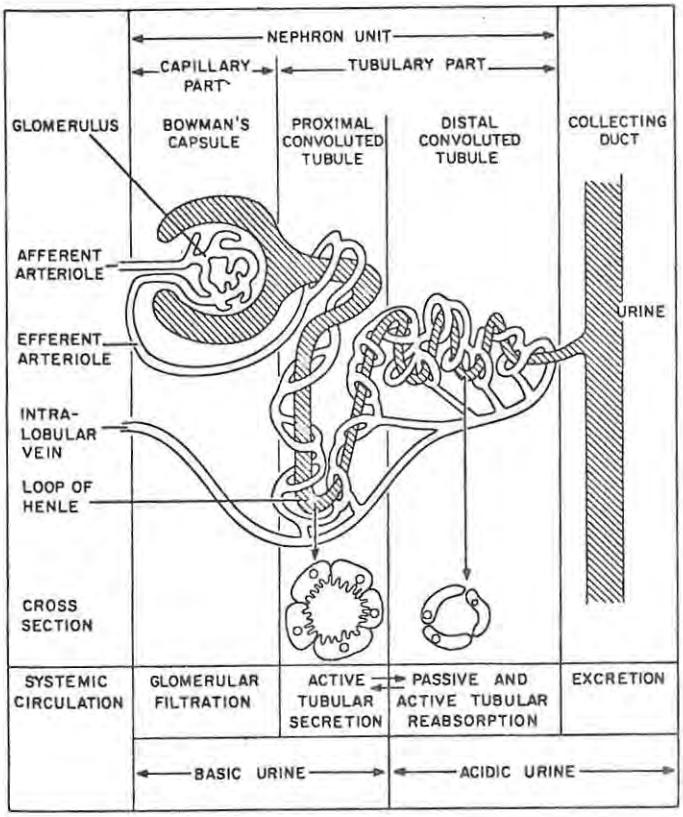


Fig. 3 Schematic Diagram of the Structure of a Nephron Unit and Its Function

There is a strong relationship between renal excretion and the pKa of a derivative. Blood is filtered through the glomerular membranes, which act as though they had pores 10 nm wide and 50 nm long (47), thus preventing excretion of very large molecules, including the plasma bound derivatives; therefore the concentration of the non-ionised, readily diffusible drug in the glomerular filtrate is very low. During the passage of the filtrate through the proximal convoluted tubule approximately 99% of the water is re-absorbed (47), thereby increasing the concentration of the drug and encouraging its re-absorption into the peritubular fluid. This re-absorption has been termed non-ionic back diffusion (24). If the drug is ionised, there is a very low concentration of unionised molecules in the fluid and secretion from the peritubular fluid to the tubular fluid can occur. Therefore, derivatives with a low pKa value should be ionised in the basic fluid of the proximal convoluted tubules and excretion would be expected. In the distal convoluted tubules, where the fluid is acid, there is minimal ionisation, thus re-absorption is favoured. These theories were confirmed by experimentation in dogs (24). Peak secretion occurred in the proximal tubular segments, and re-absorption took place principally from the distal tubular segments, though less acidic drugs were also re-absorbed in the proximal segments (24). As stated earlier, it was found that the majority of the derivatives of phenylbutazone were very strongly bound to plasma protein in man (24, 44), therefore the quantities in the glomerular filtrate were uniformly small and differences in the rate of renal excretion must

depend on differences in the rate of tubular secretion and/or re-absorption. The derivatives with a low pKa value would be ionised in the basic urine of the proximal tubules, thus active secretion would be facilitated and the rate of excretion increased. In fact it has been shown that such changes in the structure of phenylbutazone which decreased the pKa value markedly enhanced urinary excretion (3, 24, 44). Phenylbutazone had a pKa value of 4,5 and was excreted at the rate of less than 1% of the dose in 24 hours in man (24). Substitution of a hydroxyl group in the R_1 position did not reduce the pKa value or the rate of excretion. But substitution in the R_1 and R_2 positions with the acidic methylsulphone group reduced the pKa value to 2,6 and caused an increase in the rate of excretion to 40% of the dose in 24 hours (24).

Similarly, substitution of acidic groups, such as γ-keto, ethylsulphonyl-phenyl and ethylsulphoxyphenyl, in the side chain decreased the pKa values to 2,0, 2,7 and 2,8 respectively (24). Substitution of an acidic group into the p-hydroxy derivative of phenylbutazone also had the effect of reducing the pKa value and increasing the rate of excretion. For example the α-keto derivative of oxyphenbutazone was found to have a pKa value of 2,3, and 41% of the dose was excreted in 24 hours (48). Derivatives with a high pKa value would be unionised in the proximal tubules, therefore the concentration gradient would favour secretion. In fact, it has been reported that some non-ionic back diffusion took place in the proximal tubules and was considerably greater in the distal tubules, resulting in a low rate of excretion.

Thus, the isopropyl and p-p'-difluoro derivatives of phenylbutazone and prenazone with pKa values of 5,8, 4,5 and 5,1 had rates of excretion of less than 1% of the dose in 24 hours (Table 2).

The biological half-life and rate of excretion of the derivatives of phenylbutazone have been correlated, to a certain extent, with their partition co-efficient between peanut oil and buffer of pH 7,4; this value was referred to by Perel as Kp (44). It was shown that, of the derivatives tested, in general those with a Kp value less than 0,6 had a biological half-life of under twelve hours.(Table 2).

It would appear from the foregoing that a low pKa value led to a short biological half-life. There were certain anomalies to this as can be seen from Table 2. For example the t-butyl-p-hydroxy derivative of phenylbutazone had a pKa value of 7,1 and therefore should have had a long half-life. In fact, its half-life was extremely short (1 hour). However, this derivative was excreted mainly as a glucu= ronide (44), and it has been shown that glucuronides have pKa values of the same low order as glucuronic acid, viz 3,2 (25). In addition this t-butyl-p-hydroxy derivative had the high Kp value of 8,0 (44) giving a high lipid solubility which facilitated entry into the liver cells, where conjugation produced a compound with a low pKa value which could be rapidly excreted. On the other hand, the iso-propyl-p-hydroxy derivative of phenylbutazone was also excreted as a glucuronide but had a much longer half-life. This could be explained by the fact that its Kp value (0,4) was much less than that of the

t-butyl-p-hydroxy derivative; therefore its entry into liver cells would be hindered and thus the rate of glucuronide formation reduced. The p-γ-dihydroxy derivative of phenylbutazone also proved an exception to the recognised relationship between pKa, half-life and rate of excretion. This derivative proved to have a relatively high pKa value of 4,3, but was extensively excreted in the urine and had the short half-life of four hours.(44). However, it was found to have a very low Kp (0,01); this low lipid solubility would prevent facile non-ionic back diffusion in the tubules. Also, as aforementioned, only 83% of this derivative was plasma bound, therefore its concentration in the glomerular filtrate would be higher than that of the more highly bound derivatives.

Thus it is apparent that the biological half-life and the rate of excretion of derivatives of phenylbutazone are dependent on the inter-relationship of a number of different factors, such as plasma binding, pKa and Kp values.

Considerable differences in the pharmacological activities of phenylbutazone and its derivatives have been observed (15). It was shown that substitution of a chloro, methyl, nitro or hydroxyl group in the para position of the benzene ring resulted in derivatives possessing potent anti-rheumatic and sodium retaining activities, whereas substitution in the meta position, or in the butyl side chain, produced drugs which lacked these therapeutic properties. For example the p-hydroxy and p-nitro derivatives of phenylbutazone were found to be

as potent oral anti-inflammatory agents as phynylbutazone itself. It has also been shown that uricosuric activity was enhanced as the pKa value of the derivative increased (15, 24, 44). It was noted (13) that derivatives with high pKa values, such as the p-p'-dimethyl and isopropyl derivatives of phenylbutazone with pKa values of 4,9 and 5,5 respectively, had no uricosuric effect in the intravenous dose of 1 200 mg. Phenylbutazone (pKa value 4,5) and its p-hydroxy derivative (pKa value 4,7) required intravenous doses of 800 to 1 200 mg to elicit a 100% increase in uric acid clearance. The γ-hydroxy (pKa value 4,0) and the 4-phenylthioether (pKa value 3,9) derivatives were effective in doses ranging from 300 to 500 mg. The very acidic ethylsulphoxyphenyl derivative (pKa value 2,8) was active in doses as low as 30 mg. A derivative of particular interest was the p-nitro derivative (56). As already stated it proved to have potent anti-rheumatic and sodium retaining effects. In addition, having a pKa value of 3,2, it had pronounced uricosuric activity in dosage as low as 30 to 100 mg.

It thus appears that pharmacological activity can be predicted to a large extent from the nature of the substituents in the basic ring structure of the pyrazolidine 3,5-diones. Furthermore by processes of enzymatic metabolism, the substituents in this basic ring structure are modified to give metabolites with little or no pharmacological activity and physiological properties favouring their excretion.

CHAPTER II

PHYSICAL PROPERTIES AND DECOMPOSITION OF PHENYLBUTAZONE

1. SOLUBILITY OF PHENYLBUTAZONE

Phenylbutazone is almost insoluble in water, but at pH 7,0 the solubility of the sodium salt is 2,2 $mg \cdot ml^{-1}$ (10). It is readily soluble in some organic solvents as shown in Table 3.

Table 3 SOLUBILITIES OF PHENYLBUTAZONE IN SOME ORGANIC SOLVENTS

Solvent	Solubility
Ethyl alcohol	1:28
Ether	1:15
Chloroform	1:1,25
Dichloromethane	> 1:1
Dichloroethane	> 1:1

2. POLYMORPHISM OF PHENYLBUTAZONE

Phenylbutazone has been proved to exist in at least three polymorphic forms (I, II, III) with different Differential Scanning Calorimetry

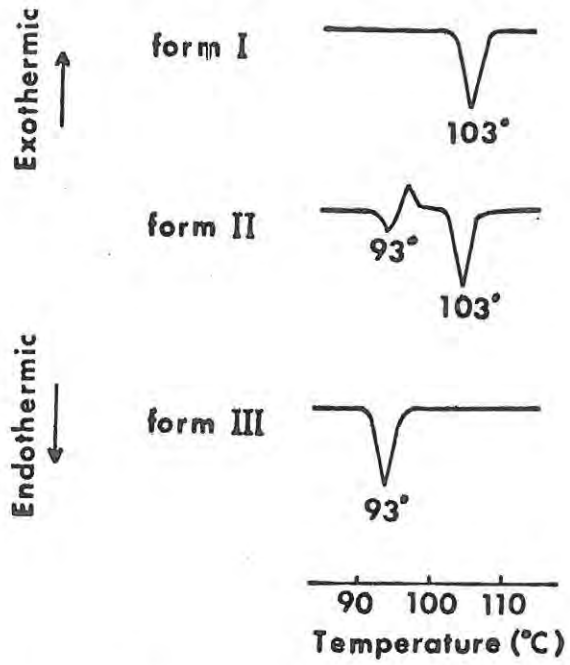


Fig. 4 DSC Curves of Phenylbutazone Polymorphs

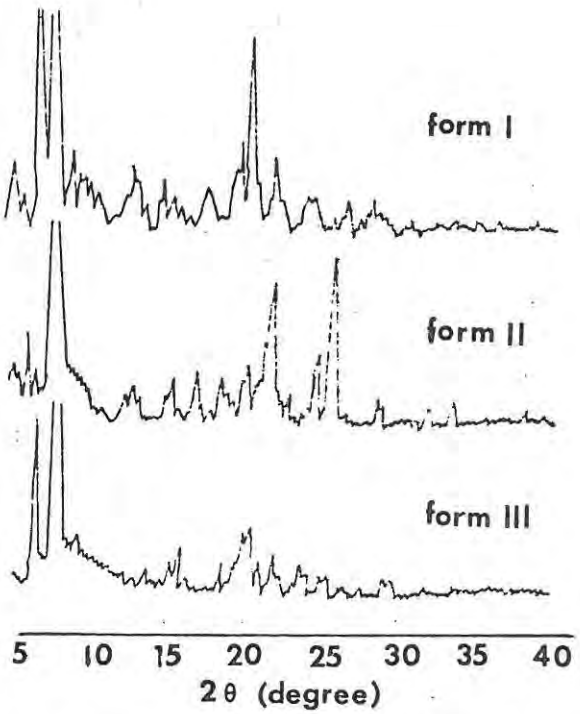


Fig. 5 Powder X-Ray Diffraction Patterns of Phenylbutazone Polymorphs

curves and different powder X-ray diffraction patterns (33). (Figures 4 and 5)

Mutual transition was observed during heating among the polymorphs, as illustrated in Figure 6.

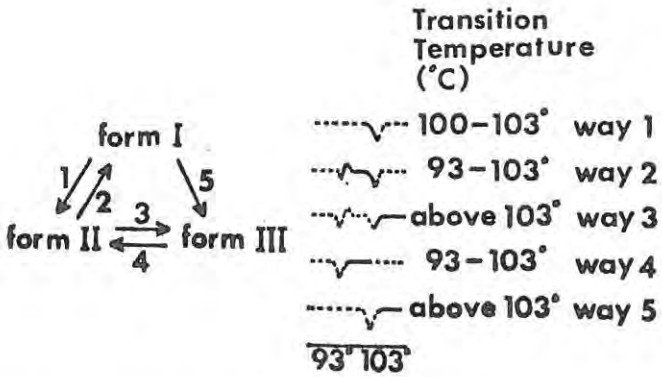


Fig. 6 Mutual Transition of Phenylbutazone Polymorphs

These findings were important because Forms I and II proved to have different dissolution rates and therefore different bio-availabilities. For technical reasons, the dissolution rate for Form III could not be determined, but an intermediate form of Form II and form III, desig= nated 'pseudo Form III', was tested and had the fastest dissolution rate. These dissolution curves are shown in Figure 7.

The commercially available phenylbutazone is considered to be made up of Forms I and II with a melting point of 104 to 107^{o} C (30).

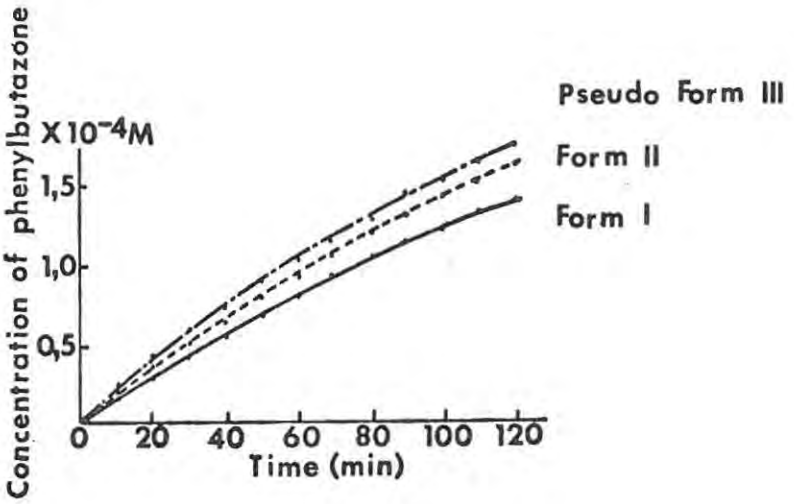


Fig. 7 Dissolution Curves of Phenylbutazone Polymorphs in Phosphate Buffer Solution, pH 7,5, at 30° C by the Stationary Disc Method

3. DECOMPOSITION OF PHENYLBUTAZONE

3.1 Forcing Methods of Oxidation of Phenylbutazone

Workers realised that air oxidation of phenylbutazone occurred and the following forcing methods of oxidation were applied to determine the products of oxidation formed under varying conditions as shown in Figures 8, 9 and 10 (3, 23).

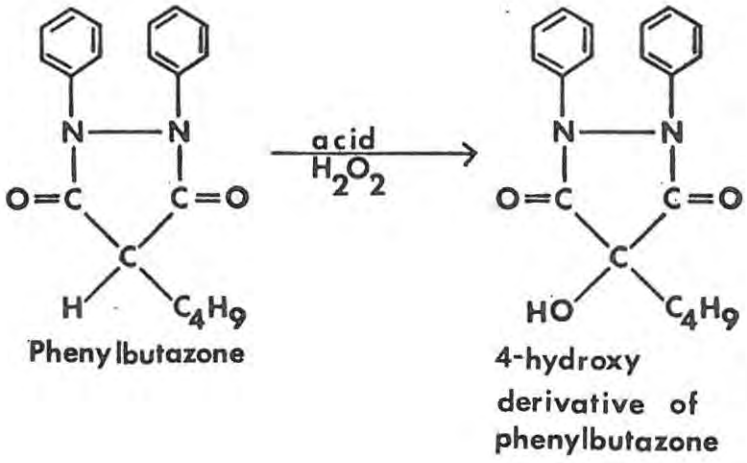


Fig. 8 Acid Oxidation of Phenylbutazone with Hydrogen Peroxide (23)

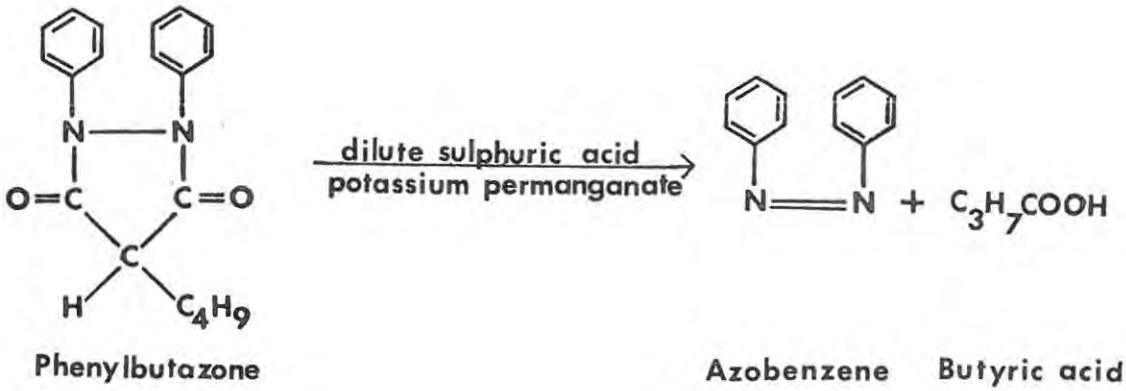


Fig. 9 Acid Oxidation of Phenylbutazone with Potassium Permanganate (3)

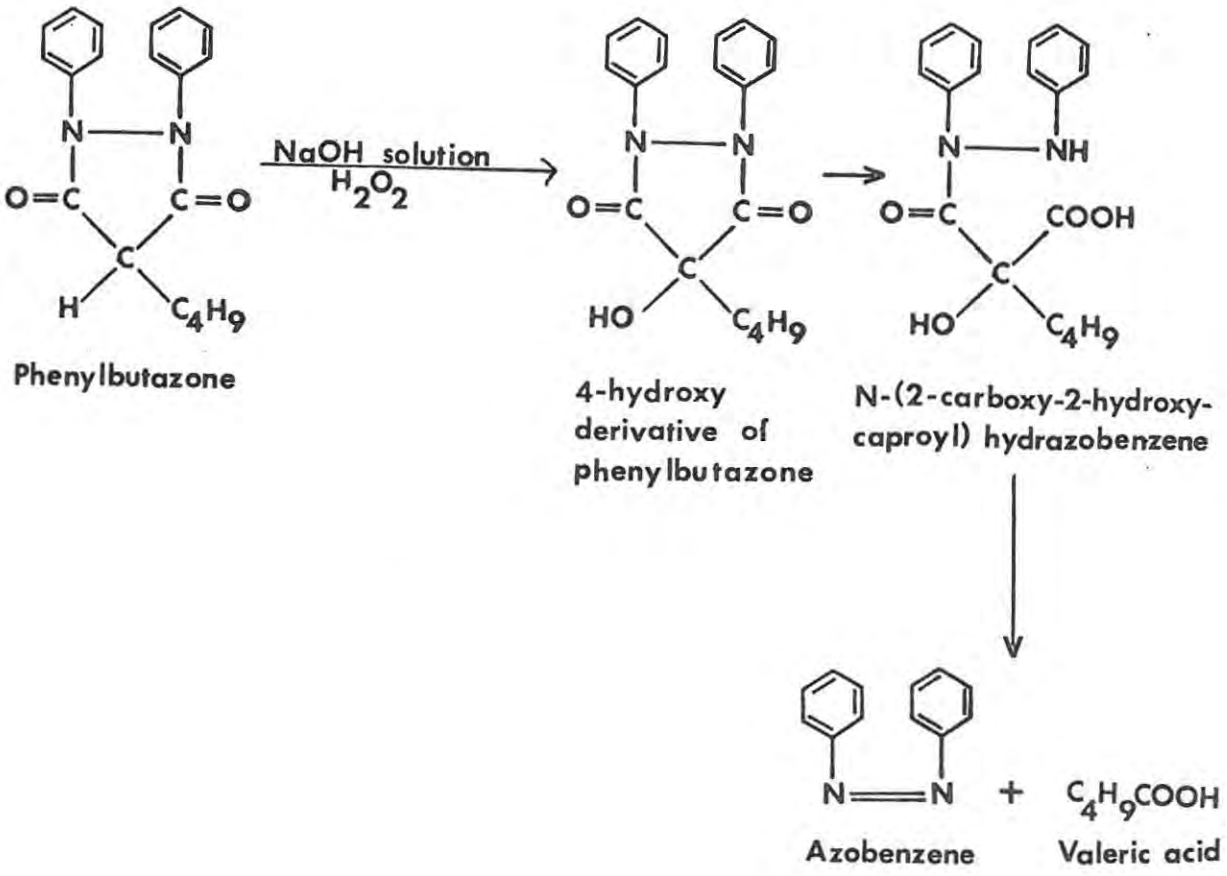
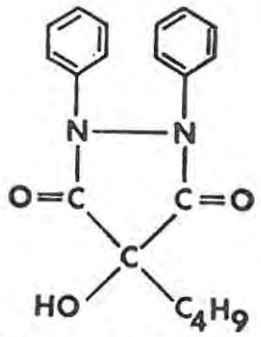


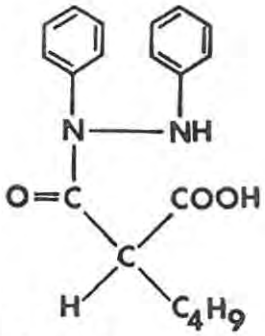
Fig. 10 Alkaline Oxidation of Phenylbutazone with Hydrogen Peroxide (23)

3.2 Products of Decomposition of Phenylbutazone Preparations

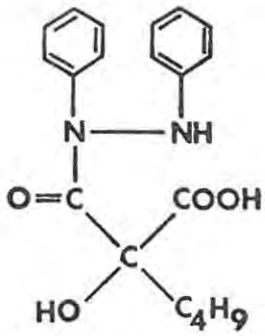
In a study of the stability of phenylbutazone in injection and suppository form, TLC techniques were used to isolate the various decomposition products shown in Figure 11 (40, 41).



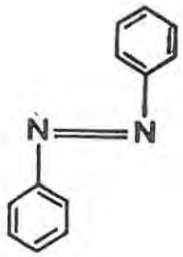
4-hydroxy derivative of phenylbutazone



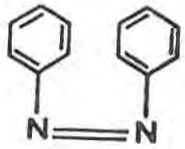
N-(2-carboxycaproyl) hydrazobenzene



N-(2-carboxy-2-hydroxycaproyl) hydrazobenzene



Trans azobenzene



Cis azobenzene

Fig. 11 Products of Decomposition of Phenylbutazone

3.3 Routes of Decomposition

It was found that decomposition products were formed through two routes (42).

3.3.1 Hydrolysis

This is the principal route of decomposition of phenylbuta=

zone in injections and the minor route in suppositories (Figure 12).

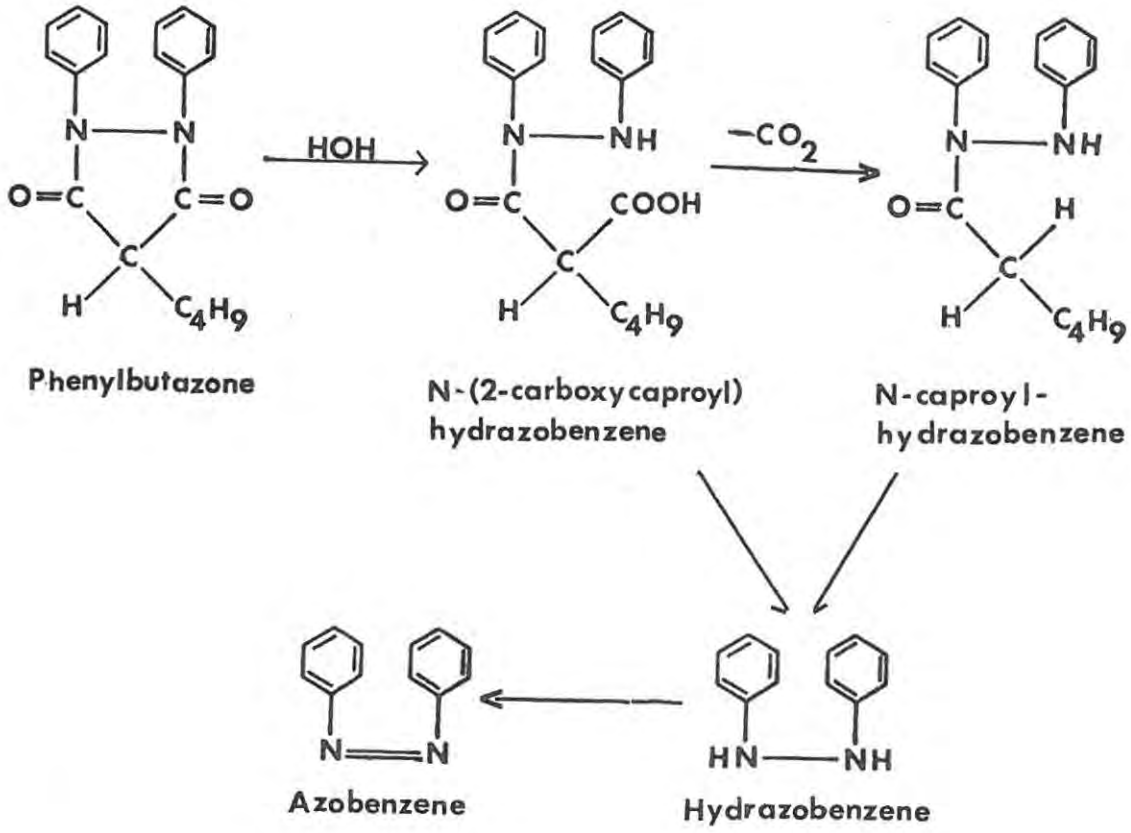


Fig. 12 Route of Decomposition of Phenylbutazone by Hydrolysis

3.3.2 Oxidation

This is the principal route of decomposition of phenylbuta= zone in suppositories and the minor route in injections (Figure 13).

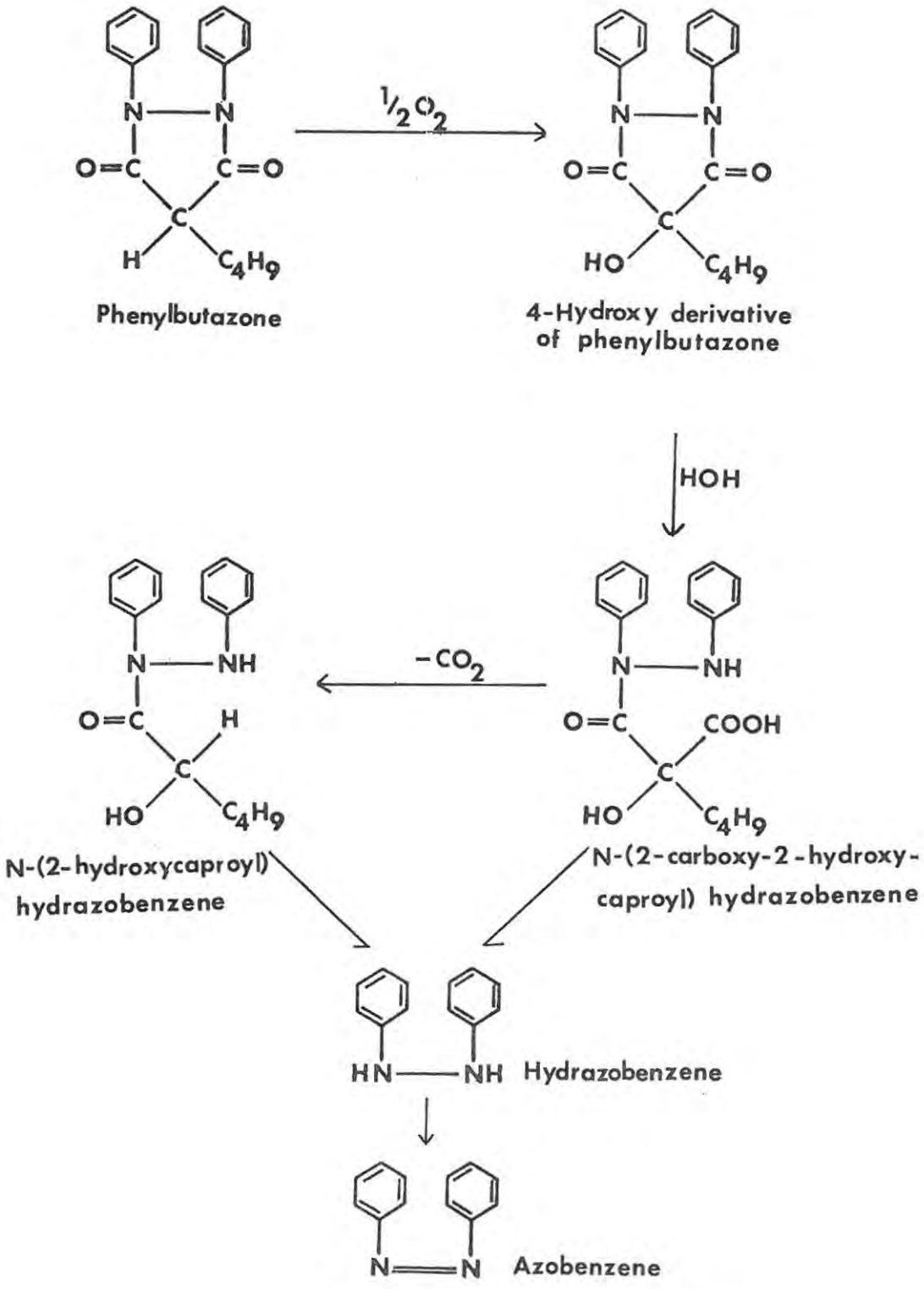


Fig. 13 Route of Decomposition of Phenylbutazone by Oxidation

3.4 Overall Scheme of Decomposition of Phenylbutazone Preparations Under Normal Storage Conditions

Under the relatively mild conditions of normal storage the pattern of decomposition has been found to take place as outlined in Figure 14 (2).

3.5 Oxidation of Phenylbutazone on TLC Plates

Oxidation of Phenylbutazone was shown to take place on TLC plates (2, 4). Two products were observed (2), the major one being the 4-hydroxy derivative of phenylbutazone and the minor one N-(α-keto-caproyl) hydrazobenzene. The fact that none of the products in the pathway II to VII (Figure 14) were identified indicated that N-(α-keto-caproyl) hydrazobenzene was produced directly from the unstable hydroperoxide intermediate shown in Figure 15.

A number of methods have been used for the reduction or prevention of on-plate oxidation. One consists of making up the slurry with McIlvaine buffer (2), or adding an anti-oxidant such as sodium thiosulphate to the slurry (22). Another has been to equilibrate TLC plates overnight in sealed plastic bags filled with nitrogen (4).

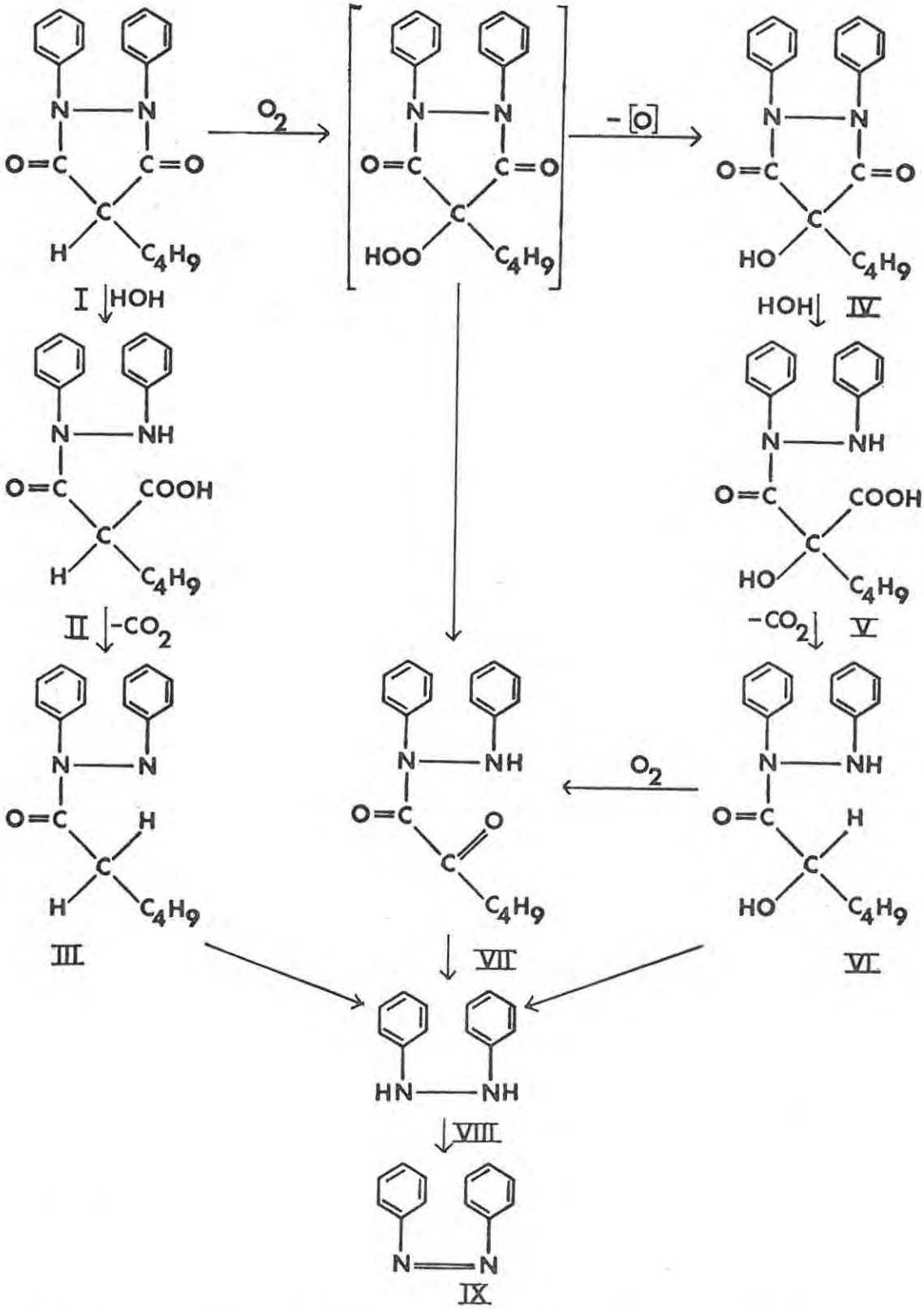


Fig. 14 Scheme of Decomposition of Phenylbutazone During Storage

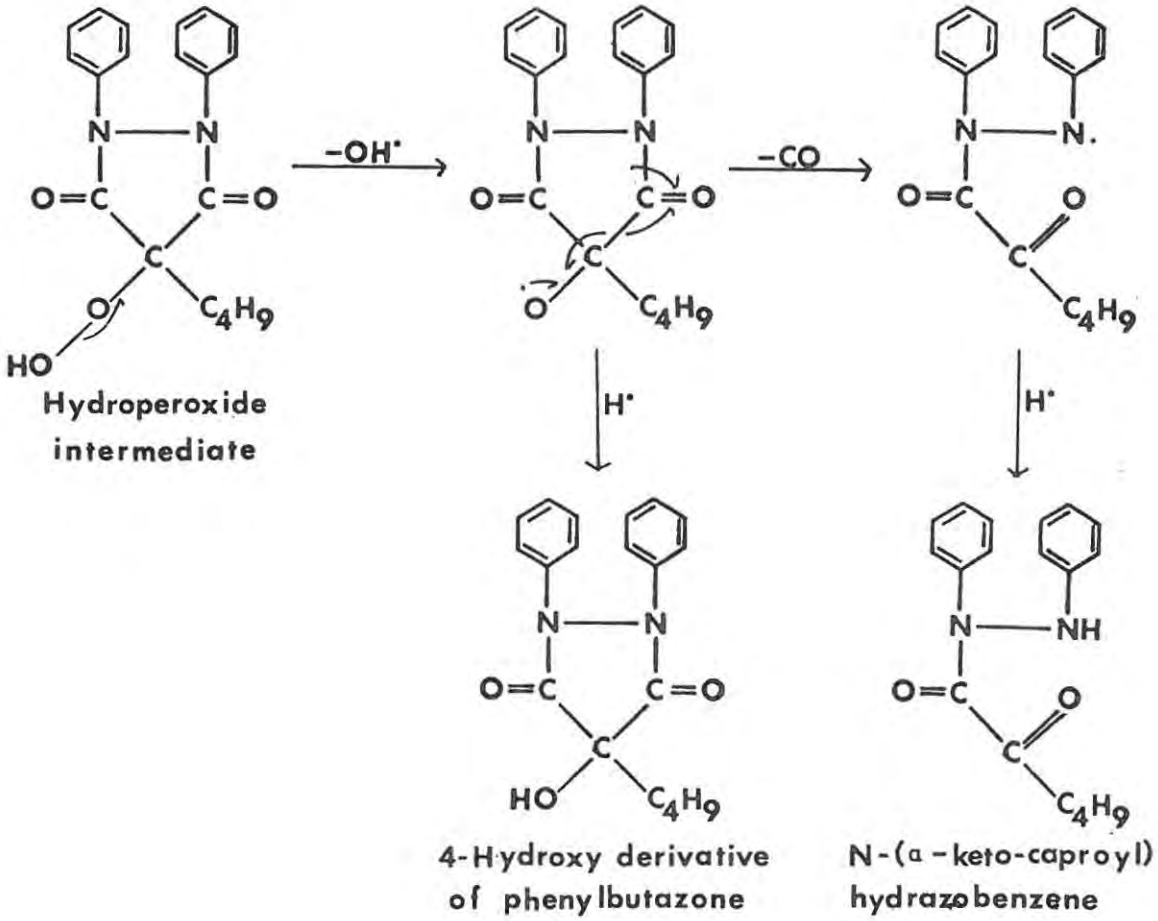


Fig. 15 Products of Oxidation of Phenylbutazone on TLC Plates

CHAPTER III

PHYSIOLOGICAL PARAMETERS OF PHENYLBUTAZONE

1. ABSORPTION

1.1 Extent of Absorption

In a study of the absorption of phenylbutazone in man (11) plasma levels of 8 patients were compared following oral and intramuscular administration of single doses of 800 mg of drug. Twenty-four hours later the levels were found to be approximately the same irrespective of the route of administration, which indicated that absorption from the gastro-intestinal tract was essentially complete (Figure 16).

In an analysis of stools collected, over a 72 h period, from subjects receiving 800 mg phenylbutazone daily less than 5% of the daily dose of the drug was found.

1.2 Rate of Absorption

The rate of absorption through the gastro-intestinal tract was found by measuring plasma levels of patients who received single

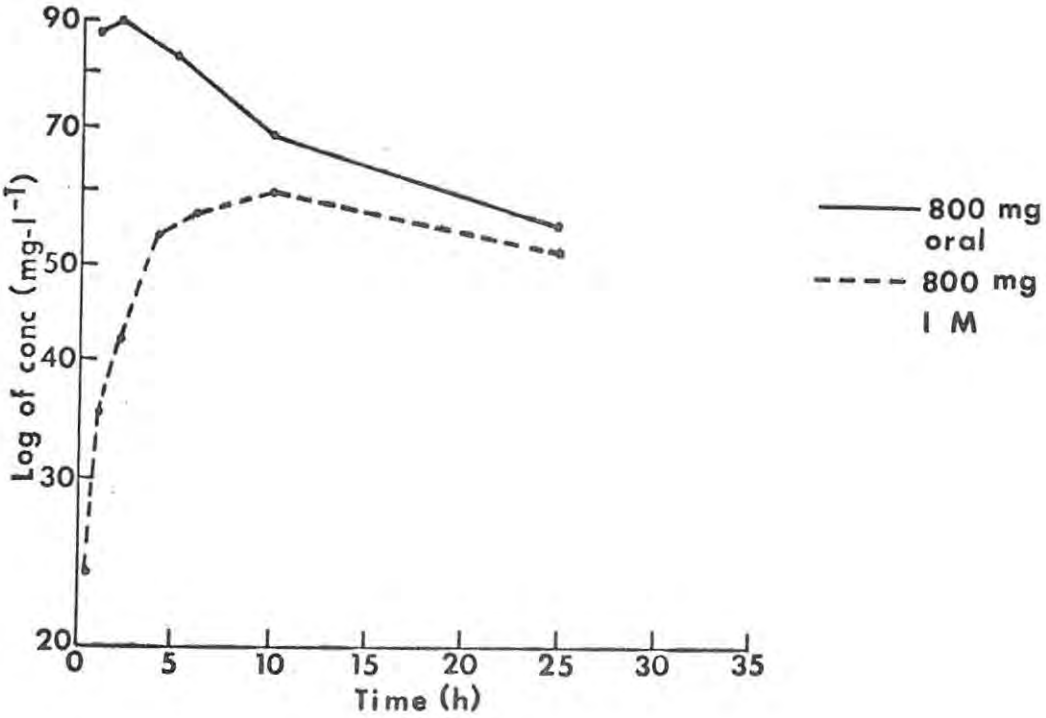


Fig. 16 Plasma Levels of Phenylbutazone after Oral and Intra-muscular Administration of the Drug to the Same Subject

doses of 800 mg of drug orally. Peak levels were usually achieved within 2 h (Figure 16). When the same patients were dosed by the intra-muscular route, peak plasma levels were only achieved within 6 to 8 h of injection (Figure 16) (11). This slow absorption was proved to be due to localisation of the drug at the site of injection due to protein binding in the tissues (11).

2. PLASMA PROTEIN BINDING

Studies in man (24) showed that, at plasma levels within the therapeutic range of concentration (60 - 150 $\mu g \cdot ml^{-1}$), 98 - 99% of the drug was bound to the plasma proteins. At higher levels (250 $\mu g \cdot ml^{-1}$), only 88% of the drug was bound. Comparative work with dogs proved that in this species protein binding was lower than in humans (92%) (Table 2) (44).

3. TISSUE DISTRIBUTION

Distribution studies were undertaken in a dog given 50 $mg \cdot kg^{-1}$ phenylbutazone intravenously and sacrificed 3 h later. The concentration of drug in all tissues was appreciably less than in plasma (Table 4).

Although tissue concentrations were lower than those in whole plasma they were considerably higher than those of unbound drug in plasma. This indicated a considerable interaction between the drug and tissue proteins; hence, the localisation of the drug at the site of intramuscular injection (11).

The tissues of a human subject who received the drug orally for 8 days prior to death also showed an appreciably higher concentration in the plasma than in organ tissue (11) (Table 5).

Table 4 DISTRIBUTION OF PHENYLBUTAZONE IN DOG TISSUES

(Dog received 50 $mg \cdot kg^{-1}$ intraveneously and the tissued were examined three hours later)

Tissue	Concentration of Phenylbutazone
	$mg \cdot kg^{-1}$
Plasma	79
Plasma water	2,0
Red blood cells	9,2
Lung	15
Kidney	48
Liver	64
Heart	30
Muscle	8,1
Brain	15
Lumbodorsal fat	23

Table 5 DISTRIBUTION OF PHENYLBUTAZONE IN HUMAN TISSUES

(Patient received 800 mg phenylbutazone orally for several days prior to death, and received the last dose of 200 mg about 12 h before death. The tissues were removed for analysis within 3 h post mortem)

Tissues	Concentration of Phenylbutazone
	$mg \cdot kg^{-1}$
Plasma	115
Plasma water	2,3
Adrenals	80
Heart	55
Spleen	37
Kidney	70
Lung	78
Muscle	45

4. PLASMA LEVELS

A group of 60 subjects was given 800 mg of phenylbutazone daily for 14 days or more. It was found that during the first 3 or 4 days plasma levels progressively increased, reached a plateau and thereafter were constant from day to day for a given individual. This pleateau level varied from 60 to 150 $mg \cdot l^{-1}$ (11).

5. EXCRETION

Seventy-two hour urine samples from human subjects, who had received 800 mg phenylbutazone by intra-muscular injection, were found to contain only traces of the administered drug (11). Further work showed that less than 1% of the dose was excreted as phenylbutazone in 24 h urine (24).

In order to measure the rate of disappearance 16 human subjects were given intra-muscular injections of phenylbutazone, and their drug plasma levels measured 24 h later and at various times thereafter. The rate of disappearance per person ranged from 10 to 35% per day with an average of 21% (11).

As stated above, less than 1% of the dose was excreted as phenylbutazone in 24 h urine, but the rate of disappearance of the drug from the plasma was proved to be approximately 21%, therefore it can be inferred that biotransformation takes place and the products of

metabolism are excreted. This was proved to be the case. Studies using counter-current distribution methods, accounted for less than 10% of the dose of the phenylbutazone administered (13). However, the introduction of radio-chemical methods greatly clarified the picture. Experiments in rats, using carbon labelled phenylbutazone, showed that most of the excretion occurred in urine within 24 h. No excretion was detected in expired air (43). This was later confirmed by workers who stated that in rats more than half the administered dose was excreted in the urine during the first 24 h. Thereafter urinary excretion was less important, and the mean total excretion in 48 h amounted to 60,8% of the dose. Faecal excretion was reported as 22,9% in the first 24 h and 11,9% in the second 24 h, a total of 34,7%. Again no excretion was detected in the expired air (4). Great variation was shown between individual animals with 48 h urinary excretion varying from 47,2% to 74,3% and faecal excretion from 31,3% to 40,8%.

Studies in humans showed a similar pattern. After 21 days 88% of the dose was recovered, 61% from urine, 27% from faeces and none from expired air (22).

6. BIOLOGICAL HALF-LIFE

The biological half-life of phenylbutazone in man is 72 h (11). Comparative studies with animals showed that in all other species tested the rate of disappearance of the drug from the plasma was faster

than in man (11). Phenylbutazone was administered intravenously to dogs and rabbits in doses of 50 $mg \cdot kg^{-1}$ and 100 $mg \cdot kg^{-1}$ respectively, and intraperitoneally to Wistar rats and Beltsville guinea pigs in doses of 100 $mg \cdot kg^{-1}$. The half-lives were found to be as follows: dog 6 h, rabbit 3 h, rat 6 h, guinea pig 5 h.

It is thought that sex may also affect half-lives, but, so far, the rat is the only species in which this has been proved (9).

CHAPTER IV

PHENYLBUTAZONE METABOLISM

1. MECHANISM OF METABOLISM

It would appear that phenylbutazone is mainly metabolised by liver microsomes through Phase I and Phase II reactions (4, 13, 22, 32, 43).

1.1 Phase I Reactions

The Phase I reactions are largely hydroxylations effected by the mixed function oxidase system following the possible mechanism shown in Figure 17.

Two mechanisms of hydroxylation occur in phenylbutazone, either hydroxylation of the carbon atom in the para position in the aromatic ring to give oxyphenbutazone as a metabolic product, or hydroxylation of the ω-1 carbon atom in the butyl side chain to give the γ-hydroxy derivative of phenylbutazone as a metabolite. These mechanisms of hydroxylation possibly follow independent pathways and utilise different enzyme systems. The results of studies suggest that butyl side chain hydroxylation is the major pathway of Phase I metabolism in phenylbutazone

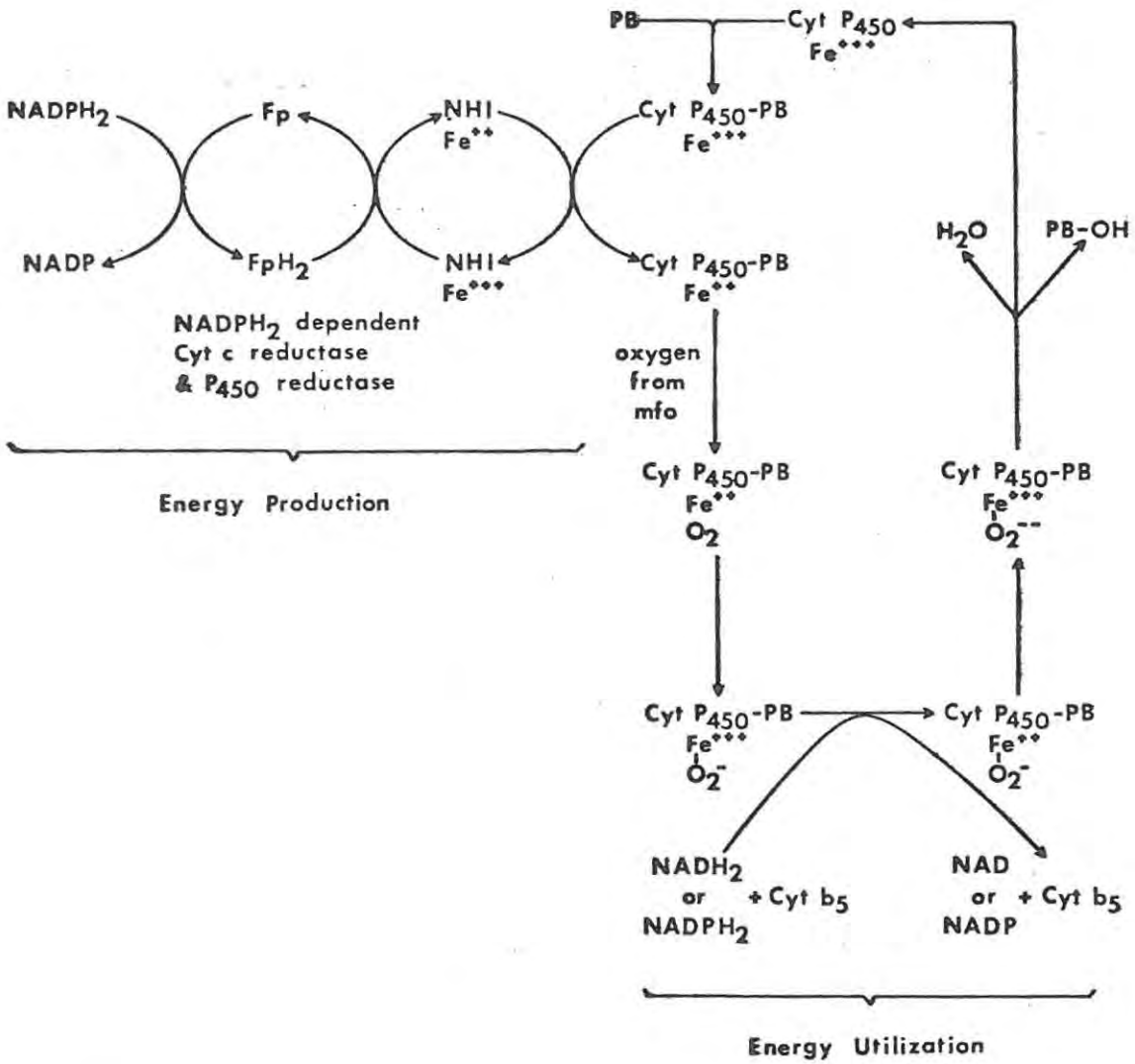


Fig. 17 Possible Mechanism of Phenylbutazone Hydroxylation

(4, 13, 22, 32). The p-γ-dihydroxy derivative of phenylbutazone has also been detected as a metabolite (4, 22).

The products of Phase I hydroxylation may then be either excreted or further metabolised.

1.2 Phase II Reactions

Metabolic products which could be hydrolysed by β-glucuronidase/arylsulphatase were detected in urine and faeces (4). These metabolites were therefore β-glucuronides or sulphates synthesised by liver microsomes utilising Phase II conjugation mechanisms. Such conjugations require an agent, which must be in an active form, and a transferring enzyme.

1.2.1 O-Glucuronide Conjugation

In O-glucuronide formation the agent, glucuronic acid, is activated to form uridine diphosphate glucuronic acid (UDPGA) and the concentration of the enzyme, UDP glucuronyl transferase, is elevated in the vicinity of cytochrome P450, where the mfo reactions are taking place (27). The mechanisms of glucuronide formation of the hydroxy derivatives of phenylbutazone are shown in Figure 18. It is possible that different glucuronyl transferases take part in glucuronide formation as shown in Figure 18 1 and 2.

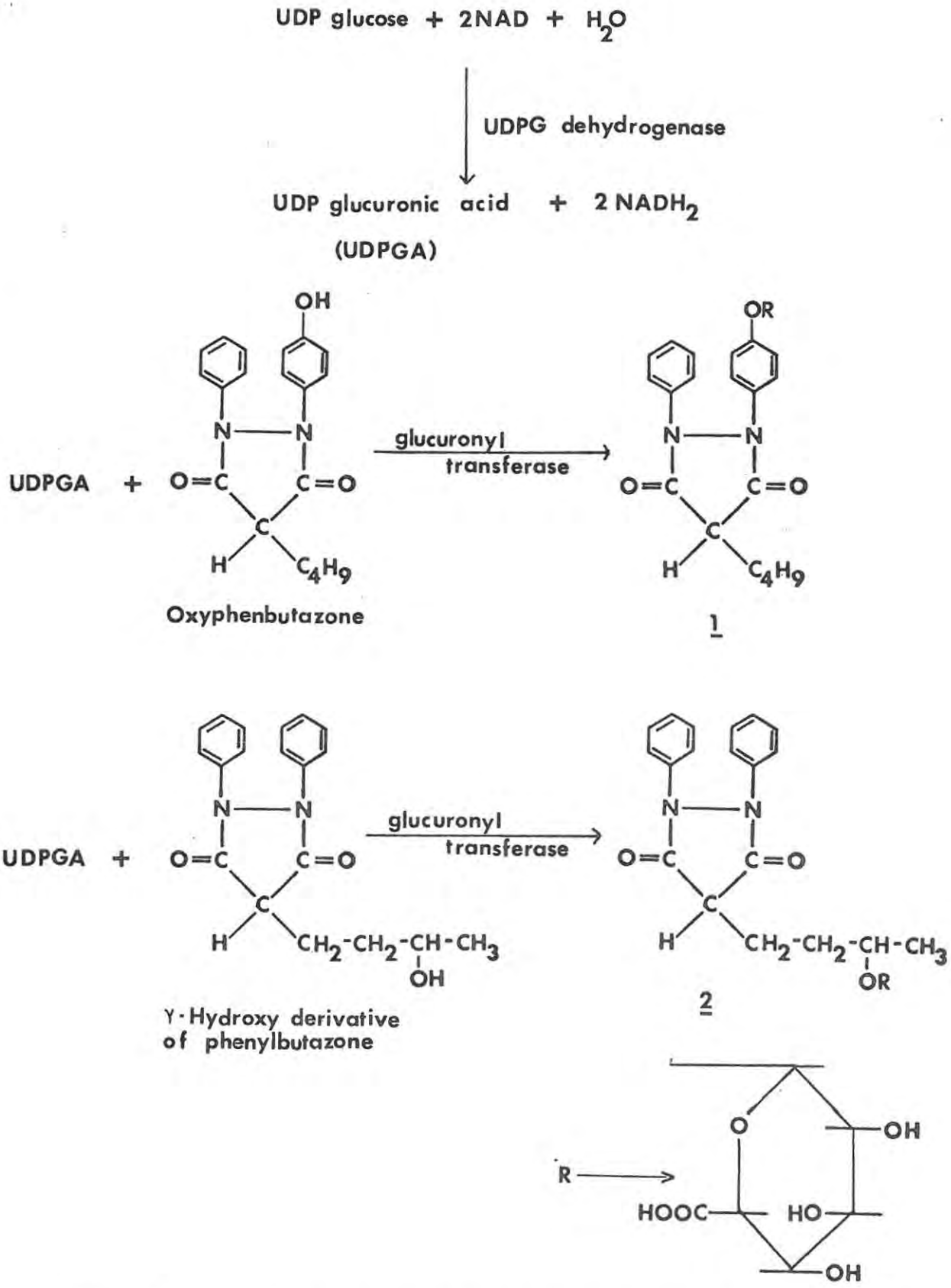


Fig. 18 Mechanisms of O-Glucuronide Formations

1.2.2 O-Sulphate Conjugation

In sulphate conjugation the agent, $SO_4^{=}$, is activated by a series of actions involving ATP to form 3'-phosphoadenosine-5'-phosphosulphate (PAPS) which, in the presence of the enzyme sulphokinase, acts with the product of Phase I metabolism as shown in Figure 19 (28). Similarly, it is possible that different sulphokinases are involved in the conjugation of the phenolic hydroxyl group and the alcoholic group in the butyl side chain.

1.2.3 C-4-Glucuronide Conjugation

Another mechanism of conjugation results in the formation of C-4-glucuronides (21, 22). This mechanism has not yet been elucidated.

1.2.4 Excretion of the Products of Phase II Reactions

Excretion of the products of conjugation can be via the urine or through the enterohepatic circulation in which substances of high molecular mass are transported to the intestine in the bile.

2. REPORTED METABOLITES OF PHENYLBUTAZONE

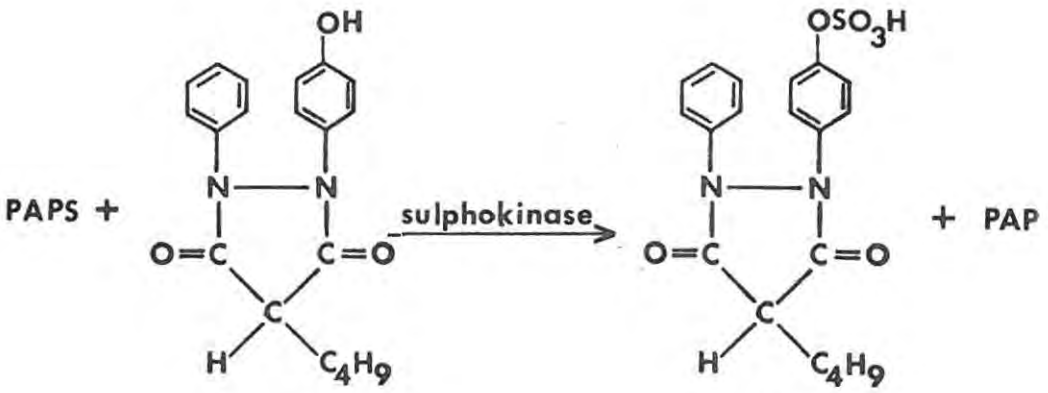
Early work on the metabolism of phenylbutazone showed the presence of two urinary metabolites, oxyphenbutazone and the γ-hydroxy derivative (12) (Figure 20).

$SO_4^{=}$ + ATP $\xrightarrow[\text{sulphurylase}]{\text{ATP}}$ adenosine-5'-phosphosulphate + p p

(APS)

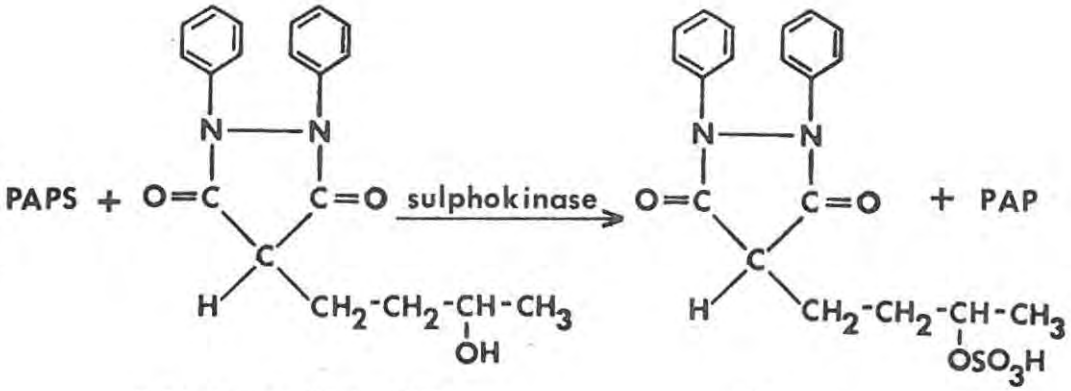
APS + ATP $\xrightarrow[\text{sulphurylase}]{\text{APS}}$ 3'-phosphoadenosine-5'-phosphosulphate + ADP

(PAPS)



Oxyphenbutazone

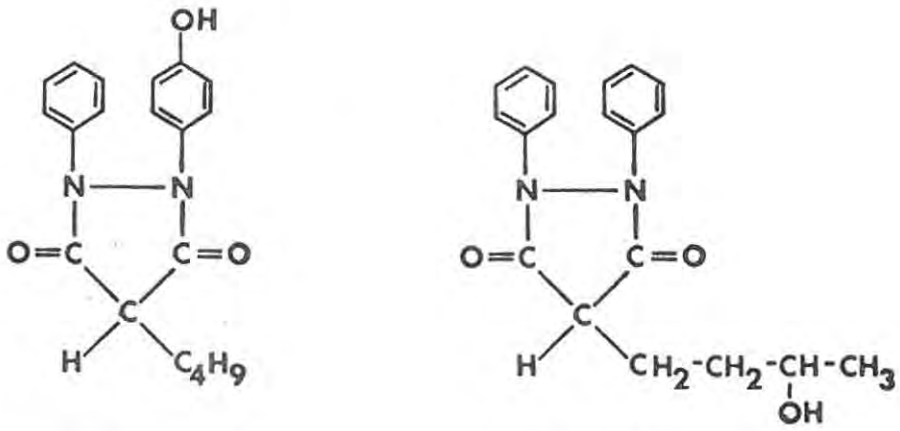
1



γ-Hydroxy derivative of phenylbutazone

2

Fig. 19 Mechanisms of O-Sulphate Formations



Oxyphenbutazone

γ-Hydroxy derivative of phenylbutazone

Fig. 20 Structure of Oxyphenbutazone and the γ-hydroxy Derivative of Phenylbutazone

The presence of these metabolites was confirmed in later studies in rats and humans using labelled drugs (1, 4, 22, 35). Following studies on the metabolism of keto-phenylbutazone (38) where the dihydroxy derivative was proved to be a major metabolite, it was suggested that a dihydroxy derivative could also be a metabolite of phenylbutazone. Bakke (4) reported the presence of this derivative (Figure 21) as a major metabolite in the urine of rats dosed with phenylbutazone and postulated the theory that it was formed by a mechanism shown in Figure 22.

The presence of this derivative has also been reported as a minor metabolite in humans (22).

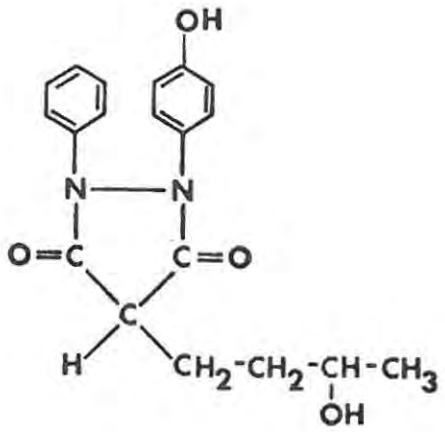


Fig. 21 Structure of the p-γ-dihydroxy Derivative of Phenylbutazone

The 4-hydroxy derivatives of phenylbutazone and oxyphenbutazone (Figure 23) have been isolated from the urine of rats, but it has not been determined whether they are metabolites or artifacts (4).

Reports on the presence of metabolic conjugates in both rats and humans have been mentioned in literature.

In experiments in rats the presence of small amounts of O-conjugates, chiefly glucuronides, of oxyphenbutazone and the γ-hydroxy and p-γ-dihydroxy derivatives of phenylbutazone were reported in the urine, amounting to not more than about 2,7% of the administered dose. In the bile, conjugates amounted to about 4,2% of the dose (Figure 25) (4).

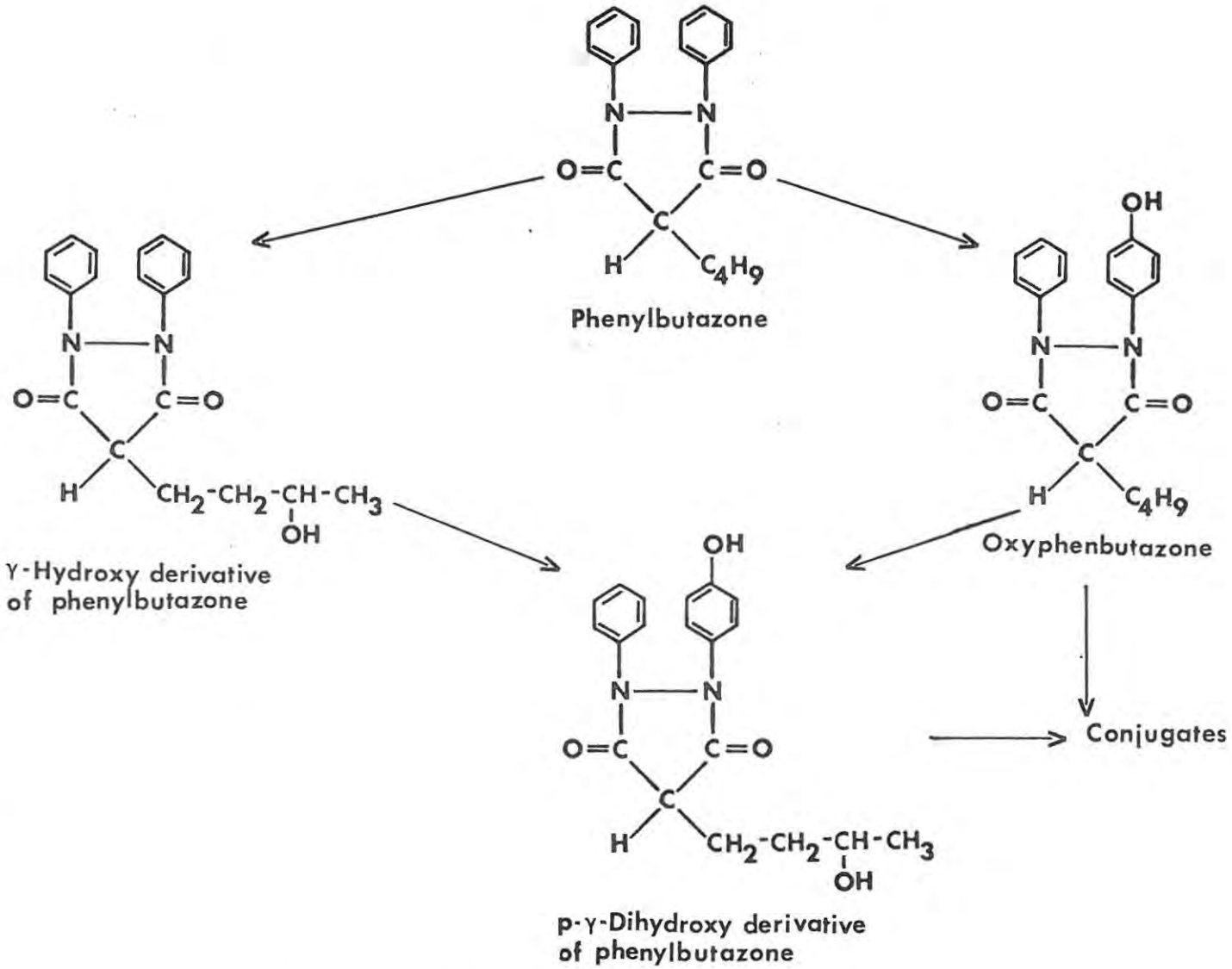
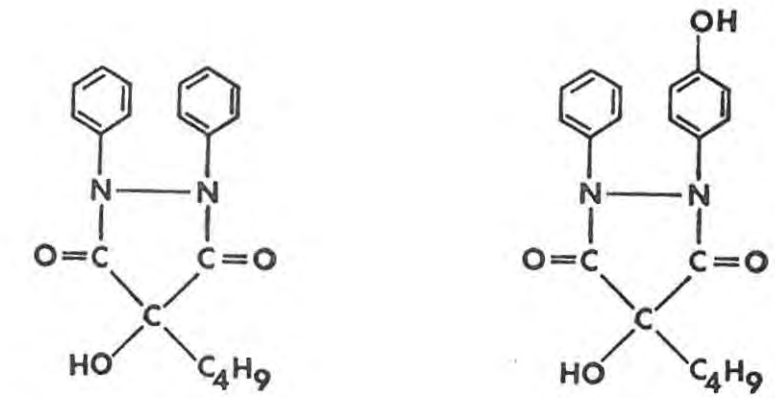


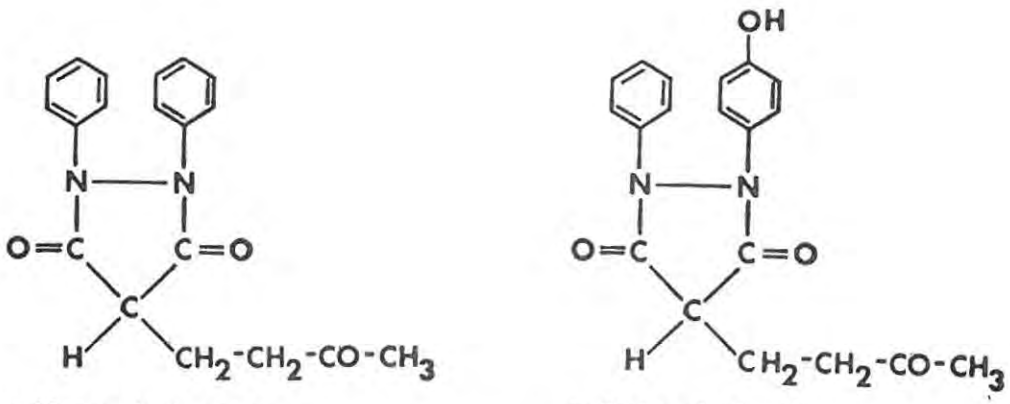
Fig. 22 Formation of the Dihydroxy Derivative of Phenylbutazone



4-Hydroxy derivative of phenylbutazone

4-Hydroxy derivative of oxyphenbutazone

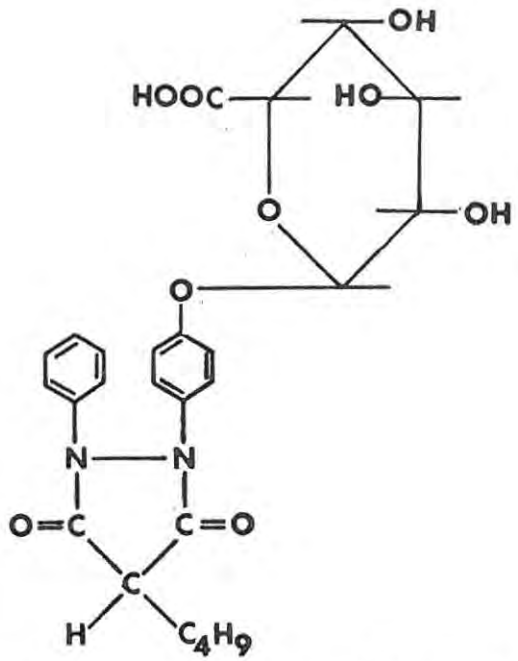
Fig. 23 Structure of the 4-Hydroxy Derivatives of Phenylbutazone and Oxyphenbutazone



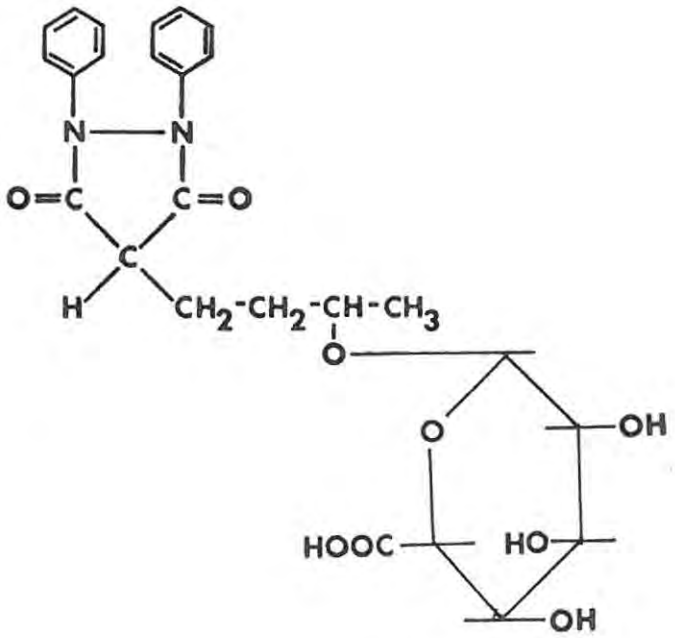
γ-Keto derivative of phenylbutazone

γ-Keto derivative of oxyphenbutazone

Fig. 24 Structure of the γ-Keto Derivatives of Phenylbutazone and Oxyphenbutazone



O-Glucuronide of oxyphenbutazone



O-Glucuronide of the γ-hydroxy derivative of phenylbutazone

Fig. 25 O-Glucuronides of Derivatives of Phenylbutazone

In experiments on humans the presence of an O-glucuronide of oxy= phenbutazone was reported (22) but only in small amounts. However, large amounts of C-4-glucuronides of phenylbutazone and the γ-hydroxy derivative of phenylbutazone were found. These metabolites contained pyrazolidine rings directly attached to glucuronic acid by a C-C bond, representing a novel class of drug metabolites (Figure 26).

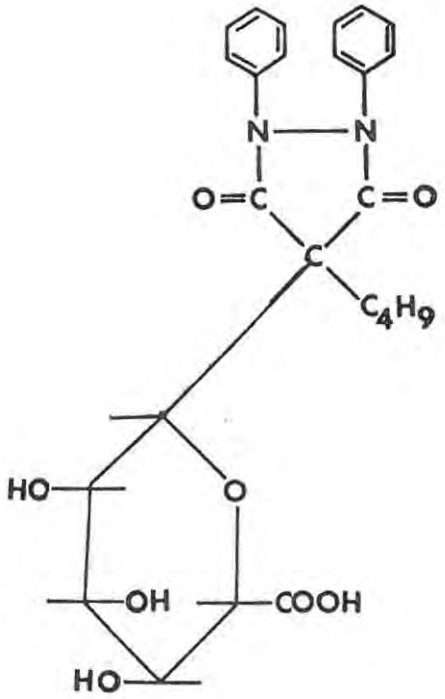


Fig. 26 Structure of C-4-Glucuronide of Phenylbutazone

3. HUMAN STUDIES

3.1 Urine

Early workers (13) reported that only 8% of the dose of

phenylbutazone was excreted in the urine in the form of the me= tabolites, oxyphenbutazone and the γ-hydroxy derivative of phenylbutazone. Later workers (22), using radio-chemical techniques, recovered 61% of the dose in 21 days, made up of non-conjugated metabolites, hydrolysable conjugates and non-hydrolysable conjugates (Table 6). The major portion of this radioactivity was in the form of C-4 conjugates, which would not have been apparent to the earlier workers with their less sophisticated techniques.

Table 6 RADIOACTIVITY RECOVERED FROM THE URINE OF HUMANS DOSED WITH PHENYLBUTAZONE

Compound	% of dose administered
Phenylbutazone	less than 0,7
Oxyphenbutazone	less than 0,7
γ-Hydroxy derivative of phenylbutazone	2,1
p-γ- Dihydroxy derivative of phenylbutazone	4,0
Hydrolysable conjugates	0,7
C-4 conjugates	39,0
Unidentified water solubles	14,0

3.2 Faeces

The same workers (22) recovered 27% of the dose from the faeces collected during 21 days, giving a total recovery of 88% (Figure 27).

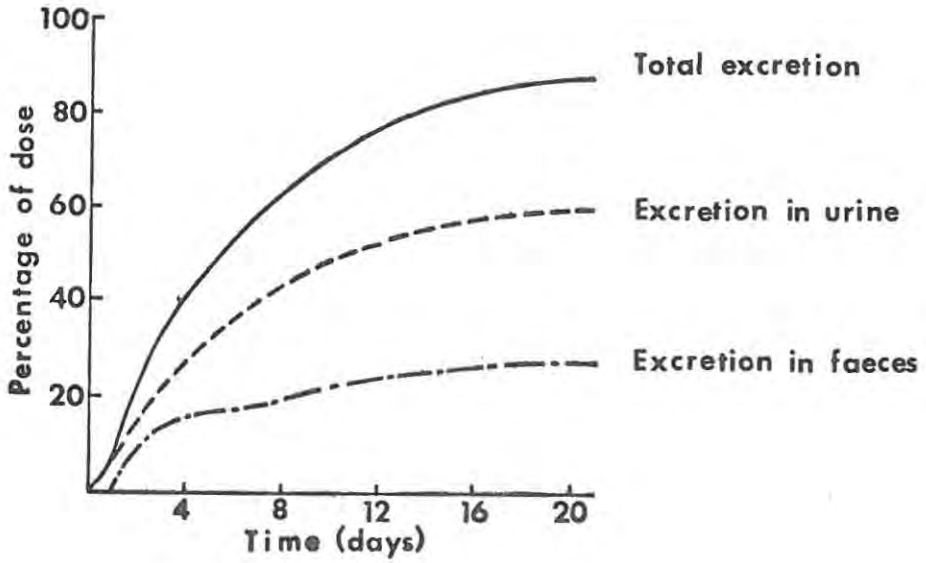


Fig. 27 Cumulative Urinary and Faecal Excretion of Radioactive Substances After Oral Administration of ^{14}C Labelled Phenylbutazone to a Male Human

4. STUDIES IN THE RAT

4.1 Urine

A report that rats dosed with ^{14}C phenylbutazone excreted most of the radioactivity in 0 - 24 h urine (43) was confirmed by

later workers (4, 32). In both cases over 50% of the total radioactivity was recovered in the first 24 h; thereafter the radioactivity dropped considerably (Tables 7 & 8).

4.2 Faeces

Workers studying excretion of radioactivity via the faeces presented different pictures. In one case (4), twice as much radioactivity was excreted in the first 24 h than in the second 24 h, and a total recovery of 34,7% was recorded over 48 h (Table 7). In another case (32) slightly more radio= activity was excreted in the second 24 h period, and a total recovery of 9,4% was recorded over 120 h (Table 8).

4.3 Expired Air

Early studies in which rats were dosed with ^{14}C phenylbutazone indicated that the drug was not metabolised to carbon dioxide (42). This was corroborated by other workers (4, 32), who reported no detectable excretion of carbon dioxide.

Table 7 EXCRETION IN RATS DOSED POST-ORALLY WITH ^{14}C-PHENYLBUTAZONE (4)

Route	% of Administered Dose		
	0 - 24 h	24 - 48 h	0 - 48 h
Urine	54,3 (44,0-67,4)	6,5 (3,2- 8,9)	60,8 (47,2- 74,3)
Faeces	22,9 (16,3-34,0)	11,8 (6,8-17,2)	34,7 (31,3- 40,8)
Total Recovery	77,2 (60,3-89,2)	18,3 (10,0-26,1)	95,5 (86,4-105,6)

Table 8 EXCRETION IN RATS DOSED POST-ORALLY WITH ^{14}C- PHENYLBUTAZONE (32)

Route	% of Administered Dose				
	0 - 24 h	24 - 48 h	48 - 72 h	72 - 120 h	0 - 120 h
Urine	50,2 (46,8-52,5)	12,9 (12,8-13,1)	2,4 (1,9-2,8)	1,8 (1,0-3,0)	67,4 (65,4-68,6)
Faeces	3,6 (3,3- 4,2)	4,3 (3,7- 5,4)	0,9 (0,8-1,2)	0,4 (0,3-0,5)	9,4 (8,5-11,0)

CHAPTER V

DETECTION AND QUANTITATION OF PHENYLBUTAZONE AND ITS METABOLITES

1. INTRODUCTION

Early work by Burns (13) on the metabolism of phenylbutazone in man, using transfer counter current distribution techniques, showed the presence of two metabolites in the urine of human subjects who had been dosed with phenylbutazone. These were the p-hydroxy and γ-hydroxy derivatives of phenylbutazone. These metabolites were identified by melting point determinations, ultraviolet spectrophotometry in sodium hydroxide solution (m_{NaOH} = 2,5 mol·kg^{-1}) and infrared spectrophotometry in chloroform. The method of quantitation was based on isolation of the metabolites by counter current distribution, using equal volumes of ethylene dichloride and aqueous phosphate buffer, crystallisation of the precipitate recovered from specified funnels and weighing the dried and washed crystals. Variation of the pH of the phosphate buffer determined which metabolite would be precipitated. The total amounts of the metabolites present were calculated by application of the binominal expansion in the manner described by Williams and Craig (1947). By this procedure it was found that the p-hydroxy derivative constituted 3% and the

γ-hydroxy derivative 5% of the total dose. In the same year (1955) these workers (12) studied the metabolism of phenylbutazone in rats using 4-^{14}C-phenylbutazone and obtained evidence for the presence of other, more water soluble, transformation products of the drug. This work was confirmed by Perel (43) in 1961 who, in similar experiments using ^{14}C-phenylbutazone, found most of the activity in 0 to 24 h urine; part of this activity was in the form of unknown polar metabolites. In later studies in 1964 (44) they showed that the glucuronide of the p-hydroxy derivative was one of these polar metabolites. No further work was reported on the metabolism of phenylbutazone until after 1974 when more specific and accurate techniques, using radio-chemical methods, were utilised by Bakke (4), Dieterle (22), McGilveray (35) and Aarbakke (1).

2. ULTRAVIOLET SPECTROPHOTOMETRY

UV absorption spectra have been used for many years in the quantitative analysis of phenylbutazone, oxyphenbutazone and the γ-hydroxy derivative of phenylbutazone. The aqueous alkali extract of the compounds showed pronounced peaks at 265 nm, 255 nm and 262 nm respectively (11).

On binding to human serum albumin, the absorption maxima of phenylbutazone was shifted to 270 nm (16), and the difference spectra generated by this shift showed a positive maximum at 285 nm (Figure 28).

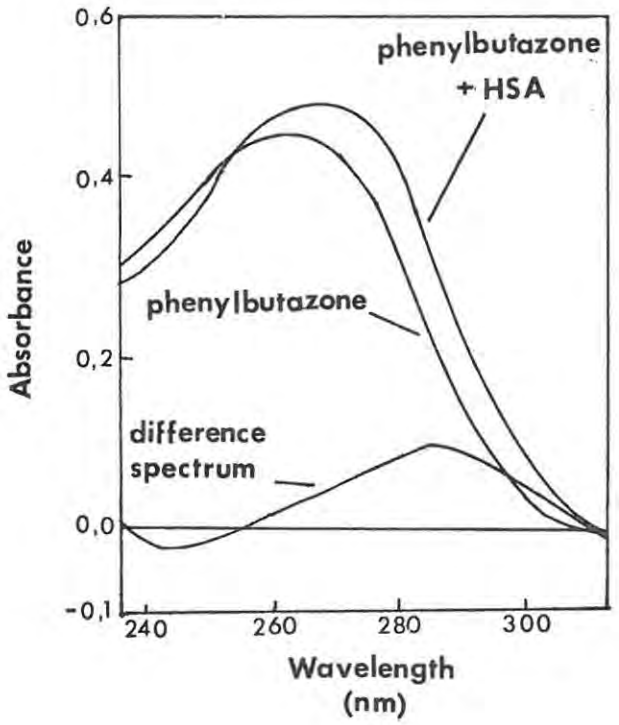


Fig. 28 UV Absorption Spectra of Phenylbutazone

The absorption spectra of phenylbutazone and oxyphenbutazone were also shifted when they were dissolved in organic solvents; ethanolic (16) and methanolic (22) solutions were both found to have their maxima at 270 nm.

3. THIN LAYER CHROMATOGRAPHY

TLC has been used to determine the products of decomposition of phenylbutazone, as reported in Chapter II. In 1974 Bakke (4) used this technique to characterise and isolate the metabolites of phenylbutazone. In order to minimise the effects of oxidation TLC

was carried out under nitrogen as follows: Pre-coated Merck silica gel 60 F_{254} plates were equilibrated overnight in sealed plastic bags filled with nitrogen. Dichloroethane extracts of urine from rats dosed with ^{14}C-phenylbutazone were applied to the plates with a Hamilton syringe through the plastic bags, and the spots dried in a stream of nitrogen. The plates were transferred to chromatographic tanks equilibrated with solvent and nitrogen using the following solvents:

A - Cyclohexane:chloroform:methanol:acetic acid (60:30:5:5)

B - Dichloroethane:acetone (9:1).

The metabolites were characterised by comparison with authentic standards as shown in Figure 29 and Table 9.

The 4-hydroxy derivative of phenylbutazone was said to be the main decomposition product of phenylbutazone and the 4-hydroxy-oxy derivative the major decomposition product of oxyphenbutazone. After hydrolysis of the aqueous extract of the urine with β-glucuronidase/arylsulfatase the conjugates of oxyphenbutazone, and the γ-hydroxy and di-hydroxy derivatives of phenylbutazone were isolated.

4. THIN LAYER CHROMATOGRAPHY AND AUTORADIOGRAPHY

In his studies on the metabolism of phenylbutazone in man, Dieterle (22) combined TLC with autoradiography to isolate the metabolites

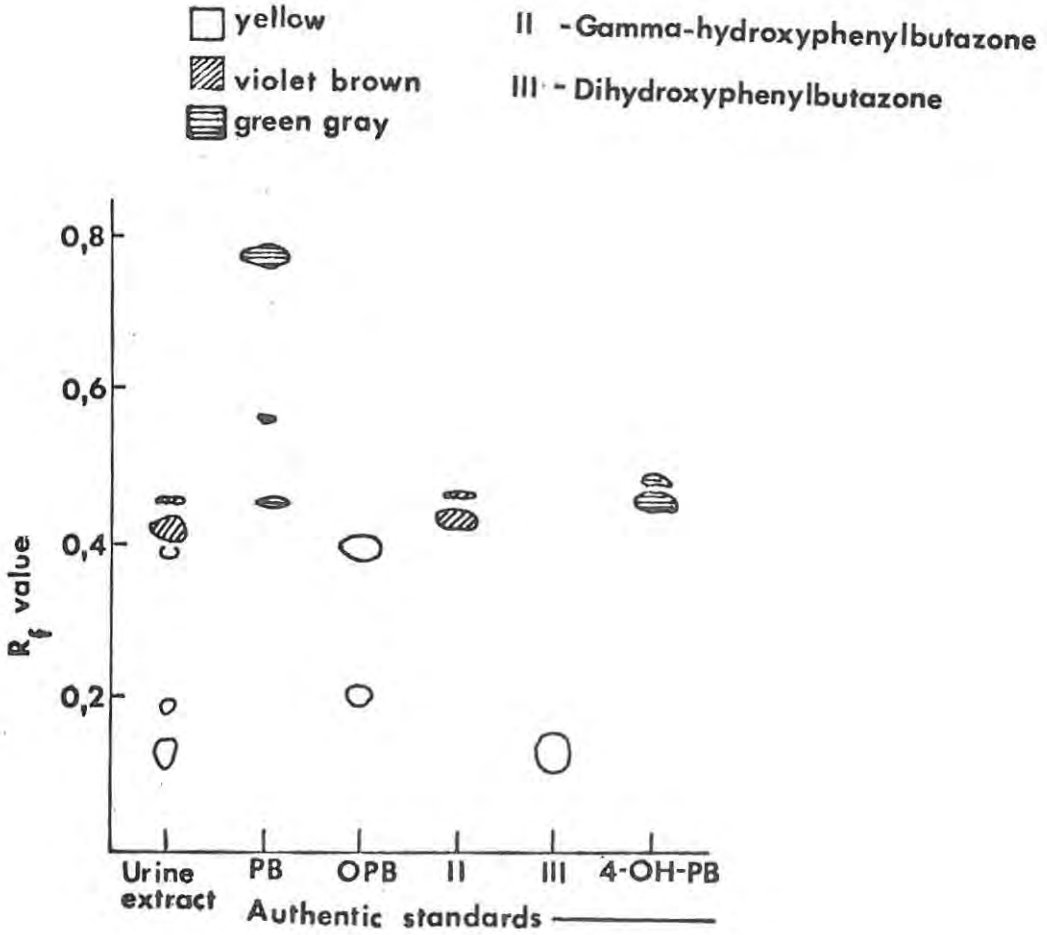


Fig. 29 Chromatogram of Urine Extract and Authentic Standards Developed in Solvent A (4) Spray - 0,5% potassium dichromate in 20% sulphuric acid

as follows:

Urine from a human male was pre-concentrated by column chromatography on Amberlite XAD-2 resin to produce a metabolite mixture. Samples of this mixture were spotted on commercial silica gel plates (Antec SL 254) and developed two-dimensionally, in a nitrogen

Table 9 R_f VALUES AND COLOUR REACTIONS OF PHENYLBUTAZONE, ITS METABOLITES AND POSSIBLE DECOMPOSITION PRODUCTS (4)

Compounds	R_f Values		Colour Reactions	
	Solvent A	Solvent B	$K_2Cr_2O_7$ and heat	Chlorine
Phenylbutazone	0,77	0,82	Green grey	None
Oxyphenbutazone	0,39	0,50	Yellow	Yellow
γ-Hydroxyphenylbutazone	0,43	0,05	Violet brown	Brown
Dihydroxyphenylbutazone	0,13	0,00	Yellow	Yellow
4-Hydroxyphenylbutazone	0,46	0,57	Green grey	None
4-Hydroxyoxyphen= butazone	0,19	0,10	Yellow	Yellow

atmosphere to prevent on-plate oxidation, with cyclohexane:chloroform: methanol:acetic acid (60:30:5:5) in one direction followed by chloroform:acetone:methanol (70:20:10) at right angles. The radio= active spots on the plates were visualised by autoradiography and compared with the positions of similarly chromatographed reference compounds (Figure 30). This method had the advantage of characteri= sing the polar, including the non-hydrolysable C-4 glucuronides, and the non-polar metabolites concurrently.

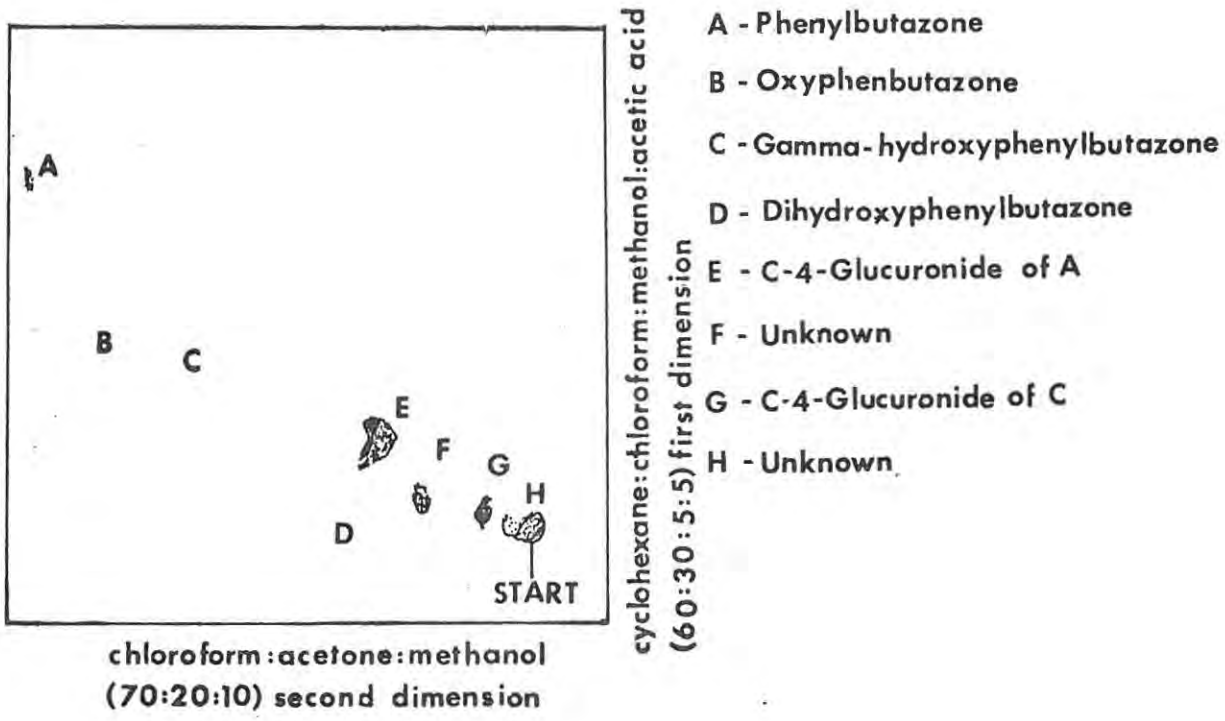


Fig. 30 Two Dimensional TLC Combined with Autoradiography of Radioactive Substance in Human Urine

5. GAS CHROMATOGRAPHY

Gas chromatography has proved a useful tool in the identification (4, 50) and quantitation (1, 34, 36, 37, 53) of phenylbutazone and some of its metabolites. The compounds were converted either to their trimethylsilyl (TMS) derivatives by reaction with N,O-bis (trimethylsilyl) acetamide (BSA) in pyridine (2:1) (1, 4, 50), or to their methyl derivatives by reaction with trimethylanilinium hydroxide (36, 37) (Figure 31) and their retention times compared with those of standards (Table 10).

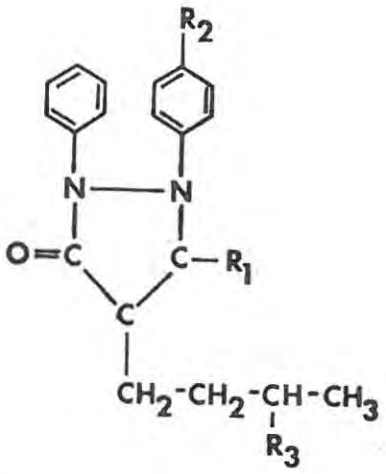


Fig. 31 General Formula of TMS and Methyl Derivatives of Phenylbutazone and Its Metabolites

Table 10 FORMULAE AND RETENTION TIMES OF DERIVATIVES OF PHENYLBUTAZONE AND SOME OF ITS METABOLITES

Compound	TMS Derivative				Methyl Derivative			
	R_1	R_2	R_3	Retention Time	R_1	R_2	R_3	Retention Time
Phenylbutazone	OTMS	H	H	3,5	OCH_3	H	H	7,1
Oxyphenbutazone	OTMS	OTMS	H	8,2	OCH_3	OCH_3	H	9,3
γ-Hydroxy derivative of phenylbutazone	OTMS	H	OTMS	6,5	OCH_3	H	OCH_3	10,6
p-γ-Dihydroxy derivative of phenylbutazone	OTMS	OTMS	OTMS	14,6	OCH_3	OCH_3	OCH_3	

For quantitation peak height ratios were calculated and compared with calibration curves constructed from the results obtained from spiked control plasma samples (34, 36, 37).

6. MASS SPECTROMETRY

Several workers have studied the fragmentation pattern of phenylbuta= zone and some of its metabolites, and these patterns have proved to be useful means of identification (2, 49, 51).

6.1 Phenylbutazone and Oxyphenbutazone

The mass spectra of phenylbutazone and oxyphenbutazone are shown in Figures 32 and 33.

Locock (29) studied the fragmentation pattern of phenylbutazone and oxyphenbutazone and reported the following results with m/e for oxyphenbutazone given in parenthesis.

The compounds were observed to undergo the McLafferty re-arrangement (51) to give radical ions at m/e 252 (258). This was a direct fragmentation of the molecular ion with the loss of the elements of butene (Figure 34).

A minor fragmentation pathway for the molecular ions of these compounds which was substantiated by the presence of metastable

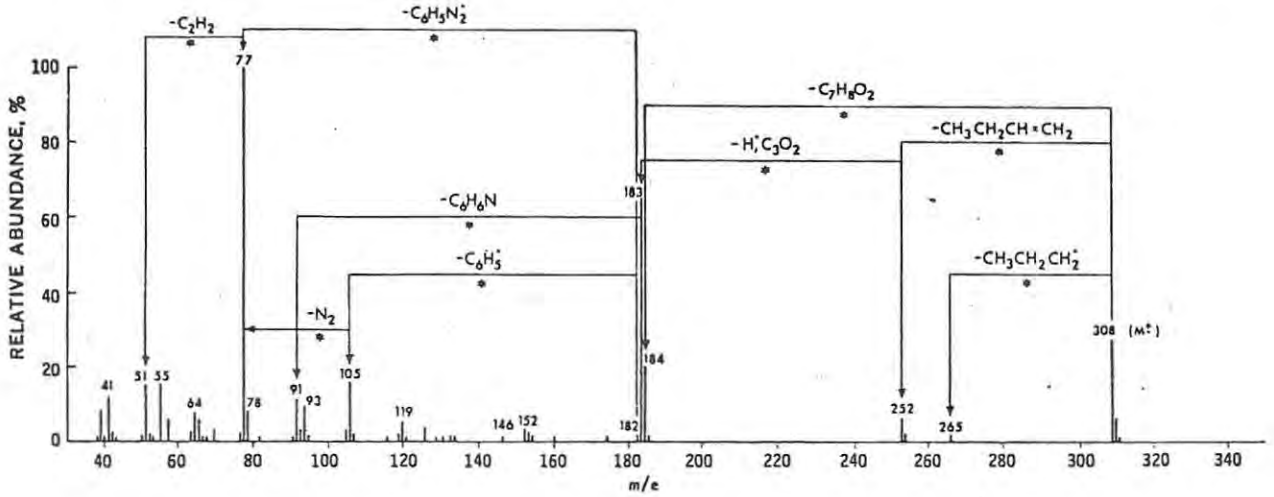


Fig. 32 Mass Spectrum of Phenylbutazone

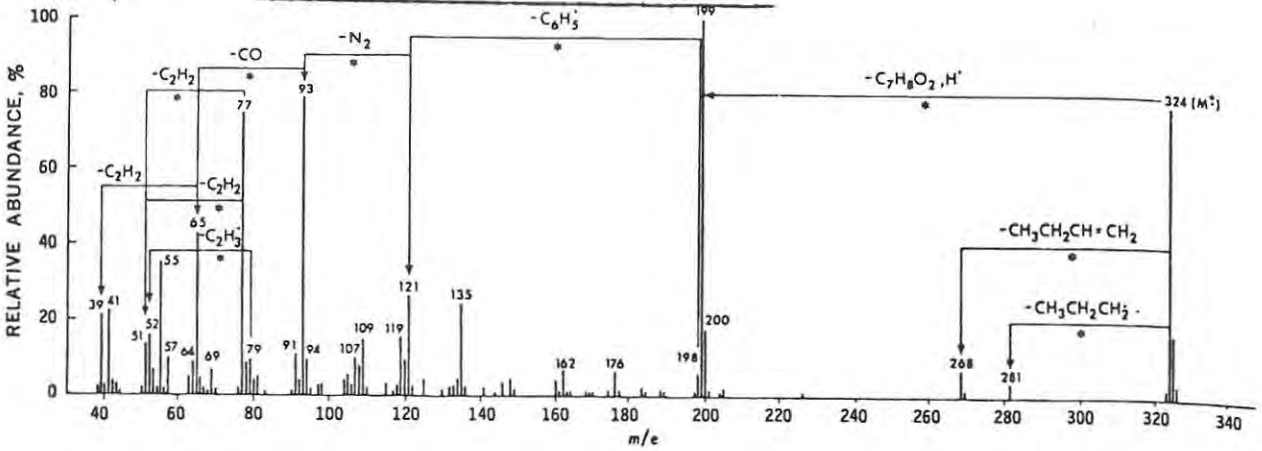


Fig. 33 Mass Spectrum of Oxyphenbutazone

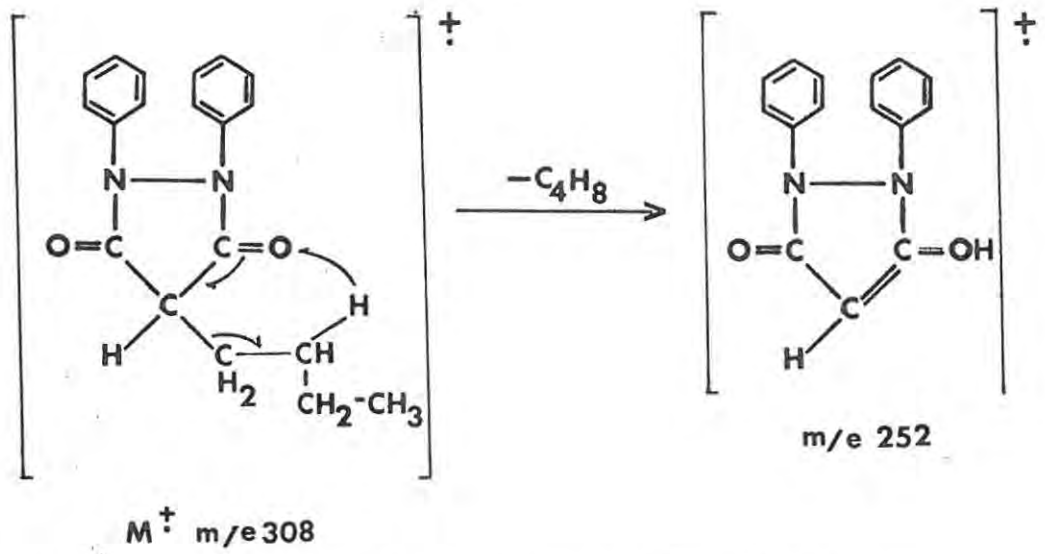


Fig. 34 McLafferty Re-arrangement of Phenylbutazone

ions, was the loss of a propyl radical from the butyl side chain of the molecular ion to give ions at m/e 265 (281).

The most characteristic fragments in the mass spectrum of phenylbutazone (oxyphenbutazone) were a series of peaks at m/e 182, 183, 184 and 185 (198, 199, 200). Table 11 shows the relative abundance of these peaks.

The peak at m/e 182 (198) was attributed to the formation of the azobenzene radical ion $(C_6H_5N_2C_6H_5)^+$.

The ion at m/e 183 (199) could originate from the molecular ion in the McLafferty re-arrangement radical ion (m/e 252 (324)) by hydrogen transfer as depicted in Figure 35.

Table 11 RELATIVE ABUNDANCE (%) OF FRAGMENT IONS IN THE RANGE m/e 182-185 (198, 199, 200)

Phenylbutazone		Oxyphenbutazone	
m/e	Relative abundance %	m/e	Relative abundance %
182	2,3	198	6,8
183	65,1	199	100,0
184	19,8	200	17,7
185	2,1		

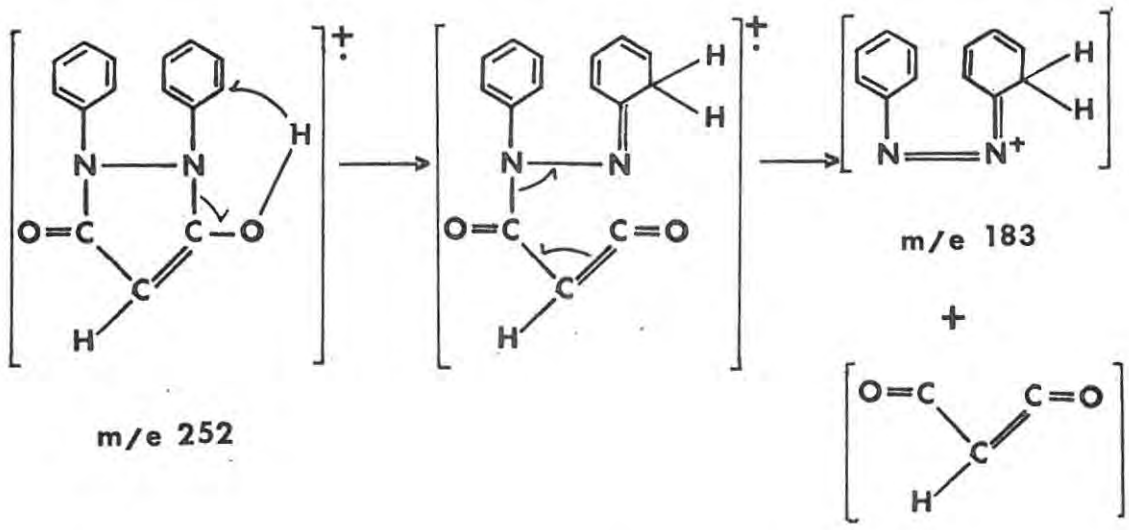


Fig. 35 Mechanism for Formation of Ion at m/e 183 (199)

A further characteristic fragmentation was the loss of the elements of phenyl isocyanate to form the radical ion $(C_6H_5NCO)^+$ m/e 119 or as a neutral molecule (Figure 36).

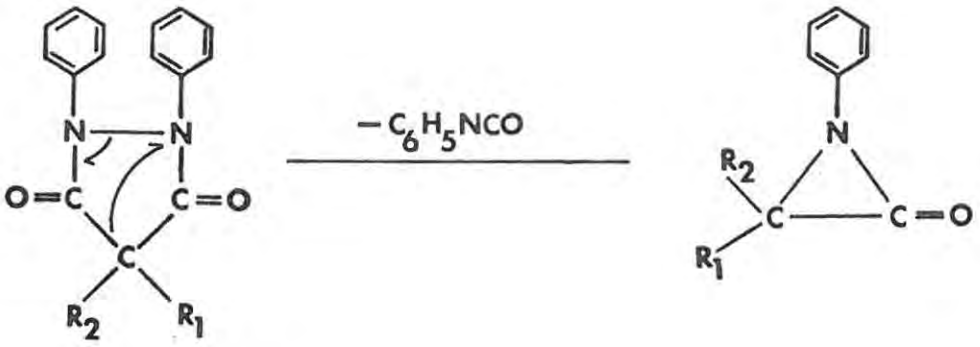


Fig. 36 Fragmentation Involving Loss of C_6H_5NCO as a Neutral Molecule

6.2 C-4 Glucuronides

In the structure elucidation of the non-hydrolysable water soluble metabolites present in the urine of humans treated with ^{14}C-phenylbutazone, Dieterle (22) used the technique of mass spectrometry. The compounds were converted to their methyl ester derivatives and these were subjected to mass spectrometry. The presence of the glucuronyl residue was evidenced by molecular ions at m/e 498 and 514 together with aglycone fragments at 308 and 324.

7. GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Several workers (1, 4, 36, 37) have used the combination of GC/MS. The m/e of the molecular ion and the most abundant ion of phenylbuta=

zone, its metabolites and their TMS and methyl derivatives are shown in Table 12.

Table 12 MOLECULAR ION AND ABUNDANT ION OF PHENYLBUTAZONE, ITS METABOLITES AND THEIR TMS AND METHYL DERIVATIVES

Compound	Molecular Ione m/e	Most Abundant Ion m/e
Phenylbutazone	308	183/184
Phenylbutazone - TMS derivative	381	183/184
- CH_3 derivative	322	183/184
Oxyphenbutazone	324	199/200
Oxyphenbutazone - TMS derivative	468	271/272
- CH_3 derivative	352	213/214
γ-Hydroxy derivative of phenylbutazone	324	183/184
γ-OTMS Derivative of phenylbutazone	460	183/184
γ-CH_3 Derivative of phenylbutazone	352	183/184
p-γ-diOH Derivative of phenylbutazone	340	199/200
p-γ-diOTMS Derivative of phenylbutazone	556	271/272
p-γ-di-CH_3 Derivative of phenylbutazone	366	213/214

It can be seen from Table 12 that, with the knowledge of the m/e of the molecular ion and most abundant ion, it is possible to distinguish one of these compounds from another. A closer inspection of the mass spectra would give a positive identification.

8. LIQUID SCINTILLATION SPECTROMETRY

8.1 Crystallisation and Inverse Isotope Dilution

In his studies on the metabolism of phenylbutazone in rats Bakke (4) used the following technique, combining crystallisation and inverse isotope dilution, to isolate and quantitate the phenylbutazone and oxyphenbutazone present in urine.

A sample of urine (5 ml) from rats dosed with ^{14}C labelled phenylbutazone was added to a buffered (pH 8) aqueous solution of 200 mg unlabelled phenylbutazone. A second 5 ml sample of urine was added to a buffered solution of 200 mg of unlabelled oxyphenbutazone. The compounds were precipitated with hydrochloric acid (m_{HCl} = 1 mol·kg^{-1}), the mixture centrifuged and the residues dissolved in hot methanol. The solutions were filtered and repeated crystallisations were carried out from aqueous methanol. Constant specific activity was attained after 6 to 7 recrystallisations and it was calculated that phenylbutazone and oxyphenbutazone accounted for only 0,5 - 1% and 2 - 4% of the urinary activity respectively (Table 13).

8.2 TLC and Liquid Scintillation Spectrometry

In order to quantitate their major metabolites, the γ-hydroxy

and p-γ-dihydroxy derivatives of phenylbutazone, and the artifact, 4-hydroxy derivative of oxyphenbutazone, Bakke (4) eluted these compounds from thin layer chromatograms, which had been developed in the solvent cyclohexane:chloroform:methanol:acetic acid (60:30:5:5), and counted their radioactivity in the liquid scintillation spectrometer. Table 13 lists the % radioactivity in the urine and % of the dose of these compounds.

Table 13 ESTIMATION OF RADIOACTIVE COMPOUNDS IN 0-24 h URINE AFTER DOSING WITH ^{14}C PHENYLBUTAZONE BY STOMACH TUBE

Compound	% Activity in Urine	% of Dose
Phenylbutazone	0,5 - 1	approx: 0,4
Oxyphenbutazone	3	1,6
γ-Hydroxy derivative of phenylbutazone	46,7	25,4
p-γ-Dihydroxy derivative of phenylbutazone	18,7	10,2
4-Hydroxy derivative of oxyphenbutazone	3,3	1,8

8,3 TLC, Spectrophotometry and Inverse Isotope Dilution Assay

In his studies on the metabolism of phenylbutazone in humans, Dieterle (22) evolved the following effective method of isolation and quantitation of metabolites which permitted simultaneous

specific analysis of phenylbutazone, oxyphenbutazone and the γ-hydroxy and p-γ-dihydroxy derivatives of phenylbutazone in the same sample of urine.

Urine (10 ml) was mixed with 20 ml of sodium thiosulphate ($m_{Na_2S_2O_3}$ = 0,1 mol·kg^{-1}) and 5 ml of a methanol solution of 10 mg of each of the 4 non-labelled compounds to be determined. The isotopically diluted compounds were extracted with methylene chloride after evaporation of the methanol, then separated and purified by preparative chromatography on thin layer plates (0,55 mm) prepared from silica gel 60 PF_{254} (Merck) containing 10% sodium thiosulphate. The plates were successively developed in solvent system 1 - chloroform:acetone (40:10) - and, after drying, with solvent system 2 - heptane:acetone:chloroform: acetic acid (55:35:30:1) - in the same direction. The indivi= dual substances were eluted with methanol and re-chromatographed with solvent system 2. From the measurement of absorbance at 270 nm and radioactivity the amount of individual metabolites present in the urine was calculated.

It was found that the contents of phenylbutazone and oxyphenbu= tazone both accounted for less than 1,1% of the urinary activity and the p-γ-dihydroxy and γ-hydroxy derivatives occured in maximum amounts of 6,5 and 3,4% respectively so that the sum of phenylbutazone and its non-water soluble metabolites amounted to only about 10% of the urinary activity.

METHODS

CHAPTER VI

APPARATUS AND MATERIALS USED IN EXPERIMENTAL WORK

1. APPARATUS USED IN EXPERIMENTAL WORK

1.1 Liquid Scintillation Spectrometer

Intertechnique liquid scintillation spectrometer Model SL 30, with three analyses windows, A, B and C, which have the following three settings:

A	-	$^{3}H/^{14}C$	$^{3}H/^{32}P$	$^{14}C/^{32}P$
B	-	no pre-setting		
C	-	^{3}H	^{14}C	^{32}P

1.2 Teletypewriter

Teletype teletypewriter (RO) set connected to liquid scintillation spectrometer.

1.3 Spectrophotometer

Hitachi spectrophotometer Model 101 UV-VIS.

1.4 Centrifuge

Chriss centrifuge type UJIS.

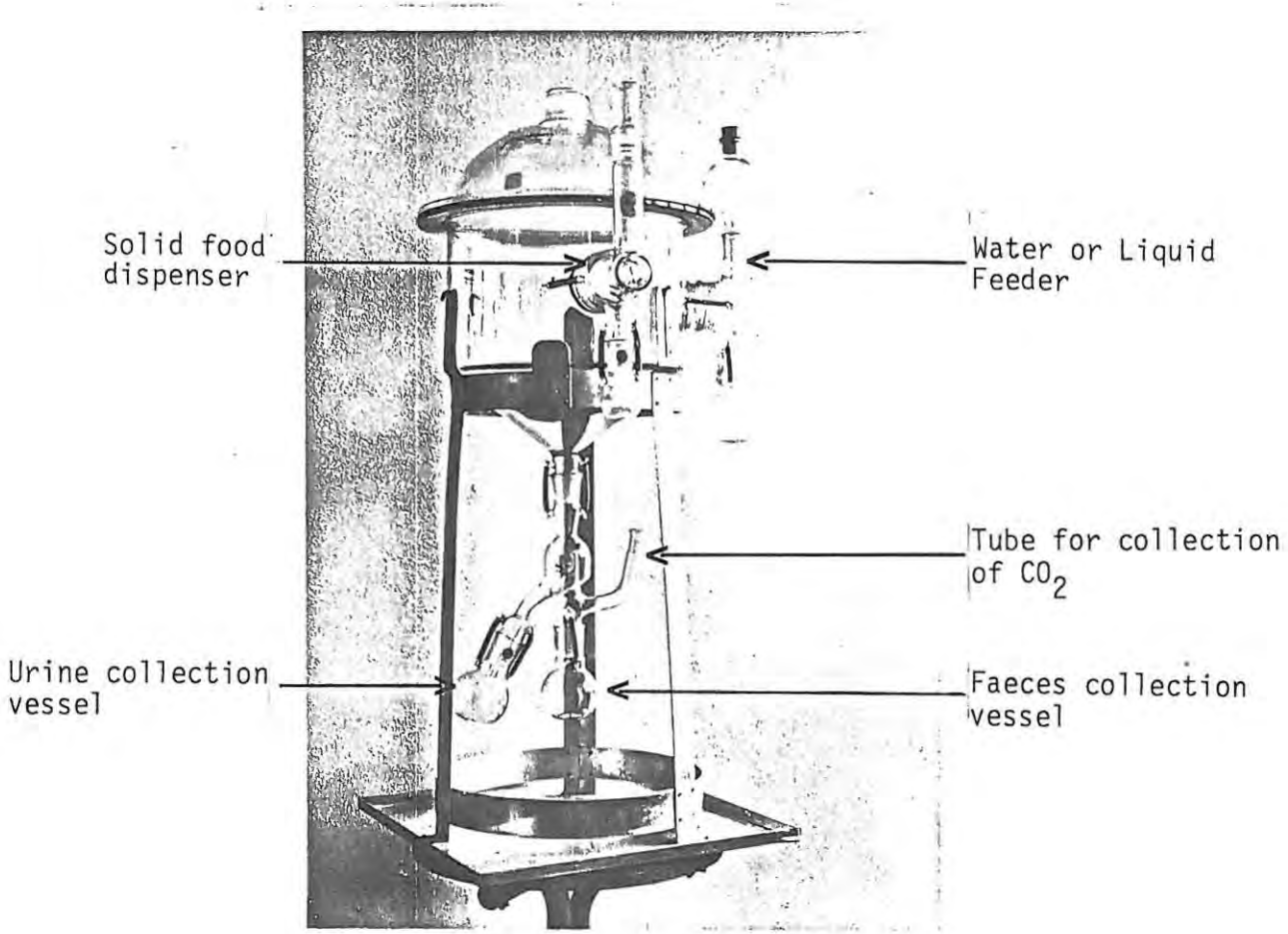


Fig. 37 Jencon Metabowl

1.5 Rotavator

Herdolph rotavator type 51111.

1.6 Homogeniser

Herdolph homogeniser type 50111.

1.7 Chromatocharger

Camag chromatocharger.

1.8 Freeze Dryer

Chriss gamma freeze dryer type 260.

1.9 Animal Cage

Jencon 'Metabowl'. (Fig. 37)

2. MATERIALS USED IN EXPERIMENTAL WORK

2.1 Reference Standards

2.1.1 Phenylbutazone donated by Ciba Geigy, Basel.

2.1.2 Oxyphenbutazone donated by Ciba Geigy, Basel.

2.1.3 γ-Hydroxy derivative of phenylbutazone synthesised by Dr. H Igel in the Pharmacy Department of the University of Durban-Westville.

2.1.4 p-γ-Dihydroxy derivative of phenylbutazone donated by Ciba Geigy, Basel.

2.1.5 4-Hydroxy derivative of phenylbutazone synthesised by Professor G.E.A. Mathew.

2.1.6 ^{14}C Labelled phenylbutazone synthesised by Professor G.E.A. Mathew.

2.2 Scintillants

2.2.1 Bray's scintillant prepared according to the method of Bray.

2.2.2 Aquagel™ produced by Chemlab (Pty) Ltd.

2.3 Enzymes

2.3.1 β-Glucuronidase/arylsulfatase produced by Boehringer Mannheim.

2.3.2 Phenolphthalein mono-β-glucuronic acid cinchonidine salt produced by Sigma Chemical Company.

2.4 TLC Materials

2.4.1 Pre-coated TLC Plates

Merck pre-coated TLC Plates Silica gel 60.F_{254}.

Pre-coated Cherrie Erzeugnisse AG DSF A plates.

2.4.2 Materials for preparing TLC Plates

Merck Silica gel GF_{254}.

Merck Silica gel PF_{254}.

3. ANIMALS USED IN EXPERIMENTAL WORK

Female Wistar rats weighing between 180 and 200 g obtained from the Natal Institute of Immunology, certified a pure bred strain.

CHAPTER VII

THIN LAYER CHROMATOGRAPHY

1. PREPARATION OF THE PLATES

For the initial thin layer chromatography experiments 20 x 20 cm pre-coated Merck Silica gel 60 F_{254} plates were cut into squares of 5 x 5 cm using a perspex template as shown in Figure 38.

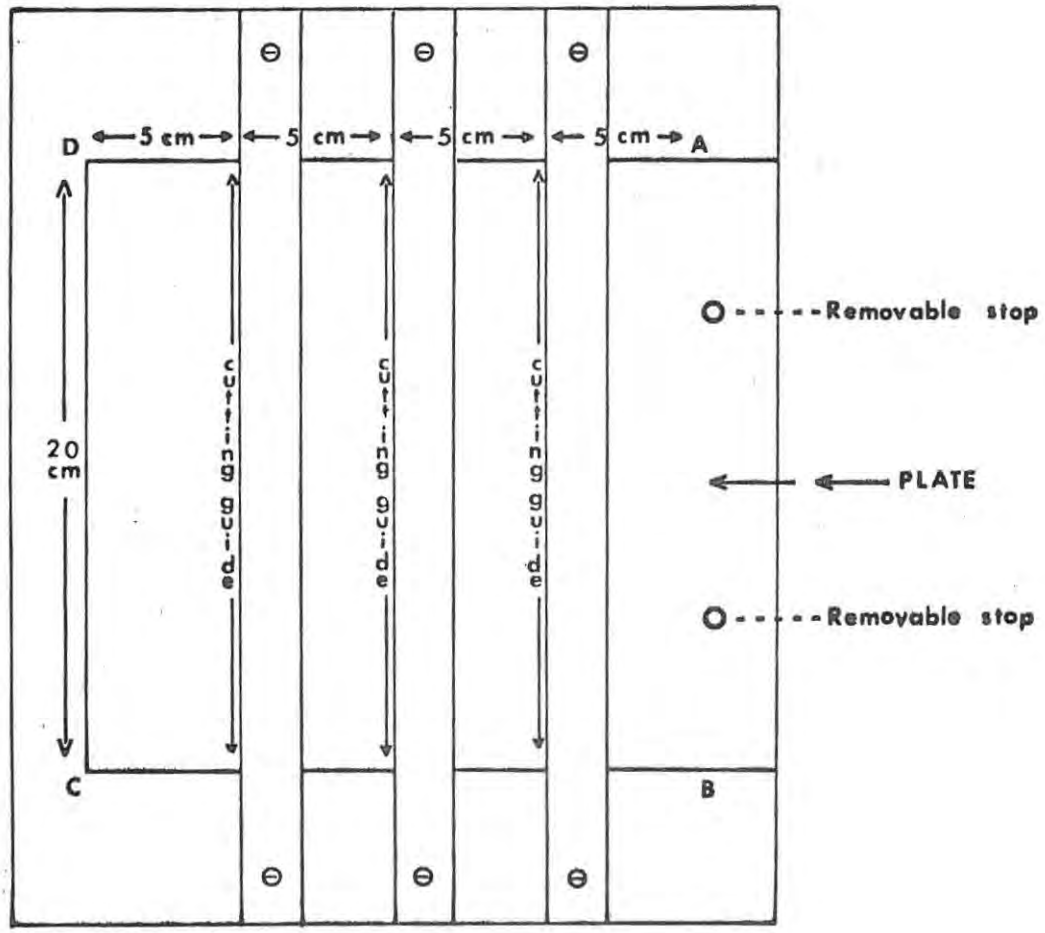


Fig. 38 Template Used to Prepare 5 x 5 cm TLC Plates

The plate was slid into the area ABCD under the cutting guides and kept in position by removable stops. It was cut along the cutting guide with a diamond cutter and the resultant 20 x 5 cm plates removed, replaced at right angles in the template and cut again, thus forming 5 x 5 cm plates.

For development these plates were held by jumbo clips and placed in 23 ml of solvent in a 250 ml beaker.

This method gave the required data for preliminary tests and was economical in the use of plates and solvents. However, it was not considered to be sufficiently accurate to give consistent R_f values. In order to improve this technique the following method was evolved and, except when otherwise stated, adopted as standard procedure in all subsequent experiments using TLC techniques.

One per cent solutions of reference standards in dichloromethane were applied 1 cm from the base of chromatographic plates, 20 cm in length, which were developed in the relevant solvent system until the solvent front had advanced to 15 cm from the origin. The plates were then dried with the aid of a hair dryer at room temperature and sprayed with the selected spray reagent. The colours of the developed spots were observed and the R_f values calculated and recorded.

2. EVALUATION OF SUITABILITY OF SPRAY SYSTEMS

The spray systems listed in Table 14 were investigated to determine the best method of characterising phenylbutazone and oxyphenbutazone on chromatograms.

Table 14 SPRAY SYSTEMS TESTED TO CHARACTERISE PHENYL= BUTAZONE AND OXYPHENBUTAZONE

A	5% Ferric chloride aqueous solution Acetic acid (m_{CH_3COOH} = 5 mol·kg^{-1})	equal parts
B	5% Potassium ferricyanide aqueous solution 10% Ferric chloride aqueous solution Water	1 part 2 parts 8 parts
C	1% Potassium permanganate aqueous solution 2% Sodium carbonate aqueous solution	equal parts
D	Potassium permanganate (m_{KMnO_4}=0,1 mol·kg^{-1}) Acetic acid (m_{CH_3COOH} = 5 mol·kg^{-1})	equal parts
E	Spray with 0,5% potassium permanganate solution, followed by 1% Ferric nitrate solution	
F	5% Potassium dichromate in 40% aqueous sulphuric acid, then heat at 105°C for 3 minutes	
G	0,5% Potassium dichromate in 20% sulphuric acid, followed by concentrated sulphuric acid	
H	0,5% Potassium dichromate in 20% sulphuric acid, then heat at 105°C for 3 minutes	

I Ehrlich's reagent

J Folin Ciocalteau reagent

K 1% Ferric chloride aqueous solution

L 0,1% 7-Chloro-4-nitrobenzofuran in chloroform

M Dragendorff's reagent

N Iodoplatinate reagent

O FPN reagent

P Mandelin's reagent

Q Marquis' reagent

One per cent solutions of phenylbutazone and oxyphenbutazone in dichloromethane (5 µl) were applied as individual small spots on the base line of activated pre-coated Merck Silica gel 60 F_{254} cm plates. The plates were air dried, developed in ethyl acetate:benzene (1:4) solvent system, examined under ultra-violet light and the spots lightly outlined with a soft lead pencil. Individual plates were then sprayed with the spray systems A to Q (Table 14) and the colour reactions noted. The results are shown in Table 15.

The colour reactions with spray systems G, H and J were similar to those reported by Awang (2), Bakke (4) and Beckstead (6) respectively and confirmed their findings.

Table 15 COLOUR REACTIONS WITH SPRAY SYSTEMS LISTED IN Table 14

Spray	Colour reaction	
	Phenylbutazone	Oxyphenbutazone
A	No visible colour reaction	No visible colour reaction
B	Blue stippled	Blue stippled
C	Pink yellow	Yellow
D	Yellow	Yellow
E	Brown yellow	Pale yellow
F	Brown	Yellow
G	Yellow orange	Yellow
H	Green gray	Yellow
I	Yellow	Yellow
J	Pale yellow on standing	Pale yellow
K	No colour	No colour
L	Pale yellow	Pale yellow
M	Pale yellow	Pale yellow
N	No colour	No colour
O	Yellow	Yellow
P	Purple brown	Yellow
Q	Pale lemon	Purple

Spray system H gave the best colour differentiation between phenyl= butazone and oxyphenbutazone. It was also found to give effective colour reactions with other possible metabolites and decomposition products of phenylbutazone as shown in Table 16.

Table 16 COLOUR REACTIONS OF PHENYLBUTAZONE AND SOME OF ITS METABOLITES AND DECOMPOSITION PRODUCTS

Phenylbutazone	green grey
Oxyphenbutazone	yellow
γ-Hydroxy (ring lactone form) derivative	purple
γ-Hydroxy (straight chain hydroxyl form) derivative	orange brown
4-Hydroxy derivative	green grey
p-γ-Dihydroxy derivative	yellow
p-4-Hydroxy derivative	yellow

This spray system was therefore used in all subsequent experiments unless otherwise stated. The method consisted of spraying the plate evenly with finely atomised spray reagent H, heating at 105^{o}C for three minutes and noting the colour formed.

3. CHROMATOGRAPHIC PLATES USED FOR TLC

3.1 Evaluation of Chromatographic Plates Used for TLC

Table 17 lists the TLC plates of 0,25 mm layer thickness that were tested.

Table 17 TLC PLATES TESTED FOR SEPARATION AND AUTORADIOGRAPHY OF PHENYLBUTAZONE, ITS METABOLITES AND DECOMPOSITION PRODUCTS

1	Laboratory prepared Alumina G plate
2	Laboratory prepared Silica gel GF_{254} plate
3	Laboratory prepared Silica gel GF_{254} in Bismuth nitrate solution ($m_{Bi(NO_3)_3}$ = 0,05 mol·kg^{-1}) in 1,5% nitric acid
4	Prepared Cherrie Erzeugnisse AG DSF A plate
5	Pre-coated Merck Silica gel 60 F_{254} plates

One per cent solutions of phenylbutazone, oxyphenbutazone and the γ-hydroxy derivative (ring lactone form) in dichloromethane and the extract of the urine from a rat dosed with phenylbutazone were spotted on each of the plates listed in Table 17. The plates were dried, developed and sprayed.

The results showed that there was considerable streaking between the spots on the Alumina G plate and only the strongly non-polar compounds were moved from the origin. Separation was efficient on both the laboratory prepared Silica gel plates and the Cherrie Erzeugnisse plates, but results were found to vary; also the plates powdered, a property which made them unsuitable for autoradiography. These experiments showed that only pre-coated Merck Silica gel 60 F_{254} plates were suitable for autoradiography, therefore these plates were used for all subsequent thin layer chromatography, unless otherwise stated.

3.2 Preparation of Preparative Plates

Slurries of Silica gel 60 PF_{254} (Merck) were prepared in 10% sodium thiosulphate solution to determine the optimum quantities required to prepare 5 plates of 0,55 mm layer thickness. These optimum quantities were found to be 60 g of Silica gel in 150 ml of 10% sodium thiosulphate solution. It was found that 10% sodium thiosulphate inhibits on-plate oxidation during the long development process used on preparative plates. (*vide* 5.3.2)

4. EVALUATION OF SOLVENT SYSTEMS

The solvent systems listed in Table 18 were investigated to determine the most effective method of separating phenylbutazone, its decompo= sition products and possible metabolites.

Table 18 SOLVENT SYSTEMS TESTED FOR THE SEPARATION OF PHENYL= BUTAZONE, ITS DECOMPOSITION PRODUCTS AND POSSIBLE METABOLITES

		Parts by volume
I	Ethyl acetate	85
	Methanol	10
	Ammonia 25%	5
II	Cyclohexane	60
	Chloroform	30
	Methanol	5
	Glacial acetic acid	5
III	Chloroform	10
	Ethanol	1
IV	Ethyl acetate	1
	Benzene	4
V	Dichloromethane	9
	Acetone	1
VI	Butanol	4
	Acetic acid	1
	Water	1
VII	Chloroform	10
	Ethanol	0,5
VIII	Chloroform	10
	Ethanol	0,25
IX	Chloroform	9,5
	Ethanol	0,5
X	Chloroform	10
	Ethanol	0,1
XI	Cyclohexane	60
	Chloroform	30
	Methanol	4
	Acetic acid	4
	Acetone	2

Table 18 (continued)

XII	Cyclohexane	60
	Chloroform	30
	Methanol	5
	Acetic acid	4
	Acetone	1
XIII	Dichloromethane	10
	Acetone	3
XIV	Dichloromethane	9
	Acetone	1,5
XV	Chloroform	9
	Benzene	8
	Methanol	1
XVI	Cyclohexane	9
	Dichloromethane	8
	Acetone	3
XVII	Chloroform	10
	Ethanol	1,5
XVIII	Chloroform	10
	Ethanol	2
XVIX	Acetone	7
	Chloroform	3
XX	Acetone	8
	Chloroform	3
XXI	Acetone	7
	Chloroform	4
XXII	Acetone	6
	Methanol	4
XXIII	Dichloromethane	5
	Acetone	2
XXIV	Butanol	5
	Methanol	1
	Acetic acid	3,6
	Water	0,8
XXV	Butanol	4
	Acetic acid	1
	Water	0,5

Table 18 (continued)

XXVI	(a) Chloroform Acetone followed in the same direction by (b) Heptane Acetone Chloroform Acetic acid	4 1 55 35 30 1
XXVII	(a) Cyclohexane Chloroform Methanol Acetic acid followed at right angles by (b) Chloroform Acetone Methanol	60 30 5 5 7 2 1

4.1 Preliminary Tests with Solvent Systems to Separate Phenylbutazone and Low Polarity Metabolites and Decomposition Products

4.1.1 Separation of Phenylbutazone and Oxyphenbutazone

As can be seen from the R_f values given in Table 19 solvent system IV separated Phenylbutazone from Oxyphenbutazone most effectively and also moved the compounds to an intermediate position on the plate between the origin and solvent front.

Table 19 R_f VALUES OF PHENYLBUTAZONE AND OXYPHENBUTAZONE IN SOLVENT SYSTEMS I TO VI

	I	II	III	IV	V	VI
Phenylbutazone	0,84	0,4	0,93	0,83	0,87	Too polar
Oxyphenbutazone	incomplete separation	0,17	0,73	0,43	0,5	Too polar

Unless otherwise stated, this solvent system was selected for use in subsequent tests involving these two substances in TLC examinations, provided no other compounds were present.

4.1.2 Choice of Reference Standard

Experiments showed that there were variations in the R_f values of individual substances from one experiment to another and for this reason it seemed advisable to refer all R_f values to a suitable standard. Oxyphenbutazone was chosen as the reference standard and given an R_f value of 1. Relative R_f values quoted hereafter are related to this standard.

4.1.3 Separation of Phenylbutazone, Its γ-Hydroxy (Ring Lactone Form) and 4-Hydroxy Derivatives and Oxyphenbutazone

One per cent solutions of the compounds in dichloromethane (5 µl) were spotted on plates prepared from Silica gel GF_{254} slurry in McIlvaine buffer as recommended by Awang (2), the plates dried, developed in solvent systems II, III, VII and VIII, and sprayed. The relative R_f values are shown in Table 20.

Table 20 RELATIVE R_f VALUES OF PHENYLBUTAZONE, ITS γ-HYDROXY (RING LACTONE FORM) AND 4-HYDROXY DERIVATIVES

Solvent System	Phenylbutazone	γ-Hydroxy Derivative (Ring Lactone Form)	4-Hydroxy Derivative
II	1,96	1,1	1,16
III	1,09	1,03	1,07
VII	2,05	1,38	1,15
VIII	2,18	1,79	1,21

As can be seen from Table 20 these solvent systems, with the exception of III, separated phenylbutazone from its derivatives. However, the 2 derivatives were not well separated from each other.

4.1.4 Separation of the γ-Hydroxy (Ring Lactone Form) and 4-Hydroxy Derivatives of Phenylbutazone and Oxyphenbutazone

Relative R_f values, using solvent systems IX to XVI are shown in Table 21.

Table 21 RELATIVE R_f VALUES OF THE γ-HYDROXY (RING LACTONE FORM) AND 4-HYDROXY DERIVATIVES OF PHENYLBUTAZONE

Solvent System	γ-Hydroxy Derivative (Ring Lactone Form)	4-Hydroxy Derivative
IX	1,33	1,16
X	1,86	1,31
XI	1,81	1,38
XII	1,53	1,39
XIII	1,17	1,10
XIV	1,19	1,15
XV	1,81	1,41
XVI	1,15	1,27

It can be seen that solvent systems X and XV separated the metabolites adequately. The actual R_f values obtained with these two solvent systems show that solvent system XV eluted these metabolites to approximately midway between the origin and the solvent front, and was therefore selected

for all subsequent work for the separation of these compounds. (Table 22)

Table 22 R_f VALUES OF THE γ-HYDROXY (RING LACTONE FORM) AND 4-HYDROXY DERIVATIVES OF PHENYLBUTAZONE AND OXYPHENBUTAZONE

Solvent System	γ-Hydroxy Derivative (Ring Lactone Form)	4-Hydroxy Derivative	Oxyphenbutazone
X	0,48	0,29	0,11
XV	0,58	0,44	0,32

4.2 Preliminary Tests with Solvent Systems to Separate Oxyphenbutazone and the More Polar Metabolites and Decomposition Products of Phenylbutazone

4.2.1 Separation of Oxyphenbutazone and the γ-hydroxy (Straight Chain Hydroxyl Form) and p-γ-Dihydroxy Derivatives of Phenylbutazone

The R_f and relative R_f values obtained using various solvent systems are shown in Table 23.

As can be seen from this Table the most effective solvent systems for the separation of these com=

Table 23 R_f AND RELATIVE R_f VALUES OF OXYPHENBUTAZONE AND THE MORE POLAR METABOLITES OF PHENYLBUTAZONE

Solvent System	Oxyphenbutazone		γ-Hydroxy Derivative (Straight Chain Form)		p-γ-Dihydroxy Derivative	
	R_f	Relative R_f	R_f	Relative R_f	R_f	Relative R_f
III	0,53	1,00	0,32	0,55	0,15	0,29
VII	0,49	1,00	0,13	0,27	0,07	0,15
VIII	0,39	1,00	0,08	0,22	0,03	0,07
XVII	0,54	1,00	0,32	0,58	0,15	0,27
XVIII	0,52	1,00	0,24	0,46	0,12	0,22
XIX	0,43	1,00	0,28	0,65	0,06	0,01
XX	0,50	1,00	0,32	0,64	0,07	0,01
XXI	0,44	1,00	0,25	0,57	0,04	0,009
XXII	0,40	1,00	0,36	0,57	0,08	0,18
XXIII	0,38	1,00	0,05	0,012	0,00	0,00
XXIV	NO CLEAR RESULTS					
XXV	NO CLEAR RESULTS					

pounds are the chloroform:ethanol solvent systems III and XVII. Experiments using multiple develop= ments with either the same or different solvent systems showed that it was possible to effect a slightly better separation, but this advantage was outweighed by the effects of on-plate oxidation.

4.2.2 Assessment of Solvent Systems Using Extracts of Urine from Rats Dosed with Phenylbutazone

As a result of these preliminary tests the following tests were undertaken with extracts of rat urine obtained by the recommended extraction procedure outlined in the following chapter.

Three plates, numbered (a), (b) and (c) were prepared by applying small spots of the following solutions on the origin, 1 cm apart.

i) Extract of urine from an untreated rat.

ii) Extract of urine from an untreated rat + a solution of oxyphenbutazone.

iii) Extract of urine from a rat dosed with phenylbutazone.

iv) One per cent solution of oxyphenbutazone in dichloromethane.

Immediately after the spots had dried Plate (a) was developed in solvent system XVII, Plate (b) in solvent system III and Plate (c) in solvent system XXV, to a solvent front 15 cm from the origin. They were then dried and sprayed.

As can be seen from Figure 39 solvent system III moved the γ-hydroxy (straight chain hydroxyl form)

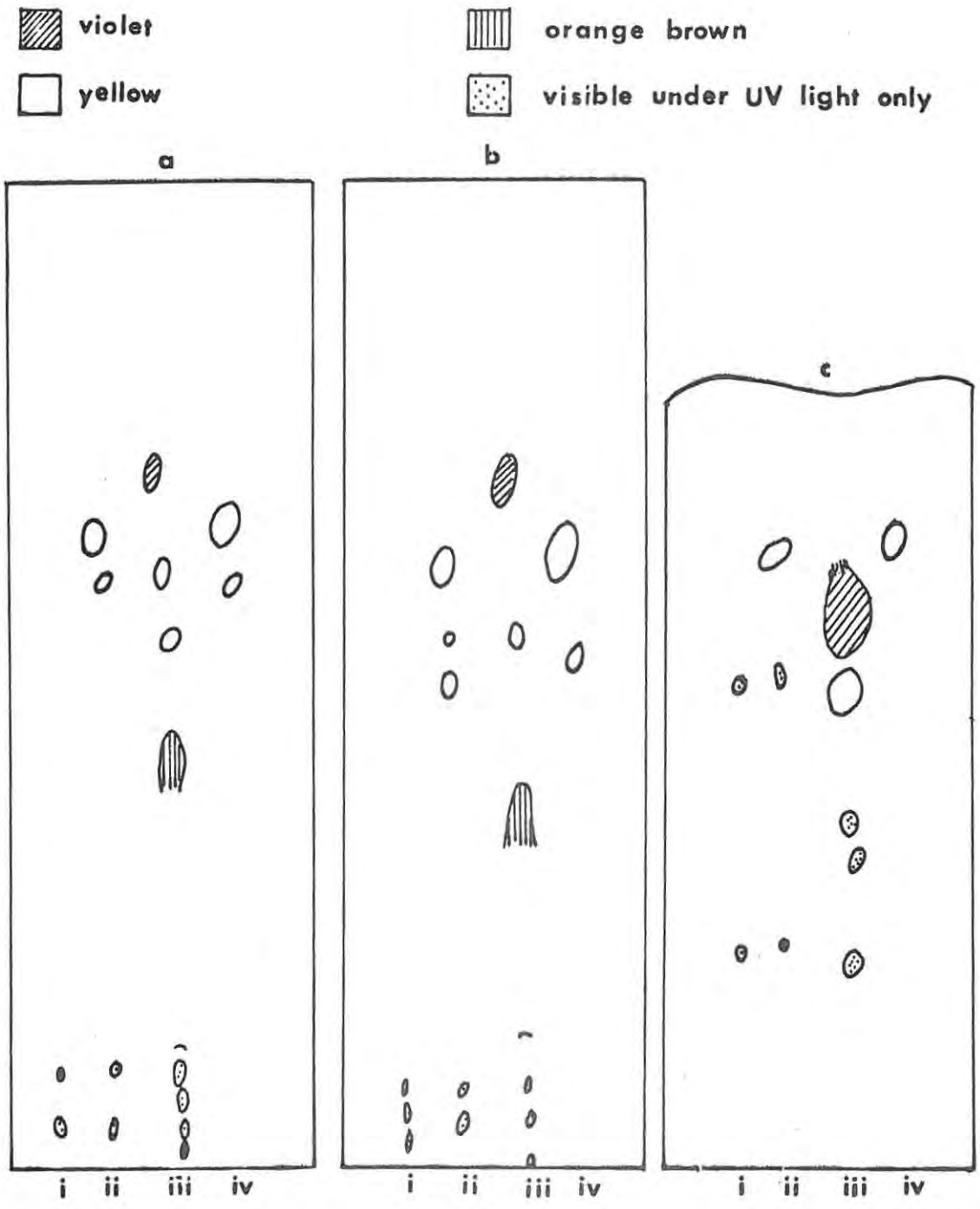


Fig. 39 Chromatograms Showing separation of Polar Metabolites of Phenylbutazone in the Extract of Rat Urine

derivative, recognised by its orange brown colour, well above the origin (R_f value 0,41) and separated oxyphenbutazone and its metabolites or decomposition products. Solvent system XVII also moved the γ-hydroxy (straight chain hydroxyl form) derivative well above the origin (R_f value 0,42) but did not separate oxyphenbutazone and its metabolites or decomposition products. Solvent system XXV lifted all the material from the origin and separated the highly polar metabolites.

4.3 Solvent Systems Selected for Chromatographic Separations

On the basis of this preliminary experimental work it was decided to use the following solvent systems for chromatographic separations unless otherwise stated:

(a) Separation of phenylbutazone from its γ-hydroxy (ring lactone form) and 4-hydroxy derivatives and oxyphenbutazone: Solvent System XV.

(b) Separation of oxyphenbutazone, its metabolites and decomposition products and the γ-hydroxy (straight chain hydroxyl form) and p-γ-dihydroxy derivatives of phenylbutazone: Solvent System III.

(c) Separation of highly polar substances (probably conjugates): Solvent System XXV

Table 24 lists the R_f values of phenylbutazone and its possible metabolites in two of the above solvent systems.

Table 24 RELATIVE R_f VALUES OF PHENYLBUTAZONE AND ITS POSSIBLE METABOLITES

Solvent	Substance	Relative R_f Value
XV	Phenylbutazone	2,00
	γ-Hydroxy derivative (ring lactone form) of PB	1,81
	4-Hydroxy derivative of phenylbutazone	1,41
III	4-Hydroxy derivative of oxyphenbutazone	0,80
	γ-Hydroxy (straight chain hydroxyl form) of PB	0,55
	p-γ-Dihydroxy derivative of phenylbutazone	0,31

5. DECOMPOSITION OF PHENYLBUTAZONE AND OXYPHENBUTAZONE

Experiments were conducted to establish the extent of decomposition of phenylbutazone and oxyphenbutazone in solution, as well as during on-plate oxidation in TLC procedures, and methods which would either minimise or prevent such decomposition were assessed. Throughout these experiments solvent system IV was used to elute the drugs.

5.1 Decomposition of Phenylbutazone and Oxyphenbutazone in Aqueous Solution

Samples of 1% solutions of the drugs in sodium hydroxide solution (m_{NaOH} = 0,1 mol·kg^{-1}) were allowed to stand in uncovered beakers for 3 days, acidified with hydrochloric acid and extracted with dichloromethane. The dichloro= methane extract was spotted on activated 5 x 5 cm plates, the plates developed for 3 cm, dried, examined under ultra= violet light and sprayed.

Freshly prepared solutions in dichloromethane were immedia= tely spotted on plates and treated similarly to act as controls. No evidence was found that either drug had decomposed on standing in solution over a period of 3 days in the form of its sodium salt.

5.2 Decomposition of Phenylbutazone and Oxyphenbutazone on TLC Plates

One per cent solutions of the drugs in dichloromethane were spotted on activated 5 x 5 cm plates and the plates allowed to stand in the air for 24 h. Freshly prepared solutions were then spotted on these plates, which were immediately developed for 3 cm, examined under ultraviolet light and sprayed. In order to clarify the interpretation of the

chromatogram the spot produced by the applied drug was given an R_f value of 1 and the R_f values of the decomposition products calculated relative to this.

It was found that freshly prepared solutions which were developed immediately underwent no decomposition, whereas the 24 h samples were completely decomposed into a major product and minor products as indicated by Table 25.

Table 25 R_f Values of On-plate Degradation Products of Phenylbutazone and Oxyphenbutazone

Relative R_f Values of Phenylbutazone Decomposition Products	Relative R_f Values of Oxyphenbutazone Decomposition Products
0,77 (minor spot) 0,06 (major spot)	0,31 (minor spot) 0,2 (minor spot) 0,0 (major spot)

5.3 Prevention of On-plate Oxidation of Phenylbutazone and Some of Its Metabolites

The following experiments were carried out to ascertain whether it was possible to prevent or reduce on-plate oxidation.

5.3.1 Incorporation of McIlvaine Buffer

Test plates were prepared from Silica gel GF_{254} (20 parts), Silica gel G (20 parts) in McIlvaine Buffer (80 parts). McIlvaine buffer was prepared by adding 37 ml of citric acid solution ($m_{citric\ acid} = 0,1\ mol \cdot kg^{-1}$) to 63 ml disodium phosphate solution ($m_{Na_2HPO_4} = 0,2\ mol \cdot kg^{-1}$) (2).

Pre-coated Merck Silica gel 60 F_{254} plates were used as control plates.

Solvent II was used as it was reported to be effective in separating phenylbutazone and its decomposition products (6). Five microlitres of a 1% solution of phenylbutazone in dichloromethane was spotted on the test and control plates at 15 min intervals over a period of 60 min, the plates dried, developed and sprayed. Figure 40 shows that decomposition started within 15 min and the inclusion of McIlvaine buffer gave a minimal degree of protection.

5.3.2 Incorporation of 10% Sodium Thiosulphate Solution (22)

A 20 x 20 cm test plate was prepared from a slurry containing 1 part Merck Silica gel PF_{254} in 2 parts 10% sodium thiosulphate solution (Plate 1) and

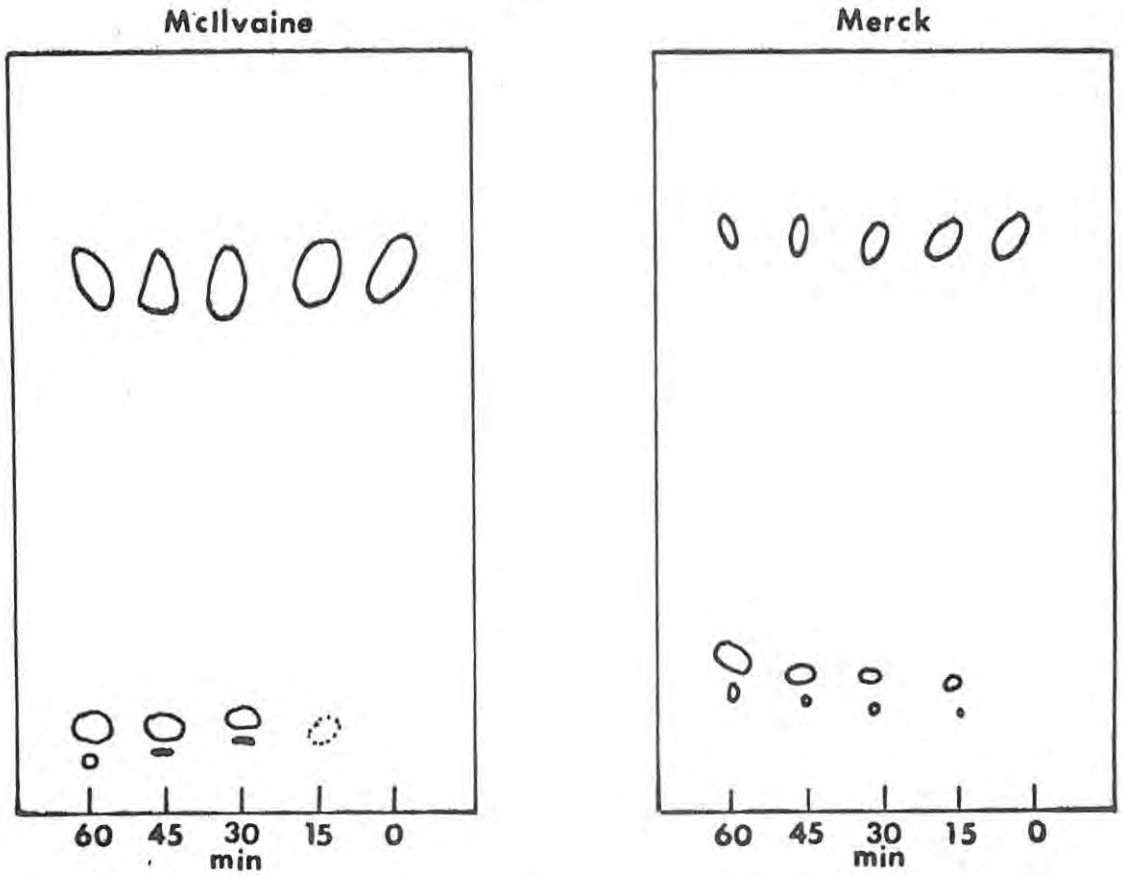


Fig. 40 Comparison of Decomposition with Time of Phenylbutazone on Two Different Plates

and another 20 x 20 cm plate was prepared using water as the vehicle to act as a control (Plate 2). Five microlitres each of 1% solutions of phenyl= butazone, oxyphenbutazone and the γ-hydroxy (ring lactone form) derivative of phenylbutazone in dichloromethane were spotted on the plates 3 times at 30 min intervals.

Using solvent system XXVI, which included solvents (a) and (b), plates 1 and 2 were developed in

solvent (a), the plates dried and developed in the same direction in solvent (b). The plates were examined under ultraviolet light and the spots marked. The spray reagent was not used as sodium thiosulphate was found to interfere with its action.

Figure 41 shows, firstly, that a product of decomposition of phenylbutazone was present in plate 2 after 60 min exposure to air, but was not present in plate 1; secondly, that the size of the spots (indicative of concentration), of the decomposition products of oxyphenbutazone increased with time in plate 2, but not in plate 1, and thirdly, there did not appear to be any difference in the decomposition of the γ-hydroxy (ring lactone form) in plates 1 and 2. Evidently it equilibrated with its straight chain hydroxyl form and formed a decomposition product to the same extent in both plates.

The results of these tests indicated that the degree of protection from decomposition given by McIlvaine buffer did not warrant its use.

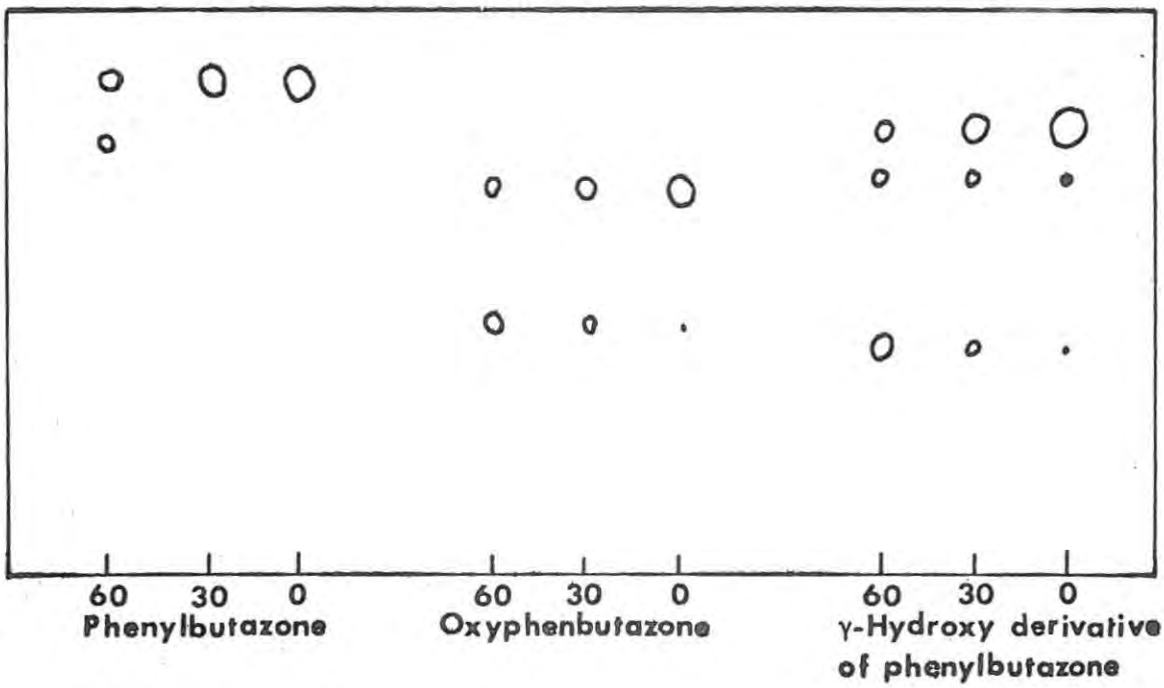


Plate 2

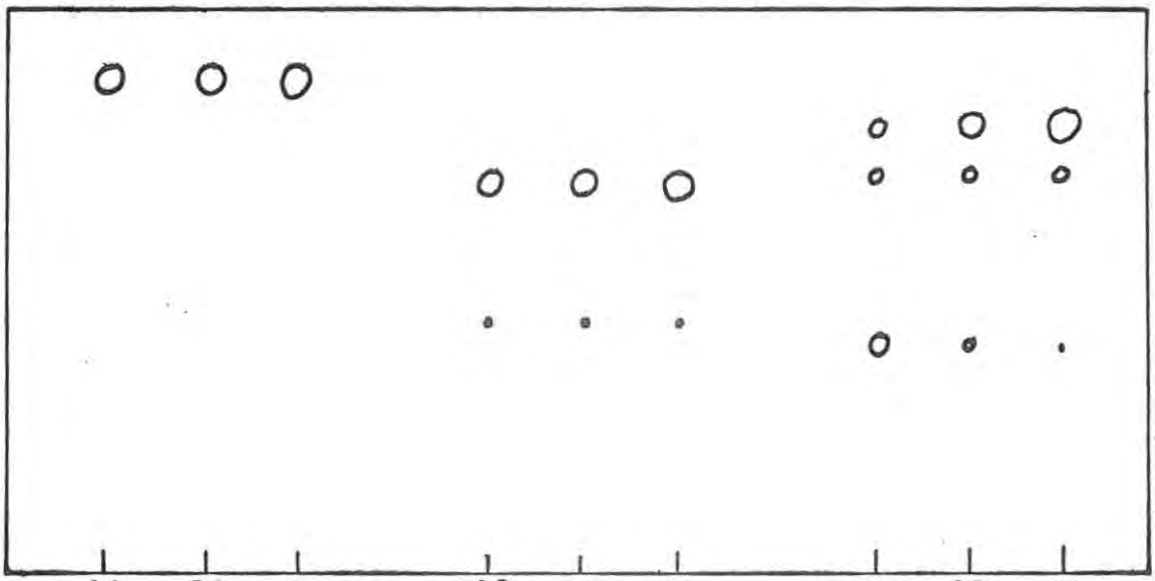


Plate 1

Fig. 41 Prevention of On-plate Oxidation by Inclusion of Sodium Thiosulphate in Preparation of Plates

CHAPTER VIII

PREPARATION OF SAMPLES FROM THE RAT

1. COLLECTION OF SAMPLES

After dosing, the rat was placed in a Metabowl whereby the faeces and urine could be collected separately. The contamination of excreta was a serious problem when rat cubes were used as food and the many methods tried to overcome this problem proved unsatisfactory. A liquid food consisting of glucose/electrolyte mixture was introduced into the drinking container and this arrangement resolved the contami= nation problem, and at the same time gave the rat adequate nourishment. However, the 48 h volume of urine increased from 15 - 20 ml to over 70 ml. This, in turn, affected the efficiency of the extraction methods, and, therefore, in experiments intended for the quantitative determination of metabolites in specimens excreted over a period of 48 h, rats were provided with drinking water, but were given no solid or liquid food.

1.1 Prevention of Decomposition of Metabolites During Collection Time

It was found that the decomposition of metabolites could be reduced to a minimum by collecting the excretion products

at a low temperature. To accomplish this, the collecting vessels of the Metabowl were placed in methanol at -20°C. Great care had to be taken to ensure that the urine did not freeze in the tubes leading to the collecting vessels and on subsequent melting overflow into the faeces collecting flask. This problem was overcome by blowing warm air on to the glass connections leading to the collection vessel.

2. EXAMINATION OF URINARY PRODUCTS

2.1 Preliminary Procedures Tested for the Extraction of Metabolites from Urine of Rats with Organic Solvents

Many methods were tried for the extraction of metabolites from samples of urine with organic solvents. Two solvents showed promise, dichloromethane and dichloroethane. Dichloro= methane has a lower boiling point, which is an advantage in that the time of evaporation decreases, thus reducing the possibility of decomposition of metabolites during the extraction process.

Well known methods used to improve the extraction process, such as salting out with ammonium sulphate, gave little advantage. Different extraction procedures were also tried. In certain cases a small quantity of solvent was used and the contents well mixed in a nipple tube on a vortex shaker.

In others, a larger proportion of solvent was added and shaken with urine on a mechanical shaker. The latter method appeared to give more quantitative extraction.

The 2 extraction processes, finally selected, were as follows:

2.1.1 Volumes of 5 ml and less

Dichloromethane (15 ml) was added to urine (5 ml), the mixture was acidified to pH 1-3 with sulphuric acid and shaken in a rotary shaker for 20 min before centrifuging at 3 000 r.p.m. for 10 min. The solvent layer was pipetted off and the extraction repeated twice using further 15 ml of dichloro= methane. The volume of the combined extracts was reduced to about 0,3 ml under nitrogen.

2.1.2 Volumes Between 5 and 40 ml

A volume of dichloromethane equal to half the volume of urine to be extracted was added to the urine, the mixture acidified with sulphuric acid to pH 1-3 and shaken for 5 min. The solvent layers were separated by centrifuging at 3 000 r.p.m. for 15 min and the organic layer removed. The urine was extracted with further volumes of solvent until extraction was complete. The

solvent extracts were mixed and the volume reduced by evaporation under nitrogen at 45^{o} C.

2.1.3 Estimation of the Number of Extractions Required to Obtain Complete Extraction of Metabolites from the Urine of Rats Dosed with Phenylbutazone

(a) TLC Procedure

Samples of urine from 3 rats dosed with phenylbutazone were extracted by method 2.1.1. After each extraction a 5 x 5 cm TLC plate was spotted with the extract, dried, developed and sprayed.

In two samples, it was found that extraction was complete after six extractions and in the third sample, after five extractions.

(b) Liquid Scintillation Counting Procedure

Samples from 2 rats treated with ^{14}C labelled phenylbutazone (s.a. 10,84 KBq-mg^{-1}) were extracted by method 2.1.2 and after each extraction 10 µl was mixed with 10 ml of scintillant and counted.

As can be seen from Table 26, it was found that, after 5 extractions, the count was low and steady.

Table 26 RADIOACTIVITY, MEASURED IN CPM, OF EXTRACTS OF URINE FROM RATS DOSED WITH ^{14}C PHENYLBUTAZONE

Extract	Rat No. 1	Rat No. 2
1st - 3rd	Not counted	Not counted
4th	355	348
5th	141	152
6th	150	140

These results indicated that at least five extrac= tions should be undertaken to ensure near complete extraction of the metabolites from the urine of rats dosed with phenylbutazone.

2.1.4 Test for Decomposition of Phenylbutazone and Oxyphenbutazone During Extraction Process

Phenylbutazone and oxyphenbutazone (1 mg of each) were added to separate 5 ml quantities of urine from an untreated rat. These urine samples were then extracted using method 2.1.1, and the extracts spotted on to TLC plates. Separate spots of 5 µl of freshly prepared solutions of phenylbutazone and oxyphenbutazone in dichloromethane were also applied to the plates which were then developed and sprayed.

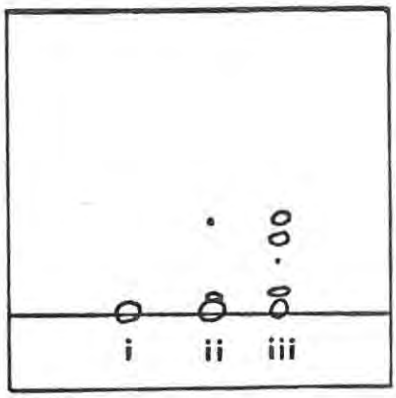
It was found that both the phenylbutazone and oxyphenbutazone, after extraction, produced a spot due to a decomposition product which was barely visible, and thus indicated that decomposition during extraction was insignificant provided the extraction process was carried out rapidly.

2.2 Establishment of Time After Dosing for First Collection of Urine from Rats Dosed with Phenylbutazone

Samples of 0 - 6 h urine and 6 - 24 h urine from a rat dosed with phenylbutazone and a sample of urine from an untreated rat were collected. Five millilitres of each of these samples was extracted by method 2.1.1 and spotted onto a 5 x 5 cm TLC plate which was dried, developed and sprayed.

As can be seen from Figure 42 the 24 h urine contained more metabolites than the 6 h urine, and these were present in higher concentrations.

These results indicated that 6 h urine samples give an incomplete picture of metabolism of phenylbutazone in rats, and consequently for these studies urine samples should not be taken less than 24 h after dosing.



i - extract of urine from an untreated rat

ii - extract of 0-6 h urine from a treated rat

iii - extract of 6-24 h urine from a treated rat

Fig. 42 Chromatogram of the Extracts of 0 - 6 h and 6 - 24 h Urine Samples from a Rat Dosed with Phenylbutazone Together with Control

2.3 Determination of the Effect of Variations in the Dose on the Metabolism of Phenylbutazone

Two rats were dosed with phenylbutazone, one with 12 $mg\text{-}kg^{-1}$ and the other with 50 $mg\text{-}kg^{-1}$, and urine samples collected after 24 h. About the same volume of urine was produced in each case.

Samples of urine (5 ml) were extracted by method 2.1.1 and 5 µl of each extract was spotted on to TLC plates, which were dried, developed and sprayed.

There was no apparent difference in the number and size of spots produced in each case, showing that differences in dosage at this level did not alter the pattern of excretion in 24 h urine.

2.4 Extraction of Water Soluble Metabolites from the Urine of Rats Dosed with Phenylbutazone

2.4.1 Test for Hydrolysable Conjugates in Urine

Initially the activity of the β-glucuronidase/arylsulfatase (Boehringer/Mannheim) enzyme was established as follows:

Twenty milligram of the chemically stable cinchonidine complex of phenolphthalein glucuronide was treated with 0,5 ml hydrochloric acid (m_{HCl} = 2 $mol \cdot kg^{-1}$). This left the phenolphthalein glucuronide as a sticky precipitate and formed the soluble cinchonidine hydrochloride. Ethyl acetate (1 ml) was added, the mixture shaken, separated and the ethyl acetate fraction passed through a cottonwool plug. This extraction was repeated 6 times, the ethyl acetate fractions mixed and evaporated under nitrogen to yield a colourless sticky residue, which was dissolved in 0,5 ml warm distilled water. The pH was adjusted to 5,0 with sodium hydroxide solution (m_{NaOH} = 0,1 $mol \cdot kg^{-1}$), the volume made up to 1,25 ml with distilled water and the solution clarified by adding charcoal and filtering. A portion of this phenolphthalein glucuronide solution (0,1 ml) was

mixed with 0,9 ml of β-glucuronidase/sulfatase in acetate buffer of pH 4,5 and incubated at 37°C for 45 min. A second portion of phenolphthalein glucuronide solution (0,1 ml) in acetate buffer (pH 4,5) to which no β-glucuronidase/sulfatase was added was similarly incubated to act as a control. At the end of the experiment sodium hydroxide solution (m_{NaOH} = 0,1 mol·kg^{-1}) was added to both tubes.

A pink colour was produced in the test solution and not in the control, indicating that the enzyme was active and had hydrolysed the glucuronide leaving the phenolphthalein to act as an indicator.

This active enzyme was used to ascertain whether samples of urine from rats dosed with phenylbutazone contained O-glucuronide or O-sulphate conjugates as follows:

Urine (5 ml) from a rat dosed with ^{14}C phenylbutazone was extracted according to method 2.1.1 and 10 µl spotted onto a pre-coated Merck 5 x 5 cm TLC plate (spot i). The aqueous residue (1 ml) was then incubated with 0,1 ml β-glucuronidase/ sulfatase and 0,9 ml acetate buffer (pH 4,5) at

37° C for 3 h. Ten microlitres of the hydrolysate was spotted on the TLC plate (spot ii) and 1,2 ml dichloromethane used to extract the remainder. Ten microlitres of this extract was spotted on the plate (spot iii). At the same time oxyphenbutazone was added to urine from an untreated rat and 5 ml of the urine extracted by method 2.1.1. Ten microlitres of the extract was spotted onto the plate (spot iv). The plate was dried, developed in solvent system XV and sprayed.

Figure 43 shows that urine, after treatment with β-glucuronidase/sulfatase, gives a major spot corresponding to the 4-hydroxyl derivative of oxyphenbutazone which could either be an artifact due to decomposition of oxyphenbutazone during the incubation period, or an authentic product of metabolism. A minor spot corresponding to oxyphenbutazone was also evident.

2.4.2 Test for Water Soluble, Non-hydrolysable Compounds in Urine

After extraction with dichloromethane of the incubation mixture, as described in 2.4.1, 0,5 ml of the aqueous layer was mixed with 10 ml aquagel and counted in a scintillation spectrometer. The

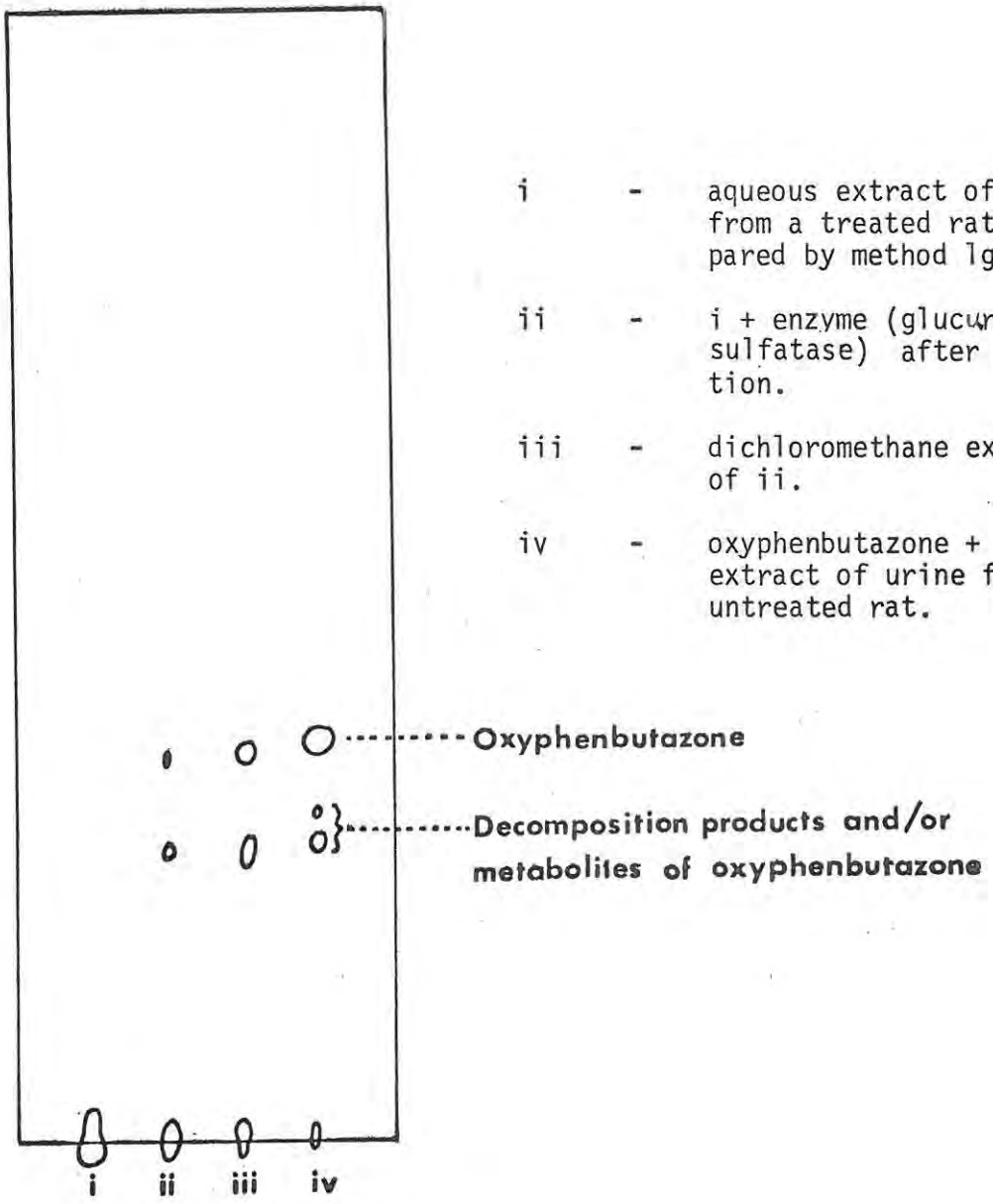


Fig. 43 Chromatogram of Extracts of Urine from Rats Dosed with Phenylbutazone Before and After Treatment with Glucuronidase/Sulfatase

result indicated that non-hydrolysable compounds accounted for about 14% of the water soluble meta= bolites.

A further portion of the aqueous layer (10 µl), as described above, was also spotted on a TLC plate prepared with sodium thiosulphate, the plate dried, developed in solvent system XXVII and examined under ultraviolet light. Spots were visible which could be due to metabolites, but it was not possible to identify them.

2.5 Assessment of the Degree of Decomposition of Phenylbutazone, Its γ-hydroxy Derivative and Oxyphenbutazone in Urine During a 24 h Incubation Period at 37° C

This experiment was carried out to assess whether possible decomposition of the metabolites could take place in the rat's bladder. Freshly collected urine (5 ml) from un= treated rats was placed in each of 4 test tubes and the following substances added:

tube i - 1 mg phenylbutazone

tube ii - 1 mg oxyphenbutazone

tube iii - 1 mg γ-hydroxy derivative of phenylbutazone

tube iv - nil (control)

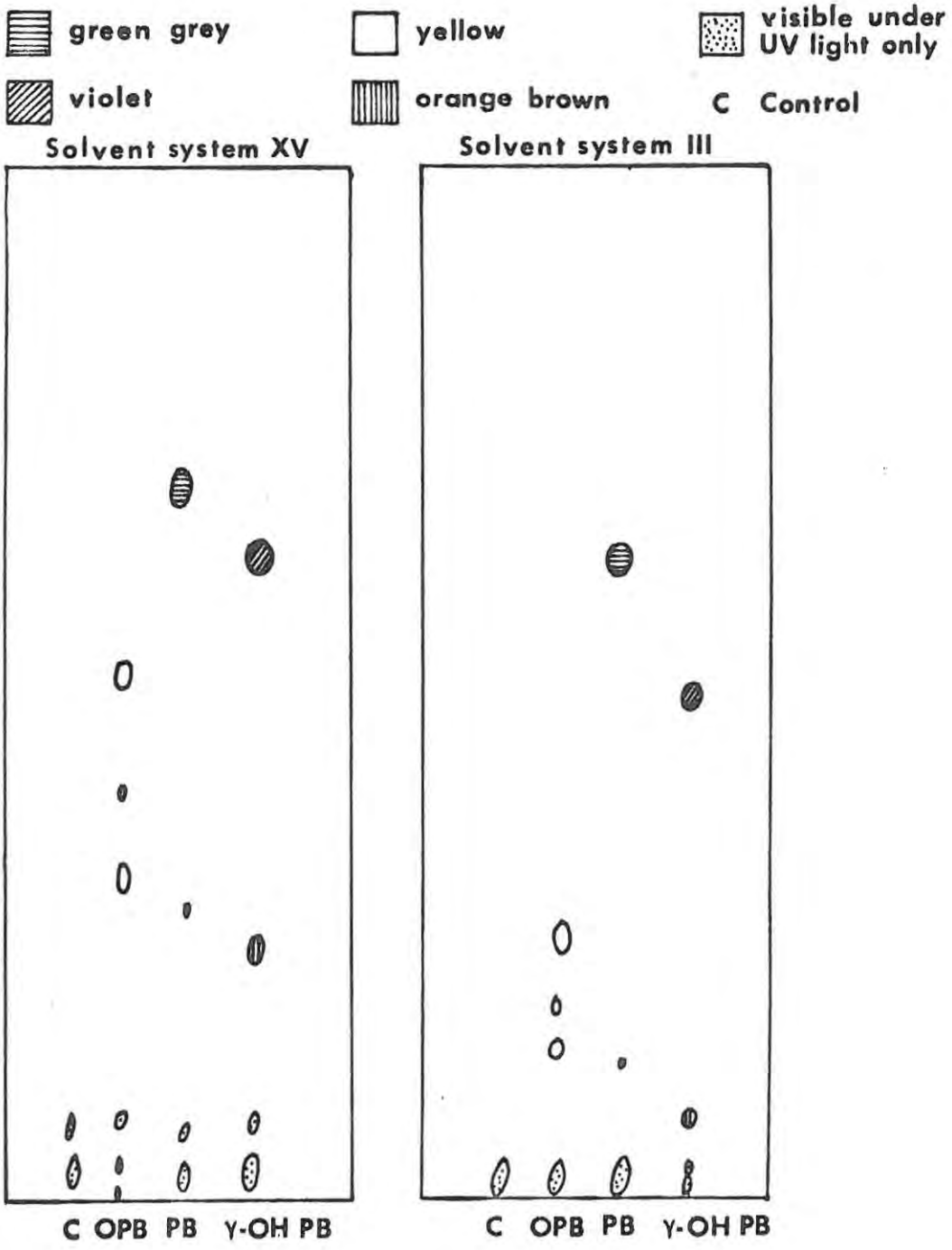


Fig. 44 Chromatogram of Extracted Rat Urine after Incubation at 37°C with Phenylbutazone, its γ-hydroxy Derivative and Oxyphenbutazone

The tubes were incubated at 37° C for 24 h, extracted according to method 2.1.1, spotted on 2 TLC plates, the plates dried, developed in solvent systems XV and III and sprayed. Figure 44 shows the degree of decomposition of each substance.

These results indicate that phenylbutazone gave rise to one minor decomposition product, oxyphenbutazone to two decomposition products, and the γ-OH derivative an equilibration mixture of the ring lactone and the straight chain hydroxyl forms, and a minor decomposition product. As it is unlikely that the urine would remain in the bladder for as long as 24 h possible decomposition of phenylbutazone and its γ-OH derivative appeared to be of little significance. However, the results showed that oxyphenbutazone had a much faster rate of decomposition, therefore the presence of these additional spots in chromatograms of the urine could be either true products of metabolism or decomposition products formed during excretion.

3. EXAMINATION OF FAECES

3.1 Measurement of Radioactivity in Faeces from Rats Dosed with ^{14}C Labelled Phenylbutazone

Lack of reproducibility was the cause of possible error in the

measurement of radioactivity in the faeces of rats dosed with ^{14}C labelled phenylbutazone. This was due to the difficulty of forming an homogenous sample, and quenching produced by the colour of the faeces. For these reasons various methods to digest and bleach samples of faeces were tried in order to produce an homogenous, colourless product which would give maximal counting efficiency.

The best conditions for counting were obtained by using the following method:

To 24 h faeces 60% perchloric acid (15 ml) was added and the mixture allowed to stand for 3 h before homogenisation. A measured volume of 0,5 to 1 ml of this homogenate was bleached by alkalising the mixture with 40% sodium hydroxide, warming and then adding 5 drops of 100 volume hydrogen peroxide. The mixture was boiled for 5 min, care being taken to avoid loss from effervescence. It was then carefully adjusted to pH 7 with concentrated hydrochloric acid.

This method produced a bleached, well homogenised sample which showed little quenching when measured for radioacti= vity in the liquid scintillation spectrometer.

3.2 Extraction of Metabolites Present in the Faeces from Rats Dosed with ^{14}C Phenylbutazone

A 24 h specimen of faeces (3 g) from a rat treated with ^{14}C phenylbutazone was homogenised in 15 ml of distilled water, centrifuged at 3 000 r.p.m. for 15 min and the mixture filtered. The filtrate was transferred to a round bottomed flask and freeze-dried overnight. It was reconstituted in 3 ml acetate buffer (pH 4,5) and examined for the presence of hydrolysable and non-hydrolysable conjugates by the methods used for examining urine.

CHAPTER IX

SYNTHESIS, ASSESSMENT OF PURITY AND PREPARATION OF CALIBRATION CURVES OF REFERENCE STANDARDS

1. PHENYLBUTAZONE

1.1 Confirmation of Relative Chromatographic Purity

Solutions of phenylbutazone and oxyphenbutazone were freshly prepared in dichloromethane, spotted on five 5 x 20 cm plates, dried, developed in the solvent systems listed in Table 18, examined under ultraviolet light, then sprayed and re-examined for coloured spots.

Table 27 shows that in each case the solution of phenylbutazone only produced one spot visible under ultraviolet light and after spraying. Thus it was assumed that the sample of phenylbutazone was chromatographically pure.

1.2 Preparation of Calibration Curve

Solutions of phenylbutazone in chloroform:methanol (9:1) were prepared in dilutions ranging from 10 $\mu g \cdot ml^{-1}$ to 60 $\mu g \cdot ml^{-1}$ and the absorbance measured at 270 nm. The results are shown in Table 28.

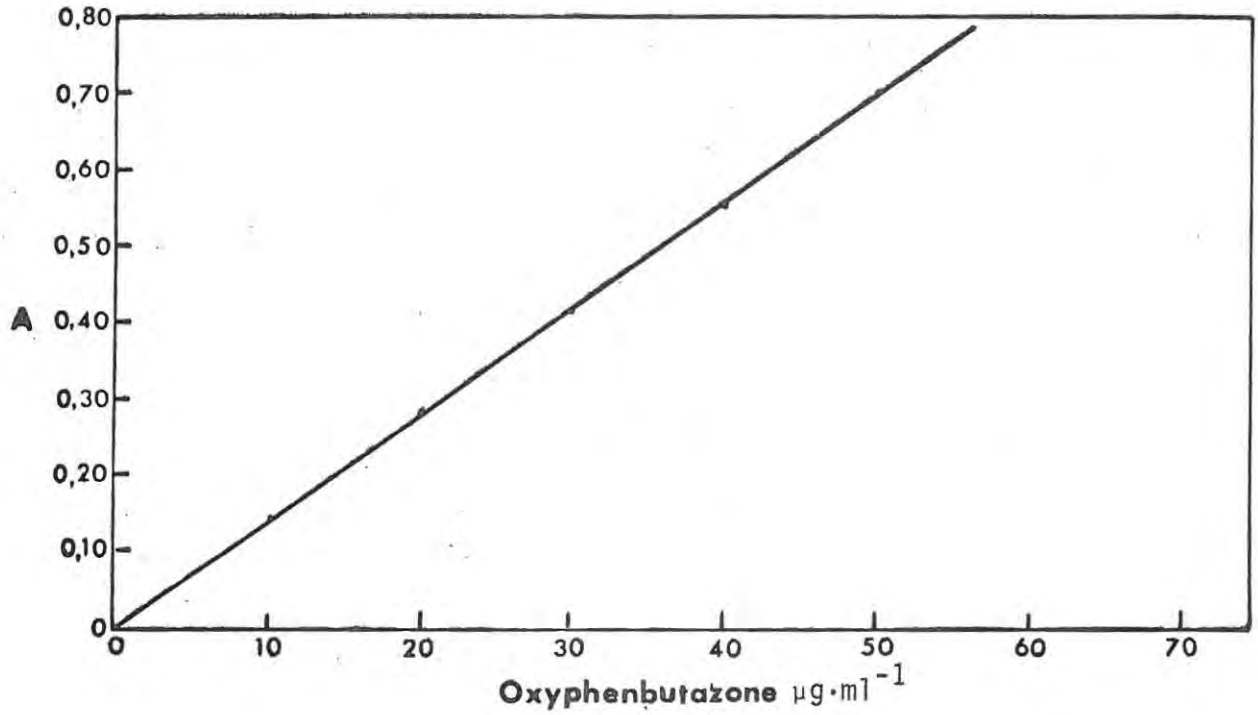
Table 27 RELATIVE R_f VALUES OF PHENYLBUTAZONE IN VARIOUS SOLVENTS

Solvents	Relative R_f value
XV	2,00
III	1,26
II	2,35
XXVI (a)	1,17
XXVI (b)	1,42

Table 28 ABSORBANCE OF SOLUTIONS OF PHENYLBUTAZONE IN CHLOROFORM:METHANOL (9:1) AT 270 nm

Absorbance	Dilution ($\mu g\ ml^{-1}$)
0,14	10
0,26	20
0,39	30
0,51	40
0,64	50
0,76	60

A graph (Figure 45) was prepared from these results. It conforms to Beer-Lambert law.



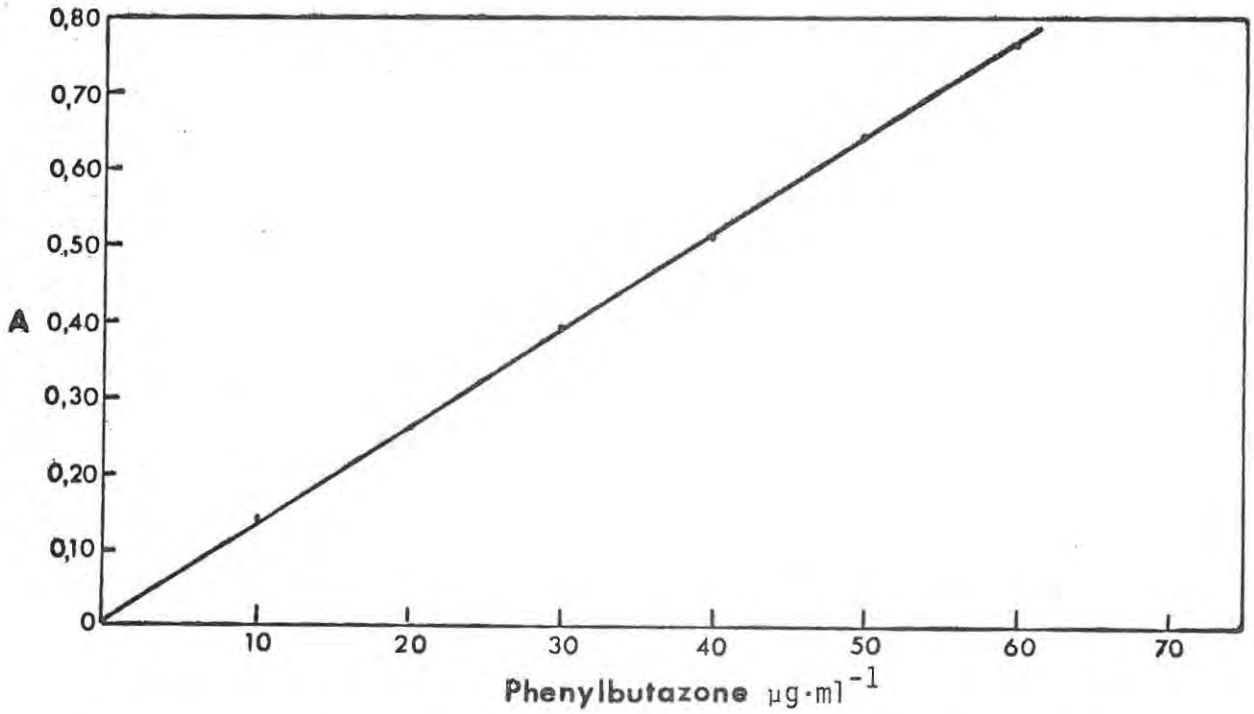


Fig. 45 Calibration Curves for Phenylbutazone and Oxyphenbutazone

2. PHENYLBUTAZONE LABELLED WITH ^{14}C IN THE 4 POSITION

^{14}C Phenylbutazone was synthesised by Professor G E A Mathew using 2-^{14}C diethylmalonate (55,5 KBg : s.a. 1443 KBg- mg^{-1}) as the source of radioactivity. The method was as follows:

The radiochemical in hexane was transferred to the reaction flask, containing inactive diethyl malonate. The hexane was evaporated off using a nitrogen stream.

N-butyl bromide, hydrazobenzene and sodium iodide were added to the flask, the apparatus was flushed with nitrogen and the flask heated to 70^{o} C with stirring of the reactants. A calculated volume of sodium ethoxide was added over 3 h and the mixture heated under reflux for 2 h. This procedure resulted in 1-butylation of di-ethyl malonate.

To form the 3,5-dioxo-pyrazolidine ring the apparatus was then conver= ted to distillation and the ethanol slowly distilled off with gradual increase of temperature of the heating bath to 120^{o} C. The reaction mass set solid and the heating was continued at 150^{o} C for 2 h.

The product was isolated by transferring the entire contents of the flask, using alternate portions of water (5 parts) and chloroform (1 part), into a separating funnel. The chloroform layer was re= moved and the aqueous layer extracted several times with chloroform

and once with ether before being run into a second 50 ml separating funnel. It was acidified with hydrochloric acid to pH 2 producing a yellow precipitate which was extracted several times with chloroform. The extracts were combined and the solvent evaporated to leave the crude product, A. This crude product was dissolved in a minimum volume of chloroform and applied to a Pharmacia SR 25/100 column packed with Mallinckrodt Silica AR CC4 (100 - 200 mesh). Fractions were collected and analysed by TLC.

The phenylbutazone fraction was collected until the emergence of a following coloured band and the solvent removed by rotary evaporation at 50^{o} C leaving a white solid, B, which was dried under vacuum. Inactive phenylbutazone was added to B and low temperature crystallisation carried out from ether to give product C.

The mother liquors were evaporated and more inactive phenylbutazone was added to the residue, D. Low temperature recrystallisation from ether gave product E.

C and E were dissolved in solvent which was evaporated off leaving a final product with a s.a. of 10,93 KBq-mg^{-1}.

3. OXYPHENBUTAZONE

3.1 Confirmation of Relative Chromatographic Purity

A freshly prepared solution of oxyphenbutazone in dichloro=methane was spotted on 2 pre-coated Merck 5 x 20 cm plates, the plates dried, developed in solvent systems III and XV, examined under ultraviolet light and sprayed.

In addition to the major spot a small, weakly stained spot, with relative R_f values of 0,78 in solvent system III and 0,59 in solvent system XV, was evident on both plates. This impurity was probably the 4-hydroxy derivative of oxyphenbutazone. In the light of these findings attempts were made to prepare a pure sample of oxyphen=butazone.

3.2 Preparation of Pure Oxyphenbutazone

Purification by re-crystallisation from ether-petroleum ether (B P 40-60^{o} C) proved ineffective. It was therefore decided to use the following TLC technique to prepare a chromatographically pure solution of oxyphenbutazone.

Oxyphenbutazone (100 mg) was dissolved in 2 ml of dichloro=methane and applied to a preparative plate of Silica gel

60 PF_{254} with the aid of a chromatocharger. The plate was developed in solvent system III to a height of 15 cm and examined under ultraviolet light. A very wide band was visible with an R_f value in the region of 0,5, which was taken to be oxyphenbutazone, and a narrow band (R_f 0,41) which was considered to be a decomposition product.

In order to guard against contamination by the decomposition product, only the upper three-quarters of the top band was scraped off, eluted with 10 ml chloroform:methanol (9:1) and the eluate centrifuged. TLC, in solvent systems III and XV, showed no evidence of the sample containing impurities. To an aliquot of this solution (2,5 ml) chloroform:methanol (9:1) was added to form a 1:2 dilution (solution A). A sample of this solution (0,05 ml) was diluted to form a 1:2 000 dilution (solution B) and 3 ml of solution B was transferred to a cuvette and the absorbance measured (0,125). This absorbance value was applied to the calibration curve which showed that solution B had a concentration of 18 $\mu g\ ml^{-1}$. Solution A was used in the quantitative procedure using multiple inverse isotope dilution.

3.3 Preparation of Calibration Curve

As the crystalline sample supplied by Ciba Geigy showed an insignificant content of impurities, it was used for the

preparation of the Calibration Curve.

Solutions of oxyphenbutazone in chloroform:methanol (9:1) were prepared in dilutions ranging from 10 to 50 $\mu g\ ml^{-1}$ and the absorbance measured at 270 nm. A Calibration Curve was prepared (Figure 45) from the results shown in Table 29.

Table 29 ABSORBANCE OF SOLUTIONS OF OXYPHENBUTAZONE IN CHLOROFORM:METHANOL (9:1) AT 270 nm

Absorbance	Dilution ($\mu g \cdot ml^{-1}$)
0,140	10
0,285	20
0,420	30
0,555	40
0,700	50

4. γ-HYDROXY DERIVATIVE OF PHENYLBUTAZONE

4.1 Synthesis

The γ-OH derivative of phenylbutazone was synthesised in this laboratory according to the method of Denss (20) as

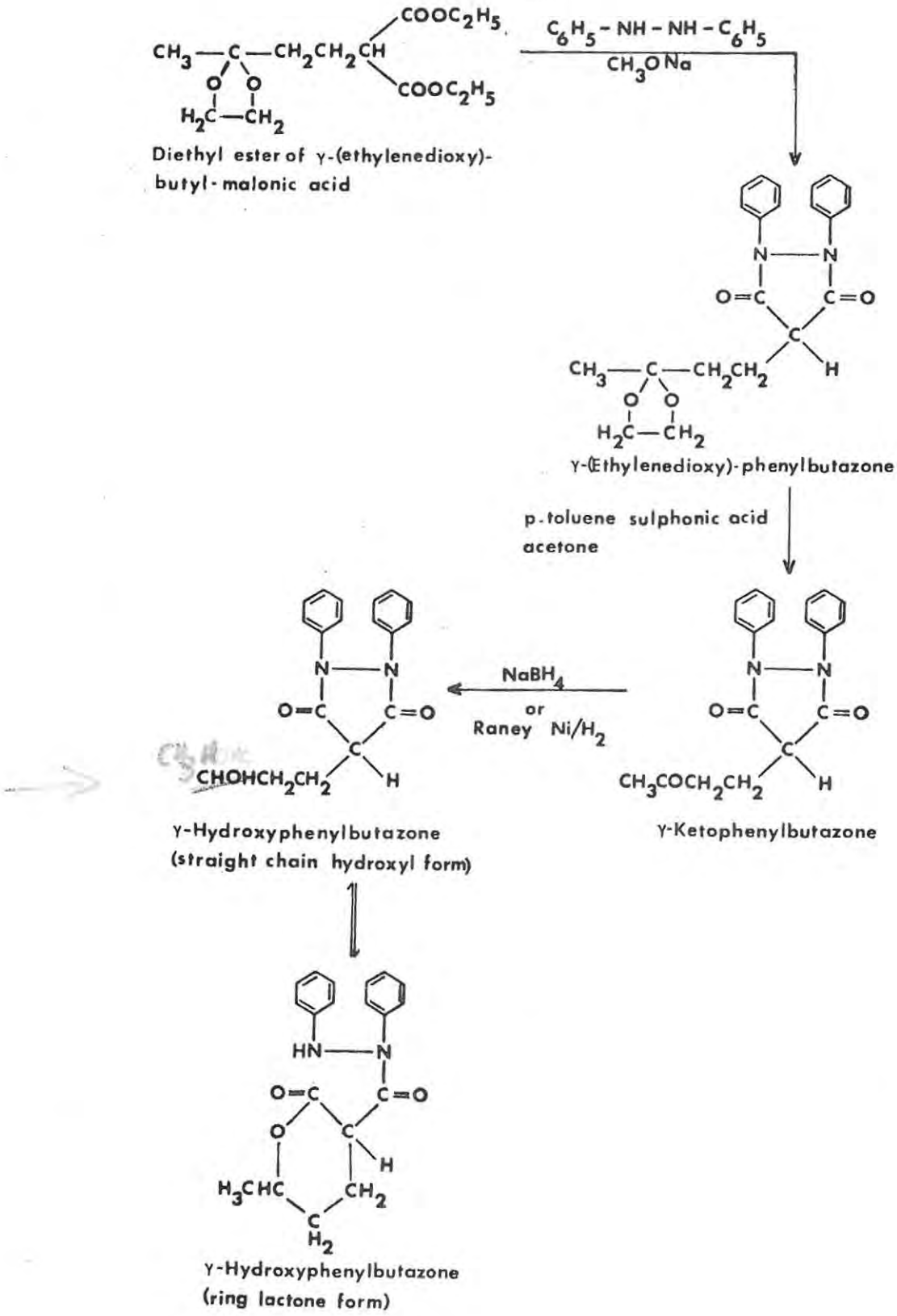


Fig. 46 Synthesis of γ-Hydroxy Derivative of Phenylbutazone

shown in Figure 46. The ring lactone form was used in the experimental work, as it remained pure in the crystalline state. The melting point of this form was 165° C.

4.2 Confirmation of Relative Chromatographic Purity

Freshly prepared solutions of the γ-hydroxy derivative of phenylbutazone and oxyphenbutazone were prepared in dichloromethane, spotted on 5 pre-coated Merck 5 x 20 cm plates, the plates dried and immediately developed in the solvent systems listed in Table 30. It was important to complete these steps in the shortest possible time to prevent formation of the straight chain hydoxyl form of the γ-hydroxy derivative. The plates were examined under ultraviolet light and then sprayed. Relative R_f values of the γ-hydroxy derivative in various solvents are shown in Table 30.

Table 30 RELATIVE R_f VALUES OF THE γ-HYDROXY DERIVATIVE OF PHENYLBUTAZONE

Solvents	Relative R_f values
XV	1,80
III	1,17
X	1,86
II	1,05
XXVI (a)	1,15

The solution of the γ-hydroxy derivative of phenylbutazone produced only one spot visible under ultraviolet light and after spraying, thus it was assumed that the sample was chromatographically pure.

4.3 Preparation of a Calibration Curve

Solutions of the γ-hydroxy derivative of phenylbutazone in chloroform:methanol (9:1) were prepared in dilutions ranging from 5 to 40 $\mu g \cdot ml^{-1}$, and their absorbance measured at 270 nm.

A Calibration Curve was prepared from the results listed in Table 31 and is shown in Figure 47. It conforms to Beer-Lambert law.

Table 31 ABSORBANCES OF SOLUTIONS OF THE γ-HYDROXY DERIVATIVE OF PHENYLBUTAZONE IN CHLOROFORM:METHANOL (9:1) AT 270 nm

Absorbance	Dilution ($\mu g \cdot ml^{-1}$)
0,11	5
0,22	10
0,41	20
0,60	30
0,78	40

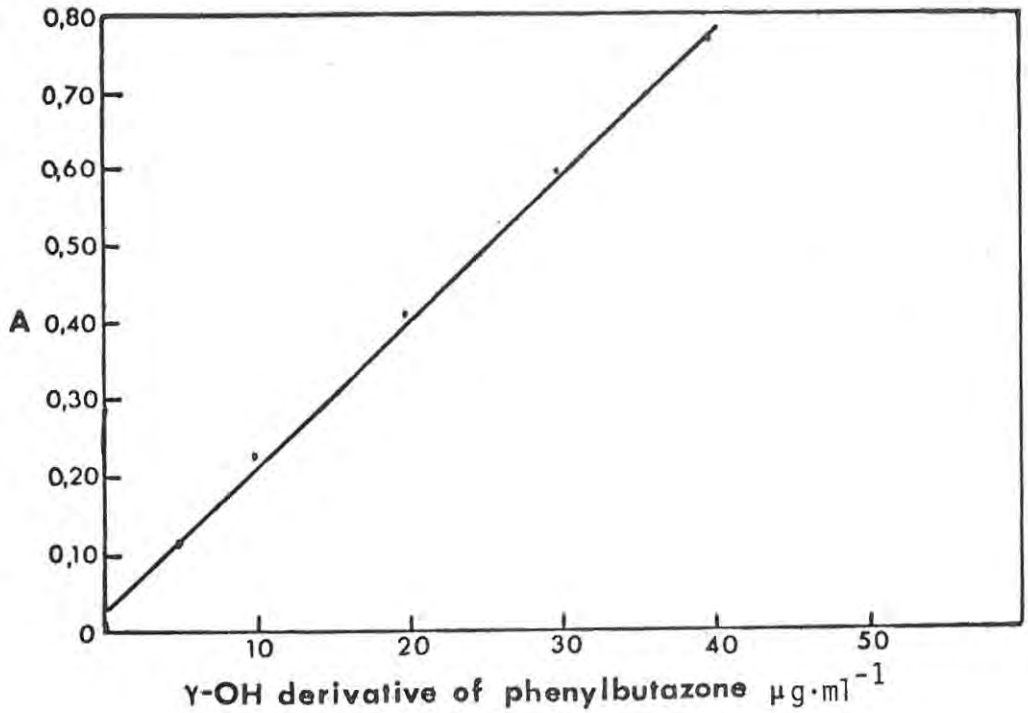


Fig. 47 Calibration Curve of the γ-hydroxy Derivative of Phenylbutazone in Chloroform:Methanol Solvent

5. p-γ-DIHYDROXY DERIVATIVE OF PHENYLBUTAZONE

5.1 Confirmation of Relative Chromatographic Purity

Freshly prepared solutions of the p-γ-dihydroxy derivative of phenylbutazone and oxyphenbutazone, which later was used as a standard, were spotted on three Merck prepared plates, dried, the plates developed in the solvent systems listed in Table 32, examined under ultraviolet light, then sprayed and re-examined. Table 32 shows that, in each case, the solution of the p-γ-dihydroxy deri=

vative of phenylbutazone produced only one visible spot under ultraviolet light and after spraying; thus it was assumed that the sample was chromatographically pure.

Table 32 RELATIVE R_f VALUES OF THE p-γ-DIHYDROXY DERIVATIVE OF PHENYLBUTAZONE

Solvents	Relative R_f values
III	0,30
VII	0,15
II	0,19

6. 4-HYDROXY DERIVATIVE OF PHENYLBUTAZONE

6.1 Synthesis

The method of G Papa (39) for the synthesis of 4-hydroxy-4-phenyl-1, 2-diphenyl-3, 5-pyrazolidinedione was modified and used by Professor Mathew to synthesise the 4-hydroxy derivative of phenylbutazone as follows:

Phenylbutazone was dissolved in a solution of sodium methoxide in methanol and a solution of 35% w/w hydrogen peroxide in methanol added dropwise. Sodium hydroxide

solution (m_{NaOH} = 6 mol·kg^{-1}) was gradually added to the cooled solution and the mixture stirred for 30 h. The solution was diluted with water, acidified with diluted sulphuric acid and the resultant precipitate taken up in ether and shaken with sodium bicarbonate solution. The alkaline extract was separated, reacidified and extracted with a large volume of ether. The ether was evaporated off and a white precipitate formed which was filtered and washed. The melting point of the crystals was 131^{o} C.

6.2 Confirmation of Chromatographic Purity

Freshly prepared solutions of the 4-hydroxy derivative of phenylbutazone and oxyphenbutazone (used as a reference) were spotted on 5 pre-coated Merck 5 x 20 cm plates, which were dried and developed in the solvent systems listed in Table 33, examined under ultraviolet light, and sprayed. Table 33 shows that in each case the solution of the 4-hydroxy derivative of phenylbutazone produced only one spot visible under ultraviolet light and after spraying and thus it was assumed that the sample was chromatographically pure.

Table 33 RELATIVE R_f VALUES OF THE 4-HYDROXY DERIVATIVE OF PHENYLBUTAZONE

Solvents	Relative R_f values
XV	1,42
III	1,07
X	1,31
II	1,20
XXVI (a)	1,27

CHAPTER X

RADIO-CHEMICAL TECHNIQUES

1. PREPARATION OF EFFICIENCY GRAPH FOR LIQUID SCINTILLATION SPECTROMETER

The efficiency graph was prepared using a series of 6 standard samples of the same activity, one being unquenched and the others having increasing quenching. The unquenched sample had a dpm value of 51×10^4. The C channel of the spectrometer was set at the ^{14}C setting and the A channel at L→U. Starting with the least quenched standard sample the counts of these samples in channels A and C were recorded. The upper and lower levels of A channel were adjusted until a combination was arrived at which gave a straight line graph (Figure 48) when the ratio of the counts in channel A against the counts in channel C were plotted against efficiency (counts in channel C $\times (51 \times 10^4)$.

This graph was used to provide efficiency figures required to convert cpm to dpm in the liquid scintillation spectrometer experimental work which follows.

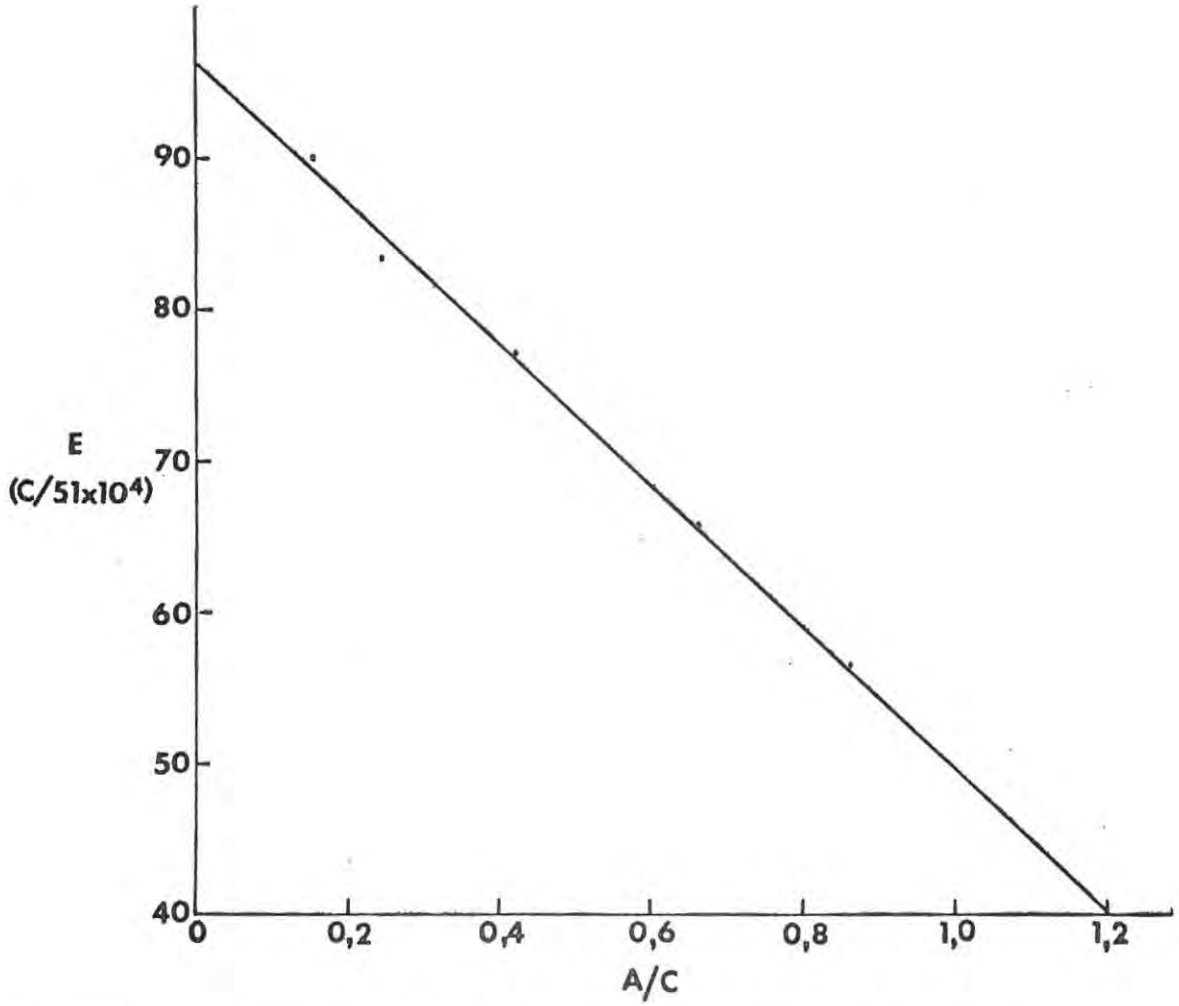


Fig. 48 Efficiency Graph for Scintillation Spectrometer.

2. METHODS OF MEASURING RADIOACTIVITY OF SAMPLES

2.1 Liquids

Samples (maximum volume 1 ml) were pipetted into l.s.c. vials and mixed with 10 ml of scintillant. In some cases,

where it was necessary to use larger samples, 15 ml of scintillant was required to give a clear solution for counting. The background count was determined using scintillant only. In all cases samples were counted for 10 min and the counts in channels A and C were recorded. These were converted into dpm using the formula shown in Figure 49.

$$dpm = \frac{\text{Number of counts in channel C (sample)}}{10 \times E} - \frac{\text{Number of counts in channel C (background)}}{10 \times E}$$

where dpm = Disintegrations per minute

E = Efficiency (from graph, Figure 48)

Fig. 49 Calculation of Disintegrations per Minute

2.2 TLC Plates

The area containing the radioactive material was marked under ultraviolet light and scraped off into a test tube. A known volume of solvent was added and the contents mixed in a vortex shaker. The tube was centrifuged at 3 000 r.p.m. for 15 min and an aliquot transferred to an l.s.c. vial. The solvent was evaporated under nitrogen, scintil= lant added and the radioactivity measured.

3. CONFIRMATION OF SPECIFIC ACTIVITY OF ^{14}C PHENYLBUTAZONE

A solution of unlabelled phenylbutazone was prepared by dissolving phenylbutazone (140 mg) in 10 ml methanol. A second solution of radioactive phenylbutazone was prepared by dissolving ^{14}C phenylbutazone (15 mg) in 1,5 ml of dichloromethane. A portion of the radioactive solution (0,1 ml) was evaporated under nitrogen and 1 ml of the solution of unlabelled drug added to give a solution of phenylbutazone containing 15 $mg \cdot ml^{-1}$.

A TLC plate was prepared from a slurry of Silica gel GF_{254} in 10% sodium thiosulphate solution, activated at 100^{o} C for 15 min and 4 spots of about 0,5 µl of the phenylbutazone solution applied to the plate. It was then dried, developed in solvent system XXVI and examined under ultraviolet light. The spots were marked, scraped off into 5 ml of chloroform:methanol (9:1) which was shaken and filtered. An aliquot of the filtrate (1 ml) was mixed with 2 ml chloroform:methanol (9:1), its absorbance was measured and the concentration of phenylbutazone obtained from the calibration curve. A further aliquot of the filtrate (2 ml) was transferred to an l.s.c. vial, the solvent evaporated off under nitrogen, Bray's scintillant (10 ml) added and the radioactivity measured in the liquid scintillation spectrometer. An equal area of the plate which showed no fluoresence under ultraviolet light was similarly treated to act as a blank. From the results shown in Table 34 the average s.a. of the ^{14}C phenylbutazone used was 10,84 $KBq\text{-}mg^{-1}$.

Table 34 SPECIFIC ACTIVITY OF ^{14}C LABELLED PHENYLBUTAZONE

Absorbance	$\mu g/ml^{-1}$ (1 in 3 solution)	Total weight of Phenylbutazone in l.s.c. vial in µg	Weight of ^{14}C Phenylbutazone in l.s.c. vial in µg	Radioactivity in dpm in vial	Radioactivity in dpm in 1 mg	Specific Activity $KBq \cdot mg^{-1}$
0,26	20	120	8	516	$6,45 \cdot 10^4$	10,74
0,125	9,5	57	3,8	261	$6,87 \cdot 10^4$	11,47
0,15	11,2	67,2	4,48	292	$6,52 \cdot 10^4$	10,85
0,295	22,8	136,8	9,12	563	$6,17 \cdot 10^4$	10,28
					$6,50 \cdot 10^4$	1 0,84

4. AUTORADIOGRAPHY

4.1 Preparation of TLC Plate

The radioactive material was spotted on a pre-coated Merck Silica gel F_{254} plate, developed in the appropriate solvent, examined under ultraviolet light and the spots lightly out= lined with a soft pencil. Dots of coloured radioactive ink were spotted on the edge of the plate.

4.2 Preparation of Cassette to Hold Plate

A cassette was made as shown in Figure 50 (45) which proved to be absolutely light proof and held the film against the plate in a non-skid fashion with even pressure.

4.3 Photographic Procedure

In the dark room Kodak Tri X PAN film was placed on the TLC plate in the cassette, the lid was sealed and the box stored in a drawer. After 28 days the film was removed and developed.

The radioactive material showed up as shadows against a clear background; the intensity of the shadows gave an indication of the amount of radioactive material present.

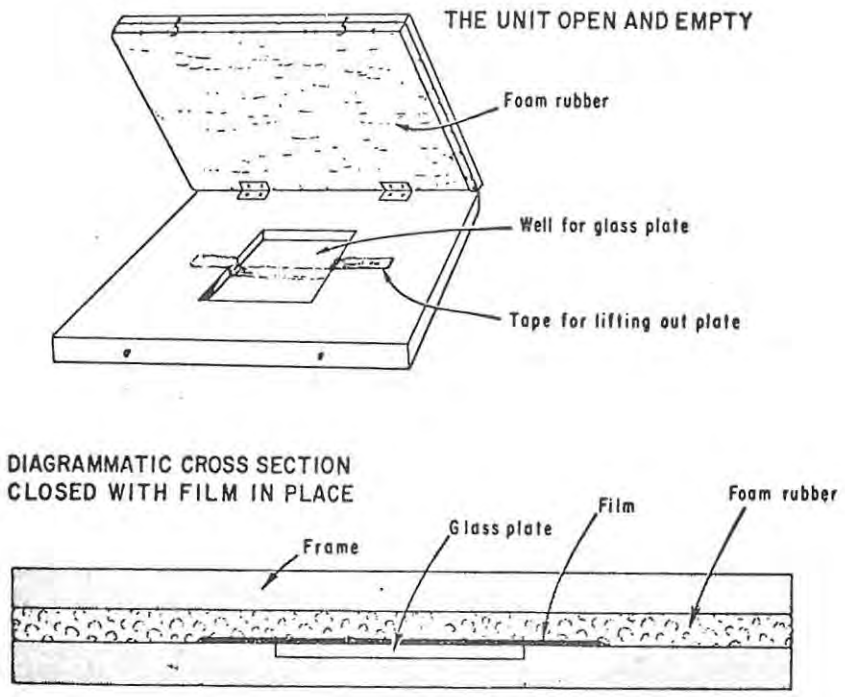


Fig. 50 Cassette for Autoradiography

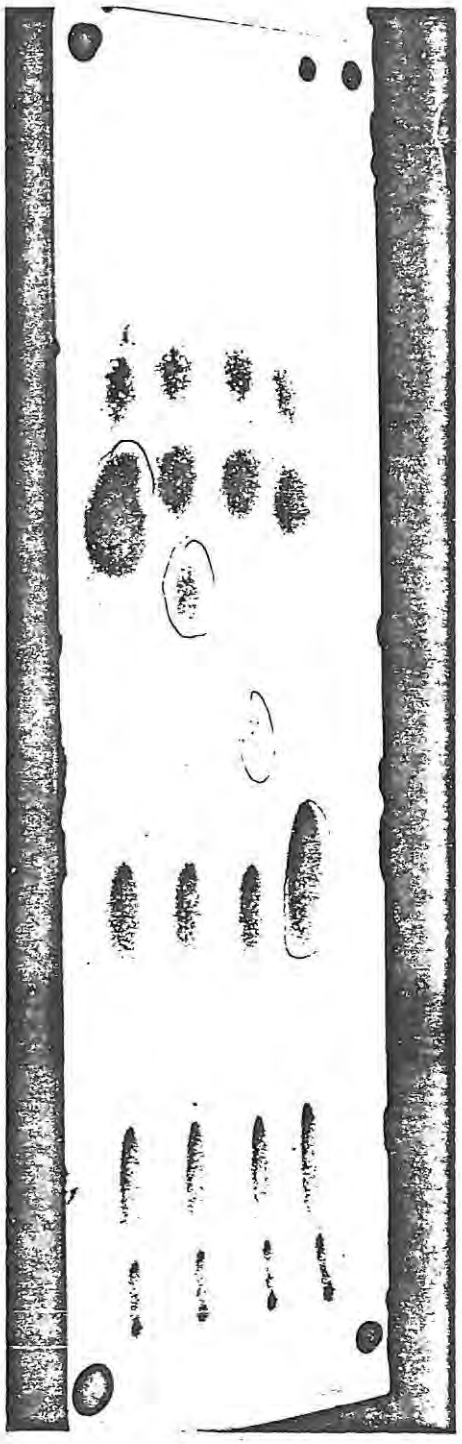
4.4. Photography Under Ultraviolet Light of TLC Plates Used for Autoradiography

The plate was placed under an ultraviolet light and photo= graphed using an ultraviolet filter that obstructed the ultraviolet light but not the fluorescent light. It was planned to use the resultant photograph as a template for the identification of the radioactive spots on the auto= radiograph.

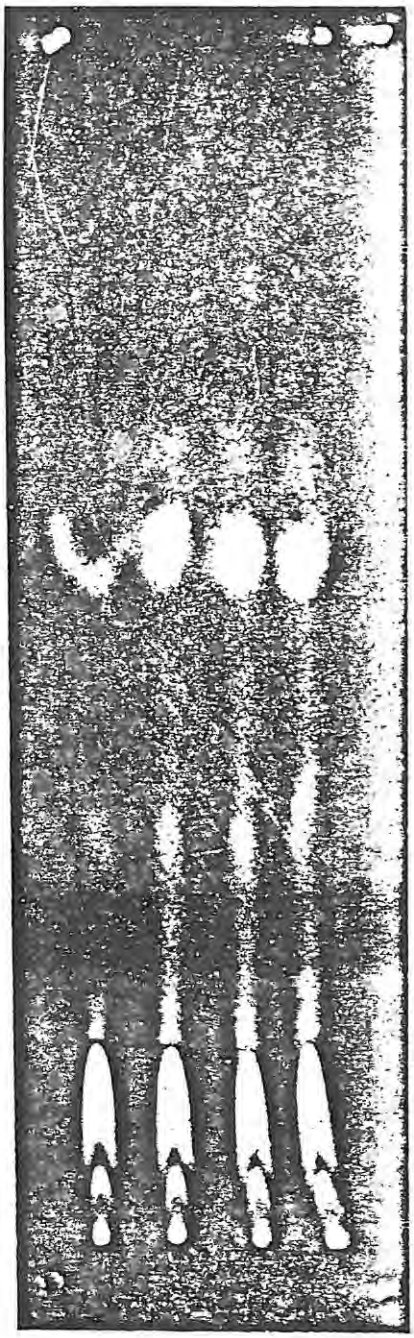
However, this was found to be impracticable as the

photograph had to be taken from an angle which, as Figure 51 illustrates, distorted the image.

As an alternative a piece of tracing paper was placed on top of the TLC plate and a tracing made of the spots observed under ultraviolet light, as well as the marker ink spots. By superimposing this tracing on the photographic film and making certain that the marker ink spots were in apposition it was possible to ascertain which spots on the chromatogram showed radioactivity.



TLC Plate
Photograph



Autoradiograph

Fig. 51 Comparison of a Photograph of Autoradiograph taken under Ultraviolet Light and by Ordinary Light

EXCRETION AND METABOLISM

OF

PHENYLBUTAZONE

CHAPTER XI

EXCRETION STUDIES

1. DOSING THE RAT

Excretion studies were carried out on 4 rats, designated Rats 1, 2, 3 and 4. Rat 1 was dosed with radioactive drug only, whereas Rats 2, 3 and 4 were dosed with a mixture of radioactive and non-radioactive drug for the purpose of conserving the limited quantity of radioactive drug available. The point of a Luer Lock needle (0 guage) was ground down to a smooth surface and protected with a small length of polythene tubing. The combined length of the needle and tubing was such that it could enter the stomach of the rat post-orally without causing injury. The needle was fitted to a 2 ml syringe, and the unit was used to dose the rats post-orally.

1.1 Rat 1

^{14}C labelled phenylbutazone (12 mg - s.a. 10,84 $KBq \cdot mg^{-1}$) was dissolved in 0,9 ml of sodium carbonate solution ($m_{Na_2CO_3}$ = 1 $mol \cdot kg^{-1}$) and 0,3 ml of distilled water added to make a 10 $mg \cdot ml^{-1}$ solution. A female Wistar rat, weighing 189 g was dosed post-orally with 1 ml of the

solution and placed in a glass Metabowl cage. A portion of the labelled phenylbutazone solution (0,1 ml) was diluted to 10 ml with distilled water; to duplicate samples of this solution (0,5 ml) 10 ml Bray's scintillant was added and the radioactivity counted to determine the activity of the dosing solution (Solution A - Table 35).

1.2 Rats 2, 3 and 4

^{14}C labelled phenylbutazone (7 mg - s.a. 10,84 KBq·mg^{-1}) and unlabelled phenylbutazone (43 mg) were dissolved in 3,6 ml sodium carbonate solution ($m_{Na_2CO_3}$ = 1 mol·kg^{-1}). The solution was made up to 5 ml with distilled water to give 1,4 mg labelled phenylbutazone per ml (Solution B). Rats 2, 3 and 4, weighing 183, 190 and 187 g respectively, were dosed post-orally with 1 ml of solution B and placed in individual glass Metabowl cages. Duplicate samples, each containing 0,05 ml of solution B and 10 ml of Bray's scintillant, were counted to determine the activity of the dosing solution (Table 35).

2. COLLECTION OF SPECIMENS AND THEIR PREPARATION FOR LIQUID SCINTILLATION COUNTING

Urine and faeces from each rat were collected at 24 h intervals, and, after collection, the Metabowls washed with a minimum volume of

Table 35 DETERMINATION OF RADIOACTIVITY IN THE DOSE ADMINISTERED

Sample taken for Counting	Counts per 10 min		Efficiency E	Radioactivity in Sample Taken for Counting in dpm	Total Radioactivity in dose in dpm
	Channel A	Channel C			
Solution A	110 073	161 127	0,648	24 487	4 947 000
	109 638	159 437	0,646	24 643	
Solution B	101 058	244 552	0,764	31 974	682 530
	113 645	282 889	0,779	36 279	
Background	94	352	0,827	38	

distilled water. The volumes of the urines and washings were recorded. The samples of faeces were treated as follows in order to prepare a solution suitable for the measurement of radioactivity. Perchloric acid (15 ml of a 60% solution) was added to the samples and the mixtures allowed to digest for 3 h before homogenising. The volumes of the homogenates were measured and aliquots, in l.s.c. vials, made alkaline with 40% sodium hydroxide and warmed. Five drops of 100 volume hydrogen peroxide were added and the mixture boiled for 5 min, care being taken to avoid spilling from effervescence. Finally they were neutralised to pH 7 with concentrated hydrochloric acid, scintillant added and the radioactivity measured. Scintillant was added to the samples of urine and Metabowl washings and the radioactivity measured. The background count of the scintillant used was also measured. The results are recorded in Table 36.

3. RADIOACTIVITY OF SAMPLES

3.1 Solutions of ^{14}C Labelled Phenylbutazone Used for Dosing

The samples of solutions of labelled phenylbutazone ad= ministered to the rats were counted for 10 min in the liquid scintillation spectrometer, and the total radio= activity in the dose calculated (Table 35).

Table 36 RADIOACTIVITY OF SAMPLES OF EXCRETION PRODUCTS OF RATS DOSED WITH ^{14}C PHENYLBUTAZONE

Sample taken for counting	Volume of Specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Bray's Scintillant Background		94	352	0,827	38	
Rat 1: 0,1 ml 24 h urine	8,2 ml	123 083	250 212	0,743	34 051	2 818 627
		128 496	245 221	0,706	34 696	
Rat 1: 0,1 ml 48 h urine	3,0 ml	102 285	193 360	0,719	26 885	796 395
		100 853	188 137	0,716	26 238	
Rat 1: 0,1 ml 72 h urine	2,25 ml	31 476	53 496	0,674	7 899	173 580
		30 896	52 862	0,676	7 881	
Rat 1: 1,0 ml 24 h cage washing	24,54 ml	29 982	39 778	0,614	6 441	159 164
		30 456	40 461	0 614	6 552	

Table 36 (continued)

Sample taken for counting	Volume of Specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Rat 1: 1,0 ml 48 h cage washing	12,0 ml	60 698	87 946	0,642	13 661	175 434
		71 081	98 070	0,628	15 578	
Rat 1: 1,0 ml 72 h cage washing	12,5 ml	13 838	18 827	0,623	2 984	37 144
		13 713	18 702	0,624	2 959	
Rat 1: 1,0 ml 24 h faeces homogenate	4,6 ml	27 803	37 937	0,624	6 042	27 986
		28 150	38 215	0,620	6 164	
Rat 1: 1,0 ml 48 h faeces homogenate	84,0 ml	32 688	34 250	0,520	6 549	567 420
		34 730	35 976	0 514	6 961	
Rat 1: 1,0 ml 72 h faeces homogenate	50,0 ml	28 498	30 335	0,532	5 664	282 080
		27 973	29 579	0,523	5 619	

Table 36 (continued)

Sample taken for counting	Volume of Specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Bray's Scintillant Background		73	281	0,846	33	
Rat 2: 0,1 ml 24 h urine	13,2 ml	10 434	21 562	0,740	2 881	372 900
		10 018	20 788	0,742	2 769	
Rat 3: 0,1 ml 24 h urine	7,0 ml	17 841	35 902	0,735	4 988	347 620
		17 986	36 634	0,736	4 944	
Rat 4: 0,1 ml 24 h urine	5,6 ml	19 750	39 153	0,730	5 330	300 132
		20 391	39 473	0,728	5 389	
Rat 2: 1 ml 24 h cage washing	19,0 ml	4 856	7 811	0,678	1 119	21 147
		4 848	7 621	0,668	1 107	
Rat 3: 1 ml 24 h cage washing	17,0 ml	7 041	11 247	0,674	1 636	28 076
		7 264	11 359	0,668	1 667	

Table 36 (continued)

Sample taken for counting	Volume of Specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Rat 4: 1 ml 24 h cage washing	20,0 ml	6 584	10 965	0,686	1 565	31 310
		6 466	11 096	0,694	1 566	
Rat 2: 0,1 ml 48 h urine	9,8 ml	3 209	6 354	0,731	836	77 469
		2 795	5 734	0,737	745	
Rat 3: 0,1 ml 48 h urine	7,0 ml	4 411	8 181	0,718	1 106	78 750
		4 386	8 569	0,728	1 144	
Rat 4: 0,1 ml 48 h urine	4,5 ml	4 405	6 182	0,632	945	60 255
		4 438	6 140	0,632	939	
Rat 2: 1 ml 48 h cage washing	17,0 ml	1 355	2 350	0,714	296	5 270
		1 456	2 470	0,692	324	
Rat 3: 1 ml 48 h cage washing	19,0 ml	2 578	4 024	0,668	570	11 163
		2 632	4 377	0,686	605	
Rat 4: 1 ml 48 h cage washing	20,0 ml	4 405	6 182	0,632	945	18 840
		4 438	6 140	0,632	939	

Table 36 (continued)

Sample taken for counting	Volume of specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Bray's Scintillant Background		111	331	0,820	40	
Rat 2: 0,1 ml 72 h urine	15,0 ml	953	1 911	0,733	221	32 700
		957	1 861	0,730	215	
Rat 3: 0,1 ml 72 h urine	6,5 ml	1 302	2 499	0,722	306	19 565
		1 261	2 437	0,726	296	
Rat 4: 0,1 ml 72 h urine	5,8 ml	1 504	2 869	0,706	366	19 401
		1 200	2 554	0,744	303	
Rat 2: 1 ml 72 h cage washing	23,0 ml	549	1 021	0,720	102	2 507
		605	1 131	0,714	116	
Rat 3: 1 ml 72 h cage washing	19,0 ml	1 081	1 707	0,670	215	4 104
		1 111	1 706	0,664	217	
Rat 4: 1 ml 72 h cage washing	19,0 ml	736	1 280	0,698	143	3 268
		1 024	1 623	0,670	202	

Table 36 (continued)

Sample taken for counting	Volume of specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Bray's Scintillant		132	302	0,764	39	
Background		115	300	0,788		
Rat 2: 0,1 ml 96 h urine	18,5 ml	241	469	0,726	26	5 180
		230	524	0,762	30	
Rat 3: 0,1 ml 96 h urine	6,5 ml	330	614	0,715	47	3 055
		325	618	0,720	47	
Rat 4: 0,1 ml 96 h urine	5,5 ml	290	557	0,724	38	2 778
		357	762	0,749	63	
Rat 2: 1 ml 96 h cage washing	32,0 ml	271	533	0,730	34	1 073
		267	521	0,727	33	
Rat 3: 1 ml 96 h cage washing	22,0 ml	626	1 002	0,673	110	2 464
		634	1 037	0,679	114	
Rat 4: 1 ml 96 h cage washing	23,0 ml	829	1 207	0,645	148	3 473
		839	1 266	0,657	154	

Table 36 (continued)

Sample taken for counting	Volume of specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Rat 2: 0,5 ml 24 h faeces homogenate	40,0 ml	2 281	2 487	0,542	420	33 880
		2 340	2 307	0,495	427	
Rat 3: 0,5 ml 24 h faeces homogenate	29,0 ml	3 737	3 839	0,512	711	42 601
		3 935	3 706	0,465	758	
Rat 4: 0,5 ml 24 h faeces homogenate	30,0 ml	1 023	1 265	0,589	176	8 760
		740	850	0,560	113	
Rat 2: 0,5 ml 48 h faeces homogenate	35,0 ml	3 360	3 606	0,531	640	44 940
		3 411	3 294	0,482	644	
Bray's Scintillant Background		140	355	0,798	45	
		143	348	0,776		
Rat 3: 0,5 ml 48 h faeces homogenate	28,0 ml	2 998	3 589	0,576	578	33 348
		3 157	3 808	0,578	613	
Rat 4: 0,5 ml 48 h faeces homogenate	36,0 ml	4 864	4 952	0,498	949	59 328
		3 614	4 256	0,572	699	

Table 36 (continued)

Sample taken for counting	Volume of specimen	Counts per 10 min		Efficiency E	Radioactivity in dpm of sample taken for counting	Radioactivity in dpm of specimen
		Channel A	Channel C			
Rat 2 0,5 ml 72 h faeces homogenate	38,0 ml	1 457	1 453	0,492	250	17 176
		1 195	1 387	0,560	202	
Rat 3 0,5 ml 72 h faeces homogenate	36,0 ml	1 522	1 615	0,523	264	19 296
		1 579	1 523	0,480	272	
Rat 4 0,5 ml 72 h faeces homogenate	32,0 ml	1 977	2 169	0,539	357	23 168
		2 041	2 134	0,518	367	
Rat 2 0,5 ml 96 h faeces homogenate	29,0 ml	806	1 043	0,605	127	7 424
		896	1 097	0,631	129	
Rat 3 0,5 ml 96 h faeces homogenate	26,0 ml	426	613	0,642	51	2 600
		390	646	0,682	49	
Rat 4 0,5 ml 96 h faeces homogenate	27,0 ml	510	715	0,636	67	4 509
		619	968	0,668	100	

3.2 Specimens of Urine and Faeces

The samples of urine and faeces prepared for liquid scintillation counting were counted for 10 min and the counts converted to radioactivity in dpm. Table 36 shows the radioactivity in each sample and the total radio= activity.

4. RATE OF EXCRETION IN URINE AND FAECES

The rate of excretion of phenylbutazone and its metabolites in the urine and faeces used in this study was calculated as a combined total from the measurement of the radioactivity of the samples recorded in Table 36. The radioactivity of the Metabowl cage washings was com= bined with that of the appropriate urine sample, as it was considered that this radioactivity emanated from urine which had dried on the surface of the glass Metabowl between collection times. The rate of excretion in urine and faeces over a period of 96 h is shown in Table 37.

Table 37 RATE OF EXCRETION OF DOSE

Specimen	RAT 1		RAT 2		RAT 3		RAT 4	
	dpm	% of dose	dpm	% of dose	dpm	% of dose	dpm	% of dose
24 h urine	2 818 627	60,2	372 900	57,7	347 620	55,0	300 132	48,6
48 h urine	796 395	19,8	77 469	12,2	78 750	13,1	60 255	11,6
72 h urine	173 580	4,3	32 700	5,2	19 565	3,3	19 401	3,3
96 h urine	-	-	5 180	1,0	3 055	0,8	2 778	0,4
TOTAL URINE		84,3		76,1		72,2		64,4
24 h faeces	27 986	0,6	33 880	5,0	42 601	6,2	8 760	1,3
48 h faeces	567 420	11,5	44 940	6,6	33 348	4,9	59 328	8,7
72 h faeces	28 208	0,6	17 176	2,5	19 296	2,8	23 168	3,4
96 h faeces	-	-	7 424	1,1	2 600	0,4	4 509	0,7
TOTAL FAECES		12,6		15,2		14,3		14,1
TOTAL RECOVERY		96,8		91,2		86,5		78,5

CHAPTER XII

IDENTIFICATION OF PHENYLBUTAZONE AND ITS METABOLITES IN URINE AND FAECES

1. COLLECTION OF SPECIMENS

A female Wistar rat, weighing 201 g, was dosed post-orally with 1 ml of a solution containing 10 $mg \cdot ml^{-1}$ of ^{14}C labelled phenylbutazone (s.a. 10,84 $KBq \cdot mg^{-1}$). The rat was placed in a Metabowl, which had its collection flasks suspended in methanol at $-20^{o}C$. The apparatus was examined regularly and frozen urine in the glass tube connected to the collection flask was carefully melted using a hot air blower. The urine (17,6 ml) and faeces (6,43 g) were collected after 48 h.

2. EXTRACTION OF NON-WATER SOLUBLE METABOLITES AND DECOMPOSITION PRODUCTS FROM URINE

After acidification of the urine with 20% sulphuric acid 8 ml of dichloromethane was added and the mixture shaken and centrifuged. The solvent layer was removed and extraction continued until the radioactivity in the urine layer remained steady. The solvent

fractions were combined, evaporated to dryness under nitrogen and dissolved in 1,0 ml of dichloromethane. The aqueous layer was retained for examination of water soluble metabolites.

3. EXAMINATION OF THE NON-AQUEOUS EXTRACT FOR METABOLITES AND DECOMPOSITION PRODUCTS

3.1 TLC Analysis

Two Merck 5 x 20 cm plates were prepared by applying to each plate small spots (10 µl) of the dichloromethane extract of urine and 1% oxyphenbutazone in dichloromethane on a base line approximately 1 cm apart. One plate was developed in solvent system XV and the other in solvent system III. They were then dried and sprayed. The relative R_f values and the colour reactions of the eluted spots were compared with those obtained for phenylbutazone and its possible metabolites (Table 38).

These results indicated that the following substances were present in the urine of rats treated with phenylbutazone:

Phenylbutazone

Oxyphenbutazone

γ-OH Derivative of phenylbutazone as the ring lactone isomer and the straight chain hydroxyl isomer

p-γ-Dihydroxy derivative of phenylbutazone

Table 38

THE RELATIVE R_f VALUES AND COLOUR REACTION OF ELUTED SPOTS OF URINE EXTRACT COMPARED WITH THOSE OF PHENYLBUTAZONE AND POSSIBLE METABOLITES

Solvent	Relative R_f value and colour reactions obtained from chromatograms of standard compounds			Relative R_f values and colour reactions obtained from chromatograms of urine extract from rat dosed with ^{14}C phenylbutazone		
		Relative R_f value	Colour Reaction	Spot	Relative R_f value	Colour Reaction
XV	Phenylbutazone	2,00	Green grey	1	2,10	Green grey
	γ-OH (Ring lactone form) derivative of of phenylbutazone	1,81	Violet	2	1,85	Violet
	4-OH Derivative of phenylbutazone	1,41	Green grey	3	1,45	Green grey
	Oxyphenbutazone	1,00	Yellow	4	1,00	Yellow
III	Oxyphenbutazone	1,00	Yellow	1	1,00	Yellow
	4-OH Derivative of oxyphenbutazone	0,80	Yellow	2	0,79	Yellow
	γ-OH (Straight chain hydroxyl form) derivative of phenylbutazone	0,55	Brown Orange	3	0,53	Brown Orange
	p-γ-DiOH derivative of phenylbutazone	0,31	Yellow	4	0,30	Yellow

4-OH Derivative of phenylbutazone

4-OH Derivative of oxyphenbutazone

In order to confirm these findings autoradiographic studies were undertaken.

3.2 Autoradiographic Analysis

Four light pencil marks, 1 cm apart were made on the base line of a Merck 5 x 20 cm TLC plate (Plate a) and a dichloromethane extract of urine (10 µl) was applied as a small spot on each of these marks. After drying 5 µl of a 1% solution in dichlorome= thane of one of the following was superimposed on each of these spots to act as a reference standard:

1 - phenylbutazone

2 - γ-hydroxy (ring lactone form) derivative of phenylbuta= zone

3 - 4-hydroxy derivative of phenylbutazone

4 - oxyphenbutazone

After drying the plate (Plate a) was developed in Solvent System XV.

A second Merck 5 x 20 cm TLC plate (Plate b) was similarly spotted with a dichloromethane extract of urine, and reference standards superimposed as follows:

5 - oxyphenbutazone

7 - γ-hydroxy (straight chain hydroxyl form) derivative of phenylbutazone

8 - p-γ-dihydroxy derivative of phenylbutazone

After drying, this plate (Plate b) was developed in solvent system III.

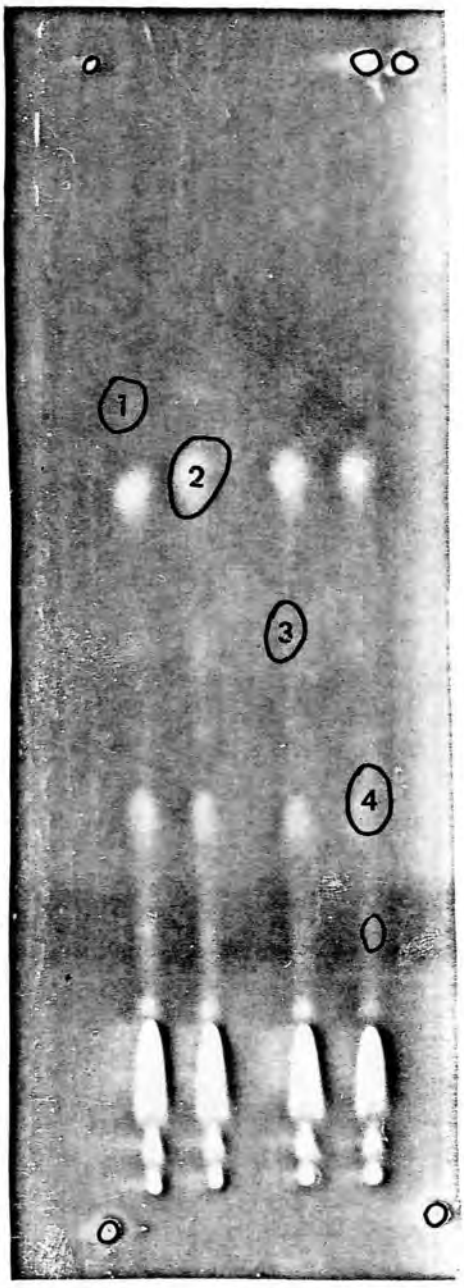
The plates were examined under ultraviolet light and the fluorescent spots lightly outlined with a soft pencil. Dots of radioactive ink were spotted at the corners of each plate to act as markers. The plates were then placed in the casette and, in the dark room, covered with Kodak Tri-X Pan film and allowed to stand for 28 days. The film was then removed and developed. A tracing of the outlined pencil spots (Figures 52 & 53) together with the ink spots on each plate was made with thin tracing paper. The tracing paper was then placed on top of the photographic film to act as a template, making sure that the ink spots were superimposed. Figures 52 & 53 show a photographic reproduction of these autoradiographs and Table 39 lists the metabolites identified in the dichloromethane extract of the urine, together with an indication of their concentrations as judged by the size and density of the spot on the photographic film.

These results (Table 39) confirmed the identification of phenylbutazone and its metabolites and decomposition products as indicated by thin layer chromatography.

As these results did not agree with those reported by Bakke it was considered important to repeat his chromatographic

Plate a

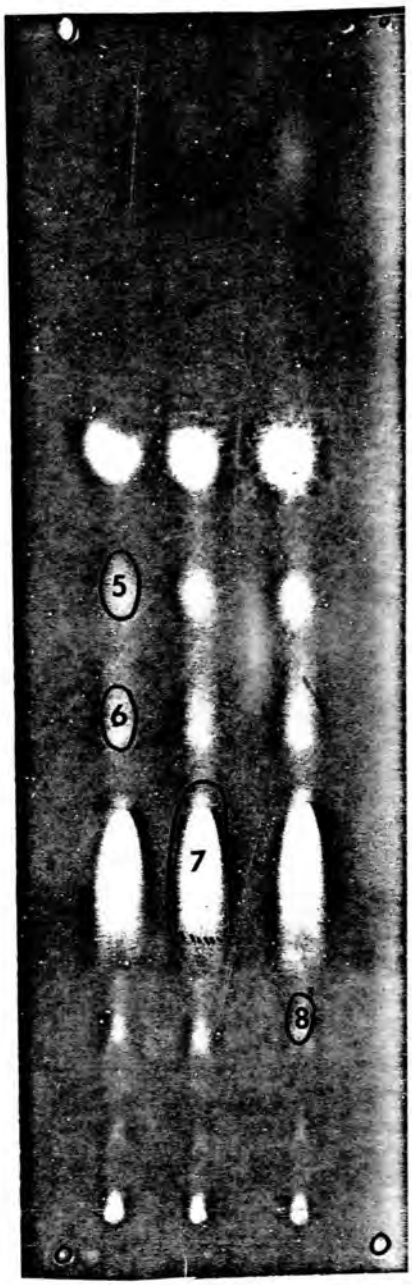
Solvent System XV



1. Phenylbutazone
2. γ-hydroxy derivative (ring lactone form) of Phenylbutazone
3. 4-hydroxy derivative of Phenylbutazone
4. Oxyphenbutazone

Fig. 52 Autoradiograph of Organic Solvent Extract of Urine from a Rat Treated with ^{14}C Phenylbutazone with Superimposed Tracings of Marker Spots of Official Standards

Plate b



Solvent System III

5. Oxyphenbutazone

6. 4-hydroxy derivative of Oxyphenbutazone

7. γ-hydroxy derivative (straight chain hydroxyl form) of Phenylbutazone

8. Para-γ-dihydroxy derivative of Phenylbutazone

Fig. 53 Autoradiograph of Organic Solvent Extract of Urine from a Rat Treated with ^{14}C Phenylbutazone, with Superimposed Tracings of Marker Spots of Official Standards

Table 39 PHENYLBUTAZONE AND ITS POSSIBLE METABOLITES IDENTIFIED ON THE AUTORADIOGRAPH REPRODUCED IN FIGURES 52 & 53

γ-OH (Straight chain hydroxyl form) derivative of phenylbutazone	+++++
γ-OH (Ring lactone form) derivative of phenylbutazone	++++
Oxyphenbutazone	+++
4-OH Derivative of oxyphenbutazone	+++
p-γ-diOH Derivative of phenylbutazone	++
Phenylbutazone	+
4-OH Derivative of phenylbutazone	+

The number of + indicate the approximate concentration shown by the size of the spots and the intensity of the radioactivity.

study using his solvent systems. The results of this experiment are shown in Table 40.

4. EXAMINATION OF URINE FRACTION AFTER EXTRACTION WITH DICHLOROMETHANE

The urine fraction remaining after extraction with dichloromethane was evaporated on a Rotavator at 60°C to 1,07 ml and 10 µl spotted on each of 2 pre-coated Merck 5 x 20 cm plates (Plates 1 and 2) (Spot i). The balance of the extract was incubated at 37°C for 3 h with 0,1 ml β-glucuronidase/arylsulfatase and 1,0 ml acetate buffer (pH 4,5). After hydrolysis the mixture was acidified to pH 1 with sulphuric

Table 40 RELATIVE R_f VALUES OF STANDARD SAMPLES OF PHENYLBUTAZONE AND ITS METABOLITES

Standard	Solvent Systems Used in This Study		Solvent Systems Used by Bakke	
	III	XV	Dichloromethane: acetone (9:1)	Cyclohexane: chloroform: methanol: acetic acid (60:30:5:5)
Phenylbutazone	-	2,15	2,10	2,55
γ-OH (Ring lactone form) derivative of phenylbutazone	-	1,79	1,40	1,05
4-OH Derivative of phenylbutazone	-	1,43	1,27	1,21
Oxyphenbutazone	1,0	1,0	1,0	1,0
4-OH Derivative of oxyphenbutazone	0,77	-	0,34	0,56
γ-OH (Straight chain hydroxyl form) derivative of phenylbutazone	0,56	-	0,24	0,19
p-γ-Dihydroxy derivative of phenylbutazone	0,29	-	0,00	0,19

acid, and 1,5 ml transferred to a nipple tube with 1,2 ml of dichloro= methane, mixed on the vortex shaker, centrifuged and 10 µl of the supernatant dichloromethane layer spotted on plates 1 and 2 (Spot ii). The same volume of the aqueous layer was also spotted on plates 1 and 2 (Spot iii). Plate 1 was used for TLC analysis and plate 2 for autoradiography.

4.1 Examination for Metabolites Hydrolysed by β-Glucuronidase/arylsulfatase

4.1.1 Chromatographic Examination

A third pre-coated Merck 5 x 20 cm plate (control plate) was spotted with standard oxyphenbutazone solution which con= tained the 4-hydroxy derivative as a decomposition product.

Plate 1 and the control plate were developed in solvent sys= tem III, sprayed and tracings of the spots on the plates were prepared. The R_f values are listed in Table 41. The chromatogram of plate 1 was identical to that of plate 2 shown in the tracing in Figure 54.

Compounds 1 and 2 had similar R_f values to oxyphenbutazone and its 4-hydroxy derivative respectively and exhibited the same colour reactions. Compound 3 could not be identified and did not have the characteristics of any of the recorded metabolites or decomposition products of phenylbutazone *(vide infra)*.

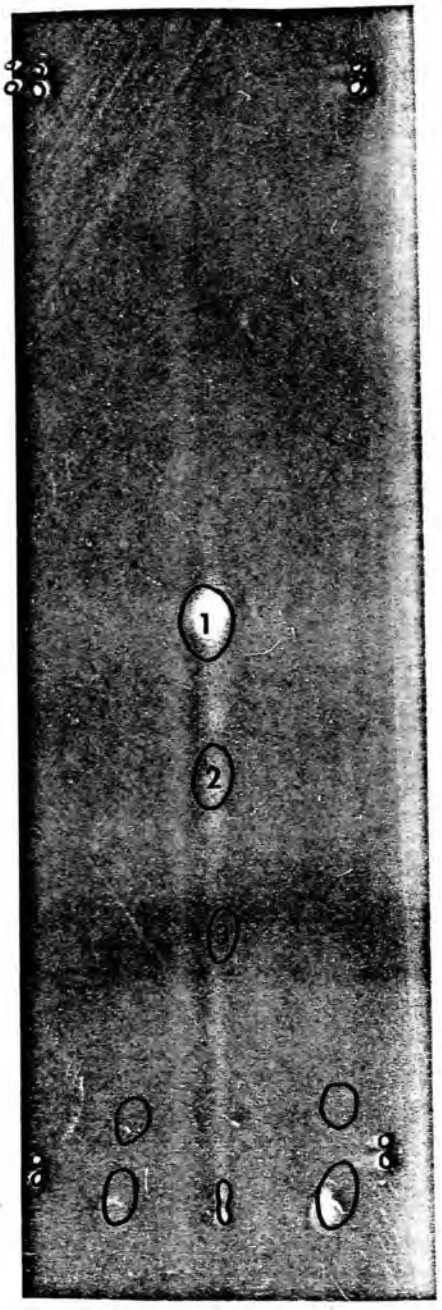
Table 41 R_f VALUES AND COLOUR REACTIONS OF OXYPHENBUTAZONE, ITS 4-HYDROXY DERIVATIVE AND THE COMPOUNDS PRESENT IN THE DICHLOROMETHANE EXTRACT OF THE HYDROLYSATE

Compound	R_f Value	Colour Reaction
Oxyphenbutazone	0,53	Yellow
4-Hydroxy derivative of oxyphenbutazone	0,40	Yellow
Compound 1	0,52	Yellow
Compound 2	0,39	Yellow
Compound 3	0,24	Orange

4.1.2 Autoradiographic Examination

Plate 2, spotted with the aqueous layer after the extraction of the urine with dichloromethane, the dichloromethane extract of the hydrolysate of this aqueous layer and the aqueous layer remaining after the extraction of this hydrolysate, was developed with solvent system III, and the plate dried. A tracing of the chromatogram visualised under ultraviolet light and a photographic negative of the autoradiograph were prepared as previously described. The tracing superimposed on the print of the autoradiograph is shown in Figure 54. The R_f value of the spots were the same as those given in Table 41.

Plate 2



i. Aqueous extract of urine from rat dosed with ^{14}C Phenylbutazone

ii. Dichloromethane extract of the hydrolysate of the aqueous extract i

iii. Aqueous layer from ii

Fig. 54 Autoradiograph of Aqueous Layer of Urine After Solvent Extraction Before and After Enzyme Hydrolysis With Superimposed Tracings of Fluorescence Visible Under Ultraviolet Light

These results confirmed that conjugates of oxyphenbutazone were metabolites of phenylbutazone. The presence of the 4-hydroxy derivative of oxyphenbutazone in the dichlorome= thane extract of the hydrolysate indicated that the conjugate of this derivative could also be a metabolite or it could be a decomposition product. However, radioactivity was not detected in the position of Compound 3 (Table 41) indica= ting that it was not a metabolite or decomposition product of phenylbutazone. On the autoradiograph there was evidence of radioactivity at a spot whose R_f value was similar to the second decomposition product of oxyphenbutazone. There was also evidence of considerable radioactivity at the origin of the plate where the aqueous residue had been spotted.

4.2 Examination for Metabolites not Hydrolysed by β-Glucuronidase/arylsulfatase

A 20 x 20 cm TLC plate was prepared from a slurry of Silica gel PF_{254} in 10% sodium thiosulphate solution, activated at 100°C for 15 min and spotted with 15 µl of the aqueous layer remaining after extraction of the hydrolysate with dichloromethane. The plate was subjected to two dimensional development according to the method of Dieterle (22) using solvent system XXVII, examined under ultraviolet light, the fluorescent spots marked and a tracing prepared (Figure 56).

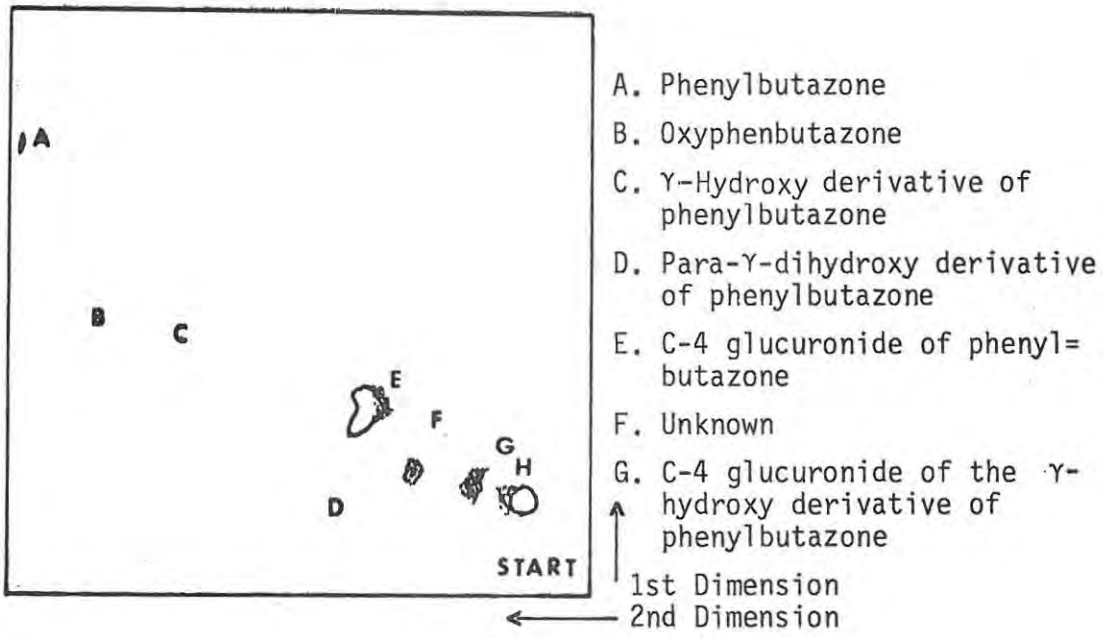


Fig. 55 Copy of an Autoradiograph of 2 Dimensional TLC of Radio= active Substances in Human Urine

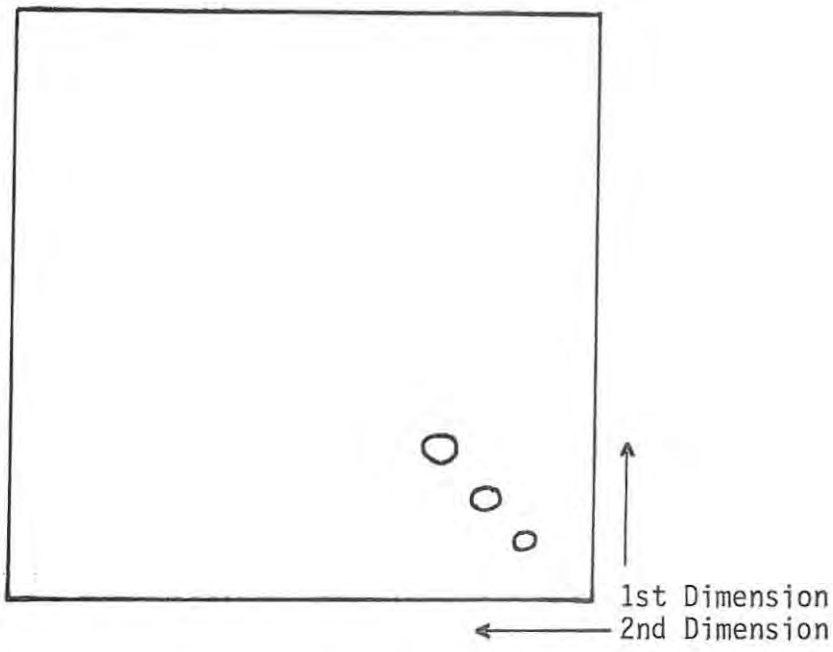


Fig. 56 Chromatogram of Non-hydrolysable Water Soluble Metabolites of Phenylbutazone in Urine of Rats Treated with Phenyl= butazone

A comparison of the relative positions of the spots present on this chromatogram with the position of the spots present in the chromatogram reported by Dieterle (22) (Figures 55 and 56) which were prepared by the same procedure suggests that these metabo= lites could be, either the C-4 glucuronide of the γ-hydroxy derivative of phenylbutazone or some unidentified polar metabolite. As Dieterle did not report the R_f values obtained from his chromatogram, and as standard compounds were not available, no definite conclusions could be drawn from this chromatographic procedure.

5. EXAMINATION OF METABOLITES PRESENT IN THE FAECES OF RATS DOSED WITH ^{14}C LABELLED PHENYLBUTAZONE

5.1 Extraction and Enzymatic Hydrolysis of Faeces

A portion of faeces collected from a rat dosed with 10 mg ^{14}C labelled phenylbutazone was homogenised in water, the specimen filtered, the filtrate (24,7 ml) freeze dried and reconstituted in 3 ml acetate buffer (pH 4,5). A portion of this solution (2 ml) was incubated at 37° C for 3 h with 0,2 ml acetate buffer (pH 4,5) and β-glucuronidase/sulphatase. Following incubation the hydrolysate was shaken with 1,2 ml of dichloromethane and centrifuged. An aliquot of the dichloromethane layer (0,9 ml) was evaporated to dryness, the residue dissolved in 0,2 ml of dichloromethane and 2 samples of 50 µl each were added to 10 ml quantities of Aquagel for

Table 42 RADIOACTIVITY IN AQUEOUS AND DICHLOROMETHANE FRACTIONS OF THE HYDROLYSATE OF THE EXTRACT OF FAECES FROM A RAT DOSED WITH ^{14}C PHENYLBUTAZONE

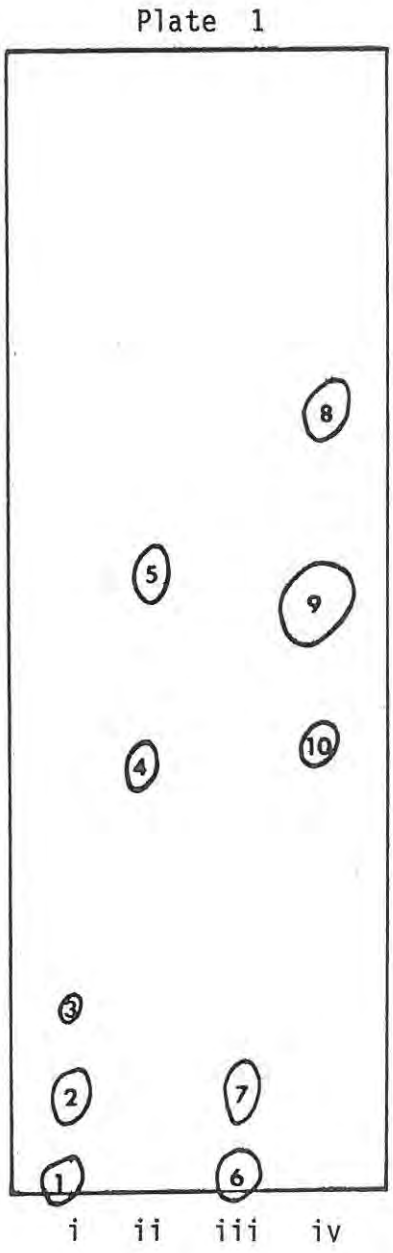
	Radioactivity (in dpm) in vial	Volume in vial	Volume of Sample	Radioactivity (in dpm) in Sample	Total Volume	Total Radioactivity (in dpm)
Dichloromethane fraction	4 250 3 618	50 µl	200 µl ex 0,9 ml	18 000 14 472	1,2 ml	20 928
Aqueous fraction	13 550 14 595	0,5 ml	4 ml	108 400 116 760	4,0 ml	112 576

measurement of radioactivity. Also 2 samples each of 0,5 ml of the aqueous fraction of the hydrolysate remaining after extraction with dichloromethane were added to 10 ml quantities of Aquagel for measurement of radioactivity. From the measurement of the radioactivity of these samples the proportion of the radioactivity emanating from the hydrolysable and non-hydrolysable metabolites present in the faeces was calculated (Table 42).

From the radioactive measurements given in Table 42, it can be seen that the major proportion of the metabolites in the faeces consists of compounds which are not hydrolysed under these conditions by β-glucuronidase/arylsulfatase enzyme and therefore are not likely to be O-glucuronides or O-sulphates.

5.2 Chromatographic Examination of the Organic and Aqueous Fractions of Extracts of Faeces Following Enzymic Hydrolysis

Two 5 x 20 cm TLC plates (1 and 2) were spotted with the reconstituted filtrate of the faeces homogenate (spot i), the dichloromethane extract of the hydrolysate (spot ii), the aqueous fraction remaining after this extraction (spot iii) and a solution of standard oxyphenbutazone containing an impurity of its 4-hydroxy derivative (spot iv). They were developed in solvent system III and dried. A tracing of the fluorescence visible on plate 1 was prepared (Figure 57) before the plate was sprayed. This plate was used to estimate the distribution of the radio-



i - reconstituted filtrate of faeces homogenate

ii - dichloromethane extract of hydrolysate of (i)

iii - aqueous residue from (ii)

iv - solution of standard oxyphenbutazone

8 - non-fluorescent control for l.s.c.

9 - oxyphenbutazone

10 - 4-OH derivative of oxyphenbutazone

Colour reactions

4
5
9
10 — Yellow

Fig. 57 Chromatogram of Fractions of Homogenate of Faeces from a Rat Dosed with ^{14}C Phenylbutazone

activity in the spots. Plate 2 was subjected to autoradio= graphy.

As can be seen from Figure 57 the dichloromethane extract of the hydrolysate contained substances with R_f values and colour reactions similar to oxyphenbutazone and its 4-hydroxy derivative. In order to ascertain whether the spots on the plate actually represented metabolites in the faeces, spots 1, 2, 3, 6 and 7 were scraped off into 5 ml water and spots 4, 5 and 8 into 5 ml chloroform:methanol (9:1). Spot 8 was used as a control. The test tubes were shaken, centrifuged and 3,5 ml of the filtrate evaporated to dryness in an l.s.c. vial. Aquagel (10 ml) was added to each vial and the radioactivity measured. The results are recorded in Table 43.

Table 43 DPM OF ELUATES FROM SPOTS SHOWN IN THE CHROMATOGRAM REPRODUCED IN FIGURE 57

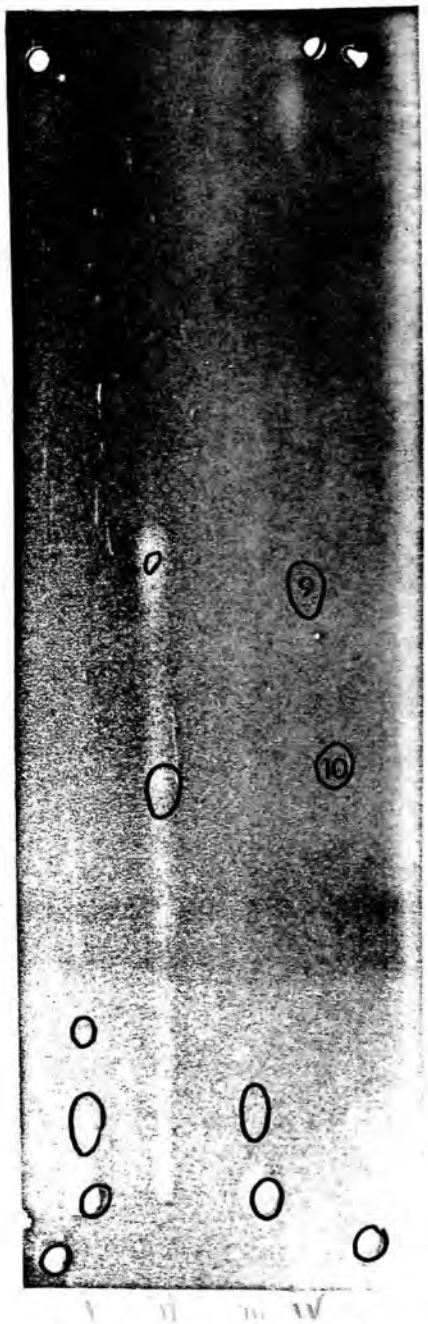
Spot	Radioactivity
1	634
2	392
3	387
4	779
5	1 062
6	509
7	290
8	386

These results showed that spot 5, corresponding to oxyphenbutazone and spot 4, corresponding to the 4-hydroxy derivative of oxyphen= butazone, both contained radioactivity and were, therefore, meta= bolites of the ^{14}C phenylbutazone given to the rat. Radioactivity was also apparent at the origin of the spot of the aqueous sample.

5.3 Autoradiographic Examination of the Organic and Aqueous Fractions of Faeces Following Enzymic Hydrolysis

Plate 2 (*vide* 5.2) was examined under ultraviolet light and a tracing prepared of the fluorescent spots, before it was placed in the cassette with the Kodak Tri-X Pan film for 28 days. At the end of this period it was developed. The autoradiograph was compared with the superimposed tracing, as illustrated in Figure 58, and showed a clearly defined spot with an R_f value similar to oxyphenbutazone and a weaker spot with an R_f value similar to the 4-hydroxy derivative of oxyphenbutazone. There was also considerable radioactivity at the origin of the aqueous fractions.

Plate 2



i. Reconstituted filtrate of faeces homogenate

ii. Dichloromethane extract of hydrolysate of i

iii. Aqueous residue from ii

iv. Solution of standard Oxyphenbutazone

9. Oxyphenbutazone

10. 4-hydroxy derivative of Oxyphenbutazone

Fig. 58 Autoradiograph of Fractions of Homogenate of Faeces Before and After Enzymatic Hydrolysis, With Superimposed Tracings of Fluorescence Visible Under Ultraviolet Light

6. CONCLUSION

The results of these experiments indicated that after dosing rats with phenylbutazone the following compounds were present in their urine:

- Phenylbutazone
- Oxyphenbutazone
- γ-Hydroxy (straight chain hydroxyl form) derivative of phenylbutazone
- γ-Hydroxy (ring lactone form) derivative of phenylbutazone
- Para-γ-dihydroxy derivative of phenylbutazone
- 4-Hydroxy derivative of oxyphenbutazone
- Conjugates of oxyphenbutazone (either O-glucuronide or O-sulphate or a mixture of O-glucuronide and O-sulphate)
- Conjugates of the 4-hydroxy derivative of oxyphenbutazone (either O-glucuronide or O-sulphate or a mixture of O-glucuronide and O-sulphate) were possibly present
- C-4 glucuronide of the γ-hydroxy derivative of phenylbutazone was possibly present but its identity could not be confirmed
- Other non-hydrolysable, water soluble compounds were isolated but could not be identified.

In the faeces the following compounds were found:

- Conjugates of oxyphenbutazone (either O-glucuronide or O-sulphate or a mixture of O-glucuronide and O-sulphate)
- Conjugates of the 4-hydroxy derivative of oxyphenbutazone (either O-glucuronide or O-sulphate or a mixture of O-glucuronide and

O-sulphate) were possibly present.

- Non-hydrolysable, water soluble compounds were isolated, but not identified.

CHAPTER XIII

QUANTITATIVE STUDIES

1. COLLECTION AND TREATMENT OF URINE FOR QUANTITATION OF METABOLITES

Quantitative studies were carried out on water insoluble metabolites in the 0 - 48 h urine of rats dosed with ^{14}C phenylbutazone, as it was found from the excretion studies that urinary excretion was almost complete within this period. A female Wistar rat was dosed post-orally with 10 mg ^{14}C labelled phenylbutazone (s.a. 10,84 $KBq \cdot mg^{-1}$). The urine was transferred to a vacuum flask containing 2 ml of sodium thiosulphate solution ($m_{Na_2S_2O_3} = 1\ mol \cdot kg^{-1}$) and 5 ml of a methanolic solution containing 8 $mg \cdot ml^{-1}$ phenylbutazone, 6 $mg \cdot ml^{-1}$ oxyphenbu= tazone and 4 $mg \cdot ml^{-1}$ of the γ-hydroxy derivative of phenylbutazone was added. The methanol was removed by bubbling nitrogen through the mixture overnight, care being taken to ensure that the tube from the nitrogen remained deep in the solution throughout this period. After acidification with 20% sulphuric acid, the urine mixture was shaken with 14 ml of dichloromethane and then centrifuged. The solvent layer was removed and extraction continued until the radioactivity of the urine layer remained constant. The solvent fractions were

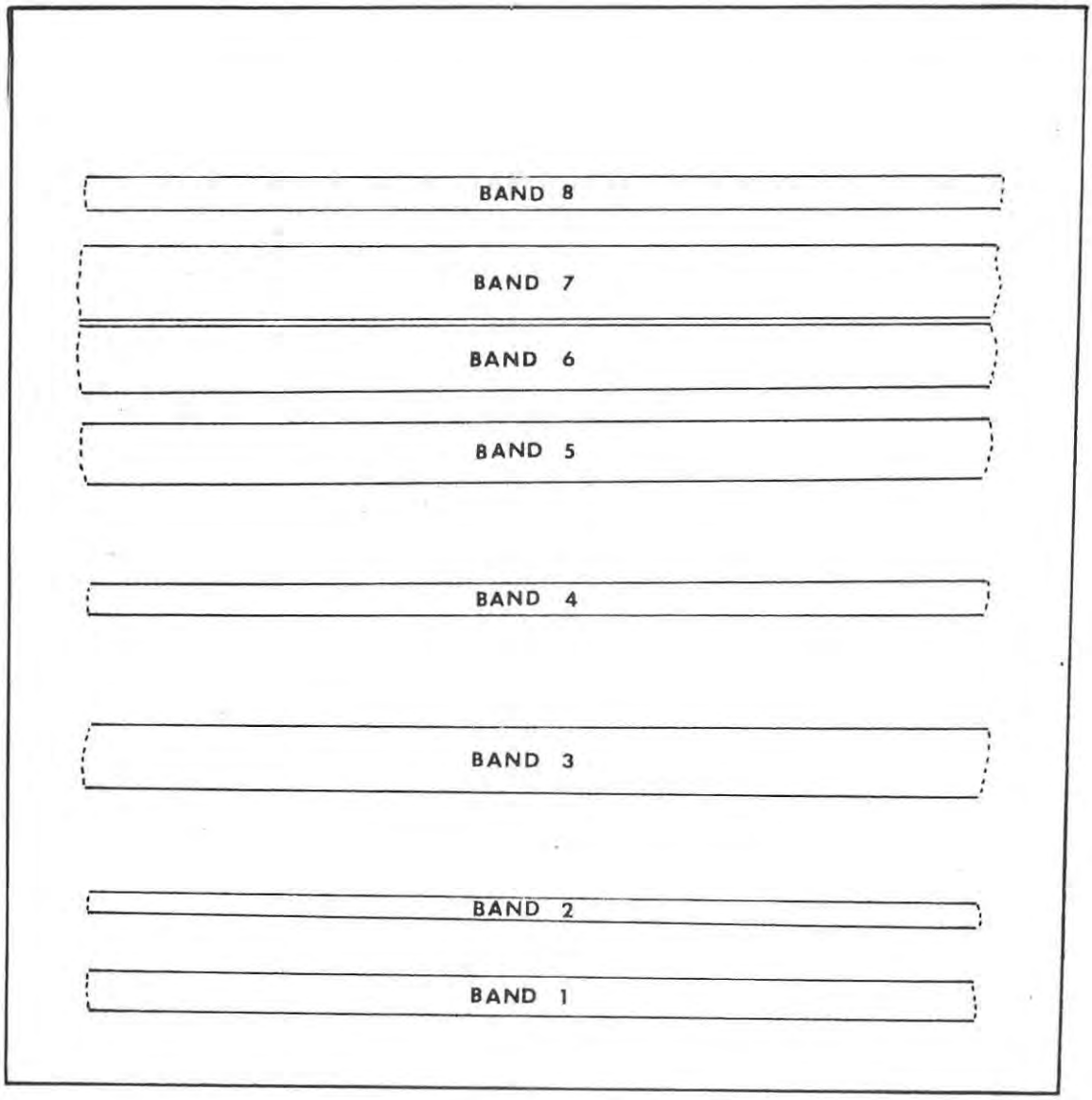
combined, evaporated to dryness under nitrogen and re-dissolved in 1,1 ml dichloromethane.

2. TLC OF DICHLOROMETHANE EXTRACT

One ml of the dichloromethane extract was applied, by means of a chromatocharger, to an activated preparative plate containing 10% sodium thiosulphate. The plate was dried, developed in solvent system XXVI, and examined under ultraviolet light. Eight bands were visible, as shown in Figure 59, and their positions marked.

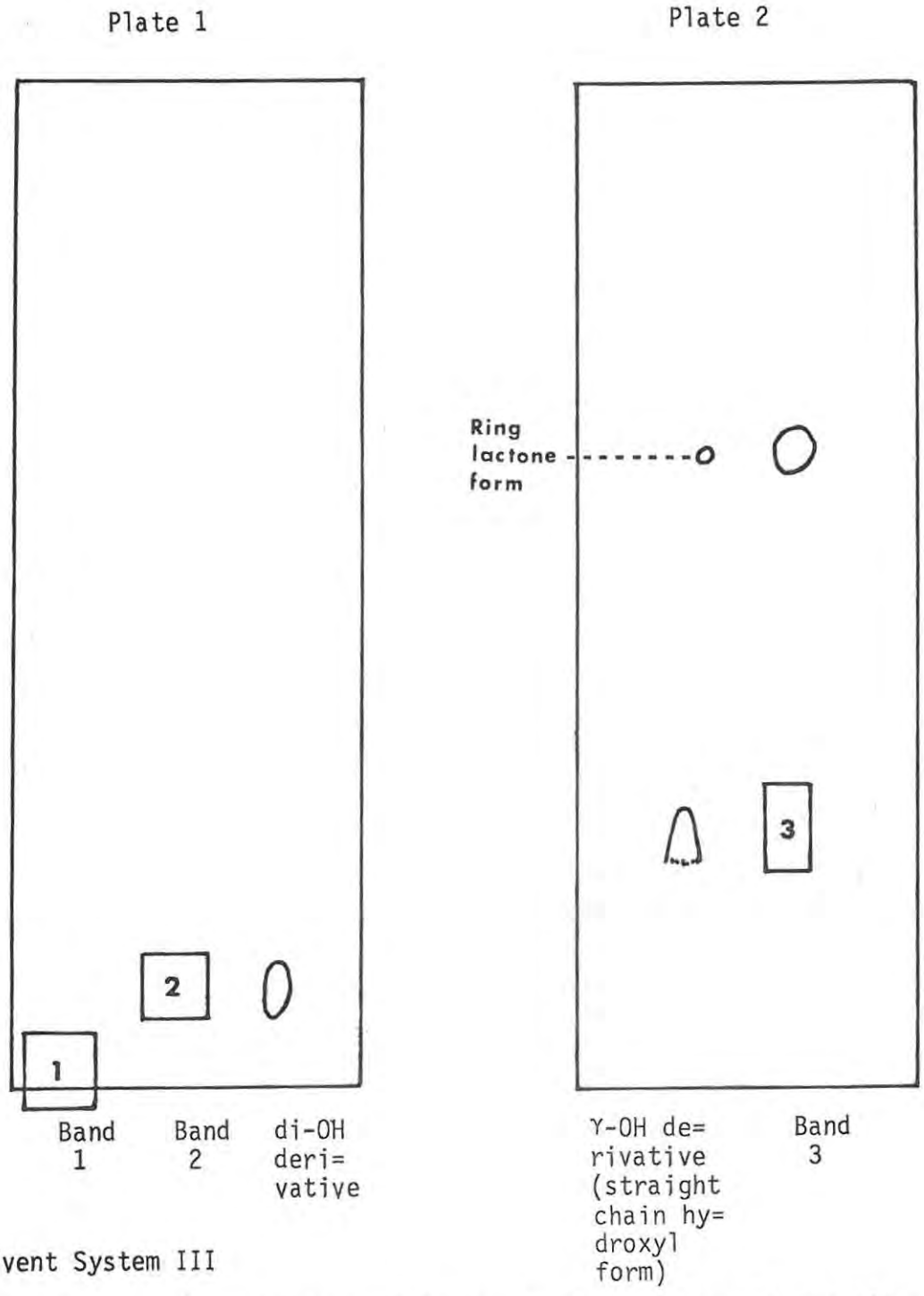
3. ISOLATION OF COMPOUNDS IN BANDS ON THE PREPARATIVE PLATE

The silica gel in each of the bands was scraped off into separate test tubes, eluted with 4 ml of chloroform:methanol (9:1) by mixing on a vortex shaker and centrifuging at 3 000 r.p.m. for 10 min. An aliquot of the filtrate (3,5 ml) was evaporated to dryness and re-dissolved in 0,2 ml of dichloromethane. Five pre-coated Merck 5 x 20 cm plates were spotted with 10 µl of each of these solutions according to the plan shown in Figures 60 and 61. Plates 1, 2 and 3 were developed in solvent system III and plates 4 and 5 were de=veloped in solvent system XV. They were examined under ultraviolet light and the fluorescent spots lightly outlined with a soft lead pencil as illustrated in Figures 60 and 61.



Solvent System XXVI

Fig. 59 Chromatogram of Preparative Plate of Dichloromethane Extract of Urine from Rat Dosed with ^{14}C Phenylbutazone

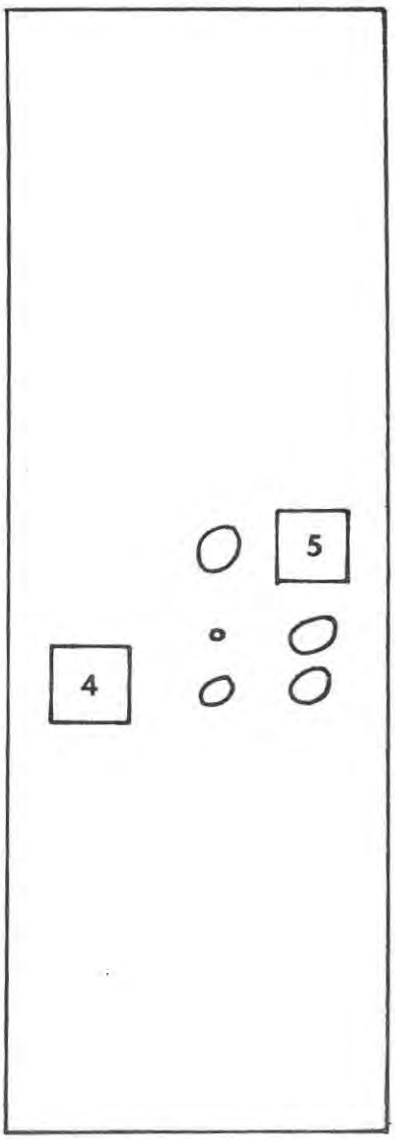


Solvent System III

Fig. 60 Chromatogram of Compounds Eluted from Preparative Plate Bands 1 - 5 Compared to Standards

Fig. 60 (continued)

Plate 3



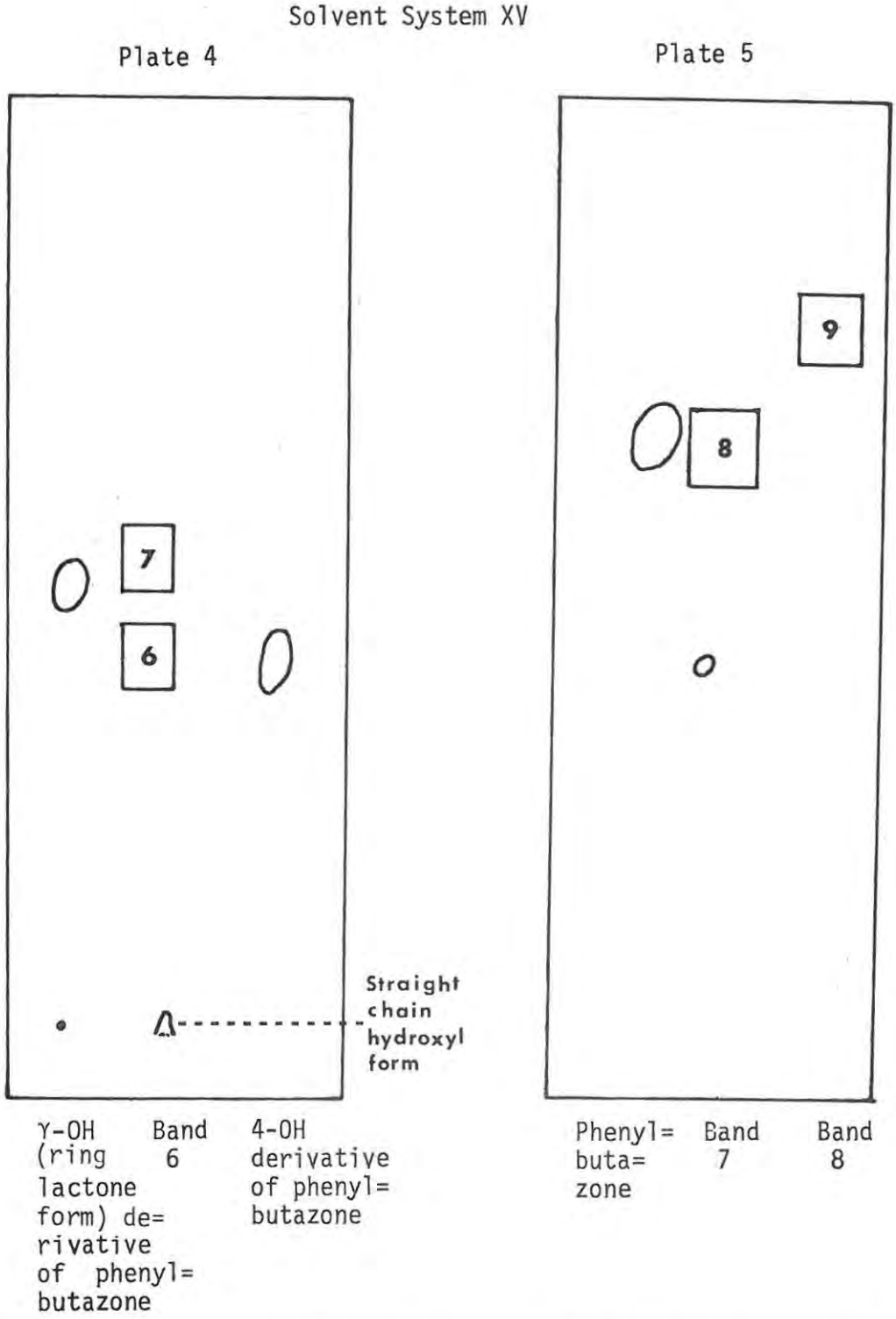


Fig. 61 Chromatogram of Compounds Eluted from Preparative Plate Bands 6 - 8 Compared to Standards

As demonstrated in Figures 59, 60 and 61 good separation had been achieved on the preparative plate except in Band 6 which contained a mixture of the γ-OH (ring lactone form) and 4-OH derivatives of phenylbutazone and these were separated by development in solvent system XV. Figures 60 and 61 also show the equilibration that took place between the ring lactone and straight chain hydroxyl forms of the γ-OH derivative of phenylbutazone. For this reason it was found feasible to quantitate the two forms collectively and not as two different entities.

4. QUANTITATIVE DETERMINATION OF METABOLITES FROM TLC PLATES

4.1 Quantitative Determination of Phenylbutazone, Its γ-hydroxy Derivative and Oxyphenbutazone

The silica gel coating on the plate in each of the areas shown as outlined numbered spots 8, 5, 7 and 3 in Figures 60 and 61 was scraped off into separate test tubes, eluted with 4 ml chloroform:methanol (9:1) by mixing on the vortex shaker and centrifuging at 3 000 r.p.m. for 10 min. An aliquot (3 ml) of each of the filtrates was pipetted into spectrophotometric cuvettes and the absorbance measured at 270 nm. The contents of the cuvettes were transferred to l.s.c. vials together with the solvent used to wash out the cuvettes. Great care was taken to ensure that no radioactivity was lost during these transfers.

The solutions were evaporated to dryness, 10 ml of Aquagel added to each of the vials and the radioactivity of each measured. Table 44 records the radioactivity of spots 8, 5, 7, and 3, representing phenylbutazone, oxyphenbutazone and the two molecular forms of the γ-hydroxy derivative of phenylbutazone respectively, together with their concentrations as calculated from spectrophotometric data.

Table 44 CONCENTRATION AND RADIOACTIVITY OF ELUTED COMPOUNDS ON CHROMATOGRAMS

Compound	Spot Number	Absorbance (270 nm)	$\mu g \cdot ml^{-1}$	$\mu g \cdot 3\ ml^{-1}$	Radioactivity (in dpm) in 3 ml
Phenylbutazone	8	47,5	37	111	205
Oxyphenbutazone	5	51,5	37	111	929
γ-Hydroxy derivative of phenylbutazone (ring lactone form)	7	9,0	3	9	1 045
γ-Hydroxy derivative of phenylbutazone (straight chain hydroxyl form)	3	32,0	16	48	4 535

The weights of the radioactive phenylbutazone, oxyphenbutazone and the γ-hydroxy derivative of phenylbutazone in the 48 h urine of the rat dosed with ^{14}C phenylbutazone were calculated using the formula in Figure 62.

$$W = W_r\left(\frac{S_{PB}}{S_r} - 1\right)$$

W Weight of unlabelled compound

W_r Weight of labelled compound

S_{PB} Specific activity of labelled phenylbutazone ($10{,}84\ KBq \cdot mg^{-1}$)

S_r Specific activity of mixed labelled and unlabelled compound

Fig. 62 Formula used to Calculate Weights of Radioactive Compounds by Inverse Isotope Dilution Analysis

4.2 Quantitative Determination of Minor Metabolites and Decomposition Products of Phenylbutazone

The silica gel in the areas shown as numbered spots 1, 2, 4, 6 and 9 in Figures 60 and 61 was extracted as described above, and 3 ml of each of the filtrates evaporated to dryness in l.s.c. vials, re-dissolved in 10 ml of Aquagel and their radioactivity measured. The weights of these minor metabolites and decompo= sition products in 48 h urine were calculated by comparing their radioactivity with that of the major metabolite, the γ-hydroxy derivative of phenylbutazone.

The weights of metabolites and decomposition products found in

the urine of rats dosed with ^{14}C labelled phenylbutazone together with the percentage of the administered dose are shown in Table 45.

Table 45 % DOSE RECOVERED OF COMPOUNDS ISOLATED FROM THE URINE OF A RAT TREATED WITH ^{14}C LABELLED PHENYLBUTAZONE

	Radioactivity in dpm in 3 ml eluate	Weight in mg in 48h urine mg	Percentage of dose in 48h urine
Phenylbutazone	205	0,115	1,15
Oxyphenbutazone	929	0,394	3,94
γ-OH Derivative of phenylbutazone	5 570	3,570	35,70
p-γ-DiOH derivative of phenylbutazone	34	0,022	0,22
4-OH Derivative of phenylbutazone	76	0,049	0,49
4-OH Derivative of oxyphen= butazone	296	0,18	1,8
Compounds remaining at origin	35	0,022	0,22
TOTAL		4,352	43,52

DISCUSSION

CHAPTER XIV

DISCUSSION

Although many derivatives of Pyrazolidine-3,5-dione have been extensively investigated as therapeutic agents (5,8,11,14,15, 24, 31, 44, 48, 52, 54, 55, 56), surprisingly little comprehensive work has been done on their metabolism in various animal species and man (11, 12, 13, 19, 26, 43). This was particularly true of phenylbutazone, one of the drugs of choice for the treatment of rheumatoid arthritis, as in early 1974, when this study was instituted, no fresh information had been published since the work of Burns (13) in 1955 and Perel (43) in 1961. For this reason it was decided to investigate the pattern of metabolism of this drug in the rat. During the course of this work, Bakke (4) published a paper on the metabolism of phenylbutazone in the rat, and Dieterle (22), McGilveray (35) and Aarbakke (1) published their findings on the metabolism of phenylbutazone in humans, so it has been possible to compare their results with those in this paper.

Early workers (13) in the field of metabolism of phenylbutazone did not have the advantages of the radio-chemical techniques now available; nevertheless, they did establish the formation of two metabolites, the p-hydroxy and the γ-hydroxy derivatives of phenylbutazone,

and their methods of quantitation for these metabolites gave results reasonably close to those of Dieterle (Table 46).

Table 46 COMPARATIVE FIGURES OF % RECOVERY OF THE P-HYDROXY AND γ-HYDROXY DERIVATIVES OF PHENYLBUTAZONE IN THE URINE OF MAN DOSED WITH PHENYLBUTAZONE

Metabolite of Phenylbutazone	% of Administered Drug	
	Burns (1955)	Dieterle (1976)
p-Hydroxy derivative of phenylbutazone (oxyphenbutazone)	3	1,6
γ-Hydroxy derivative of phenylbutazone	5	5,6

Perel (43) reported the presence of the glucuronide of oxyphenbutazone, but was not able to isolate the C-4 glucuronides identified by Dieterle (22).

A problem that arose during this investigation was the tendency to decomposition of phenylbutazone and oxyphenbutazone during the course of experimentation. However, it was proved that phenylbutazone did not decompose detectably at room temperature in alkaline solution in 3 days, and decomposition was minimal during an incubation period of 24 h in urine. The R_f value of the product of decomposition during incubation differed from that of the 4-hydroxy derivative which formed during chromatography on TLC plates within 15 min of spotting.

Bakke (4) overcame problems of oxidation by performing all tests in an atmosphere of nitrogen, but in this study it was found to be an unnecessary precaution provided plates were developed immediately after spotting. In preparative chromatography, using double development where compounds were in contact with the slightly acidic coating of TLC plates for a prolonged period of time, it was found that some decomposition occurred. The addition of sodium thiosulphate to the slurry used for the preparation of these plates reduced the decomposition to a minimum by acting as an anti-oxidant (22). On the other hand, although oxyphenbutazone did not decompose in alkaline solution, two products of decomposition, one of which was its 4-hydroxy derivative, were produced during incubation at 37^{o} C. These results indicated that the 4-hydroxy derivative of phenylbutazone found in the urine was probably a product of metabolism, whereas the 4-hydroxy derivative of oxyphenbutazone could be either an artifact formed by decomposition of the oxyphenbutazone in the bladder or a metabolite. It was found that oxidation of metabolites during the collection of urine and faeces could be reduced to a minimum by immersing the collection vessels of the Metabowl in methanol at -20^{o} C.

An early report (43) to the effect that rats dosed with ^{14}C phenylbutazone excreted >50% of their radioactivity in 0 - 24 h urine was confirmed by other workers (Bakke (4), Mathew (32)) and in this study. Table 47 shows that there was close agreement in the % dose recovered in the 0 - 48 h urine in these later studies. However, the table

also shows considerable disparity in the percentage dose recovered from the faeces by these workers.

As can be seen in Table 47, Bakke (4) recovered a much higher % of the dose from the faeces. This higher result is surprising since his results were estimated in the dichloroethane extract of homogenised faeces and no account was taken of possible water soluble metabolites present in the aqueous fraction of the faeces homogenate remaining after extraction. Furthermore, one would expect faecal excretion to consist mainly of water soluble conjugates of relatively high molecular mass. He also reported that excretion in faeces collected over a period of 24 h after dosing was almost twice as high as that in faeces collected from 24 to 48 h (22,9% and 11,8% respectively), whereas these studies showed the opposite to be the case; 3,3% in faeces collected up to 24 h after dosing and 7,9% in 24 - 48 h faeces. The higher value of excretion in the 24 - 48 h faeces found in these studies suggests that enterohepatic circulation is significant, a fact substantiated by studies in man (22) illustrated in Figure 27.

Bakke carried out excretion studies in male Wistar rats whereas the results by other workers shown in Table 47 are given for female Wistar rats. This could account for these discrepancies as it is recognised that there are sex differences in metabolism in rats (9).

Unfortunately the facilities of gas chromatography and GC/MS were not

Table 47 COMPARISON OF RESULTS OF EXCRETION STUDIES ON RATS TREATED WITH ^{14}C PHENYLBUTAZONE

Worker	Specimen	Percentage of Dose				
		0 - 24 h	24 - 48 h	48 - 72 h	72 - 96 h	TOTAL
Mathew (3 rats)	Urine	50,2 (46,8-52,5)	12,93 (12,8-13,1)	3,07 (1,9-2,8)	1,8 (1,0-3,0)	68
	Faeces	3,7 (3,6- 4,2)	4,33 (3,7- 5,4)	0,96 (0,8-1,2)	0,43 (0,3-0,5)	9,42
Bakke (5 rats)	Urine	54,3 (44,0-67,4)	6,5 (3,2- 8,9)			60.8
	Faeces	22,9 (16,3-34,0)	11,8 (6,8-17,2)			34,7
This study (4 rats)	Urine	53,53 (48,6-60,2)	14,18(11,6-19,8)	4,03 (3,3-5,2)	0,7 (0,4-1,0)	72,44
	Faeces	3,28 (0,6- 6,2)	7,92 (4,9-11,5)	2,33 (0,6-3,4)	0,73(0,4-1,1)	14,26

available in this laboratory. However, the metabolites in urine were identified chromatographically on TLC plates and their R_f values and colour reactions compared with those of standard reference compounds. Two solvent systems were found to be necessary to separate the metabolites satisfactorily. These results were further confirmed using autoradiography.

Table 48 RELATIVE R_f VALUES AND COLOUR REACTIONS OF STANDARD COMPOUNDS

Solvent	Standard Compounds	Relative R_f Value	Colour Reaction
XV	Phenylbutazone	2,00	Green grey
	γ-Hydroxy (ring lactone form) derivative of phenylbutazone	1,81	Violet
	4-Hydroxy derivative of phenylbutazone	1,41	Green grey
	Oxyphenbutazone	1,00	Yellow
III	Oxyphenbutazone	1,00	Yellow
	4-Hydroxy derivative of oxyphenbutazone	0,80	Yellow
	γ-Hydroxy (straight chain hydroxyl form) derivative of phenylbutazone	0,55	Brown Orange
	p-γ-Dihydroxy derivative of phenylbutazone	0,31	Yellow

Table 48 shows that the major metabolite in urine, the γ-hydroxy derivative of phenylbutazone, is present in both its molecular forms. The product of microsomal metabolism is probably the straight chain hydroxyl form, part of which lactonises to form an equilibrium mixture. In this study it has been shown that this equilibrium mixture can be separated chromatographically into the more polar straight chain hydroxyl form and the less polar ring lactone form. Autoradiography showed conclusively that both forms were present in the urine of rats treated with ^{14}C phenylbutazone. Although it is well known that γ-hydroxy compounds exist in two molecular forms (20) no cognisance was taken by other workers of the fact that they have different polarities and therefore different R_f values on TLC plates.

The aqueous fraction remaining after extraction of urine with dichloromethane was found to contain hydrolysable and non-hydrolysable compounds. The water insoluble compounds obtained from the enzymatic hydrolysate of this aqueous fraction were oxyphenbutazone and its 4-hydroxy derivative. The latter was most probably an artifact formed during hydrolysis. As the enzyme system used for hydrolysis was a mixture of beta-glucuronidase and aryl-sulphatase, it was not possible to determine whether the conjugates were beta-glucuronides or sulphates. By comparison with the findings of other workers they were probably mainly glucuronides (4). Unhydrolysable water soluble compounds remaining in the aqueous fraction were detected but could not be identified.

Faeces was also found to contain metabolites and decomposition products. Enzymatic hydrolysis of faeces homogenates showed that the greater percentage of these products could not be hydrolysed by beta-glucuronidase/aryl-sulphatase. The hydrolysable products, however, were found to be either the beta-glucuronide or aryl-sulphate of oxyphenbutazone and its 4-hydroxy derivative.

For quantitation of metabolites, the method of multiple inverse isotope dilution analysis reported by Dieterle (22) was used. This proved to be a useful and accurate method of quantitating the major metabolites, using a combination of spectrophotometry and liquid scintillation spectrometry. The minor metabolites and decomposition products were quantitated by comparing their radioactivity with that of the γ-hydroxy derivative of phenylbutazone, the major metabolite.

In the method of Bakke (4), phenylbutazone and oxyphenbutazone were quantitated by crystallisation and liquid scintillation spectrometry, and the amounts of other metabolites and decomposition products were estimated by measurement of the radioactivity of the individual compounds and comparing these with the total radioactivity in the urine (Table 49). Table 49 shows that there was close agreement in the total percentage of metabolites recovered by Bakke (4) and in this study.

However, there is a significant difference between the amounts of

Table 49 QUANTITATION OF COMPOUNDS PRESENT IN THE URINE OF RATS TREATED WITH ^{14}C LABELLED PHENYLBUTAZONE, EXPRESSED AS PERCENTAGE RECOVERY OF DOSE

Compound	Bakke	This Study
Phenylbutazone	0,4	1,15
Oxyphenbutazone	1,6	3,94
γ-Hydroxy derivative of phenylbutazone	25,4	35,70
p-γ-Dihydroxy derivative of phenylbutazone	10,2	0,22
4-Hydroxy derivative of phenylbutazone	-	0,49
4-Hydroxy derivative of oxyphenbutazone	1,8	1,80
	39,4	43.32

individual metabolites reported by him and those found in the present study. He reported the p-γ-dihydroxy derivative of phenylbutazone as a major urinary metabolite (10,2%) whereas these studies indicate that it is only a minor metabolite (0,22%) (Table 49). Repetition of the chromatographic procedure reported by Bakke for the identifi= cation of metabolites showed that the substance he reported as a major metabolite and named the p-γ-dihydroxy derivative of phenylbu= tazone was in all probability the straight chain hydroxyl isomer of the γ-hydroxy derivative of phenylbutazone (Table 50).

It can be seen from Table 50 that there would be practical difficulties in identifying these two compounds using the solvent systems he has

Table 50 RELATIVE R_f VALUES OF STANDARD COMPOUNDS USING BAKKE'S SOLVENT SYSTEMS (4)

Standard Compound	Dichloro=ethane: acetone (9:1)	Cyclohexane: chloroform: methanol: acetic acid (60:30:5:5)
Phenylbutazone	2,10	2,55
γ-Hydroxy (ring lactone form) derivative of phenylbutazone	1,40	1,00
4-Hydroxy derivative of phenylbutazone	1,27	1,21
Oxyphenbutazone	1,00	1,00
4-Hydroxy derivative of oxyphenbutazone	0,34	0,56
γ-Hydroxy (straight chain hydroxyl form) derivative of phenylbutazone	0,24	0,19
p-γ-Dihydroxy derivative of phenylbutazone	0,00	0,19

quoted. Further evidence in support of this view is that the com=bined % of the γ-hydroxy and p-γ-dihydroxy derivatives of phenylbuta=zone reported by him is almost identical to the % of the combined isomers of the γ-hydroxy derivatives found in this study (Table 49). Furthermore, it would seem unlikely that microsomal hydroxylation would follow a pathway leading to the dihydroxylated derivative as a major metabolite when an easily excreted monohydroxylated metabolite can be formed by a single metabolic process.

There is a disparity between the % dose recovered in 48 h urine (69,5%) found in the excretion studies and the % dose recovered by the method outlined for quantitation of metabolites (43,5%). This difference can be explained by the fact that only water insoluble metabolites were quantitated by the latter method.

In his studies on the metabolism of phenylbutazone in man, Dieterle (22) reported the major metabolites in the urine to be C-4-glucuronides of phenylbutazone and its γ-hydroxy derivative. They were responsible for about 40% and 12% respectively of the urinary radioactivity. In contrast, the present study of the metabolism in rats has shown that the non-conjugated compounds were the major urinary metabolites. This is a good example of species variation in drug metabolism and confirms that, though the study of drug metabolism in animals is important, the results of these studies cannot be taken as representative of the metabolic pattern in man.

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