Journal of Chromatography, 576 (1992) 103–109
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6260

Separation and identification of the 4-hydroxyantipyrine sulphoconjugate

J. Moreau, C. Palette, P. Cordonnier, E. Naline, C. Advenier and M. Pays*

Département de Biochimie, de Pharmacologie et de Toxicologie, Centre Hospitalier de Versailles, 177 Rue de Versailles, F 78157 Le Chesnay Cedex (France)

(First received March 5th, 1991; revised manuscript received December 27th, 1991)

ABSTRACT

In a previous study we observed, during separation of total antipyrine metabolites by high-performance liquid chromatography and after enzymatic hydrolysis, an unidentified peak corresponding to an ionic compound with pyrazolinone features. In the present study, this compound was identified as the 4-hydroxyantipyrine sulphoconjugate, and its structure was definitively confirmed by gas chromatographic–mass spectrometric analysis and by the use of pure synthetic substance. We also demonstrated the inhibitory effect of sodium metabisulphite, a necessary preservative of urinary samples, on hydrolysis of this conjugate in the presence of sulphatases from *Helix pomatia* or *Aerobacter aerogenes*. This inhibitory effect makes it impossible to perform a global assay of antipyrine metabolites after enzymatic or chemical hydrolysis and confirms the valuc of direct assay of the 4-hydroxyantipyrine sulphoconjugate.

INTRODUCTION

Studies of antipyrine biotransformation are of considerable interest in the evaluation of oxidative metabolism. However, all pharmacokinetic studies devoted to the urinary excretion of antipyrine and its metabolites show discrepancies between the amount excreted and the dose administered. Using analytical methods [hydrolysis, extraction, high-performance liquid chromatography (HPLC)], the total recovery ranges from 60 to 70% for the major metabolites, norantipyrine (nor-AP), 3-hydroxymethylantipyrine (3-CH_2OH-AP), 4-hydroxyantipyrine (4-OH-AP), 4-4'-dihydroxyantipyrine [4-4'-$(OH)_2$-AP] and 3-carboxyantipyrine (3-COOH-AP) [1–3]. In contrast, after administration of antipyrinc labelled with ^{14}C on the pyrazolone ring, the radioactivity excreted over 120 h reaches at least 95% of that in the dose administered [4,5].

There are several possible reasons for this discrepancy: (1) there is no method capable of measuring all phase I and phase II antipyrine metabolites; (2) free and deconjugated nor-AP and 4-OH-AP are particularly unstable, owing to their volatility, their oxidation (usually prevented by sodium metabisulphite [6,7]) and their tautomerism [8]; (3) conjugates are very sensitive to cleavage in both acidic and enzymatic media [8–11], so that there is no single procedure that can provide total hydrolysis and good conservation of the primary metabolites obtained; (4) the existence of as yet unknown metabolites cannot be excluded [12].

In a previous study [3], we described the conditions for simultaneous determination by HPLC of the three major antipyrine metabolites after enzymatic hydrolysis (3-CH_2OH-AP, 4-OH-AP, nor-AP) and of the 3-COOH-AP metabolite. Our attention was drawn to an unassigned liquid chromatographic peak which was attributed to an antipyrine derivative. The aim of the present

0378-4347/92/$05.00

study was to identify this compound, which was found by gas chromatography–mass spectrometry (GC–MS) and the use of pure synthetic substance to be the sulphoconjugate of 4-OH-AP (4-OH-S-AP) and to measure it. We also wanted to demonstrate that assaying this derivative after enzymatic hydrolysis was impossible because of the inhibitory effect of sodium metabisulphite on enzyme activity. However, adding this antioxygen to urinary samples is necessary to maintain the stability of the free forms of nor-AP and 4-OH-AP.

EXPERIMENTAL

Chemicals and reagents

Glacial acetic acid, orthophosphoric acid (84%), sodium acetate trihydrate, Tris and diethyl ether were purchased from Prolabo (Paris, France) and were of Normapur quality. Acetonitrile, methylene chloride and methanol were purchased from Merck (Darmstadt, Germany) and were of LiChrosolv quality. Sodium metabisulphite, *n*-decylamine, lyophilised type H-S powder of β-glucuronidase plus sulphatase from *Helix pomatia* (ref. G-1512) and sulphatase solution from *Aerobacter aerogenes* type H-VI (ref. S-1629) were purchased from Sigma (St.Louis, MO, USA). Tetrabutyl ammonium hydrogensulphate (TBA) was purchased from Fluka (Mulhouse, France). N-Methyl-N'-nitroguanidine and diazomethane generator were purchased from Chrompack (Les Ulis, France). Antipyrine, nor-AP, 4-OH-AP, 3-CH_2OH-AP and 3-COOH-AP were as previously described [3]. 4-Hydroxyantipyrine sulphate was a gift from Dr. F. X. Jarreau (Centre de Recherche Delalande, Rueil Malmaison, France).

Stock solutions

For extraction, 0.25 *M*, acetate buffer, pH 5.2, 0.10 m*M* *n*-decylamine solution, pH 3.8, in 0.10 *M* acetate and 2 m*M* *n*-decylamine solution, pH 3.5, in 0.09 *M* acetic acid were used [3].

For enzymatic hydrolysis, 0.5 *M* phosphate or acetate buffer, pH 6, or 0.2 *M* Tris buffer, pH 7.4 (20°C), was used. For inhibition experiments, acetate and Tris buffers were loaded with 2% (w/v) sodium metabisulphite (final pH 4.8 and 5.6, respectively).

Apparatus and chromatographic conditions

HPLC. The HPLC system was as previously described [3]. The mobile phase was the same as for the determination of 3-COOH-AP: 5% (v/v) aqueous solution of glacial acetic acid containing 2 m*M* (400 μl/l) *n*-decylamine (pH 3.5) and acetonitrile (77:23, v/v). It was run at a rate of 1 ml/min. The column effluent was monitored at a wavelength of 242 nm.

GC and GC–MS. Gas chromatograms were obtained with a Varian 3300 gas chromatograph equipped with methylphenyl siloxane capillary columns. The initial pressure of the carrier gas (helium) was 0.4 atm. For GC alone a J&W macrobore DB5 column was used (15 m × 0.53 mm I.D.; film thickness 1.5 μm). The temperature programme was 80°C for 1 min, rising to 150°C at 20°C/min, then to 220°C at 4°C/min. Direct injection of 1 μl was performed at 250°C, and the thermionic detector was set at 280°C. The chromatograms were recorded on a Servotrace recorder (SEFRAM) running at 5 mm/min.

For GC–MS, the chromatograph was coupled to a Finnigan Mat ion trap detector (ITD) consisting of an IBM PC/AT with version 4.00 software. The column was a wide-bore CP Sil8CB from Chrompack (25 m × 0.32 mm I.D.; film thickness 0.12 μm) connected to an on-column injector. Temperature programmes were 50°C for 1 min, rising to 250°C at 200°C/min for the injector, and 50°C for 1 min rising to 150°C at 20°C/min, then gradually heated to 250°C at 5°C/min for the column oven. The column exit was connected to the ITD heated transfer line (250°C).

Mass spectra were obtained after electron impact (EI mass spectra, 70 eV) or, in order to confirm molecular masses, after chemical ionization (CI) with methane as reagent gas, in the full-scan mode (1 s per scan).

Derivatization procedure for GC–MS. When necessary, dry extracts were subjected to cold methylation with 1 ml of freshly prepared diazo-

methane in diethyl ether in the presence of 0.1 ml of methanol. The tubes were allowed to stand for 30 min at room temperature and then evaporated to dryness. Methylated samples were redissolved in 0.1 ml of methanol, and injected.

Extraction procedure

Exhaustive extraction of the unknown compound was obtained with an ion-pairing solution consisting of *n*-decylamine (2 m*M*) or TBA (5 m*M*) in acetic acid solution (pH 3.5–4). Equal volumes (1 ml) of untreated urine and ion-pairing solution were gently mixed together with dichloromethane. After decanting and evaporation of the organic layers, the dry extracts were redissolved in 100 μl of mobile phase and injected into the HPLC system for collection. The eluted fraction of interest was saturated with sodium chloride powder, and the unknown compound was directly extracted with dichloromethane.

To reduce *n*-decylamine contamination, the dry extract was dissolved in 500 μl of aqueous orthophosphoric acid solution (2 m*M*) saturated with sodium chloride (pH 0.5), and re-extracted with dichloromethane. This extract was ready for GC–MS.

Hydrolysis of the unknown metabolite

A concentrated dry extract of the purified unknown compound was dissolved in 2 ml of acetate buffer pH 6. For the following assays, 100-μl aliquots were used.

Acid hydrolysis. Each aliquot was incubated for 1 h at 80°C in the presence of 1 ml of 5 *M* hydrochloric acid, and then chromatographed.

Enzymatic hydrolysis. Optimum or inhibitory conditions for sulphatase activity were chosen. A 1-ml aliquot of acetate or phosphate buffer pH 6 was added to two aliquots before incubation (3 h, 37°C) with enzymatic preparation (β-glucuronidase plus sulphatase from *Helix pomatia*, 150 μl). Then, proteins were precipitated with 1 ml of methanol, and the tubes centrifuged at 2000 *g* for 10 min. A 50-μl aliquot of the supernatant was directly injected for the determination of 4-OH-AP formed and/or residual unknown compound.

Effect of urine and sodium metabisulphite on the activity of the sulphatase. This effect was tested by dissolving the unknown compound in 2 ml of control urine without sodium metabisulphite. Aliquots of 100 μl were analysed with: (a) β-glucuronidase plus sulphatase from *Helix pomatia* (50 μl, 2875 mU/min) in acetate buffer (1 ml) pH 6 or 4.7, or freshly prepared 2% metabisulphite acetate buffer (final pH 4.8); (b) Sulphatase from *Aerobacter aerogenes* (20 μl, 290 mU/min) in Tris buffer (1 ml) pH 7.4 or 5.6, or freshly prepared 2% metabisulphite Tris buffer (final pH 5.6).

After 2 h at 37°C, the aliquots were treated as above.

Human experiment

Urinary 4-OH-S-AP excretion was measured over 48 h in five healthy volunteers (25.6 ± 2.1 years; 66 ± 3.4 kg) after intravenous infusion of 1 g of antipyrine. These volunteers were included in a pharmacokinetic study approved by the local ethics committee. This as yet unpublished study concerns the effect of an enzymatic inhibitor of antipyrine metabolism. Urine was recovered on sodium metabisulphite.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profiles of the metabolites studied, before and after methylation, obtained with direct GC on a methylphenyl siloxane macrobore capillary column. Note that only 3-COOH-AP needs methylation to be eluted and that derivatization increases the height of the peaks corresponding to the other polar metabolites. This satisfactory behaviour during GC makes it possible to study the antipyrine derivatives by MS after coupling to GC. The broadening of the peaks observed on Fig. 2 is due to adsorption of the polar compounds on the coupling glass interface in GC–MS.

The EI mass spectra obtained (Table I) show that most compounds have an abundant molecular peak. Only 4-OH-AP undergoes drastic fragmentation, leading to the basal peak at *m/z* 56 which is not very specific; the molecular peak at *m/z* 204 accounts for only 10%; chemical ionization is usually necessary to confirm the identity of this molecule.

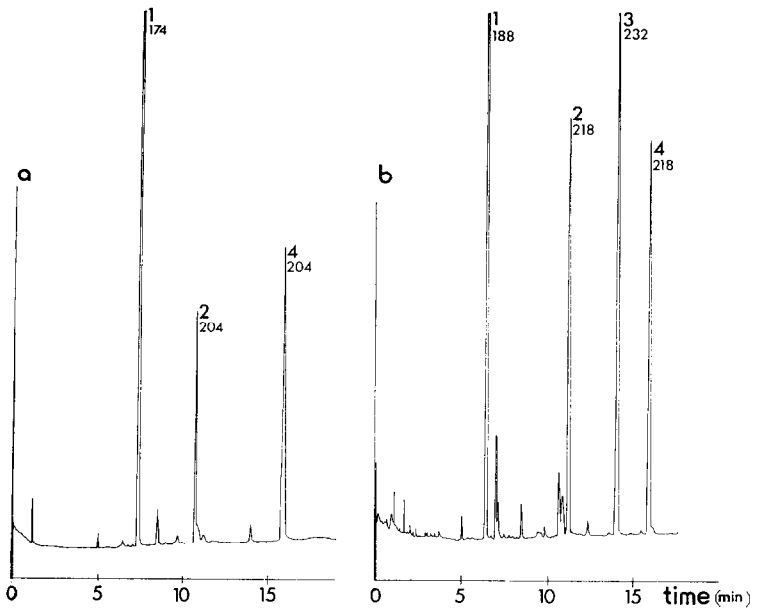

Fig. 1. Gas chromatographic separation of antipyrine metabolites (a) untreated and (b) after methylation with diazomethane. The molecular masses of crude and derivatized molecules are indicated. Peaks: 1 = nor-AP; 2 = 4-OH-AP; 3 = 3-COOH-AP; 4 = 3-CH_2OH-AP. Conditions: column, 15 m × 0.53 mm I.D. megabore DB-5 (film thickness 1.5 μm); temperature, 80°C for 1 min, 20°C/min to 150°C, then 4°C/min to 220°C; carrier gas, helium 0.4 atm; direct injection (1 μl) at 250°C; thermionic detector; $8 \cdot 10^{-11}$ a.u.f.s.; chart speed, 5 mm/min.

The physicochemical properties of the unknown compound also are very helpful in determining its identity. Its behaviour during HPLC (elution in reversed-phase ion pairing) (Fig. 3) and its extractability, shown in Table II, show that it is an anionic compound, even at pH 1. Its extraction recovery by dichloromethane is only 50% at pH 0.5, but it is 89% at pH 4 in the presence of 2 m*M* *n*-decylamine as the cationic counterion. These characteristics were used for exhaustive extraction of the compound in the presence of *n*-decylamine, and for its purification at pH 5 after liquid chromatographic collection.

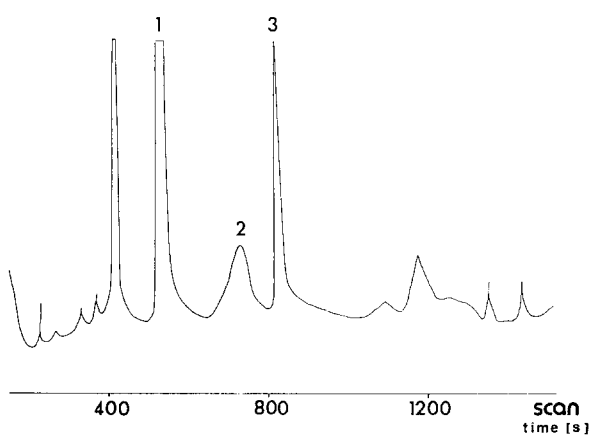

Fig. 2. GC–ITD reconstructed ion chromatogram of urinary extract containing an unknown compound, after methylation. Peaks: 1 = methylated hippuric acid; 2 = unknown compound with characteristic *m/z* 56 and 204 as 4-OH-AP; 3 = methylated 3-COOH-AP (*m/z* 232). Data acquisition in the full-scan mode.

Another important property of the unknown compound is its non-methylation under the conditions used for standards. Injection into the GC–MS system of urinary extract rich in the unknown metabolite shows an important peak of hippuric acid, an endogenous urinary metabolite and a compound with a mass spectrum identical to that of 4-OH-AP. After methylation the unchanged mass spectrum of 4-OH-AP persists, whereas the methylated hippuric acid separates and 3-COOH-AP appears (Fig. 2). This result is confirmed by analysis of the isolated product (Fig. 4), which shows a single peak of low intensity (relative abundance 2.3%) with the parent ion at *m/z* 204. Chemical ionization clearly gives the expected mass MH^+ 205.

TABLE I

MOST IMPORTANT FRAGMENTS IN THE ELECTRON IMPACT MASS SPECTRA FRAGMENTATION OF ANTIPYRINE DERIVATIVES

The *m/z* ratios in the first column correspond to the parent peak. The figures in parentheses are the percentage relative abundance.

Compound	*m/z*						
Antipyrine	188 (100)	96 (69)	77 (41)	56 (32)	105 (19)	173 (6)	
Nor-AP	174 (100)	77 (59)	91 (54)	105 (34)			
Nor-AP (Me)	188 (100)	77 (97)	173 (35)	91 (23)	105 (18)	106 (17)	
Nor-AP $(Me)_2$	202 (100)	77 (98)	173 (20)	187 (20)	118 (19)	91 (14)	
3-COOH-AP (Me)	232 (100)	77 (55)	82 (48)	105 (35)	112 (27)	91 (14)	145 (12)
3-CH_2OH-AP	204 (100)	56 (85)	93 (65)	77 (52)	112 (30)	82 (30)	
3-CH_2OH-AP (Me)	218 (100)	120 (90)	188 (40)	105 (40)	93 (35)	126 (12)	
3-CH_2OH-AP $(Me)_2$	232 (100)	130 (52)	77 (50)	173 (50)	201 (45)	98 (45)	91 (13)
4-OH-AP	204 (8)	56 (100)	77 (9)	176 (5)			
4-OH-AP (Me)	218 (33)	56 (100)	91 (6)	77 (5)	203 (3)	175 (2)	

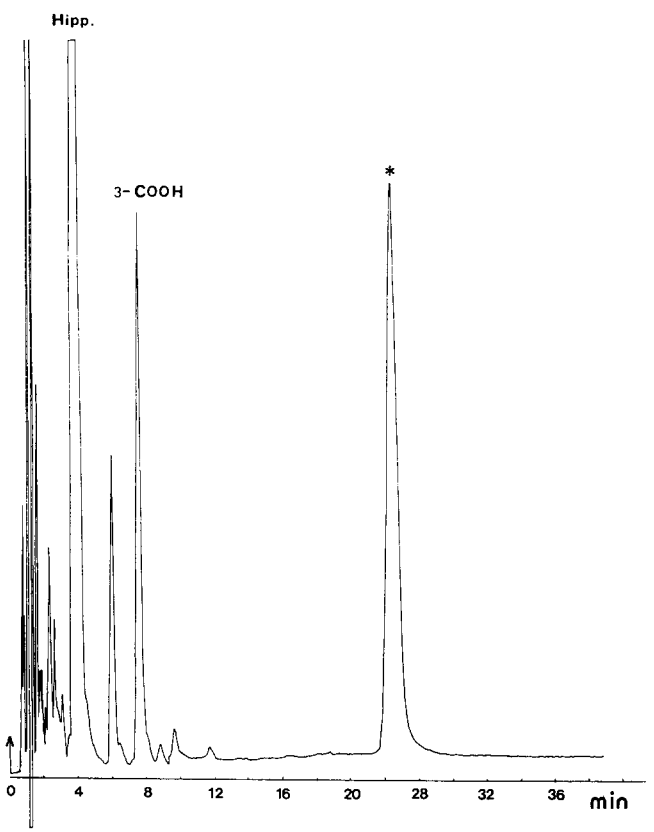

Fig. 3. Typical liquid chromatogram of urinary extract containing unknown compound (*), 3-COOH-AP and hippuric acid (Hipp). Conditions as described in the Experimental section except that the flow-rate was set at 1.3 ml/min.

TABLE II

RECOVERY OF THE UNKNOWN COMPOUND FROM HUMAN URINE INTO THE ORGANIC LAYER, UNDER VARIOUS CONDITIONS

A 100-μl aliquot of urine containing the unknown compound was mixed with 1 ml of the extraction medium. The solutions were saturated with sodium chloride powder, then the pH was brought to the value indicated with phosphoric acid or sodium hydroxide. After extraction with 8 ml of dichloromethane, the organic layers were separated and evaporated. The residues were redissolved in mobile phase and injected. The remaining aqueous phases were also chromatographed and the relative recoveries evaluated by comparing corrected peak areas.

Medium	pH	Percentage of unknown compound in organic layer
0.5 *M* Acetate	1	17
Sodium chloride	1	39
(0.9%, w/v)	0.5	50
	6	0
0.10 m*M* *n*-Decylamine	1	39
in 0.10 *M* acetate	6	5
2 m*M* *n*-Decylamine	1	81
in 0.09 *M* acetate	4	89
	6	30

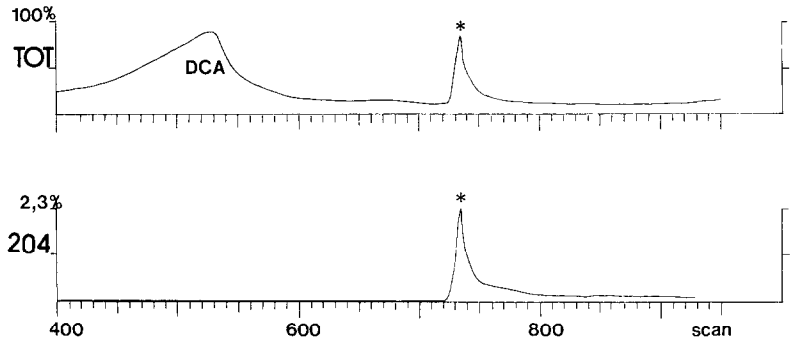

Fig. 4. Chromatograms of the isolated unknown compound (*). GC–ITD shows little contamination by *n*-decylamine. The chromatogram reconstructed at *m/z* 204 displays a single peak attributed to 4-OH-AP.

All these results point to a 4-OH-AP compound with a hydroxyl function that is blocked by an anionic group; this blockage is labile under the high temperature of GC. Similar behaviour has been described by Walle *et al.* [13] for propranolol sulphate. The hypothesis that this metabolite is 4-OH-S-AP was confirmed by specific enzymatic hydrolysis, using a sulphatase. Under specific sulphatase activity conditions (acetate buffer pH 6), an appreciable amount of 4-OH-AP was produced, unambiguously identified by its HPLC retention time, while in the presence of phosphate ions, which are known to inhibit sulphatase activity, no 4-OH-AP was formed. Cleavage of the sulphonic group from the conjugated metabolite can also be obtained chemically by heating the compound in the presence of 5 *M* hydrochloric acid (1 h at 80°C).

In our previous study using reversed-phase HPLC [3] after enzymatic hydrolysis, chromatograms of urinary extracts revealed an unknown peak now identified as 4-OH-S-AP (Fig. 3), but why this sulphoconjugate was not hydrolysed when the mixture of enzymes we used was perfectly active on the isolated product, even at a less than optimum pH, remains to be elucidated. Since urinary compounds or sodium metabisulphite [13,14] have been held responsible for such inhibition, we tried to evaluate it by the experiment reported in Table III. No inhibitory effect of urine could be demonstrated, but the sodium metabisulphite utilized to prevent oxidation of the primary metabolites, notably 4-OH-AP, totally inhibited, at the concentration used, the sulphatase activity.

There is, therefore, a fundamental incompatibility between hydrolysis of sulphoconjugates and preservation of the primary metabolites

TABLE III

INHIBITION OF SULPHATASE ACTIVITY

Untreated urine was spiked with purified 4-OH-S-AP, then aliquots of 100 µl were diluted with 1 ml of acetate buffer pH 6 (1) or pH 4.8 (2) or (3) containing 2% (w/v) sodium metabisulphite (MBS), final pH 4.8 or with 1 ml of Tris buffer pH 7.1 (4) (37°C) or pH 5.3 (5) or (6) containing 2% (w/v) sodium metabisulphite, final pH 5.6. A 50-µl aliquot of glucuronidase from *Helix pomatia* (2875 mU in sulphatase) was added to tubes 1–3; a 20-µl aliquot of sulphatase from *Aerobacter aerogenes* (290 mU) was added to tubes 4–6. After incubation (2 h at 37°C) samples were diluted with 1 ml of methanol and centrifuged. Aliquots of 20 µl were injected into the HPLC system for 4-OH-AP or 4-OH-S-AP evaluation.

Hydrolysis conditions	Relative amounts (mean ± S.E.M., n = 2)	
	4-OH-AP	4-OH-S-AP
Before hydrolysis	0	100 ± 0.3
β-Glucuronidase + sulphatase from *Helix pomatia*		
Acetate pH 6.0 (1)	97.9 ± 2.6	0
Acetate pH 4.8 (2)	100 ± 1.7	0
Acetate, MBS, pH 4.8 (3)	0	98.2 ± 0.5
Sulphatase from *Aerobacter aerogenes*		
Tris pH 7.1 (4)	91.1 ± 1.5	0
Tris pH 5.3 (5)	99.3 ± 0.6	0
Tris, MBS, pH 5.6 (6)	0	97.6 ± 1.6

formed. The other antioxygens tested (vitamin C, dithiothreitol) were not very effective at protecting antipyrine metabolites against oxidation, thereby confirming the data reported in the literature [6,7]. Only a direct assay of phase II metabolites could solve this problem. Already, the HPLC technique described here enables the sulphoconjugates to be assayed by simple dilution of urine in methanol. As we now possess a standard 4-OH-S-AP, we tested this method on the urine (1:20 dilution in methanol) of five healthy volunteers who had received 1 g of antipyrine intravenously. An additional 4.3 ± 0.6% of the dose was recovered as 4-OH-S-AP over 48 h, raising the total amount of 4-OH-S-AP recovered from 23.7 to 28%.

We conclude that the study of antipyrine metabolites requires the protection of their free forms by an antioxygen, the most efficacious of these agents being sodium metabisulphite. Our demonstration of a hitherto unknown peak identified as the sulphoconjugate of 4-OH-AP highlights the fact that using sodium metabisulphite inhibits the action of sulphatase. Determining the phase I and phase II metabolites would improve our knowledge of antipyrine metabolism.

REFERENCES

1 M. Danhof, M. W. E. Teunissen and D. D. Breimer, *Pharmacology*, 24 (1982) 181.
2 M. Eichelbaum, B. Sonntag and H. J. Dengler, *Pharmacology*, 23 (1981) 192.
3 C. Palette, P. Cordonnier, E. Naline, C. Advenier and M. Pays, *J. Chromatogr.*, 563 (1991) 103.
4 H. Uchino, J. Inaba and W. Kalow, *Xenobiotica*, 13 (1983) 155.
5 J. Bottcher, H. Bassman and R. Schuppel, *Arch. Pharmacol.*, 316 (1981) R17.
6 M. Danhof, E. De Grot-Van der Vis and D. D. Breimer, *Pharmacology*, 18 (1979) 210.
7 M. Eichelbaum, *Pharmacology*, 23 (1981) 192.
8 J. Bottcher, H. Bassman and R. Schuppel, *J. Pharm. Pharmacol.*, 36 (1984) 391.
9 J. Bottcher, H. Bassman and R. Schuppel, *J. Pharm. Pharmacol.*, 34 (1982) 168.
10 J. Bottcher, H. Bassman and R. Schuppel, *Drug Metab. Dispos.*, 10 (1982) 90.
11 M. W. E. Teunissen, J. E. Meerburg-Van der Torren, N. P. E. Vermeulen and D. D. Breimer, *J. Chromatogr.*, 278 (1983) 367.
12 H. Cardy and E. Poquet, *Tetrahedron*, 37 (1981) 2279.
13 T. Walle, U. K. Walle, D. R. Knapp, E. C. Conradi and M. Bargar, *Drug Metab. Dispos.*, 11 (1983) 344.
14 P. M. Harrisson, A. M. Tonkin, C. M. Cahill and A. J. McLean, *J. Chromatogr.*, 343 (1985) 349.