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Quantitation and urinary pattern of 4,4′-dihydroxy-antipyrine, 4-hydroxy-antipyrine and 3-hydroxymethyl-antipyrine, as main metabolites of antipyrine in man and rat

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A t.l.c.-assay has been developed for the simultaneous determination from the urine of man and animals of three major hydroxylated metabolites of antipyrine (4,4′-dihydroxy-antipyrine, 4-hydroxy-antipyrine, 3-hydroxymethyl-antipyrine). The methodology is also applicable to bile fluid, liver perfusate and liver homogenate. Genuine conjugates are cleaved by acid hydrolysis and free, acid stable metabolites are extracted. Extracts are subjected to t.l.c. and the chromatograms analysed quantitatively by u.v.-reflectance measurements using authentic materials as standards. Calibration curves are linear with a correlation coefficient $r > 0{\cdot}990$. Recovery for each metabolite is >95%. Reproducibility of the method is good, with variation coefficients in the range of 3–7%, depending on concentration. The sensitivity of the method is sufficient for practical needs. The specificity of the procedure was confirmed using radio-labelled antipyrine. In man, 4-hydroxy-antipyrine is the principal hydroxylation product in this series, accounting for about 35–40% of the dose. 3-Hydroxymethyl-antipyrine makes up for about 13–17% and 4,4′-dihydroxy-antipyrine represents 3–6% of the dose of antipyrine. In the rat, 4-hydroxy-antipyrine accounts for about 15–31%, 3-hydroxymethyl-antipyrine for 22–28% and 4,4′-dihydroxy-antipyrine for up to 11–18% of the dose. Variation of this pattern in different strains is moderate. In both species, the major portion of phase-I-metabolites is excreted as conjugates. Part of them appears in a free form.

Elimination kinetics of antipyrine have been extensively used as a probe for assessing hepatic mixed function oxidase (MFO) activity in man and animals (Vesell 1979). More information about the activity of hepatic MFO may be derived from the determination of hydroxylated metabolites of antipyrine being formed and excreted (e.g. in the urine). This approach, which allows the analysis of distinct segments of hepatic MFO, as has been shown for different experimental conditions in man and rat (Schüppel 1969; Petruch et al 1974; Andreasen & Greisen 1976; Schüppel et al 1977; Schüppel et al 1980), has been hampered by the lack of precise and selective assay procedures for the main urinary phase-I-metabolites of antipyrine as well as by the incomplete knowledge of the biotransformation pattern of this drug.

By detecting aromatic ring hydroxylation of antipyrine as an additional pathway of biotransformation, metabolic disposition of the drug has now been nearly brought to completion (Bässmann et al 1979; Böttcher et al 1980). Antipyrine thus undergoes four different hydroxylation reactions leading to either 4-hydroxy-antipyrine (Brodie & Axelrod 1950), norantipyrine (Schüppel 1966), 3-hydroxymethyl-antipyrine (Yoshimura et al 1968) and 4,4′-dihydroxy-antipyrine (Bässmann et al 1979), which makes antipyrine an ideal model drug for the purpose mentioned above (Fig. 1).

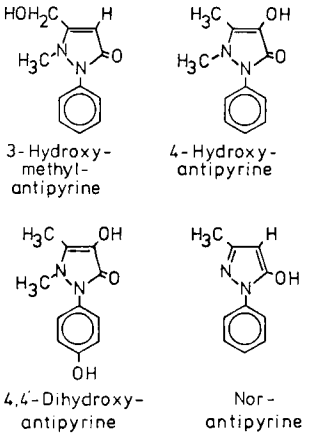

FIG. 1.

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On the other hand, there have been several studies in the past aimed at establishing the profiles of urinary metabolites of antipyrine in man and rat, using, in part, ^{14}C-labelled antipyrine (Eichelbaum et al 1976; Aarbakke 1978; Kellermann & Luyten-Kellermann 1978; Böttcher et al 1979; Schüppel et al 1980; Böttcher et al 1981). Efforts have now led to the development and publication of selective assay procedures, which allow for the simultaneous determination of several hydroxylation products of unlabelled antipyrine from human urine (Inaba & Fischer 1980; Danhof et al 1979a) and from rat urine (Danhof et al 1979b). Results, however, show notable variation, which—at least in part—is likely to be due to different analytical methods (Danhof 1980).

Phase-I-metabolites of antipyrine are mainly excreted as conjugates. In man, glucuronidation has been shown to represent the principal conjugation reaction, whereas in the rat, the leading route of conjugation is sulphation (Bässmann & Böttcher 1980). Stability of all conjugates towards acid as well as enzymic cleavage definitely differs to a wide extent, which makes any hydrolytic step to be included in the assay procedure a highly critical one.

We now describe a method for the simultaneous determination of 4,4′-dihydroxy-antipyrine, 4-hydroxy-antipyrine and 3-hydroxymethyl-antipyrine from urine after acid hydrolysis using quantitative t.l.c. In addition, results of a quantitative study in man and in two strains of rats are reported.

MATERIALS AND METHODS

Chemicals

Ethyl-[3-^{14}C]acetoacetate was purchased from Amersham-Buchler, Braunschweig, FRG; 4-methoxy-phenylhydrazine-HCl was from EGA, Steinheim, FRG; antipyrine was from Sigma, München, FRG. Enzymes were obtained from Boehringer, Mannheim, FRG (β-glucuronidase Nr. 127·051, arylsulphatase Nr. 102·890). Phenylhydrazine, dimethylsulphate, other chemicals, reagents, solvents (reagent grade) and silica gel (Kieselgel-60, 70–230 mesh) used for conventional column chromatography were from Merck, Darmstadt, FRG. Chloroform ($CHCl_3$), ethanol (EtOH) and methanol (MeOH) were of technical grade and were distilled before use. Pre-coated t.l.c. and h.p.t.l.c. glass plates (10 × 20 cm) and t.l.c. aluminium sheets (20 × 20 cm), Kieselgel 60 F-254 were used (Merck). Wheaton micro-vials (0·3 ml) were from Zinsser, Frankfurt, FRG. Samples and standards were spotted on t.l.c.-plates by disposable micropipettes (end to end 1–5 μl) Brand, Wertheim, FRG.

Synthesis

[3-^{14}C]Antipyrine was prepared on a micropreparatory scale. 44·0 mg (338 μmol, 1 mCi) ethyl-[3-^{14}C]acetoacetate was dissolved in 150 μl glacial acetic acid and 37·0 mg (341 μmol) freshly distilled phenylhydrazine was added (Knorr 1887). The solution was heated for 4 h at 110 °C. The acetic acid was evaporated nearly to dryness under a stream of nitrogen in a water bath at 25 °C yielding crude norantipyrine. Methylation was with 130 μl dimethylsulphate at 165 °C for 5 h (FIAT Final Report 1947). 250 μl distilled water was added and heated at 90 °C for 5 h. Sodium hydroxide, 150 mg in 500 μl distilled water, was added and heated for an additional 4 h at 80 °C. The solution was evaporated (Rotavapor, 40 °C) and extracted with chloroform. This extract, being free of norantipyrine as checked by t.l.c., was further purified by sublimation (84 °C at 5×10^{-3} Torr) yielding 43·59 mg (231 μmol) antipyrine (= 68·5%). M.p. 108–109 °C (uncorr.), specific activity 107 M Bq mmol^{-1} = 2·9 mCi mmol^{-1}. Both chemical and radiochemical purity was proven by t.l.c. assay.

3-Hydroxymethyl-antipyrine was synthesized according to Yoshimura et al (1971), starting from antipyrine. M.p. was 142–143 °C (uncorr.). Analytical data (n.m.r., m.s., u.v., i.r.) were in keeping with the literature and the substance was pure as judged by t.l.c.-assay.

4,4′-Dihydroxy-antipyrine was synthesized from 4-methoxy-phenylhydrazine as follows: condensation with ethyl-acetoacetate to give 4′-methoxy-norantipyrine, methylation with dimethylsulphate (see above) and extraction from alkaline solution with chloroform to give 4′-methoxy-antipyrine (I) which was subjected to a hydroxylation procedure using the ascorbic acid reagent of Brodie et al (1954) except that 0·1 M phosphate-buffer pH 6·7–methanol 7:3 (v/v) was used as solvent. Hydroxylation was allowed to proceed for 3 h at 37 °C. The medium was then made alkaline (pH 11, NaOH) and quickly extracted with chloroform for separation from the starting material. After acidification, phenols were extracted into chloroform, which, after evaporation, yielded a crude fraction of 4-hydroxy-4′-methoxy-antipyrine (yield: 10–25%), which gave an immediate blue colour with Folin's reagent. After cleavage with hydrobromic acid the mixture was extracted at pH 5 (NaCl saturated) with chloroform-ethanol (9:1) and further purified on a silica

gel column (Bässmann et al 1979) to give pure 4,4′-dihydroxy-antipyrine. The product was crystallized from ethanol and had a m.p. of 237–241 °C (decomp.). Synthesis can also start from 4-Br-4′-methoxy-antipyrine, which is superior with respect to the purity of the end-product.

4-Hydroxy-antipyrine was prepared according to Knorr & Pschorr (1896) or using the ascorbic acid system (Brodie et al 1954) starting from antipyrine. M.p. was 181–182 °C for products of either method.

Isolation of glucuronides

Lyophilized rat urine was thoroughly extracted with methanol and concentrated under a stream of nitrogen. The extract was transferred to t.l.c.-aluminium sheets (20 × 20 cm), which were developed in two subsequent runs using the following solvent systems: I chloroform–ethanol (50:50), II chloroform–methanol–glacial acetic acid (30:20:2·5). R_F-values were for 3-hydroxymethyl-antipyrine glucuronide 0·25 and for 4-hydroxy-antipyrine glucuronide 0·36. T.l.c.-sheets were cut into strips corresponding to u.v.-absorption of glucuronides and were eluted with methanol using the line-elution technique.

Instrumentation

T.l.c.-scans were performed with a chromatogram-spectrophotometer KM-3 Zeiss, Oberkochen, FRG operated at 270 nm in the reflectance mode. ^{14}C-Radioactivity peaks on t.l.c. were identified using a t.l.c.-Radioscanner II, LB 2723, Berthold, Wildbad, FRG. Radioactivity was measured by LSC using a Betaszint-5000 Spectrometer, Berthold, Wildbad, FRG. Optical density was measured using a spectrophotometer. Melting points (uncorr.) were determined using a melting point microscope.

Partition coefficients

Partition coefficients were determined by u.v.-spectrophotometry using two partitioning systems: 1. chloroform and 0·05 M phosphate buffer pH 6·3, 2. chloroform–ethanol (9:1) and 0·05 M phosphate buffer pH 6·3, which was saturated with NaCl.

Concentration in the aqueous phase for each metabolite and for antipyrine was 150–200 μg/10 ml, which was equilibrated with the organic phase (10 ml) by mixing on a vortex mixer for 2 min.

Analytical procedure

Urine, 1·0 ml, after addition of 6·0 ml toluene, was evaporated to dryness in a glass-stoppered flask (30 ml) connected to a Rotavapor. The residue was dissolved in 500 μl 3 M HCl, hydrolysed for 60 min in a boiling water bath and adjusted to pH 4–5 by adding 500 μl of a saturated solution of sodium acetate. The mixture was further saturated with NaCl and extracted with 6·0 ml of a mixture of chloroform–ethanol (9:1). Organic phase, 5·0 ml, was transferred to a small beaker in a water bath (25–30 °C) and solvent was evaporated with a stream of air.

The residue was taken up in chloroform–ethanol (9:1) and transferred to a micro-vial. This solute was brought to dryness under a stream of nitrogen and redissolved in a volume of 100 μl of chloroform–ethanol–methanol mixture (85:10:5). 1–3 μl of each sample was spotted on t.l.c.-plates along with the appropriate calibration standards. For the determination of the free metabolites and unchanged antipyrine, a 1·0 ml sample of urine was saturated with sodium chloride, extracted and processed as described.

Chromatography and evaluation

A standard solution containing authentic 3-hydroxymethyl-antipyrine, 4-hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine was prepared in a mixture of chloroform–ethanol–methanol (85:10:5) in the concentration range of 100–350 ng μl^{-1} for each metabolite. The solution is stable for three to six months when kept at −18 °C in the dark. On each plate, 4 standards were applied by spotting 1, 2, 3, 4 μl of the stock solution along with eight urine samples in the range of 100–1500 ng for each metabolite to be determined. Plates were developed twice, first in chloroform–ethanol (45:5) for 3·5–4·0 cm, and, after drying, in chloroform–ethanol (45:10) for 6·5–7 cm. Separation obtained was checked under u.v.-light before scanning. R_F-values were for 1, 3-hydroxymethyl-antipyrine 0·28; 2, 4,4′-dihydroxy-antipyrine 0·40; 3, 4-hydroxy-antipyrine 0·65.

Evaluation of chromatograms was based on either peak height or of square peak height. Both parameters were found to be linearly correlated to the concentration of the standards. Calibration curves fitted by linear regression yielded correlation coefficients better than 0·990.

Acid hydrolysis of isolated glucuronides

Isolated glucuronides were subjected to acid hydrolysis as described above for urine samples, for t = 0 (control), 5, 15, 30, 45 and 60 min in duplicates. In each sample, the amount of phase-I-metabolites being formed was determined as well as

the remnant of glucuronides present. For this purpose, samples were neutralized, saturated with NaCl and extracted for the determination of free metabolites as described. The aqueous solutes containing the intact glucuronides were then evaporated to dryness and thoroughly extracted with methanol, which was concentrated under reduced pressure. Glucuronides so obtained were spotted on h.p.t.l.c.-plates and developed as in the isolation procedure. Concentration of glucuronides was assayed by u.v.-scanning (KM-3 Zeiss) and expressed as percent recovery vs control.

Human experiments

Four healthy volunteers, mean age 31 years, 62–80 kg, received 1200 mg antipyrine orally, each with 150 ml of tap water. All were non-smokers and had not taken any medication for at least one month before the study. In an additional experiment a volunteer (62 kg) received 1200 mg antipyrine by mouth with an additional label of [3-^{14}C]antipyrine (50 μCi). Total urines were collected for 48 h after dosage, volumes measured and samples stored at −18 °C until analysed.

Animal experiments

Rats of two strains, 300–350 g, were kept on a standard chow (Altromin, Laage, FRG). Male Wistar rats were purchased from Lippische Versuchstieranstalt, External, FRG, male Sprague-Dawley rats were from Gro kreutz, Unfinden, FRG. Animals were dosed with 15 mg antipyrine (45–52·5 mg kg^{-1}) i.p. and received an additional water load of 10 ml by mouth. One animal of strain I received a dose of 15 mg antipyrine i.p. with a label of [3-^{14}C]antipyrine (6 7 μgCi). During the experiments animals had free access to tap water. Total urine was collected in all-glass metabolic cages for 24 h after application. Each urine was diluted to 50·0 ml and stored at −18 °C until used for analysis.

RESULTS

Partition coefficient

Partition coefficients of the three hydroxylated metabolites of antipyrine clearly reflect increasing hydrophilicity in this series as opposed to the lipophilicity of the parent compound (Table 1). Complete extraction of 4,4′-dihydroxy-antipyrine and 3-hydroxymethyl-antipyrine from aqueous media with chloroform is therefore difficult. In contrast, use of a mixture of chloroform–ethanol (9:1) substantially improved recovery, when the aqueous phase is saturated with sodium chloride.

Table 1. Partition coefficients of antipyrine and three hydroxylated metabolites as determined by u.v.-spectrometry in two different systems.

Wavelength, nm	$CHCl_3$ vs phosphate buffer 0·05 M, pH 6·3	$CHCl_3$–ethanol (9:1) vs phosphate buffer 0·05 M pH 6·3, NaCl saturated
Antipyrine		
242	21	50
4-Hydroxy-antipyrine		
242	2·0	38
3-Hydroxymethyl-antipyrine		
260	0·12	5·5
4,4′-Dihydroxy-antipyrine		
265	0·05	2·0

Under the analytical conditions used, the amount extracted is calculated to be better than 90% which is in total keeping with the recoveries obtained.

Stability of phase-I-metabolites

Authentic phase-I-metabolites in a concentration of 100 μg ml^{-1} were tested for stability in aqueous buffered solution at pH 3·0 and 6·3 at 37 °C for 3 and 24 h. Metabolites were also incubated in rat blank urine, to which arylsulphatase (1 IU ml^{-1}) and β-glucuronidase (1 IU ml^{-1}) had been added. As shown in Table 2, 3-hydroxymethyl-antipyrine was stable throughout as opposed to either 4-hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine, which readily decomposed under the conditions tested. In strongly acidic solution, as is present during acid hydrolysis of conjugates, all three phase-I-metabolites were stable (Table 3, Fig. 2).

Table 2. Decomposition of phase-I-metabolites of antipyrine. Effect of incubation in buffered aqueous solution or in rat blank urine, expressed as percentage recovery. Concentration was 100 μg ml^{-1}, temperature: 37 °C.

	3 h incubation			24 h incubation		
	Phosphate buffer 0·1 M		Rat blank urine %	Phosphate buffer 0·1 M		Rat blank urine %
	pH 3·0 %	pH 6·3 %		pH 3·0 %	pH 6·3 %	
3-Hydroxymethyl-antipyrine	100	100	100	100	100	100
4-Hydroxy-antipyrine	69	77	17	31	0	0
4,4′-Dihydroxy-antipyrine	41	60	12	27	0	0

Hydrolysis of isolated conjugates

Acid hydrolysis, as described, resulted in cleavage of all sulphates present in less than 15 min. 4-Hydroxy-antipyrine glucuronide was hydrolysed to better than 90% within 45 min and cleavage was complete after 60 min (Fig. 2). 3-Hydroxymethyl-antipyrine glucuronide was hydrolysed up to 70% within 60 min.

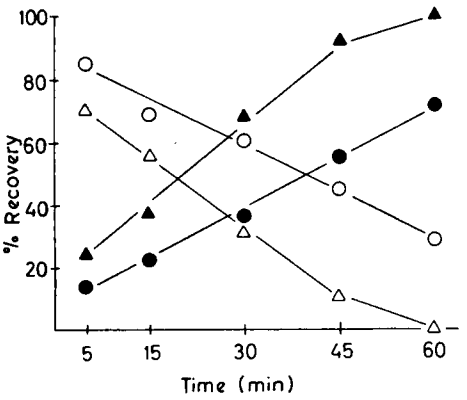

FIG. 2. Kinetics of the acid hydrolysis of isolated glucuronides of 4-hydroxy-antipyrine and 3-hydroxy-methyl-antipyrine. Percent recovery both for the conjugates (open symbols) and for the phase-I-metabolites being formed during hydrolysis (solid symbols) vs. time of incubation is shown. (△): 4-hydroxy-antipyrine glucuronide, (○): 3-hydroxymethyl-antipyrine glucuronide, (▲): 4-hydroxy-antipyrine, (●): 3-hydroxymethyl-antipyrine. For details see under methods.

Recovery

Analytical recovery was examined using rat urine samples spiked with known amounts of authentic materials within the range of concentration usually found after antipyrine dosage. Samples were carried in duplicate through the analytical procedure including acid hydrolysis. Using a set of six concentrations for each metabolite within the range of concentration, indicated in Table 3, constant recoveries were found to amount >95%. This result is interpreted to show adequate stability of 4-hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine under the conditions.

Precision

The precision of the method was determined using rat urine. One animal was dosed with 15 mg antipyrine (i.p.) and urine collected for 24 h. The urine sample was adjusted to a volume of 30·0 ml (first sample = high concentration) and further diluted with rat blank urine (1:5) (second sample = low concentration). Ten samples of each of the two urine dilutions so obtained were subjected to the complete analytical procedure. Results are given in Table 4.

Table 3. Recoveries for three hydroxylated metabolites of antipyrine from rat urine. A set of six concentrations (in duplicate) was used for the assay of each metabolite. Calibration curves were calculated by linear regression.

Concentration range, μg ml^{-1}	Linear regression coeff.	Mean recovery %
3-Hydroxymethyl-antipyrine 20–170	0·998	95·6
4-Hydroxy-antipyrine 10– 90	0·997	98·8
4,4′-Dihydroxy-antipyrine 4– 40	0·993	99·5

Table 4. Reproducibility of the assay of antipyrine metabolites from rat urine. Two urine samples have been assayed with a high and a low concentration of metabolites.

	Actual concn μg ml^{-1}	Variation coefficient %
I. sample = high concentration range (n = 10)		
3-Hydroxymethyl-antipyrine	100	3·1
4-Hydroxy-antipyrine	175	3·1
4,4′-Dihydroxy-antipyrine	75	2·9
II. sample = low concentration range (n = 10)		
3-Hydroxymethyl-antipyrine	20	6·6
4-Hydroxy-antipyrine	35	4·7
4,4′-Dihydroxy-antipyrine	15	5·9

Sensitivity

The sensitivity of the method allowed an exact determination of all metabolites down to a concentration of 5 μg ml^{-1} of biological material to be made. It is thus adequate for any metabolite present at least at a 1% level of the dose used in this study.

Specificity

The specificity of the method was monitored by comparison of urine samples after dosage with radiolabelled antipyrine. Urine was analysed as described and chromatograms were developed to a path length of up to 15 cm. Chromatograms were scanned for both radioactivity and u.v. reflectance. Radioactivity peaks in the radioscan were identical with those in the u.v. scans of urine samples as well as of authentic material (Fig. 3). In addition, chromatograms were sprayed with either Folin's reagent, yielding an instant blue colour for 4-hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine or Ehrlich's reagent, which gave a red colour for 3-hydroxymethyl-antipyrine after heating at 110 °C for 10 min.

Urinary pattern of phase-I-metabolites

Quantitative determination showed that in man 4-hydroxy-antipyrine is the predominant hydroxylation product of antipyrine, accounting for about 35–40% of the dose. 3-Hydroxymethyl-antipyrine is equivalent to 13–17% and 4,4′-dihydroxy-antipyrine to 3–6% of the dose of antipyrine given (Table 5).

In the rat, 4-hydroxy-antipyrine is also the principal phase-I-metabolite of antipyrine, which rep-

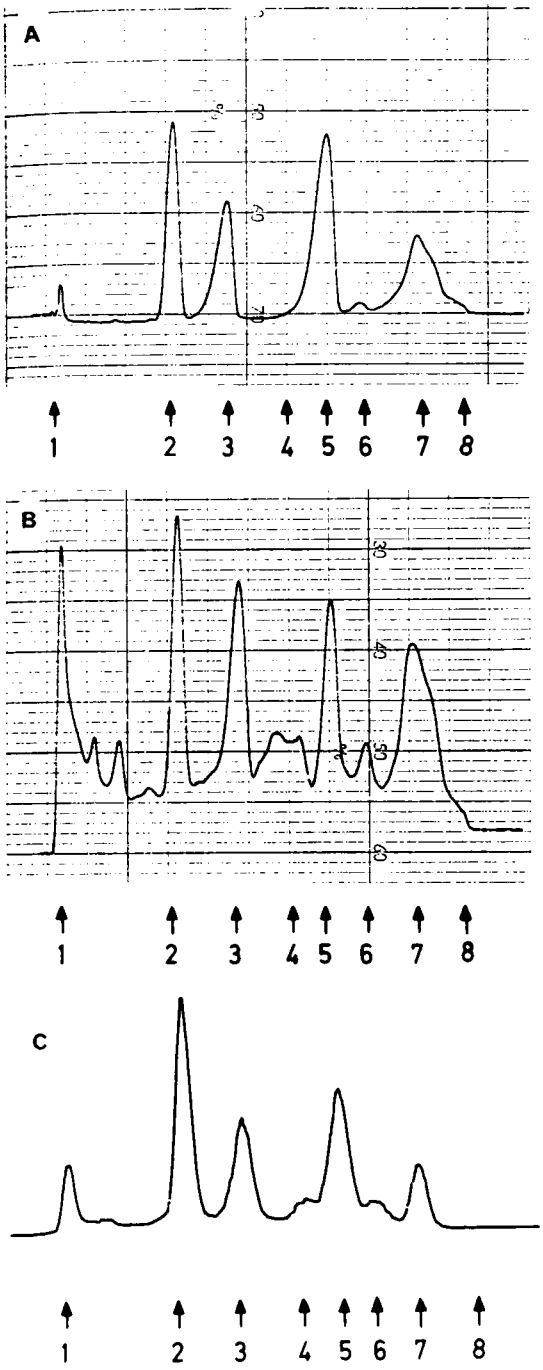

FIG. 3. T.l.c.-separation of three phase-I-metabolites of antipyrine A. T.l.c.-scan of authentic material. B. T.l.c.-scan of an extract from hydrolysed rat urine. C. T.l.c.-radioscan of an extract from hydrolysed rat urine. 1. Origin, 2. 3-hydroxymethyl-antipyrine, 3. 4,4′-dihydroxy-antipyrine, 4. antipyrine, 5. 4-hydroxy-antipyrine, 6. nor-antipyrine, 7. unknown material, 8. front of solvent.

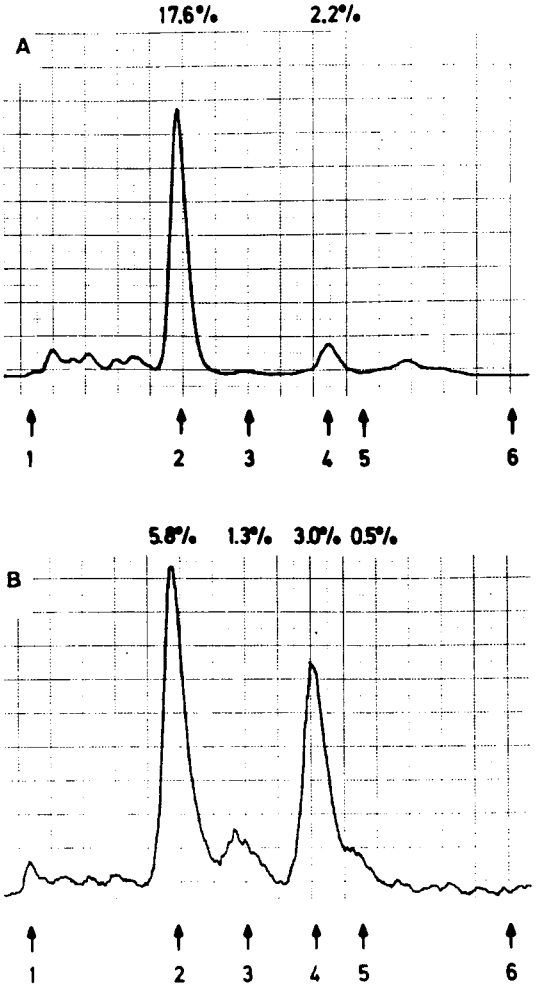

FIG. 4.T.l.c.-separation of free metabolites and unchanged antipyrine. A radioscan is shown of an extract obtained from rat urine (A) or human urine (B) after dosage with [3-^{14}C]antipyrine. Percentage of dose formed in the individual experiment is given at the upper margin of the panel. 1. Origin, 2. 3-hydroxymethyl-antipyrine, 3. 4,4′-dihydroxy-antipyrine, 4. antipyrine, 5. 4-hydroxy-antipyrine, 6. front of solvent.

resents up to 15–31% of the dose. 3-Hydroxymethyl-antipyrine is formed up to 22–28% and 4,4′-dihydroxy-antipyrine will go up to 11–18% of the dose, the latter clearly at variance with the amount formed in man (Table 5). Irrespective of the pattern given in Table 5, formation of 4-hydroxy-antipyrine may exhibit noticeable variation within substrains of rats obtained from different breeders. This variation is much less pronounced for 3-hydroxymethyl-antipyrine and 4,4′-dihydroxy-antipyrine.

The main portion of the three hydroxylated metabolites appeared as conjugates in the urine. A

Table 5. Urinary pattern of antipyrine and three hydroxylated metabolites in man and in two strains of rats. Percent of dose excreted: mean (with s.d.)

		Male volunteers n = 4 %	Male rats (Sprague-Dawley) n = 6 %	Male rats (Wistar) n = 6 %
Antipyrine, unchanged		3·1 (0·6)	0·7 (0·4)	4·0 (1·0)
3-Hydroxymethyl-antipyrine	total	14·9 (1·3)	25·6 (1·2)	24·4 (1·8)
	free	6·2 (1·1)	18·1 (4·4)	20·4 (3·6)
4,4′-Dihydroxy-antipyrine	total	4·3 (1·1)	14·0 (2·3)	13·8 (0·9)
	free	1·2 (0·2)	not detected	not detected
4-Hydroxy-antipyrine	total	38·3 (2·0)	23·1 (4·3)	26·7 (4·0)
	free	0·5	not detected	not detected
Total of three metabolites under study		57·5 (2·1)	62·7 (5·4)	64·9 (6·3)

Sampling period: man: 0–48 h, rat: 0–24 h

considerable amount of the 3-hydroxymethyl-antipyrine was excreted in a free form both in man and rat (Fig. 4 and Table 5). For 4,4′-dihydroxy-antipyrine and 4-hydroxy-antipyrine the amount of free form was small or even absent.

DISCUSSION

Analytical methodology to be adopted for the determination of urinary metabolites of antipyrine has to take into account: (1) the stability of urinary conjugates towards hydrolysis and (2) the instability of some of the phase-I-metabolites which were either excreted in a free form or freed from their conjugates by hydrolysis. In fact, all three metabolites, the assay of which is described in the present paper, exhibit substantial variation in both instability of free phase-I-metabolites and stability of their conjugates. Both aspects are of crucial importance especially with respect to the variations found in the phase-I- and phase-II-metabolism pattern of antipyrine in man and rat (Böttcher et al 1980, 1981).

4-Hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine are eliminated in man and rat as sulphates and glucuronides, which are completely cleaved under the conditions used. Stability of both free hydroxylated metabolites is limited, however, as is shown in Table 2, confirming earlier reports for 4-hydroxy-antipyrine (Brodie & Axelrod 1950; Danhof et al 1979a; Inaba et al 1980). The unconjugated portion of 4-hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine excreted in the urine (Fig. 4) will also undergo decomposition. Suitable precautions therefore have to be taken to counteract decomposition of these free metabolites during collection and storage of urine samples and during analytical work-up.

Recovery of these metabolites will be markedly compromised by any time-consuming hydrolytic procedure under unfavourable conditions, e.g. enzymic hydrolysis in buffered solution. The same is true for the evaporation of large volumes of organic extractants at elevated temperatures. In contrast, all conjugates have been shown to be stable and their hydrolysis under strongly acidic conditions prevents phase-I-metabolites from decomposition, as is seen from the direct quantitative assay of authentic conjugates which disappear during hydrolysis, phase-I-metabolites being formed (Fig. 2). Recoveries reported for all metabolites in this study confirms this finding (Table 3).

3-Hydroxymethyl-antipyrine is eliminated in both species as a glucuronide and to a considerable part in an unconjugated form, which makes up for 30–60% of total amount excreted (Table 3 and Fig. 4). Free 3-hydroxymethyl-antipyrine is stable in aqueous media or urine and does not present any analytical problems. In contrast, 3-hydroxymethyl-antipyrine glucuronide has been found to be highly resistant to acid and enzymic hydrolysis (Zietz 1976).

There is an increase in hydrophilicity in the series of phase-I-metabolites from 4-hydroxy-antipyrine < 3-hydroxymethyl-antipyrine <4,4′-dihydroxy-antipyrine, as is reflected by the partition coefficients (Table 1). Extractability of such metabolites from an aqueous matrix is limited therefore. Complete extraction of all metabolites under study was achieved using a mixture of chloroform–ethanol (9:1), when the aqueous phase was initially saturated with sodium chloride.

Specificity of the assay procedure was proved by comparison with radiolabelled material (Fig. 3). It has been shown, that unchanged antipyrine and other metabolites present in the biological material, including norantipyrine and its decomposition products, will not interfere with the analysis.

By determining the three urinary hydroxylated metabolites, the activity of three different hydroxylating processes within the hepatic MFO can be assessed in vivo including aliphatic, aromatic and quasiaromatic C-hydroxylation. Together they will account for 57·5 (2·1)% (mean (s.d.)) of the dose of antipyrine given in man and for 66·0–75·7% in the rat. Data obtained in this study are in total agreement with our own results reported earlier using [3-^{14}C]antipyrine in man (Böttcher et al 1981) and rat (Böttcher et al 1979). *N*-Demethylation, by the formation of norantipyrine, will further add a major phase-I-metabolite to the biotransformation pattern of antipyrine (Schüppel 1966; Schüppel et al 1981).

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