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Quantitation of norantipyrine sulphate and norantipyrine glucuronide as major metabolites of antipyrine in man and rat

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Two assay procedures are described for the quantitation of norantipyrine sulphate and norantipyrine glucuronide present in biological material. One, a selective acid hydrolytic assay procedure that affords the discriminative determination of both conjugates without prior separation, measures free norantipyrine by tlc-uv. The other is a tlc separation procedure for intact norantipyrine conjugates, which, in conjunction with radiolabelled material, derived from [3-^{14}C]antipyrine, enables the direct quantitation of both conjugates independently. In man, about 25% of dose of antipyrine (1200 mg) was excreted as norantipyrine glucuronide within 48 h. The amount of norantipyrine sulphate was small. In the rat, norantipyrine sulphate represented about 15–20% of the dose of antipyrine (40–50 mg kg^{-1}); no norantipyrine glucuronide was formed. Free norantipyrine has not been detected in urine of either species after antipyrine dosage. Pretreatment with 3-methylcholanthrene in the rat substantially enhanced excretion of norantipyrine sulphate, whereas induction with phenobarbitone was without effect on the microsomal *N*-demethylation of antipyrine. The lability of free norantipyrine was examined under different conditions and contrasted with the relative stability of norantipyrine conjugates. Two of the main degradation products of norantipyrine were identified as 4-phenylazo-norantipyrine and a 4,4′-bipyrazole-derivative.

A substantial part of antipyrine undergoes *N*-demethylation in man and rat (Schüppel 1966; Baty & Price Evans 1973). The norantipyrine is always excreted in a conjugated form, in the rat as the sulphate, in man as the glucuronide, when antipyrine is given in a normal dose (Böttcher et al 1980, 1981b, 1982c). The actual extent of the *N*-demethylation remains equivocal. Quantitative data on the formation of norantipyrine show variation that is beyond inter- or intra-species differences usually found in the biotransformation pattern of this drug. This finding questions the validity of the analytical procedures used. Therefore, a comparative study on the analytical profiles of norantipyrine and its urinary conjugates seemed warranted. For this purpose, two assay procedures have been devised and both have been used to measure excretion of norantipyrine conjugates in urine of man and rat after dosage of antipyrine. In addition, the effects of either 3-methylcholanthrene or phenobarbitone pretreatment on the *N*-demethylation of antipyrine have been studied in rats.

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MATERIALS AND METHODS

Chemicals

Antipyrine (Phenazon Ph. Eur. III, 2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) was purchased from Sigma, München, FRG; norantipyrine (3-methyl-1-phenyl-2-pyrazolin-5-one, techn. grade) was from Merck-Schuchardt, Hohenbrunn, FRG and was recrystallized from hot ethanol. 4-Amino-antipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) was from Merck, Darmstadt, FRG. [3-^{14}C]Antipyrine and antipyrine phase-I-metabolites were synthesized as described (Böttcher et al 1982a). Urease was from Boehringer, Mannheim, FRG. All other chemicals, reagents, scintillators, solvents (reagent grade) and precoated tlc plates (Kieselgel 60 F-254, 20 × 20 cm, 20 × 10 cm) were purchased from Merck. Norantipyrine sulphate and norantipyrine glucuronide were isolated from rat and human urine, respectively, after oral dosing with antipyrine (rats: 40 mg kg^{-1}, man: 15 mg kg^{-1}) as described (Böttcher et al 1982c). Standard solutions of norantipyrine in chloroform are stable over 4–8 weeks at −18 °C, when decomposition becomes demonstrable.

Synthesis

Pyrazolin-4,5-dione (3-methyl-1-phenyl-2-pyrazolin-4,5-dione), $C_{10}H_8N_2O_2$, 118·19, was prepared according to Knorr & Pschorr (1896) or by acid hydrolysis of methylrubazonic acid (Emerson & Beagle 1943).

4-Phenylazo-norantipyrine (3-methyl-1-phenyl-4-phenylazo-2-pyrazolin-5-one) $C_{16}H_{14}N_4O$, 278·32, was synthesized according to Knorr (1887).

4,4′-Bipyrazol-derivative (5-hydroxy-3-methyl-1-phenyl-4(4′-hydroxy-3′-methyl-5′-oxo-1′phenyl-Δ^2-pyrazolin-4′-yl)-pyrazole), $C_{20}H_{18}N_4O_3$, 362·39, was prepared from norantipyrine (1 mmol in 1·0 ml $CHCl_3$) and pyrazolin-4,5-dione (1 mmol in 1·0 ml $CHCl_3$). 24 h after mixing, a white crystalline precipitate had been formed, which was washed with $CHCl_3$. M.p. (uncorr.) 173–175 °C.

Elementary analysis: Calc. for $C_{20}H_{18}N_4O_3$: C, 66·28; H, 5·01; N, 15·46. Found: C, 66·26; H, 5·03; N, 15·42.

Instrumentation

Tlc-plates were scanned on a chromatogram-spectrophotometer KM-3 Zeiss, Oberkochen, FRG, operated at 250 nm in the reflectance mode. Radioactivity on tlc-plates was identified using a tlc-Radioscanner II, type LB 2723, Berthold, Wildbad, FRG. Radioactivity was measured by lsc using a Betaszint 5000 spectrometer, Berthold. Optical density was determined in a spectrometer PMQ-2 Zeiss, Oberkochen, FRG. Mass spectra were recorded using a MS-9 mass spectrometer (AEI, Manchester, UK) with direct inlet system, electron impact: 70 eV. Melting points were determined using a melting point microscope type 350 Leitz, Wetzlar, FRG. Wheaton microvials were from Zinsser, Frankfurt, FRG. Disposable micropipettes were from Brand, Wertheim, FRG (end-to-end 1–5 µl).

Partition coefficients

Partition coefficients were determined by uv-spectrophotometry as described (Böttcher et al 1982a) using the following systems: 1. chloroform/acetate buffer 0·05 m, pH 4·5; 2. chloroform/phosphate buffer 0·05 m, pH 12·0; 3. chloroform/potassium chloride-HCl buffer 0·05 m, pH 1·0.

Assay of norantipyrine conjugates by selective acid hydrolysis of norantipyrine sulphate

Urine (1·00 ml) was mixed with 3 M HCl (200 µl) in a glass-stoppered conical centrifugation tube (10 ml). After 5 min at room temperature, a saturated sodium acetate solution (200 µl) was added to bring to pH 4–5. Further $CHCl_3$ (1·50 ml) was added and the mixture was agitated on a whirl-mix for 30 s. After centrifugation, 1·00 ml of the organic layer was transferred to a micro-vial and the solvent was evaporated in a stream of nitrogen whilst the vial was allowed to cool. Residue was taken up in 100 µl of mixture of $CHCl_3$–EtOH (90 : 10) and a sample (1–3 µl) was spotted on a tlc-plate.

Norantipyrine glucuronide

Hydrolysis of an identical sample for 5 min at 90 °C (water bath) and processing as above will yield the amount of total norantipyrine corresponding to norantipyrine sulphate and norantipyrine glucuronide. The amount of norantipyrine glucuronide present is calculated from the difference. After spotting, tlc-plates were developed in $CHCl_3$–EtOH (45 : 3) (solvent I), dried and scanned at 250 nm for evaluation as described (Böttcher et al 1982a).

Direct assay of [3-^{14}C]norantipyrine conjugates by lsc

In a glass-stoppered flask (30 ml) 1·0 ml of radiolabelled urine was mixed with 1 mg urease and was incubated at room temperature (23 °C) for 3 h. After decomposition of urea, 6·0 ml of a mixture of ethanol–toluene (2 : 1) was layered onto the sample and was brought to dryness on a Rotavapor (40–50 °C). The residue was extracted with 3 × 300 µl anhydrous methanol, the extracts combined and evaporated to dryness in a stream of nitrogen. The residue was taken up in a known volume of methanol (50–100 µl) and a sample (5–15 µl) was lined onto a tlc-plate for a path width of 30 mm. Authentic norantipyrine sulphate and glucuronide, respectively, were used as reference (Fig. 4). Plates were developed in solvent II ($CHCl_3$–EtOH–NH_3 25% (50 : 50 : 2), path length: 15 cm) and subsequently in solvent III ($CHCl_3$–MeOH–glacial acetic acid (60 : 40 : 5), path length: 10 cm). Separation of conjugates was checked under uv-light and zones of radioactivity were identified by radioscanning. Appropriate zones were scraped off and transferred to scintillation vials for lsc.

Stability of norantipyrine conjugates

Known amounts of authentic norantipyrine sulphate (100 µg), norantipyrine glucuronide (100 µg) and of a mixture of both conjugates (100 µg each) were dissolved in 1·0 ml samples of rat blank urine and underwent selective hydrolysis as appropriate. The amount of norantipyrine liberated was determined. After extraction, samples were brought to dryness and residues were checked for conjugates eventually

remaining unhydrolysed under the conditions used. For the single conjugate experiments, results were invariably negative. With the conjugate mixture, the expected amount of norantipyrine liberated from norantipyrine sulphate was found after the first step of selective hydrolysis, with the norantipyrine glucuronide left intact, which, in turn was entirely cleaved in the second hydrolytic step. Each assay was performed fourfold.

Stability of norantipyrine

Unlabelled norantipyrine was incubated (100 μg ml^{-1}) in either phosphate-buffer (0·1 M, pH 3·0 and 6·3) or rat blank urine at 37 °C. After 3 and 24 h, recovery was determined by tlc-assay (Table 1). In a second experiment, using radiolabelled urine from a rat dosed with [3-^{14}C]antipyrine; a set of four samples of 1·5 ml were each extracted with 2 × 20 ml $CHCl_3$ for separation of unchanged antipyrine and free 3-hydroxymethyl-antipyrine. Then all specimens underwent acid hydrolysis of norantipyrine sulphate as given above. Two samples were immediately assayed for norantipyrine as described. The other two samples were left for 3 h at room temperature (23 °C) at pH 4·5 before extraction with $CHCl_3$. Extracts were left for further 3 h before tlc-assay was performed for norantipyrine and its degradation products 4,4′-bipyrazol-derivative and 4-phenylazo-norantipyrine (Fig. 1).

Human experiments

Four healthy volunteers, mean age 31 years, 62–80 kg, each ingested 1200 mg antipyrine dissolved in 150 ml tap water. Participants were non-smokers and had not taken any medication for at least one month. One volunteer (J.B., 62 kg) also received 1200 mg antipyrine with an additional label of [3-^{14}C]antipyrine (50 μCi). Total urine was collected for 48 h after dosing and samples stored at −18 °C until analysed.

Animal experiments

Male Sprague-Dawley rats (Lippische Versuchstieranstalt, Extertal, FRG), 300–350 g, were fed a lab chow (Altromin Nr. 1324, Lage, FRG) and had free access to tap water.

Induction experiments of drug biotransformation. 3-Methylcholanthrene was given as 1 × 50 mg kg^{-1} i.p. in corn oil, phenobarbitone-Na was given in isotonic solution 100 mg kg^{-1} i.p. for 3 consecutive days. Controls were treated with appropriate vehicle alone. Five days after the onset of pretreatment animals received a test dose of antipyrine (15 mg/animal i.p.). One rat received a dose of 15 mg antipyrine with a label of [3-^{14}C]antipyrine (9 μCi i.p.). Total urine was collected in all-glass metabolic cages for 24 h after dosage. Each urine sample was diluted to 50 ml and stored at −18 °C until analysed.

Table 1. Recovery of norantipyrine from aqueous solution (in %) by tlc-assay (see text) (100 μg ml^{-1}). Effect of incubation at 37 °C.

Incubation time h	Norantipyrine incubated in Rat blank urine %	Phosphate buffer 0·1 M pH 3·0 %	pH 6·3 %
3	30	98	103
24	0	95	97

RESULTS

Stability of conjugates of norantipyrine

Both conjugates are stable in neutral aqueous and methanolic solution. However, by virtue of their enolic structure (Böttcher et al 1982b, 1982c), both exhibit a characteristic acid lability compared with

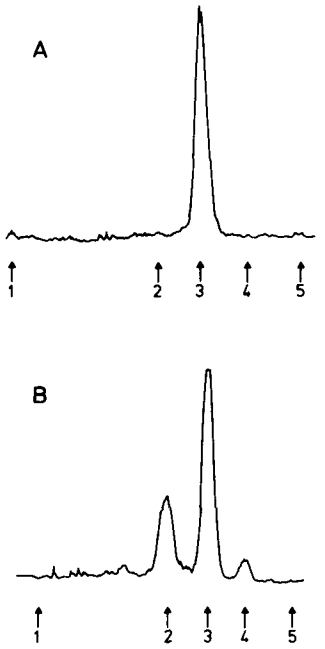

FIG. 1. Degradation of norantipyrine. Radioscan of [3-^{14}C]-norantipyrine isolated in the assay procedure after selective hydrolysis of rat urine obtained after dosage of [3-^{14}C]antipyrine. A: Immediate tlc-separation, B: tlc-separation after 2 × 3 h standing at room temperature: extract contains 4,4′-bipyrazol-derivative and 4-phenylazo-norantipyrine as decomposition products. 1. Origin, 2. 4,4′-Bipyrazol-derivative, 3. Norantipyrine, 4. 4-Phenylazo-norantipyrine, 5. Front of solvent.

the other conjugates in the metabolism of antipyrine (Böttcher et al 1982a). Nevertheless, there exists a distinct difference between norantipyrine sulphate and glucuronide in that stability against acid (Böttcher et al 1982c). Selective hydrolysis, as used in the above assay procedure, is based on this finding, enabling the discriminative determination of norantipyrine sulphate and glucuronide in the same sample without separation.

Stability of norantipyrine

Norantipyrine is stable in slightly acidic or neutral buffer solution, but is unstable in rat and human urine. It is also unstable in organic solvents, e.g. in chloroform. Starting from [3-^{14}C]norantipyrine, liberated from radiolabelled norantipyrine sulphate in rat urine after a dose of [3-^{14}C]antipyrine, it was possible to identify 4-phenylazo-norantipyrine and 4,4'-bipyrazol-derivative as the main degradation products of norantipyrine (Fig. 1). Both degradation products (Fig. 2a, b) have been identified by tlc and mass spectrum against authentic material (Fig. 3a, b).

Further, norantipyrine undergoes decomposition, when treated with dilute or strong acid at elevated temperature. Identification of decomposition products was not attempted, nevertheless, these are hydrophilic, as is concluded from experiments using [3-^{14}C]norantipyrine. Furthermore, norantipyrine has been found to be highly volatile. Therefore, uncontrolled evaporation of great volumes of organic extractants containing norantipyrine, even at room temperature, will cause significant or even total loss of norantipyrine. At 30 °C ambient temperature, analytical amounts of norantipyrine

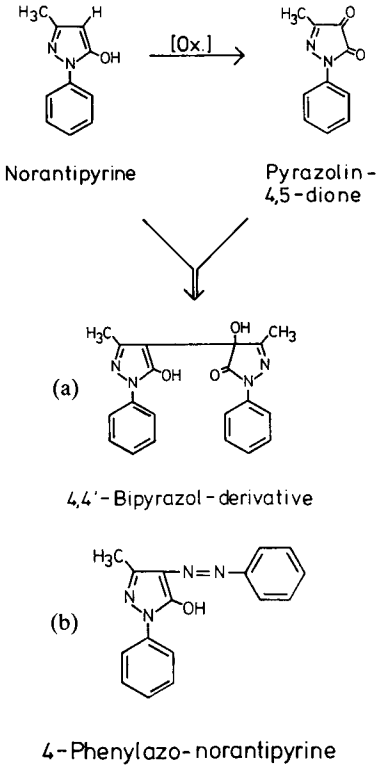

FIG. 2. (a) Suggested pathway for the formation of the 4,4'-bipyrazol-derivative from norantipyrine via pyrazolin-4,5-dione by autoxidation and dimerization. (b) Structure of 4-phenylazo-norantipyrine as a decomposition product of norantipyrine.

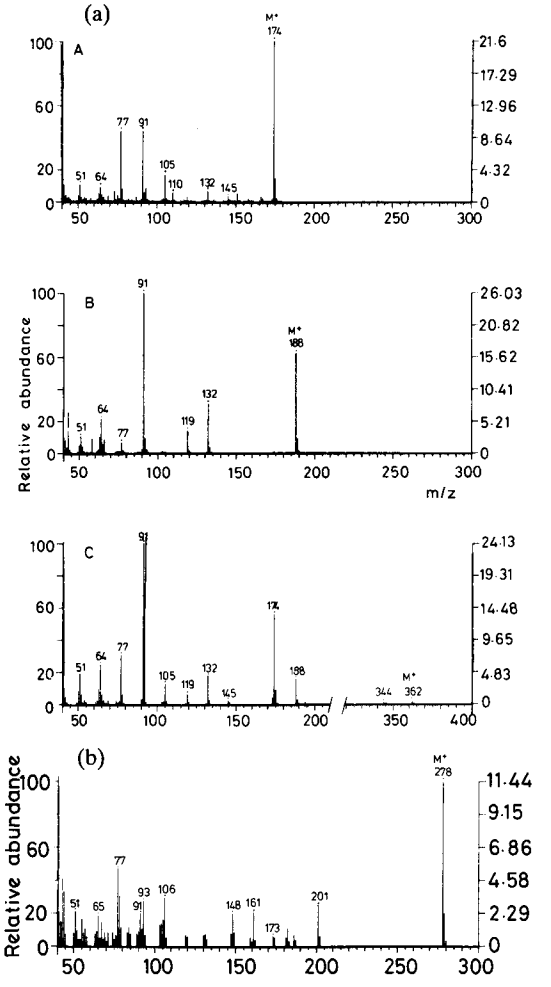

FIG. 3. EI-mass spectra of norantipyrine, pyrazolin-4,5-dione and of two identified decomposition products of norantipyrine, obtained from authentic material. (a) A: norantipyrine, B: pyrazolin-4,5-dione, C: 4,4'-bipyrazol-derivative; (b): 4-phenylazo-norantipyrine.

(10–100 μg) will thus be totally lost from open vials within 24 h.

Partition properties

The partition coefficients determined in different systems for norantipyrine, norantipyrine conjugates and antipyrine are shown in Table 2. Norantipyrine is a highly lipophilic compound at neutral or weakly acidic conditions and is remarkable in that norantipyrine is the only phase-I-metabolite of antipyrine, which is much more lipophilic than its parent compound. Norantipyrine is thus totally extracted from aqueous media at slightly acidic conditions by a minute volume of e.g. chloroform.

Table 2. Partition coefficients of norantipyrine, norantipyrine conjugates and antipyrine as determined by uv-spectrophotometry in three different systems.

	$CHCl_3$/ acetate buffer 0·05 M, pH 4·5	$CHCl_3$/ KCl buffer 0·05 M, pH 1·0	$CHCl_3$/ phosphate buffer 0·05 M, pH 12·0
Norantipyrine	50	16	<0·01
Norantipyrine sulphate	<0·01	Hydrolysis	<0·01
Norantipyrine glucuronide	<0·01	<0·01	<0·01
Antipyrine	20	16	17

Direct assay of urinary conjugates of norantipyrine

By use of the tlc-separation technique for both intact norantipyrine conjugates (Fig. 4), a direct quantitation of norantipyrine sulphate from rat urine and of norantipyrine glucuronide from a volunteer's urine after dosage of [3-^{14}C]antipyrine, respectively, was performed using lsc, in parallel with the assays by selective hydrolysis as described above. Results are in Table 3.

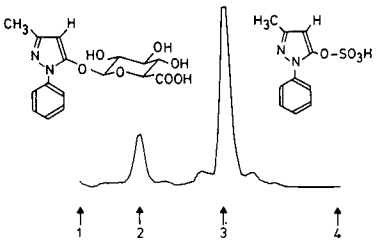

FIG. 4. Tlc-separation of norantipyrine conjugates. Radioscan of a mixture of isolated radiolabelled norantipyrine sulphate and norantipyrine glucuronide after tlc-separation (solvent system I: $CHCl_3$–EtOH (50:50) path length 15 cm, solvent system II: $CHCl_3$–MeOH–glacial acetic acid (60:40:5) path length 10 cm). 1. Origin, 2. Norantipyrine glucuronide, 3. Norantipyrine sulphate, 4. Front of solvent.

Assay for norantipyrine conjugates by selective acid hydrolysis

The assay procedure for both conjugates is characterized by a short hydrolytic step followed by a rapid and complete extraction in a small volume of organic solvent, which is quickly evaporated under controlled conditions, thus accounting for both the lability and volatility of free norantipyrine. The quality parameters of the assay are as follows:

(a) *Precision*. Reproducibility of the assay was tested for each of the norantipyrine conjugates in two sets of test samples (n = 6) containing 10 and 100 μg ml^{-1} of either conjugate, respectively, which were run through the assay. Variation coefficients of these determinations are as follows (n = 6): sulphate 10 μg ml^{-1} ± 3·1%, 100 μg ml^{-1} ± 1·8%, glucuronide 10 μg ml^{-1} ± 3·7%, 100 μg ml^{-1} ± 2·4%. Precision of the hydrolytic assay was further substantiated by comparing the results obtained by the direct assay of urinary conjugates of [3-^{14}C]norantipyrine using lsc in identical samples (Table 3).

Table 3. Urinary pattern of norantipyrine conjugates in antipyrine metabolism of man and rat (% of the dose, mean ± s.d.). Comparison of two assay methods: method I: selective acid hydrolysis, method II: direct determination of [3-^{14}C]norantipyrine conjugates by lsc after tlc-separation (see text). Sampling period: man: 48 h, rat: 24 h p.appl.

Pretreatment Number of animals/ individuals	Method used	Norantipyrine sulphate %	Norantipyrine glucuronide %
Rat			
Saline			
6	I	16·4 ± 2·1	---
1	II	18·3	---
Phenobarbitone-Na			
6	I	15·6 ± 3·8	---
1	II	17·2	---
Corn oil			
6	I	18·6 ± 2·5	---
1	II	17·9	---
3-Methylcholanthrene			
6	I	26·4 ± 2·4	---
1	II	25·6	---
Man			
4	I	---	25·6 ± 2·6
1	II	1·5	24·3

(b) *Recovery*. Recovery for norantipyrine was determined directly from authentic norantipyrine sulphate and glucuronide 10–200 μg ml^{-1}. Appropriate amounts of conjugates were dissolved in rat blank urine. Samples were run in duplicate through the assay. As is shown in Table 4, recovery is linear within the concentration range studied and yields up to 96% for sulphate and up to 92% for glucuronide.

(c) *Sensitivity*. The procedure described will cover concentrations down to 5·0 μg ml^{-1} of medium, when conventional precoated tlc-plates are used. This, in our hands, is sufficient to exactly quantitate norantipyrine as a main phase-I-metabolite of antipyrine, even when small doses of antipyrine have been employed. If needed, sensitivity may be raised by using hptlc-plates.

(d) *Specificity*. The specificity of the selective hydrolytic step is high. Cleavage of other conjugates in antipyrine metabolism is negligible. Use of solvent mixture I ($CHCl_3$–EtOH 45:3) affords a reliable separation from unchanged antipyrine and free 3-hydroxymethyl-antipyrine usually being present (Fig. 5a, b). Absorption maximum of norantipyrine is at 240 nm on tlc-plates with a sharp decrease below 270 nm, which is characteristic for norantipyrine in the series of antipyrine phase-I-metabolites. Norantipyrine gives a bright red colour with Ehrlich's reagent after heating at 110 °C for 10 min.

Specificity of the assay has further been checked using rat pooled urine from experiments with [3-^{14}C]antipyrine. Samples were run through the assay procedure and uv-scans of chromatograms were compared with appropriate radioscans (Fig. 5a–c). Authentic material was used as reference. Complete correlation was found.

Urinary pattern of norantipyrine conjugates in man and rat

As can be taken from Table 3, norantipyrine is representing a principal phase-I-metabolite in the biotransformation of antipyrine in man and rat, confirming earlier reports (Schüppel 1966). At the actual dosage studied, sulphation of norantipyrine is the single conjugation reaction found in the rat. In man, norantipyrine glucuronide is predominantly formed (Table 3). Free norantipyrine has never been detected in fresh or frozen samples of urines of either species, which is at variance with another report (Danhof 1980). In the rat, formation of norantipyrine is significantly enhanced by pretreatment with 3-methylcholanthrene, indicating an increase in hepatic *N*-demethylation of antipyrine. In contrast induction by phenobarbitone is without effect on the *N*-demethylation of antipyrine (Böttcher et al 1982d).

DISCUSSION

The results obtained show, that norantipyrine conjugates are relatively stable compounds, which can be used for direct quantitation. In contrast, free norantipyrine is a rather unstable phase-I-metabolite, posing considerable problems in its valid quantitation; even under mild conditions, it undergoes degradation. An autoxidative process is presumably involved, as can be taken from the formation of a 4,4′-bipyrazol-derivative with an oxygen function at the 4′-position. This view is in keeping with the observation, that antioxidants exert, at least in part, a stabilizing effect on norantipyrine, e.g. during enzymic hydrolysis of norantipyrine conjugates in urine samples (Danhof et al 1979a; Kahn et al 1981; Eichelbaum et al 1981).

Under strongly acidic conditions at elevated temperature, decomposition of norantipyrine proceeds via other pathways, leading to more hydrophilic by-products, which are not extracted by lipophilic solvents, thereby not interfering with the hydrolytic assay procedure described for the other main phase-I-metabolites of antipyrine (Böttcher et al 1982a). Under moderate acidic conditions, as are used in the present assay procedures, norantipyrine is sufficiently stable to allow analytical work-up without loss (Schüppel 1968). There are further indications,

Table 4. Recovery of norantipyrine from authentic norantipyrine glucuronide or norantipyrine sulphate after selective hydrolysis. Concentration range: 10–200 μg ml^{-1} of norantipyrine equivalent. Assays (see text) were run in duplicate. Correlation coefficient for the standard curve obtained: r_1/r_2.

	Norantipyrine liberated from the glucuronide		Norantipyrine liberated from the sulphate	
μg added	μg found	Recovery %	μg found	Recovery %
10	8·6	86	9·0	90
50	49·4	98·8	52·1	104·2
100	91·2	91·2	94·6	94·6
200	184·0	92·0	194·5	97·3
$\bar{X} \pm s_x$		92·0 ± 4·6		96·5 ± 5·1
r_1	0·9997			
r_2			0·9995	

Table 5. Synopsis of quantitative data on urinary excretion of norantipyrine in man and rat following antipyrine dosage (as % of the dose).

Species	Sampling period h	Urinary excretion %	Methodology used	References
Rat	24	10–20	Colorimetry	Schüppel 1966
Man	24	6	Gc-ms	Baty & Price Evans 1973
Man	120	3–5	Hplc/[3-^{14}C]Antip.	Eichelbaum et al 1976
Man	48	18	Gc-ms	Tschanz et al 1977
Rat	24	2·6	*N*-$^{14}CH_3$-Antip.	Aarbakke 1978
Man	48	9	Hplc	Kellermann & Luyten-Kellermann 1978
Rat	24	15–32	Tlc	Böttcher et al 1979
Rat	24	6·2–8·6	Hplc	Danhof et al 1979b
Man	48	14·5	Hplc	Danhof et al 1979a
Man	52	16·5/17·3	Hplc	Danhof & Breimer 1979
Rat	6	13–15	[*N*-$^{14}CH_3$]Antip.	Inaba et al 1980
Man	36	15	Gc	Inaba & Fischer 1980
Man	48	11·3	Gc-ms	Toverud et al 1981
Man	48	27·5	Tlc/[3-^{14}C]Antip.	Böttcher et al 1981a
Man	72	18	Hplc	Eichelbaum et al 1981
Man	60	13·5	Gc-ms	Tang et al 1982

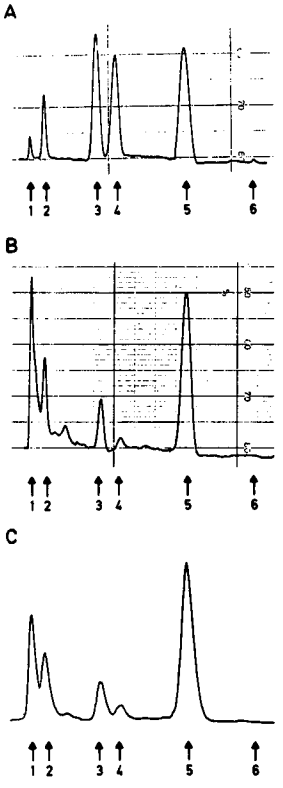

FIG. 5. Tlc-separation of norantipyrine from other phase-I-metabolites of antipyrine, solvent $CHCl_3$–EtOH (45:3). A: Tlc-scan of a mixture of authentic material, B: Tlc-scan of an extract obtained after selective acid hydrolysis of rat urine after dosage of [3-^{14}C]antipyrine, C: radioscan of the same extract. 1. Origin, 2. 3-Hydroxymethyl-antipyrine, 3. Antipyrine, 4. 4-Hydroxy-antipyrine, 5. Norantipyrine, 6. Front of solvent.

that norantipyrine is also unstable in chloroform, as can be taken from rapid deterioration of standard solutions of norantipyrine in chloroform, when not strictly kept cold and dark. The use of chloroform is thus only acceptable for isolation, when small volumes are used, which can quickly be evaporated under controlled conditions. Lipophilicity of norantipyrine facilitates such processing. Norantipyrine solutions are also sensitive to light, giving rise to yellow discoloration. Further, the volatility of norantipyrine deserves mentioning again.

To its conjugates, norantipyrine represents a substantial portion in the metabolic disposition of antipyrine in man and rat (Table 3). The wide range of data, reported so far for the excretion of norantipyrine in man and, in particular, in the rat, is remarkable (Table 5). This variation is most likely due to poor recovery of norantipyrine from its urinary conjugates, owing to incomplete cleavage of urinary conjugates, to partial decomposition of norantipyrine being liberated, or to loss by evaporation. In this context, enzymic hydrolysis of conjugates, e.g. in urine samples, seems to be particularly critical. The use of arylsulfatases from different sources (*Helix pomatia* vs *E. coli*) for example tends to give different results, as these enzymes seem to exhibit different sensitivities towards various inhibitors present in urine from man or rat (Dodgson & Spencer 1953; Levvy & Conchie 1966). In contrast, mild acid hydrolysis uniformly yields high recovery of norantipyrine of about 90–95% (Table 4). Selective acid hydrolysis thus offers a simple and rapid means for the differential quantitation of norantipyrine conjugates in biological material, enabling the

detection of interspecies differences in phase-II-metabolism of xenobiotics, as they are present between man and rat. At the dose level used in this study, in the rat only norantipyrine sulphate is formed. In man, norantipyrine glucuronide is the predominant or even sole conjugate excreted. This conjugation pattern resembles that found for 4-hydroxy-antipyrine and 4,4′-dihydroxy-antipyrine (Böttcher et al 1982a). For isoprenaline, a β-sympathicomimetic agent, a reverse conjugation pattern has been reported for man and rat (Davies 1977).

As might be expected from its high lipophilicity (Table 2), free norantipyrine has not been detected in fresh urine specimens after antipyrine dosage in either species. It is only after conjugation, that norantipyrine can be eliminated by the kidney. This is at variance with the other three phase-I-metabolites which are excreted as free metabolites to varying degrees, reflecting their hydrophilicity (Böttcher et al 1982a).

Pretreatment with 3-methylcholanthrene enhanced *N*-demethylation of antipyrine in the rat, as is evidenced by an increase of urinary norantipyrine sulphate output, at the expense of 3-hydroxymethyl-antipyrine and its glucuronide (Böttcher et al 1982d), confirming earlier reports (Inaba & Fischer 1980). A similar enhancement by 3-methylcholanthrene-induction has been reported for the microsomal C-4-hydroxylation of antipyrine (Danhof et al 1979b, Böttcher et al 1982d). Phenobarbitone induction was without effect on the *N*-demethylation of antipyrine in rats. From these results, it is concluded, that 3-methylcholanthrene-inducible isoenzymes of hepatic cytochrome P-450 play a major role in the biotransformation of antipyrine in the rat. Urinary metabolite profiles for both norantipyrine and 4-hydroxy-antipyrine will thus serve as tools for assessing activities of these segments of hepatic mixed-function oxidase in the rat.

REFERENCES

Aarbakke, J. (1978) Acta Pharmacol. Toxicol. 43: 64–68

Baty, J. D., Price Evans, D. A. (1973) J. Pharm. Pharmacol. 25: 83–84

Böttcher, J., Bässmann, H., Schüppel, R. (1979) Naunyn-Schmiedeberg's Arch. Pharmacol. 307 (Suppl.): R 8

Böttcher, J., Bässmann, H., Schüppel, R. (1980) Ibid. 311 (Suppl.): R 17

Böttcher, J., Bässmann, H., Schüppel, R. (1981a) Ibid. 316 (Suppl.): R 5

Böttcher, J., Bässmann, H., Schüppel, R. (1981b) in: Gustafsson, J. Å. et al (eds) Biochemistry, Biophysics and Regulation of Cytochrome P-450, Elsevier/North-Holland Biomedical Press, Amsterdam, pp 81–83

Böttcher, J., Bässmann, H., Schüppel, R. (1982a) J. Pharm. Pharmacol. 34: 168–175

Böttcher, J., Bässmann, H., Schüppel, R. (1982b) Drug Metab. Dispos. 10: 90–91

Böttcher, J., Bässmann, H., Schüppel, R., Lehmann, W. D. (1982c) Naunyn-Schmiedeberg's Arch. Pharmacol. 321: 226–233

Böttcher, J., Bässmann, H., Schüppel, R. (1982d) in: R. Sato et al (eds) Microsomes, Drug Oxidations, and Drug Toxicity, Japan Scientific Societies Press, Tokio, pp 441–442

Danhof, M. (1980) Antipyrine metabolite profile as a tool in the assessment of the activity of different drug oxidizing enzymes in man, Proefschrift University Leiden

Danhof, M., Breimer, D. D. (1979) Br. J. Clin. Pharmacol. 8: 529–537

Danhof, M., de Groot-van der Vis, E., Breimer, D. D. (1979a) Pharmacology 18: 210–223

Danhof, M., Krom, D. P., Breimer, D. D. (1979b) Xenobiotica 9: 695–702

Davies, D. S. (1977) in: Parke, D. V., Smith, R. L. (eds) Drug metabolism—from microbe to man. Taylor & Francis Ltd, London p 358

Dodgson, K. S., Spencer, B. (1953) Biochem. J. 55: 315–320

Eichelbaum, M., Schomerus, M., Spannbrucker, N., Zietz, E. (1976) Naunyn-Schmiedeberg's Arch. Pharmacol. 293 (Suppl.): R 63

Eichelbaum, M., Sonntag, B., Dengler, H. J. (1981) Pharmacology 23: 192–202

Emerson, E., Beagle, L. C. (1943) J. Org. Chem. 8: 429–437

Kahn, G. C., Boobis, A. R., Blair, I. A., Brodie, M. J., Davies, D. S. (1981) Analyt. Biochem. 113: 292–300

Kellermann, G. H., Luyten-Kellermann, M. (1978) Life Sci. 23: 2485–2490

Knorr, L. (1887) Liebigs Ann. Chem. 238: 137–219

Knorr, L., Pschorr, P. (1896) Ibid. 293: 49–55

Inaba, T., Fischer, N. E. (1980) Can. J. Physiol. Pharmacol. 58: 17–21

Inaba, T., Lucassen, M., Kalow, W. (1980) Life Sci. 26: 1977–1983

Levvy, G. A., Conchie, J. (1966) in: Dutton, G. J. (ed.) Glucuronic Acid, free and combined. Academic Press, N.Y.

Schüppel, R. (1966) Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol. 255: 71–72

Schüppel, R. (1968) Pharmakokinetische Untersuchungen an der alkoholisierten Ratte, Diss. Math.-Naturw. Fakultät Universität Tübingen

Schüppel, R. (1969) Naunyn-Schmiedeberg's Arch. Pharmacol. 265: 156–169

Tang, B. K., Uchino, H., Inaba, T., Kalow, W. (1982) Biomed. Mass Spectrom. 9: 425–428

Toverud, E.-L., Boobis, A. R., Brodie, M. J., Murray, S., Bennett, P. N., Whitmarsh, V., Davies, D. S. (1981) Eur. J. Clin. Pharmacol. 21: 155–160

Tschanz, C., Hignite, C. E., Huffman, D., Azarnoff, D. L. (1977) Cancer Res. 37: 3881–3886