Journal of Chromatography, 563 (1991) 103–113
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5627

High-performance liquid chromatographic method for the determination of the three main oxidative and 3-carboxylic antipyrine metabolites in human urine

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 May 30th, 1990; revised manuscript received August 30th, 1990)

ABSTRACT

The oxidative metabolism of xenobiotics is usually explored using the antipyrine test, which consists of determining the production clearances and urinary percentages of three major antipyrine metabolites 4-hydroxyantipyrine, norantipyrine and 3-hydroxymethylantipyrine. The total forms of these compounds are generally determined by high-performance liquid chromatography (HPLC). However, the 3-carboxylic acid metabolite (3-carboxyantipyrine), which is the ultimate oxidation form in the 3-hydroxylation pathway, should also be taken into account, but so far its determination by HPLC has not been reported. A simple and accurate HPLC method has now been developed to determine the three major metabolites plus 3-carboxyantipyrine. In this method, all compounds are extracted in an aprotic non-polar solvent, at pH 3.5 for the major metabolites and unchanged antipyrine, then at pH 0.9 for 3-carboxyantipyrine. Total forms are evaluated after enzymatic hydrolysis. Throughout the procedure, attention is paid to the relative instability of norantipyrine and 4-hydroxyantipyrine. Recovery, accuracy and precision are discussed. The method has been applied to the determination of relative amounts (percentage of the dose administered) excreted in the urine of ten adult subjects 48 h after ingestion of antipyrine (600 mg). The proportion of 3-carboxyantipyrine excreted was 4.5 ± 0.2%, which is in agreement with published values obtained by gas chromatography. The excretion rates of the major metabolites also were similar to those reported in the literature, thereby confirming that the reported method is valid. 3-Carboxyantipyrine is totally excreted as the free form and norantipyrine almost completely as glucuroconjugate.

INTRODUCTION

Antipyrine is widely used to study hepatic monooxygenase activity in man [1]. Its use as a marker to investigate the potential of new drugs to alter microsomal oxidation is based on the determination of total forms of its urinary excreted metabolites and variation of their production clearance [2–9].

The phase I antipyrine metabolites known in man are 4-hydroxyantipyrine (4-OH) [10], 3-hydroxymethylantipyrine (3-CH_2OH) [11] and norantipyrine (NOR) [12,13], these being called the major metabolites. Other known metabolites are antipyrine 3-carboxylic acid (3-COOH) [14], formed from 3-CH_2OH,

0378-4347/91/$03.50

and the aromatic hydroxylation derivatives 4′-hydroxyantipyrine (4′-OH) [15] and 4,4′-dihydroxyantipyrine (4,4′ diOH) [16].

The antipyrine metabolites 4-OH, 3-CH_2OH and NOR have been studied using thin-layer chromatography (TLC) [15–22] and high-performance liquid chromatography (HPLC), usually in the reversed-phase mode [23–30]. Numerous publications indicate the difficulties encountered owing to the diversity of metabolites and the instability of some of them (NOR, 4-OH, phenol derivatives), and to hydrolytic cleavage, which varies according to the conditions of hydrolysis and the nature of the conjugates [20,22], this nature itself being dependent on the dose of antipyrine administered in man (after oral administration of less than 15 mg/kg body weight, only 5% of the dose is recovered in urine as sulphoconjugated 4-OH, and there is no sulphoconjugated NOR [17]. As cumulative excretion of radioactivity in urine after ingestion of ring-labelled [3-^{14}C]antipyrine over 48 h is about 95% of the dose [18,21], the urinary recovery of major metabolites and unchanged antipyrine over the same period is only about 60–70% of the dose [4–7,23,24,31,32]. The metabolites that are not accounted for are arylhydroxyl derivatives and 3-COOH, representing together 10–13% of the dose ingested [15,18,24]. Other undetected metabolites might consist of oxidation products (as yet unidentified) and possibly pyrazolone degradation products [21,33].

In pharmacokinetic studies, evaluation of the percentages of antipyrine metabolites excreted in the urine means that the total forms (*i.e.* free plus conjugated) of these metabolites must be determined after hydrolysis. Enzymatic hydrolysis at pH 4.5–5.0 ensures deconjugation of the major metabolites present in man, but these pH values preclude the determination of arylhydroxyl metabolites [19,26]. The 3-COOH metabolite, which is stable at all pH levels, is excreted as a free compound [34]. It has been determined by gas chromatography [34] and by TLC [18,35], but quantification was not accurate [35] and, so far, all attempts at determination by HPLC have failed [23,34]. During studies of the antipyrine oxidative metabolism by standard HPLC methods, the 3-hydroxylation pathway is underestimated if excretion as the 3-COOH derivative is not taken into account.

For this reason, we propose a reversed-phase HPLC method which makes it possible to determine the major metabolites and the 3-COOH metabolite of antipyrine. Owing to the initial enzymatic hydrolysis, the arylhydroxyl derivatives cannot be determined by this method.

EXPERIMENTAL

Chemicals

Antipyrine and pyramidon [used as an internal standard (I.S.)] were purchased from Sigma (Paris, France), NOR and 4-OH from Jansen Chimica (Beerse, Belgium) and benzofuran-2-carboxylic acid (used as an I.S. for 3-COOH determination) from Aldrich (Strasbourg, France). 3-CH_2OH was synthesized according to the method described by Danhof *et al.* [36]. 3-COOH, synthesized according to

the method described by Yoshimura *et al.* [14], was a gift from Professor M. Robba (Laboratoire de Chimie Thérapeutique, UFR des Sciences Pharmaceutiques, Université de Caen, Caen, France).

Glacial acetic acid, orthophosphoric acid (84%), sodium acetate (trihydrate), sodium chloride and disodium hydrogenphosphate (dihydrate) of Normapur quality were purchased from Prolabo (Paris, France). Acetonitrile, dichloromethane and methanol of LiChrosolv quality were purchased from Merck (Darmstadt, F.R.G.). Sodium metabisulphite, *n*-decylamine and lyophilized powder of *β*-glucuronidase plus sulphatase from *Helix pomatia* (ref. G-1512) were purchased from Sigma.

Reference and stock solutions

Standards. Working standard solutions were prepared from a stock standard solution in methanol containing antipyrine (100 μg/ml), 3-COOH (200 μg/ml), 3-CH_2OH (500 μg/ml), NOR (600 μg/ml) and 4-OH (1000 μg/ml) by stepwise dilution with methanol to half, fifth and twentieth concentrations). All solutions were kept at −30°C.

Internal standards. Benzofuran-2-carboxylic acid was used for the determination of 3-COOH and pyramidon for the determination of all other metabolites.

Acetate buffer. A 0.25 *M* stock solution of acetate buffer (pH 5.2) consisting of 27 g/l sodium acetate trihydrate and 3 ml/l glacial acetic acid in distilled water was kept for several months at 4°C. A working acetate buffer solution (pH 4.9) was prepared every day by addition of 2% (w/v) of sodium metabisulphite to the stock solution.

Washing buffer. A washing buffer solution of 0.5 *M* disodium hydrogenphosphate (pH 8.8) containing 35 g/l sodium chloride was adjusted to pH 7.8 with 38% potassium hydroxide solution.

Apparatus and chromatographic conditions

The HPLC system used consisted of a Varian Model 5020 pump with a Valco manual injector equipped with a 50- or 20-μl loop (Varian, Les Ulis, France) and coupled with a Kratos Spectroflow 773 spectrophotometer (Cunow, Cergy, France) and a Shimadzu CR-4A integrator (Touzart et Matignon, Vitry sur Seine, France) set at 5 mm/min. Chromatography was carried out on a column (150 mm × 4.6 mm I.D.) packed with C_8 Ultrasphere (particle size 5 μm) (Beckman, Gagny, France). The device was completed with a precolumn (C_8, 10 μm) (20 mm × 4.6 mm I.D.).

The mobile phase for the determination of 3-COOH was 0.5% (v/v) glacial acetic acid containing 2 m*M* (400 μl/l) *n*-decylamine (pH 3.5)–acetonitrile (77:23, v/v). The mobile phase for the determination of antipyrine and the others metabolites was 15% (v/v) acetonitrile in an aqueous buffer solution (pH 3.8–3.9) prepared with 5 ml/l acetic acid, 3 g/l sodium acetate trihydrate and containing 0.10 m*M* (20 μl/l) *n*-decylamine. The flow-rate of the mobile phases was 1.0 ml/min. The column effluent was monitored at 242 nm.

Human experiments

Eleven healthy volunteers (five men and six women) who had not received any medication during the previous seven days participated in the project. After an overnight fast, antipyrine (600 mg) was administered orally with 150 ml of water.

Urine samples were collected on sodium metabisulphite (1 g per fraction) over 48 h in four fractions of 12 h. Samples were stored at −30°C until analysed.

Extraction procedures

All extractions were carried out in glass-stoppered centrifuge tubes (15 ml).

Hydrolysis. To 100 μl of centrifuged urine sample, 1 ml of working acetate buffer (pH 4.9) and 50 μl of a reconstituted aqueous solution of β-glucuronidase–sulphatase (corresponding to 5000–6000 IU of β-glucuronidase and 150–200 IU of sulphatase in the 50 μl) were added.

Extraction of antipyrine, NOR, 3-CH_2OH and 4-OH. The mixture was extracted with 8 ml of dichloromethane and 1 g of sodium chloride for 10 min on a linear agitator and then centrifuged (5 min, 1500 *g*). The organic layer was collected, 1 ml of washing buffer solution was added and the mixture was mixed on a linear agitator for 5 min, then centrifuged (5 min at 1500 *g*) and 500 μl of water were added to the organic phase (to prevent NOR sublimation). Only the dichloromethane present in this mixture was evaporated to dryness at 37°C under a stream of nitrogen and 500 μl of methanol were immediately added. After homogenization, 20 μl of the mixture were injected into the chromatograph to determine unchanged antipyrine and total forms of NOR, 3-CH_2OH and 4-OH.

Extraction of 3-COOH. To 500 μl of the previous aqueous layer, 2 μg of benzofuran-2-carboxylic acid [as internal standard in 100 μl of methanol–water (50:50, v/v)], 100 μl of aqueous orthophosphoric acid solution (1:4, v/v) and 0.5 g of sodium chloride were added and the mixture was extracted with 6 ml of methylene chloride for 10 min on a linear agitator and then centrifuged (5 min, 1500 *g*). The organic layer was collected and evaporated to dryness at 37°C under a stream of nitrogen. The residue was dissolved in 500 μl of the mobile phase for the determination of 3-COOH and 50 μl were injected into the chromatograph.

Quantification of free forms

The determinations of antipyrine and metabolites excreted in urine as the free form can be performed with the same procedure without addition of β-glucuronidase and the incubation step.

Calibration graphs

Calibration graphs were obtained by spiking drug-free urine samples (100 μl) with working stock solutions (50 μl). To each sample, 1 ml of working acetate buffer (pH 4.9), 50 μl of water, 20 μg of pyramidon (as I.S. in 100 μl of water) and 1 g of sodium chloride were added. All samples were extracted with 8 ml of dichloromethane and analysed according to the procedure described above.

Four-point calibration graphs were obtained for each compound. For the determination of the total forms, the 3-CH_2OH concentration ranged from 12.5 to 250 μg/ml, NOR from 15 to 300 μg/ml and 4-OH from 25 to 500 μg/ml. The determination of antipyrine and metabolites excreted in urine as the free form can be performed with the same procedure without addition of β-glucuronidase and the incubation step.

For the free forms, the concentrations for the calibration graphs ranged from 2.5 to 50 μg/ml for antipyrine, 5 to 50 μg/ml for 3-CH_2OH, 1 to 10 μg/ml for 4-OH and 5 to 100 μg/ml for 3-COOH.

Controls were also analysed with the hydrolysis step.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 1 shows the chromatograms obtained after extraction of antipyrine and the three major metabolites (3-CH_2OH, 4-OH and NOR) from a 24-h human urine sample obtained after ingestion of 600 mg of antipyrine. All the derivatives identified before or after hydrolysis and the internal standard are well separated and no interfering peaks are present.

Fig. 2 shows the separation of the 3-COOH metabolite after extraction of the same human urine sample. The chromatogram also shows an early peak corresponding to hippuric acid extracted from urine and an unidentified peak that is probably related to antipyrine metabolism (*cf. Analytical variables*).

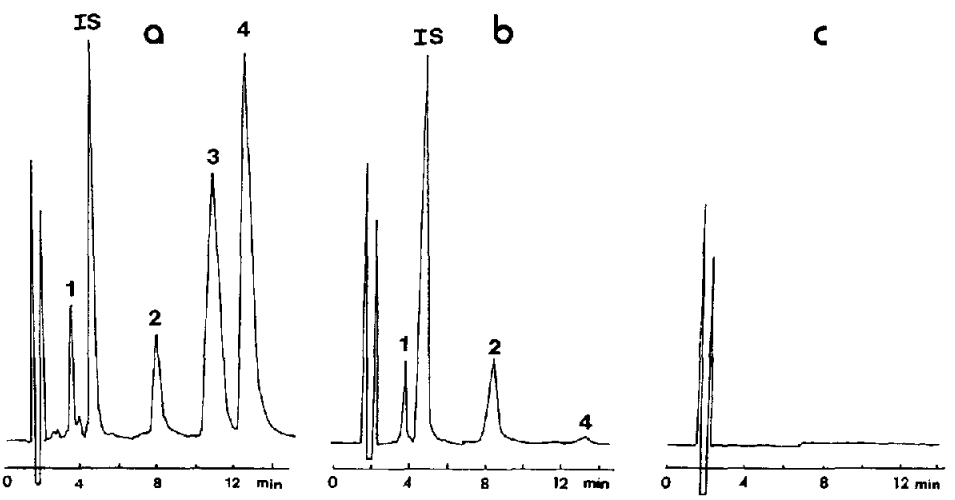

Fig. 1. Chromatographic separation of (2) antipyrine and major metabolites, (1) 3-CH_2OH, (3) NOR, (4) 4-OH and pyramidon (IS) after extraction of a 24-h urine sample obtained after intake of 600 mg of antipyrine *per os* by a volunteer. (a) After hydrolysis (total forms): 1 = 58.6; 2 = 13.3; 3 = 67.2; 4 = 78.8 mg/l. (b) Without hydrolysis (free forms). (c) Blank urine after hydrolysis.

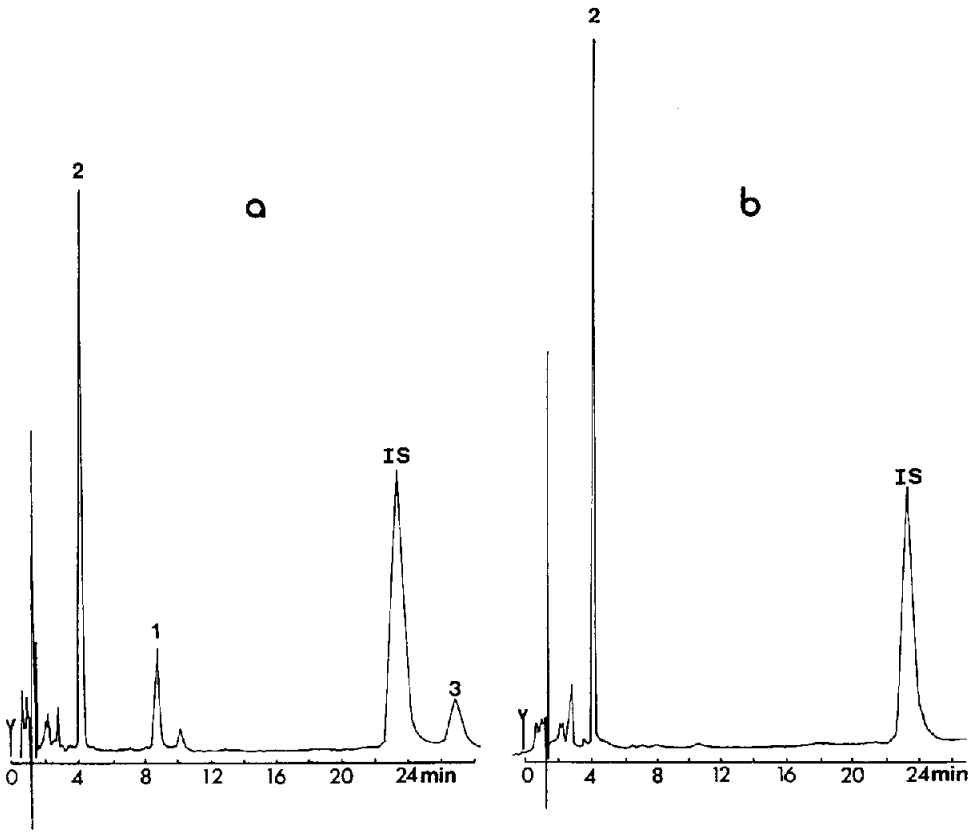

Fig. 2. Chromatogram obtained after urinary extraction of 3-COOH metabolite from the same volunteer as in Fig. 1. (a) 24 h after antipyrine ingestion, (b) blank urine before antipyrine ingestion. 1 = 3-COOH (16.3 mg/l); IS = benzofuran-2-carboxylic acid; 2 = hippuric acid; 3 = unidentified.

Recovery

The absolute recoveries were calculated by dividing the peak height for the extracted solution by the peak height obtained by injecting equivalent amounts of unextracted standard solution. The values were 74.2 ± 0.37% for 3-COOH (n = 7), 84.4 ± 0.38% for 3-CH_2OH, 83.9 ± 1.37% for NOR, 96.3 ± 1.37% for 4-OH and 95.8 ± 1.37% for antipyrine (n = 6). The recoveries were not affected by the washing procedure.

Linearity

Linearity was observed in the ranges 2.5–100 μg/ml for antipyrine, 25–1000 μg/ml for 4-OH, 12.5–500 μg/ml for 3-CH_2OH, 15–600 μg/ml for NOR and 5–200 μg/ml for 3-COOH. The linear regression equations and correlation coefficients are given in Table I.

TABLE I

LINEARITY OF CALIBRATION GRAPHS

Compound	$y = ax + b$		Correlation coefficient (r)
	a	b	
Antipyrine	13.46	−0.0074	0.9998
3-CH_2OH	23.45	−0.0740	0.9998
3-COOH	4.28	−0.0257	0.9994
4-OH	14.50	−0.1200	0.9998
NOR	26.75	+0.0779	0.9998

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF ANTIPYRINE AND ITS METABOLITES IN SPIKED URINE SAMPLES

Compound	Concentration given (μg/ml)	Concentration observed (mean ± S.D.) (μg/ml)	R.S.D.[a] (%)	Relative error (%)
Day-today variation (n = 11)				
Antipyrine	0.5	0.46 ± 0.02	3.8	−8.0
	5	4.81 ± 0.08	1.8	−3.8
3-CH_2OH	2	2.06 ± 0.05	2.3	+0.0
	20	19.8 ± 0.42	2.1	−1.0
3-COOH	0.75	0.73 ± 0.04	5.8	−2.7
	7.5	7.39 ± 0.15	2.1	−1.5
4-OH	4	3.88 ± 0.12	3.2	−3.0
	40	39.5 ± 0.41	1.0	−1.25
NOR	2.5	2.30 ± 0.07	3.2	−8.0
	25	25.1 ± 0.70	2.8	+0.4
Within-day variation (n = 10)				
Antipyrine	0.5	0.47 ± 0.01	1.9	−6.4
	5	4.88 ± 0.05	1.1	−2.4
3-CH_2OH	2	2.09 ± 0.03	1.7	+4.5
	20	20.1 ± 0.15	0.8	+0.8
3-COOH	0.75	0.73 ± 0.03	4.2	−2.6
	7.5	7.59 ± 0.08	1.1	+1.2
4-OH	4	3.91 ± 0.03	0.7	−2.2
	40	40.0 ± 0.60	1.5	+0.0
NOR	2.5	2.21 ± 0.03	2.2	−11.6
	25	25.4 ± 0.30	1.0	+1.6

[a] Relative standard deviation.

Detection limit

The detection limits corresponding to a signal-to-noise ratio of 3:1 were 0.5 μg/ml for antipyrine and 3-COOH and 1 μg/ml for 3-CH_2OH, 4-OH and NOR.

Accuracy

To assess the accuracy of the procedure, reproducibilities for both day-to-day and within-day variations were determined (Table II). The relative standard deviations for two concentrations of antipyrine and each metabolite in the within-day study varied between 0.7 and 4.2% and in the day-to-day study varied between 1 and 5.8%. Accuracy was evaluated by comparing the given amounts of the substances with those determined. The observed concentrations were in good agreement with the actual concentrations; the relative error ranged from −3.8 to 1.6% for the upper concentrations of all substances and from −8.0 to 4.5% for the lower concentrations of the substances, except for NOR, the relative error for which ranged from −11.6 to −8%, attesting to the instability of this metabolite.

Analytical variables

The necessity of a highly acidic medium (pH 0.9) for the extraction of 3-COOH and the presence of endogenous urinary interfering compounds requires a prior extraction of antipyrine and the main metabolites before that of 3-COOH. The former are extracted from the aqueous phase from hydrolysis at pH 3.5 and the organic phase from extraction must be washed with a buffer solution at pH 7.8 to eliminate the endogenous urinary interfering compounds. The 3-COOH solute is then extracted from the residual acidified aqueous phase from hydrolysis. If this is done, the organic phase from 3-COOH extraction is clean. This phase still contains an endogenous urinary compound, identified as hippuric acid by coupled gas chromatography–mass spectrometry, and an as yet unidentified compound with a pyrazol-5-one structure. These extraction pH values are obtained after the addition of large amounts of sodium chloride, which facilitates the extraction of soluble compounds in dichloromethane. They differ from the pH values of buffers resulting from an increased ionic strength and from a retrogressive hydrolysis of the ionic species constituting the buffer solution (acetates, phosphates, sulphites).

The proposed one-stage global extraction procedure with pH modulation is simpler and faster than the usual methods previously reported, which require mixed solvents [20,23,26] and which also have the disadvantage of not extracting 3-COOH.

For antipyrine and its major metabolites, chromatographic separations are performed by a reversed-phase mechanism with acetate buffer (pH 4.2) as the mobile phase. *n*-Decylamine at a low concentration (0.1 m*M*) limits the access to free silanol sites and ensures regular chromatographic peaks. For the 3-COOH metabolite, chromatographic separations are performed by a reversed-phase mechanism with ion pairing by circulation of a mobile phase (pH 3.5) containing

2 m*M* *n*-decylamine, which ensures equilibrated decylammonium–carboxylate paired ions for the metabolite and its internal control. This double separation procedure is necessary for optimum resolution of the different metabolites and to obtain clean and reproducible chromatograms.

Stability of antipyrine metabolites

The main difficulties encountered in determining antipyrine metabolites are due to the instability of NOR and 4-OH in the free forms, whereas 3 CH_2OH, 3-COOH and the conjugated forms of 4-OH and NOR are stable [20, 26, 34]. Protection against oxidative degradation at pH > 6 is ensured by addition of sodium metabisulphite [20,23,24,26]. We found that adding 1 g of sodium metabisulphite to each of the urine fractions collected over 12 h was sufficient to protect free 4-OH. Protecting NOR requires, on top of this, the addition, before deconjugation, of sodium metabisulphite to the buffer solution which sets the pH of hydrolysis.

We found no adsorption of NOR on the glass walls [23,24], even non-silanized, but we observed a loss of NOR by sublimation after complete desiccation of the extracts under nitrogen at 37 or 30°C. Controlled evaporation of dichloromethane in the presence of water and redissolving this water extract in methanol ensures a suitable stability of the 4-OH and NOR solutes.

Urinary excretion pattern in human antipyrine metabolism

The urinary metabolite pattern observed after oral administration of 600 mg of antipyrine is shown in Table III. The cumulative urinary excretion is about 72% of the dose, or 67–68% if the 3-COOH metabolite is excluded. These values, and also individual excretion values of the major metabolites, are in agreement with those previously reported [4–6,21,23,36], although some workers [3,7,8,24,25] have reported cumulative excretion values of 40–60% and lower individual values for 4-OH [8], NOR [3,8,25] and 3-CH_2OH [7,25]. The excretion values of 3-COOH (4.5 ± 0.2% of the dose of antipyrine administered, 6–6.5% of the cumulative excretion) are concordant with those reported in the literature after determination by gas chromatography [5,34] or radiometric TLC assay [18]. Determinations of the free and conjugated forms also confirmed the results of previous studies [18,19,22,28,34] and revealed that 3-COOH is excreted only as the free metabolite, whereas conjugated forms constitute 60% of 3-CH_2OH excretion, 98–99% of 4-OH excretion and the totality of NOR excretion. The NOR value observed in this study is consistent with the value reported for the determination of the nor-antipyrine glucuronide derivative [28] in urine, and it confirms that NOR is totally excreted as the glucuroconjugate.

The proposed method is suitable for evaluating hepatic microsomal activity by determination of the antipyrine metabolites excreted in urine. Simultaneous determination of the 3-COOH metabolite should provide a better approach to biotransformations by 3-hydroxylation.

TABLE III

URINARY METABOLITE PROFILES OF ANTIPYRINE FOLLOWING ORAL ADMINISTRATION OF 600 mg OF ANTIPYRINE (% DOSE EXCRETED IN 48 h)

Compound	Species	Percentage dose excreted in 24 h											
		1	2	3	4	5	6	7	8	9	10	11	Mean ± S.E.M.[a]
3-CH_2OH	Total	17.0	20.9	18.2	18.4	18.6	14.6	12.5	16.9	13.5	13.7	12.9	16.1 ± 0.8
	Conjugate	11.1	15.5	12.1	10.8	11.5	8.8	4.5	7.5	8.4	9.3	8.7	9.8 ± 0.9
	Free	5.9	5.4	6.1	7.6	7.1	5.8	8.0	9.4	5.1	4.4	4.2	6.3 ± 0.5
4-OH	Total	28.7	36.1	31.8	34.4	29.3	32.5	21.4	30.0	28.7	28.9	27.0	29.9 ± 1.2
	Conjugate	28.4	35.9	31.3	34.0	28.9	32.0	21.1	29.5	28.4	28.5	26.8	29.5 ± 0.7
	Free	0.3	0.2	0.5	0.4	0.4	0.5	0.3	0.5	0.3	0.4	0.2	0.36 ± 0.03
NOR	Conjugate	17.8	24.0	22.3	17.0	17.6	23.3	22.5	14.2	19.7	19.5	12.5	19.1 ± 1.1
3-COOH	Free	3.8	3.4	3.4	5.4	5.2	4.2	6.0	4.0	4.7	4.6	4.5	4.5 ± 0.2
Antipyrine	Free	1.6	3.4	4.3	1.9	3.5	6.2	5.8	2.8	3.1	2.7	2.5	3.4 ± 0.4
Total		68.9	87.8	80.0	77.1	74.2	70.8	68.2	67.9	69.7	69.4	59.4	72.1 ± 2.2

[a] Standard error of the mean.

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