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Separation and identification of phase I and phase II [^{14}C]antipyrine metabolites in rat and dog urine

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Abstract

A simple and accurate HPLC procedure was developed to quantify, in a single run, all phase I and phase II [^{14}C]antipyrine metabolites that occur in rat and dog urine. All metabolites were subjected to thermospray-LC–MS and EI-MS in order to establish their structure. The rat metabolizes antipyrine to eight major metabolites, six of which are conjugated; 1.4% of the dose was excreted unchanged, 18.9% in a free form, 30.6% as sulfates and 21.1% as glucuronides. The dog metabolizes antipyrine to four metabolites, all as sulfate (61.0% of the dose) or glucuronide conjugates (16.2% of the dose).

1. Introduction

Antipyrine, one of the classic antipyretic and analgesic drugs, has been extensively used to study the influence of age, diseases, drugs, hereditary and environmental factors on human and animal in vivo and in vitro oxidative hepatic enzyme activity [1,2]. Antipyrine is metabolized by several forms of cytochrome P-450 into four major metabolites (Fig. 1), i.e. 3-hydroxymethylantipyrine (HMA), 4-hydroxyantipyrine (OHA), 4,4′-dihydroxyantipyrine (DOHA) and norantipyrine (NORA), all of which are further extensively metabolized by conjugation.

Several groups have investigated the effects of

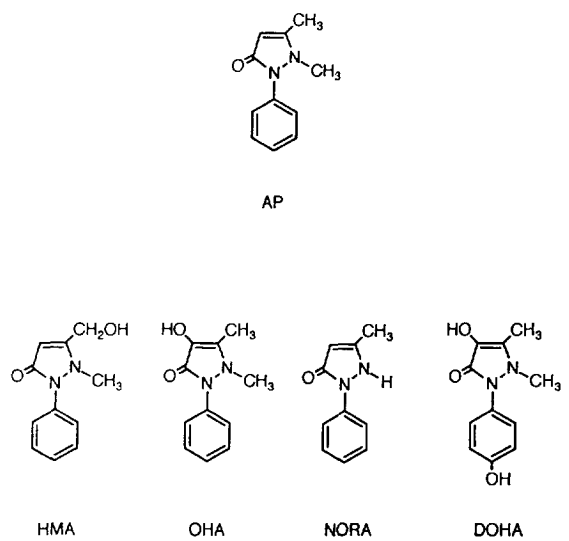

Fig. 1. Structures of antipyrine and four major phase I metabolites.

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cytochrome P-450 induction and inhibition upon the pattern of phase I metabolites of antipyrine in rat urine [3–6], human urine [7–9], and isolated and cultured hepatocytes [10,11], while no data are available for the metabolism of antipyrine in the dog.

A considerable number of methods for the determination of antipyrine and its phase I metabolites have been published [12–14]; more recently, phase II metabolites have been identified [11,15,16].

However, studies of antipyrine metabolism are still hampered by the lack of a convenient determination method for all phase I and phase II metabolites as they appear in biological materials. Hydrolysis of conjugates, either enzymatic or chemical, still represents the standard approach. Yet problems arise due to the stability of some conjugates with regard to hydrolysis, the particular instability of free and deconjugated NORA and OHA owing to oxidation, and the volatility of NORA after desiccation.

In the present study, a reversed-phase, ion-pairing HPLC assay procedure was developed for the separation of all phase I and phase II antipyrine metabolites that occur in rat urine after intravenous dosing with [^{14}C]antipyrine. This procedure can be used to characterize cytochrome P-450 enzymes in the liver. In order to establish reliably the identity of the antipyrine metabolites, all peaks eluting from the HPLC system were collected separately, purified and analyzed using thermospray-LC–MS and EI-MS.

The metabolic profile obtained for rat urine was compared with the metabolic pattern in dog urine.

2. Experimental

2.1. Chemicals and reagents

Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazoline-5-one) was from Merck (Darmstadt, Germany). 3-[^{14}C]-Antipyrine was purchased from Sigma (St. Louis, MO, USA) and had a radiochemical purity of 99% as determined by thin layer chromatography. Deuterated antipyrine ($^{2}H_5$-phenyl) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). PIC A reagent (tetrabutylammoniumphosphate; TBAP) and SepPak C_{18} cartridges were purchased from Millipore (Eschborn, Germany). Glucuronidase/arylsulfatase from *Helix pomatia* was from Boehringer Mannheim (Mannheim, Germany). All solvents used were of analytical grade and were from Merck.

2.2. Animal experiments

Male Sprague–Dawley rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany) weighing 250–300 g were kept on a standard diet (Altromin 1324, Laage, Germany) and tap water throughout the experiment. Antipyrine was dissolved in saline. Three rats received an intravenous dose of 30 mg/kg [^{14}C]antipyrine (specific activity of 10.85 MBq/mmol; [^{14}C]antipyrine: [^{2}H]antipyrine, w/w = 2:1). The animals were placed in individual metabolism cages and urine and faeces were collected separately after 24 and 48 h. A portion of each urine sample, containing ca. 3000 Bq, was subjected immediately to HPLC; the remainder was frozen and stored at −20°C.

Male beagle dogs weighing 9–10 kg were obtained from Hazleton (Hazleton, UK). They were fed a commercial diet (Expan, Eggersmann, Rinteln, Germany) and had free access to water during the entire course of the experiment. Three dogs were administered an intravenous dose of 10 mg/kg [^{14}C]antipyrine (specific activity of 8.6 MBq/mmol). They were placed in individual metabolic cages where urine and faeces were collected separately for 24 and 48 h. A portion of each urine sample, containing ca. 3000 Bq, was analyzed immediately; the remainder was frozen and stored at −20°C.

2.3. Apparatus and chromatographic conditions

HPLC

Urine was injected directly onto the chromatographic system. The HPLC system I consisted of a L6200 Intelligent pump (Merck), a variable-wavelength UV detector (L4000, Merck) oper-

ated at 254 nm and a flow-through radioactivity monitor filled with YG-150-Yttriumglass solid scintillator (effective cell volume: 150 μl; LB 506, Berthold, Wildbad, Germany). A 12.5 cm × 4 mm I.D. LiChroCart column packed with LiChrospher 60 RP Select B, 5 μm (Merck) was used, preceded by a 2.5 cm × 4 mm I.D. LiChrospher 100 RP 18 precolumn (5 μm) (Merck). The mobile phase consisted of solution A (0.005 *M* TBAP in water) and solution B (0.005 *M* TBAP in methanol), run using the following linear gradient segments: 0 min: 10% B; 10 min: 13% B; 26 min: 33% B; 35 min: 40% B; 45 min: 100% B; 50 min: 100% B; and 55 min: 10% B. The flow-rate was set at 0.7 ml/min, resulting in a pressure of about 60 bar. The system was operated at room temperature.

Isolated metabolites from rat urine were purified under the following conditions: a 12.5 cm × 4 mm I.D. LiChroCart column packed with LiChrospher 100 RP 18, 5 μm (Merck) was used. The mobile phase was: solution A = 0.02 *M* triethylammonium acetate in water (pH 4.0); solution B = ethanol. The following linear gradient segments were run: 0 min: 5% B; 40 min: 20% B; 45 min: 100% B; 50 min: 100% B; and 55 min: 5% B. The flow-rate was set at 0.8 ml/min.

Thermospray-LC–MS

Purified metabolites were dissolved in methanol and subjected to thermospray-LC–MS. Spectra were recorded on a SSQ 70 Finnigan MAT (Bremen, Germany). In order to optimize TSP performance, ammonium acetate (0.05 *M*) at 2.1 ml/min was added between the radio-detector and the TSP interface. The jet temperature was 250°C, the vaporizer temperature 105°C, and scan mode was *m/z* 160–600 in 2 s for positive ions and *m/z* 182–600 in 2 s for negative ions. HPLC conditions: the mobile phase consisted of solution A [0.02 *M* ammonium acetate in water (pH 6.8)] and solution B (methanol), run with the following linear gradient segments: 0 min: 0% B; 30 min: 100% B; 35 min: 100% B; and 40 min: 0% B at a flow-rate of 0.7 ml/min.

The HPLC system (II) consisted of a Waters 600 MS pump (Millipore), a Novapak NV C_{18}, 4 μm, 300 × 3.9 mm I.D. column (Millipore), a Waters U6K injector (Millipore), a Waters 490 MS detector (Millipore) set at 254 nm and a Ramona radioactivity monitor (Raytest, Straubenhardt, Germany) operated in the heterogenous mode with a 160-μl flowcell filled with glass scintillator.

EI-MS

A double focusing VG 7035 (Fisons Instruments, Mainz, Germany) was used in the electron-impact ionization mode: electron energy: 80 eV, emission: 90 μA (trap current) and source temperature 140°C. Samples were introduced with a vacuum-lock direct insertion probe.

Characterization of dog metabolites was achieved by cochromatography with characterized rat metabolites.

2.4. Hydrolysis of conjugates

Isolated conjugated metabolites were enzymatically hydrolyzed using β-glucuronidase/arylsulfatase (pH 4.6, 37°C for 24 h and then at pH 6.2, 37°C for another 24 h) and purified by SepPak C_{18} extraction. SepPak C_{18} cartridges (sorbent: 360 mg; particle size: 80 μm; hold-up volume: 0.85 ml) were conditioned with 10 hold-up volumes of methanol and flushed with 10 hold-up volumes of water. A 0.5-ml volume of the sample, dissolved in water was loaded and unwanted components eluted with water. The samples were eluted with methanol.

3. Results and discussion

Rats excreted 69–73% of the dose in the urine within 24 h after administration, followed by 2–4% within the next 24 h. Dogs excreted 80–82% of the dose in the urine within 24 h, whereas 2–3% was excreted in the next 24 h.

A new analytical method for the examination of native urine had to be developed in order to avoid all problems and losses that arise through hydrolysis of conjugates. Urine was applied to columns with different phases (C_8, C_{18}, Cyano, Amino, Select B) or a combination of columns (C_{18} and amino) using different buffers (am-

monium acetate, potassium phosphate, triethylammonium acetate) and strong solvents (methanol, ethanol, acetonitrile). None of these conditions tested resulted in a good separation of the metabolites, although best results were obtained using the LiChrospher Select B column, so all following chromatography was done using a Select B column. Introduction of the ion-pairing reagent tetrabutylammonium acetate (TBAP) dramatically improved the separation using a linear gradient from 0 to 100% methanol. The selectivity was further improved by dividing the linear gradient into linear gradient segments resulting in our final ion-pairing HPLC system.

Fig. 2 shows chromatograms of the antipyrine metabolites in rat and dog 24-h urine samples after direct injection of 100 μl and 300 μl, respectively, onto the chromatographic system. The HPLC method allowed a very good separation of all metabolites without interfering peaks. However, the presence of non-volatile ion-pairing agents makes on-line coupling with MS impossible since the ion-pairing agents clog the vaporizer [21]. In order to establish the structure of the metabolites, all peaks occurring in rat urine were isolated using the ion-pairing HPLC system I granting the best separation. All isolated metabolites were then chromatographed on a C_{18} column using triethylammonium acetate in order to get rid of TBAP and minor contaminations. They were subjected to thermospray-LC–MS using a volatile ammonium acetate buffer which enabled the identification of underivatized metabolites.

As representative examples, Figs. 3 and 4 show the mass spectra of the two compounds isolated from peaks 4 and 7, respectively, that were identified as OHA glucuronide (peak 4) and OHA sulfate (peak 7).

After establishing the molecular ions, conjugated metabolites were subjected to enzymatic hydrolysis and their hydrolyzed versions analyzed by EI-MS.

Table 1 summarizes the spectrometric results obtained.

The results prove that the isolated peaks 1, 2, 4 and 7 are hydroxylated antipyrine derivatives ($[A + H]^+ = 205$, molecular mass = 204). EI-MS

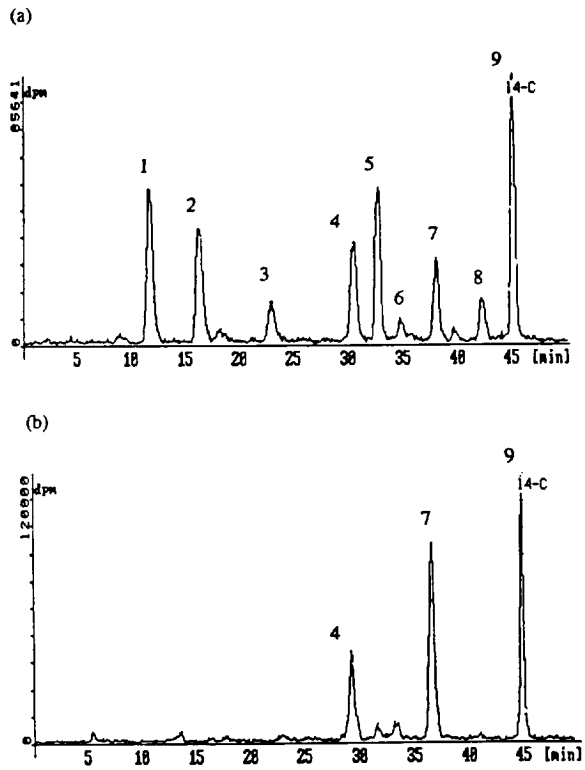

Fig. 2. (a) Typical liquid radio-chromatogram of rat urine from a [^{14}C]antipyrine treated rat; 100 μl injected, corresponding to ca. 60 μg antipyrine. (b) Typical liquid radio-chromatogram of canine urine from a [^{14}C]antipyrine treated dog; 300 μl injected, corresponding to ca. 60 μg antipyrine. Conditions are described in the Experimental section. Peaks were identified as follows: 1 = HMA; 2 = HMA glucuronide; 3 = antipyrine; 4 = OHA-glucuronide; 5 = DOHA-sulfate; 6 = NORA-glucuronide; 7 = OHA-sulfate; 8 = OH-NORA; 9 = NORA-sulfate.

analysis can be used to differentiate between the two hydroxylated metabolites HMA and OHA since HMA gives the key fragments m/z 188 and m/z 82 whereas OHA fragments to m/z 202 and m/z 56 [17,18]. In our study, EI-MS proved that the peaks 1 and 2 represent HMA while peaks 4 and 7 are OHA. Peak 1 is unconjugated HMA ($[A + H]^+ = 205$, $[A + CH_3COO]^- = 263$), peak 2 is glucuronidated HMA ($[M + H]^+ = 381$, $[M - H]^- = 379$). Peak 4 was identified as OHA-glucuronide ($[M + H]^+ = 381$, $[M - H]^- = 379$) and peak 7 as OHA-sulfoconjugate ($[M + NH_4^+] = 302$, $[M - H]^- = 283$).

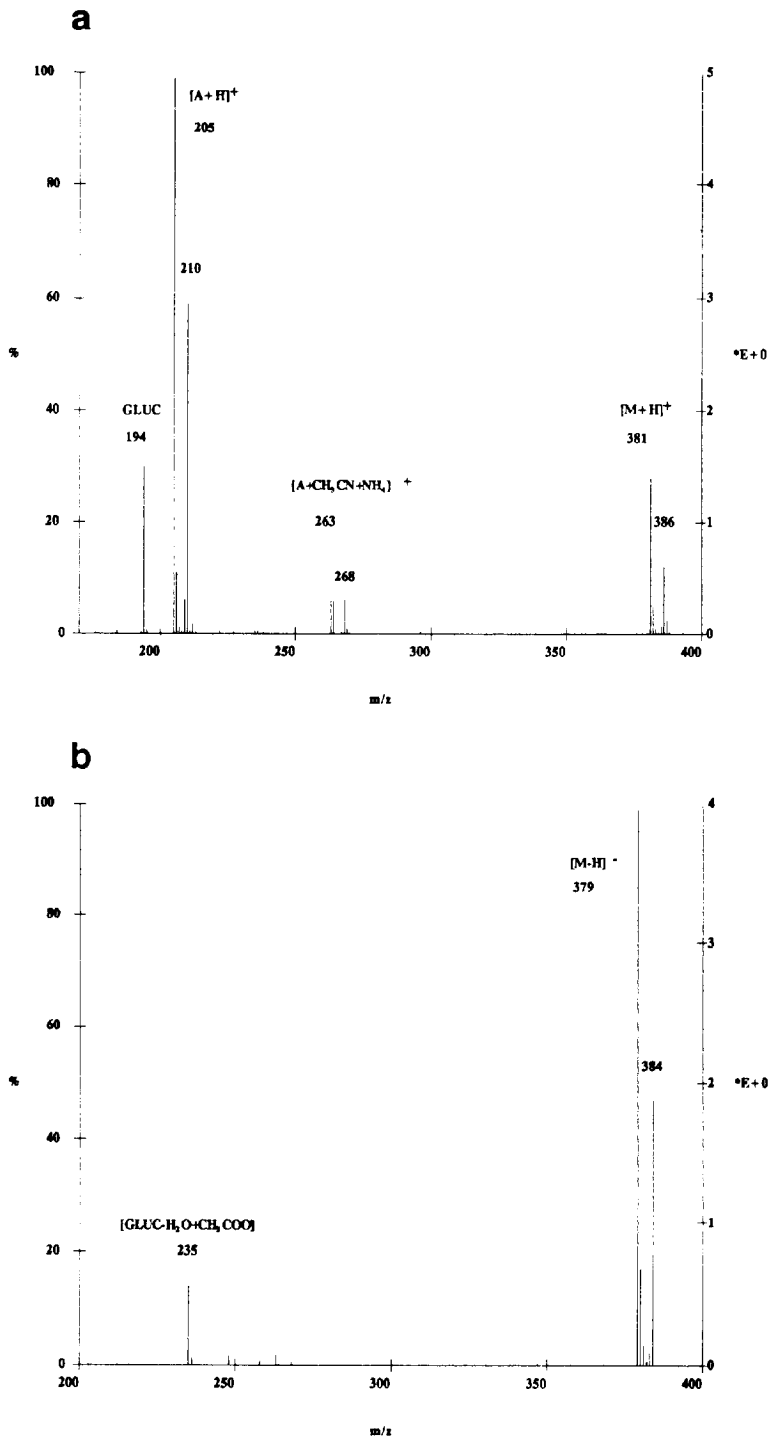

Fig. 3. Thermospray mass spectra of peak no. 4 (= 4-hydroxy-antipyrine glucuronide). (a) Positive-ion mode, (b) negative-ion mode. Conditions are described in the Experimental section.

Table 1
MS-data of antipyrine metabolites

Peak No.	$^{2}H^{a}$	LC-MS						EI-MS		Structure
		(+)Ions			(−)Ions			A	M/Z^{b}	
		$[M+H]^{+}$	$[A+H]^{+}$	$[Gluc]^{+}$	$[M-H]^{-}$	$[A-H]^{-}$	$[Gluc]^{-}$			
1	5	–	205 (100)	–	–	263 [+AcOH] (100)	–	204 (4)	188 (1) 82 (6)	HMA
2	5	381 (38)	205 (100)	194 (20)	379 (100)	–	235 [+AcOH] (12)	204 (4)	188 (1) 82 (4)	HMA–G
3	5		189 (100)	–	–	–	–	188 (83)	56 (45)	AP
4	5	381 (15)	205 (100)	194 (32)	379 (100)	–	235 [+AcOH] (17)	204 (3)	202 (3) 56 (88)	OHA–G
5	4	318 $[M+NH_4]^{+}$ (6)	221 (100)	–	299 (100)	219 (15)	–	220 (2)	218 (6) 56 (40)	DOHA-S
6	5	351 (1)	175 (48)	194 (67)	349 (1)	–	235 [+AcOH] (100)	174 (1)		NORA-G
7	5	302 $[M+NH_4]^{+}$ (15)	205 (100)	–	283 (100)	–	–	204 (6)	202 (2) 56 (100)	OHA-S
8	5	–	191 (100)	–	–	189 (100)	–	190 (3)	174 (1)	OH-NORA
9	5	–	175 (80)	–	253 (62)	233 [+AcOH] (100)	–	174 (100)		NORA-S

M = molecular mass; A = aglycon mass; $[Gluc]^{+} = [\text{glucuronic acid} - H_2O + NH_4^{+}]$; $[Gluc]^{-} = [\text{glucuronic acid} - H_2O + CH_3COO^{-}]$, relative intensity in parentheses; G = glucuronide; S = sulfate.

[a] Number of deuterium atoms incorporated in the phenyl ring.

[b] Key fragments according to Refs. [17,18].

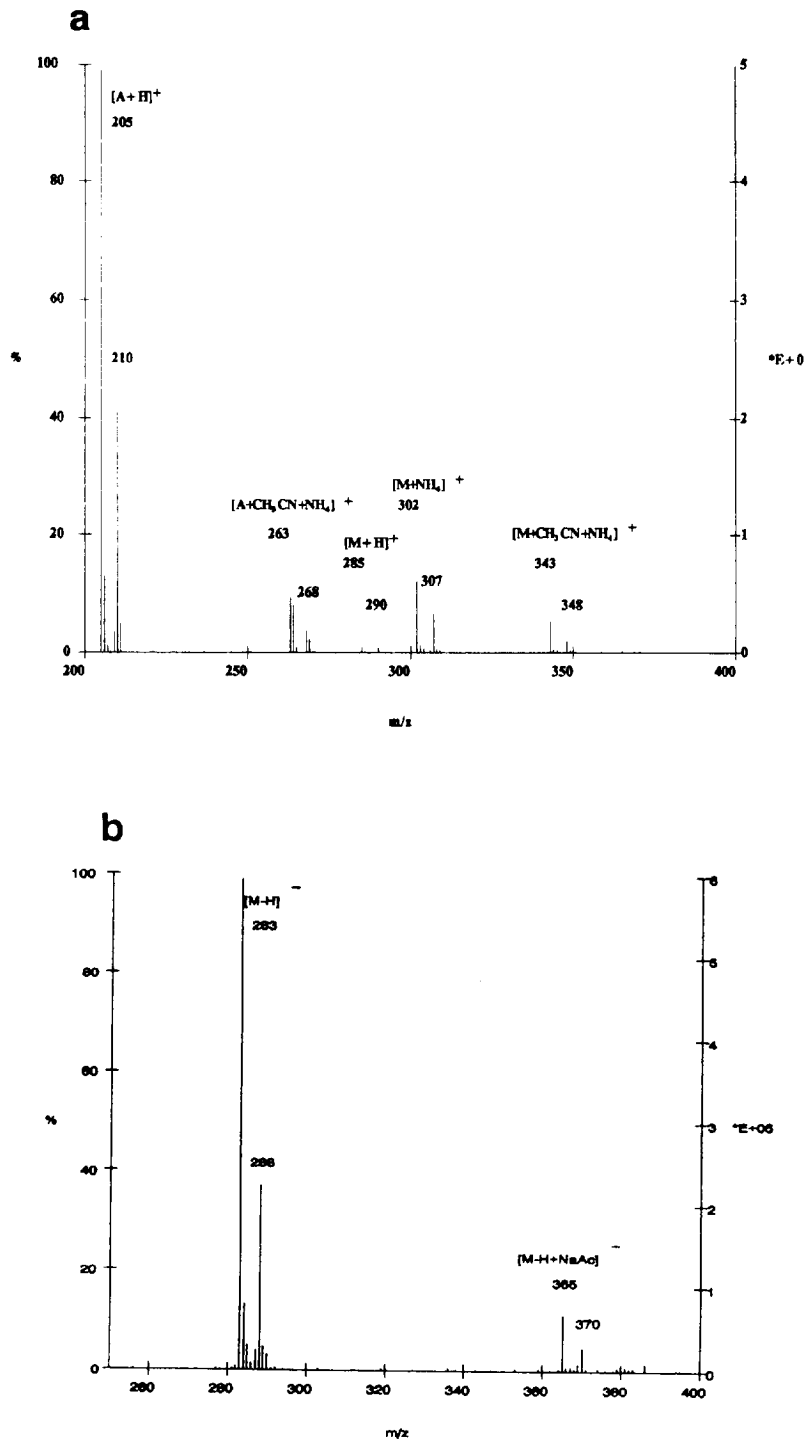

Fig. 4. Thermospray mass spectra of peak no. 7 (= 4-hydroxy-antipyrine sulfate). (a) Positive-ion mode, (b) negative-ion mode. Conditions are described in the Experimental section. Acetonitrile/ammonium adduct ions [M + 59] in thermospray-MS are formed by dehydration of ammonium acetate [19,20].

Peak 3 was found to be unchanged antipyrine ($[A + H]^+ = 189$). Peak 5 represents a double hydroxylated metabolite ($[A + H]^+ = 221$, $[A - H]^- = 219$). The lack of one deuterium atom and the fragmentation to m/z 56 prove that it is hydroxylated on the benzene ring and at position 4 of the pyrazolone ring, making it DOHA which is conjugated to sulfuric acid ($[M + NH_4]^+ = 318$, $[M - H]^- = 299$). Hydroxylation of the benzene ring is assumed to occur at position 4, since 4,4'-dihydroxy-antipyrine is a metabolite that has been reported previously [9,15].

Peaks 6 and 9 are deprived of the methyl group, making them nor-antipyrine ($[A + H]^+ = 175$, molecular mass = 174). Peak 6 is clearly a glucuronide ($[M + H]^+ = 351$, $[M - H]^- = 349$) while peak 9 is a sulfate ($[M - H]^- = 253$).

Peak 8 is hydroxylated and demethylated antipyrine ($[A + H]^+ = 191$, $[A - H]^- = 189$, molecular mass = 190). Due to its fragmentation in EI-MS, it must be hydroxylated at position 3 of the pyrazolone ring. This phase I metabolite is identified here for the first time using the HPLC system described above.

The identified peaks therefore correspond to two unconjugated metabolites (HMA, OH-NORA), three sulfates (OHA, DOHA, NORA), three glucuronides (HMA, OHA, NORA) and antipyrine itself. After a single intravenous dose, the main portion of the metabolites appears as conjugates in rat urine. After 48 h, $30.6 \pm 1.4\%$ of the dose is excreted as sulfates, $21.1 \pm 0.4\%$ as glucuronides and $18.9 \pm 1.6\%$ unconjugated. Expressed as the sum of phase I and phase II metabolites (percent of dose) HMA accounts for $25.7 \pm 1.5\%$, OHA for $16.9 \pm 1.2\%$, DOHA for $11.0 \pm 0.9\%$, NORA for $15.2 \pm 0.8\%$ and hydroxy-norantipyrine for $3.9 \pm 0.3\%$. Unchanged antipyrine accounts for $1.4 \pm 0.4\%$ of the dose. The values for the particularly unstable metabolites NORA and OHA are higher than those reported before [2,3].

In order to confirm the advantages of this HPLC system, frozen rat urine was thawed and analyzed after one month, six months and one year. No differences, either qualitative or quantitative, could be detected when the chromatograms were compared with the original ones, proving that no decomposition of the conjugated metabolites had occurred.

The metabolic profile obtained in rat urine was compared with the metabolic pattern in dog urine by cochromatography of the isolated rat metabolites with dog urine, in which only four metabolites were detected: OHA-glucuronide ($16.2 \pm 1.5\%$ of dose), DOHA-sulfate ($1.0 \pm 0.1\%$ of dose), OHA-sulfate ($25.2 \pm 1.5\%$ of dose) and NORA-sulfate ($34.3 \pm 1.9\%$ of dose). No unconjugated metabolites and no unchanged antipyrine could be detected. Phase I metabolite OHA accounts for $40.1 \pm 1.2\%$ of the dose, DOHA for $1.0 \pm 0.1\%$ and NORA for $34.3 \pm 1.9\%$.

The HPLC system described here allowed, in a single run, a simultaneous and accurate determination of all antipyrine metabolites that are found in the urine of rats and dogs, enabling for the first time a complete picture of antipyrine metabolism in these species. This procedure can be easily applied for characterizing cytochrome P-450 inductions and inhibitions in the liver of treated animals.

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