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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTIPYRINE AND ITS MAIN METABOLITES IN PLASMA, SALIVA AND URINE, INCLUDING 4,4'-DIHYDROXY-ANTIPYRINE

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SUMMARY

A rapid, selective and sensitive method was developed for the determination of antipyrine and its main metabolites in plasma, saliva and urine by an automated high-performance liquid chromatographic system. Using a MOS-Hypersil reversed-phase column with a phosphate buffer—acetonitrile mobile phase, baseline separation of antipyrine, its metabolites 3-hydroxymethylantipyrine, norantipyrine and 4-hydroxyantipyrine, and the internal standard, phenacetin, was achieved within 6 min. Factors regarding the accuracy and precision of the method and the stability of phase I metabolites during sample preparation are discussed, taking into account certain drawbacks of previously published methods.

Based on the same chromatographic system a method was developed for the assay of 4,4'-dihydroxyantipyrine in urine. This compound is an important metabolite of antipyrine in the rat, representing 12.6 ± 1.8% of the administered dose (n = 18).

INTRODUCTION

Antipyrine is commonly used as a model compound to study the influence of disease, drugs and environmental factors on hepatic drug-metabolizing enzyme activity in vivo [1, 2]. In such studies this activity was mostly assessed on the basis of plasma or saliva elimination kinetics.

Recently, the value of the antipyrine test has been improved by also assessing the antipyrine metabolite profile [3], since the formation of the main metabolites of antipyrine was shown to be regulated by different forms of cytochrome *P*-450 [4—8]. In rats, the formation of 3-hydroxymethylantipyrine (HMA) is associated with the phenobarbital (PB) type of cytochrome

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P-450, that of 4-hydroxyantipyrine (OHA) with the methylcholanthrene (MC) type, while norantipyrine (NORA) is probably also formed by an MC-inducible type of cytochrome *P*-450. Hence the assessment of rates of formation of antipyrine metabolites enables the quantitation of different drug-oxidizing enzymes in one test [9].

Aromatic ring hydroxylation was recently found to be an additional pathway in the biotransformation of antipyrine in man and rat [10]. Of these aromatic ring hydroxylated products, 4,4′-dihydroxyantipyrine (DOHA) appears to be a relatively important metabolite, since it represents 11—18% of the dose in rats and 3—6% in man [11]. 4′-Hydroxyantipyrine (pOHA) was found to be a minor metabolite of antipyrine, representing 2—4% of the dose in man and less than 1% in the rat [12]. For investigations in man, only HMA, NORA and OHA are relevant, since the amounts of the other metabolites do not exceed 5% of the administered dose [12, 13]. In rats however, DOHA is also an important metabolite [11].

In this study, an automated high-performance liquid chromatographic (HPLC) method for the determination of antipyrine in plasma, saliva and urine, and its metabolites HMA, NORA and OHA in urine is described, taking into account certain drawbacks of previously published methods [3, 14—20]. Special emphasis is put on the stability of phase I metabolites during sample preparation. Furthermore, an HPLC method was developed for the determination of DOHA in urine.

MATERIALS AND METHODS

Chemicals

Antipyrine (AP) was purchased from Brocacef (Maarssen, The Netherlands). 4-Hydroxyantipyrine (OHA), norantipyrine (NORA) and 3-hydroxymethylantipyrine (HMA) were synthesized according to previously described methods [21—24]. 4,4′-Dihydroxyantipyrine (DOHA) was a gift from Dr. Schüppel and co-workers, Institut für Pharmakologie und Toxikologie der Technischen Universität Braunschweig (F.R.G.). Organic solvents were obtained from Baker Chemicals (Deventer, The Netherlands) and limpet acetone powder type I (glucuronidase—sulphatase) from Sigma (St. Louis, MO, U.S.A.).

Instrumentation and chromatographic conditions

The liquid-chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of an M-45 pumping device, a WISP 710B automatic sample injector and an M-440 UV detector at 254 nm. The chromatographic data were processed by a Hewlett-Packard 3390 reporting integrator.

Columns (100 × 3.0 mm) were packed with spherical 5 μm MOS-Hypersil (Shandon, Southern Instruments, Astmoor, U.K.). Evaporation of extraction solvents was achieved under reduced pressure by a Büchler vortex evaporator.

The eluent for the separation of antipyrine and its metabolites, HMA, NORA, and OHA (assays A, B and C) consisted of a mixture of 0.02 *M* phosphate buffer, pH 7.2, and acetonitrile (100:10) containing sodium pyrosulphite 2 g/l. For the assay of DOHA in urine (assay D) the eluent consisted of a mixture of 0.02 *M* phosphate buffer, pH 6.5, and acetonitrile (100:8).

The eluent for the determination of antipyrine in blood, plasma and saliva (assay E) consisted of a mixture of 0.0067 *M* phosphate buffer, pH 7.2, and acetonitrile (100:18). For all eluents, flow was set at 2 ml/min, resulting in a pressure of about 135 bars.

Calibration graphs

Calibration graphs were prepared by spiking blank samples with antipyrine or the metabolites to be measured and carrying the samples through the analytical procedure. Evaluation of chromatograms was based on peak area ratios as calculated by the internal standard procedure of the HP-3390 reporting integrator. Calibration graphs for antipyrine in human plasma and saliva ranged from 1.0 to 20.0 μg/ml and in rat blood from 2.5 to 50.0 μg/ml; in urine for antipyrine from 2.5 to 30.0 μg/ml, for HMA and DOHA from 12.5 to 100.0 μg/ml and for NORA and OHA from 12.5 to 150.0 μg/ml.

Analytical procedures

Assay A: HMA and AP in urine. To 0.50 ml of urine, 0.50 ml of 0.2 *M* acetate buffer pH 4.5 and 10 mg of limpet acetone powder were added. After incubation of this mixture for 3 h at 37°C, 10 μg of phenacetin (as internal standard in 50 μl of ethanol), 200 mg of sodium chloride and 0.10 ml of 4 *M* sodium hydroxide were added successively and mixed on a whirlmixer for 15 sec. The mixture was extracted with 7 ml of dichloromethane and the organic layer was collected and evaporated to dryness with a vortex evaporator. The residue was disssolved in 0.20 ml of methanol and diluted with 0.50 ml of eluent. Of this solution 15 μl were injected into the HPLC system.

Assay B: NORA and OHA in urine. To 0.50 ml of urine, 0.50 ml of 0.5 *M* acetate buffer pH 4.5 containing 40 mg of $Na_2S_2O_5$ and 10 mg of limpet acetone powder were added. After incubation and addition of internal standard (see above) the mixture was extracted with 5 ml of dichloromethane—*n*-pentane (3:7, v/v) by mixing on a whirlmixer for 15 sec. After centrifugation the organic phase was collected and evaporated in a vortex evaporator under reduced pressure at room temperature. The residue was dissolved in 0.20 ml of methanol and diluted with 0.50 ml of 0.01 *M* acetate buffer containing 12.5 mg of $Na_2S_2O_5$ (freshly prepared). Of this solution 15 μl were injected into the HPLC system.

Assay C: simultaneous assay of HMA, NORA, AP and OHA. Sample handling was identical to that described for the assay of NORA and OHA (assay B), except that after enzymatic hydrolysis the mixture was saturated with NaCl (200 mg) and extracted with 5 ml of chloroform—ethanol (9:1, v/v).

Assay D: DOHA and OHA in urine. A mixture of 0.25 ml of urine and 0.10 ml of concentrated hydrochloric acid was heated in a boiling water-bath for 90 min. Adjustment to pH 4—5 was achieved by addition of 0.20 ml of 4 *M* sodium hydroxide and 200 mg of sodium acetate. The solution was saturated by adding 200 mg of sodium chloride. After addition of the internal standard phenacetin (10 μg in 50 μl of ethanol), the mixture was extracted with 7 ml of chloroform–ethanol (9:1). The organic phase was collected and evaporated to dryness in a vortex evaporator under reduced pressure at 40°C. The residue was dissolved in 0.10 ml of methanol and diluted with 0.25 ml of 0.01 *M*

hydrochloric acid containing 1 mg of $Na_2S_2O_5$ to ensure stability of OHA. Of this solution 25 μl were injected into the HPLC system.

Assay E: antipyrine in blood plasma and saliva. To 0.50 ml of blood, plasma or saliva 0.10 ml of 4 *M* sodium hydroxide and 4 μg of phenacetin (as internal standard in 50 μl of ethanol) were added. After extraction with 5 ml of dichloromethane–*n*-pentane (1:1, v/v) on a whirlmixer for 15 sec, the organic layer was collected and evaporated to dryness under reduced pressure (Büchler vortex evaporator) or by standing overnight at room temperature. The residue was dissolved in 0.20 ml of methanol and diluted with 0.50 ml of eluent. Of this solution 15 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

Hydrolysis of metabolite conjugates

The metabolites of antipyrine are excreted in urine mainly as conjugates [11]. In man, the major conjugation route is glucuronidation. HMA is conjugated to an extent of 30—75%, while the remainder is excreted as free metabolite [25]. OHA and NORA are glucuronidated almost completely, about 2% being conjugated to sulphate or excreted as free metabolite [11]. In rats, sulphation is the major conjugation reaction [26].

Since these conjugates are very stable, it would be desirable to determine them directly without cleavage. Selective extraction and separation of such hydrophilic compounds, however, is very difficult, while reference compounds are not available. Thus far separation and quantitation of the intact conjugation has only been achieved by thin-layer chromatography with radiolabelled compounds [11, 27] or by an off-line combination of liquid chromatography and field desorption mass spectrometry [28]. Therefore a hydrolysis step has to be included when antipyrine metabolites are to be determined routinely by commonly used HPLC detectors like UV spectrophotometry.

Hydrolysis under strong acidic conditions [17] was not satisfactory for the assay of HMA and NORA, since NORA decomposes under these conditions, while HMA glucuronide is only hydrolysed up to 70%. Therefore, enzymatic hydrolysis with glucuronidase—sulphatase was preferred for the assay of HMA, NORA and OHA, since it resulted in complete cleavage of their conjugates [3, 16]. Addition of sodium pyrosulphite (16 mg/ml) was necessary to prevent decomposition of NORA [16] and OHA [3].

Tabarelli-Poplawski and Uehleke [29] reported that OHA irreversibly binds to protein, resulting in a loss of 0.25 μg/mg protein. Our experiments also indicated that small amounts of OHA and NORA were bound to the hydrolysing enzyme powder. As a result, calibration graphs showed negative intercepts, though good linearity was obtained between 10 and 200 μg/ml and slopes were the same with and without enzyme incubation. The loss of NORA and OHA may be less significant if purified enzymes are used, though Eichelbaum et al. [16] reported decreased recoveries of OHA and NORA when more than 10,000 Fishman units or purified *Helix pomatia* enzyme were used per ml of urine.

For the assay of DOHA conjugates, only acidic hydrolysis as described by Böttcher et al. [17] gave satisfactory results, since DOHA decomposed readily

at pH 4.5, which is required for enzymatic hydrolysis. OHA conjugates were also hydrolysed quantitatively with this method [17]. The optimal hydrolysis time was 90 min (boiling water-bath). Continuation of hydrolysis after 90 min resulted in slightly decreased DOHA recoveries, while OHA recovery remained the same (Fig. 1).

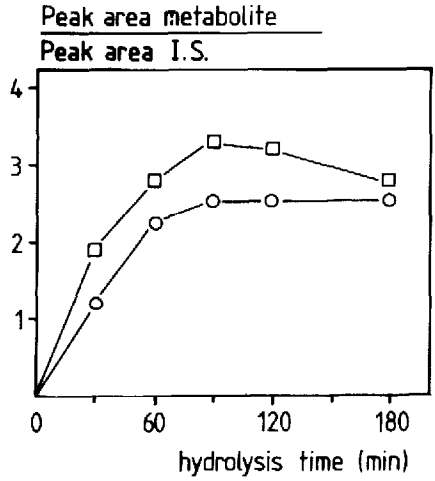

Fig. 1. Peak area ratios of DOHA (□) and OHA (○) after acidic hydrolysis as a function of time. Each point represents the mean of three determinations.

Stability of antipyrine metabolites

One of the most difficult problems in the determination of antipyrine metabolites is the relative instability of NORA. Some authors claim that NORA adsorbs to glass and that silanisation of glassware solves this problems [3, 14, 16]. A more likely explanation of higher recoveries after silanisation of glassware is that NORA dissolves in the silanised layer because of its lipophilicity and is thus protected from further decomposition. However, back-extraction from this layer will be incomplete, and will depend on the thickness of the layer. As a consequence silanisation of glassware leads to irreproducible results and non-linear calibration graphs of NORA.

A better approach to obtain reproducible results is to take the causes of NORA loss into account, i.e. volatility and decomposition due to pH influences and oxidation. NORA is volatile at room temperature [27] and readily disappears when evaporation to dryness of the extraction solvent is achieved by a stream of air or nitrogen. This problem was solved by evaporation of the extraction solvents under reduced pressure (e.g. Büchler vortex evaporator) or by addition of 0.25 ml of methanol before evaporation and stopping the evaporation when about 0.1 ml of solvent (methanol) remains. In both cases evaporation should take place without heating.

Loss of NORA due to oxidation in aqueous media (e.g. urine) can be prevented by addition of sodium pyrosulphite at concentrations of 16 ml/ml [16]. Our observations confirm this result and further indicate that NORA decomposes at pH $>$ 6 (Fig. 2) as well as at pH $<$ 2. OHA is also unstable at pH $>$ 6. Oxidation of OHA can be prevented by adding sodium pyrosulphite

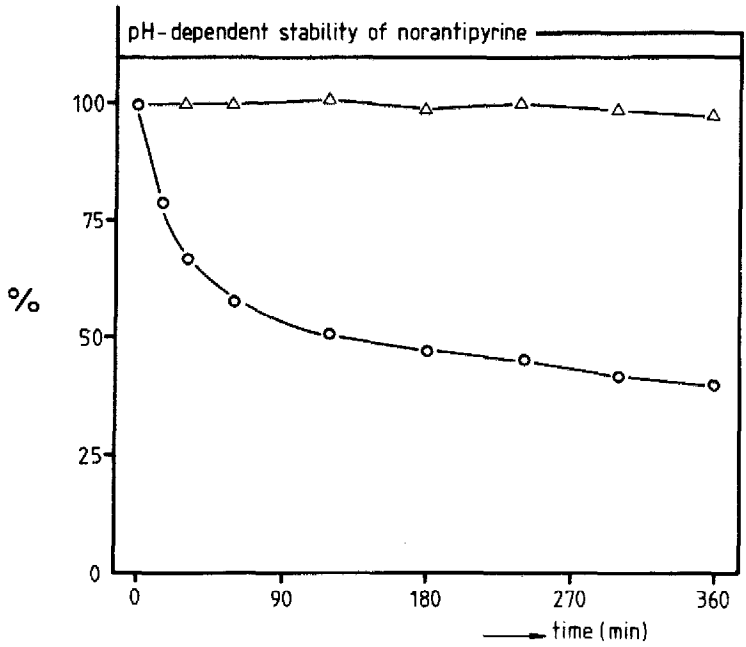

Fig. 2. Stability of NORA in solutions of different pH: (△), in 0.01 *M* acetate buffer pH 4.5 containing sodium pyrosulphite 16 mg/ml; (○), in eluent (0.02 *M* phosphate buffer pH 7.2 containing 10% acetonitrile) containing sodium pyrosulphite 16 mg/ml. Each point represents the mean of three determinations.

[3]. As a result, a solution of OHA and NORA in 0.01 *M* acetate buffer at pH 4.5 containing 16 mg/ml sodium pyrosulphite was found to be stable for at least 6 h (for NORA this is shown in Fig. 2). By addition of an equal volume of 0.5 *M* acetate buffer containing 40 mg/ml sodium pyrosulphite to 0.5 ml of urine, the same results were obtained during enzymatic hydrolysis for 3 h at 37°C.

DOHA is unstable in aqueous solutions at pH > 2. Stability of DOHA at pH 4.5 was not improved by addition of sodium pyrosulphite.

So far, no stability problems have been observed for HMA. The parent compound antipyrine is volatile (J. Böttcher and H. Bässmann, personal communication). Therefore loss of antipyrine may occur when evaporation of extraction solvents takes place at temperatures above 30°C.

Extraction procedures

For extraction of the phase I metabolites of antipyrine from urine, obtained after enzymatic hydrolysis (HMA, NORA and OHA), the systems described by Danhof et al. [3], Eichelbaum et al. [16] and Böttcher et al. [17] were compared. The extraction recoveries obtained with different systems are shown in Table I. NORA, OHA and unchanged antipyrine gave a sufficient extraction yield with all three systems at pH 4.5, whereas NORA and OHA were not extracted at pH > 10. For extraction of HMA, saturation of the aqueous layer was necessary, while pH had little influence. The highest recoveries were obtained when chloroform—ethanol (9:1) was used. The recovery of the

internal standard phenacetin was higher with increasing lipophilicity. Considering the low selectivity of the single-step extraction procedures [16, 17], several other compounds were co-extracted (Fig. 3), resulting in considerably higher background in the chromatograms than after the more selective extraction procedures [3]. This may lead to overestimation of peak areas (Table II) and shortening of column life-times. Interference of other drugs or metabolites may also occur.

We therefore recommend the more selective dual extraction procedure described by Danhof et al. [3] whenever antipyrine is given simultaneously with other drugs. When antipyrine is given alone, the less selective extraction described by Böttcher et al. [17] may be preferred, since it requires only one extraction step and one chromatographic run. Eichelbaum's method [16] gives lower HMA recoveries (Table I), while the chromatographic background is similar. For extraction of DOHA after acidic hydrolysis, the hydrolysate had to be saturated with sodium chloride. Optimal pH for extraction of DOHA was pH 4—5 [17]. This pH adjustment was achieved by adding 0.20 ml of 4 *M*

TABLE I

EXTRACTION RECOVERIES OF ANTIPYRINE AND METABOLITES FROM HUMAN URINE

Extraction solvents	pH	NaCl saturation	Percentage recovery ± S.D. (n = 5)			
			HMA	NORA	OHA	AP
Dichloromethane (DCM)	>10	+	54 ± 3	n.d.*	n.d.	100 ± 1
DCM—*n*-pentane (3:7)	4.5	—	n.d.	90 ± 2	90 ± 2	98 ± 1
DCM—isopropanol (9:1)	4.5	+	58 ± 3	88 ± 2	87 ± 2	98 ± 1
Chloroform—ethanol (9:7)	4.5	+	96 ± 2	82 ± 3	99 ± 1	99 ± 1

*n.d. = not detected.

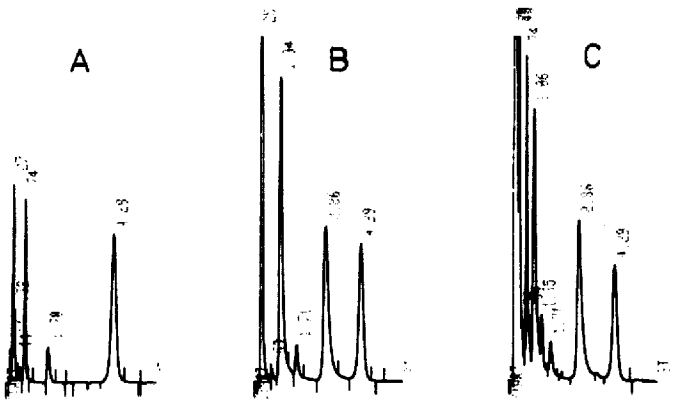

Fig. 3. Chromatograms of the same urine sample of a human volunteer, obtained after different extraction procedures. A, B and C correspond to the assay methods as described in Materials and methods section. Retention times (in min): 0.74 = HMA, 1.04 = NORA, 1.71 = AP, 2.86 = OHA, 4.29 = internal standard.

TABLE II

ACCURACY AND PRECISION OF THE ANALYSIS OF ANTIPYRINE AND ITS METABOLITES. COMPARISON OF OBTAINED CONCENTRATIONS (μg/ml ± S.D., $n = 5$) FROM THE SAME HUMAN URINE SAMPLE AFTER DIFFERENT ASSAY METHODS*

Extraction solvents	Method	HMA	NORA	OHA	AP
Dichloromethane (DCM)	A	16.0 ± 0.6	n.d.**	n.d.	2.9 ± 0.1
DCM—*n*-pentane (3:7)	B	n.d.	48.6 ± 1.2	54.7 ± 0.9	2.9 ± 0.1
Chloroform—ethanol (9:1)	C	22.5 ± 1.5	51.7 ± 1.5	55.9 ± 0.9	2.8 ± 0.1

*For corresponding chromatograms, see Fig. 3.
**n.d. = not detected.

sodium hydroxide and 200 mg of sodium acetate to the hydrolysate. After extraction with 7 ml of chloroform—ethanol (9:1) the recovery of DOHA was 90 ± 2%, which is in agreement with previous results [17]. Since both acidic hydrolysis and extraction (Table I) of OHA are satisfactory with the procedure described for DOHA, OHA can be quantified simultaneously with DOHA.

Dissolution of residues

After evaporation of the organic solvents, the residues are usually dissolved in eluent before injection. However, at the pH of the eluent (7.2) OHA and NORA are unstable (Fig. 2). Furthermore, NORA, OHA and phenacetin (internal standard) dissolve slowly in the eluent.

To overcome these problems, we dissolved the residues in 0.20 ml of methanol and diluted then with 0.50 ml of 0.01 *M* acetate buffer pH 4.5 containing sodium pyrosulphite (25 mg/ml). Under these conditions, all metabolites and phenacetin readily dissolved and were stable for at least 6 h, except DOHA. The residue obtained in the DOHA assay could not be dissolved in eluent (pH 6.5) due to instability of DOHA at pH > 2. Therefore the residue was dissolved in 0.10 ml of methanol and diluted with 0.25 ml of 0.01 *M* hydrochloric acid. This medium provided sufficient stability of DOHA: after 3 h more than 95% was left. Since only 15—25 μl of these solutions were injected, neither methanol nor the difference in pH caused any change in column equilibrium.

Chromatographic system

Recently three HPLC systems have been described that provide baseline separation of HMA, NORA, OHA and unchanged antipyrine [3, 16, 18]. The method described by Kahn et al. [18] is time-consuming, since it includes a derivatization step and a gradient elution, resulting in an injection cycle of 36 min. Eichelbaum et al. [16] described a straight-phase system in which we find it difficult to keep the composition of the mobile phase constant, due to volatility of ammonia. Therefore NORA retention times were not constant with time. The reversed-phase system described by Danhof et al. [3] was preferred, since it requires no derivatization, an isocratic run takes no longer than 10 min and the mobile phase (0.05 *M* phosphate buffer pH 6.5

containing acetonitrile, 100:5) is very stable. Improvement was achieved by using MOS-Hypersil instead of LiChrosorb RP-2, resulting in a decrease of pressure from 200 to 100 bars at a flow-rate of 1.5 ml/min. At an eluent pH of 7.2, an excellent separation was achieved between HMA, NORA, OHA, unchanged antipyrine and phenacetin, while an increase in the acetonitrile content in the eluent from 5% to 10% shortened the run time to 6 min (Fig. 4). Typical chromatograms of the metabolites of antipyrine after extraction from a human urine sample (methods A, B and C) are shown in Fig. 3.

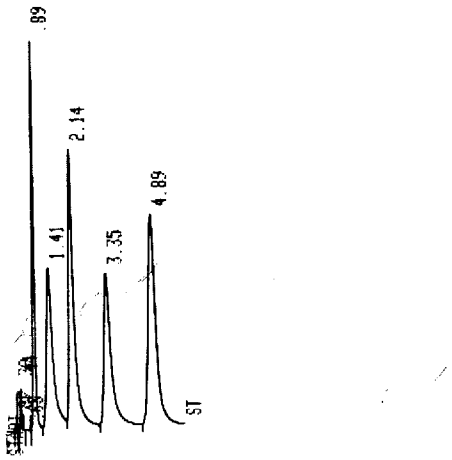

Fig. 4. Chromatogram obtained after injection of a standard solution containing HMA (retention time = 0.89 min), NORA (1.41), AP (2.14), OHA (3.35) and phenacetin (4.89). Abbreviations and conditions given in Materials and methods section.

The chromatographic systems for all assays described in this paper were identical except for small changes in composition of the eluents. In the assay of antipyrine in blood, plasma and saliva, acetonitrile content was 18%, resulting in very short retention times and excellent resolution (Fig. 5).

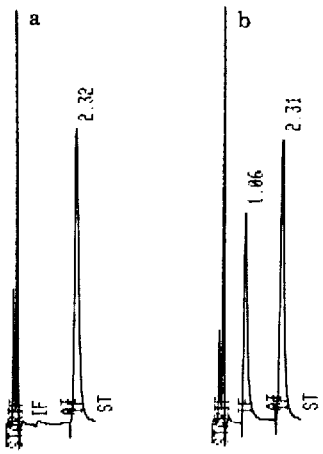

Fig. 5. Chromatograms obtained after extraction of blank plasma (a) and of plasma containing 4.8 μg/ml antipyrine (b) (retention time = 1.06 min). Both samples contained the internal standard phenacetin (retention time = 2.31 min).

Since antipyrine and phenacetin are not readily oxidized, sodium pyrosulphite was omitted from the eluent. As to the assay of DOHA, separation was optimal with the eluent at pH 6.5 containing 8% acetonitrile. Fig. 6 shows a typical chromatogram of a rat urine sample carried through the analytical procedure D as described. Finally the life-time of the pump seals was markedly increased by lowering the molarity of the phosphate buffer from 0.05 to 0.02 *M* in the metabolite assays and to 0.0067 *M* in the assay of antipyrine in blood, plasma and saliva. These decreased molarities proved to be sufficient to maintain constant pH.

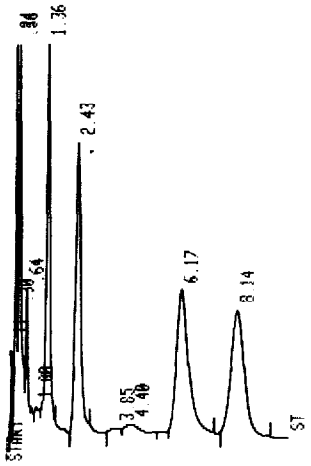

Fig. 6. Chromatogram obtained with assay method E of rat urine. Retention times (in min): 2.43 = DOHA, 4.40 = AP, 6.17 = OHA and 8.14 = internal standard. Other antipyrine metabolites: 1.36 = HMA (not fully hydrolysed) and 3.85 = NORA (partly decomposed).

Accuracy and precision

Linear calibration graphs with correlation coefficients better than 0.995 were obtained for all assay procedures, with standard deviations of 3—5% in the lower concentration ranges and 1—3% in the higher ones (n = 5). A calibration graph for DOHA in urine is shown in Fig. 7. Table II shows the concentrations (μg/ml ± S.D.) of antipyrine and its metabolites in the same human urine sample as assayed by the methods A, B and C. Corresponding chromatograms are shown in Fig. 3. The urine sample was a 0—24 h sample from a male volunteer who had received 500 mg of antipyrine intravenously, resulting in metabolite concentrations of 25—35% of the highest calibration graph concentration.

All assay methods showed good reproducibility, with standard deviations of 2—3% (n = 5), except for HMA after dichloromethane extraction (4%). The latter was due to the low extraction recovery of this method and may be improved by repeated extraction. Concentrations of NORA and especially HMA seemed to be higher with the one-step extraction than after two separate extractions (Table II). This difference was attributed to increased peak areas, caused by interference of co-extracted compounds that were not sufficiently separated from the peaks to be measured (Fig. 3C), despite baseline separation of the metabolites (Fig. 4). Therefore the precision of the selective dual extraction method described by Danhof et al. [3] was found to be better than that of

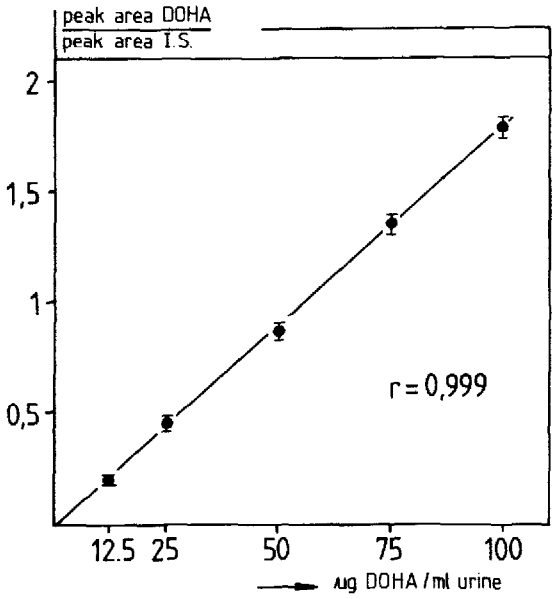

Fig. 7. Calibration graph of 4,4'-dihydroxyantipyrine (DOHA) in urine in a concentration range of 12.5—100.0 µg/ml. Each point represents the mean ± S.D. of five observations.

the single-step extraction method. The lowest detectable concentration for all compounds was about 400 ng/ml. Detection limits were easily improved to 25 ng/ml by increasing the injection volume from 15 to 200 µl.

CONCLUSIONS

The phase system described in this paper for separation of antipyrine and its metabolites is an improvement on previously described methods, since it is highly reproducible and stable, while run times are short. The same system, with small changes in eluent composition can be used for all assays. In the assay procedures no derivatization step is included, though hydrolysis of metabolite conjugates is still required. For HMA, NORA and OHA enzymatic hydrolysis is to be preferred, while for DOHA acidic hydrolysis gives better results.

With the new assay for DOHA, it is now possible to quantify another important metabolite of antipyrine in urine with a rapid and simple HPLC method. In male Wistar rats (n = 18), DOHA formation accounted for 12.6 ± 1.8% of the administered dose (10 mg of antiyprine) [7, 8]. These results are in good agreement with those obtained by Böttcher et al. [17].

Improved stability of metabolites during the assay made all assays suitable for automatic injection. Automatic injection enabled the assay of the samples of twelve experiments with one chromatographic system in one day, using eight blood or saliva samples and one urine sample in each experiment. For human experiments a dose of 2 mg/kg antipyrine is sufficient to allow reliable determination of antipyrine in plasma or saliva and of antipyrine and its metabolites in urine, while for rat experiments 10 mg/kg is the minimum dose. The procedure described in this paper is quite suitable for detailed studies on antipyrine metabolism in man [30] and rat [7, 8].

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