Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 12, pp. 1783–1790, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Assay of antipyrine and its main metabolites 3-hydroxymethylantipyrine, norantipyrine and 4-hydroxyantipyrine in urine*

E. BRENDEL,† I. MEINEKE and C. DE MEY

SK&F Institute for Applied Clinical Pharmacology, Hildebrandstrasse 10, D-3400 Göttingen, FRG

Abstract: Two assay methods for antipyrine (AP) and its main metabolites 3-hydroxymethylantipyrine (3-HMA), norantipyrine (NORA) and 4-hydroxyantipyrine (4-HA) in urine samples have been compared. Method I involved a 3 h incubation at 37°C with β-glucuronidase, whereas method II used acid hydrolysis with 3 M hydrochloric acid to convert NORA glucuronide to the aglycone and 24 h incubation at 37°C with β-glucuronidase for hydrolysis of 3-HMA and 4-HA glucuronides. The precision of both sample preparation procedures was evaluated by means of HPLC with UV detection. The relative standard deviation (RSD) for the metabolites were considerably greater than 10% with method I. Application of method II, however, led to intra-assay and inter-assay RSD of less than 10%.

Keywords: *Antipyrine; antipyrine metabolites; urine; high-performance liquid chromatography.*

Introduction

In recent years antipyrine has been used to detect changes in the hepatic mixed function oxidase system produced by investigational drugs. Frequently metabolite formation rates are determined by multiplying the total plasma clearance of antipyrine by the fraction of the dose ultimately excreted into the urine as the respective metabolite [1]. This approach requires a sufficiently accurate and precise analytical method for the determination of antipyrine and its main metabolites in urine. The metabolites are excreted predominantly as conjugates, which in man are mainly the glucuronides [2, 3]. Because these conjugates are not commercially available as standards, their assay involves the preliminary hydrolysis to the unconjugated metabolites, a step which cannot be controlled directly.

Several high-performance liquid chromatographic (HPLC) assays for antipyrine (AP) and its main metabolites 3-hydroxymethylantipyrine (3-HMA), norantipyrine (NORA) and 4-hydroxyantipyrine (4-HA) have been developed [4–8]. In these publications, the instability and volatility of NORA and the hydrolysis of the conjugates have been identified as the two most critical factors and the hydrolysis has been carried out enzymically by incubating urine samples with various glucuronidase/sulphatase prepar-

*Presented at the "Third International Symposium on Drug Analysis", May 1989, Antwerp, Belgium.
†To whom correspondence should be addressed.

ations at 37°C for 2.5–3 h. This incubation time was reported to be necessary for complete hydrolysis of 4-HA glucuronide. However, under these conditions NORA begins to decompose despite the addition of the antioxidant sodium bisulphite [5].

During the development of the analytical procedure in this laboratory a 3 h incubation time was found to be insufficient for some samples. Prolonged incubations, however, resulted in a pronounced decomposition of NORA. This paper describes an assay method for AP and its main metabolites in urine which ensures complete hydrolysis of 4-HA glucuronide without the decomposition of NORA.

Experimental

Reagents and standards

AP, NORA and 4-HA were purchased from Aldrich Pharmaceuticals (Milwaukee, Wisconsin, USA) and 3-HMA was synthesized by Smith Kline & French Laboratories (Welwyn Garden City, Herts, UK). Phenacetin (internal standard) was obtained from Fluka (Buchs, Switzerland) and β-glucuronidase (G-8132) from limpets (*Patella vulgata*) was from Sigma (Deisenhofen, FRG). All solvents were of HPLC grade (Merck, Darmstadt, FRG). Concentrated stock solutions (1 mg ml^{-1}) of AP, NORA, 3-HMA, 4-HA and phenacetin were made in methanol. Appropriate working standard solutions were obtained from these stock solutions by dilution with methanol. All standard solutions were stored at −20°C.

Procedures

Method I. Method I is based on the procedure described by Danhof *et al.* [4] which has been slightly modified. Urine (1 ml) was mixed with 100 μl freshly prepared aqueous ascorbic acid solution (10 mg ml^{-1}) and then with 3 ml 0.1 M acetate buffer pH 4.5 containing 5 mg β-glucuronidase. After incubation at 37°C for 3 h, 1 ml of this solution was mixed with 15 μl internal standard solution (100 μg ml^{-1}), 0.5 ml water and 0.5 ml 0.5 M acetate buffer pH 4.5. The reaction mixture was then extracted with 5 ml dichloromethane–n-hexane (30:70, v/v) and the organic layer was separated (extract I).

The pH of the aqueous layer was adjusted to 11–12 by addition of 4 M sodium hydroxide. The mixture was extracted with 5 ml dichloromethane, the organic layer separated (extract II) and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residue was dissolved in 200 μl mobile phase and then extract I was added. This mixture was concentrated as described above under nitrogen to 200 μl of which 20–30 μl were injected into the HPLC system.

Method II.

(a) *Assay for AP and NORA.* Urine (0.1 ml) and 0.9 ml of water were mixed with 200 μl 3 M hydrochloric acid in a glass-stoppered glass vial and incubated at 90°C in a water-bath for 5 min. The solution was then mixed with 50 μl internal standard solution (10 μg ml^{-1}) and 200 μl saturated aqueous sodium acetate solution and then extracted with 3 ml chloroform for 30 s. The organic layer was evaporated to dryness under a gentle stream of nitrogen at ambient temperature and the residue reconstituted in 200 μl mobile phase. Then 30–50 μl of this solution were injected into the HPLC system.

(b) *Assay for 3-HMA and 4-HA.* Urine (0.1 ml) and 0.9 ml of water were mixed with 100 μl freshly prepared aqueous ascorbic acid solution (10 mg ml^{-1}) and 3 ml 0.1 M

acetate buffer pH 4.5 containing 5 mg β-glucuronidase. This solution was incubated at 37°C for 24 h. Then 1 ml of the reaction mixture was mixed with 50 μl internal standard solution (10 μg ml^{-1}) and 250 mg sodium chloride and extracted with 5 ml chloroform–ethanol (90:10, v/v) for 30 s. The organic layer was evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted in 200 μl mobile phase. Then 30–50 μl of this solution were injected into the HPLC system.

Instrumental conditions

For the HPLC system a M6000A pump connected to a WISP 710B autosampler (both from Millipore/Waters, Eschborn, FRG) was used. The chromatographic separation was performed on a 10 μm μBondapak C-18 column (300 × 4.6 mm i.d.; Millipore/Waters) with a mobile phase consisting of 0.05 M phosphate buffer (pH 5.5)–methanol (80:20, v/v) delivered at 1.5 ml min^{-1}. For detection of the analytes a Spectroflow 773 variable wavelength detector (Kratos, Karlsruhe, FRG) set at 244 nm was used. The chromatograms were recorded and integrated by a Data Module 730 B integrator (Millipore/Waters).

Calibration and evaluation of precision and accuracy

Standard samples for both methods were obtained by spiking water with appropriate volumes of standard working solutions. These samples were processed as described above. Calibration curves were calculated as a function of the respective area ratio of analyte to internal standard versus concentration. These calibration curves were determined daily.

Urine samples from healthy volunteers who had received an oral dose of 5 mg kg^{-1} AP were assayed several times on different days using methods I and II in order to assess the precision of both methods. The accuracy of the assay for AP and the unconjugated metabolites was determined by spiking water with appropriate amounts of the analytes. These samples were then assayed by method II.

Results and Discussion

In the development of the assay method for AP and its main metabolites in urine, the method described by Danhof *et al.* [4] was followed but it was modified slightly (method I). For example, both organic extracts (the acidic extract I and the alkaline extract II) were combined to allow the simultaneous assay of all the components. Since the validity of this assay could not be assessed due to the fact that the glucuronides of 3-HMA, NORA and 4-HA are not commercially available, the precision was investigated with a 0–48 h urine sample from a subject who had received an oral dose of 5 mg kg^{-1} antipyrine. The results in Table 1 show that good precision was achieved for AP only; the RSD of the assay of all main metabolites exceeded 10%. The high RSD for NORA can be partly explained by the relative instability and volatility of this compound whereas the large variation in the 3-HMA and 4-HA concentration results can be explained by incomplete hydrolysis.

Stable recoveries of 4-HA were given only after 12–16 h of incubation. As NORA is almost completely decomposed under these conditions, alternative methods for the hydrolysis were investigated. Böttcher *et al.* [2, 3] showed that NORA glucuronide is completely hydrolysed under strongly acidic conditions at 90°C within 5 min whereas the glucuronides of 3-HMA and especially 4-HA need considerably longer hydrolysis times.

Table 1
Precision of method I assessed by repeated analyses of a 0–48 h urine sample from subject T.E.

	Concentration (μg ml^{-1})			
Date of analysis	AP	3-HMA	NORA	4-HA
day 1	7.44	30.5	24.3	103
day 2	6.00	31.0	26.7	78.6
day 3	6.21	23.7	30.8	61.9
day 4	5.92	33.8	15.9	83.8
day 5	6.04	37.0	17.1	86.1
day 6	5.71	28.4	20.8	67.2
day 7	6.05	30.8	21.8	80.7
Mean	6.20	30.7	22.5	80.2
RSD (%)	9.2	13.5	23.4	16.7

Table 2
Mean concentrations and relative standard deviation ($n = 5$) of AP, 3-HMA, NORA and 4-HA (μg ml^{-1}) by method II in urine samples from subjects T.E. and E.B.

Urine sample	T.E. 0–48 h				E.B. 0–48 h			
	AP		NORA		AP		NORA	
Date of analysis	Conc.	RSD (%)	Conc.	RSD (%)	Conc.	RSD (%)	Conc.	RSD (%)
day 1	5.65	3.5	39.7	9.1	6.00	5.1	16.4	5.0
day 2	5.03	13.4	39.0	2.1	5.08	9.1	17.4	3.7
day 3	4.76	8.8	38.7	4.5	5.26	3.0	17.6	3.0
day 4	4.90	5.4	38.0	4.5	4.88	4.8	17.3	4.8
inter-assay	5.09	7.7	38.9	1.8	5.31	9.2	17.2	3.1

Urine sample	T.E. 0–48 h				E.B. 0–48 h			
	3-HMA		4-HA		3-HMA		4-HA	
Date of analysis	Conc.	RSD (%)	Conc.	RSD (%)	Conc.	RSD (%)	Conc.	RSD (%)
day 1	33.5	3.9	94.4	2.6	23.0	2.7	64.6	2.0
day 2	35.7	2.0	103	2.6	26.3	4.0	66.7	4.8
day 3	36.6	5.2	110	4.3	26.4	4.0	69.9	3.2
day 4	36.1	6.6	107	1.8	27.2	7.9	78.7	3.9
day 5	39.2	3.8	110	1.9	28.6	2.6	78.1	1.5
inter-assay	36.2	5.6	105	6.2	26.3	7.8	71.6	9.1

These findings were confirmed in the present work. Urine samples had to be hydrolysed with hydrochloric acid for 3–4 h in order to achieve reproducibly high recoveries of 4-HA. Under these drastic hydrolysis conditions NORA again proved to be unstable. Consequently, two different hydrolysis procedures were chosen for NORA and for 3-HMA and 4-HA. AP can be determined either together with 3-HMA and 4-HA or with NORA. However, the latter option proved to be more favourable, because fewer interfering peaks were observed in the chromatogram. By using method II the analysis of the same urine sample that was used for the precision testing of method I was repeated. The precision data are shown in Table 2. Intra-assay RSD was significantly lower than

10% for all metabolites and was above 10% only once, for AP. The inter-assay RSD was below 10% for all analytes. A comparison of the data from Tables 1 and 2 indicates that the results for NORA obtained with method I were not only imprecise but also too low due to its instability and volatility. Furthermore, only once (out of seven analyses) was a satisfactory recovery for 4-HA obtained with that method.

The analyses by method II were performed 1 year after the analyses by method I. During this period the urine sample had been stored at −20°C. The data show that AP and the conjugated metabolites are stable in urine for at least 1 year under these conditions.

The accuracy of method II could be investigated only by analysing samples spiked with the unconjugated compounds. The recovery and the % error are given in Table 3. The % error was calculated as the difference between the mean concentration found and the theoretical concentration of the spiked samples, expressed as a percentage of the theoretical concentration. These data cannot demonstrate the completeness of the hydrolysis. However, they show that the unconjugated analytes are stable during the hydrolysis and that the accuracy of the extraction procedure is acceptable.

Calibration graphs were linear between 0.02 and 2.0 μg ml^{-1} for AP, 0.1–10.0 μg ml^{-1} for 3-HMA and NORA and 0.5–20.0 μg ml^{-1} for 4-HA. These ranges are suitable for the analysis of diluted urine samples (diluted 1:10 with water) which have been collected for up to 48 h after an oral dose of 5 mg kg^{-1} AP. Chromatograms of urine samples after acid hydrolysis (assay for AP and NORA) are shown in Fig. 1. These samples were collected from a healthy volunteer before (Fig. 1A) and between 0–12 h after (Fig. 1B) an oral dose of 5 mg kg^{-1} AP. The chromatograms in Fig. 2 were obtained after enzymic hydrolysis of further aliquots of the same samples.

The limits of quantification are 0.02 μg ml^{-1} for AP, 0.1 μg ml^{-1} for 3-HMA and NORA and 0.5 μg ml^{-1} for 4-HA in the diluted sample. These levels allow the analysis of urine samples collected for up to 48 h after dosage. The method is precise and sufficiently fast allowing the analysis of about 40 samples per day per person.

Table 3
Accuracy of method II

Analyte	Theoretical conc. (μg ml^{-1})	n	Conc. found (μg ml^{-1})	Error (%)
AP	0.1	6	0.09	−10
	0.5	6	0.48	−4.0
	2.0	6	2.05	2.5
NORA	0.5	4	0.39	−22
	2.0	6	2.08	4.0
	10.0	6	10.24	2.4
3-HMA	0.5	6	0.51	2.0
	2.0	6	2.09	4.5
	10.0	6	9.97	−0.3
4-HA	0.5	6	0.58	16
	2.0	6	1.96	−2.0
	10.0	6	9.56	−4.4

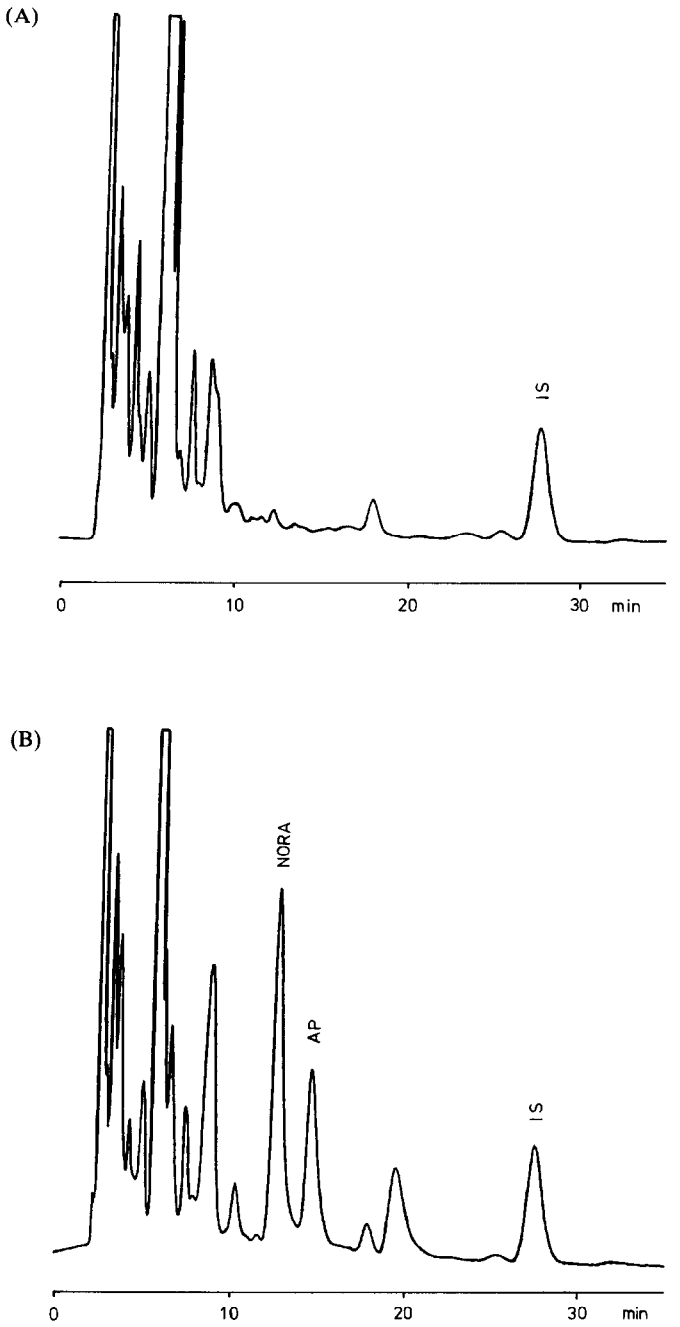

Figure 1
High-performance liquid chromatogram of a sample of human urine (diluted 1 + 9 with water) after acid hydrolysis (method II) (A). Before administration of antipyrine (B). 0–12 h sample collected after administration of 5 mg kg^{-1} of antipyrine.

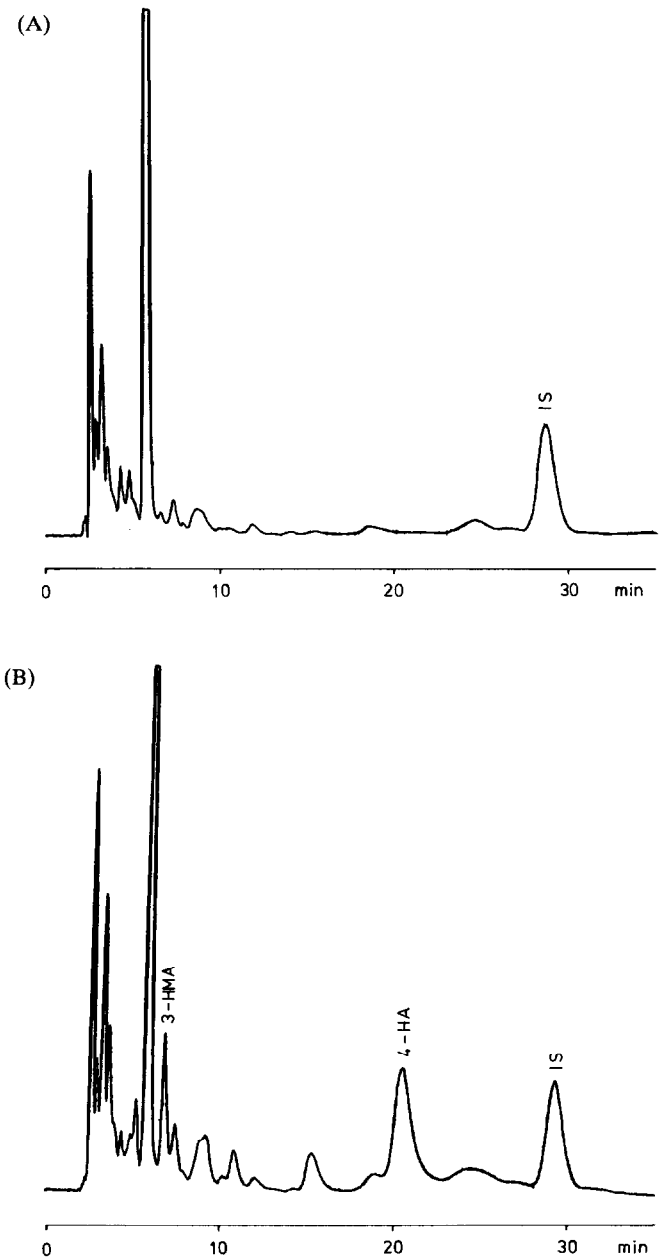

Figure 2
High-performance liquid chromatogram of a sample of human urine (diluted 1 + 9 with water, after 24 h incubation with β-glucuronidase (A). Before administration of antipyrine (B). 0–12 h sample collected after administration of 5 mg kg^{-1} of antipyrine.

References

[1] M. Danhof, A. van Zuilen, J. K. Boeijinga and D. D. Breimer, *Eur. J. Clin. Pharmac.* **21**, 433–441 (1982).
[2] J. Böttcher, H. Bässmann and R. Schüppel, *J. Pharm. Pharmac.* **34**, 168–175 (1982).
[3] J. Böttcher, H. Bässmann and R. Schüppel, *J. Pharm. Pharmac.* **36**, 391–398 (1984).
[4] M. Danhof, E. de Groot-van der Vis and D. D. Breimer, *Pharmacology* **18**, 210–223 (1979).
[5] M. Eichelbaum, B. Sonntag and H. J. Dengler, *Pharmacology* **23**, 192–202 (1981).
[6] M. W. E. Teunissen, J. E. Meerburg-van der Torren, N. P. E. Vermeulen and D. D. Breimer, *J. Chromatogr.* **278**, 367–378 (1983).
[7] J. Moncrieff, *J. Chromatogr.* **383**, 425–431 (1986).
[8] M. A. Mikati, G. K. Szabo, R. J. Pylito, B. W. LeDuc, T. R. Browne and D. J. Greenblatt, *J. Chromatogr.* **433**, 305–311 (1988).

[*Received for review 18 May 1989; revised manuscript received 9 June 1989; final revision received 1 July 1989*]