Journal of Chromatography, 421 (1987) 198–205
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3798

Note

Simple high-performance liquid chromatographic method for simultaneous determination of aminopyrine and its metabolites

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(First received April 3rd, 1986; revised manuscript received May 27th, 1987)

Aminopyrine (4-dimethylaminoantipyrine, AM) is an analgesic, antipyretic and anti-inflammatory drug used in many countries as its derivative, sodium N-(1,5-dimethyl-3-oxo-2-phenyl-pyrazolin-4-yl)-N-methylaminomethanesulfonate (dipyrone, metamizol, sulpyrine) [1]. AM is rapidly bio-transformed to an active metabolite, 4-monomethylaminoantipyrine (MAA) [2–4], which is further metabolized to 4-aminoantipyrine (AA) [5]. It was recently reported [6,7] that another metabolite of MAA, 4-formylaminoantipyrine (FAA) [6–8], is formed by the oxidation of the N-methyl side-chain of MAA (Fig. 1) [3].

Aminopyrine has been used extensively as a model substrate to monitor the ability of the liver to oxidize foreign compounds. This is achieved by determining the clearance and elimination rate of AM following oral administration or by aminopyrine breath test (ABT) [8]. Simultaneous measurement of the unchanged AM and its metabolites requires chromatographic analysis. Although several methods have been described for monitoring AM and its metabolites by high-performance liquid chromatography (HPLC) [9–11], none have attempted to assay AM and its metabolites, MAA, AA and FAA, simultaneously without elaborate extraction procedures.

In this paper we report a sensitive, selective and rapid HPLC assay for quantitating AM and its metabolites in rabbit plasma after simple protein precipitation with acetonitrile. This method is used to study the kinetics of AM and its

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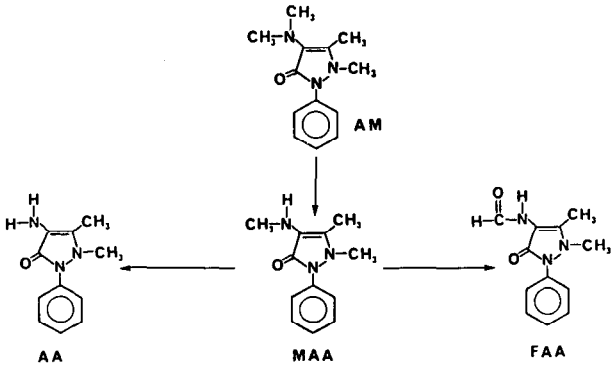

Fig. 1. Metabolic degradation pathway of aminopyrine.

metabolites (MAA, AA and FAA) in rabbits. AM and its metabolites are also quantitated after in vitro incubation of AM with liver microsomes.

EXPERIMENTAL

Chemicals and reagents

AM, AA, MAA, FAA, and the internal standard, 4-propylaminoantipyrine (PAA) were provided by Hoechst (Frankfurt, F.R.G.). All the solvents used were of HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Chromatography

The HPLC system consisted of a Waters Model M-45 pump, with a Waters U6K injector and a Model 441 Waters UV detector. A 10 cm × 6 mm I.D. Radial-Pak C_{18} (10 μm particle size) column (Waters Assoc., Milford, MA, U.S.A.) was used to separate AM from its metabolites and the internal standard. A guard column packed with C_{18} packing (30–40 μm particle size) was used before the analytical column. The mobile phase used (pH 5.4) was methanol–triethylamine–glacial acetic acid–water (34.6:0.9:0.5:64.0). The column was maintained at room temperature and the mobile phase flow-rate was 2.0 ml/min. Column effluents were monitored at 254 nm (λ_{max} of AM and the metabolites in the mobile phase used was 257 nm).

Standard solutions

Stock solutions of AM, FAA, AA and MAA (250 μg/ml) were prepared in distilled water. PAA solution (10 μg/ml in acetonitrile) was used as the internal standard. Blank plasma was spiked with known amounts of AM and metabolites over the concentration range 0.20–10.0 μg/ml. Since AM is light-sensitive and its metabolites adhere to glass containers, all solutions were stored in amber siliconized glass containers.

Sample preparation

To 200 μl of plasma in a 1.5-ml centrifuge tube 200 μl of acetonitrile and 100 μl of internal standard solution were added. The tubes were vortexed for 30 s and centrifuged (2000 *g*) for 2 min. A 50-μl volume of the supernatant was injected into the column and AM and metabolite concentrations were quantitated by the peak-height ratio method.

Accuracy, coefficient of variation and recovery

The accuracy of the method was studied by analyzing samples at two different concentrations (0.6 and 5.0 μg/ml). The concentrations in these samples were calculated from calibration curves run at the same time. The reproducibility of the method was determined by calculating the coefficient of variation of six sequentially analyzed samples with identical concentrations. The recovery was determined by comparing the response of pure samples with the response of plasma containing known concentrations (0.6 and 5.0 μg/ml) of AM and its metabolites.

In vivo rabbit experiment

Six healthy male New Zealand white rabbits (Green Meadows Rabbitry, Pittsburgh, PA, U.S.A.), weighing between 2.5 and 3.0 kg received 20 mg/kg AM (in 1.5 ml of water) by injection into the right marginal ear vein. Blood samples were drawn at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 h. The samples were centrifuged immediately and the plasma was frozen in silanized amber glass vials until analysis.

Model-independent parameters were computed for aminopyrine after intravenous administration. The clearance (Cl), the disposition half-life ($t_{1/2}$) and the steady-state volume of distribution (V_{ss}) were calculated according to standard techniques [12]. Since MAA, AA and FAA were not administered intravenously, the clearance and V_{ss} could not be calculated for these compounds and only $t_{1/2}$ is reported.

In vitro rabbit experiment (microsomal incubates)

Rabbits were killed by administration of 10–15 ml of a solution of potassium chloride and potassium citrate (4% of each), directly into the heart so as to cause rapid cardiac arrest. Livers were excised from the rabbit and washed with Tris–KCl buffer (0.02 *M* Tris–HCl, pH 7.4 containing 1.15% potassium chloride). About 10 g of the right lobe was homogenized in two volumes of Tris–KCl buffer with a Polytron homogenizer. The homogenates were centrifuged at 5 °C and 9000 *g* for 20 min (Sorvall RC-5 centrifuge); the supernatant was decanted and centrifuged at 100 000 *g* for 60 min (Beckman L8-70 ultracentrifuge). The pellet (microsomes) from the latter centrifugation was resuspended in Tris–KCl buffer to a final volume equal to twice the original liver weight, such that the protein content of the microsomes, as measured by the biuret method [13] with bovine serum albumin standards, was about 10 mg/ml of microsomes. Microsomes were kept on ice and used on the same day they were prepared.

Aminopyrine metabolism was determined in a 3.0-ml incubation mixture consisting of: (a) 1 ml of a mixture of 5 m*M* magnesium chloride; 0.43 m*M* nicotin-

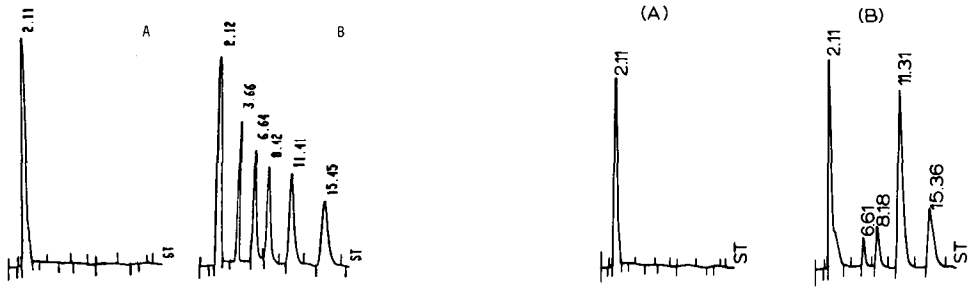

Fig. 2. Chromatograms from the analysis of: (A) blank rabbit plasma sample, (B) rabbit plasma sample obtained 1 h after intravenous administration of aminopyrine (20 mg/kg).

Fig. 3. Chromatograms from the analysis of: (A) blank rabbit microsomal incubate, (B) rabbit microsomal incubate obtained 5 min after incubation with 0.1 m*M* aminopyrine.

amide–adenine dinudeotide phosphate; 10 m*M* glucose 6-phosphate; 1.5 enzyme unit of glucose 6-phosphate dehydrogenase; 50 m*M* Tris–HCl (pH 7.4); (b) 1 ml of microsomes (protein = 10 mg/ml); and (c) 1 ml of 0.3 m*M* aminopyrine as substrate. The mixtures were incubated in a Dubnoff metabolic incubator at 37°C. Samples from the incubates were removed at 0, 5, 10, 15 and 30 min, and the protein was precipitated with an equal volume of acetonitrile. The N-demethylation of AM (0.1 m*M*) was measured in the described incubation mixture by measuring the change in the concentration of AM and by following the generation of MAA and AA in the mixture with time.

RESULTS

Fig. 2 illustrates the chromatogram of a blank plasma sample (panel A) and a plasma sample obtained 1 h after intravenous AM administration (panel B). No peaks derived from endogenous compounds were observed in blank plasma at times that would interface with the assay. Good resolution between peaks of interest was obtained in the plasma sample. Under the chromatographic conditions described, FAA, AA, MAA, AM and the internal standard were euluted with retention times of 3.66, 6.64, 8.42, 11.41 and 15.45 min, respectively. Under these chromatographic conditions, a new sample could be analyzed every 18 min. No interfering peaks were observed in blank microsomal incubates (Fig. 3A). Retention times for AM, the metabolites and the internal standard in microsomal incubates at 5 min after incubation (Fig. 3B) were comparable to those observed in plasma.

In the concentration range studied (0.2–10.0 μg/ml) linear relationships with correlation coefficients greater than 0.990 were observed between peak-height ratios and the concentrations of AM and the metabolites in rabbit plasma (Table I). Similar linear relationships and correlation coefficients were observed between peak-height ratios and the concentrations of AM and the metabolites in the rabbit microsomal incubate. Table II lists the accuracy, coefficient of variation and recovery of AM and the metabolites in rabbit plasma spiked at two different concentrations (0.6 μg/ml and 5.0 μg/ml).

TABLE I

CHARACTERISTICS OF PLASMA STANDARD CURVES

y = peak-height ratio, x = spiked plasma concentration, r = correlation coefficient.

Compound	Regression line	r	Concentration range (μg/ml)
AM	$y=0.297x-0.017$	0.997	0.20–10.0
MAA	$y=0.277x-0.009$	0.991	0.20–10.0
AA	$y=0.433x-0.102$	0.997	0.20–10.0
FAA	$y=0.267x-0.044$	0.996	0.20–10.0

TABLE II

COEFFICIENT OF VARIATION, ACCURACY AND RECOVERY ($n=6$)

Compound	Coefficient of variation (%)		Accuracy (%)		Recovery (%)	
	0.6 μg/ml	5.0 μg/ml	0.6 μg/ml	5.0 μg/ml	0.6 μg/ml	5.0 μg/ml
AM	7.6	5.8	101.3	99.5	96.5	98.5
MAA	7.5	5.1	101.7	100.9	95.4	99.8
AA	9.3	1.3	104.2	99.6	95.0	96.8
FAA	8.7	6.6	99.5	100.4	97.2	98.2

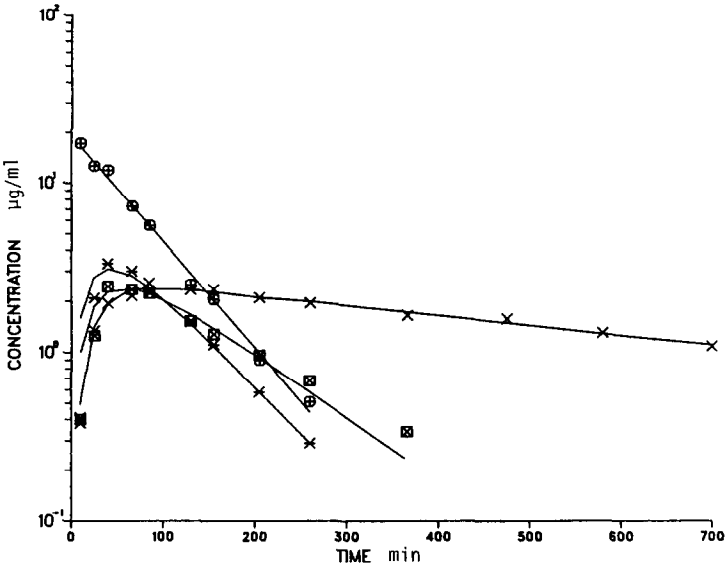

Fig. 4. Profile of aminopyrine and the metabolites in rabbit plasma vs. time, after intravenous administration of aminopyrine (20 mg/kg) to a rabbit. × = 4-formylaminoantipyrine, ⊠ = 4-aminoantipyrine, ⋇ = 4-methylaminoantipyrine and ⊕ = aminoantipyrine.

TABLE III

AMINOPYRINE PHARMACOKINETIC PARAMETERS AFTER INTRAVENOUS ADMINISTRATION

Pharmacokinetic parameter	Rabbit code						Mean ± S.D.
	1	2	3	4	5	6	
Disposition rate constant (min^{-1})	0.01	0.01	0.04	0.009	0.009	0.013	0.015 ± 0.012
Disposition half-life (min)	68.5	68.8	71.5	76.9	73.5	52.6	68.6 ± 8.5
Area under the curve ($\mu g \cdot min/ml$)	1099	1087	1212	1299	1183	1156	1172 ± 78.3
V_{ss} (l/kg)	1799	1827	1702	1709	1792	1313	1690 ± 191.7
Clearance (ml/min/kg)	18.2	18.4	16.5	15.4	16.9	17.3	17.1 ± 1.1

TABLE IV

ELIMINATION HALF-LIVES ($t_{1/2}$) OF MAA, AA AND FAA FOLLOWING INTRAVENOUS DOSE OF AMINOPYRINE

Rabbit code	$t_{1/2}$ (min)		
	MAA	AA	FAA
1	145.4	350.6	572
2	122.0	327.8	546
3	216.6	473.7	508
4	155.9	370.1	598
5	135.9	163.5	563
6	119.5	418.6	543
Mean	149.2	350.7	555
S.D.	35.8	105.5	30.4

The coefficient of variation for AM and the metabolites ranged between 1.3 and 9.3%. The accuracy ranged between 99.5 and 104.2%. Similar values of coefficient of variation ($< 10\%$) and accuracy (> 99–105%) were obtained for spiked microsomal incubates. The recoveries in both cases (0.6 μg/ml and 5.0 μg/ml) were between 95 and 99.8%.

AM and the metabolite levels in plasma samples after intravenous administration of AM (20 mg/kg) to a rabbit are shown in Fig. 4. AM and the metabolites could be quantitated over five half-lives by the described procedure. However, the assay sensitivity can be increased further by increasing the injection volume from 50 to 100 μl. Tables III and IV list the pharmacokinetic parameters calculated for AM and its metabolites in the rabbits following administration of a single intravenous dose of aminopyrine (20 mg/kg).

AM and the metabolites were also quantitated during in vitro incubation of AM with rabbit liver microsomes. Fig. 3B illustrates the concentration of AM

and the concentration of two of the AM metabolites 5 min after incubation. The third metabolite of AM, FAA, appears to be formed by a non-microsomal enzyme, since it did not appear in the microsomal incubates even after 60 min of incubation.

DISCUSSION

The use of a Radial-Pak column is an integral component of this assay. Among the many advantages, the use of Radial-Pak columns allows for flow-rates greater than 2.5–3.0 ml/min with low-pressure drops. The use of a guard column enabled us to analyze as many as 1500 samples without changing the Radial-Pak column. However, the guard column was changed after injecting about 100 samples, as pressure build up was noticed. The use of 0.9% triethylamine was essential for optimal resolution between AM and the metabolites. An increase or decrease in the percentage of triethylamine resulted in poor resolution between AM and its metabolites.

The HPLC method described is a rapid and reproducible procedure, in contrast to earlier assays [9–11], which required extensive extraction procedures. Also, the assay described requires a relatively short run time of 18 min and a small plasma volume of 0.2 ml, as opposed to the method of Katz et al. [10] which requires a run time of 50 min and a plasma volume of 1 ml (coupled with an extensive extraction procedure). The current assay procedure produces sharp, well separated peaks of AM and the metabolites unlike the assay described by Asmardi and Jamali [11], which used two different extraction procedures, with two different internal standards and two different wavelengths (254 nm and 280 nm) to assay dipyrone and MAA in plasma and urine. Inoue et al. [9] reported good separation between AM and the metabolites in rat liver microsomes, but an extensive extraction procedure was involved and a run time of 20–25 min was required. In conclusion, this simple and rapid assay can be successfully applied to quantitate AM and its metabolites in many biological fluids such as plasma and liver homogenates.

ACKNOWLEDGEMENTS

We wish to thank Dr. P. Hajdu' of Hoechst Laboratories (F.R.G.) for the gift of aminopyrine, its metabolites and the internal standard. The services of Miss Judy Cherevka for typing this manuscript are gratefully acknowledged.

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