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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATIONS OF AMINOPYRINE AND ITS METABOLITES IN MAN

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SUMMARY

A quantitative high-performance liquid chromatographic method, using a polystyrene—divinyl benzene (Hitachi No. 3010 gel) column and aqueous methanol as the mobile phase, was employed for the determination of aminopyrine and its related compounds, 4-acetylaminoantipyrine, 4-aminoantipyrine and 4-monomethylaminoantipyrine. Baseline separation could be achieved within 25 min. The method was applied to the recovery of these materials from control urine and human urine. Before separation human urine was adjusted to pH 9 and extracted with ethyl acetate, chloroform and diethyl ether.

INTRODUCTION

Aminopyrine (AM) has widely been used as an analgesic and antipyretic drug. The following compounds are known as the main metabolites of aminopyrine in human urine: 4-acetylaminoantipyrine (4-AcAA), 4-aminoantipyrine (4-AA) and 4-monomethylaminoantipyrine (4-MAA).

Common methods for the analysis of aminopyrine and its metabolites are based on spectrophotometric [1—4], gas chromatographic [5] and mass fragmentographic [6] determinations. High-performance liquid chromatography (HPLC) has been applied to the determination of antipyrine in biological fluids [7].

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In the present paper, rapid HPLC was introduced for the determination of AM metabolites without any derivatization using a porous polymer gel column. We report the separation and individual quantitation of these metabolites in less than 25 min and the application of the method to human urine and the recovery test. The technique can also provide a simple and inexpensive method for the HPLC analysis of aminopyrine and its metabolites in biological fluids.

MATERIALS AND METHOD

Apparatus

An Hitachi Model 635 high-pressure liquid chromatograph equipped with an Hitachi spectrophotometer Model 200-10, and Model 834 chromatoprocessor was used for the analyses. The detector wavelength was 254 nm. Two grams of Hitachi gel No. 3010 porous liquid chromatographic packing, particle size 15—20 μm, were suspended in 10 ml of methanol. After brief vigorous shaking of the suspension, the packing was swollen by allowing to stand for 1 h. Two-thirds of the supernatant was discarded, then the remaining suspension was shaken vigorously and introduced into the stainless-steel column (2.1 mm × 500 mm) through the packing apparatus. Samples (1—2 μl) were injected through a septum using a Hamilton HP 305 syringe. The mobile phase was methanol—water (1:1) and was degassed by sonicating for 10 min before use. The column was maintained at 30° by a regulated water jacket, and the flow-rate was 1.2 ml/min.

Reagents

JP★ grade aminopyrine was recrystallized from ligroin (b.p. 77—80° fractions). 4-Aminoantipyrine and 4-acetylaminoantipyrine were obtained from Wako Pure Chemical (Tokyo, Japan) and purified by recrystallization with chloroform for 4-AA and with diluted ethanol for 4-AcAA. 4-Monomethylaminoantipyrine was prepared by Morita's method [8].

Pyrrole 2-carboxylic acid was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and recrystallized from diluted ethanol before use.

TEK-CHEK® No.1 (control urine for routine analysis) was purchased from Ames (Elkhart, Ind., U.S.A.), and reconstituted by the addition of 15 ml of water before use.

Procedure for recovery test

To 10 ml of human fresh urine excreted from healthy adults, 312.5 μg each of authentic 4-AcAA, 4-AA, 4-MAA and AM were added, and the urine sample was brought to pH 9 with 1 *N* NaOH. The urine was then concentrated to a small volume, and extracted successively with 30 ml each of ethyl acetate, chloroform, and ethyl ether. The combined extracts were dehydrated over anhydrous sodium sulfate.

To the dried extract, 250 μg of pyrrole 2-carboxylic acid as an ethanolic solution were added as an internal standard. The organic phase was evaporated to dryness in vacuo. A stream of nitrogen gas was passed through the residue, and 50 μl of methanol were added to dissolve AM and its metabolites. A 1—2-μl aliquot of this solution was injected into the chromatograph which was

★ JP = Pharmacopoeia of Japan

equipped with an ultraviolet (UV) spectrophotometer (254 nm).

AM and its metabolites were eluted with methanol—water (1:1). The quantities of these materials were determined by comparing the peak areas with that of pyrrole 2-carboxylic acid.

RESULTS AND DISCUSSION

The structures of aminopyrine and its major metabolites are shown in the metabolic pathway in Fig. 1. As can be seen from Fig. 2, a typical HPLC elution pattern was observed after the injection of a mixture of 4-acetylaminoantipyrine, 4-aminoantipyrine, 4-monomethylaminoantipyrine, aminopyrine and pyrrole 2-carboxylic acid as internal standard. An almost complete separation of these compounds was accomplished in less than 25 min, the retention times (min) being 4-AcAA 3.4, 4-AA 7.3, 4-MAA 12.1, AM 19.8. Therefore, the baseline separation obtained using Hitachi gel No. 3010 with aqueous methanol as the mobile phase allows for the individual quantitation of these compounds.

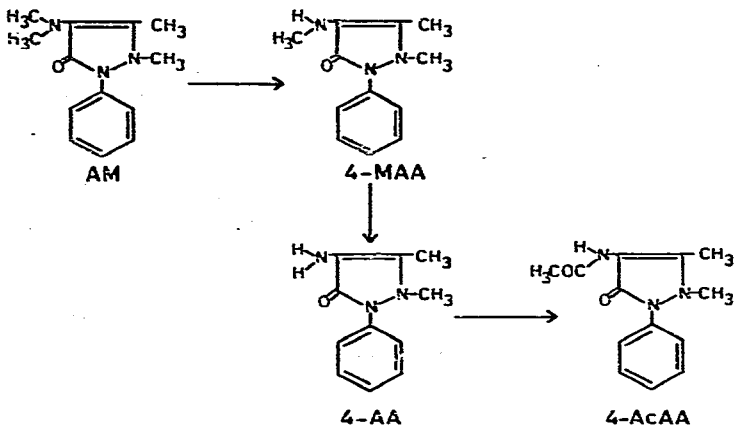

Fig. 1. Structures of aminopyrine and related compounds.

The linear relationship between the peak area ratio for aminopyrine, its metabolites and pyrrole 2-carboxylic acid and the amount of these compounds present per injection is shown in Fig. 3. The areas of the chromatographic peaks were calculated by chromatoprocessor.

The accuracy of determining AM and its metabolites by this method was found to be better than ± 2.5%, with correlation coefficients between 0.997 and 0.999.

Table I shows the results of analysing known amounts of AM and its related compounds by HPLC. The results show that these compounds are well determined in the range 0.44—20 μg for 4-AcAA, 4-AA and 4-MAA, and 5.0—20 μg for AM. The coefficients of variation for the method are below 5% (n=5) for 4-AcAA and 4-AA at 0.45 μg, for 4-MAA at 0.56 μg, and for AM at 5.0 μg. The detection limits were 25, 50, 70 and 120 ng for 4-AcAA, 4-AA, 4-MAA and AM, respectively, with a signal-to-noise ratio of 2:1.

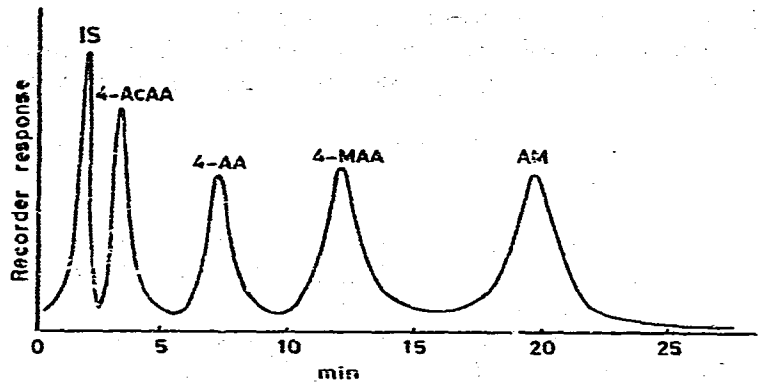

Fig. 2. Typical HPLC elution pattern for a standard mixture of 4-acetylaminoantipyrine (4-AcAA, 5.0 μg), 4-aminoantipyrine (4-AA, 5.0 μg), 4-monomethylaminoantipyrine (4-MAA, 5.0 μg) and aminopyrine (AM, 25.0 μg), with pyrrole 2-carboxylic acid (IS, 2.5 μg) as an internal standard.

The method was examined for the possible interference of urinary constituents. Human urine has many constituents such as uric acid, urea, creatine, hippuric acid, indican, hormones and enzymes. It is crucial that such compounds are not extracted from the urine by the organic solvents. Urine was therefore adjusted to various pH values in the range 2—9, and the solvent extraction for AM and its metabolites carried out. Fig. 4 shows chromatograms of AM and its metabolites extracted from human urine at various pH values. The extracts at pH 2 or 3 contained significant amounts of urine constituents, and each peak overlapped with the peak of the internal standard and 4-AcAA.

With the absorption measured at 254 nm, and the pH of the urine gradually raised to 4, 5 and 6, significant amounts of urine constituents were similarly ex-

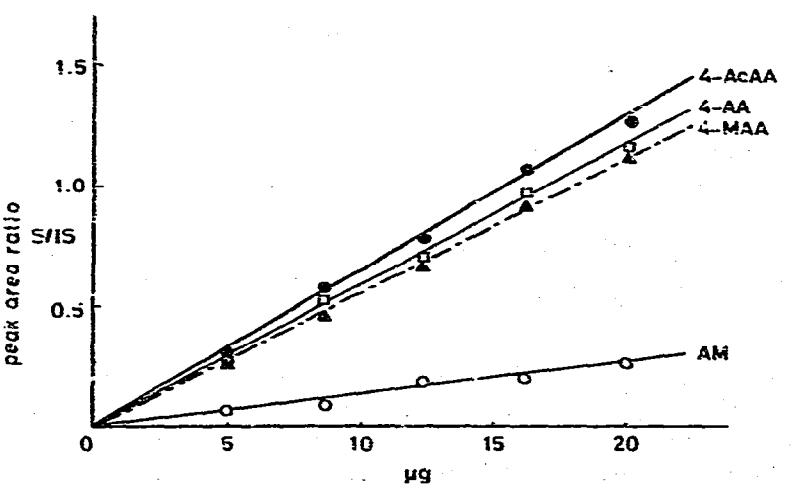

Fig. 3. Calibration curves for aminopyrine and its metabolites with pyrrole 2-carboxylic acid as an internal standard. Each point represents the average of at least five determinations.

TABLE I

DETERMINATION OF AMINOPYRINE AND ITS RELATED COMPOUNDS

$n = 5$.

Amount taken (μg)	4-AcAA		4-AA		4-MAA		AM	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
20.00	98.9	1.0	99.7	2.4	96.9	2.6	100.9	2.2
16.25	100.4	1.0	103.2	2.0	98.9	4.3	101.0	1.5
12.50	95.2	0.7	99.8	2.3	99.6	3.3	100.0	1.5
8.75	101.4	0.6	95.4	1.1	95.5	2.6	99.8	2.2
5.00	95.6	2.5	92.4	2.5	99.2	1.9	109.4	2.3
1.11	97.3	3.7	100.0	2.7	96.8	3.4	96.4	5.6
0.56	98.2	3.6	99.6	2.9	98.0	4.6		
0.444	97.7	4.7	96.4	2.7	95.9	5.6		
0.088	94.3	6.0	90.9	8.0	90.9	8.8		

tracted by the solvent. The extracts at pH 8 also contained a small amount of the urine constituents, but the extracts at pH 9 contained almost none.

On the basis of the analytical results and the properties of the AM metabolites, the following method was used in extraction procedures from urine. The pH of human urine was adjusted to 9, then the urine was concentrated to approx. 1 ml for the solvent extraction, because 4-AcAA is highly soluble in water [9].

In the light of the amount of aminopyrine given as a single dose from a Pharmacopeia of Japan, approximately 300 μg each of aminopyrine and its metabolites were added to 10 ml of human urine from healthy volunteers. After ad-

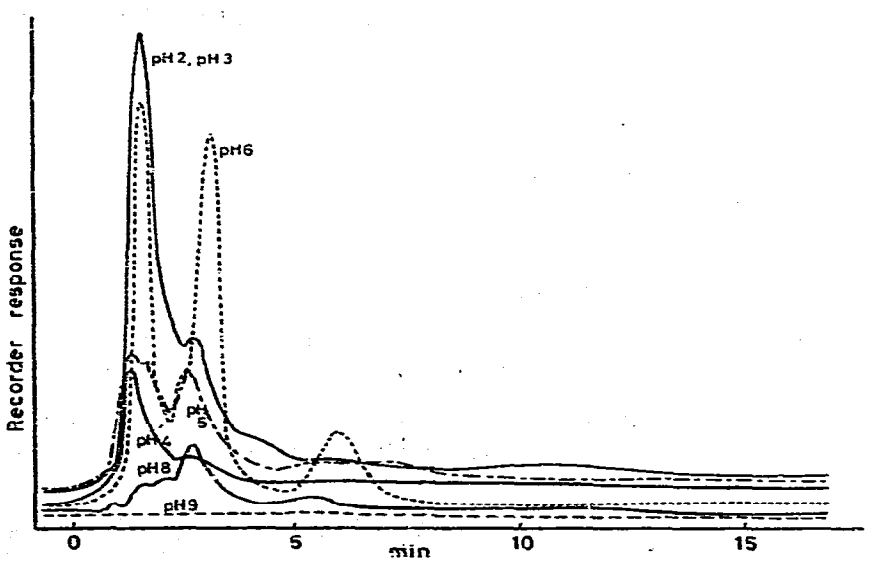

Fig. 4. HPLC of the extracts obtained by solvent extraction of freshly voided human urine at several pH values without the addition of any materials to the urine.

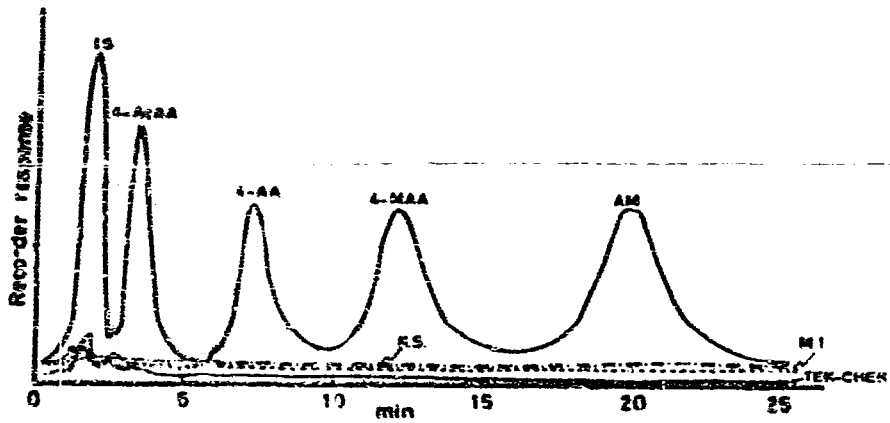

Fig. 5. HPLC of aminopyrine and its metabolites after they had been added to human urine and extracted with the organic solvents at pH 9.

justing the pH of the urine to 9, ethyl acetate, chloroform and ethyl ether extractions were carried out by mechanical shaking for 10 min with 30 ml of each solvent. The HPLC chromatograms of the combined extracts are shown in Fig. 5. The two chromatograms drawn with dotted and broken lines represent those of the blank test, in which AM and its metabolites had not been added to the urine. Furthermore, as the control experiment, the chromatogram of the "control" urine, which is usually utilized as a sensitivity control of the urinary test, is shown by a solid line. The chromatogram thus obtained showed contamination by a small amount of endogenous urinary constituents, which appeared as small peaks and shoulders on the chromatogram. The values for determination were not corrected in this case.

Table II shows the results of the recovery test for AM and its metabolites added to human urine.

TABLE II

RECOVERY OF AM AND ITS METABOLITES ADDED TO HUMAN URINE

Values are given as mean ± S.D. (n=5).

Urine sample	4-AcAA (31.25 μg)	4-AA (31.25 μg)	4-MAA (31.25 μg)	AM (31.25 μg)
TEK-CHEK®	29.87 ± 0.81 (95.5 ± 2.62%)	27.13 ± 1.32 (86.8 ± 4.23%)	23.71 ± 0.70 (75.9 ± 2.25%)	27.53 ± 5.77 (88.1 ± 18.5%)
K.S.	30.00 ± 0.24 (96.1 ± 0.78%)	26.88 ± 1.76 (86.0 ± 5.64%)	28.95 ± 1.44 (92.6 ± 4.60%)	30.11 ± 8.19 (96.3 ± 26.2%)
M.I.	22.40 ± 1.46 (73.1 ± 5.0%)	24.25 ± 2.97 (77.6 ± 9.5%)	25.64 ± 1.41 (82.0 ± 2.8%)	26.42 ± 7.9[illegible] (84.6 ± [illegible])
T.N.	30.80 ± 1.46 (98.5 ± 4.65%)	30.97 ± 1.42 (99.1 ± 4.55%)	27.78 ± 2.8[illegible] (88.9 ± 9.00%)	[illegible]

The idiosyncrasies of the volunteers seem to be revealed as differences in recoveries. The percentage recovery of all AM related compounds is found to be 89% on the average, when the recovery data are included for TEK-CHEK®.

The determination described here for AM and its metabolites has advantages that include complete baseline separation and specific identification of AM metabolites but do not involve a partial and time-consuming procedure. In addition, the method can also be applied to the determination of aminopyrine and its metabolites in urine without further purification and derivatization.

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