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SIMULTANEOUS DETERMINATION OF AMINOPYRINE HYDROXYLATION AND AMINOPYRINE N-DEMETHYLATION IN LIVER MICROSOMES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Aminopyrine and its metabolites, including 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazoline-5-one which is a hydroxylated metabolite of aminopyrine, were separated on a reversed-phase (C_8) Radial-Pak column using a mobile phase of methanol—triethylamine—water (30:1:69) adjusted to pH 5.40 with acetic acid. Detection of the peak was performed by an ultraviolet detector at 254 nm. By the rapid and simple method, aminopyrine hydroxylation as well as aminopyrine N-demethylation in liver microsomes can be examined simultaneously.

INTRODUCTION

Aminopyrine (AM) has been widely used as an analgesic and antipyretic drug or a model substrate for in vitro and in vivo investigation of drug metabolism. Brodie and Axelrod [1] have demonstrated that the major metabolic route is two sequential N-demethylations to give first 4-monomethylaminoantipyrine (MAA) and then 4-aminoantipyrine (AA). This route in liver microsomes was confirmed by Gram et al. [2] in the rat and rabbit. Recently, Iguchi et al. [3, 4] demonstrated that 4-formylaminoantipyrine (FAA) is a noticeable metabolite of aminopyrine in man and animals. Nigam et al. [5] found that FAA is formed from AM in the liver microsomes of phenobarbital-treated rats. Another route of AM disposition is the oxidation of the 3-methyl group to give 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazoline-5-one (AM-OH), which was demonstrated by Yoshimura et al. [6]. This is a minor but very important route because the aldehyde intermediate of

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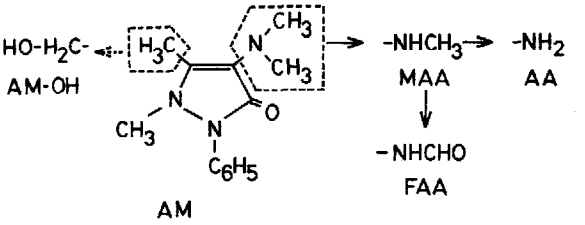

Fig. 1. Illustration of aminopyrine metabolic pathway found and postulated in liver microsomes. The abbreviations are described in the Introduction.

the further metabolite of AM-OH binds irreversibly to tissue macromolecules or proteins and forms stable complexes [7]. It has not been confirmed that AM-OH is formed in liver microsomes.

Although several methods have been described for monitoring AM and its metabolites by high-performance liquid chromatography (HPLC) [5, 8, 9], none has attempted to assay AM-OH as well as MAA, AA, and FAA simultaneously. Fig. 1 shows the metabolic pathway of aminopyrine found and postulated (the left pathway) in liver microsomes.

In this paper we report a rapid and simple assay method that separates the metabolites of aminopyrine, and determines AM-metabolizing enzyme activity in liver microsomes.

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system and Model U6K injector. Separations were carried out on a 10 cm × 5 mm I.D. Radial-Pak C_8 (10 μm particle size) column (Waters Assoc.). The mobile phase was methanol—triethylamine—water (30:1:69) adjusted to pH 5.40 with acetic acid. The flow-rate was 1 ml/min. Detection was performed by a Model 440 UV detector (Waters Assoc.) at 254 nm. Areas of peaks were calculated by a Waters data module.

Materials

Aminopyrine was purchased from Daiichi Seiyaku (Tokyo, Japan). AM-OH was kindly supplied by Professor Yoshimura (Kyushu University). FAA and MAA were kindly supplied by Dr. Yoshioka and Dr. Sakai (National Institute of Hygienic Sciences). All other chemicals used were of reagent grade.

Procedure

Rat liver microsomes were obtained according to the method described previously [10, 11]. The standard incubation mixture consisted of the following components in a total volume of 0.5 ml: NADPH 4 m*M*, $MgCl_2$ 5 m*M*, microsomal suspension 0.1 ml, 0.2 *M* sodium phosphate buffer (pH 7.4), and AM 4 m*M*. Incubation was carried out at 37°C; the reaction was stopped by mixing with 1 ml of cold chloroform, then the mixture was centrifuged at 1800 *g* for 5 min. Aqueous and chloroform layers were applied to Extrelut 1 (E.

Merck, Darmstadt, G.F.R.). After 5 min, 9 ml of chloroform were poured onto the column. The eluate was evaporated to dryness in vacuo and dissolved in 100 μl of methanol. Then 10 μl of the solution was injected into the HPLC system.

RESULTS

A typical chromatogram from a single 10-μl injection of a standard solution of AM and four metabolites is presented in Fig. 2. The separation and baseline assay could be achieved within 20 min.

Linearity was evaluated at the concentration range described in Table I. Calibration curves of all compounds were fitted to each regression line and the correlation coefficients were larger than 0.9990. In the case of AM, which is a

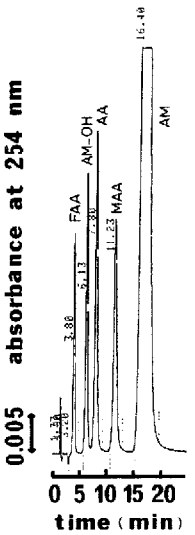

Fig. 2. Chromatogram of a standard solution containing AM, MAA, AA, FAA, and AM-OH.

TABLE I

CALIBRATION CURVE OF AMINOPYRINE AND ITS METABOLITES

The relative area (y) calculated by Waters data module and concentration (x, nmol per 10 μl) were fitted to a function of the regression line $y = ax + b$.

Compound	Concentration range of calibration curve (nmol per 10 μl)	Parameter a	Parameter b	Coefficient of correlation
FAA	0.14— 4.6	2533.2	68.1	0.9993
AM-OH	0.31— 9.9	1947.4	22.3	0.9994
AA	0.26— 8.4	2681.1	25.2	0.9996
MAA	0.26— 16.7	2535.0	20.8	0.9995
AM	0.32— 10.3	2076.1	—21.0	0.9995
	25.0 —200.0	2268.1	—663.2	0.9990

TABLE II

REPRODUCIBILITY OF THE ASSAY

Reproducibility was calculated by the equation

$$\frac{\text{amount found (nmol)}}{\text{amount taken (nmol)}} \times 100 = \text{reproducibility (\%)}.$$

Each value represents the mean ± S.E. of three determinations.

Compound	Amount taken (nmol)	Reproducibility (mean ± S.E. %)		Coefficient of variation (%)
FAA	0.14	95.3	6.08	11.1
	0.29	102.8	3.90	6.6
	0.58	107.2	0.82	1.3
	1.15	110.3	0.96	1.5
	2.30	104.4	1.76	2.9
	4.60	100.0	1.71	3.0
AM-OH	0.14	90.1	4.63	8.9
	0.31	85.9	4.17	8.4
	0.62	91.6	0.63	1.2
	1.24	96.1	0.86	1.6
	2.48	105.4	0.44	0.7
	4.96	101.4	1.52	2.6
	9.91	99.4	1.32	2.3
AA	0.26	83.7	4.45	9.2
	0.52	95.1	2.15	3.9
	1.05	95.8	2.05	3.7
	2.10	105.1	1.02	1.7
	4.19	101.2	1.63	2.8
	8.38	99.5	0.90	1.6
MAA	0.26	82.1	1.21	2.6
	0.52	83.6	2.92	6.0
	1.04	92.2	1.76	3.3
	2.08	96.0	1.79	3.2
	4.17	105.7	0.69	1.1
	8.33	101.3	1.48	2.5
	16.67	99.4	0.98	1.7
AM	0.32	80.6	0.95	2.0
	0.65	81.0	4.00	8.6
	1.29	92.6	3.85	7.2
	2.58	104.2	0.55	0.9
	5.16	99.2	1.70	3.0
	10.32	99.6	0.33	0.6
	25.0	98.9	0.21	0.4
	50.0	100.8	0.89	1.5
	100	99.9	1.60	2.8
	200	98.5	1.71	3.0

TABLE III

RECOVERY

Compounds were added to incubation mixtures without NADPH, incubated for 10 min, and extracted as described in the procedure.
Recovery was calculated by the equation

$$\frac{\text{(amount found at single injection)} \times 10}{\text{amount added to incubation mixture}} \times 100 = \text{recovery (\%)}.$$

Compound	Amount added to incubation mixture (nmol)	Recovery (mean ± S.E. %)		Coefficient of variation (%)
FAA	5 (n = 5)	92.4	2.28	6.0
	20 (n = 6)	97.0	2.34	5.4
AM-OH	10 (n = 5)	95.0	1.85	4.8
	40 (n = 6)	101.4	3.02	6.7
AA	10 (n = 5)	82.0	3.84	10.5
	40 (n = 6)	81.2	2.81	8.5
MAA	10 (n = 5)	83.4	2.13	5.7
	40 (n = 6)	81.6	2.07	6.2
AM	500 (n = 5)	98.3	3.16	7.2
	1500 (n = 6)	97.8	2.67	6.7

substrate, the concentration of unchanged AM is so high that we have to use the parameters a and b in the range (25—200 nmol per 10 μl) to calculate the concentration.

Reproducibility is as described in Table II. The coefficients of variation of all compounds were very small. When FAA 0.14 nmol was injected, the coefficient of variation was 11.1%. But at higher amounts the precision improved.

Recovery studies were carried out by the addition of AM and metabolites to incubation mixtures which contained liver microsomal fraction as described in the procedure. The result is given in Table III. The amount of AM added to incubation mixtures was larger than that of metabolites because it must remain at high concentration. Recoveries of all compounds were constant as shown in Table III.

Fig. 3 shows a typical chromatogram of the chloroform extract of the incubation mixture with liver microsomal enzymes of untreated rat. The main metabolites were MAA (retention time: t_R = 11.36 min), AA (t_R = 8.06 min) and AM-OH (t_R = 6.23 min). The peak at t_R = 5.06 min was not FAA since FAA added to this extract gave both the FAA peak and the peak at 5.06 min.

Fig. 4A shows the relationship between the formation of these three metabolites and incubation time. Linearity was observed from 0.5 to 10 min. AM disposition rate in liver microsomal fractions as a function of enzyme concentration is shown in Fig. 4B. Linearity was observed between 10 and 100 μl of liver microsomal fractions.

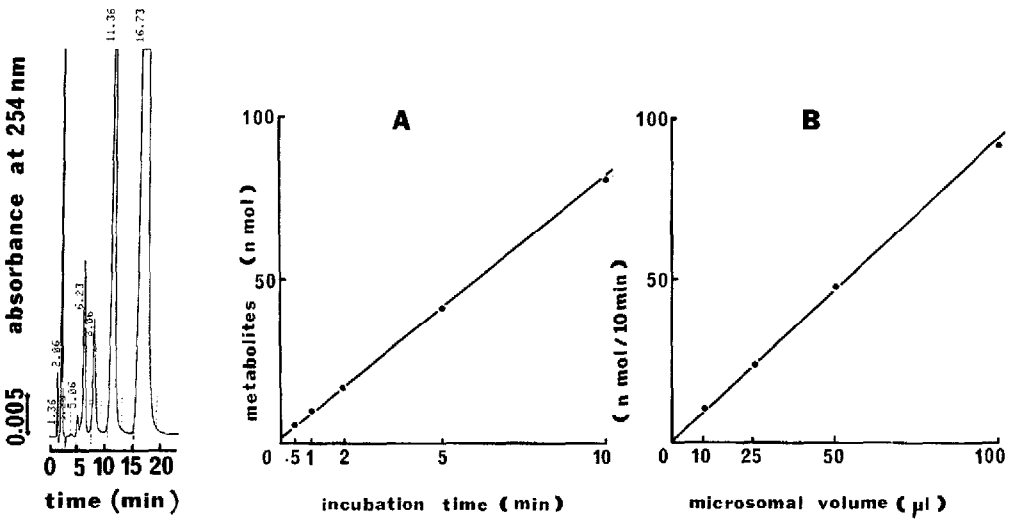

Fig. 3. Chromatogram of the chloroform extract of the incubated mixture containing AM and its metabolites with liver microsomal enzymes of untreated rat. Volume of the extract injected was 10 μl.

Fig. 4. (A) Relationship between the amount of metabolites (MAA + AA + AM-OH) in the liver microsomal enzymes measured by the HPLC assay and incubation time. Incubation mixture contained 100 μl of liver microsomal suspension of rat treated with phenobarbital (60 mg/kg intraperitoneally, 72, 48 and 24 h before sacrifice). Incubations were carried out at 37°C. (B) Relationship between the amount of metabolites and microsomal volume as enzyme. Incubations were carried out for 10 min at 37°C.

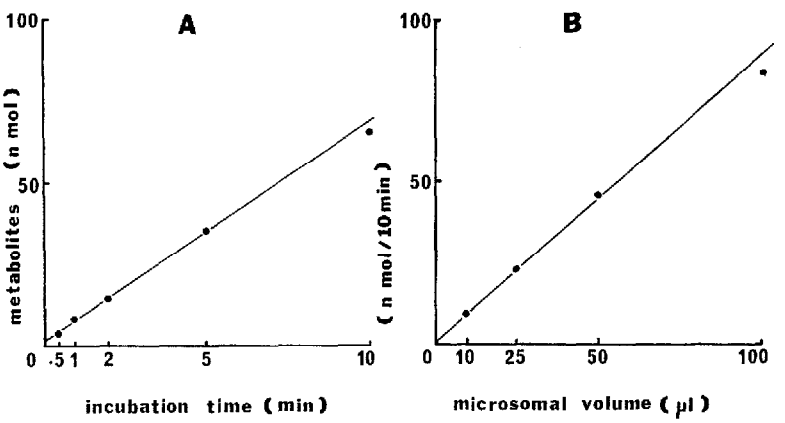

Fig. 5. (A) Relationship between the amount of MAA + AA formed and incubation time. The incubation conditions were the same as in Fig. 4A. (B) Relationship between the amount of MAA + AA formed and microsomal volume as enzyme. Incubations were carried out for 10 min at 37°C.

Fig. 5A shows the relationship between the total formation of MAA and AA, and incubation time. Linearity was observed from 0.5 to 10 min. The formation of MAA and AA under these conditions is regarded as a result of AM N-demethylation. The AM N-demethylation rate in liver microsomal fractions as a function of enzyme concentration is shown in Fig. 5B. Linearity was observed between 10 and 100 μl of liver microsomal fractions.

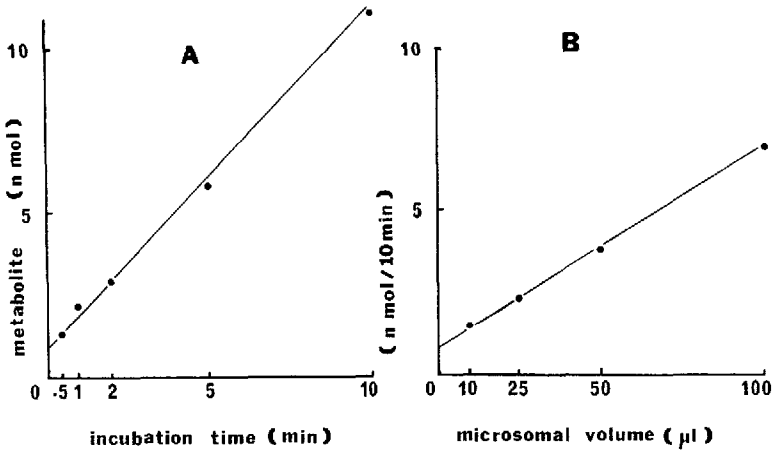

Fig. 6. (A) Relationship between the amount of AM-OH formed and incubation time. The incubation conditions were the same as in Fig. 4A. (B) Relationship between the amount of AM-OH formed and microsomal volume as enzyme. Incubations were carried out for 10 min at 37°C.

Fig. 6A shows the relationship between the formation of AM-OH and incubation time. Linearity was observed from 0.5 to 10 min. AM hydroxylation activity in liver microsomes as a function of enzyme concentration is shown in Fig. 6B. Linearity was observed between 10 and 100 μl of liver microsomal fractions.

DISCUSSION

A multi-faceted approach to the analysis of AM metabolism in liver microsomes has been presented. The primary advantage of this method is to determine simultaneously AM hydroxylation as well as AM N-demethylation activity.

The hydroxylation pathway has been thought to be the minor one in AM disposition [6, 7]. But the percentage of AM-OH of the total metabolites was higher than 15% in liver microsomes of phenobarbital-treated rats (calculated from Figs. 4 and 6). This value can not be ignored in the study of AM disposition. Moreover, the further metabolite of AM-OH binds irreversibly to macromolecules. This phenomenon is very important because the stable complex formed is thought to be the main factor behind AM allergy [7]. It is possible that this metabolite is concerned with other toxicities of AM. Therefore the examination of AM-hydroxylation is very necessary and the HPLC method to separate AM-OH has proved to be very useful.

The simultaneous separation of AM-OH, AA and/or MAA has not been previously reported. The separation of AM-OH from AA and/or MAA depends on the property of the column and the pH of the mobile phase. We used a Radial-Pak C_8 column and a mobile phase consisting of methanol—triethylamine—water (30:1:69) at pH 5.40. A good separation was achieved by these conditions.

This method is very simple and rapid, and will be a useful tool for an all-round examination of AM metabolism in liver microsomes.

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