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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 6-AMIDINO-2-NAPHTHYL [4-(4,5-DIHYDRO-1*H*-IMIDAZOL-2-YL)AMINO]BENZOATE DIMETHANESULPHONATE AND ITS METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

6-Amidino-2-naphthyl [4-(4,5-dihydro-1*H*-imidazol-2-yl)amino]benzoate dimethanesulphonate has been developed for the therapy of pancreatitis. A reversed-phase high-performance liquid chromatographic assay of the levels of this drug and its metabolites in biological fluids was investigated. Fluorescence detection with post-column alkaline degradation was used for the determination of the intact drug and the amidinonaphthol moiety metabolite, and ultraviolet detection at 254 nm was used to determine the levels of the benzoic acid moiety metabolite. Satisfactory recoveries and variabilities of the intact drug and its metabolites from biological fluids were obtained. The detection limits for the intact drug and amidinonaphthol were 0.5 ng/ml at a signal-to-noise ratio of 12 in plasma and 10 ng/ml at a signal-to-noise ratio of 32 in urine and homogenized faeces, and those of benzoic acid were 5 ng/ml at a signal-to-noise ratio of 3 in plasma and 50 ng/ml at a signal-to-noise ratio of 7 in urine and homogenized faeces.

INTRODUCTION

6-Amidino-2-naphthyl [4-(4,5-dihydro-1*H*-imidazol-2-yl)amino]benzoate dimethanesulphonate (A, FUT-187) is a new derivative of amidinonaphthol, developed as an oral therapeutic agent for use in pancreatitis [1,2].

Compound A is metabolized in the body to 6-amidino-2-naphthol (AN) and [4-(4,5-dihydro-1*H*-imidazol-2-yl)amino]benzoic acid (IABA), which can then form the respective glucuronide conjugates, as shown in Fig. 1.

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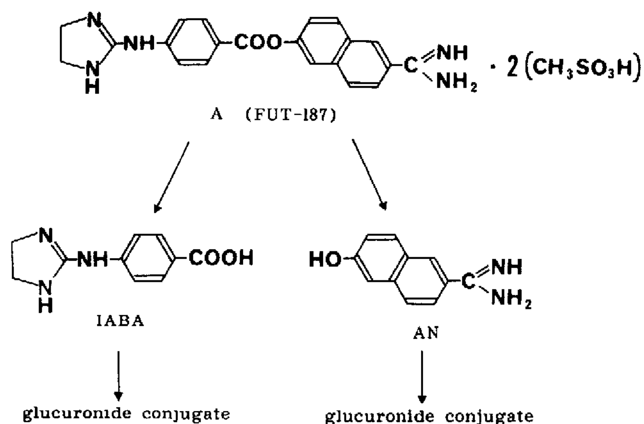

Fig. 1. Metabolic pathway of compound A.

In order to study the metabolism of compound A, a quantitative method for the determination of A and its metabolites in biological materials is required. The spectrofluorometric method, based on trypsin-inhibition activity, has been used to determine the level of nafamostat mesilate, a compound related to A, in blood samples [3]. However, this method is not suitable for the determination of A because of its lower inhibitory potential to trypsin. Furthermore, there are no reports of the determination of AN and IABA in biological materials.

Accordingly, two high-performance liquid chromatographic (HPLC) methods were developed for the determination of compound A and its metabolites in biological fluids.

EXPERIMENTAL

Reagents and chemicals

Compound A and its metabolites, AN and IABA, were supplied by Torii (Chiba, Japan). β-Glucuronidase (Type IX) was purchased from Sigma (St. Louis, MO, U.S.A.). The ion-pair chromatographic reagent, sodium 1-hexanesulphonate, and other chemicals used were all purchased from Wako (Osaka, Japan). Methanol, ethanol and acetonitrile were of liquid chromatographic reagent grade.

The Sep-Pak C_{18} cartridge used for the extraction was obtained from Waters Assoc. (Milford, MA, U.S.A.). The cartridge was activated before use with consecutive 5-ml volumes of distilled water, methanol and distilled water. Milex-HA and -HV filter units (0.45 μm) were obtained from Millipore (Bedford, MA, U.S.A.).

The 0.1 *M* acetate buffer (pH 5.5) used for the enzymic degradation was prepared by dissolving 8.203 g of sodium acetate in 1.0 l of ion-exchanged water; the pH was adjusted with acetic acid.

Instrumentation and chromatographic conditions

The liquid chromatographic system (Shimadzu, Kyoto, Japan) consisted of two LC-6A pumps, an RF-535 variable-wavelength fluorescence detector, an SPD-

2AS variable-wavelength UV detector, a CTO-6A column oven, a CRB-6A reaction oven, a SIL-6A automatic injector and a C-R3A Chromatopac data system and a third LC-6A pump, used for post-column reagent addition.

A Shim-pack CLC ODS column (150 mm×6 mm I.D., 5 μm particle size) (Shimadzu) was used for the separation. For determination of A and AN, the mobile phase was prepared with acetonitrile and 0.1 *M* acetic acid solution (pH 3.0) containing 0.03 *M* sodium 1-hexanesulphonate (18:82, v/v); the flow-rate was 1.2 ml/min. For post-column alkaline degradation, 2.0 *M* potassium hydroxide solution was added through a low-dead-volume T-connector at a flow-rate of 0.4 ml/min. The reaction-coil size was 2.0 m×0.8 mm I.D. The column and reaction coil were maintained at 45 and 70°C, respectively. The eluted compounds were measured using the fluorescence detector set at 320 nm for excitation and 485 nm for emission; the sensitivity (*H*) was set at 32.

For separation of IABA, the mobile phase was prepared with methanol and 0.1 *M* acetic acid solution (pH 3.0) containing 0.03 *M* sodium 1-hexanesulphonate (35:65, v/v); the flow-rate was 1.5 ml/min. The column was maintained at 45°C, and the eluted compound was measured using the UV detector set at 254 nm; the attenuator was set at 0.01 a.u.f.s.

Sample preparation

Blood samples were collected in heparinized containers and immediately centrifuged for 15 min at 2000 *g* at 5°C in a refrigerated centrifuge in order to separate the plasma. The plasma was then instantly transferred to a 10-ml test-tube containing 20 μl of 1.0 *M* hydrochloric acid. After collection, urine samples were transferred to a 1-l bottle and acidified with 10% (v/v) formic acid to pH 3–4 (ca. 30 ml for 1 l of urine), then placed in an ice-bath. Faeces samples were immediately homogenized with four times the volume of 1% (v/v) formic acid, and 10 ml of the homogenate were collected. The acidified plasma, urine and faeces homogenate samples were frozen at −20°C until analysis.

Acidified plasma samples (0.5 ml) were mixed and centrifuged, then transferred to a 10-ml glass centrifuge tube and diluted with 1 ml of distilled water and 0.1 ml of 1.0 *M* hydrochloric acid. The samples were applied to a Sep-Pak C_{18} cartridge, washed with 4 ml of 0.01 *M* hydrochloric acid and then eluted with 5 ml ethanol containing 1% (v/v) of 0.5 *M* formic acid. The ethanol eluate was dried under nitrogen gas at 15°C and then dissolved in 0.2 ml of the mobile phase.

Acidified urine samples (0.5 ml) were filtered with a Milex-HA filter unit (0.45 μm).

To 0.5 ml of acidified faeces homogenate, 2.5 ml of ice-cold methanol containing 2.5% (v/v) of 0.5 *M* formic acid were added. The solution was mixed well for a few minutes and centrifuged for 15 min at 2000 *g* in a refrigerated centrifuge (5°C). The resulting supernatant was filtered with a Milex-HV filter unit (0.45 μm).

Finally 20 μl of each preparation were analysed by the two HPLC methods, equipped with fluorescence detection by post-column alkaline degradation and UV detection, respectively.

For the determination of glucuronide conjugates of AN and IABA, the acidified

plasma, urine and supernatant of homogenized faeces were dissolved in 0.1 *M* acetate buffer (pH 5.5), and 1000 units of β-glucuronidase were added to the solution. The mixture was then incubated at 37°C for 6 h to hydrolyse the conjugates. After cooling, the incubate was subjected to further hydrolysis at 25°C for 10 min by addition of 0.2 ml of 1.0 *M* sodium hydroxide; 0.4 ml of 1.0 *M* hydrochloric acid was then added. Each preparation was treated by the above-mentioned procedures and then analysed by both HPLC methods.

Calibration curves

A series of standard solutions of compound A and its metabolites, AN and IABA, containing 5, 20, 100, 400, 1000, and 2000 ng/ml for plasma samples and 0.5, 2, 10, 40 and 100 μg/ml for urine and faeces homogenate samples in 1 ml of 0.01 *M* hydrochloric acid solution were prepared. Each standard solution was found to be stable at 5°C for up to one month. A 20-μl sample of each standard solution was analysed using the HPLC methods, and calibration curves were obtained by plotting the peak areas of A, AN and IABA against the concentration of each compound. Each calibration curve was linear: A, $y=2.217\cdot10^5x-3689$ ($r=0.9999$); AN, $y=4.859\cdot10^5x-2237$ ($r=0.9999$); IABA, $y=1.491\cdot10^3x-0.023$ ($r=0.9999$).

RESULTS AND DISCUSSION

Sample preparation

Since compound A is biologically hydrolysed, particularly in blood, the collected plasma and urine samples were immediately acidified with hydrochloric acid or formic acid to stabilize A until analysis. The faeces samples were homogenized with methanol containing formic acid.

A Sep-Pak C_{18} cartridge containing μBondapak C_{18}/Porasil (R/B) was used to clean up plasma samples. It was possible to isolate A and its metabolites on this cartridge using a diluted hydrochloric acid solution and subsequent elution with organic solvents, i.e. methanol, ethanol, acetonitrile and these solvents containing formic acid. Various solvents were tested as eluents, and ethanol containing 1% (v/v) of 0.5 *M* formic acid was found to give the highest recoveries of A, AN and IABA.

Thus, the Sep-Pak C_{18} cartridge was used to extract the plasma samples, this form of preparation being simple and fast.

In addition, extraction with organic solvents was attempted for plasma samples; however, this method proved to be unsatisfactory owing to the poor recoveries of AN and IABA. The deproteinization of plasma samples by the addition of various solvents was also examined. The use of ethanol containing 1% (v/v) of 0.5 *M* formic acid to prepare a protein-free filtrate from plasma gave highly reproducible recoveries; however, the resulting supernatant gave poor resolution of IABA on the chromatogram.

Filtration with a Milex filter unit was used for the preparation of urine and homogenized faeces samples. No significant adsorption of A, AN and IABA on this filter was observed.

Glucuronide conjugates of AN and IABA in plasma, urine and faeces could be determined using the same preparation procedure and under the same HPLC conditions as non-conjugated compounds after the following treatment. Each sample was diluted with 0.1 *M* acetate buffer (pH 5.5) containing β-glucuronidase, and the solution was incubated at 37°C for 6 h to hydrolyse the glucuronide conjugates. Subsequently, the resulting solution was hydrolysed with 0.2 ml of 1.0 *M* sodium hydroxide to decompose compound A into AN and IABA, since A was partially hydrolysed to AN and IABA during the enzymic degradation procedure. Using this method, concentrates of AN and IABA were obtained in the form of total non-conjugates. Acetate buffer at various pH values was tested for enzymic degradation and a pH of 5.5 was found to be the most suitable, giving the highest recovery for all compounds. The concentrations of conjugated AN and IABA were calculated by subtracting the concentrations of non-conjugates plus those derived from hydrolysis of A from those measured by the above procedure.

Chromatography

It was difficult to establish a sensitive method for the simultaneous assay of compound A and its metabolites, AN and IABA, owing to their different polarities. Thus separate methods for the assay of A and AN, and of IABA were investigated.

Metabolite AN could be monitored by a fluorescence detector with high detection limit, whereas compound A could not be detected by this method. Furthermore, the UV detection of A showed poor sensitivity. Consequently, a post-column technique using hydrolysis with potassium hydroxide, to convert A into AN was used to measure A. The most suitable size for the reaction coil and reaction temperature were found to be 2.0 m×0.8 mm I.D. and 70°C, respectively. Simultaneous assay of A and AN was carried out using post-column alkaline degradation with fluorimetric detection. UV detection was used to monitor IABA.

Compounds A, AN and IABA were well separated from biological constituents when most reversed-phase chromatographic columns were used. After various tests, a Shim-pack CLC ODS column was chosen. An isocratic solvent system consisting of acetonitrile and 0.1 *M* acetic acid solution (pH 3.0) containing 0.03 *M* sodium 1-hexanesulphonate (18:82, v/v) was chosen for separation of A and AN, and a system of methanol and 0.1 *M* acetic acid solution (pH 3.0) containing 0.03 *M* sodium 1-hexanesulphonate (35:65, v/v) for separation of IABA. These ion-pair procedures were found to be suitable for the separation of A and AN or IABA from biological materials. Other conditions, e.g. the use of a Unisil Q C_{18} column (250 mm×4.6 mm I.D., 10 μm particle size) (Gasukuro kogyo, Tokyo, Japan) and acetonitrile and 0.1 *M* acetic acid solution (pH 3.0) containing 0.03 *M* sodium 1-hexanesulphonate (30:70, v/v for plasma and faeces and 28:72, v/v for urine), also resulted in good chromatographic separation of A and AN in biological materials.

Typical chromatograms showing the separation of blank, A, AN and IABA prepared from human plasma and urine following addition of each compound and chromatograms of blank, total AN and IABA prepared from human plasma and

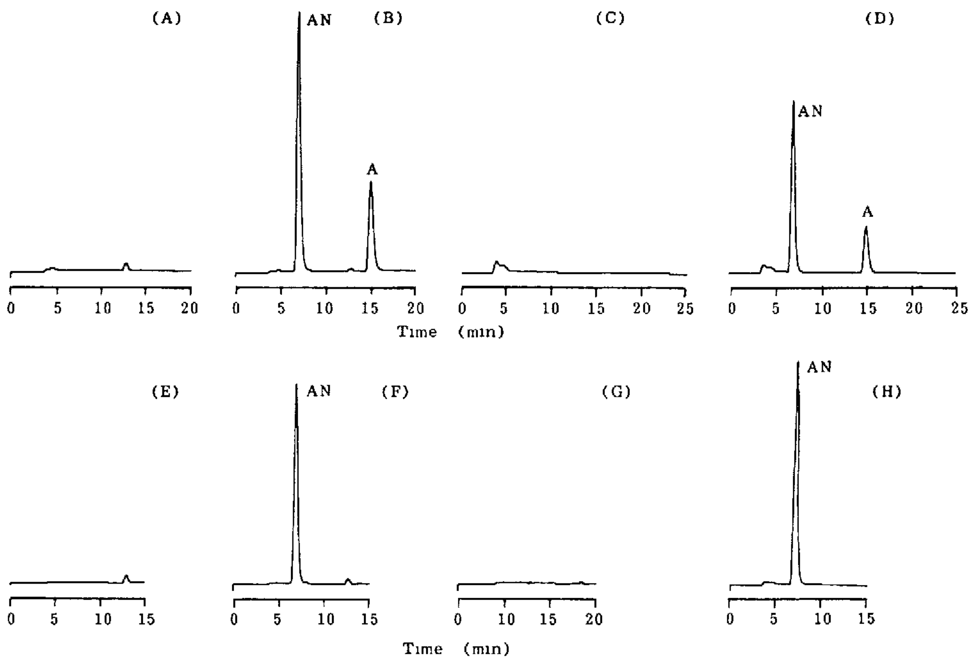

Fig. 2. Chromatograms showing the separation of compounds A and AN prepared from human plasma (B) and urine (D) and blank plasma (A) and urine (C), and the chromatograms of total AN prepared from human plasma (F) and urine (H) and blank plasma (E) and urine (G) after enzymic degradation. Details of the post-column HPLC conditions with fluorescence detection are described in the text.

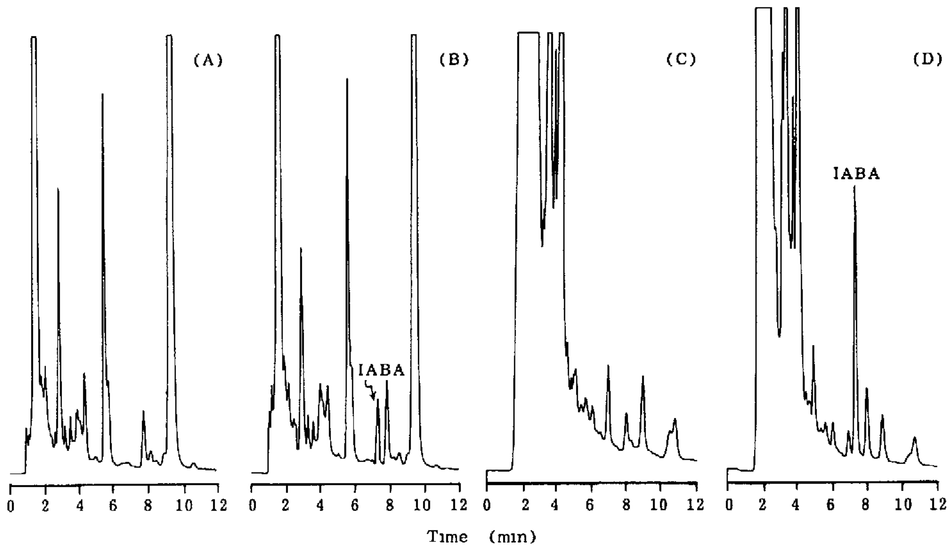

Fig 3. Chromatograms showing the separation of IABA prepared from human plasma (B) and urine (D) and blank plasma (A) and urine (C). Details of the HPLC conditions with UV detection are described in the text

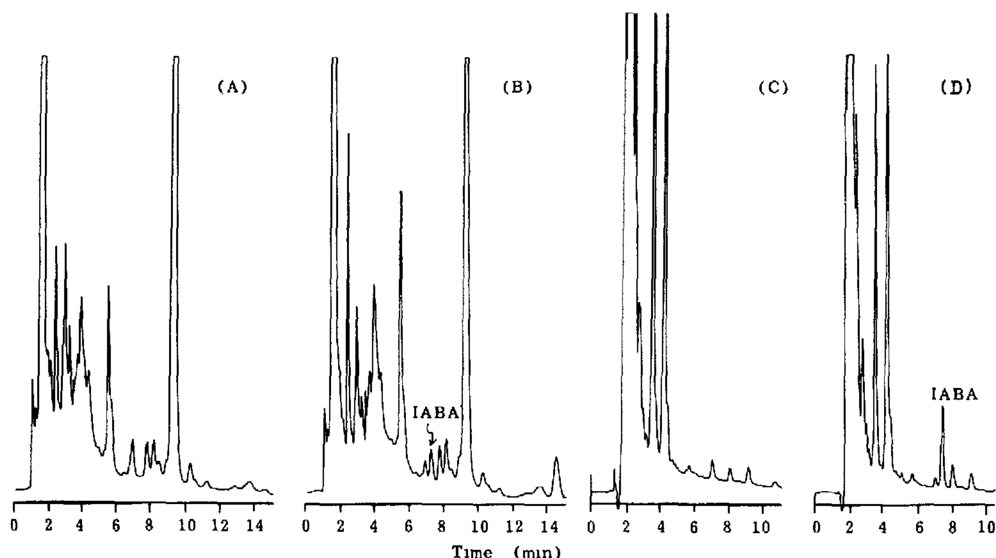

Fig. 4. Chromatograms showing the separation of total IABA prepared from human plasma (B) and urine (D) and blank plasma (A) and urine (C) after enzymic degradation. Details of the HPLC conditions with UV detection are described in the text.

urine after enzymic degradation are shown in Figs. 2–4. The retention times of A, AN and IABA, under these HPLC conditions were 14.9, 6.9 and 7.3 min, respectively. The prepared faeces sample showed a similar chromatographic pattern. The chromatographic interferences from constituents endogenous to all biological materials were negligible. Thus, these methods appear to be satisfactory for the determination of A and its metabolites in human biological materials when A is administered.

Recovery, variability and sensitivity

Known amounts (5 ng/ml to 100 μg/ml) of compounds A, AN and IABA were added to blank samples of human plasma, urine and faeces homogenate, and the recoveries of these compounds were determined by the proposed methods. As indicated in Table I, satisfactory recoveries and variabilities of A, AN and IABA from plasma and urine were obtained with good intra-assay coefficients of variation (C.V.). The recoveries from faeces were 87.9 $\pm$ 2.8% for A, 93.4 $\pm$ 1.9% for AN and 93.3 $\pm$ 3.8% for IABA. Enzymic degradation and subsequent hydrolysis to produce total non-conjugates of AN and IABA also resulted in good recoveries.

The detection limits for A and AN using the post-column HPLC method were 0.5 ng/ml at a signal-to-noise ratio (S/N) of 12 in plasma and 10 ng/ml at S/N = 32 in urine and faeces homogenate, and those for IABA using an UV detector were 5 ng/ml at S/N = 3 in plasma and 50 ng/ml at S/N = 7 in urine and homogenized faeces. The time required for these assays was 25 min for A and AN and 30 min for IABA. The intra-assay reproducibility was $\pm$2.8%. No significant difference was observed when the recoveries of A and AN from plasma using the

TABLE I

RECOVERIES OF COMPOUNDS A, AN AND IABA FROM HUMAN PLASMA AND URINE

Each value is the mean ± S.D of three determinations. No value indicates below detection limit.

Biological fluid	Added (μg/ml)	Recovery (%)		
		A	AN	IABA
Plasma	2.00	99.1±0.4	95.0±0.0	90.9±3.0
	1 00	94.7±1.4	91.2±1.4	85.2±1.3
	0.40	97.5±1.4	96.6±1.5	85.6±5.1
	0 20	94.4±0.1	96.9±0.2	95.4±6 5
	0.10	102.6±1.1	103.1±2.6	104.0±6.9
	0.02	99.7±3.7	100.5±0.8	90.8±1.9
	0.005	85.2±1.5	86.2±2.4	98.3±0.7
	0.002	96 1±4.4	104.5±8.2	–
C.V. (%)		5.3	6.4	7.6
Urine	100.00	100.3±3.1	100.1±2.8	97 3±0.7
	40.00	100.5±4.7	99 1±4.7	88.4±1.0
	10.00	96.7±2.6	102.4±2.8	90.0±2.6
	2.00	93.7±3.4	105.7±3.7	84.4±1.7
	0.50	101.7±5.6	102.6±5.9	106.0±1.1
	0.05	99.8±2.3	101.4±3.8	100.9±2.2
C.V. (%)		4.6	4.2	8.0

present preparation procedure and from blood deproteinized by the addition of ethanol containing formic acid were compared.

Stability

Compounds A, AN and IABA were found to be stable in the final prepared solutions at room temperature (25°C) for up to 15 h following the preparation procedure described; hence, the present method can be used with an automatic injector for overnight analysis.

The stability of A and its metabolites in ethanol solution containing formic acid was also assessed at both room temperature (25°C) and 5°C. No significant degradation was observed.

Subsequently, the stability of A and its metabolites was evaluated in human plasma and urine acidified with hydrochloric acid or formic acid at 5°C and −20°C. No significant drug degradation in frozen samples was observed during two weeks or after standing at 5°C for one day.

Application

The present HPLC methods were also applied to animal plasma and urine. The results obtained under the following procedure for the chromatographic separation, recovery, sensitivity and precision were in good agreement with those obtained with human plasma and urine. For the assay of A and its metabolites in visceral tissue, 0.5 g of visceral tissue was homogenized in an ice-bath with 4 ml of methanol solution containing 5% (v/v) of 0.5 *M* formic acid and then centri-

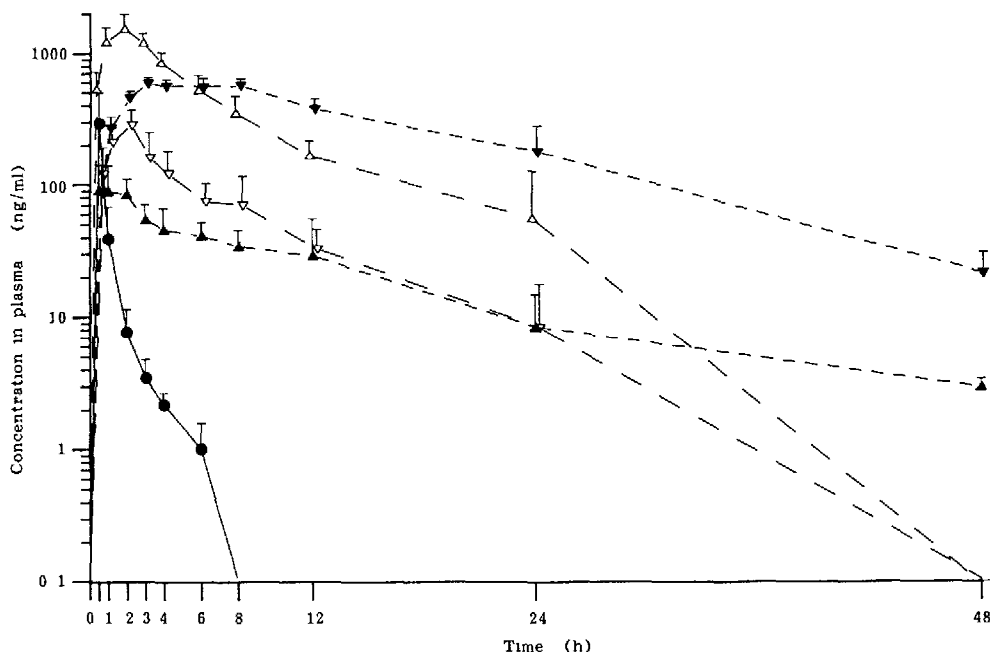

Fig. 5. Plasma levels of compound A and its metabolites after oral administration of A at a dose of 300 mg to beagle dogs. Each point is presented as the mean ± S.D. of five dogs. (●) A; (▲) AN; (▼) AN conjugate; (△) IABA; (▽) IABA conjugate.

fuged at 2000 *g* at 5 °C for 15 min. This homogenization was repeated three times, and the combined supernatants were dried under a stream of nitrogen. The residue was dissolved in distilled water acidified with 20 μl of 1.0 *M* hydrochloric acid. This preparation was then treated by the same procedure as for plasma samples and analysed by the HPLC methods described.

To determine the levels of A and its metabolites in pancreatic juice, an acidified sample was filtered with a Milex-HA filter unit (0.45 μm) and subjected to two HPLC analyses. Compound A was most unstable in pancreatic juice, therefore the collected sample was immediately acidified with 1.0 *M* hydrochloric acid. No degradation of A was observed in this acidified pancreatic juice for two weeks at −20 °C and for one day at 5 °C and room temperature (25 °C).

Compound A was given orally at a dose of 300 mg to five beagle dogs, and the time course of changes in the concentrations of A and its metabolites in plasma were measured by the HPLC method described (Fig. 5). Clinical pharmacological studies on A are now under way and the results will be reported elsewhere.

The specification, precision and sensitivity of these methods appear to be satisfactory for the determination of the levels of A and its metabolites in plasma, urine, faeces and other biological materials. It should be useful for basic and clinical pharmacological studies on compound A.

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