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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF [3,4-DI-(4-METHOXYPHENYL)-5-ISOXAZOLYL]ACETIC ACID AND ITS METABOLITES IN HUMAN PLASMA AND URINE

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SUMMARY

A high-performance liquid chromatographic method is described for the simultaneous determination of the non-steroidal analgesic and anti-inflammatory agent [3,4-di-(4-methoxyphenyl)-5-isoxazolyl]acetic acid and its three metabolites in plasma and urine. Deproteinized plasma (with acetonitrile) or urine was applied to a Sep-Pak C_{18} cartridge, washed with distilled water and then eluted with methanol. The methanol eluate was reduced to dryness. The resulting residues from the plasma and urine were redissolved in methanol and 40% methanol aqueous solution, respectively. Aliquots of each solution were chromatographed on a reversed-phase column using a mobile phase of methanol–20 m*M* potassium dihydrogenphosphate (pH 6.4) (linear gradient from 0 to 100% methanol at 3%/min with a flow-rate of 1.5 ml/min) on a liquid chromatograph equipped with an ultraviolet absorbance detector (254 nm). Detection was limited to 10 ng/ml in plasma and 100 ng/ml in urine for each compound. An accurate and sensitive assay for the determination of [3,4-di-(4-methoxyphenyl)-5-isoxazolyl]acetic acid and its metabolites was established.

INTRODUCTION

[3,4-Di-(4-methoxyphenyl)-5-isoxazolyl]acetic acid (I) is a new derivative of isoxazol [1] that was developed as a non-steroidal analgesic and anti-inflammatory agent [2].

Compound I is metabolized in the body mainly to [3-(4-hydroxyphenyl)-4-(4-methoxyphenyl)-5-isoxazolyl]acetic acid (II) and [3-(4-methoxyphenyl)-4(4-hydroxyphenyl)-5-isoxazolyl]acetic acid (III), with metabolites II and III then proceeding to [3,4-di-(4-hydroxyphenyl)-5-isoxazolyl]acetic acid (IV), as shown in Fig. 1. These metabolites undergo further changes to the respective conjugated compounds.

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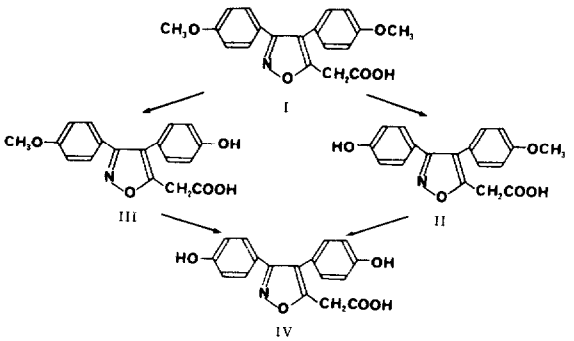

Fig. 1. Main metabolic pathways of compound I.

For a clinical elucidation of the metabolism of I, i.e. absorption and excretion, it seems important to establish a quantitative method for the determination of the concentrations of I and its metabolites in human biological fluids. As yet, there are no reports of such a method. Therefore, we examined various methods for the assay of I and its metabolites in human biological fluids, and established a successful analytical method employing high-performance liquid chromatography (HPLC) using a reversed-phase chromatographic column with a UV absorbance detector.

EXPERIMENTAL

Materials and reagents

Compound I and its metabolites, II, III and IV, were synthesized and purified in our laboratory. Other chemicals used were all purchased from Wako Pure Chemicals (Osaka, Japan). Acetonitrile and methanol were of liquid chromatographic reagent grade.

The 10 m*M* phosphate buffer (pH 7.0) used for the dilution of urine samples was prepared by dissolving 35.81 g of disodium hydrogenphosphate 12-hydrate and 13.61 g of potassium dihydrogenphosphate in 1 l of distilled water, adjusted with sodium hydroxide or phosphoric acid if necessary.

Instruments

A Shimadzu Model LC-6 high-performance liquid chromatograph equipped with a Model SPD-6AV variable-wavelength detector, a Model C-R3A Chromatopac data system and a Model SIL-6A automatic injector (Shimadzu, Kyoto, Japan) was used.

A Shimadzu Shim-pack CLC-ODS chromatographic column (150×6 mm I.D., 5 μm particle size) was used for the separation. The mobile phase was a linear gradient (3%/min) of 0% methanol in 20 m*M* potassium dihydrogenphosphate (pH 6.4) as the initial concentration and 100% methanol as the final concentration; the flow-rate was 1.5 ml/min. The column was maintained at 35 °C, with the eluted compound thus being recorded by the detector at a constant wavelength of 254 nm; the attenuator was set at 0.04 a.u.f.s.

Preparation procedure

Blood samples were collected in heparinized containers and centrifuged for 15 min at 2000 *g* in a refrigerated centrifuge in order to separate the plasma. The plasma and urine were frozen at −20°C until taken for analysis.

Ice-cold acetonitrile (4 ml) was added to 0.5 ml of plasma in a 10-ml centrifuge tube, which was then well vortexed for 30 s. The sample was placed on ice for 30 min and then centrifuged for 15 min at 2000 *g* in a refrigerated centrifuge to remove proteins. The clear supernate was transferred to a 10-ml centrifuge tube and concentrated to dryness under nitrogen gas at water temperature, with the resulting residue being redissolved in 2 ml of distilled water. Subsequently, this solution was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.), which was activated with consecutive 5-ml volumes of methanol and distilled water before use, washed with 3 ml of distilled water and then eluted with 4 ml of methanol. The methanol eluate was reduced to dryness under nitrogen gas at water temperature and redissolved in 0.1 ml of methanol. Then 20 μl of this final solution were injected into the liquid chromatograph.

Urine was treated by the same procedure, except that a 1.0-ml sample was diluted with 1 ml of 10 m*M* phosphate buffer (pH 7.0) and then applied to a Sep-Pak C_{18} cartridge. The reduced residue of the methanol eluate was dissolved in 0.2 ml of 40% methanol aqueous solution.

Calibration curves

Calibration curves for the determination of I and its metabolites by HPLC were prepared by adding known amounts (0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml for plasma and 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml for urine) of these compounds to 1.0-ml aliquots of plasma and urine, and then assaying the mixture using the same preparation procedure; the peak areas were plotted against the concentrations of these compounds. All these calibration curves were linear.

RESULTS AND DISCUSSION

Chromatographic separation of I and the three metabolites was first examined using authentic samples and a reversed-phase chromatographic system or adsorption chromatographic system. When a reversed-phase column for the HPLC separation and a gradient system with methanol–water as the mobile phase were used, metabolites II and III were not separated, even when the initial methanol concentration was varied. Linear gradient and isocratic systems of methanol–20 m*M* potassium dihydrogenphosphate (mobile phase A), methanol–10 m*M* phosphate buffer (pH 7.0) (mobile phase B) or methanol–10 m*M* sodium acetate buffer (pH 4.0) (mobile phase C) were found to be suitable for the separation of authentic I and the three metabolites. Among reversed-phase columns tested, Shim-pack CLC-ODS using gradient and isocratic systems separated the four authentic compounds very well, whereas other reversed-phase columns using either gradient or isocratic system, i.e. μBondapak C_{18} (Waters Assoc.), Nova Pak C_{18} (Waters Assoc.), Nucleosil C_{18} (Chemco, Osaka, Japan), Finepak SIL C_{18}-5 (Nihon Bunko, Tokyo, Japan), Unisil Pack QC18-10 (Gaschro Kogyo,

Tokyo, Japan), Divelosil ODS (Nomura Kagaku, Aichi, Japan) or TSKgel ODS-120T (Toyosoda, Tokyo, Japan), did not separate metabolites II and III very well. On the other hand, the adsorption column, i.e. Zorbax SIL (Du Pont, Wilmington, DE, U.S.A.) or LiChrosorb Si-100 (E. Merck, Darmstadt, F.R.G.) could also be used to separate the four authentic compounds.

Next, the preparation procedure of I and its metabolites from human plasma and urine was examined. The extraction with chloroform under acidic conditions with hydrochloric acid gave quantitative recoveries of I and its metabolites, but the same extract exhibited peaks due to biological components overlapping with one or more of the metabolites during analysis with either a reversed-phase or an adsorption chromatographic system. The preparation of plasma and urine samples using a Sep-Pak C_{18} cartridge, washed with distilled water and subsequently eluted with methanol, resulted in no interfering peaks from biological components following chromatography on a Shim-pack CLC-ODS column with a gradient system of mobile phase A. Although this system achieved high recoveries of I and the three metabolites from urine, the identically prepared eluate from plasma showed low recoveries (ca. 60–70%) because of stronger protein binding. On the other hand, the deproteinization of plasma with acetonitrile gave a quantitative recovery of each compound, but this preparation gave a poor separation on HPLC analysis both with reversed-phase and with adsorption chromatography.

On the basis of these findings, the deproteinization with acetonitrile and subsequent application to the Sep-Pak C_{18} cartridge were used for the preparation procedure of plasma. A chromatographic column, Shim-pack CLC-ODS, and a linear gradient system (mobile phase A, 0 to 100% methanol at 3%/min, flow-rate 1.5 ml/min) were used for the reversed-phase HPLC. This preparation procedure, together with the above HPLC conditions, achieved the highest recovery and best separation of compound I and the three metabolites from plasma components. Urine was treated by the same procedure, with the exception of the deproteinization step and an extra dilution with 10 m*M* phosphate buffer (pH 7.0) before application to the Sep-Pak C_{18} cartridge.

Since compound I and its metabolites are all readily soluble in methanol, it was used to redissolve the residue obtained from the concentration to dryness of the methanol eluate of plasma with a Sep-Pak C_{18} cartridge. On the other hand, a 40% methanol aqueous solution was used to redissolve the urine sample residue to obtain the best chromatographic separation of metabolites II, III and IV from urine components. No decomposition of I or its three metabolites was observed in either methanol or 40% methanol aqueous solution.

Typical chromatograms of I and its metabolites prepared from human plasma and urine following administration of I, and chromatograms of the respective control human plasma and urine extracts, are shown in Figs. 2 and 3. The retention times of I, II, III and IV, under the stipulated HPLC conditions, were 23.9, 20.6, 20.8 and 17.5 min, respectively, and the detection limits were 10 ng/ml in plasma and 100 ng/ml in urine for each compound. The method had an accuracy of 2.1–3.3% and very good reproducibility.

Known amounts of I and the three metabolites were added to human plasma and urine samples (1.0 ml) and the recovery of each compound was determined.

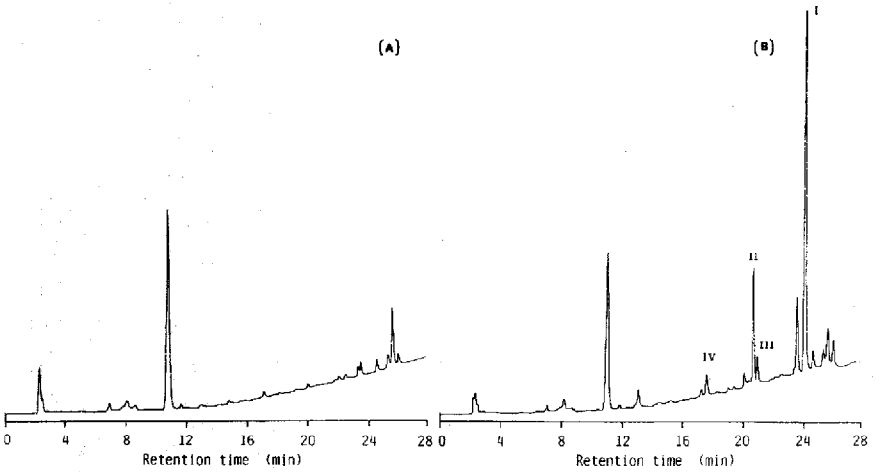

Fig. 2. HPLC profiles of (A) human plasma control and (B) human plasma extracts following administration of compound I to healthy men. For peak identification, see Fig. 1.

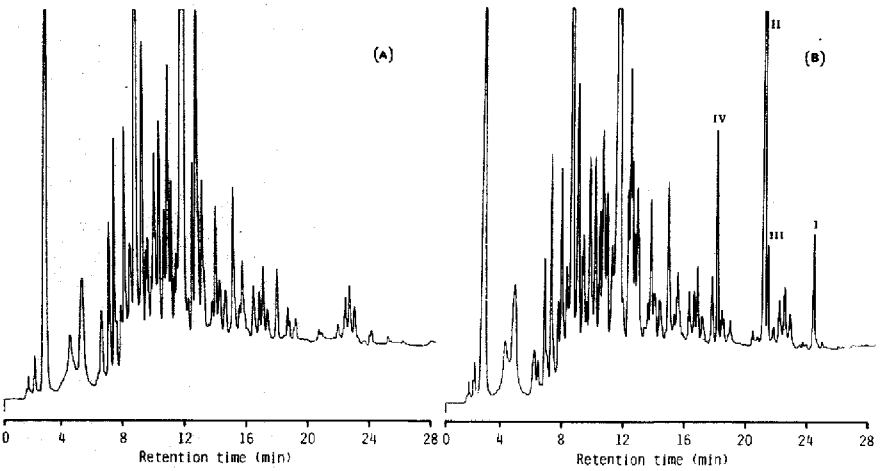

Fig. 3. HPLC profiles of (A) human urine control and (B) human urine extracts following administration of compound I to healthy men. For peak identification, see Fig. 1.

As given in Table I, the respective overall recoveries of I, II, III and IV from plasma were 88.1 ± 7.8, 93.1 ± 7.0, 83.8 ± 4.5 and $82.5 \pm 8.0\%$, and those from urine were 86.3 ± 5.4, 90.2 ± 9.8, 98.8 ± 2.7 and $96.0 \pm 4.2\%$.

The conjugates of I and its metabolites in urine could be determined using the same preparation procedure and under the same HPLC conditions as non-conjugated compounds after the following treatment. Urine (1.0 ml) was diluted with 1.0 ml of 2 *M* hydrochloric acid and the solution was incubated at 100°C for 10 min to convert the conjugates into non-conjugated compounds. After cooling, the resulting solution was applied to a Sep-Pak C_{18} cartridge. Using this method, the concentrate of each compound was obtained in the form of total non-conjugates. Thus, the concentrations of conjugated compounds were determined by subtracting the concentrations of non-conjugates from those measured by the above pro-

TABLE I

RECOVERIES OF COMPOUND I AND ITS METABOLITES (II, III AND IV) FROM HUMAN PLASMA AND URINE

Each value is the mean of three determinations.

Biological fluid	Added (μg/ml)	Recovery (%)			
		I	II	III	IV
Plasma	10.0	90.0 ± 7.5	89.2 ± 6.7	90.9 ± 1.4	78.4 ± 3.5
	5.0	93.9 ± 1.1	94.4 ± 1.7	85.0 ± 2.0	78.2 ± 3.3
	1.0	87.0 ± 4.9	92.2 ± 5.7	83.3 ± 2.2	78.6 ± 2.0
	0.5	94.4 ± 1.9	104.1 ± 2.3	77.5 ± 3.5	80.7 ± 0.7
	0.1	75.2 ± 3.5	85.7 ± 2.4	83.3 ± 3.9	96.8 ± 3.5
Mean ± S.D.		88.1 ± 7.8	93.1 ± 7.0	83.8 ± 4.5	82.5 ± 8.0
Urine	50.0	86.3 ± 6.5	93.0 ± 7.0	100.2 ± 2.0	102.2 ± 1.9
	10.0	93.4 ± 1.4	105.7 ± 1.0	98.3 ± 3.2	94.3 ± 4.2
	5.0	89.6 ± 4.1	87.8 ± 2.6	96.3 ± 4.4	94.2 ± 5.0
	1.0	82.2 ± 2.0	80.8 ± 2.8	102.6 ± 0.7	91.4 ± 6.4
	0.5	80.0 ± 3.7	83.9 ± 7.0	96.4 ± 14.6	97.9 ± 10.2
Mean ± S.D.		86.3 ± 5.4	90.2 ± 9.8	98.8 ± 2.7	96.0 ± 4.2

cedure. The recoveries of I and its metabolites under this preparation procedure showed good results (85–95%) for each compound.

Furthermore, the following additional experiments were conducted. The stabilities of I and its metabolites in the frozen-stock plasma and urine, and those

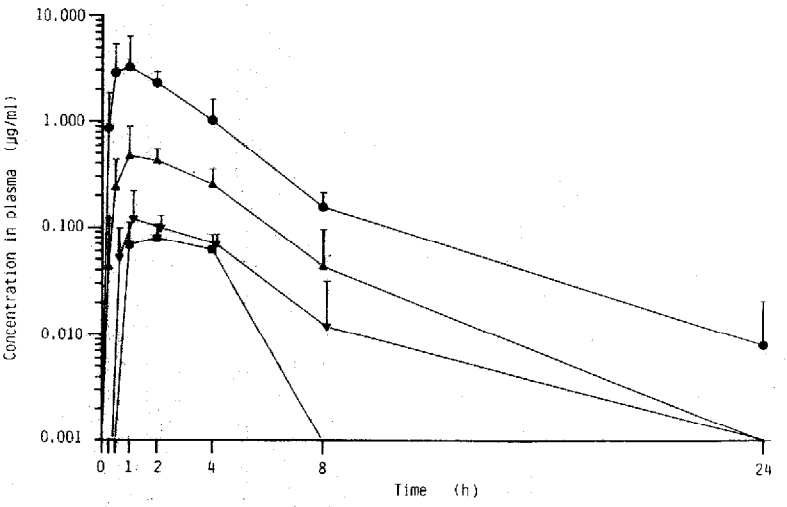

Fig. 4. Plasma levels of compound I and its metabolites (II, III and IV) after oral administration of 100 mg of I to healthy volunteers. Each point is the mean ± S.D. of six men. Symbols: ● = I; ▲ = II; ▼ = III; ■ = IV.

TABLE II

CUMULATIVE URINARY EXCRETION OF COMPOUND I AND ITS METABOLITES (II, III AND IV), AND THEIR CONJUGATES AFTER ORAL ADMINISTRATION OF I TO HEALTHY VOLUNTEERS

Each value is the mean ± S.D. of six determinations. Dose administered was 100 mg.

Compound	Excretion (mol.% of dose)		
	0–6 h	0–12 h	0–24 h
I	5.81 ± 0.72	6.83 ± 0.73	6.90 ± 0.74
I conjugate	0.89 ± 0.85	1.01 ± 0.86	1.02 ± 0.87
II	18.48 ± 2.35	21.18 ± 3.17	21.53 ± 3.32
II conjugate	1.33 ± 1.10	2.03 ± 1.34	2.24 ± 1.49
III	3.24 ± 0.43	4.38 ± 0.45	5.15 ± 0.30
III conjugate	0.16 ± 0.22	0.34 ± 0.27	0.40 ± 0.32
IV	6.14 ± 1.25	7.76 ± 1.96	7.94 ± 2.04
IV conjugate	0.37 ± 0.61	0.65 ± 0.79	1.05 ± 1.08

of the respective compounds in the cooled-stock plasma and urine extracts were examined. No significant decomposition was observed.

Finally, a 100-mg dose of I was administered orally to each of six healthy volunteers. The concentrations of I and its metabolites in the plasma and urine were determined using the present HPLC method. The results obtained are shown in Fig. 4 and Table II.

The present assay method was also applied to plasma and urine of other animals. The results obtained for the chromatographic separation, recovery and sensitivity correlated well with those obtained with human plasma and urine. The present method is simple and has a high accuracy and sensitivity and should be useful for basic and clinical pharmacological investigations on compound I.

REFERENCES

1 R.G. Michtich, C.C. Shaw and R.B. Rastogi, U.S. Pat., 4 327 222 (1982).
2 N. Ono, N. Yamamoto, A. Sunami, Y. Yamasaki, H. Ajioka and H. Miyake, Abstracts of 10th International Congress of Pharmacology, Sydney, August 25, 1987, Abstract No. P520.