Determination of the Antiplatelet Agent, KB-3022, and Its Metabolite by High-Performance Liquid Chromatography

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A rapid, accurate and reproducible high-performance liquid chromatographic method for the simultaneous determination of a new antiplatelet agent, ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate (KB-3022), and its main metabolite, 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetic acid (desethyl KB-3022), in biological fluids has been developed. KB-3022 and desethyl KB-3022 in plasma or urine were extracted with a mixture of *n*-hexane and dichloromethane (1 : 1), separated on a reversed-phase C_{18} column with a mixture of 0.01 M $(NH_4)_2HPO_4$ (pH 3.0), acetonitrile and isopropyl alcohol as a mobile phase, and quantitated by ultraviolet absorbance measurement at 340 nm. 4-[2-{4,5-bis(4-methoxyphenyl)thiazol-2-yl}pyrrol-1-yl]butyric acid was used as an internal standard.

The detection limit of KB-3022 in plasma was 3 ng/ml, and that of KB-3022 in urine and desethyl KB-3022 in plasma or urine was 1 ng/ml. The coefficients of variation for the determination of KB-3022 or desethyl KB-3022 in plasma or urine were below 5.6%. This method was applied to the determination of the plasma concentration of KB-3022 and desethyl KB-3022 after intravenous administration to rats.

Keywords antiplatelet agent; ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate; metabolite; determination; HPLC; plasma; urine

Introduction

Ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate, KB-3022, is a new antiplatelet agent[1] structurally belonging to the series of substituted diphenylthiazoles (Fig. 1).

The platelet aggregation inhibitory activity of KB-3022 is mediated through inhibition of cyclooxygenase activity and it is therefore considered to have an inhibitory effect on the production of prostaglandins.

This paper describes a rapid, accurate and reproducible high-performance liquid chromatographic (HPLC) method for the simultaneous determination of KB-3022 and its main metabolite, 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]-pyrrol-1-ylacetic acid (desethyl KB-3022) in biological fluids (Fig. 1). The validity of the present method was demonstrated by the determination of KB-3022 and desethyl KB-3022 in plasma after intravenous administration of KB-3022 to rats.

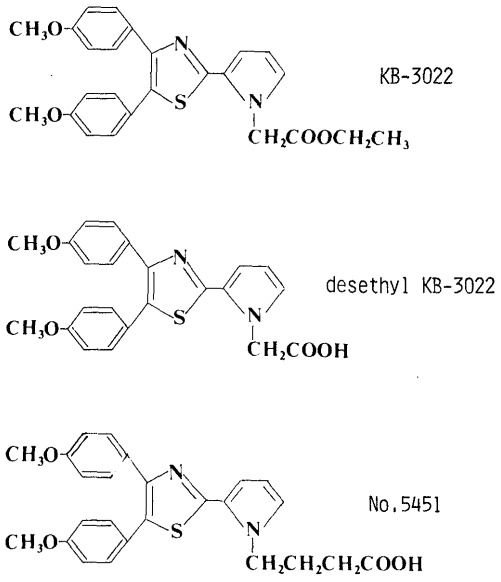

Fig. 1. Structure of KB-3022, Desethyl KB-3022 and No. 5451 (Internal Standard)

Experimental

Materials KB-3022, desethyl KB-3022 and 4-[2-{4,5-bis(4-methoxyphenyl)thiazol-2-yl}pyrrol-1-yl]butyric acid (No. 5451) (Fig. 1), used as an internal standard, were synthesized in this laboratory. Acetonitrile of HPLC grade was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and all other chemicals were of reagent grade.

Instruments and Analytical Conditions The HPLC system consisted of Model 510 constant flow pump, a Model U6K injector, a Model 481 UV detector (Millipore Co., Waters Chromatography Div. Milford, MA, U.S.A.) and a Model U-228 recorder (Nippon Denshi Kagaku Co., Ltd., Tokyo, Japan). An Inertsil ODS 5-150 column (5 μm, 4.6 mm × 150 mm, Gasukuro Kogyo Inc., Tokyo, Japan) was used. The mobile phase of KB-3022 and desethyl KB-3022 was a mixture of 0.01 M $(NH_4)_2HPO_4$ (adjusted to pH 3.0 with 5 N HCl), acetonitrile, and isopropyl alcohol (35 : 40 : 25, v/v/v, for plasma samples, 30 : 45 : 25, v/v/v, for urine samples). Chromatography was done at 30 °C and the flow-rate was 0.9 ml/min for plasma samples and 0.7 ml/min for urine samples.

Analytical Procedure for Assay To 0.4 ml of plasma were added 0.4 ml of 0.1 N HCl, 0.4 ml of acetonitrile, 3 μl of the internal standard solution (4 μg/ml, in acetonitrile), and 6.3 ml of a mixture of *n*-hexane and dichloromethane (1 : 1). For the urine samples, to 2.0 ml of urine were added 0.1 ml of 1 N HCl, 5 μl of the internal standard solution, and 6.3 ml of a mixture of *n*-hexane and dichloromethane (1 : 1). The mixture was shaken for 10 min and centrifuged for 5 min at 800 × ***g***. After the centrifugation, 5 ml of the organic supernatant was transferred to a clean tube and dried under a stream of nitrogen gas at 50 °C. The residue was redissolved in 50 μl (for plasma samples) or 100 μl (for urine samples) of acetonitrile, and a 20 μl (for plasma samples) or 30 μl (for urine samples) aliquot was injected onto the HPLC column. The absorbance of the eluate at 340 nm was monitored. The linear calibration curves within a range of 2 (for urine samples) or 5 (for plasma samples) to 50 ng/ml were obtained by plotting the peak height ratio of KB-3022 or desethyl KB-3022 to No. 5451 against the amount of KB-3022 or desethyl KB-3022 added. All samples were stored in the dark at 4 °C until use.

Animal Treatments Male rats (Wistar, 8 weeks) fasted overnight were used. KB-3022 dissolved in a mixed solution of polyethyleneglycol 400, ethanol and water (1 : 1 : 1, v/v/v) was administered intravenously to rats at a dose of 1 mg/ml/kg. The blood samples were withdrawn from the abdominal vena cava with a heparinized plastic syringe at 2, 5, 10, 20, 40 min, 1, 2, 3 and 5 h after dosing. The blood samples were then centrifuged (4 °C), and 0.4 ml of each plasma sample was used for the assay.

Inactivation of the Glass Vessel Surface The surface of all glass vessels was treated with 3% (w/v) trimethylchlorosilane in benzene to protect the adsorption of KB-3022 and desethyl KB-3022. After the treatment, the glass surface was washed 5 times with 5 ml of MeOH, and air-dried at room temperature.

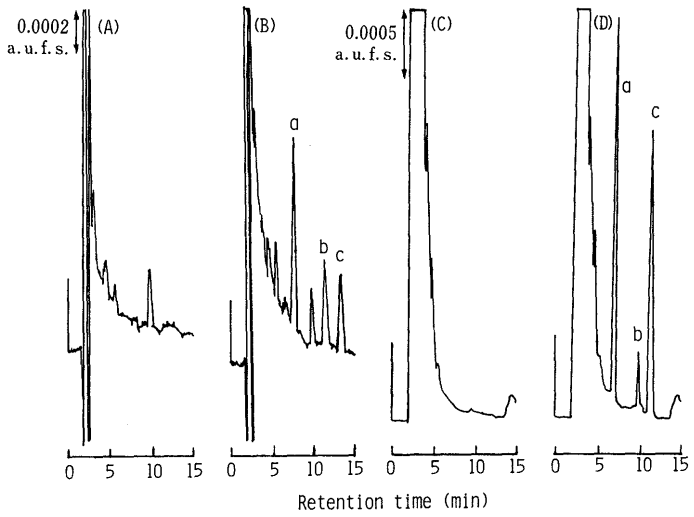

Fig. 2. Chromatograms of Extracts Obtained from Rat Plasma or Urine

(A) Blank plasma. (B) Plasma spiked with 8 ng of desethyl KB-3022 (a), 4.8 ng of No. 5451 (b), and 8 ng of KB-3022 (c). (C) Blank urine. (D) Urine spiked with 30 ng of desethyl KB-3022 (a), 6.0 ng of No. 5451 (b), and 30 ng of KB-3022 (c).

Results and Discussion

Several mobile phases were examined to obtain the complete separation of KB-3022 and desethyl KB-3022. The mixture of 0.01 M $(NH_4)_2HPO_4$ and acetonitrile was used as a mobile phase because both compounds were separated satisfactorily from the peaks of the endogenous components in plasma or urine. Furthermore, the addition of isopropyl alcohol to the mobile phase gave sharper peaks than those without isopropyl alcohol. Thus the mixtures of 0.01 M $(NH_4)_2HPO_4$, acetonitrile and isopropyl alcohol were adopted at the ratio of 35 : 40 : 25 for plasma samples and 30 : 45 : 25 for urine samples. The chromatograms obtained from blank rat plasma and rat plasma spiked with 8 ng of KB-3022, 8 ng of desethyl KB-3022 and 4.8 ng of No. 5451 (I.S.) are shown in Fig. 2A, B. The chromatograms obtained from blank rat urine and rat urine spiked with 30 ng of KB-3022, 30 ng of desethyl KB-3022 and 6.0 ng of No. 5451 are also shown in Fig. 2C, D, respectively.

No interfering peak was observed in the plasma and urine extracts and the separation of the three compounds added was complete. The present method allowed us to detect KB-3022 in plasma at a concentration as low as 3 ng/ml, and KB-3022 in urine and desethyl KB-3022 in plasma or urine at a concentration as low as 1 ng/ml ($S/N=2$).

Good linearity was obtained with the correlation coefficient of 0.9992 (5—50 ng/ml, $Y=0.0193X+0.0124$, $n=5$) for KB-3022 and 0.9999 (2—50 ng/ml, $Y=0.0400X+0.0033$, $n=6$) for desethyl KB-3022 in rat plasma, and 0.9999 (1—50 ng/ml, $Y=0.1021X-0.0156$, $n=7$) for KB-3022 and 0.9999 (1—50 ng/ml, $Y=0.1357X+0.1856$, $n=7$) for desethyl KB-3022 in rat urine. Good linearity was also obtained with the correlation coefficient of 0.9990 (5—50 ng/ml, $Y=0.0182X+0.0229$, $n=5$) for KB-3022 and 0.9993 (1—50 ng/ml, $Y=0.0505X+0.0119$, $n=7$) for desethyl KB-3022 in human plasma, and 0.9999 (1—50 ng/ml, $Y=0.1066X-0.0113$, $n=7$) for KB-3022 and 0.9999 (1—50 ng/ml, $Y=0.1412X-0.0122$, $n=7$) for desethyl KB-3022 in human urine, respectively.

Table I. Accuracy and Reproducibility for the Determination of KB-3022 and Desethyl KB-3022 Added to Rat or Human Plasma and Urine ($n=5$)

	Amount added (ng/ml)		Recovery (%) (Mean ± S.D.)	
			KB-3022	Desethyl KB-3022
Plasma	Rat	5.0	100.4 ± 3.6 (3.6)[a]	99.6 ± 4.3 (4.3)
		10.0	102.4 ± 5.5 (5.4)	99.8 ± 3.1 (3.1)
	Human	5.0	100.0 ± 5.5 (5.5)	99.6 ± 3.6 (3.6)
		10.0	103.8 ± 4.5 (4.3)	98.6 ± 2.2 (2.2)
Urine	Rat	2.0	102.0 ± 2.7 (2.6)	99.0 ± 4.2 (4.2)
		10.0	101.4 ± 1.3 (1.3)	99.6 ± 1.5 (1.5)
	Human	2.0	102.0 ± 5.7 (5.6)	97.0 ± 4.5 (4.6)
		10.0	99.0 ± 1.7 (1.7)	99.6 ± 1.7 (1.7)

a) Numbers in parentheses are coefficients of variation (%).

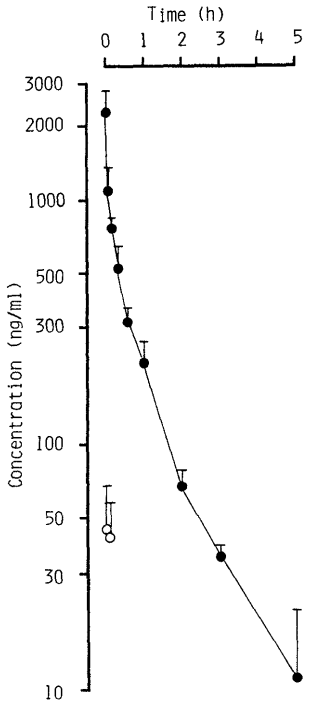

Fig. 3. Plasma Concentration of KB-3022 (○) and Desethyl KB-3022 (●) in Rats after Intravenous Administration of KB-3022 at a Dose of 1 mg/kg

Each point represents the mean ± S.D. ($n=3$).

The accuracy and reproducibility of the present method were evaluated at concentration 2 or 5, and 10 ng of KB-3022 and desethyl KB-3022 per 1 ml of the biological fluids. The data for plasma and urine samples are summarized in Table I.

In the intra-assay study, the overall recoveries of KB-3022 from plasma samples ($n=10$) averaged 101.4 ± 4.5% (S.D.) for rats, and 101.9 ± 5.2% (S.D.) for human, and the coefficient of variation was below 5.4%. The overall recoveries of desethyl KB-3022 from plasma samples ($n=10$) averaged 99.7 ± 3.6% (S.D.) for rats, and 99.1 ± 2.8% (S.D.) for human, and the coefficient of variation was below 4.4%. And the overall recoveries of KB-3022 from urine samples ($n=10$) averaged 101.7 ± 2.1% (S.D.) for rats, 100.5 ± 4.3% (S.D.) for human, and the coefficient of variation was below 5.5%. The overall recoveries of desethyl KB-3022 from

urine samples ($n=10$) averaged $99.3 \pm 3.0\%$ (S.D.) for rats, $98.3 \pm 3.5\%$ (S.D.) for human, and the coefficient of variation was below 4.7%.

The applicability of the present method was tested by analyzing plasma samples obtained after intravenous administration of KB-3022 at a dose of 1 mg/kg to rats. The plasma levels of KB-3022 were 45 ng/ml at 2 min, 42 ng/ml at 5 min, but KB-3022 was not detected at other sampling points, as shown in Fig. 3. The plasma level of desethyl KB-3022 was 2247 ng/ml at 2 min and decreased almost bi-exponentially with the half-life of about 0.03 h for α phase and 0.6 h for β phase.

Because of its sensitivity, simplicity and the simultaneous determination of KB-3022 and its metabolite, desethyl KB-3022, the present method is well suited for the routine analysis of a large number of plasma and urine samples in animal experiments or clinical studies.

References

1) N. Seko, K. Yoshino, K. Yokota, A. Yamashita, K. Ito and G. Tukamoto, Abstracts of Papers, 9th Symposium on Medicinal Chemistry, Tokyo, November 1988, p. 131; K. Yokota, N. Yamamoto, Y. Morimoto, A. Yamashita and K. Ito, *Jpn. J. Pharmacol.*, **46** (Supr), 190P (1988).