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Note

High-performance liquid chromatographic procedure for the determination of a new positive inotropic agent, 3,4-dihydro-6-[4-(3,4-(dimethoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone, in human plasma and urine

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3,4-Dihydro-6-[4-(3,4-(dimethoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone (I; OPC-8212) has been reported to have a positive inotropic action [1—3]. Compound I preferentially increases myocardial contactile force without affecting heart rate [2]. Heart failure has been treated with digitalis and catecholamines, but digitalis elicits a variety of responses from different individuals, has a very narrow therapeutic margin and involves the risk of occasional fatal arrhythmia [4—6], and catecholamines are available only in intravenous dosage forms [7, 8]. Compound I does not present these problems and so was selected for further evaluation [2]. It was found to elicit pharmacological activities different from those of other compounds of similar clinical application. It is also devoid of an arrhythmia effect and is considered to be a potential selective, positive inotropic agent for oral use [9, 10]. We have already reported the pharmacokinetics of I in several animals [11]. Therefore, to study the pharmacokinetics in humans, an attempt was made to develop a method to quantitate I in human plasma and urine that would be simple, suitable for routine analysis and highly sensitive.

In this paper, we describe a simple method for the determination of I in human plasma and urine using high-performance liquid chromatography (HPLC). The results of the HPLC assay for plasma and urine concentrations of I after a single oral dose to healthy male subjects are also described.

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MATERIALS AND METHODS

Drug and chemicals

Compound I (Fig. 1) and the internal standard, 3,4-dihydro-6-[4-(4-methoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone (Fig. 1), were supplied by the Laboratories of Medicinal Chemistry, Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). A standard solution of I was prepared in a hydrochloric acid—methanol solution (1 mg/ml) and was diluted with methanol as required before use. The internal standard solution was prepared in a methanol solution. Acetonitrile, chloroform, methanol, acetic acid, sodium hydroxide and potassium nitrate were of analytical-reagent grade and were purchased from Wako (Tokyo, Japan). Human control plasma was supplied by the Japan Red-Cross Blood Supply Center, Tokushima Branch (Tokushima, Japan).

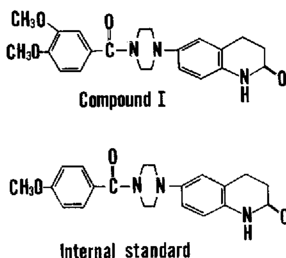

Fig. 1. Chemical structure of I and internal standard.

Chromatography

The HPLC separations were carried out using a Waters Assoc. ALP/GCP 204 compact routine system (Model 6000A pump, Model 440 absorbance detector equipped with 280-nm filter kit, WISP 710B autosampler; Waters Assoc., Milford, MA, U.S.A.). The peak height ratio was calculated by a Shimadzu Chromatopac C-R1B integrator system (Shimadzu Seisakusho, Kyoto, Japan). A μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters Assoc.) was used with acetonitrile—0.01 *M* potassium nitrate—acetic acid (30:70:1) as the mobile phase at a flow-rate of 1.0 ml/min.

Sample preparation

The internal standard (400 ng for plasma, 2 μg for urine) was added to 1 ml of human plasma or 0.5 ml of human urine (in the case of high concentrations, plasma or urine was diluted with control plasma or urine, respectively). The contents were stirred on a vortex mixer followed by the addition of 1 ml of 1 *M* sodium hydroxide and 5 ml of chloroform and shaken mechanically for 10 min. The mixture was centrifuged for 10 min at 1700 *g* and 4 ml of the chloroform layer were evaporated to dryness under a stream of air. The residue was redissolved in methanol (100 μl), and an aliquot (20 μl) was injected into the HPLC system and analysed.

Preparation of the calibration curve

The diluted standard I solution was added to a portion of plasma or urine.

Four replicate plasma samples were prepared in the concentration ranges of 20–1000 ng/ml for plasma and 0.2–5.0 μg/ml for urine, and extracted using the extraction procedures described above. After HPLC analysis the peak height ratio of I to the internal standard calculated from the chromatograms was plotted on the ordinate, and unchanged I concentrations were plotted on the abscissa. The calibration curve was constructed by a least-squares regression method from the peak height ratio versus the corresponding unchanged concentration of I in the test samples.

RESULTS AND DISCUSSION

Chromatograms obtained by the above procedures using plasma or urine samples with and without I and the internal standard are given in Figs. 2 and 3. No significant interference was observed in the regions for I and the internal standard on the chromatogram. Good chromatographic separation was obtained with distinct retention times for I and the internal standard of 8.5 and 12.0 min, respectively. The time required for analysis of a single sample was 14 min. The linearity, reproducibility and precision of the calibration curves are given in Tables I and II. Calibration curves were linear in concentrations of 20–1000 ng/ml of plasma and 0.2–5 μg/ml of urine, and passed through the origin. At a plasma concentration of 100 ng/ml the peak height ratio was 0.37

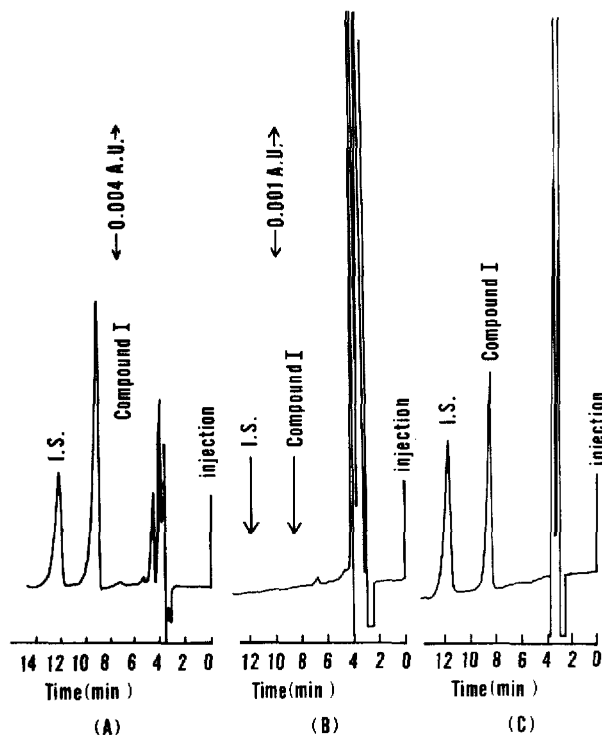

Fig. 2. (A) Representative chromatogram of human plasma spiked with internal standard (400 ng/ml) after the administration of the drug. (B) Chromatogram of blank human plasma. (C) Chromatogram of I (20 ng) and internal standard (20 ng).

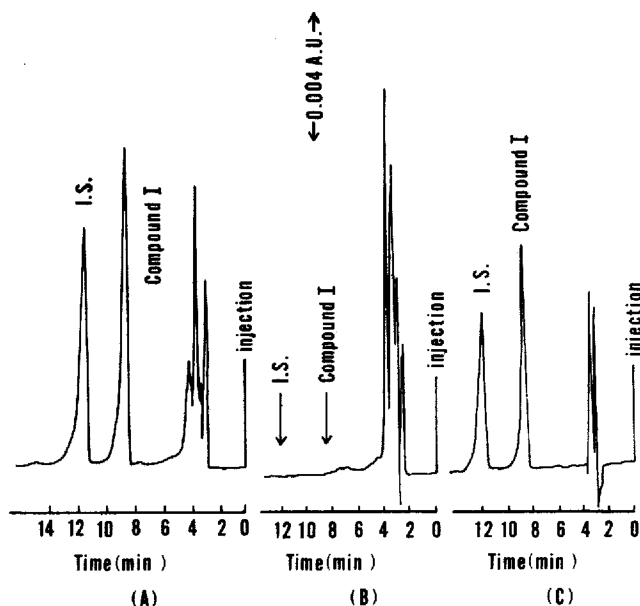

Fig. 3. (A) Representative chromatogram of human urine spiked with internal standard (2 μg/ml) after the administration of the drug. (B) Chromatogram of blank human plasma. (C) Chromatogram of I (100 ng) and internal standard (100 ng).

± 0.02 with a coefficient of variation (C.V.) of 4.1%. At other concentrations the C.V. was smaller than 4.1% indicating little deviation of the peak height ratio. The equation for the resulting line was $y = 271.9945x + 1.8947$ with a correlation coefficient of 0.9995. At a urine concentration of 1.0 μg/ml the peak height ratio was 0.37 ± 0.01 with a coefficient of variation of 1.3%. At other concentrations the C.V. was smaller than 1.3% indicating little deviation from the peak height ratio. The equation for the resulting line was $y = 2.5988x + 0.0278$ with a correlation coefficient of 0.9999. The plasma and urine concentrations of I calculated from the calibration curve were comparable to the corresponding amount of the drug added to the plasma, being 97—118% and

TABLE I

LINEARITY AND PRECISION OF HPLC ASSAY OF I IN HUMAN PLASMA WITH THE INTERNAL STANDARD METHOD

A 1-ml volume of plasma was used.

Concentration of the drug added to plasma (ng/ml)	Peak height ratio (mean ± S.D.)*	C.V. (%)	Recalculated concentration (ng/ml)	Percentage of theoretical concentration
20	0.08 ± 0.00	0.0	23.7	118
50	0.19 ± 0.01	2.7	52.8	106
100	0.37 ± 0.02	4.1	101.7	102
500	1.77 ± 0.05	2.9	483.9	97
1000	3.70 ± 0.05	1.4	1007.5	101

*Results from four replicate samples were used.

TABLE II

LINEARITY AND PRECISION OF HPLC ASSAY OF I IN HUMAN URINE WITH THE INTERNAL STANDARD METHOD

A 0.5-ml volume of urine was used.

Concentration of the drug added to urine (μg/ml)	Peak height ratio (mean ± S.D.)*	C.V. (%)	Recalculated concentration (μg/ml)	Percentage of theoretical concentration
0.2	0.07 ± 0.00	0.0	0.21	105
1.0	0.37 ± 0.01	1.3	0.99	99
2.0	0.76 ± 0.01	0.7	1.99	100
5.0	1.92 ± 0.02	1.0	5.00	100

*Results from four replicate samples were used.

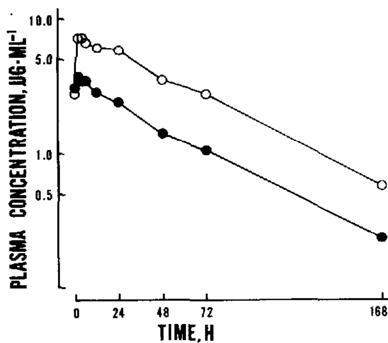

Fig. 4. Plasma concentration of I after oral administration of I to human male subjects. •, Single oral dose of 60 mg per body; peak level 3.77 μg/ml; half-life 44.50 h; AUC 228.93 μg h ml^{-1}. ○, Single oral dose of 120 mg per body; peak level 7.38 μg/ml; half-life 49.28 h; AUC 553.36 μg h ml^{-1}.

99—105% of amount of the drug added to the plasma and urine, respectively. The plasma and urine concentrations of I were determined as described above.

Fig. 4 shows the time course of the plasma concentration of I after single oral doses of 60 and 120 mg per body in healthy male subjects. The plasma levels 4 h after oral doses of 60 and 120 mg per body reached peak levels of 3.77 and 7.38 μg/ml and then declined with apparent biological half-lives of 44.50 and 49.28 h, respectively. The areas under the curve (AUC) until ∞ h calculated by the trapezoidal rule were 228.93 and 553.36 μg h ml^{-1}, respectively. It was recognized that the plasma concentration and urinary excretion were closely related. The detection limit (< 20 ng/ml in plasma, < 0.2 μg/ml in urine) of this procedure was considered sufficient to determine plasma and urine concentrations of I, since the dose of 120 mg per body used in the present study was smaller than the predicted clinical dose which yields plasma and urine concentrations within the range of the calibration curve constructed here. For higher concentrations of I in plasma, a smaller sample than that used in this study is required. In view of these results, it was concluded that this HPLC method is a simple, highly sensitive, reproducible procedure for the determination of plasma and urine concentrations of I, and therefore a valuable

tool in the investigation of the clinical pharmacokinetics and bioavailability of this compound.

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