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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF A NEW NOOTROPIC, N-(2,6-DIMETHYLPHENYL)-2-(2-OXO-1-PYRROLIDINYL)ACETAMIDE, IN HUMAN SERUM AND URINE

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SUMMARY

A high-performance liquid chromatographic method for the determination of a novel nootropic agent, N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl)acetamide (DM-9384, I), in human serum and urine has been developed. Compound I and the internal standard were extracted with chloroform from alkalinized serum and urine, and the organic layer was evaporated to dryness. The residue was chromatographed on a Nucleosil $7C_{18}$ reversed-phase column using 1/15 *M* potassium dihydrogenphosphate–acetonitrile (7:3, v/v) as a mobile phase. Quantitation was achieved by monitoring the ultraviolet absorbance at 210 nm. The response was linear (0–2114.0 ng/ml) and the detection limits were 30 ng/ml for serum samples and 50 ng/ml for urine samples. The utility of the assay was demonstrated by determining compound I in serum and urine samples from three healthy male subjects receiving an oral dose of 30 mg of the drug. This method is satisfactorily sensitive and accurate, and is applicable for pharmacokinetic studies of I in humans.

INTRODUCTION

A new nootropic, N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl)acetamide (I, DM-9384, Fig. 1), is a cyclic derivative of γ-aminobutyric acid (GABA) [1]. It antagonizes the amnestic effect induced with picrotoxin or bicuculline in mice [2]. Furthermore, compound I has a potent effect on the recovery of learning and memory impaired by electroconvulsive shock or scopolamine in rats. It is assumed that this effect is partly due to protection against the decrease in ATP and partly due to augmented choline acetyltransferase activity [3]. The metabolic disposition of I has been studied in the rat, dog and monkey using ^{14}C-labelled I. It was well absorbed and distributed rapidly and widely to the tissues [4]. We

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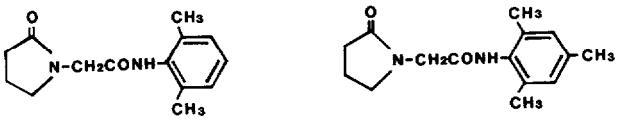

I II

Fig. 1. Chemical structures of I (DM-9384) and II (internal standard).

describe here a high-performance liquid chromatographic (HPLC) method for the quantitation of I in human serum and urine to investigate the absorption, elimination and metabolism of I in humans and to study the pharmacokinetics of I during clinical trials.

EXPERIMENTAL

Materials

DM-9384 (I) and the internal standard, N-(2,4,6-trimethylphenyl)-2-(2-oxo-1-pyrrolidinyl)acetamide (II, Fig. 1) were synthesized in the Research Institute of Daiichi Seiyaku (Tokyo, Japan).

Acetonitrile and chloroform were of HPLC grade and potassium dihydrogenphosphate and sodium hydroxide were of analytical grade.

Preparation of standard solutions

Stock solutions of I (528.50 μg/ml) and II (409.35 μg/ml) were prepared by dissolving accurately weighed samples in methanol. These solutions were found to be stable for at least four to five months when stored at 4 °C. Working solutions of appropriate concentrations were made by diluting the stock solution of I with methanol.

Instrumentation and chromatographic conditions

An SP8700 ternary solvent delivery system (Spectra-Physics, San Jose, CA, U.S.A.) equipped with an autosampler system (TOSO, Tokyo, Japan) and a variable-wavelength UV detector 638-41 (Hitachi, Tokyo, Japan) were used. Reversed-phase HPLC separation was carried out with Nucleosil $7C_{18}$ (Macherey-Nagel, Düren, F.R.G.) as the stationary phase (200 mm × 4.6 mm I.D., particle size 7 μm) and 1/15 *M* potassium dihydrogenphosphate–acetonitrile (7:3, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. The UV wavelength was set at 210 nm. All analyses were done at ambient temperature.

Assay procedure

A 5-μl volume of II (409.35 μg/ml) and 1.0 ml of 0.1 *M* sodium hydroxide were added to 1.0 ml of serum or urine. The mixture was extracted with 5 ml of chloroform by shaking for 10 min. After centrifugation for 10 min at 1800 *g*, the organic phase was transferred to a glass tube for evaporation to dryness under a stream of nitrogen. The residue was redissolved in 500 μl of mobile phase, and an aliquot of 20 μl was injected into the chromatograph through the autosampler.

Calibration curves

The calibration curve standards were prepared by adding known amounts of I to blank serum or urine to provide various concentrations, 66.1–2114.0 ng/ml in serum samples and 132.1–2114.0 ng/ml in urine samples.

The calibration curves were constructed by plotting the peak-height ratio of I to the internal standard against the concentrations of I.

Serum and urine samples

A single 30-mg dose of I was administered orally to three healthy male subjects. Serum samples were collected into glass tubes at 0 (predose), 0.5, 1, 2, 4, 8, 12 and 24 h after dosing. After centrifugation for 15 min at 1800 *g*, the serum was transferred to a plastic tube fitted with a plastic cap. Urine samples were collected at 0–2, 2–4, 4–8, 8–12 and 12–24 h after dosing. Serum and urine samples were stored at -20°C until analysis.

Data processing

The concentrations of unknown samples were calculated from the calibration curves.

The pharmacokinetic profile was studied by plotting serum concentrations and cumulative urinary excretion of I versus time. The kinetic parameters were analysed using a one-compartment open model with first-order absorption [5], and the area under the serum concentration–time curve from zero to infinity was calculated using the linear trapezoidal method.

RESULTS AND DISCUSSION

Extraction procedure

Several organic solvents were examined as extraction media. As a result, only chloroform was found to be suitable for the extraction of I from serum or urine at a high recovery rate. The efficiency of extraction of I was almost constant, irrespective of the pH of the aqueous phase (pH 4.5–10), but the endogenous

TABLE I

RECOVERIES OF I EXTRACTED WITH CHLOROFORM FROM HUMAN SERUM OR URINE

Each value represents the mean ± S.D. Either five or six replicate samples were assayed in each case.

Concentration (ng/ml)	Recovery (%)	
	Serum	Urine
70.5	90.5 ± 2.5	
140.9	94.8 ± 3.6	100.9 ± 9.4
352.3	93.1 ± 3.4	
704.5	90.9 ± 5.0	95.5 ± 5.9
1761.3	98.2 ± 3.0	
3522.5	93.5 ± 4.4	99.2 ± 2.5

matrix constituents derived from serum or urine were at a minimum when serum or urine samples were extracted after alkalinization with 1 ml of 0.1 *M* sodium hydroxide. The absolute recoveries of I from serum and urine were assessed by comparing the peak heights obtained from the direct injections of the standard solution of I with those of extracts of the alkalinized blank serum or urine spiked with the same amounts of I. The extraction recoveries of I ranged from 90.5 to 98.2% over the concentration range 70.5–3522.5 ng/ml in serum samples and from 95.5% to 100.9% over the concentration range 140.9–3522.5 ng/ml in urine samples (Table I). In addition, the mean recovery rate for 1688.8 ng/ml II was 97.2%.

HPLC conditions

Compound I has a UV absorbance peak at 262 nm, at which the absorption coefficient ($3.8 \cdot 10^2$) is not sufficiently high. Its UV absorbance at 210 nm is much greater, although interfering peaks increase to some extent. Therefore, we selected a detection wavelength of 210 nm, which resulted in an increased absolute sensitivity.

Various compositions of acetonitrile and phosphate buffer were tested as a mobile phase, and 1/15 *M* potassium hydrogenphosphate–acetronitrile (7:3, v/v) was found to provide a good separation of I and II. The retention times of I and II were 6 and 9.5 min, respectively. No peak derived from endogenous substances in serum or urine interfered significantly with the detection of I and II. Representative chromatograms obtained from serum and urine samples are shown in Fig. 2 and Fig. 3, respectively.

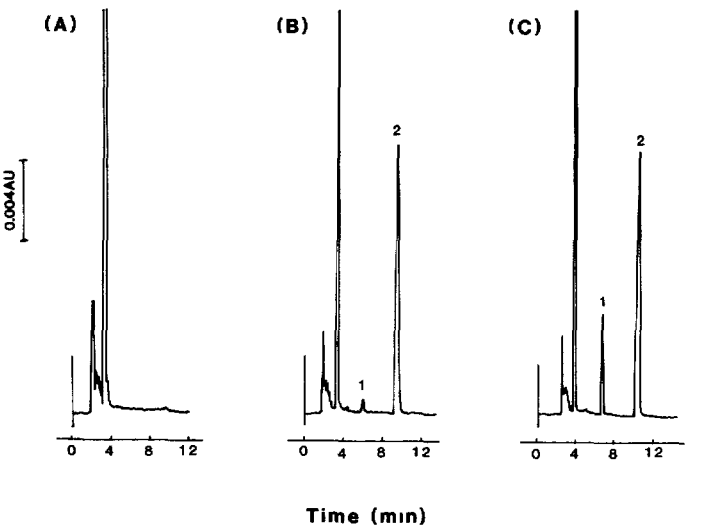

Fig. 2. Representative chromatograms of serum extracts obtained from (A) blank serum, (B) blank serum spiked with I (70.5 ng/ml) and internal standard (1688.8 ng/ml) and (C) serum from a human volunteer after an oral dose of 30 mg of I. Peaks: 1 = I, 2 = internal standard.

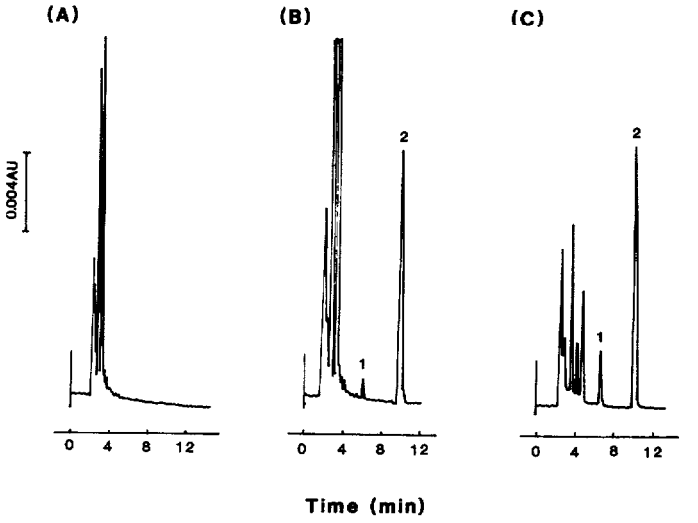

Fig. 3. Representative chromatograms of urine extracts obtained from (A) blank urine, (B) blank urine spiked with I (140.9 ng/ml) and internal standard (1688.8 ng/ml) and (C) urine from a human volunteer after an oral dose of 30 mg of I. Peaks: 1 = I; 2 = internal standard.

TABLE II

WITHIN-DAY PRECISION AND ACCURACY IN THE DETERMINATION OF I IN SERUM

Theoretical concentration (ng/ml)	Measured concentration (mean ± S.D., $n=6$) (ng/ml)	C.V. (%)	Relative error (%)
66.1	63.0 ± 2.5	3.9	−4.6
132.1	127.7 ± 3.5	2.8	−3.4
264.3	263.3 ± 3.2	1.2	−0.4
528.5	538.5 ± 16.0	3.0	1.9
1057.0	1066.8 ± 26.1	2.4	0.9
2114.0	2107.3 ± 31.9	1.5	−0.3

TABLE III

WITHIN-DAY PRECISION AND ACCURACY IN THE DETERMINATION OF I IN URINE

Theoretical concentration (ng/ml)	Measured concentration (mean ± S.D., $n=6$) (ng/ml)	C.V. (%)	Relative error (%)
132.1	135.0 ± 5.1	3.7	2.2
264.3	259.2 ± 4.3	1.7	−1.9
528.5	539.6 ± 22.9	4.2	2.1
1057.0	1046.3 ± 44.7	4.3	−1.0
2114.0	2117.0 ± 47.3	2.2	0.1

TABLE IV

BETWEEN-DAY PRECISION IN THE DETERMINATION OF I IN SERUM

Theoretical concentration (ng/ml)	Measured concentration (mean ± S.D., $n=5$) (ng/ml)	C.V. (%)	Relative error (%)
66.1	65.2 ± 2.3	3.5	−1.3
132.1	132.1 ± 3.8	2.9	0.0
264.3	264.4 ± 1.0	0.4	0.0
528.5	537.2 ± 15.6	2.9	1.6
1057.0	1051.6 ± 10.5	1.0	−0.1
2114.0	2114.5 ± 4.7	0.2	0.0

TABLE V

BETWEEN-DAY PRECISION IN THE DETERMINATION OF I IN URINE

Theoretical concentration (ng/ml)	Measured concentration (mean ± S.D., $n=5$) (ng/ml)	C.V. (%)	Relative error (%)
132.1	140.7 ± 10.1	7.2	6.5
264.3	260.9 ± 8.9	3.4	−1.3
528.5	538.3 ± 17.7	3.3	1.8
1057.0	1045.3 ± 9.7	0.9	−1.1
2114.0	2119.0 ± 3.7	0.2	0.2

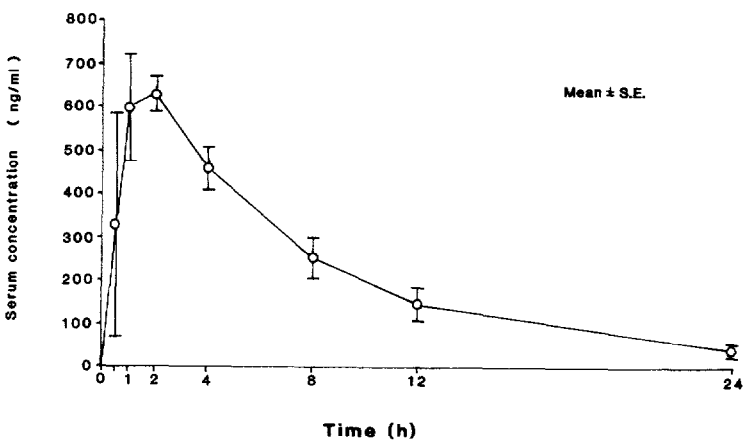

Fig. 4. Serum concentration of I following an oral dose of 30 mg of I to three healthy subjects.

Linearity and sensitivity

The calibration curves for I in serum and urine were linear over the range 0–2114.0 ng/ml. The correlation coefficients of the calibration curves for serum and

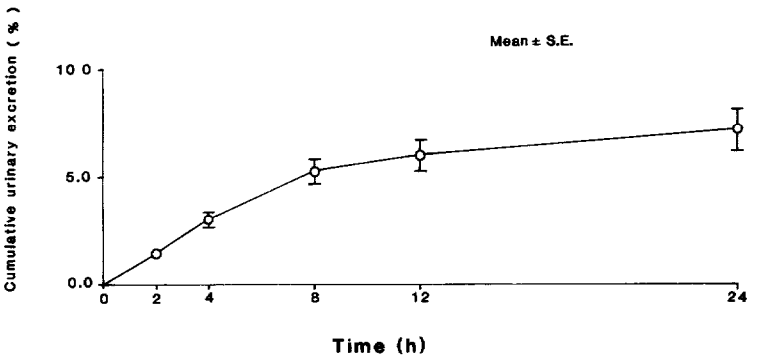

Fig 5. Cumulative urinary excretion of I following an oral dose of 30 mg of I to three healthy subjects.

urine analysis were 0.9997 and 0.9992, respectively. The detection limits (signal-to-noise ratio of 2) for I were 30 ng/ml in serum and 50 ng/ml in urine.

Precision and accuracy

Reproducibilities for both within-day assay and between-day assay were evaluated to assess the precision and accuracy of this analytical method. The results are shown in Table II–V. The coefficients of variation (C.V.) of the six independent samples at each concentration in the within-day assay were between 1.2 and 3.9% for serum samples in the concentration range 66.1–2114.0 ng/ml and between 1.7 and 4.3% for urine samples in the concentration range 132.1–2114.0 ng/ml. The C.V. values in the between-day assay were 0.2–3.5% for serum samples and 0.2–7.2% for urine samples in the same concentration ranges.

The accuracy was determined by comparing the nominal concentrations of I with those observed. The relative error in the within-day assay ranged from −4.6 to 1.9% for serum samples and from −1.9 to 2.2% for urine samples.

Application of the method

The utility of this proposed method for clinical study was demonstrated by analysing I in serum and urine samples from three healthy volunteers given an oral dose of 30 mg. Representative analytical data are illustrated in Fig. 4 and Fig. 5. The serum data were fit to one-compartment open model with first-order absorption. The serum concentration of I reached a maximum (675 ng/ml) at 1.67 h after dosing, and then decreased with a half-life of 4.43 h. The area under the serum concentration–time curve was 5.67 $\mu g \cdot h/ml$.

Approximately 7.1% of the administered dose was recovered in the urine in its unchanged form within 24 h after dosing.

In conclusion, the present HPLC method is sufficiently sensitive, rapid and simple. It has proved to be accurate and precise and is thus useful for monitoring I in pharmacokinetic studies and in clinical therapy.

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