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Picogram determination of a novel dopamine D_4 receptor antagonist in human plasma and urine by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry

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Abstract

A sensitive and specific assay for the determination of 3-{[4-(4-chlorophenyl)piperazin-1-yl]methyl}-1H-pyrrolo[2,3-*b*]pyridine (I, L-745,870), a potential antipsychotic agent, has been developed, utilizing high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) detection. The analyte and the internal standard (II, 3-{[4-(4-trifluoromethyl)piperazin-1-yl]methyl}-1H-pyrrolo[2,3-*b*] pyridine) were isolated from a basified biological matrix using liquid–liquid extraction with methyl-*tert.*-butyl ether. The organic extract was evaporated to dryness, the residue was reconstituted in a mobile phase and injected into the HPLC system. The chromatographic conditions used for the analysis were a Keystone Scientific C_{18} BDS 150×4.6 mm, 5 μm column with a mobile phase consisting of a 40:60 (v/v) mixture of acetonitrile and water containing 10 m*M* ammonium acetate and 0.1% formic acid pumped at a flow-rate of 1.2 ml/min, yielding retention times of 3.4 and 5.0 min for I and II, respectively. The MS–MS detection was performed on a PE Sciex API III Plus tandem mass spectrometer using a heated nebulizer interface. Multiple reaction monitoring using the parent→product ion combinations of m/z 327→131 and 361→131 were utilized to quantitate I and II, respectively. The assays were validated in the concentration range of 0.1 to 100 and 0.5 to 500 ng/ml for plasma and urine, respectively. The precision of the assays, expressed as coefficients of variation were less than 10% over the entire concentration range, with adequate assay accuracy, sensitivity and specificity to determine the pharmacokinetics in human subjects following a single 1-mg dose.

Keywords: Dopamine D_4 receptor antagonists

1. Introduction

The basic pharmacological property common to various drugs used as antipsychotic agents is their ability to inhibit dopaminergic activity [1]. The antipsychotic effect of these drugs is generally considered to be mediated by blockade of dopamine D_2 receptors that usually triggers neurological side effects [1–3]. A recent study showed that dopamine D_4 receptors are elevated in schizophrenics [4], and that clozapine, an atypical neuroleptic used for the treatment of schizophrenia, has much greater affinity for dopamine D_4 than D_2 receptors [5–7]. Compound I (3-{[4-(4-chlorophenyl)piperazin-1-yl]-methyl}-1H-pyrrolo[2,3-*b*]pyridine, Fig. 1A), an antagonist with high affinity and selectivity for the human dopamine D_4 receptor [8], has been considered as a potential antipsychotic agent. In order to

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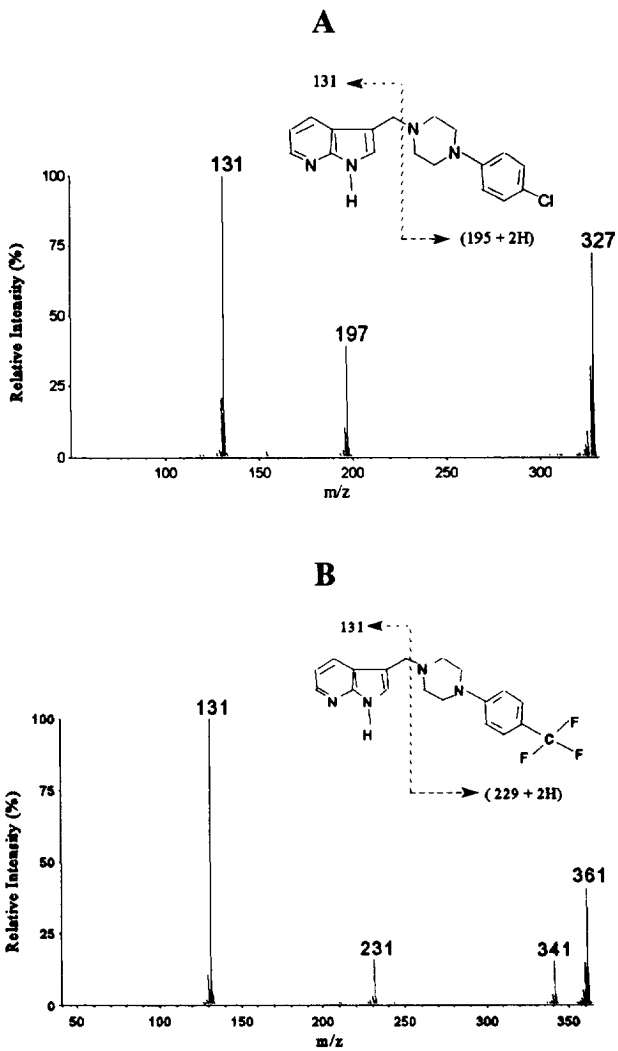

Fig. 1. Chemical structures and positive product ion mass spectra of the protonated molecules of I (A, *m/z* 327) and internal standard II (B, *m/z* 361).

support a human pharmacokinetics program, highly sensitive analytical methods for the determination of I in both plasma and urine were required, with a limit of quantification (LOQ) below 1 ng/ml. The development of these methods based on HPLC with atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS–MS) is the subject of this paper.

In the last few years, HPLC–APCI-MS–MS techniques have proven to be of great use for both the identification and quantification of drugs and metabolites in biological fluids at ng to sub-ng concentrations. Some recent examples of the application of this methodology to the quantification of drugs and metabolites in biological fluids, both from our laboratory and from others, are listed in references [9–17]. Chemical ionization of the sample molecule at atmospheric pressure results in a highly efficient ionization due to the high collision frequency [18]. The primary reagent ions, produced by gentle ionization of the solvent vapor in the ion source, ionize the analyte molecules, thus creating a high abundance of

the protonated molecular ions. Collision induced dissociation (CID) of the protonated molecular ions by a neutral gas (Ar), with monitoring of one or more product ions, provides highly specific determination of an analyte in the tandem MS–MS mode.

The subject of this paper is the development of analytical methods for the determination of I in human plasma and urine with the limit of quantification (LOQ) being 0.1 and 0.5 ng/ml, respectively. The LOQ is defined here as the lowest concentration on the calibration line for which intra-day assay precision (C.V.) was less than 10% and assay accuracy was within ±10%. The long-term performance of these methods was confirmed during the analysis of biological fluid samples from a number of clinical studies with I.

2. Experimental

2.1. Materials and reagents

Compound I (L-745,870) and the internal standard (II, 3-{[4-(4-trifluoromethyl)piperazin-1-yl]methyl}-1H-pyrrolo[2,3-*b*]pyridine, Fig. 1B) were synthesized at Merck Research Laboratories (Terlings Park, UK). Ammonium acetate and β-glucuronidase were purchased from Sigma (St. Louis, MO, USA). Formic acid was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer biocompatible binary pump 250, a WISP 715 autoinjector (Waters-Millipore, Milford, MA, USA) and an API III Plus triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer interface. During liquid–liquid extraction, the samples were rotated using a Glas-Col Laboratory Rotator (Beckman Instruments, Palo Alto, CA, USA) at a speed setting of 8.

2.3. Chromatographic conditions

The aqueous portion of the mobile phase was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water, to which 820 μl of formic acid were added. The pH of the aqueous portion was adjusted to 4.5 with ammonium hydroxide for urine analysis, while no pH adjustment was required for the plasma assay. The mobile phase consisted of 60% of the aqueous portion and 40% of acetonitrile and was pumped at a flow-rate of 1.2 ml/min. Chromatography was performed on a BDS-Hypersil C_{18} (150×4.6 mm; 5 μm particles) analytical column coupled with a BDS-Hypersil C_{18} (20×4.6 mm; 5 μm particles) guard column (Keystone Scientific, Bellefonte, PA, USA). The total run-time was 7.5 min with I eluting at 3.7 min and II at 5.0 min after injection.

2.4. Mass spectrometric conditions, data acquisition and analysis

The mass spectrometer was interfaced to the HPLC system via a heated nebulizer probe that was maintained at 500°C. The air pressure of the nebulizer was set at 80 p.s.i., nebulizer flow at 0.6 l/min and curtain gas (N_2) flow was set at 0.9 l/min. Positive chemical ionization was affected by a corona discharge needle (+4 μA) and the sampling orifice potential was set at +40 V. The first quadrupole, Q1, was set to monitor the protonated molecules $[M+H]^+$ at m/z 327 for drug and m/z 361 for II, with collision-induced fragmentation at Q2 (collision gas argon, $290 \cdot 10^{13}$ atoms cm^{-2}) and monitoring of the product ions via Q3 at m/z 131 for both I and II. The electron multiplier setting was −4.0 kV and the detector electronics were set to counts of 10. Dwell time was 400 ms.

Data acquisition and analyses were performed using RAD and MacQuan software (PE-Sciex). Unknown sample concentrations were calculated from the equation $y=mx+b$, as determined by the weighted $(1/y^2)$ linear least-squares regression of the calibration line constructed from the peak-area ratios of the drug to II vs. drug concentration.

2.5. Standard solutions

A standard stock solution of I (1.0 mg/ml) was prepared in methanol. Subsequent dilutions were made in methanol to give the following concen-

trations: 1000, 500, 250, 100, 50, 25, 10, 5, 2.5 and 1 ng/ml for the plasma assay and 5000, 2500, 1000, 500, 250, 100, 50, 10 and 5 ng/ml for the urine assay. A standard stock solution (1.0 mg/ml) of internal standard, II, was also prepared in methanol. Subsequent dilutions in methanol were made to prepare 100 and 500 ng/ml working standard solutions of II for the plasma and urine assays, respectively.

2.6. Sample preparation

2.6.1. Plasma

The drug and internal standard (II) were isolated from basified plasma using liquid–liquid extraction. To a 1-ml volume of plasma placed in a 15-ml glass centrifuge tube, 100 μl of a working standard solution of II (equivalent to 10 ng of II per ml of plasma) was added. After addition of 1 ml of carbonate buffer, pH 9.8, followed by 7 ml of methyl-*tert.*-butyl ether (MTBE), the tubes were mixed by rotation for 15 min, centrifuged at 4000 rpm for 15 min and the organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 50°C. The residue was redissolved in 500 μl of a 40:60 (v/v) mixture of acetonitrile–water (containing 0.1% formic acid), vortex-mixed for 1 min, sonicated for 15 min, vortex-mixed again and centrifuged for 5 min, and 60 μl of the sample were injected into the LC–MS–MS system.

2.6.2. Urine

A 1-ml volume of urine was pipetted into a 15-ml centrifuge tube and 1 ml of a solution containing β-glucuronidase (7200 Fishman units) in 0.2 *M* phosphate buffer (pH 6.5) was added. After incubation at 37°C for 20 h, 100 μl of a working standard solution of II (equivalent to 50 ng of II per 1 ml of urine) and 1 ml of carbonate buffer, pH 9.8, were added, followed by the addition of 7 ml of MTBE. Using a similar procedure as described for plasma samples, the residue after evaporation of the extract to dryness was redissolved in 2 ml of a 40:60 (v/v) mixture of acetonitrile–water (containing 0.1% formic acid, and with the pH adjusted to 4.5). A 60-μl volume of the urine extract was injected into the HPLC–MS–MS system.

2.7. Precision, accuracy, linearity and recovery

The precision of the method was determined by replicate analyses ($n=5$) of human plasma spiked with I at concentrations 1.0, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/ml for the plasma assay, and at concentrations of 5, 10, 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml for the urine assay. Assay accuracy was assessed by comparing the mean calculated concentrations of standards, determined by replicate analyses, to the nominal concentration. Recovery was calculated by comparison of peak areas of I extracted from plasma and/or urine to those of the directly injected standards.

Quality control (QC) samples at 0.75 and 75 ng/ml for the plasma assay and 2.0 and 400 ng/ml for the urine assay were prepared from separately weighed and diluted standard samples of I and II. An aliquot (1.25 ml) of these solutions was transferred to a 2-ml plastic tube, stored at −20°C, and analyzed daily with clinical samples. The calculated concentrations of the QC samples were compared on a day-to-day basis and allowed for the assessment of inter-day assay performance.

The linearity of each standard curve was confirmed by plotting the peak-area ratio of the drug to II versus drug concentration. A calibration line was constructed daily and the spiked samples were assayed together with QC and clinical samples.

3. Results and discussion

In order to support clinical studies with oral doses of I as low as 1 mg, an assay methodology for I in plasma with a LOQ of 0.1 ng/ml was required. The conventional methods, based on ultraviolet absorption, fluorescence and/or electrochemical detection after HPLC separation, were not considered viable detection options for the fast development of an assay at sensitivities below 1 ng/ml. Thus, initially, an attempt was made to establish an assay based on capillary gas chromatography (GC) with electron ionization (EI)-MS detection. A method based on

solid-phase extraction of I and II from plasma, GC separation and MS detection was developed [19], but the LOQ of the method (1 ng/ml) was not sufficient to support clinical studies with low doses of I. Therefore, a method based on HPLC with MS–MS detection was evaluated.

The positive ion mass spectra (Q1) of both I and II indicated the presence of predominantly the protonated molecules $[M+H]^+$ of these compounds at m/z 327 and 361, respectively. The CID product ion mass spectra of these ions showed mostly intense fragment ions at m/z 131 (Fig. 1). After examination of a broad spectrum of collision gas thickness and ion energies, the fragment ion at m/z 131 was chosen for quantification of both I and II, due to the high sensitivity and selectivity of detection. The proposed fragmentation of the protonated molecules of I and II is presented in Fig. 2.

Multiple reaction monitoring using the parent→product ion combinations of m/z 327→131 and 361→131 were used to quantify I and II, respectively.

The isolation of I and II from plasma and urine was based on liquid–liquid extraction. The efficiency of the extraction of I and II from the aqueous phase of plasma or urine into the organic solvent was greatly improved when both molecules were not protonated. Since the pK_a values for I were 2.74 and 7.22, the pH of plasma was adjusted to 9.8 by the addition of a carbonate buffer. Recovery of I from plasma and urine was more than 90% at all concentrations within the calibration curve range.

The retention times of I and II were highly dependent on the pH and on the content of the organic solvent in the mobile phase. In spite of the use of highly specific MS–MS detection, endogenous impurities from the urine of some subjects gave a response at the parent→product ion combinations used for monitoring either I and/or II, and coeluted with the peaks of interest. For the urine assay, the pH of the aqueous portion of the mobile phase had to be adjusted to 4.5 to maintain assay specificity and to effectively alter the retention times of I and II relative to the endogenous interferences extracted from urine. Under the conditions described in Section 2, assay specificity was maintained for all

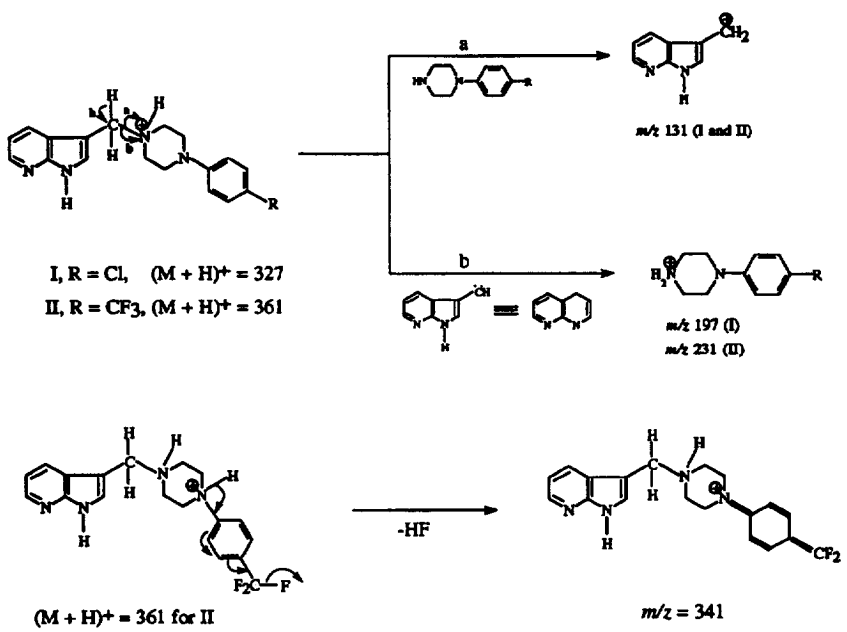

Fig. 2. Proposed mechanism of fragmentation of protonated molecules of I and II.

plasma and urine samples originating from a large number of subjects participating in clinical studies.

The validation of the assay for I in urine was performed after incubation of the urine samples with β-glucuronidase to convert the N-glucuronide(s) of I present in post-dose urine samples to I, in a manner similar to that described earlier [10]. Using this method, the total concentration of I in urine in the form of a free drug and its N-glucuronide(s) was determined. The total amount of I excreted in urine was less than 1% of the total dose.

Representative chromatograms of plasma and urine extracts analyzed using the HPLC–MS–MS method are presented in Fig. 3 Fig. 4, respectively.

The method was validated for human plasma in the concentration range of 0.1–100 ng/ml and for urine in the concentration range of 0.5–500 ng/ml. The intra-day precision of the methods was less than 10% at all concentrations within the standard curve range, with an adequate assay accuracy ranging from 92 to 105% (Table 1 Table 2, respectively).

The inter-day variability, as measured by the assay of QC samples, was less than 12 and 10% for plasma and urine samples, respectively (Table 3).

The typical equations describing calibration lines were $y=0.077507x-0.003656$ in plasma and $y=0.014047x-0.002321$ in urine, with correlation coefficients of 0.9984 and 0.9985, respectively.

The method has been applied to the determination of I in plasma samples from subjects participating in several safety and tolerability studies of I in healthy male volunteers. As an example, plasma concentrations of I from four subjects on day 1 after receiving a 1-mg dose of I are represented in Fig. 5. The method is capable of monitoring concentration levels of compound I in plasma up to 12 h after dosing with 1 mg of I.

In conclusion, a sensitive and specific LC–MS–

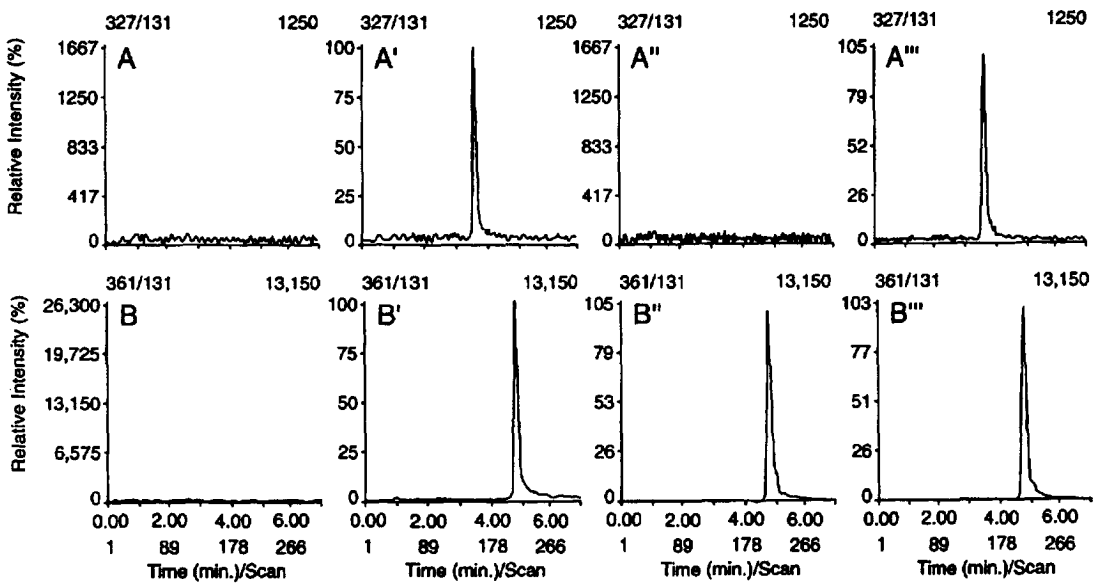

Fig. 3. Representative HPLC–MS–MS chromatograms of the plasma extracts obtained by multiple reaction monitoring at m/z 327→131 for I (channel A), and m/z 361→131 for internal standard II (channel B); chromatograms A and B=extracts of control plasma; chromatograms A′ and B′=extracts of control plasma spiked with 1.0 ng/ml of I and 10 ng/ml of II, respectively; chromatograms A″ and B″=pre-dose plasma extract, spiked with 10 ng/ml of II; chromatograms A‴ and B‴=plasma extract on day 1, 36 h after receiving a 10-mg dose of I, spiked with 10 ng/ml of II. The concentration of I is equivalent to 1.0 ng/ml. The numbers in the upper right hand corner of the chromatograms correspond to the peak heights expressed in arbitrary units.

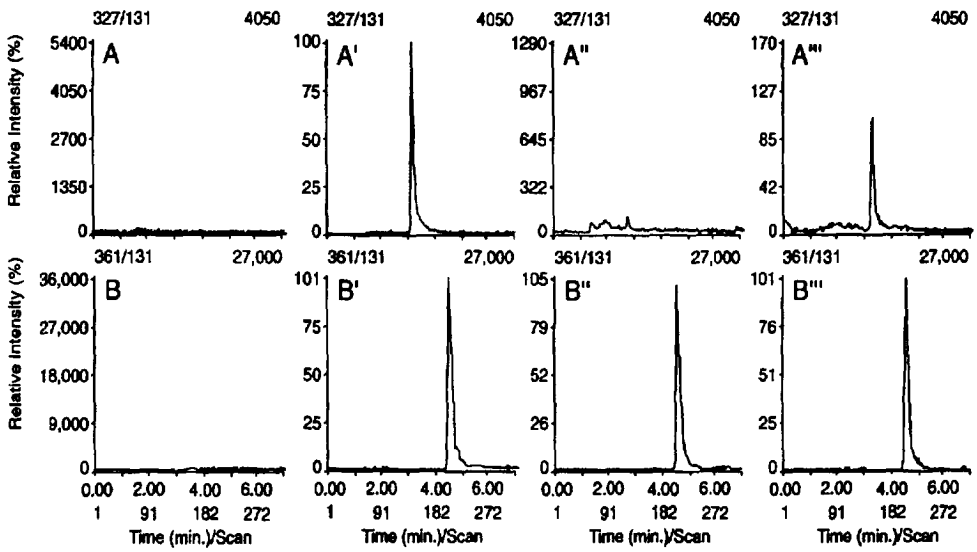

Fig. 4. Representative HPLC–MS–MS chromatograms of the urine extracts obtained by multiple reaction monitoring at m/z 327→131 for I (channel A), and m/z 361→131 for internal standard II (channel B); chromatograms A and B=extracts of control urine; chromatograms A′ and B′=extracts of control urine spiked with 10.0 ng/ml of I and 50 ng/ml of II; chromatograms A″ and B″=pre-dose urine extract, spiked with 50 ng/ml of II; chromatograms A‴ and B‴=urine extract, 24–36 h after receiving a 5-mg dose of I, spiked with 50 ng/ml of II. The concentration of I is equivalent to 5.8 ng/ml. The numbers in the upper right hand corner of the chromatograms correspond to the peak heights expressed in arbitrary units.

MS method has been developed for the determination of I in human plasma and urine in the concentration range of 0.1–100 and 0.5–500 ng/ml, respectively. The ruggedness and long-term performance of the method were confirmed during the analyses of I in plasma and urine samples originating from human subjects participating in a number of clinical trials.

Table 1
Precision[a] and accuracy data for the analysis of L-745,870 (I) in human plasma

Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	C.V. (%)	Accuracy[b] (%)
0.10	0.10	9.0	100
0.25	0.23	5.0	92
0.50	0.48	6.5	96
1.00	1.03	4.2	103
2.50	2.52	1.9	101
5.00	5.06	1.9	101
10.00	10.46	2.5	105
25.00	24.56	2.2	98
50.00	50.87	0.9	102
100.00	102.85	1.4	103

[a] Expressed as C.V. (%); $n=5$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)]×100.

Table 2
Precision[a] and accuracy data for the assay of L-745,870 (I) in human urine

Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	C.V. (%)	Accuracy[b] (%)
0.50	0.52	5.4	104
1.00	0.96	4.6	96
5.00	4.66	4.9	93
10.00	9.97	1.5	100
25.00	25.04	2.6	100
50.00	50.11	4.8	100
100.00	103.94	2.4	104
250.00	256.69	1.3	103
500.00	527.23	2.1	105

[a] Expressed as C.V. (%); $n=5$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)]×100.

Table 3
Inter-day variability of the LC–MS–MS assay of quality control samples spiked with I

Biological specimen	Spiked concentration (ng/ml)	Number of determinations	Mean calculated concentration (ng/ml)	C.V. (%)
Plasma	0.75	14[a]	0.76	11.8
Plasma	75.0	14[a]	77.1	36.9
Urine	2.0	8[b]	2.0	6.5
Urine	400	8[b]	389.2	3.7

[a] Over a period of seven days.
[b] Over a period of four days.

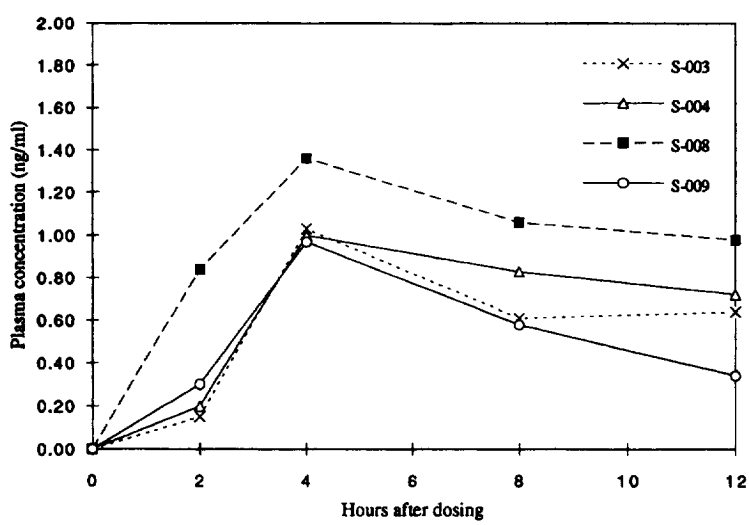

Fig. 5. Representative plasma concentration–time profiles of I after oral administration of a single 1-mg dose to human subjects (S-003, S-004, S-008, S-009).

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