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Determination of a novel selective inhibitor of type 1 5α-reductase in human plasma by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry

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Abstract

A sensitive and specific assay of human plasma for the determination of (5α,7β,16β)-16[(4-chlorophenyl)oxy]-4,7-dimethyl-4-aza-androstan-3-one (**I**), a selective inhibitor of human type 1 5α-reductase, has been developed. The method is based on high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) detection. The analyte (**I**) and internal standard, Proscar (**II**), were isolated from the basified biological matrix using a liquid–liquid extraction with methyl-*tert*.-butyl ether (MTBE). The organic extract was evaporated to dryness, the residue was reconstituted in mobile phase and injected into the HPLC system. 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 precursor→product ion combinations of m/z 430→114 and 373→305 was used to quantify **I** and internal standard (**II**), respectively. The assay was validated in the concentration range of 0.5 to 500 ng/ml in human plasma. The precision of the assay, expressed as coefficient of variation (C.V.), was less than 7% over the entire concentration range, with adequate assay specificity and accuracy. The HPLC–MS–MS method provided sufficient sensitivity to completely map the 24 h pharmacokinetic time-course following a single 0.5 mg dose of **I**.

Keywords: 5α-Reductase inhibitor

1. Introduction

Compound **I** **(**5α,7β,16β)-16[(4-chlorophenyl)-oxy]-4,7-dimethyl-4-aza-androstan-3-one (L-751 788, Fig. 1) belongs to a class of 4-azasteroids with a chemical structure similar to finasteride (Proscar), a potent 5α-reductase (5αR) inhibitor [1]. Inhibition of 5αR results in an antiandrogen effect by decreasing target-organ dihydrotestosterone (DHT) levels. Two isozymes of 5αR are known to exist, type 1 (5αR1) and type 2 (5αR2). In contrast to finasteride, which is a potent inhibitor of 5αR2, compound **I** is a potent and specific inhibitor of the human 5αR1 [2,3]. Finasteride has undergone successful clinical evaluation [4], and was approved for use in the treatment of benign prostatic hyperplasia (BPH).

The two 5αR isozymes have been characterized and both are found in human skin in a ratio that varies depending upon location. Beard and scrotal skin contain mainly 5αR2 whereas chest skin contains both isozymes. Scalp, face, arm, breast, abdomen, back and legs contain mainly 5αR1. The

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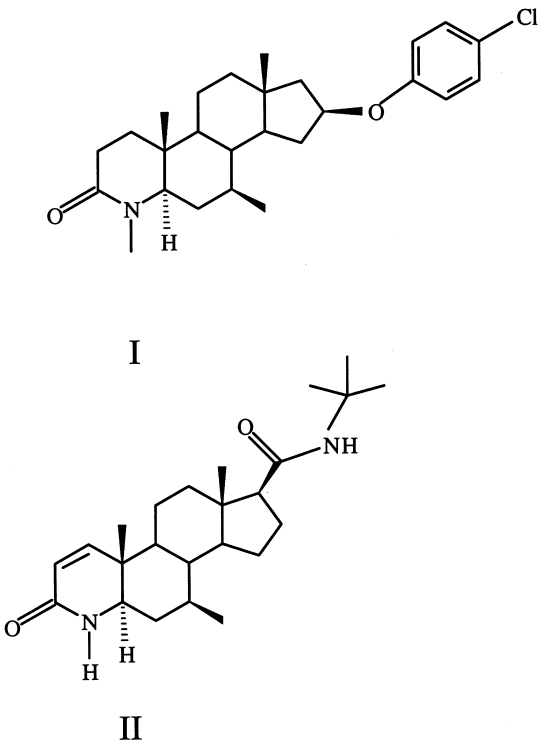

Fig. 1. Chemical structures of **I** and internal standard **II**.

localization of the 5αR1 in the sebaceous gland of skin [5] and the implication that DHT is one of the prerequisites for the onset of acne suggests that inhibition of this isozyme may be useful in the management of this disorder. Therefore, **I**, an inhibitor of 5αR1, may be useful in the prevention or treatment of acne.

In order to support initial clinical pharmacokinetic studies of **I**, it was necessary to develop a sensitive and specific method for the determination of **I** in human plasma with the limit of quantification (LOQ) of less than 1 ng/ml. The LOQ target of 1 ng/ml was established based upon historical data of finasteride and the projected oral doses of **I**.

Initially, the feasibility of development of a high-performance liquid chromatography (HPLC) assay with a conventional ultraviolet (UV) absorbance detection was evaluated. The presence of chlorophenoxy group in **I** created the possibility of an improved sensitivity of detection in the near UV region due to the enhanced absorption of **I** in comparison with other azasteroids studied earlier [6]. The UV absorbance spectra of **I** in methanol indicated the presence of two absorption bands with the maxima at 204 nm and 230 nm and the molar absorptivities (ϵ) of 18 600 and 14 700 M^{-1} cm^{-1}, respectively. In principle, the development of an assay based on HPLC with UV absorbance detection at 230 nm with the LOQ of ~1 ng/ml was feasible but would require a very highly selective and efficient off-line and on-line sample clean-up using column switching to monitor **I** at sub-nanogram concentrations without endogenous interferences from plasma matrix. Instead, we have decided to use HPLC–tandem mass spectrometry (MS–MS) to achieve the LOQ of 0.5 ng/ml using relatively simple sample preparation procedure and short analysis time. The HPLC–MS–MS method was utilized earlier for determination of structurally similar azasteroids in both clinical and preclinical studies [7–10].

The subject of this paper was the development of a highly sensitive HPLC–MS–MS assay for the determination of **I** in human plasma with the LOQ of 0.5 ng/ml. The need for careful assessment of the specificity of MS–MS based assays [11,12] was also emphasized and the absence of a matrix effect and assay specificity in the presence of metabolites was demonstrated.

2. Experimental

2.1. Materials

Compounds **I** and **II** were synthesized at the Merck Research Labs. (Rahway, NJ, USA). All solvents and reagents were of HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The drug-free, human heparinized plasma originated from Biological Specialties (Lansdale, PA, USA). Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) were purchased from West Point Supply (West Point, PA, USA).

2.2. Instrumentation

A PE Sciex (Thornhill, Canada) API III Plus tandem mass spectrometer equipped with heated

nebulizer interface, a Waters Associates (Waters-Millipore, Milford, MA, USA) WISP 715 autoinjector, and Perkin-Elmer biocompatible binary pump (Model 250) were used for all HPLC–MS–MS analyses. The data were processed using MacQuan software (PE Sciex) on a Macintosh Quadra 900 microcomputer.

2.3. Chromatographic conditions

HPLC separation was performed using a Keystone Scientific BDS Hypersil C_{18}, 50×4.6 mm I.D., 5 μm analytical column heated to 60°C and coupled with a 5-μm in-line filter. The aqueous portion of the mobile phase was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water and the addition of 820 μl formic acid. The mobile phase was a mixture of 80% acetonitrile and 20% water containing 0.1% formic acid and 10 m*M* ammonium acetate, and was delivered at a flow-rate of 1.0 ml/min. The retention times for **I** and **II** were 2.8 and 0.8 min.

2.4. HPLC–MS–MS conditions

A PE Sciex triple quadrupole mass spectrometer was interfaced via a Sciex heated nebulizer probe to the HPLC system, and gas phase chemical ionization was effected by a corona discharge needle (+4 μA) using positive ion atmospheric pressure chemical ionization (APCI). The heated nebulizer probe temperature was maintained at 500°C. The nebulizing gas (air) pressure and auxiliary flow were set at 550 kPa and 2.0 l/min, respectively. Curtain gas flow (nitrogen) was 0.9 l/min, and the sampling orifice potential was set at +65 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60°C. The mass spectrometer was programmed to admit the protonated molecules $[M+H]^+$ at *m*/*z* 430 (**I**) and *m*/*z* 373 (**II**), via the first quadrupole filter (Q1), with collision-induced fragmentation at Q2 (collision gas argon, $270 \cdot 10^{13}$ atoms cm^{-2}), and monitoring the product ions via Q3 at *m*/*z* 114 and 305 for **I** and **II**, respectively. The electron multiplier setting was −4.7 kV. Peak area ratios obtained from multiple reaction monitoring of the analyte and internal standard, (*m*/*z* 430→114)/(*m*/*z* 373→305), were utilized for the construction of calibration curves using linear least square regression of the plasma concentrations and the measured peak area ratios. Data collection, peak integration and calculations were performed using MacQuan PE-Sciex software.

2.5. Standard solutions

A stock solution of **I** (1 mg/ml) was prepared in methanol. This solution was further diluted with methanol to give a series of working standards with the concentrations of 5, 10, 50, 100, 500, 1000 and 5000 ng/ml for plasma assay. The internal standard **II** was also prepared as a stock solution (1 mg/ml) in methanol by dissolving 10 mg of solid **II** in 10 ml of methanol. A working standard of 100 ng/ml was prepared by dilution of stock standard with methanol, and was used for all analyses. All standards were prepared once a month and stored at 5°C.

A series of quality control (QC) samples at 1 and 400 ng/ml for the plasma assay were prepared. Aliquots (1.25 ml) of these solutions were placed in 2-ml plastic tubes, 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 to assess inter-day assay variability.

2.6. Sample preparation

A 1-ml aliquot of plasma was pipetted into a 15-ml centrifuge tube and 100 μl of the working standard of **II** (equivalent to 10 ng/ml of I.S.) followed by the addition of 1 ml of 0.2 *M* carbonate buffer (pH 9.8). After addition of 7 ml of methyl-*tert*.-butyl ether and capping tubes with PTFE-lined caps, the mixture was rotated and mixed for 15 min. The tubes were then centrifuged and the organic layer was transferred to a clean centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at 50°C, the residue was reconstituted in 300 μl of the mobile phase and a 150-μl aliquot was injected onto the HPLC–MS–MS system.

2.7. Precision, accuracy and recovery

The precision of the method was determined by the replicate analyses (n=5) of human plasma con-

taining **I** at all concentrations utilized for constructing calibration curves. The linearity of each standard curve was confirmed by plotting the peak area ratio of the drug to I.S. versus drug concentration. The unknown sample concentrations were calculated from the equation $y=mx+b$, as determined by weighted $(1/y^2)$ linear regression of the standard line. The standard curve was constructed daily and these standard samples were assayed with quality control and unknown samples. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)]×100. The recovery was determined by comparing the peak area of **I** extracted from biological fluids to that of standards injected directly.

2.8. Assessment of matrix effect

The assessment of matrix effect and assay reliability is critical when homologues rather then stable isotope-labeled parent compound is selected for use as internal standard [11,12]. The undetected but co-eluting endogenous impurities may affect the ionization efficiencies of the analytes. Therefore, the ionization efficiency of both analytes, as measured by the individual peak areas of **I** and **II** in different plasma matrix, was evaluated. Both **I** and **II** were spiked into five different sources of plasma and their peak areas were determined. By comparing peak areas of the same analyte in different lots of plasma, the recovery and the differences in ionization efficiency associated with a given plasma lot was assessed.

2.9. Assessment of assay specificity

Assay specificity for the clinical samples was assessed by running blank control and patients' pre-dose biological fluid samples. No endogenous interferences were observed. In addition, internal standard peak areas in plasma samples of twelve subjects participating in a clinical study were compared for reproducibility at all timepoints after dosing with **I**. The coefficient of variation (C.V.) of the internal standard peak areas was 6.3%, further indicating consistent recovery and ionization efficiency of the internal standard between various subject timepoint samples, after dosing. In addition, in order to assess assay specificity in the presence of metabolites, selected post-dose samples were assayed under HPLC gradient elution conditions, thereby greatly increasing the retention factor (k) for both analytes. No additional peaks were observed during the gradient analysis, when monitored at the precursor→product ion combinations used for the determination of **I** and **II**, indicating the metabolites of **I** have not interfered with the quantification of **I** and **II**.

3. Results and discussion

3.1. Assay validation

The positive product mass spectrum of the protonated $[M+H]^+$ molecule of **I** at m/z 430 indicated the presence of several ions at m/z 271, 253, 229 and 114. For the internal standard **II**, the fragmentation was more favorable, and the product mass spectrum of the protonated molecule of **II** at m/z 373 exhibited only two major fragments at m/z 317 and 305 (Fig. 2) that is generally much more favorable for sensitive MS–MS detection.

Initially, four different precursor→product ion combinations (m/z 430→271, 430→253, 430→229, 430→114) were evaluated for sensitive and selective determination of **I**. The intensity of response and the presence of endogenous plasma impurities in different channels and at the retention time of **I** were monitored in plasma samples originating from ten different subjects. Small peaks originating from endogenous plasma components were observed in channels m/z 430→271, 430→253, 430→229 in at least one source of plasma, but such interference was not present in the channel m/z 430→114. It was later confirmed that the method was selective at m/z 430→114 and interference peaks in pre-dose plasma samples from all subjects participating in clinical studies were not observed. Therefore, concentrations of **I** were determined at m/z 430→114 over other MS–MS channels available. For internal standard **II**, the most intense product ion at m/z 305 was selected for quantification. By monitoring the precursor→product ion combinations at m/z 430→114 for **I** and m/z 373→305 for **II** in the multiple reaction monitoring mode, a highly sensi-

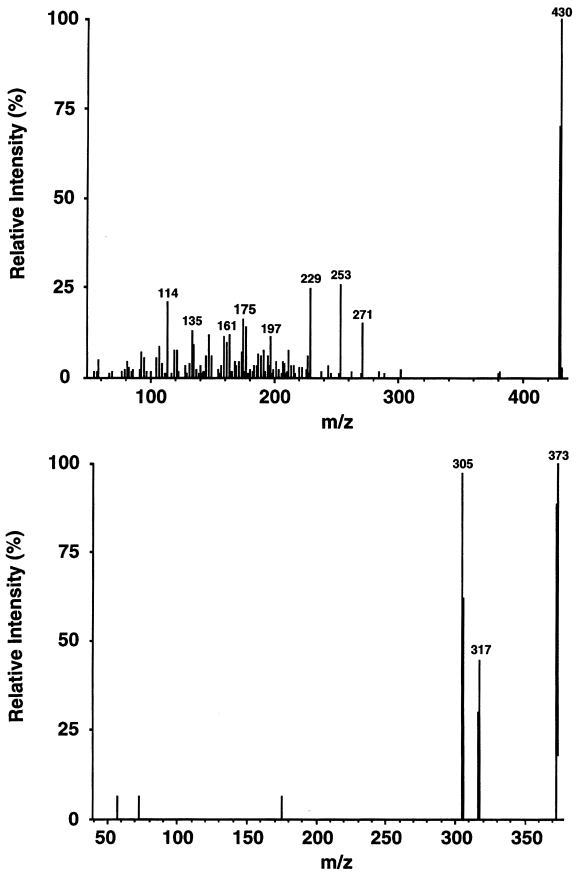

Fig. 2. Positive product ion mass spectra of the protonated molecules of **I** at m/z 430 (A) and internal standard **II** at m/z 373 (B).

tive assay for **I** in plasma with the LOQ of 0.5 ng/ml was developed.

The isolation of **I** and **II** from plasma was based on a simple liquid–liquid extraction from basified plasma, evaporation of the extract to dryness, reconstitution of the residue in mobile phase and injection into the HPLC system. The sample was basified to reduce the amount of compounds extracted from the matrix, thereby reducing the endogenous material injected onto the analytical column and increasing column lifetime and possibly reducing matrix effects.

The analytical column was heated to 60°C to reduce the retention time of analytes and to improve peak shape. Similar decrease in the retention time can be achieved by increasing acetonitrile content in the mobile phase from 80 to 95%, but under these conditions the internal standard **II** was practically unretained and eluted at 0.5 min. In addition, the efficiency of ionization of **I** and **II** was lower when a mobile phase with high organic content (95% acetonitrile) was utilized in comparison with the efficiency of ionization when a mobile phase with lower organic content (80% acetonitrile) was used. Therefore, in order to achieve the desired assay sensitivity, column heating and a mobile phase with a lower organic content rather than a mobile phase with higher organic content was employed. Heating of the analytical column had no adverse effect on its performance, as demonstrated by the analysis of more than 1200 samples from clinical studies using a single analytical column.

Following the procedure described in Section 2, the assay was validated in human plasma in the concentration range of 0.5 to 500 ng/ml. The difference between the nominal standard concentration and the back-calculated concentration from the weighted linear regression line was less than 10% for each point on the standard curve indicating that the linear regression analysis applied $(1/y^2)$ provided an adequate fit of the data. The correlation coefficient was always greater than 0.99. For ten daily runs, the mean slope and correlation coefficients of the calibration lines were 0.00777 ± 0.00059 and 0.996 ± 0.002, respectively. Representative chromatograms are shown in Fig. 3.

The potential ion suppression effect and ionization efficiencies for **I** and **II** in plasma samples originating from different individuals were closely examined. As seen in Table 1, peak areas of **I** and **II** were practically the same (C.V. of peak areas was less than 10%), strongly indicating little or no difference in ionization efficiency and consistent recovery of analytes **I** and **II** from five different plasma sources. In addition, any small changes in peak area of **I** in different lots of plasma were compensated by small similar changes in the peak areas of the internal standard **II**. The C.V. of peak area ratios of **I**/**II** was much smaller than the C.V.s of peak areas of **I** and **II**, confirming the desired compensating effect of the presence of internal standard on the precision and reliability of the quantification of **I**. In addition, the reproducibility and accuracy of the determination of internal standard was highly adequate as demonstrated in Table

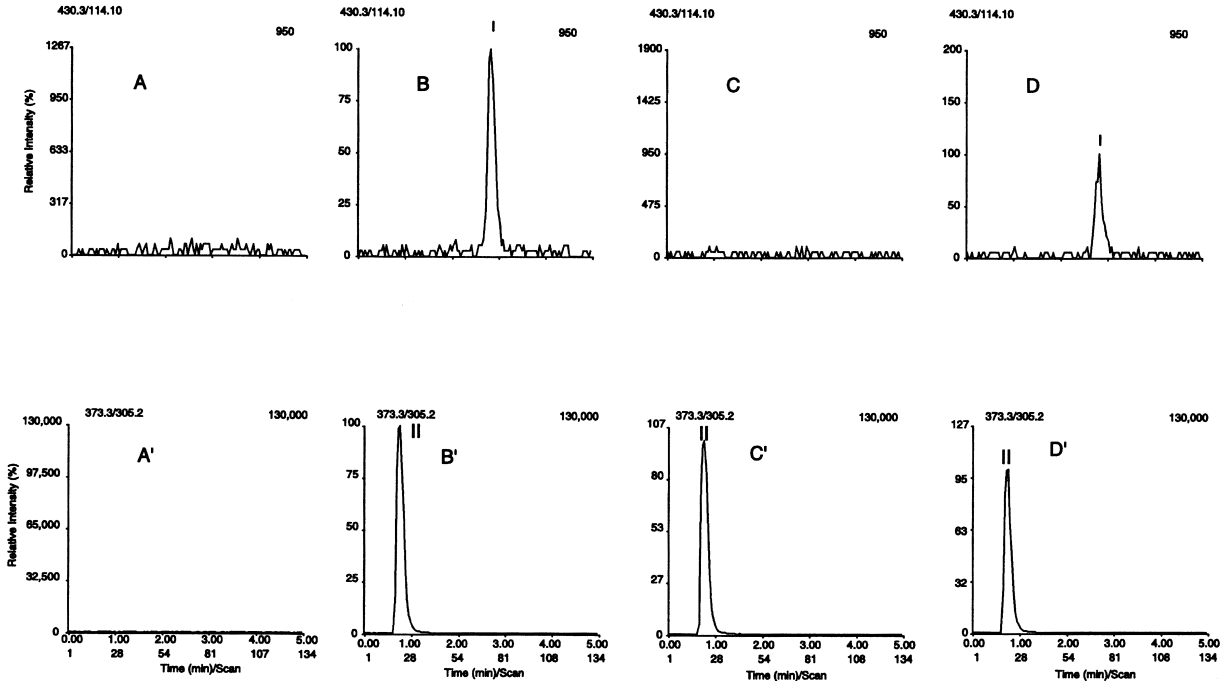

Fig. 3. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 430→114 (channel 'a') for **I** and m/z 373→305 (channel 'b') for internal standard (**II**); (A, A′) Blank control plasma monitored at channels 'a' and 'b', respectively; (B, B′) control plasma spiked with 1 ng of **I** and 10 ng of **II** monitored at channels 'a' and 'b', respectively; (C, C′) pre-dose plasma sample of a subject spiked with 10 ng **II** and monitored at channels 'a' and 'b', respectively; (D, D′) plasma sample of a subject 24 h after receiving a 0.5 mg oral dose of **I** and spiked with 10 ng of **II** monitored at channels 'a' and 'b'; concentration equivalent to 0.62 ng/ml. The numbers in the upper right hand corner correspond to peak heights expressed in arbitrary units.

1. In spite of the fact that internal standard was only modestly retained on the column (retention factor, k~1), an in-depth evaluation of a potential matrix effect using control plasma samples from a number of individuals indicated that the peak areas of **II** were practically the same (Table 2). Also, the peak areas of **II** in post-dose plasma samples were very similar to each other and to the peak areas in control plasma confirming that the potential interferences from metabolites present in post-dose biological

Table 1
Intra-day precision of peak areas of **I** and **II** spiked into five different sources of plasma

Nominal concentration (ng/ml)	Peak area of **I**[a]	Precision C.V. (%)	Peak area of **II**[b]	Precision C.V. (%)	Peak area ratios of **I**/**II** C.V. (%)
0.5	2448	5.0	590 503	3.8	2.9
1.0	4772	6.8	594 807	4.3	3.1
5.0	25 007	9.5	605 821	3.8	6.0
10.0	49 852	8.0	635 150	5.9	1.9
50.0	255 390	8.3	604 198	8.4	3.0
100.0	507 686	9.5	610 477	8.2	1.7
500.0	2 617 550	5.8	612 838	3.6	2.0

[a] **I** spiked into five different sources of plasma, in arbitrary units.
[b] **II** spiked into five different sources of plasma, in arbitrary units.

Table 2
Intra-day accuracy and precision of replicate analysis ($n=5$) for determination of **I** in human plasma

Nominal concentration (ng/ml)	Mean[a] concentration (ng/ml)	Precision[b] (C.V., %)	Accuracy[c] (%)	Recovery[d] (%)
0.50	0.51	2.9	102	96
1.00	0.98	3.1	98	94
5.00	5.00	6.0	100	98
10.0	9.58	1.9	96	98
50.0	51.0	3.0	102	100
100.0	101.8	1.7	102	100
500.0	521.0	2.0	104	103

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as coefficient of variation (C.V., %).
[c] Expressed as [(mean observed concentrations/nominal concentration)×100] ($n=5$).
[d] Recovery was determined by comparing the peak area of **I** extracted from biological fluids to that of standards injected directly.

fluids were not affecting the peak area of **II**. All of these data illustrate the adequacy of choosing **II** as an internal standard in spite of its relatively limited retention ($k\sim1$) on the column.

The intra-day precision, expressed as the C.V. was less than 9% at all concentrations within the standard curve range (Table 2). The mean recovery of **I** from plasma was 98% and was practically the same at all concentrations within the standard curve range (Table 2).

The limit of quantification (LOQ) of the assay was 0.5 ng/ml. The LOQ was defined here as the lowest concentration on the standard curve for which precision of the determination, expressed as C.V., was less than 10%, with assay accuracy within ±10% of the nominal concentrations of **I**.

The summary of the analyses of QC standards for a clinical trial are presented in Table 3. These data show good inter-assay accuracy and precision. In addition, both low and high QC samples were analyzed after three months of storage at −20°C. Mean values ($n=5$) were 1.04±0.13 and 404.2±8.23 indicating adequate stability of **I** in the biological matrix frozen at −20°C.

Stability of **I** during multiple freeze–thaw cycles was also evaluated by analyzing low and high QC samples. Following three freeze–thaw cycles the mean values ($n=2$) were 0.98±0.16 and 419.8±23.9 for low and high QC samples, respectively.

3.2. Analyses of samples from clinical studies

The performance and ruggedness of the HPLC–MS–MS assay was tested by analyzing more than 1200 plasma samples from a clinical study with **I**. As an example, representative concentrations of **I** in plasma after oral administration of a 2 mg dose of **I** to selected human subjects are shown in Fig. 4.

In conclusion, an assay for **I** in human plasma with the LOQ of 0.5 ng/ml was developed and its performance and ruggedness was tested during multi-sample analyses of samples from clinical studies.

Table 3
Inter-day accuracy and precision data for the assay of quality control samples spiked with **I**

Initial[a] assay concentration (ng/ml)	No. of determinations	Mean calculated concentrations (ng/ml)	C.V. (%)
1.00	16[b]	1.04	5.3
405.8	16[b]	441.18	4.9

[a] $n=5$.
[b] Over a period of eight days.

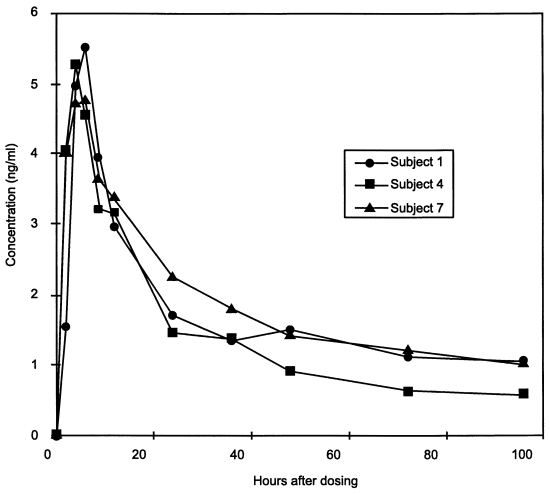

Fig. 4. Concentration of **I** in plasma (ng/ml) of selected human subjects after oral administration of 2 mg of **I**.

Assay selectivity and the absence of matrix effects, were demonstrated, as required when an analog rather than an isotopically labeled parent compound is used as an internal standard.

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