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Low level determination of dorzolamide and its de-ethylated metabolite in human plasma by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry[1]

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Abstract

A sensitive and specific method for the determination of dorzolamide (I) and its de-ethylated metabolite (II) in human plasma has been developed utilizing high pressure liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection. The analytes and internal standard (III) were isolated from the deproteinized pH 8.0 buffered plasma, using a liquid-liquid extraction with a mixture of ethyl acetate, toluene, and isopropanol. The analytes were then back extracted into 0.085% phosphoric acid (200 μl) and after washing the acidic extract with hexane, the organic layer was discarded and a fraction (50 μl) of the acid extract was injected into the LC/MS/MS system. The MS/MS detection was performed on a PE Sciex API III tandem mass spectrometer using a heated nebulizer interface. Multiple reaction monitoring of the parent → product ion combinations of m/z 325 → 199, 297 → 199, and 397 → 306 were used to quantify I, II, and III, respectively. The assay was validated in the concentration ranges of 0.5–100 and 2.5–100 ng ml^{-1} of plasma for I and II, respectively. The precision of the assays, expressed as coefficients of variation (C.V.%), were less than 10% over the entire concentration range, with adequate assay specificity and accuracy. The LC/MS/MS method provided a 10-fold increase in the sensitivity of I over the previously reported HPLC/UV method [1]. © 1997 Published by Elsevier Science B.V.

Keywords: Carbonic anhydrase inhibitors; Topically active; Assay; Plasma; HPLC; MS-MS detection

1. Introduction

Compound I [(−)-(*SS*)-4-ethylamino-5,6-dihydro-6-methyl-7,7-dioxide-4*H*-thieno(2,3-*b*)thiopyran-2-sulfonamide, Dorzolamide, MK-507, Fig. 1], a potent and selective inhibitor of human carbonic anhydrase II, is topically active for reduction of elevated intraocular pressure [2,3]. It

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has been recently approved for the treatment of glaucoma. After topical ocular administration, dorzolamide rapidly reaches the systemic circulation where it distributes preferentially to red blood cells (RBCs). A previously developed HPLC assay with ultraviolet (UV) detection (252 nm) in human whole blood, urine, and plasma had a limit of quantification (LOQ) of 5 ng ml^{-1} for both I and its de-ethylated metabolite II [1]. Because of high binding affinity of I and II to carbonic anhydrase in RBCs, concentrations of I and II at steady-state in plasma were almost three orders of magnitude lower than in whole blood, and were generally below LOQ of the existing assay. Therefore, it was desirable to develop a more sensitive analytical method with a LOQ below 5 ng ml^{-1} to monitor concentrations of I and II in plasma to support human pharmacokinetic studies. Liquid chromatography with atmospheric pressure chemical ionization (APCI) and tandem mass spectrometric (MS/MS) detection was chosen for its demonstrated sensitivity and specificity for many drug analytes. Some recent examples from our laboratories of the application of this methodology to the determination of selected new drug candidates in biological fluids are listed in references [4–7].

All clinical studies were conducted using the single stereoisomer I with the absolute configuration *SS* around the two chiral centers of the molecule. Initially, in order to assess the potential for the in vivo inversion of configuration at one or both chiral centers of I and the stereochemistry and an absolute configuration of the metabolite, chiral methods capable of separation of the four stereoisomers of I and four stereoisomers of II were developed [8,9]. Using these methods it was established that no inversion of configuration was occurring, and only *SS* isomer (I) was present in post-dose biological fluids. In addition, the formation of a metabolite was shown to be stereoselective and the absolute stereochemistry of a metabolite was *SS* (II). Based on these chiral data, non-stereoselective assays were utilized for determination of I and II in biological fluids.

The development of a non-chiral LC/MS/MS assay for I in plasma with an order of magnitude better sensitivity (0.5 ng ml^{-1}) than the previously developed method based on HPLC with UV detection (5 ng ml^{-1}), and the development of an analogous LC/MS/MS method for II with the improved LOQ from 5 to 2.5 ng ml^{-1}, is the subject of this paper. In addition, the detailed comparison of the LC/MS/MS and HPLC/UV methods is provided.

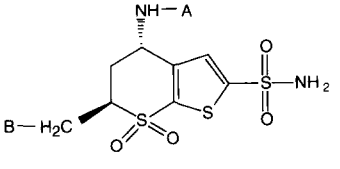
2. Experimental

2.1. Materials

Hexane, acetonitrile, methanol, ethyl acetate, toluene, isopropanol, water and phosphoric acid were all HPLC Optima grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and trichloroacetic acid were all certified grade and were also purchased from Fisher Scientific. Ammonium acetate and trifluoroacetic acid (certified grade) originated from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA), respectively. The drug free control plasma was purchased from Biological Specialities (Lansdale, PA, USA.). The hydrochloride salt of I and the free base of II were synthesized in the Medicinal Chemistry Department of Merck Research Laboratories (West Point, PA, USA).

2.2. Instrumental

A Perkin-Elmer biocompatabile binary pump 250 and ISS 200 autoinjector were used for all HPLC analyses. The chromatographic system



I; A = C_2H_5, B = H
II; A = H, B = H
III; A = C_3H_7 B = $(CH_2)_2OCH_3$

Fig. 1. Chemical structures of dorzolamide (I), its de-ethylated metabolite (II), and internal standard (III).

consisted of Keystone Scientific's (Bellefonte, PA, USA) base deactivated C_{18} Hypersil 20 × 4.6 mm, 5 μm guard column, which was changed daily to insure the peak shape integrity. The analytical column was a base deactivated cyano Hypersil 100 × 3.0 mm, 5 μm. The mobile phase consisted of a 35/65 (v/v, %) mixture of acetonitrile/water containing 10 mM ammonium acetate and 0.1% trifluoracetic acid delivered at a flow rate of 0.6 ml min^{-1}. The mobile phase was filtered through a 0.2 μm Nylon 66 filter (Rainin Instruments, Woburn, MA, USA). The runtime was 5 min. The HPLC autoinjector was set to inject a 50 μl volume of the extract. Mass spectrometric detection was carried out using a PE SCIEX API III triple-quadruple instrument (PE-SCIEX, Thornhill, Toronto, Canada) operating in positive ion APCI with heated nebulizer probe and a corona discharge needle set at +3 μA. The temperature of the heated nebulizer probe was 500°C. Multiple reaction monitoring was employed using argon as the collision gas at a thickness of 320×10^{12} molecules cm^{-2}. The nebulizing gas (nitrogen) pressure and auxiliary flow were set at 80 psi and 2 l min^{-1}, respectively. The curtain gas was nitrogen at 0.9 l min^{-1}. The orifice potential and electron multiplier settings were +55 V and −4.8 kV, respectively. 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 molecular ions $[M+H]^+$ at m/z 325 (I), 297 (II) and 396 (III) via the first quadruple filter (Q_1) with collision induced fragmentation in Q_2, and monitoring, via Q_3, the product ions at m/z 199 for both I and II and 306 for III. The output signal from the mass spectrometer was interfaced to a MacIntosh computer operating RAD and MacQuan software for data collection, peak integration and analysis. Peak area ratios obtained from multiple reaction monitoring of analytes (m/z 325 → 199 for I and m/z 297 → 199 for II) to the internal standard (m/z 396 → 306, III), were utilized for the construction of calibration curves, using weighted ($1/y^2$) linear least square regression of the measured peak area ratios vs. plasma concentrations.

2.3. Standard solutions

A stock standard solutions of I and II (1 mg ml^{-1}) were prepared in methanol. These solutions were then further diluted with methanol to yield a series of working standards with the concentrations of 1.0, 0.5, 0.2, 0.1, 0.05, 0.025, 0.01, 0.0075 and 0.005 μg ml^{-1}. The internal standard (III) stock solution (1 mg ml^{-1}) was also prepared in methanol and was diluted with methanol to 0.1 μg ml^{-1}. The later working standard of III was used for all analysis. Stock standards and working standards of I, II, and III were prepared monthly.

2.4. Sample preparation

The standard curve for I was constructed by spiking 1 ml of blank human plasma with known concentrations of I in the range of 0.5–100.0 ng ml^{-1} plus 10 ng of III. The standard curve for II was in the concentration range of 2.5–100.0 ng ml^{-1} due to lower extraction efficiency and a less sensitive instrumental response. For each sample, 100 μl solutions of the appropriate standards were added, followed by vortexing the tube for 10 s, and precipitation of plasma proteins by the addition of 0.5 ml of 10% trichloroacetic acid and vigorous vortexing for 1 min. The sample was then buffered to pH 8.0 with 5 ml of 0.2 M phosphate buffer followed by liquid-liquid extraction with 10 ml mixture of ethyl acetate:toluene:isopropanol, 50:40:10,v:v:v, respectively. The tubes were capped, rotate-mixed for 20 min and centrifuged for 5 min. The organic layer was transferred to a 15 ml centrifuge tube containing 0.2 ml of 0.085% phosphoric acid and after rotate-mixing for 15 min and centrifugation for 5 min, the organic layer was aspirated and discarded. The acidic layer containing I, II, and III was additionally washed with 2 ml of hexane and after rotate-mixing for 10 min, centrifugation for 5 min, and aspiration and discarding of the hexane layer, 50 μl of the acidic layer was injected into the LC/MS/MS system.

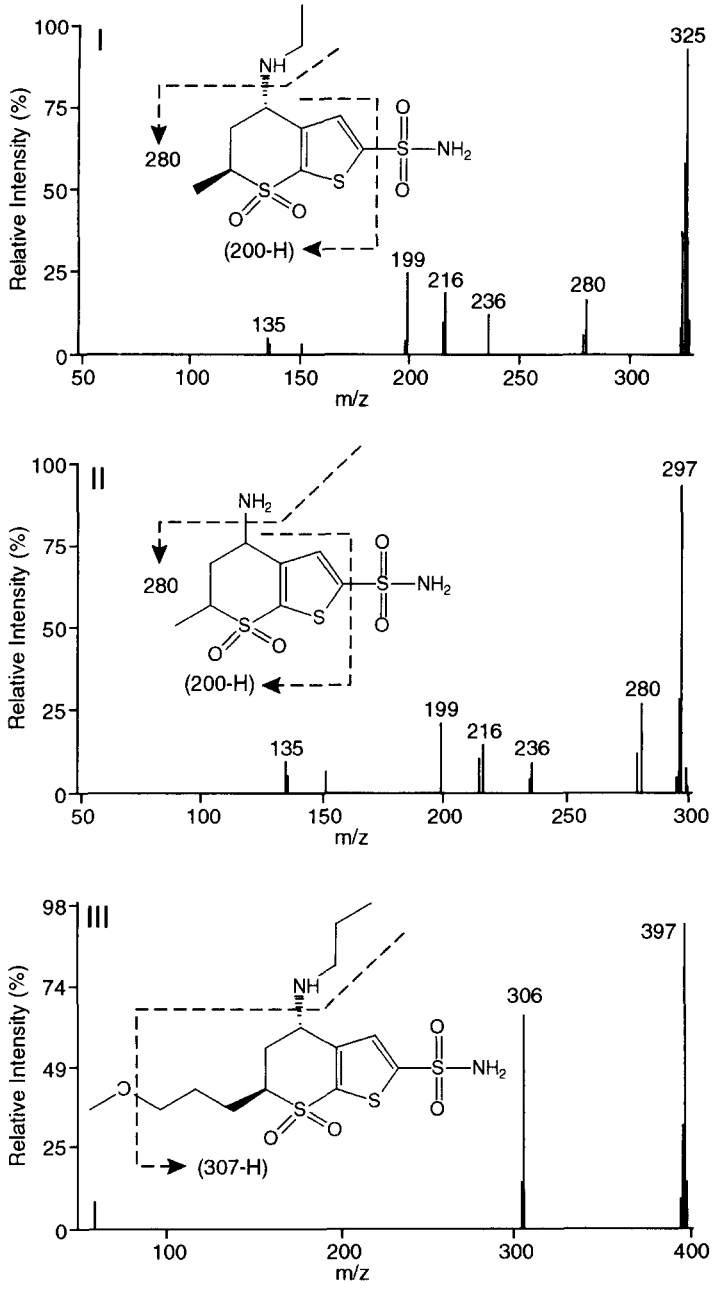


Fig. 2. The product ion mass spectra of the protonated molecules of I, II, and III.

2.5. Precision, accuracy, linearity and recovery

The precision of the assay was determined by the replicate analyses ($n = 5$) of human plasma containing I at concentrations of 0.5, 0.75, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0 and 100.0 ng ml^{-1} and II at concentrations 2.5, 5.0, 10.0, 20.0, 50.0 and 100.0 ng ml^{-1}. Each replicate standard line was constructed by spiking standard solutions of I, II, and III into a different lot of plasma to ensure

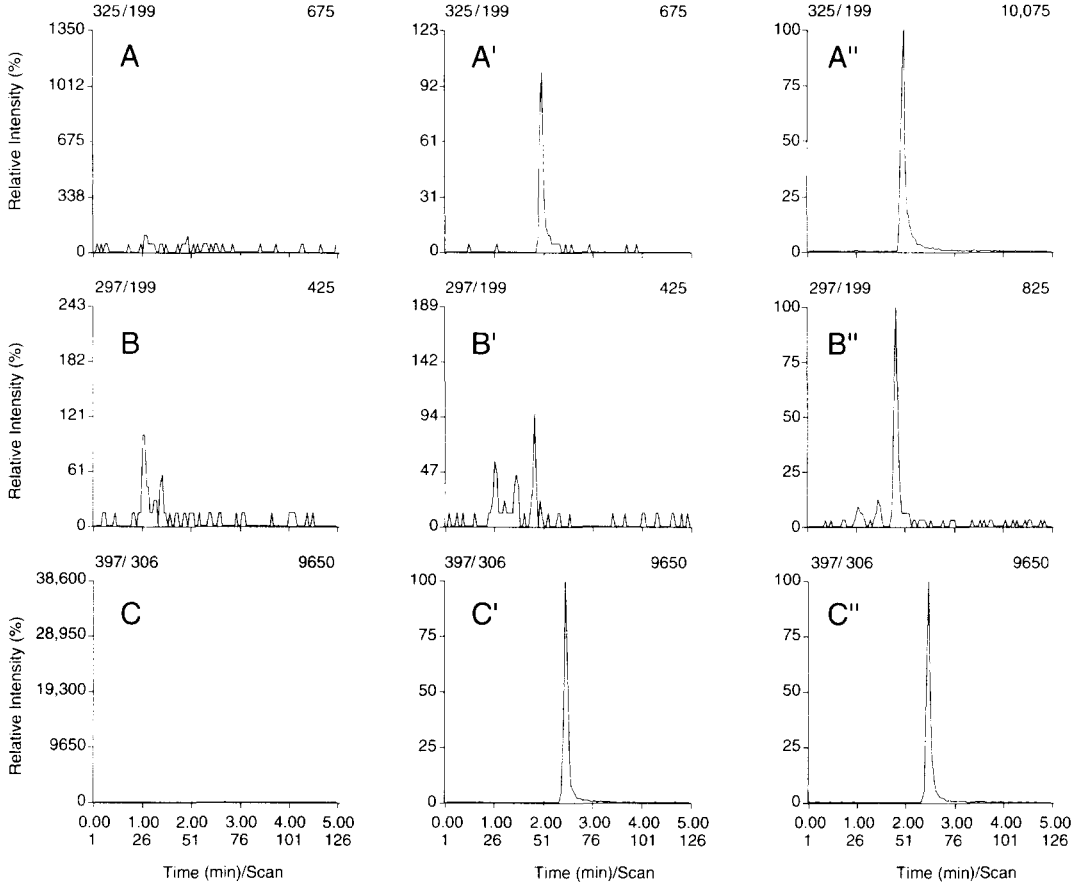


Fig. 3. Representative chromatograms of plasma extracts obtained by multiple reaction monitoring at m/z 325→199 for I (channel A), m/z 297→199 for II (channel B) and m/z 397→306 for III (channel C). Chromatograms A,B,C—extracts of blank human plasma; A',B',C'—extracts of control plasma (1 ml) spiked with 0.75 ng of I, 5 ng of II, and 100 ng of III; A",B",C'—extracts of plasma from human subject following 169 days of dosing with I, spiked with 100 ng of III, concentrations of I and II equivalent to 15.6 and 21.6 ng ml^{-1} of plasma, respectively.

consistent recoveries between plasma samples from different sources. The accuracy of the assays was checked by preparing quality control (QC) samples with known concentrations of 2.0, 7.5 and 75.0 for I, and 7.5 and 75.0 ng ml^{-1} for II. The QC samples were prepared prior to the start of analysis of clinical samples and were kept frozen at −15°C under the same storage conditions as the clinical samples. These QC standards were assayed along with unknown samples each day analyses were performed. The calculated concentrations of the QC samples were compared on a day-to-day basis as an additional check of accuracy, reproducibility, and overall assay performance. The linearity of each standard curve was confirmed by plotting the ratio of the drug to the internal standard peak areas versus drug concentration. A standard curve was prepared and assayed daily with the quality control and the unknown samples. The specificity of the assay was confirmed by analyzing blank and subject's pre-dose plasma samples. No endogenous interferences were detected. The recovery was determined by comparing the peak area of I and II extracted from plasma to that of standards injected directly.

Table 1
Intraday variability of MK-507 (I) spiked plasma; precision and accuracy of replicate analysis ($n = 5$)

Nominal concentration (ng ml^{-1})	Mean[a] concentration (ng ml^{-1})	Precision[b] (C.V.%)	Accuracy[c] (%)
0.50	0.52	12.0	104.0
0.75	0.71	4.4	94.7
1.0	1.06	3.3	106.0
2.5	2.36	7.5	96.0
5.0	4.88	6.0	94.4
10.0	10.68	6.3	97.6
20.0	21.38	2.9	95.7
50.0	47.83	2.9	95.7
100.0	103.16	3.1	103.2

[a]Mean constructions 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 calculated concentration)/(nominal concentration) × 100].

3. Results and discussion

3.1. LC/MS/MS method

Full scan positive ion (Q_1) mass spectra of I, its de-ethylated metabolite II and internal standard III yielded predominately the protonated molecular ions at m/z of 325, 297 and 397, respectively. The product ion mass spectra of these protonated molecules (Fig. 2) indicated the presence of the most intense fragment ions at m/z 199 for both I and II, and m/z 306 for III. The parent → product ion combinations of m/z of 325 → 199, 297 → 199 and 397 → 306 were selected for the quantitation of I, II, and III, respectively.

By monitoring these parent → product ion pairs in the multiple reaction monitoring (MRM) mode, 50 and 100 pg of I and II, respectively, were detected with a signal to noise ratio of 5 to 1. The recoveries of I and II were 70 ± 5 and 35 ± 4%, respectively, and were practically the same between different lots of plasma and over the entire concentration range studied, allowing reliable quantitation of both I and II. The instrumental operating parameters were optimized for sensitive detection of I at 0.5 ng per ml of plasma level, which resulted in a slightly lower instrumental response to II. The lower instrumental response of II coupled with a lower extraction efficiency of II vs. I contributed to the observed difference in the LOQ of I (0.5 ng ml^{-1}) vs. II (2.5 ng ml^{-1}).

Using the procedure described in Section 2, the plasma assay was validated for I and II in the concentration range of 0.5–100 and 2.5–100 ng ml^{-1}, respectively. The typical chromatograms are presented in Fig. 3.

The within-day method precision, expressed as the coefficient of variation (C.V.%), was less than 10% at all concentrations within the standard curve range (Table 1 and Table 2), except for I at 0.5 ng ml^{-1} where the C.V. was 12%. The typical equations describing the standard lines were $y = 0.083966x - 0.009391$ for I and $y = 0.004355x - 0.002533$ for II, with the correlation coefficients of 0.998 and 0.992 for I and II, respectively. The accuracy for both I and II at all points in the standard curve range was 94–108%.

Assay specificity was confirmed by analyses of control and pre-dose plasma samples from subjects participating in clinical studies. Analyses of these samples indicated that no response at the parent → product ion combinations used for monitoring I, II and III was observed. In addition, LC/MS/MS analyses of control plasma spiked separately with I, II, and III confirmed that the MS/MS response was limited to the parent → product ion combinations of a single analyte and no response and 'cross-talk' was observed for the other two analytes.

Inter-day variability, as measured by the concentration of QC standards, was also less than 10% (Table 3 and Table 4).

Table 2
Intraday variability of de-ethylated metabolite (II) of MK-507 spiked in human plasma; precision and accuracy of replicate analysis ($n = 5$)

Nominal concentration (ng ml^{-1})	Mean[a] concentration (ng ml^{-1})	Precision[b] C.V.%	Accuracy[c] (%)
2.5	2.58	9.8	103.0
5.0	4.78	4.6	95.6
10.0	10.81	9.3	108.0
20.0	20.00	4.3	100.0
50.0	47.16	4.6	94.3
100.0	105.78	2.4	105.8

[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 calculated concentration)/(nominal concentration) × 100].

The assay in plasma, as described, was used to analyze over 300 plasma samples from a pharmacokinetics and safety study in patients with impaired renal function, after oral (2 mg solution b.i.d. for 24 weeks) administration of I. The concentrations of I and II in plasma of ten patients were followed at biweekly intervals and for up to 91 days after last dose. Concentrations of I and II varied from 0.0 to 35.8 and 0.0 to 75.7 ng ml^{-1}, for I and II, respectively. In spite of the special care taken in the clinic to avoid any hemolysis during plasma collection, the concentrations of I and II in plasma may have been overestimated due to a not easily detectable hemolysis of whole blood and almost three orders of magnitude higher concentrations of both I and II in whole blood (up to 5 and 15 μg ml^{-1} for I and II, respectively) than in plasma.

3.2. Comparison of LC/MS/MS with HPLC-UV method [1]

The major differences between these two methods are summarized in Table 5.

The LOQ for the assay of I in plasma using LC/MS/MS was 0.5 ng ml^{-1}, which was ten times lower than that using the HPLC/UV method [1]. For the de-ethylated metabolite II, the LOQ using LC/MS/MS method (2.5 ng ml^{-1}) was two times lower than that of HPLC/UV method (5 ng ml^{-1}). Only about 25% of plasma extract was injected into the LC/MS/MS system whereas injection of 66% of the solution after extraction was necessary to achieve the reported LOQs using HPLC/UV method. Although the sample preparation procedure based on liquid-liquid extraction from basified plasma and back extraction into an acid was practically the same between the two methods, the high specificity of MS/MS detection allowed simplification of chromatography and the significant improvement in the speed of analysis from 28 to 5 min per run. Instead of a mobile phase containing an ion-pairing reagent in the HPLC/UV method (25/75, v/v, %, mixture of acetonitrile/water containing 5.5 mM of octanesulfonic acid in 0.085% phosphoric acid) and the necessity of using two analytical HPLC columns [RP-8(250 × 4.6 mm, 5 μ) and RP-18(50 × 4.6 mm, 3 μ)] in series [1], the LC/MS/MS method required only a single column and a conventional reverse-phase mobile phase pumped at a slower flow rate of 0.6 ml min^{-1} as compared with the HPLC/UV method (1 ml min^{-1}). The lower flow rate and more than 5-fold decrease in run time allowed substantial savings in the use and disposal of organic solvents, as observed earlier for other conventional vs. LC/MS/MS methods developed in our laboratories [7].

In conclusion, utilization of tandem mass spectrometric instead of UV detection for the HPLC determination of dorzolamide and its de-ethylated metabolite in human plasma allowed simplification of chromatography and led to a significant improvement in assay sensitivity and speed of analysis.

Table 3
Interday variability for the assay of quality control samples spiked with I

Nominal concentration	Initial mean[a] concentration	Mean[b] concentration	S.D.	C.V.[c] (%)
2.0	1.7	1.9	0.14	7.4
7.5	8.2	7.6	0.21	2.7
75.0	75.8	73.9	0.57	0.8

[a]Initial mean ($n = 5$) assayed concentration.
[b]Mean concentration ($n = 8$) over 4 days of analysis.
[c]Coefficient of variation.

Table 4
Inter-day variability for the assay of quality control samples spiked with II

Nominal concentration	Initial mean[a] concentration	Mean[b] concentration	S.D.	C.V.[c] (%)
7.5	7.9	7.1	0.49	7.1
75.0	82.2	73.1	0.28	0.4

[a]Initial mean ($n = 5$) assayed concentration.
[b]Mean concentration ($n = 8$) over 4 days of analysis.
[c]Coefficient of variation.

Table 5
Comparison between the LC/MS/MS vs. HPLC/UV [1] methods for the determination of I and II in human plasma

	I		II	
	LC/MS/MS	HPLC/UV	LC/MS/MS	HPLC/UV
LOQ (ng ml^{-1})	0.5	5.0	2.5	5.0
Run time (min)	5	28	5	28
Extract injected (%)	25	66	25	66
Number of HPLC columns in series	1	2	1	2
Flow rate (ml min^{-1})	0.6	1.0	0.6	1.0

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