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Determination of a cyclic hexapeptide, a novel antifungal agent, in human plasma by high-performance liquid chromatography with ion spray and turbo ion spray tandem mass spectrometric detection

C.M. Chavez-Eng*, M. Schwartz, M.L. Constanzer, B.K. Matuszewski

Merck & Co., Inc., Merck Research Laboratories, WP42-208, P.O. Box 4, West Point, PA 19486-0004, USA

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Abstract

Methods for the determination of a semi-synthetic cyclic hexapeptide (**I**, MK-0991) in human plasma based on high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) detection using pneumatically assisted electrospray (ion spray, ISP) and turbo ion spray (TISP) interfaces were developed. Drug and internal standard (**II**, an isostere of **I**) were isolated from plasma by solid-phase extraction (SPE). The eluent from SPE was evaporated to dryness, the residue was reconstituted in mobile phase and injected into the HPLC system. The use of ISP, TISP and heated nebulizer (HN) interfaces as sample introduction systems were evaluated and showed that the heated nebulizer was not adequate for analysis due to thermal instability and/or adsorption of **I** and **II** to glass surfaces of the interface. Compounds **I** and **II** were chromatographed on a wide pore (300 Å), 150×4.6 mm C_8 analytical column, and the HPLC flow-rate of 1.2 ml/min was split 1:20 prior to introduction to the ISP or TISP interface of the mass spectrometric system. The MS–MS detection was performed on a PE Sciex API III Plus tandem mass spectrometer operated in selected reaction monitoring mode (SRM). The precursor→product ion combinations of m/z 1093.7→1033.6 and 1094.7→1033.6 were used to quantify **I** and **II**, respectively, after chromatographic separation of the analytes. The assay was validated in the concentration range of 10–1000 ng/ml using ISP, and 2.5–500 ng/ml of plasma using TISP with good precision and adequate accuracy. The effects of HPLC mobile-phase components on the ionization efficiency and sensitivity of detection in the positive ionization mode, the evaluation of the matrix effect, and limitations in sensitivity of detection of **I** due to the formation of multiply charged species are presented. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Echinocandin antimicrobials represent a novel group of anti-*Aspergillus* and anti-*Candida* agents which inhibit the synthesis of 1,3-ϑ-D-glucan, an essential and major cell wall component in those pathogens. Amphotericin B is the only currently available broad-spectrum fungicidal agent for treatment of serious fungal infections, but its use is limited due to inherent toxicity [1]. Compound **I** (MK-0991, Fig. 1A), a water-soluble semi-synthetic cyclic hexapeptide is currently being evaluated as a new therapeutic agent for the treatment of various fungal infections. In vitro and in vivo studies have shown that **I** demonstrates activity against *Candida*

*Corresponding author.

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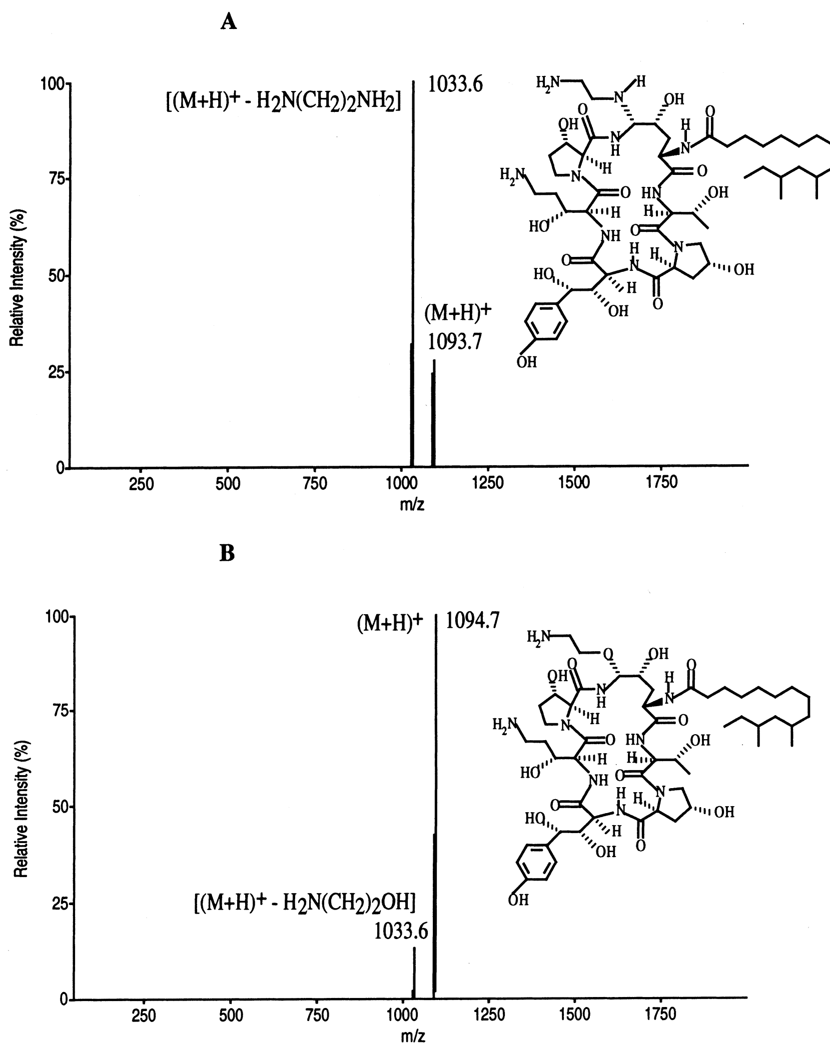

Fig. 1. Chemical structures and positive product ion mass spectra of the protonated molecules of **I** (**A**, *m/z* 1093.7) and internal standard **II** (**B**, *m/z* 1094.7).

spp., *Aspergillus fumigatus*, *Pneumocystis carinii* and *T. glabrata* [2–6].

Analyses of plasma and urine samples from clinical studies with **I** were originally performed using HPLC with fluorescence (FLU) detection [7]. This method has a concentration range of 10–1000 ng/ml of **I** in plasma or urine which was adequate for the quantification of **I** in both matrices after I.V. dosing with the drug. Single- and multiple-dose pharmacokinetics of **I** in man describing the dose regimen, half-life, degree of accumulation and time to steady state has been reported [8]. Concentrations of **I** in plasma of subjects dosed with 5 mg I.V. were measurable up to 48 h after dosing. However, it was necessary to develop an alternative method based on HPLC–MS–MS to confirm the selectivity of the HPLC–FLU method and to support studies with potentially lower doses of **I**. In addition, it was of interest to explore the applicability of the MS–MS technique for quantitative determination of small

peptides capable of forming multiply charged ions in the ionization region of the mass spectrometer. Formation of these ions may, in general, limit the sensitivity of quantitative determination of these molecules.

HPLC together with mass spectrometry (MS) has been used extensively for the characterization of peptides that are endogenous to a biological tissue or extracted from complex biological fluids [9–14]. Generally, chromatographic procedures were used for the separation or purification of each peptide, while the appropriate MS techniques were used to determine the m/z ratio of the protonated molecule, $(M+H)^+$, and the structurally related product ions of the targeted peptide. Methods for quantification or peptide mapping, based primarily on HPLC with MS detection, have been developed using HN interface [12] or by electrospray ionization [13,14]. The utilization of an HN interface is generally limited due to the thermal instability of peptides in the HN probe operating at 350–500°C required for the rapid desolvation of the nebulized droplets. Only selected, modified, small peptides that are thermally stable and volatile at these temperatures can be analyzed using the HN interface. As an example, a method for determination of a thermally stable tripeptide using HN-HPLC–MS was developed with the LOQ of 50 pg/ml [12].

For the majority of thermally labile, more hydrophilic peptides with molecular weights (MW) larger than 1000, electrospray interface (ESI) was utilized. In these cases, an extensive sample pretreatment and clean-up was required together with the post-column addition of an organic 'makeup' mixture prior to electrospray ionization [13,14]. The post-column addition of an organic solvent or an organic solvent mixture (i.e., propionic acid-2-propanol) increased volatility of the mobile phase, improved droplet desolvation, and minimized the signal suppressing effect of strong acid modifiers (trifluoroacetic acid, TFA) present in the mobile phase [14]. All of these methods have a number of limitations including the dilution of analytes after post-column addition of organic solvents [14] and the need to use smaller diameter columns that are generally not robust and rugged for routine assays in biofluids. In addition, these methods use a single MS rather than tandem MS–MS mode for detection of deprotonated [12] or protonated [13] molecular ions of the analytes, requiring an effective chromatographic separation of analytes from endogenous matrix interferences. Because of all these limitations, for example, the LOQ for the determination of a peptide with the MW of 671 was only 2 ng/ml and required processing of 8 ml of plasma [13]. Another major limitation in the use of MS detection for the analysis of peptides is the potential for formation of multiply charged ions, effectively decreasing the sensitivity of detection both in MS and MS–MS modes. The proper selection of the mobile phase and the type of the MS interface (HN, ISP, TISP) may greatly affect the efficiency of ionization and the formation of singly versus multiply charged ions that may be critical to the successful development of sensitive assays for peptides and/or other molecules with multiple sites of ionization.

The evaluation of the effect of the mobile phase composition and the selection of the MS interface for the sensitive determination of **I** in human plasma are the subject of this paper. The matrix effect and the limitations in using HPLC–MS–MS for the quantitative determination of small peptides are discussed and a comparison between the HPLC–FLU and HPLC–MS–MS methods is presented.

2. Experimental

2.1. Materials and methods

Compound **I** (Fig. 1A) and internal standard, (I.S., **II,** Fig. 1B) were synthesized at Merck Research Laboratories (Rahway, NJ, USA) as diacetate and dihydrochloride salts, respectively. Ammonium acetate (AA), was purchased from Sigma (St. Louis, MO, USA). Sequenal grade triethylamine (TEA) and TFA were supplied by Pierce (Rockford, USA). Formic acid (FA) was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

One molar potassium acetate buffer (assay buffer) was adjusted to pH 4.9 with glacial acetic acid.

Diol solid-phase extraction columns (3 ml) were obtained from Baker (Phillipsburg, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer 250 Biocompatible Binary pump, a WISP 715 Auto-injector (Waters-Millipore, Milford, MA, USA), and an API III Plus triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a HN, an ISP, or a TISP interface.

2.3. Chromatographic conditions

The aqueous portion of the mobile phase was prepared by the addition of 676 μl TFA to 1000 ml of water. The pH of the aqueous portion of the mobile phase was adjusted to 3.0 with ammonium hydroxide. The mobile phase consisted of 65% of the aqueous portion and 35% of acetonitrile and was pumped at a flow-rate of 1.2 ml/min. The eluent from the column was split 1:20 prior to introduction to the ISP or TISP interface of the mass spectrometer. Chromatography was performed on a Zorbax SB C_8 150×4.6 mm, 5 μm wide bore (300 Å) column. The total runtime was 11.5 min with **I** eluting at 5.5 min and I.S. at 6.8 min after injection. A column wash step consisting of pumping at 1.2 ml/min a mixture of 90% acetonitrile and 10% of the aqueous portion of the mobile phase for 0.9 min, 5.5 min after injection, was utilized. The column was reequilibrated with the mobile phase for 5.0 min after the column wash.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the HPLC system via a HN, ISP or TISP interface. The HN probe was held at 350–500°C. The nebulizing air pressure and auxiliary flow-rates were 80 p.s.i. and 2 ml/min, respectively. Gas-phase chemical ionization occurred via a corona discharge needle operating at +4 μA. For the ISP or TISP interface, nebulizer (air) pressure was set at 60 p.s.i. and curtain gas (N_2) flow at 1.2 l/min. Turbo ion spray temperature was set at 500°C. Positive chemical ionization was effected by high voltage (+4800 V) applied to the electrode of the ion sprayer. The sampling orifice potential was set at +85 and +55 V for ISP and TISP methods, respectively. The first quadrupole, Q1, was set to monitor the protonated molecules $(M+H)^+$ at m/z 1093.7 for **I** and m/z 1094.7 for I.S. (**II**) with collision-induced fragmentation at Q2 (collision gas argon, 275×10^{13} atoms cm^{-2}), and monitoring the product ions via Q3 at m/z 1033.6 for both **I** and **II**. Collision energy was 16.6 V ($R1$=26.6 V, $R2$=10.0 V). The electron multiplier setting was −4.2 kV and detector electronics were set to counts of 10. Dwell time was 400 ms.

2.5. Data acquisition and analysis

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-square regression of the calibration line constructed from the peak area ratios of drug to I.S. versus drug concentration.

2.6. Standard preparation

A standard stock solution of **I** (1.0 mg free base/ml) was prepared in 35:65 acetonitrile–0.1% TFA (pH adjusted to 3.0 with TEA). Subsequent dilutions were made in mobile phase to give the following concentrations: 100, 50, 25, 10, 5, 2.5, 1 μg/ml for the ISP-MS–MS method; and 50, 25, 10, 5, 2.5, 1.0, 0.5, 0.25 μg/ml for the TISP method. A standard stock solution of **II** was prepared as 1.0 mg free base/ml in dimethylsulfoxide. Subsequent dilutions were made with 35:65 acetonitrile–0.1% TFA (pH adjusted to 3.0 with TEA) to prepare 50 μg/ml working solution of I.S.

The plasma standard line was constructed using a series of control human plasma (1 ml) spiked with working standards of **I** and **II** (10 μl each) and assay buffer (250 μl, pH 4.9).

Quality control (QC) samples at 12.5, 25.0, 375, and 750 ng/ml were prepared in control plasma. Aliquots (1.25 ml) of these solutions were transferred to 2-ml plastic tubes, stored at −70°C, and analyzed to determine the freeze–thaw and long-term storage stability of **I** in plasma.

2.7. Sample preparation

Sample preparation was practically the same as described using the HPLC–FLU method [7]. Briefly, to 1 ml of plasma in a polypropylene tube, 10 μl of working I.S. solution, and 0.25 ml of 1 *M* potassium acetate buffer were added, mixed and loaded onto a pre-conditioned diol SPE column. After washing the cartridge with water and methanol, **I** and **II** were eluted using 1 ml of solution containing 0.25 *M* ammonium hydroxide and 0.1% TFA in methanol. The eluate was evaporated to dryness using a Turbo Vap evaporator at water bath temperature of 50°C. The residue was reconstituted with 100 μl mobile phase, and 50 μl (for ISP-MS–MS method) and 75 μl (for TISP-MS–MS method) were injected into the LC–MS–MS system.

3. Results and discussion

3.1. Mass spectra and the choice of the MS–MS interface, the HPLC mobile phase, and MS–MS detection conditions

Optimization of the mobile phases and mass spectrometer's acquisition parameters was performed by infusing solutions of **I** in different solvents via the ISP interface. Similar optimization procedure was initially attempted using the HN interface by flow injection of concentrated solutions (10 μg/ml) of **I** in different solvent mixtures directly into the ionization region of the mass spectrometer. Full mass spectrum (Q1) of **I** did not indicate the presence of the $(M+H)^+$ ion at *m/z* 1093.7 or a doubly charged ion $(M+2H)^{+2}$ at *m/z* 547.3. Lowering the temperature of the HN interface from 500 to 300°C or varying orifice potentials gave practically the same spectra and the formation of the singly or doubly charged protonated molecular ions of **I** was not observed. The lack of formation of these ions was probably due to the thermal instability of **I** during passage through the HN interface or the inefficiency of ionization under the corona discharge conditions utilized. Contrary to the HN interface, both mono- and doubly charged protonated molecular ions of **I** at *m/z* 1093.7 and 547.3 were observed when the ISP interface was utilized (Fig. 2).

The absolute intensity and the ratio of intensity of the singly and doubly charged protonated molecular ions of **I** changed significantly with the change in the composition of the mobile phase (Table 1). The highest $(M+H)^+$ intensities and the most favorable ionization leading to the formation of predominantly a singly charged species at *m/z* 1093.7 was observed when mobile phase consisted of a mixture of acetonitrile with 0.1% TFA (pH adjusted to 3.0) (Table 1, entry 2), or a mixture of acetonitrile with 0.1% TFA containing 10 m*M* AA (pH 3) (Table 1, entry 3). The mobile phase consisting of a mixture of acetonitrile with 0.1% TFA (pH adjusted to 3.0) was chosen for the development of an assay. Using this mobile phase, and under the HPLC conditions described in Section 2, symmetrical peaks necessary for quantification of analytes at low concentrations were obtained. The additional column washout step included in the chromatographic separation of analytes was necessary to elute from the column late eluting interferences from plasma and to regenerate the column after each injection. This washout step increased considerably the time of analyses, but was needed to preserve the required good peak shape of analytes and to prevent the decrease in the retention time of analytes during the course of analysis.

The product ion mass spectra of the protonated molecules of **I** at *m/z* 1093.7 and **II** at *m/z* 1094.7 indicated the presence of a common intense fragment ion at *m/z* 1033.6 that was probably formed by the loss of 1,2-diaminoethane and 2-aminoethanol from the protonated molecules of **I** and **II**, respectively (Fig. 1). Similar product ion mass spectra of the doubly charged species of **I** at *m/z* 547.3 showed the presence of smaller fragment ions at *m/z* 131, 392 and 538 of low intensities that were of no practical value for the sensitive detection of the analytes. On the other hand, the presence of the intense product ion at *m/z* 1033.6 originating from the protonated molecules of **I** and **II** allowed sensitive detection of **I** and **II** in SRM mode using the precursor→product ion combinations of *m/z* 1093.7→1033.6 for the drug and *m/z* 1094.7→1033.6 for the internal standard **II**. Since the *m/z* values of the precursor ions for **I** and **II** were different by only one mass unit and the same product ion for both analytes was monitored (*m/z* 1033.6), chromatographic separation of **I** and **II** was necessary to avoid any 'cross-talk' effect

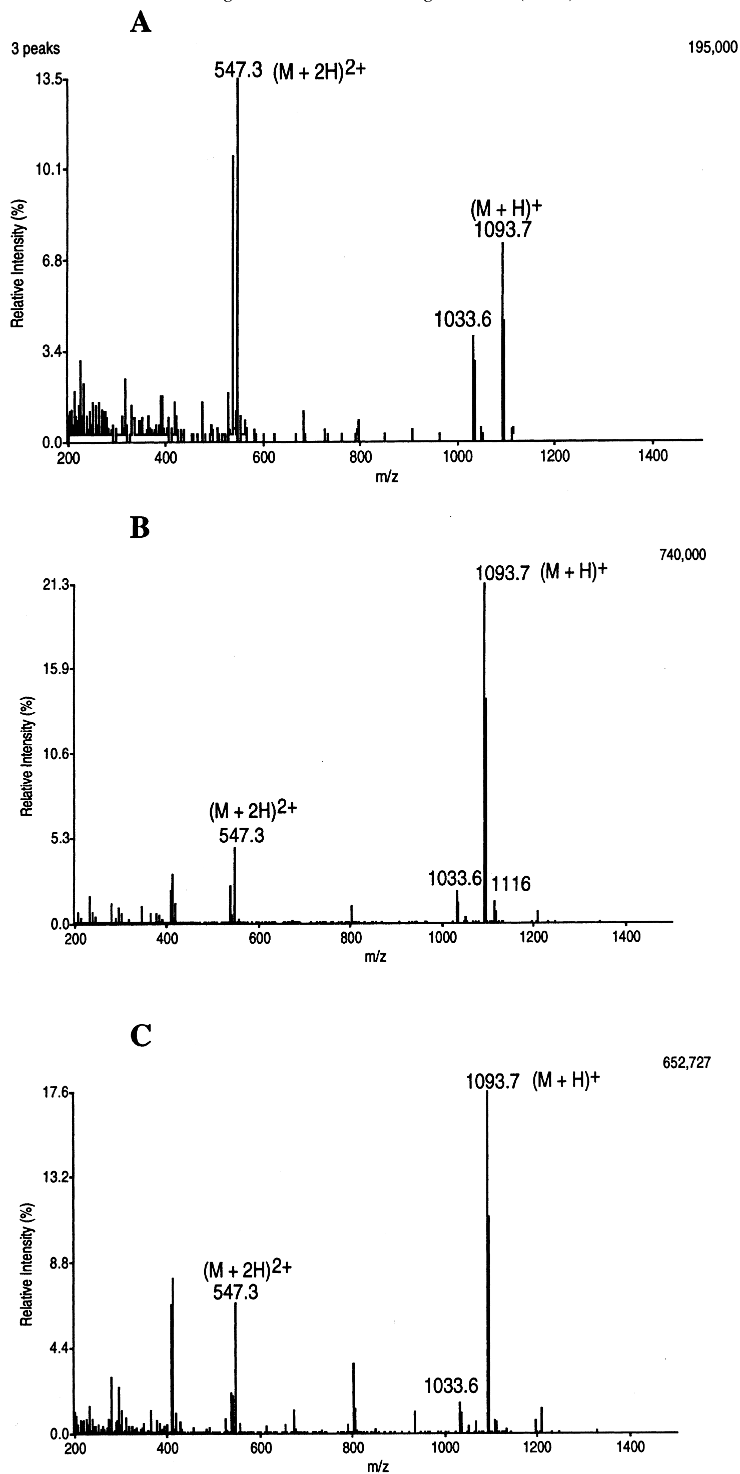

Fig. 2. Mass spectra (Q1) of **I** in different mobile phases obtained using ISP interface; 10 μl/min infusion of 10 μg/ml solution of **I** : (A) in 35:65 acetonitrile–water; (B) in 35:65 acetonitrile–0.1% TFA (pH adjusted to 3.0); (C) in 35:65 acetonitrile–0.1% TFA +10 m*M* AA. The numbers in the upper right hand corner represent peak heights expressed in arbitrary units.

Table 1
Intensities and intensity ratios of singly to doubly charged ions of **I** in various mobile phases obtained using ion spray interface[a]

(Entry no.) mobile phase[b]	Ion counts[c]		Ratio
	$(M+H)^+$ 1093.7 (A)	$(M+2H)^{+2}$ 547.3 (B)	(A/B)
(1) Acetonitrile–water	170 000	290 000	0.59
(2) Acetonitrile–water with 0.1% TFA (pH 3 adjusted with ammonium hydroxide)	840 000	210 000	4.00
(3) Acetonitrile–water with 0.1% TFA containing 10 m*M* AA, pH 3	840 000	350 000	2.40

[a] Solution of **I** (10.0 μg/ml) was infused at 10 μl/min, orifice potential and curtain gas flow were optimized for each mobile phase studied.
[b] The ratio of the content of the organic phase to aqueous phase was 35:65 (v/v).
[c] In arbitrary units.

Table 2
Precision and accuracy of the ISP-HPLC–MS–MS assay of **I** in human plasma

Nominal conc. (ng/ml)	Precision[a] C.V. (%)	Accuracy (%)[b]
Standards:		
10	9.9	101.0
25	4.6	100.0
50	5.2	98.0
100	1.7	101.9
250	1.0	99.7
500	2.4	101.2
1000	2.3	99.9
Quality controls:		
25	4.3	95.6
750	0.8	99.9

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

between the MS–MS channels used for the detection of analytes.

3.2. Assay validation

Isolation of **I** and **II** from plasma was based on solid-phase extraction and the recovery of **I** was better than 90% at all concentrations within the calibration range. The method was validated in the concentration range of 10–1000 ng/ml using ISP interface and in the range 2.5–500 ng/ml using TISP interface with adequate assay precision and accuracy (Tables 2 and 3, respectively).

Representative chromatograms are presented in Fig. 3.

The LOQ of the TISP-HPLC-MS–MS method was lower than the LOQ of the ISP-HPLC–MS–MS method mostly due to the larger fraction of final extract injected on column (50% in the ISP- and 75% in the TISP-MS–MS assay). Although the LOQ of the TISP-HPLC–MS–MS method was lower than the LOQ of the ISP-HPLC–MS–MS, the latter assay had much better precision and accuracy than TISP method and seemed to be preferable for the long-term use in support of clinical programs.

The relatively modest sensitivity of the HPLC–MS–MS methods (LOQ≥2.5 ng/ml) for the determination of **I** was mostly due to the formation of doubly charged species. The formation of doubly charged species led to the overall decrease in concentration of singly charged protonated molecular ions of **I** used as precursor ions in the SRM mode. In addition, due to thermal instability of the analytes, there was a need of utilization of ISP or TISP interfaces for determination of **I** instead of the HN interface. In the case of ISP or TISP interfaces, analytical columns with small diameters and lower mobile-phase flow-rates may be utilized, but the

Table 3
Precision and accuracy of the TISP-HPLC–MS–MS assay of **I** in human plasma

Nominal conc. (ng/ml)	Precision[a] C.V. (%)	Accuracy (%)[b]
Standards:		
2.5	11.7	104.0
5.0	6.0	100.0
10.0	9.0	96.0
25.0	9.9	100.8
50.0	4.7	93.2
100.0	7.0	100.7
250.0	9.2	106.2
500.0	7.1	113.0
Quality controls:		
12.5	3.8	91.2
375.0	3.0	104.8

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

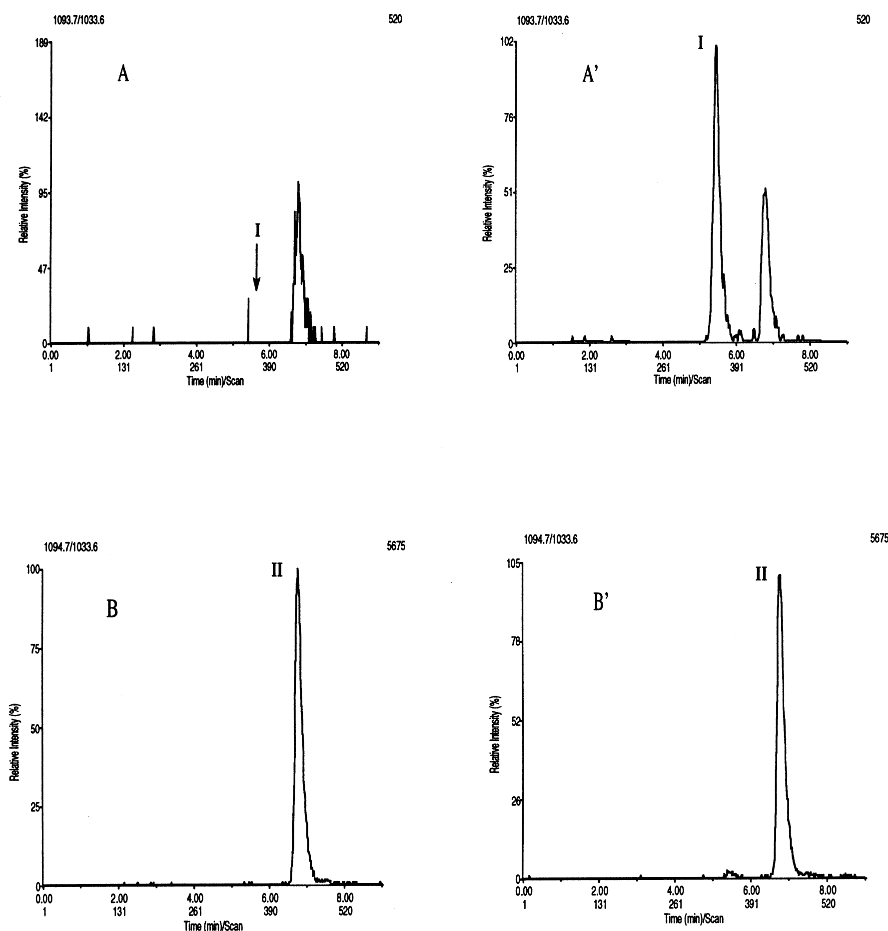

Fig. 3. Representative ISP-HPLC–MS–MS chromatograms of the plasma extracts obtained by multiple reaction monitoring at m/z 1093.7→1033.6 for **I**, channel 'A', and m/z 1094.7→1033.6 for internal standard **II**, channel 'B'. Chromatograms A and B: control plasma spiked with 500 ng/ml **II** monitored at channels 'A' and 'B', respectively. Chromatograms A′ and B′: control plasma spiked with 10 ng/ml of **I** and 500 ng/ml of **II** monitored at channels 'A' and 'B', respectively. The numbers in upper right hand corner of chromatograms correspond to the peak heights expressed in arbitrary units.

overall method sensitivity is not necessarily improved under these conditions. Columns with small diameters are not robust enough for large number of injections of biological extracts and the volume of the extract injected is also limited.

The example of **I** is illustrative of the potential limitations of the HPLC–MS–MS methods for sensitive determinations of analytes possessing multiple sites of ionization and requiring MS analysis using ISP or TISP instead of the HN interface.

3.3. Assessment of the matrix effect.

The matrix effect and ion suppression can seriously affect the reliability of quantitative determination of analytes using HPLC–MS–MS especially when ISP or TISP interfaces are utilized [15,16]. In order to assess the matrix effect during determination of **I**, several experiments were performed in which absolute peak areas of both **I** and **II** extracted from five different plasma lots originating from different in-

Table 4
Peak areas and coefficient of variations (C.V.s) of peak area ratios obtained after spiking and extraction of **I** and **II** from five different lots of control human plasma using ISP and TISP interfaces[a]

Conc. (ng/ml)	ISP					TISP				
	Mean[b]		C.V.%[c]		C.V.%[c]	Mean[b]		C.V.%[c]		C.V.%[c]
	I	**II**	**I**	**II**	Ratio **I/II**	**I**	**II**	**I**	**II**	Ratio **I/II**
2.5	—	—	—	—	—	1636	172 983	17.1	11.0	11.7
5.0	—	—	—	—	—	4067	175 088	14.3	8.9	6.0
10	5234	72 902	14.3	7.7	9.9	9278	191 280	3.0	9.2	9.0
25	16 369	77 088	6.9	6.1	4.6	24 889	183 234	14.2	11.2	9.9
50	34 566	78 640	5.7	4.4	5.2	46 447	182 588	14.9	16.3	4.7
100	72 016	76 687	3.5	4.3	1.7	91 998	165 460	11.2	9.6	7.0
250	181 417	77 769	4.9	4.6	1.0	251 863	171 265	12.4	11.6	9.2
500	352 378	74 003	1.8	2.2	2.4	567 772	179 783	18.0	13.4	7.1
1000	704 422	74 727	2.6	2.6	2.3	—	—	—	—	—

[a] Fractions of final extracts injected on column were 50 and 75% for ISP and TI SP-HPLC–MS–MS methods, respectively.
[b] Peak areas in arbitrary units.
[c] Expressed as coefficient of variation (C.V., %); $n=5$.

dividuals were examined. The data presented in Table 4 indicated that both the recovery of analytes and ion suppression, if any, were practically the same between different lots of plasma, and matrix effect was not observed. Higher variabilities in peak areas of **I** and **II** and the peak area ratios were observed using TISP interface. Since sample preparation was the same for both TISP- and ISP-HPLC–MS–MS methods, the higher variability observed using TISP interface was not due to the differences in recovery between different lots of plasma but rather due to thermal instability of the analytes and/or possibly some matrix effect during TISP ionization.

The data in Table 4 also indicated that compound **II** used as internal standard partially compensated for the variability of peak areas of **I,** and the precision of determination of the peak area ratios of **I**/**II** was much better than the precision of the determination of individual peak areas of **I** and **II**. When matrix effect is present, this compensatory effect of the internal standard may be decreased or even absent when an analog rather than isotopically labeled internal standard is utilized, since the degree of ion suppression in various lots of biofluids may be different between an analyte and an analog used as an internal standard. As the data in Table 4 indicated, this was not the case in the assay presented in this paper and the choice of **II** as internal standard and of the extraction and chromatographic conditions utilized were adequate for reliable determination of **I**.

3.4. Comparison of ISP-HPLC–MS–MS and HPLC/FLU assays

The sensitivity and sample throughput of the HPLC-MS–MS method was very similar to the HPLC–FLU assay developed earlier since both sample preparation procedure and chromatographic run times (11.5 min for MS–MS method versus 10.5 min for FLU assay) were practically the same. A relatively modest improvement in the LOQ of the ISP/TISP-HPLC–MS–MS method (10–2.5 ng/ml) versus HPLC/FLU (LOQ=10 ng/ml) was mostly due to the increase in the fraction of the final extract injected on column from 33% in the HPLC–FLU method to 50% in the ISP- and 75% in the TISP-MS–MS assay.

In addition, the comparison between the HPLC–ISP-MS–MS and HPLC–FLU method was made using selected post-dose samples of a subject dosed with **I**. In this case, when subject's samples were

Table 5
Concentrations of **I** in plasma samples of a subject after 70 mg I.V. administration of **I** determined by HPLC–FLU and ISP-HPLC–MS–MS methods

HPLC–FLU (μg/ml) **A**	ISP-HPLC–MS–MS (μg/ml) **B**	% Difference (**A**–**B**)/**A**×100
4.12	4.32	−4.8
4.65	4.53	+2.6
3.88	3.88	0.0

analyzed using HPLC–FLU method, an unusually high concentrations of **I** were originally observed. In order to confirm these results, the same subject's samples were analyzed using the HPLC–ISP-MS–MS method. The results obtained were very similar (Table 5) indicating the good correlation and good selectivity between the two assays.

In conclusion, methods for the determination of **I** in human plasma in the concentration range of 10–1000 ng/ml, using ISP-HPLC–MS–MS, and 2.5–500 ng/ml, using TISP-HPLC–MS–MS methodology, have been developed. The matrix effect was not encountered during the analysis of plasma samples originating from a number of different subjects. The sensitivity and sample throughput of the HPLC–MS–MS methods was shown to be similar to the earlier developed method based on HPLC with fluorescence detection. Relatively modest sensitivity of determination by HPLC–MS–MS of peptides and other molecules with multiple sites of ionization (LOQ≥1 ng/ml) in comparison with the majority of drugs (LOQ≤1 ng/ml) may be partially due to the formation of multiply charged species effectively decreasing the intensity of molecular ions used for quantification.

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