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Determination of a substance P antagonist in human plasma and urine using high-performance liquid chromatography with ultraviolet absorbance and tandem mass spectrometric detection[1]

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Abstract

A high-performance liquid chromatographic (HPLC) assay using ultraviolet (UV) detection was developed and compared with a HPLC method with tandem mass spectrometric (HPLC/MS–MS) detection for the determination of a substance P receptor antagonist 2(*S*)-((3,5-bis(trifluoromethyl)benzyl)-oxy)-3(*S*)-phenyl-4-((3-oxo-1,2,4-triazol-5-yl)methyl)morpholine (Fig. 1, **Ia**, L-742 694) in human plasma and urine. The drug was isolated from the biological matrix through liquid–liquid extraction. In the HPLC/UV method, the samples were initially injected onto a cyano Hypersil column, and the chromatographic region containing the peaks of interest was heart-cut onto an analytical C-18 Hypersil column via a column switching device. The analyte was quantified by monitoring absorbance at 205 nm. The limit of quantification for **I** extracted from 1 ml of plasma or urine was 2.5 ng ml^{-1}, and the assays were validated in the concentration range 2.5–500 ng ml^{-1}. The HPLC/MS–MS method was validated in the concentration range 0.2–500 ng ml^{-1}. Both assays provided data with precision, measured as coefficient of variation, better than 10% at all points within the standard curve range and with adequate accuracy. © 1997 Published by Elsevier Science B.V.

Keywords: Absorbance; Biological fluids; Column switching; High-performance liquid chromatography assay; Tandem mass spectrometric detection

1. Introduction

Compound **I**, 2(*S*) - ((3,5 - bis(trifluoromethyl) - benzyl)-oxy)-3(*S*)-phenyl-4-((3-oxo-1,2,4-triazol-5-yl)methyl)morpholine (Fig. 1, L-742 694) is a non-peptide substance P receptor antagonist which selectively acts at NK-1 cell-surface receptors [1]. This compound is being evaluated for the treatment of conditions such as migraine

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headaches and postoperative pain. Compound **I** contains two chiral centers and is one of the four stereoisomers with the absolute configuration *S* at both chiral centers. Initially, in order to assess the potential for an in vivo inversion of configuration at one or both chiral centers of **I**, a method for the chiral separation of four stereoisomers of **I** was developed [2]. It was established using this method that no inversion of configuration was occurring, and only a single isomer was present in postdose plasma samples of dogs after dosing with **I**. Based on the absence of an in vivo inversion of configuration around both chiral centers of **I**, a non-stereoselective and highly sensitive assay in plasma and urine was developed to support pharmacokinetic studies with **I**.

Recent advances in interfacing technology have led to the successful combination of mass spectrometry (MS) and liquid chromatography, giving an alternative highly sensitive and selective high-performance liquid chromatography (HPLC) detector. Compared to absorbance detection, MS is generally more sensitive, and assay selectivity may be improved through the use of tandem mass spectrometry (MS–MS). Using atmospheric pressure chemical ionization (APCI) and MS–MS detection, a number of sensitive and selective analytical methods for various drug candidates in biological fluids have been developed [3–11]. In selected cases, a direct comparison between the sensitivity of the determination of various molecules using conventional methods of detection and tandem mass spectrometric detection was made [8–10]. In these examples, the limit of quantification (LOQ) using tandem mass spectrometric detection was generally improved by a factor of five with the concurrent decrease in the sample preparation time and the chromatographic run time. The development of two separate methods for **I** based on HPLC with ultraviolet (UV) absorbance and HPLC with MS–MS detection and the comparison of assay performance using these two methods is the subject of this paper.

2. Experimental

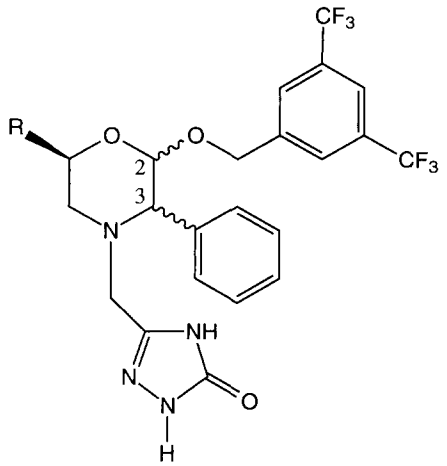
2.1. Materials

Compound **I** and an internal standard **II** (Fig. 1) were synthesized at the Merck Research Laboratories (Rahway, NJ). All solvents were HPLC grade (Fisher, Fair Lawn, NJ). Heparinized human control plasma was obtained from Sera-Tec Biologicals (North Brunswick, NJ). Water was deionized using a Milli-Q reagent water system (Millipore, Milford, MA) resulting in 18 MΩ conductivity.

2.2. Instrumentation

2.2.1. HPLC/UV method

The HPLC system (Fig. 2) consisted of a series 410 LC pump (pump 1) (Perkin–Elmer, Norwalk, CT), a model 6000A isocratic pump (pump 2) (Waters, Milford, MA), a model WISP 715 autosampler (Waters), and a model 785A absorbance detector (Applied Biosystems, Ramsey, NJ). The eluent from the analytical column (column 2) was directed into the absorbance de-



I, R=H
II, R=CH_3

Fig. 1. Chemical structures of L-742 694 (**I**) and internal standard (**II**).

tector and then to waste. A six-port column-switching valve (Valco, Houston, TX) was employed to divert the eluent from column 1 to waste or onto column 2. The switching valve was controlled via time event programming on pump 1. The times at which the events on pump 1 were set to trigger the valve were determined prior to analysis by injecting a standard solution of **I** and monitoring the retention time after elution from column 1. The timed events were then set to place the valve in position A at the beginning of the run, switch the valve to position B at 1.6 min, and return the valve to position A at 2.0 min. The chromatographic data were acquired and analyzed with an automated laboratory system (PE/Nelson Access*Chrom V 1.7, Cupertino, CA). Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted linear regression of the standard line. The standard line was constructed by plotting peak heights of **I** versus drug concentration. All calculations were performed using PE Nelson Access*Chrom software.

The mobile phase for pump 1 and 2 was composed of a 40/60 and a 50/50 (v/v) mixture of acetonitrile (ACN) and 10 mM phosphate buffer (pH 7), respectively. The mobile phases were filtered through a nylon filter (0.2 μm) and each was delivered at a flow rate of 1 ml min^{-1}. Column 1 was a Cyano BDS Hypersil column (50 × 4.6 mm, 3 μm) and was protected with a Cyano BDS Hypersil guard column. Column 2 was a C-18 BDS Hypersil column (150 × 4.6 mm, 5 μm) preceded by a C-18 BDS Hypersil guard column. All columns were purchased from Keystone Scientific (Bellefonte, PA)

2.2.2. HPLC/MS–MS

Liquid chromatography with tandem mass spectrometric detection was performed using a Sciex model API III triple quadrupole mass spectrometer (Thornhill, Ontario, Canada). The liquid chromatograph included a model ISS-200 autoinjector (Perkin–Elmer, Norwalk, CT) and a model 250 HPLC pump (Perkin–Elmer) which delivered a mobile phase consisting of a 55/45 mixture of ACN/0.1% formic acid, 10 mM ammonium acetate at a flow rate of 1 ml min^{-1}. A C-8 BDS

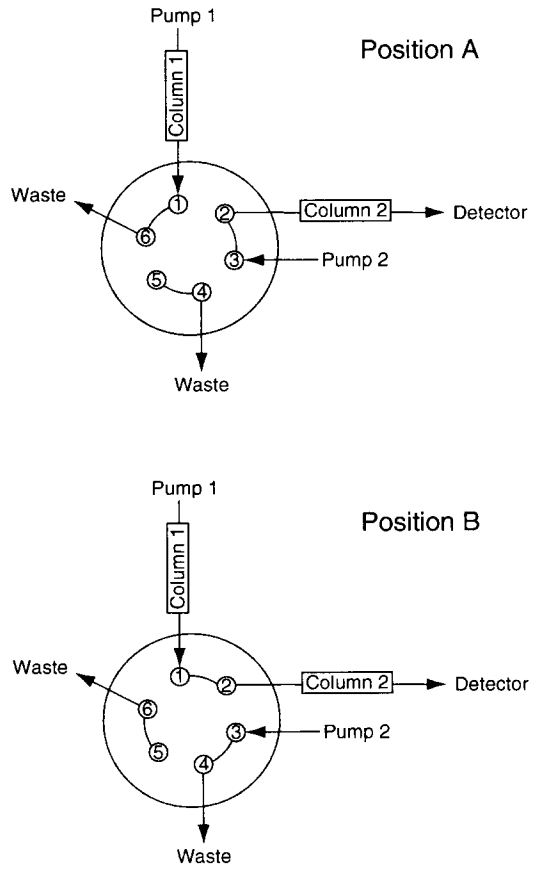


Fig. 2. HPLC system and the electronic switching valve positions used in the HPLC/UV assay.

Hypersil column (50 × 4.6 mm, 5 μm) was used as an analytical column and was protected with a C-8 BDS Hypersil guard column. Under these conditions the drug and the internal standard eluted at 2.1 and 2.5 min, respectively.

The HPLC system was interfaced to a mass spectrometer via a Sciex heated nebulizer probe held at 500°C. The nebulizing air pressure and auxiliary flow rate were 80 psi and 2 l min^{-1}, respectively. Gas-phase chemical ionization occurred via a corona discharge needle operating at 3 μA and positive ions were passed into the quadrupole mass analyzer via a 0.0045 inch aperture. The protonated molecules $(M + H)^+$ of the drug ($m/z = 503$) and the internal standard ($m/z = 517$) were drawn through the first quadrupole filter (Q1), with argon collision-induced fragmen-

tation occurring in Q2 (− 10 eV, 375 × 10^{12} atom cm^{-2}), and monitoring the product ions at m/z 161 and 175 for **I** and **II**, respectively, via Q3. The orifice potential was set at + 55 V and the electron multiplier at − 3.7 kV. The curtain gas was ultra-high-purity nitrogen flowing at a rate of 0.9 l min^{-1}. The detector electronics counted every 10th pulse with a dwell time of 400 ms.

Unknown sample concentrations were calculated from a standard line constructed by plotting peak area ratios of **I** to **II** versus drug concentration. All calculations were performed using MacQuan software (P-E Sciex).

2.3. Preparations of working standards

Separate standard stock solutions (1 mg ml^{-1}) were prepared in methanol for both the drug and the internal standard. Dilutions were made using methanol to give a series of working standards with concentrations needed to construct a calibration curve.

2.4. Preparations of plasma standards

2.4.1. HPLC/UV method

Plasma and urine standards were prepared by adding 100 μl of the corresponding working standard of **I** to 1 ml plasma (or urine) contained in 15 ml centrifuge tubes for analyte concentrations of 2.5, 5, 10, 25, 50, 100, 200 and 500 ng ml^{-1}. The pH of the solution was controlled by adding to the centrifuge tube 0.1 M, pH 9.6 carbonate–bicarbonate buffer prior to extraction with 5 ml methyl-*t*-butyl ether (MTBE). Interferences from plasma which coeluted with **I** under the chromatographic condition utilized were extracted from the organic layer into 1 ml 0.5 N HCl. The organic layer was separated from the acid and the solvent was evaporated under a stream of nitrogen at 40°C. The residue was reconstituted into 200 μl of mobile phase and 150 μl of this solution was injected into the chromatographic system.

2.4.2. HPLC/MS–MS

Plasma standards were prepared by adding 100 μl of the corresponding working standard of both **I** and **II** to 1 ml plasma for analyte concentrations of 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 50.0, 100, 250 and 500 ng ml^{-1} and 10 ng ml^{-1} of internal standard.

Due to the high selectivity of the MS–MS detection, a multi-step liquid–liquid extraction was not necessary. After the drug and the internal standard were extracted from pH 9.8-buffered plasma with 5 ml MTBE, the organic solvent was evaporated to dryness, the residue was reconstituted in 150 μl of the mobile phase, and 50 μl was injected into the HPLC/MS–MS system.

2.5. Precision, accuracy, recovery, specificity and stability

The precision of the method was determined by the replicate analyses ($n = 5$) of human plasma and urine containing **I** at all concentrations utilized for constructing calibration curves. 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. Assay specificity was assessed by analyzing blank control plasma and urine from five different sources. No endogenous interferences were observed.

The stability of the drug in plasma was determined by spiking control plasma with **I**, resulting in final concentrations of 4 and 400 ng ml^{-1}. These solutions were aliquoted into polypropylene tubes, capped, and kept at − 20°C. Five replicate samples at each concentration were extracted and assayed on 3 separate days. Concentrations were determined from a calibration curve prepared from different lots of human control plasma on each day of analysis.

3. Results and discussion

3.1. HPLC/UV method

Representative chromatograms of blank human control plasma and control plasma spiked with 5 ng ml^{-1} of **I** are shown in Fig. 3. The precision and accuracy data for the HPLC/UV assay in plasma and urine are presented in Table 1.

The intraday precision, defined as the coefficient of variation (CV), at all concentrations on the standard line, was less than 10%, and the accuracy was within 94–109%. Interday stability data were obtained by analyzing quality control samples containing 4 and 400 ng of **I** added to 1 ml of human-control plasma. Five replicate samples at each concentration were extracted and assayed on 3 consecutive days. All measured concentrations were within 20% of the nominal values, with precision better than 10%.

Assay specificity was achieved through a multistep sample preparation procedure which effectively removed the endogenous interferences from plasma. Specificity of the assay was confirmed by analyzing plasma extracts from five different lots of plasma. No endogenous interferences were observed. The recovery of the drug from plasma was about 80% at all concentrations within the standard curve range.

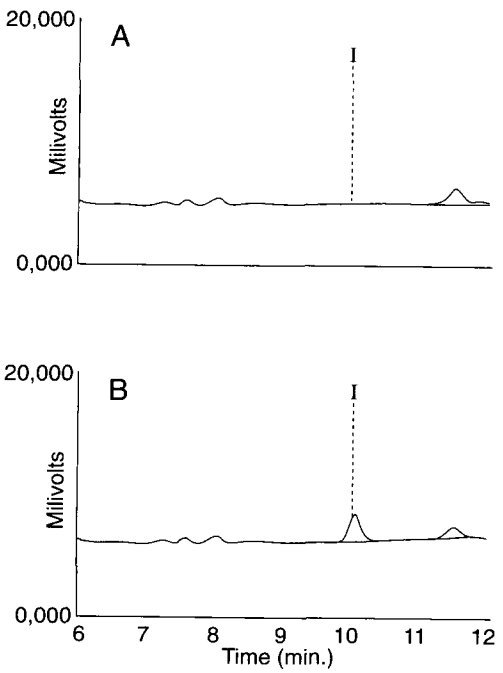


Fig. 3. Chromatograms of (A) blank human control plasma and (B) human control plasma spiked with 5 ng ml^{-1} of **I**, extracted, and monitored by UV absorbance detection at 205 nm.

Table 1
Precision[a] and accuracy[b] data for the determination of **I** in human plasma and urine using HPLC/UV

Conc. (ng ml^{-1})	Plasma		Urine	
	% CV[a]	Accuracy[b]	% CV[a]	Accuracy[b]
2.5	5.5	98	5.3	95
5.0	7.2	109	3.4	106
10	5.2	103	1.8	106
25	8.1	97	1.9	97
50	8.7	98	3.1	101
100	5.6	107	2.8	106
250	7.9	109	1.8	97
500	5.4	94	1.6	104

[a] Precision expressed as the coefficient of variation (CV, %), $n = 5$.

[b] Accuracy expressed as [(mean calculated conc.)/(spiked conc.)] × 100.

3.2. HPLC/MS–MS method

Sample preparation for the HPLC/MS–MS assay was simplified and was based on a single liquid–liquid extraction of **I** and **II** from buffered plasma (pH 9.8) using MTBE. Quantification involved monitoring the product ions at m/z 161 and 175, originating from the protonated molecules of the drug and the internal standard at m/z 503 and 517, respectively (Fig. 4).

A methyl analog (**II**) of the drug was used as an internal standard (IS). When analogs of an analyte are used as IS, instead of a stable isotope labeled compound, the absence of a matrix effect often observed in the GC/MS-based assay needs to be demonstrated. This effect was evaluated by spiking plasma extracts from various subjects and from different sources with the drug and IS, monitoring the absolute peak areas (heights) of parent compound and IS, and calculating their respective ratios. Both absolute peak areas and/or ratios were relatively constant, indicating the matrix effect was negligible. In addition, the retention times of **I** and **II** were very similar, effectively minimizing the potential for such an effect.

Using the HPLC/MS–MS method, the LOQ of the drug from human plasma was 0.2 ng ml^{-1}. Typical chromatograms obtained using this

method are presented in Fig. 5. The assay precision and accuracy data (Table 2) indicated the intraday assay precision was less than 9% at all concentrations within the standard curve range, and assay accuracy was within 90–109%. The detailed comparison of the two techniques is presented in Table 3.

In conclusion, comparison of two HPLC methods for the determination of **I** in human plasma indicated that the utilization of MS–MS detection led to greater than a 10-fold improvement in assay sensitivity (0.2 ng ml^{-1}) over similar methods based on HPLC with UV detection (2.5 ng ml^{-1}) (Table 3). The utility of HPLC/MS–MS as a sensitive and selective analytical technique for the determination of trace quantities of drugs in biological fluids has been confirmed. Selectivity enhancement obtained by monitoring selected parent → product ion combinations simplified sample extraction procedure and chromatography. Additionally, the on-line sample clean-up using column-switching technique was required in the HPLC/UV assay to achieve the required specificity at a short UV wavelength of detection (205 nm), whereas a simpler chromatographic system was sufficient when MS–MS detection was uti-

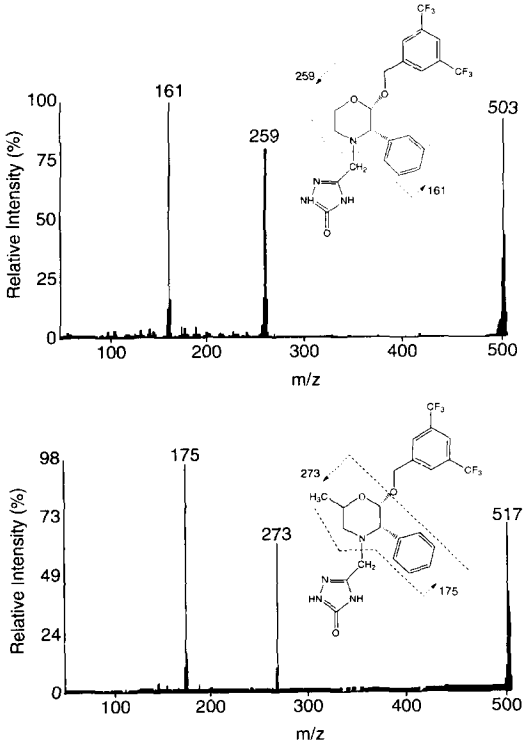


Fig. 4. Positive ion product mass spectra of the protonated molecules of **I** (A, $m/z = 503$) and an internal standard **II** (B, $m/z = 517$).

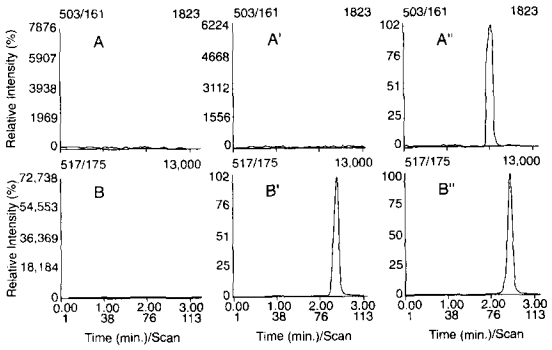


Fig. 5. Representative HPLC/MS–MS chromatograms of plasma extracts obtained by multiple reaction monitoring at m/z 503 → 161 (channel 'a') for **I**, and m/z 517 → 175 (channel 'b') for **II**; chromatograms A and B are extracts of blank plasma monitored at channels 'a' and 'b', respectively; chromatograms A′ and B′ are extracts of blank plasma spiked with 10 ng ml^{-1} of **II** monitored at channels 'a' and 'b', respectively, and chromatograms A″ and B″ are extracts of plasma spiked with 1 ng ml^{-1} of **I** and 10 ng ml^{-1} of **II**, monitored at channels 'a' and 'b', respectively. The numbers in the upper right-hand corner of chromatograms indicate peak heights expressed in arbitrary units.

Table 2
Precision and accuracy data for the determination of **I** in human plasma using HPLC/MS–MS

Conc. (ng ml^{-1})	Precision[a]	Accuracy[b]
0.20	8.3	95
0.50	5.1	108
1.00	5.1	108
2.50	8.2	109
5.00	4.5	104
10.0	4.3	101
50.0	3.7	102
100	4.2	98
250	4.6	90
500	2.9	96

[a] Precision expressed as the coefficient of variation (CV, %), $n = 5$.

[b] Accuracy expressed as [(mean calculated conc.)/(spiked conc.)] × 100.

Table 3
Comparison between the HPLC/UV and HPLC/MS–MS methods for the determination of **I** in human plasma

	HPLC/MS–MS	HPLC/UV
LOQ (ng ml^{-1})	0.2	2.5
Run time (min)	3	12
Extract injected (%)	33	75
Extraction (liq./liq.)	Single	Double and column switching
Chromatography (no. of columns)	1	2
Sample preparation	Fast, simple	Slow, multi-step

lized due to an increased specificity inherent in this technique.

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