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Determination of the substance P receptor antagonist CP-122,721 in plasma by narrow-bore high-performance liquid chromatography–ionspray tandem mass spectrometry

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Abstract

A simple, highly sensitive and specific LC–MS–MS assay was developed for the determination of CP-122,721 (**I**) in rat and human plasma. **I** and a structural analog, CP-129,943 (**II**, internal standard), were extracted from plasma with methyl *tert.*-butyl ether (MTBE). The dried MTBE extracts were reconstituted and analyzed using a narrow-bore (2.1 mm I.D.) YMC basic HPLC column and a mobile phase of acetonitrile–20 m*M* ammonium acetate, pH 5 (50:50, v/v). Column effluents were monitored by ionspray tandem mass spectrometry. Multiple reaction monitoring (MRM) using the parent to product ion combinations of m/z 381→205 and 395→219 was used to quantitate **I** and **II**, respectively. The assay exhibited a linear dynamic range of 0.2–100 ng/ml. Absolute recoveries from plasma were above 80% for both **I** and **II**. The precision and accuracy values for the method were within ±3 and ±9%, respectively. Sample analysis times were less than 5 min from one injection to the next. The assay has proved to be applicable to the pharmacokinetic study of **I** in rats. © 1997 Elsevier Science B.V.

Keywords: Substance P; CP-122,721

1. Introduction

CP-122,721, (+)-(2*S*,3*S*)-3-(2-methoxy-5-trifluoromethoxybenzylamino)-2-phenyl-piperidine (**I**, Fig. 1), is a potent, selective, apparently non-competitive and orally active substance P (SP) receptor antagonist that is being developed for the treatment of diseases characterized by excessive substance P (SP) receptor stimulation [1,2]. In vivo, **I** shows extraordinary oral potency in blocking the central and peripheral action of SP, with ID_{50} values of 0.1–0.2 mg/kg. **I** has a favorable (10:1) brain to

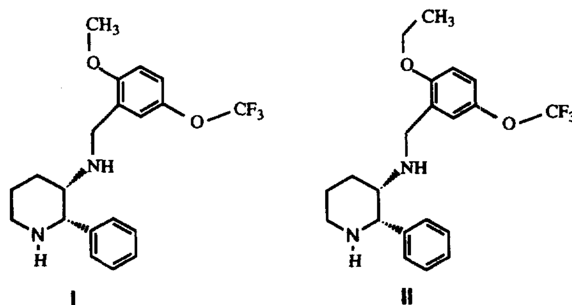

Fig. 1. Structures of parent drug, CP-122,721 (**I**) and I.S. (**II**).

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plasma ratio and a safety profile reflecting 1000-fold separation between exposure levels associated with efficacy and those producing adverse effects [1,2]. Preliminary metabolism and pharmacokinetic studies in animals and humans indicated that **I** is extensively metabolized resulting in a low drug concentrations in systemic circulation [3]. Therefore, to fully evaluate the pharmacokinetics of **I**, a highly sensitive method with a lower limit (LLOQ) of 0.2 ng/ml in plasma was required. A gas chromatography–electron capture detection (GC–ECD) method for the determination of **I** in plasma was available [4], but was not suitable for the determination of **I** pharmacokinetics in the low pg/ml range. In addition, the method requires a derivatization step and relatively longer (>10 min) GC analysis time.

Liquid chromatography (LC) coupled with either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) tandem mass spectrometry (MS–MS) is a proven technique for the rapid, sensitive and specific determination of drugs in biological fluids [5–11]. Our preliminary studies indicated that APCI was not as sensitive as ionspray for the determination of **I** in rat or human plasma. LC–MS–MS techniques using ionspray interface have been used for the identification of conjugated and non-conjugated drug metabolites [12–14]. We have recently used this technique for the identification of metabolites of **I** in rat urine, bile and plasma [3]. Therefore, it was considered of interest to explore the feasibility of ionspray MS–MS for quantitation of **I** in rat and human plasma.

This report describes the development, validation, and application of a rapid, highly sensitive and specific method utilizing narrow-bore LC-ionspray–MS–MS. The assay has an analysis time of less than 5 min and is capable of determining low plasma levels (200 pg/ml) of **I**.

2. Experimental

2.1. Materials and reagents

Compound **I**, (+)-2*S*,3*S*)-3-(2-methoxy-5-trifluoromethoxybenzylamino)-2-phenyl-piperidine, a structural analog (CP-129,943, I.S.), (+)-2*S*,3*S*)-3-(2-ethoxy-5-trifluoromethoxy-benzylamino)-2-phenyl-piperidine (**II**, Fig. 1) were synthesized at Pfizer Central Research (Groton, CT, USA). Acetonitrile (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid (GR grade) deionized water, ammonium acetate (HPLC-grades) were obtained from Fisher (Fair Lawn, NJ, USA), and methyl *tert.*-butyl ether (MTBE), HPLC-grade, was obtained from Burdick and Jackson (Muskegon, MI, USA). Human plasma with EDTA anticoagulant was obtained from Biological Specialties Corporation (Landsdale, PA, USA).

2.2. Instrumentation and chromatographic conditions

HPLC was performed using a HP-1050 solvent delivery system and a HP-1050 autoinjector. The LC was coupled to a Perkin-Elmer SCIEX API III^+ (Toronto, Canada) triple-quadrupole mass spectrometer. The analytical column was a YMC basic (150×2.1 mm, 5 μm, YMC, Greensboro, NC). The mobile phase consisted of acetonitrile–20 m*M* ammonium acetate, pH 5 (50:50, v/v). The HPLC system was operated isocratically at 300 μl/min and at room temperature. The column effluent was split and approximately 50 μl/min was introduced to the ionspray ionization source. The remaining effluent was directed into the waste. The quadrupole power supply was set for unit mass resolution. Ion signals were detected with an electron multiplier operated in the pulse-counting mode by counting every tenth pulse. The production efficiency of the protonated molecular ion (MH^+) was optimized by measuring (MH^+) abundance while varying the orifice and ionspray voltages. The ionspray and orifice voltages were set at 5000 and 60 V, respectively, and a nebulizing gas pressure was set at 60 p.s.i. (1 p.s.i.= 6894.76 Pa) of N_2. Collisionally-activated dissociation (CAD) studies were performed using argon gas at a thickness of 2.0×10^{15} atoms/cm^2 and a collision energy of 25 eV. The instrument was programmed for a scan dwell time of 200 ms. These parameters were optimized by systematically varying the argon pressure and collision energy for maximum reproducibility and the abundance of the selected product ion. The responses of **I** and **II** were measured in the positive ion mode using the technique of MRM; i.e. monitoring selected product ions of

specific parent ions. The protonated molecular ions of **I** (m/z 381) and **II** (m/z 395) were dissociated by collision in Q2 with argon. The mass spectrometer was set to selectively monitor parent to product ion fragments of m/z 381→205 for **I** and m/z 395→219 for **II**. The area ratios for calibration curves and quantitative analysis were made using the PE-SCIEX MacQuan software version 1.3.

2.3. Preparation of standard and quality control samples

All centrifuge tubes were silylated in a vacuum oven using the procedure of Fenimore et al. [15]. Stock solutions of **I** and **II** (1 mg/ml) were prepared in de-ionized water and were serially diluted with water to obtain the desired concentrations. The stock solutions were kept refrigerated and discarded 1 month after their preparation.

The plasma concentrations of calibration standards were 0, 0.2, 1, 10, 30, 50, 80 and 100 ng/ml. Three levels of quality controls (QC), at 2, 30 and 80 ng/ml (low, medium and high), were prepared at the beginning of the validation and were stored at −20°C.

2.4. Extraction procedure

An aliquot of 1 ml human plasma was placed in a centrifuge tube. To this was added 10 ng of I.S. and the sample was vortex mixed. The plasma was extracted with 5 ml of MTBE for 10 min using a vortex mixer. Following centrifugation (2440 *g*) for 5 min, the organic layer was transferred to a clean centrifuge tube, and the extract was evaporated to dryness in a nitrogen Turbo Vap, LV evaporator (Zymark, Hopkinton, MA, USA; bath temperature= 37°C, ~25 min). The dried residue was reconstituted in 100 μl of HPLC mobile phase, sonicated for 10 min, vortexed for 10 min and centrifuged. Samples were transferred to clean autosampler injection vials and a 20-μl sample volume was injected into the HPLC–MS–MS system.

2.5. Method validation

For method validation, duplicate plasma calibration curves and five replicates of the QCs were analyzed on three separate days. The peak areas generated by MRM of **I** and **II** were obtained. The ratios of the peak areas of **I** to the peak areas of **II** were calculated. The standard curves were constructed by weighted ($1/x^2$) least-squares linear regression analysis of the peak-area ratios of **I**/**II** versus the concentrations of **I**. The equations of the calibration curves were used to calculate the concentration of **I** in the samples and QCs from their peak area ratios.

The intra-assay precision and accuracy was determined by analyzing a set of QC samples ($n=6$) at each of the three levels, 2, 30 and 80 ng/ml. An inter-assay precision and accuracy study was carried out by analyzing QC samples in six replicates on three separate days.

2.6. Recovery

The extraction recoveries of **I** and **II** from human plasma were calculated by comparing the areas of peaks of the extracted and non-extracted analytes. The recovery study was carried out by spiking control human plasma samples with **I** at three concentrations (in six replicates), 2.0, 30.0 and 80.0 ng/ml, and with **II** at 10 ng/ml. These samples were extracted as described above. For nonextracted samples, appropriate amounts of **I** and **II** (same level as extracted samples) were added and the final volume made up to 100 μl with the HPLC mobile phase. Aliquots (20 μl) of both extracted and non-extracted samples were analyzed. The areas of the analyte and I.S. were obtained and the percent recovery was calculated by dividing the areas of the extracted analytes over the areas of non-extracted standards.

3. Results and discussion

3.1. Method development

The narrow-bore LC-ionspray–MS–MS for the determination of **I** in human plasma was investigated. Full-scan (Q1) mass spectrum (MS) of **I** showed a protonated molecular ion (MH^+) at m/z 381. CAD product ion spectrum for **I** yielded a high-abundance fragment ion at m/z 205 (Fig. 2a). The Q1 MS and the product ion spectrum of **II**

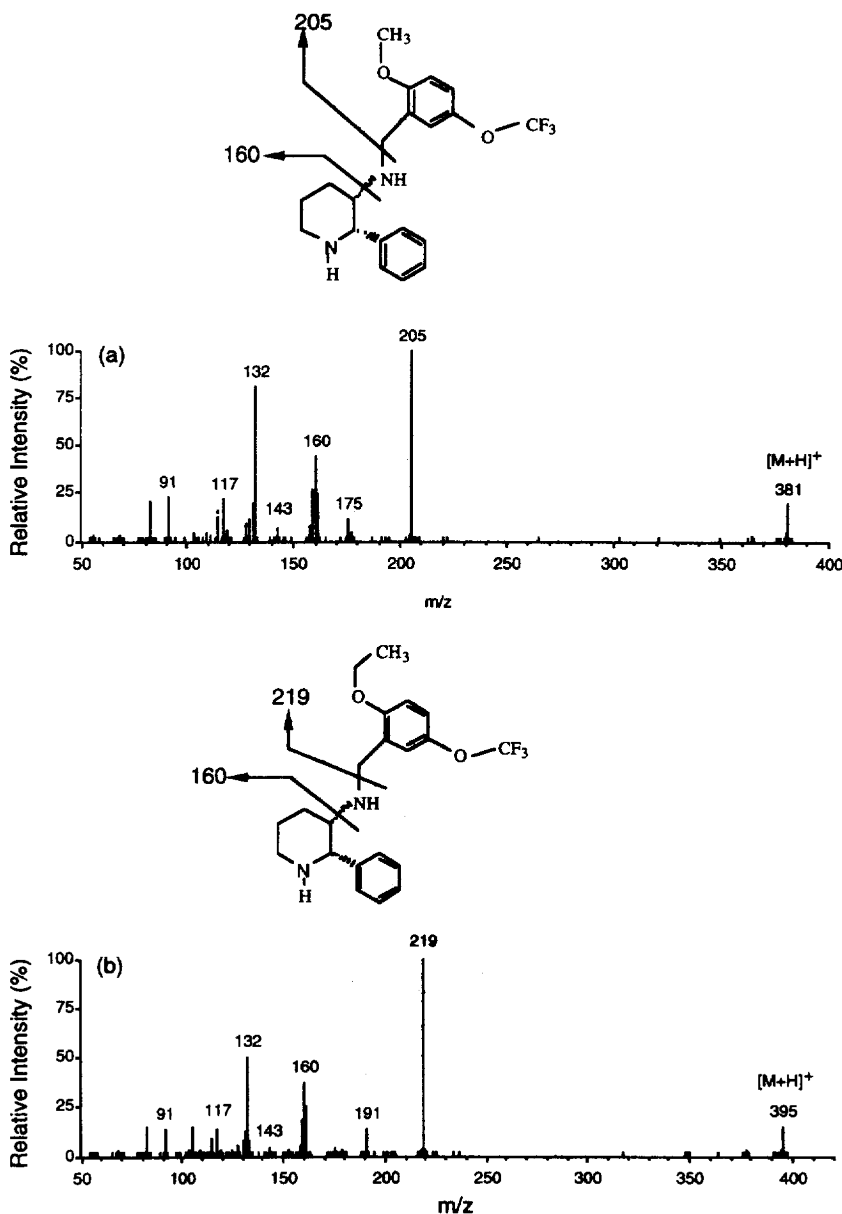

Fig. 2. CAD product ion spectra of (a) CP-122,721 (*m/z* 381) and (b) CP-129,943 (*m/z* 395, I.S.).

displayed a pattern similar to **I** (Fig. 2b). The protonated molecular ion (MH^+) was observed at *m/z* 395 and the high-abundance fragment ion was observed at *m/z* 219. The product ions at *m/z* 205 and 219 were resulted by the loss of phenylpiperidinyl amino moiety from **I** and **II**, respectively, and were used for the quantitation.

3.2. Separation and specificity

Narrow-bore column HPLC was used in this work in order to achieve greater sensitivity [16]. A short retention time of less than 4 min was achieved for both **I** and **II**. Ion chromatograms for CP-122,721 (381→205) and I.S. (395→219) from (a) blank

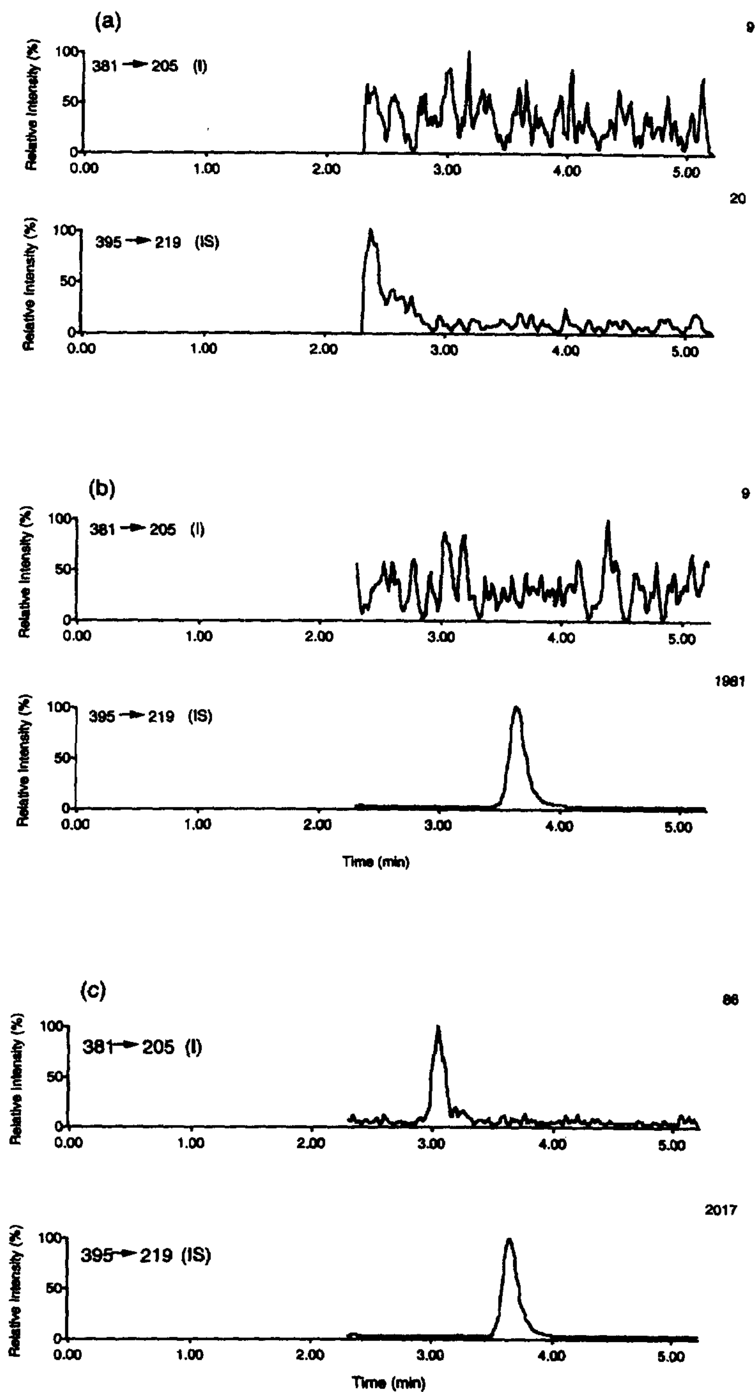

Fig. 3. Representative MRM chromatograms of (a) control plasma, (b) plasma spiked with I.S. (10 ng/ml), and (c) plasma spiked with both drug (CP-122,721, 0.2 ng/ml) and I.S. (10 ng/ml).

plasma, (b) plasma spiked with **II** (10 ng/ml), and (c) plasma spiked with **I** (0.2 ng/ml) and **II** (10 ng/ml) are shown in Fig. 3. For both the drug and I.S., the chromatograms were free of interfering peaks at their respective retention times.

3.3. Linearity, precision and accuracy

Calibration data for **I** are shown in Table 1. Calibration curves were plotted as the peak area ratio (drug/I.S.) vs. drug concentration. Although the mass spectrometer is notable for its narrow dynamic range due to saturation of the ion-counting circuitry [17], this assay has a rather extended dynamic range. The assay was linear in the concentration range of 0.2–100 ng/ml. The correlation coefficients were greater than 0.998 for all the curves.

The intra- and inter-assay precision of the analysis were examined with samples spiked with **I** (QCs). As shown in Table 2, the intra-day variability was determined at three different concentrations in six replicates, which were assayed against a single calibration curve. The intra-assay precision at these concentrations (2, 30 and 80 ng/ml) was within 2% and the accuracy was from 1 to 9%. As shown in Table 3, the inter-assay variation was determined by analysis of the spiked plasma (QC samples) on three separate occasions, relative to calibration samples that were freshly prepared each time. The inter-assay precision at three concentrations (2, 30 and 80 ng/ml) was within 3% and the accuracy was from 5 to 8%. These results indicate that the method was reliable within that range and the use of a structurally related internal standard was very effective for reproducibility by LC–MS.

Table 3
Inter-assay precision and accuracy for CP-122,721 in plasma

Analysis sequence	Mean calculated concentration (ng/ml), $n=2$		
	2.0 ng/ml[a]	30 ng/ml[a]	80 ng/ml[a]
1	2.2	32.6	76.6
2	2.1	31.6	77.6
3	2.2	30.4	73.3
Inter-assay			
R.S.D. (%)	2.7	3.5	3
Deviation (%)	8.3	5.1	−5.2

[a]Nominal concentrations.

3.4. Lower and upper limits of quantification

The lower and upper limits of quantification were determined at 0.2 and 100 ng/ml of **I** in human plasma. As shown in Table 4, plasma samples were spiked with 0.2 and 100 ng/ml of **I** ($n=6$, at each level), extracted and quantitated from a standard

Table 1
Calibration curve for CP-122,721 in plasma

Nominal conc. (ng/ml)	Calculated conc. (mean±S.D., $n=6$) (ng/ml)	R.S.D. (%)	Deviation (%)
0.2	0.20±0.01	5.5	0.5
0.5	0.48±0.02	4.1	−3.2
2.0	2.09±0.10	4.9	4.55
10.0	10.49±0.22	2.1	4.87
30.0	30.22±0.53	1.7	0.75
50.0	50.20±2.07	4.1	0.41
80.0	76.63±1.69	2.2	−4.21
100.0	93.67±2.97	3.2	−6.33

Table 2
Intra-assay precision and accuracy for CP-122,721 in plasma

Nominal conc. (ng/ml)	Calculated conc. (mean±S.D., $n=6$) (ng/ml)	R.S.D. (%)	Deviation (%)
2.0	2.1±0.04	2.0	5.08
30.0	32.76±0.62	1.9	9.19
80.0	80.68±1.87	2.3	0.85

Table 4
Precision and accuracy of lower and upper limits of quantitation for CP-122,721 in plasma

Nominal conc. (ng/ml)	Calculated conc. (mean±S.D., n=5)	R.S.D. (%)	Deviation (%)
0.2	0.19±0.01	3.2	−6.0
100.0	96.27±1.1	1.1	−3.7

curve. The precision of the lower and upper limit of quantification was within ±3% and the accuracy was from 4 to 6%. Evidently, the assay permits the quantitation of the drug with adequate accuracy and precision.

3.5. Extraction recovery

A simple liquid–liquid extraction procedure was introduced to extract **I** and **II** from plasma. As shown in Table 5, the recovery of **I** was determined at three different concentrations in five replicates. The recovery of **II** was obtained only at one level (10 ng/ml) which was used during the assay. The recoveries of **I** were 100–116%. The I.S. recovery was 80–87%.

3.6. Application

The narrow-bore LC–MS–MS method was used to provide pharmacokinetic data for **I** in rat plasma following oral administration of radiolabeled drug at a dose of 25 mg/kg [3]. Plasma concentrations were measured for 12 h following oral administration. The representative plasma concentrations–time curves for unchanged **I** after oral administration to the male and female rats are shown in Fig. 4. The plasma concentration of **I** reached a peak of 130.6 and 227.2 ng/ml for male and female rats, respectively, and declined in a log-linear manner. The plasma concentrations were similar to those found earlier using a GC–ECD method [4].

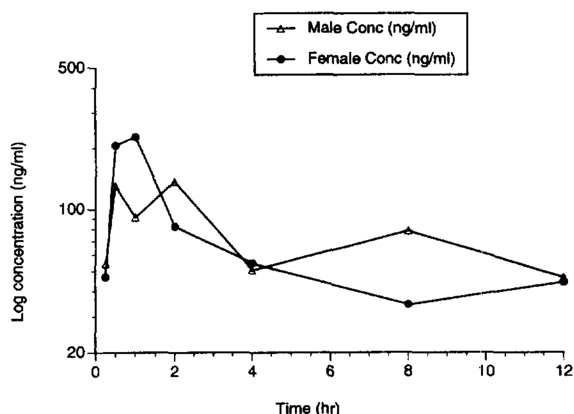

Fig. 4. Plasma concentrations of CP-122,721 in male and female rats.

4. Conclusions

A very simple, and sensitive narrow-bore LC-ionspray-MS–MS assay was developed using a liquid–liquid extraction technique. The assay has proven to be fast and rugged, with each sample requiring less than 5 min of analysis time. The method was applied successfully for the determination of **I** in rat plasma and is currently being used to support clinical studies. The assay has a very wide range of quantitation. Therefore, this assay should

Table 5
Assay recovery for CP-122,721 in plasma

Nominal conc. (ng/ml)	Mean peak area (n=5)				% Recovery (mean±S.D., n=5)	
	Non-extracted		Extracted			
	CP-122,721	I.S.	CP-122,721	I.S.	CP-122,721	I.S.
2.0	3749	22 424	3749	17 789	100.9±14.97	79.45±4.43
30.0	50 528	20 718	56 139	17 740	111.28±7.1	85.71±4.76
80.0	125 002	21 696	144 773	18 758	116.01±5.97	86.58±5.05

have wide applicability for determination of pharmacokinetics in humans following oral administration of low to high doses.

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