Determination of a novel substance P inhibitor in human plasma by high-performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometric detection using single and triple quadrupole detectors

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Abstract

Methods based on high-performance liquid chromatography (HPLC) with atmospheric-pressure chemical ionization (APCI) mass spectrometric (MS) detection using either single (MS) or triple (MS/MS) quadrupole mass spectrometric detection for the determination of (2R)-[1(R)-(3,5-bis-trifluoromethylphenyl)ethoxy]-3(S)-(4-fluoro-phenyl)morpholin-4-ylmethyl]-5-oxo-4,5-dihydro-[1,2,4]triazol)methyl morpholine (Aprepitant, Fig. 1) in human plasma has been developed. Aprepitant (**I**) and internal standard (**II**, Fig. 1) were isolated from the plasma matrix buffered to pH 9.8 using a liquid–liquid extraction with methyl-t-butyl ether (MTBE). The analytes were separated on a Keystone Scientific's Javelin BDS C-8 2 mm × 4.6 mm 3 μm guard column coupled to BDS C-8 50 mm × 4.6 mm 3 μm analytical column, utilizing a mobile phase of 50% acetonitrile and 50% water containing 0.1% formic acid and 10 mM ammonium acetate delivered at a flow rate of 1 ml/min. The single quadrupole instrument was operated in a single ion monitoring (SIM) mode analyzing the protonated molecules of Aprepitant and **II** at m/z 535 and 503, respectively. The triple quadrupole mass spectrometer was operated in multiple reaction monitoring mode (MRM) monitoring the precursor → product ion combinations of m/z 535 → 277 and 503 → 259 for Aprepitant and **II**, respectively. The linear calibration range for both single and triple quadrupole detectors was from 10 to 5000 ng/ml of plasma with coefficients of variation less than 8% at all concentrations. Both single and triple quadrupole instruments yielded similar precision and accuracy results. Matrix effect experiments performed on both instruments demonstrated the absence of any significant change in ionization of the analytes when comparing neat standards to analytes in the presence of plasma matrix. Both instruments were used successfully to support numerous clinical trials of Aprepitant.

Keyword: Substance P inhibitor

1. Introduction

Substance P is a endecapeptide, which is released from both central and peripheral terminals of small fibered sensory neurons [1–4]. It is a member of the tachykinin family of peptides responsible for the stimulation of neurokinin receptors with an especially high affinity for the NK$_1$ receptor [5]. Antagonism of the NK$_1$ receptor has potentially diverse therapeutic indications such as in the treatment of pain, depression, inflammation, migraine, and emesis [6–10].

Aprepitant, (2R)-[1(R)-(3,5-bis-trifluoromethylphenyl) ethoxy]-3(S)-(4-fluoro-phenyl)morpholin-4-ylmethyl]-5-oxo-(4,5-dihydro-[1,2,4]triazol)methyl morpholine, is a non-peptide substance P receptor antagonist, which selectively acts at NK-1 cell- surface receptors. This compound was evaluated in Merck Research Laboratories for the treatment of chemotherapy induced nausea and vomiting [11–14]. Recently, Aprepitant was approved by the United States Food and Drug Administration for the treatment of chemotherapy induced emesis and is marketed by Merck & Co. under the trade name Emend™.

For the clinical evaluation of Aprepitant it was necessary to develop a bioanalytical method with a limit of quantification of 1 ng/ml of plasma. Initially, the feasibility of

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determination of Aprepitant using HPLC with ultraviolet detection (UV) was considered. The UV absorbance spectra of Aprepitant in methanol indicated the presence of an absorption band with a maximum at 205 nm and a molar absorption coefficient (ε) of 15200 M^{-1} cm^{-1}. In principle, the development of an assay with UV detection at 205 nm with the lower limit of quantification (LLOQ) of 1 ng/ml was feasible, but would require highly selective off-line and on-line sample clean-up using column switching to monitor Aprepitant at the low ng/ml concentrations without endogenous interferences from the biological matrix. In addition, a HPLC runtime of 10 min or longer to ensure adequate separation of analytes from endogenous background impurities would probably be required. Based upon our previous experience with a similar compound [15], we have decided to utilize HPLC-MS/MS methodology to develop a rugged and selective method with a relative short run time (6 min) using a simple sample preparation allowing for the analysis of greater than 150 samples per instrument per day. Initially, an HPLC-MS/MS method was developed and validated in the concentration range of 1–500 ng/ml with an LLOQ of 1 ng/ml. The method was used for supporting the initial dose-ranging studies. After the selection of a therapeutic dose, the LLOQ was increased to 10 ng/ml with the upper standard curve range expanded from 500 to 5000 ng/ml. Due to the large volume of clinical samples and the high demands for HPLC-MS/MS support for other clinical programs, the feasibility of using a single quadrupole MS instrument to quantify Aprepitant in samples from clinical trials was also evaluated.

The subject of this paper is the development of the bioanalytical method for Aprepitant in human plasma using single or triple quadrupole mass spectrometric detectors for the determination of this compound. In addition, the detailed assessment of the selectivity of both MS and MS/MS based methods was also performed and the absence of matrix effect on the quantification of Aprepitant using both techniques was clearly demonstrated.
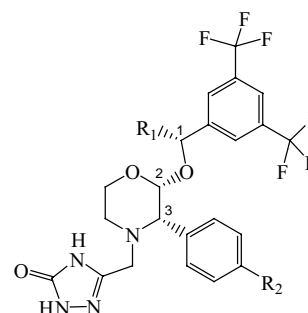
2. Experimental

2.1. Materials

Aprepitant and compound **II** (Fig. 1) were received from the Merck Research Laboratories (Rahway, NJ) sample repository. All solvents were HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ.). The different lots of drug-free EDTA treated plasma originated from Biological Specialty (Lansdale, PA.). Nitrogen (99.999%) and argon (99.999%) were purchased from West Point Supply (West Point, PA.).

2.2. Instrumental

A Perkin-Elmer (PE) Sciex (Thornhill, Canada) API III Plus triple quadrupole mass spectrometer or an API 150

Aprepitant $R_1 = CH_3$; $R_2 = F$
II $R_1 = H$; $R_2 = H$

Fig. 1. Chemical structures of Aprepitant and **II**.

single quadrupole mass spectrometer both equipped with a heated nebulizer (HN) interface, a PE 200 autoinjector, and a PE 200 Quaternary pump were used for all analyses. The data were processed using MacQuan software (PE Sciex) on a MacIntosch Quadra 900 microcomputer.

2.3. Standard solutions

Stock standard solutions (100 μg/ml) for Aprepitant and **II** were prepared in methanol. A 10 μg/ml stock solution of Aprepitant was then obtained by serial dilution. This solution was further diluted with methanol to give a series of working standards of 0.1 to 50 μg/ml. The internal standard (**II**) stock solution (100 μg/ml) in methanol was serially diluted with methanol to yield a working standard of 10 μg/ml.

2.4. Chromatographic conditions

Chromatographic separation of analytes was performed on a Keystone Scientific's C-8 analytical column (50 mm × 4.6 mm 5 μm, Keystone Scientific, Bellefonte, PA) with a mobile phase consisting of 50% acetonitrile (ACN) and 50% water containing 10 mM ammonium acetate and 0.1% formic acid, pumped at a flow rate of 1 ml/min. The same HPLC conditions were used for MS and MS/MS detection. The total runtime was 6 min. All analytes were baseline separated. The retention times of Aprepitant and **II** were 3.4 and 3.1 min corresponding to capacity factors (k') of 5.8 and 5.2, respectively.

2.5. HPLC-mass spectrometric detections conditions

A PE Sciex single (Sciex API 150) or triple (Sciex API III Plus) quadrupole mass spectrometer was interfaced via a Sciex Heated Nebulizer probe (HN) with the HPLC system. The HN probe was maintained at 500 °C and gas phase chemical ionization was effected by a corona discharge needle (+4 μA) using positive ion atmospheric pressure chemical ionization (APCI). The nebulizing gas (N_2) pressure

was set for the HN interface at 80 psi. The auxiliary flow was 2.0 l/min, the curtain gas flow (N_2) was 0.9 l/min, and the sampling orifice potential was +50 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60 °C. All mass analyzers were operated at unit mass resolution. The mass spectrometer was programmed to admit the protonated molecules $[M + H]^+$ at m/z 535 for Aprepitant and 503 for **II** via the first quadrupole filter (Q1) for both instruments. For the triple quadrupole instrument, collision induced fragmentation at Q2 (collision gas argon, 275×10^{13} atoms cm^{-2}) yielded the product ions at m/z 277 and 259 for Aprepitant and **II**, respectively, which were detected at Q3. For the single quadrupole instrument, peak area ratios from single ion monitoring (m/z 535/503) were used for the construction of the calibration lines. For the triple quadrupole instrument peak area ratios obtained from multiple reaction monitoring (MRM) of the analytes (m/z 535 $\rightarrow$ 277)/(503 $\rightarrow$ 259) were utilized for the construction of calibration lines for Aprepitant. Weighted ($1/x^2$) linear least-square regression of the plasma concentrations and measured peak area ratios were used for the quantification on both instruments. Data collection, peak integration and calculations were performed using MacQuan PE-Sciex software.

2.6. Sample preparation

2.6.1. Clinical sample preparation

The liquid–liquid extraction method consisted of basifying 1 ml of plasma, spiked with 100 µl of internal standard solution (10 µg/ml) with pH 9.8 carbonate buffer (1 ml), and then extraction with methyl-*t*-butyl ether (7 ml) in conical disposable glass tubes (15 ml) by rotating mixing. The organic extract was transferred to a clean 15-ml centrifuge tube and evaporated under a stream of nitrogen at 50 °C. The dried residue was reconstituted in mobile phase (1 ml and 300 µl for calibration lines in the concentration range of 10–5000 or 1–500 ng/ml, respectively) and injected part of this solution (15 and 30 µl for 10–5000 or 1–500 ng/ml concentration ranges, respectively) into the HPLC system.

2.6.2. Sample preparation for recovery and matrix effect evaluation experiments

Three sets of samples for the construction of five standard lines were prepared to evaluate the assay accuracy, precision, recovery, and the absence or presence of the matrix effect. The first set of samples corresponding to the five standard lines (set 1, 8 concentrations × 5 = 40 samples) was prepared to evaluate the mass spectrometric (MS and MS/MS) responses for neat standards in methanol of both analytes Aprepitant and **II** injected in the mobile phase. The second set (set 2) was prepared in plasma extracts originating from five different sources to which standards of Aprepitant and **II** in methanol were spiked *after* extraction. The third set (set 3) was prepared in plasma from the same five different

sources as in set 2, but these plasma samples were spiked with standards *before* extraction. By comparing the absolute areas of peaks Aprepitant and **II**, the peak areas ratios, and slopes of the standard lines from these three different sets of samples and in different plasma lots, the absence or presence of matrix effect, precision and accuracy of the method and recovery of the analytes were assessed. This evaluation was performed using experimental procedures described by us earlier [16,17].

2.7. Precision, accuracy, and recovery

The precision of the method was determined by the replicate analyses ($n = 5$, set 3) of Aprepitant in five different sources of human plasma at all concentrations utilized for the construction of calibration curves. The linearity of each standard curve was confirmed by plotting the peak area ratio of the drug to IS (**II**) versus drug concentration. The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted ($1/x^2$) linear regression of the standard line. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)] × 100. The recovery was determined by comparing the mean peak areas of Aprepitant in set 3 to those observed in set 2 [17].

2.8. Assessment of the matrix effect

The assessment of matrix effect and assay reliability is critical when analogs rather than stable isotope-labeled analytes are used as internal standards [16–22]. Undetected coeluting endogenous impurities may affect the ionization efficiencies of the analytes. By comparing the peak areas of analytes in different lots of plasma, the peak area ratios of analytes to an internal standard, and by analyzing plasma samples spiked before and after extraction, the recovery and ion suppression or enhancement associated with a given lot of plasma was assessed, as detailed in reference [17].

2.9. Assessment of assay selectivity

The assay selectivity was assessed by analyzing extracts from five lots of plasma originating from different sources. Endogenous peaks at the retention time of the analytes of interest were not observed in any of the plasma lots evaluated. In addition, the "cross-talk" between MS channels used for monitoring Aprepitant and **II**, was evaluated and was not observed. The "cross-talk" effect may originate from the slow removal of ions from the collision cell that was quite common in the early designed collision cells. Therefore, if the time used to remove the residual ions was insufficient, a "cross-talk interference" would occur [22]. Additionally a second form of "cross-talk" interference may occur when an analog, used as an IS and/or metabolite(s) that are not chromatographically separated from the analyte of interest,

are converted in the interface of the mass spectrometer to the analyte of interest or are contaminated chemically with each other. The potential for this type of "cross-talk" effect was eliminated by chromatographically separating the IS and metabolites from each other and **I**, and by demonstrating the absence of response in the MS channels used for monitoring **I** and **II** when individual compounds were injected at the highest concentrations of each analyte used in the assay.

2.10. Quality control sample preparation

Quality control (QC) samples at three different concentrations (50, 2500, 4500 ng/ml) were prepared from a pool of five different sources of plasma.

Aliquots (1.25 ml) of quality control plasma samples were placed in polypropylene tubes and stored frozen at $-20\,^{\circ}$C.

3. Results and discussion

3.1. Assay selectivity and method validation

Full scan positive ion mass spectra of Aprepitant and **II** yielded predominately the protonated molecules at m/z 535 and 503, respectively. The product ion mass spectra of these protonated molecules generated in the triple quadrupole system (Fig. 2) indicated the presence of intense product ions at m/z 277 and 259 for Aprepitant and **II**, respectively.

The isolation of Aprepitant and **II** was based on a liquid-liquid extraction from plasma, evaporation of the extract to dryness, reconstitution of the residue, and injection of the sample into the HPLC system.

The chromatographic conditions, which provided adequate retention of analytes (capacity factors, k' of greater than 5 for both Aprepitant and **II**) were selected. Baseline separation of both drug and internal standard was desired for the evaluation of "crosstalk" between the acquisition channels corresponding to Aprepitant and **II**. The "crosstalk" was evaluated by separately injecting the Aprepitant at the highest concentration on the standard line (5000 ng/ml) and working internal standard (**II**, 1000 ng/ml) and monitoring the response in the acquisition channel for the other analyte. It was clearly demonstrated that there was no "crosstalk" between channels used for monitoring Aprepitant and **II** and no response in the channel for **II** was seen when 5000 ng/ml of Aprepitant was injected and, also no response in the channel for Aprepitant was observed when 1000 ng/ml of **II** was injected (Fig. 3). In addition, good retention of analytes ($k' \geq 5$) allowed separation of more polar compounds and other potentially interfering compounds from the analytes, thereby potentially decreasing or eliminating the competition for ionization at the retention times of Aprepitant and **II**. However, the good retention of analytes ($k' \geq 5$) does not necessarily guarantee the absence of matrix effect on
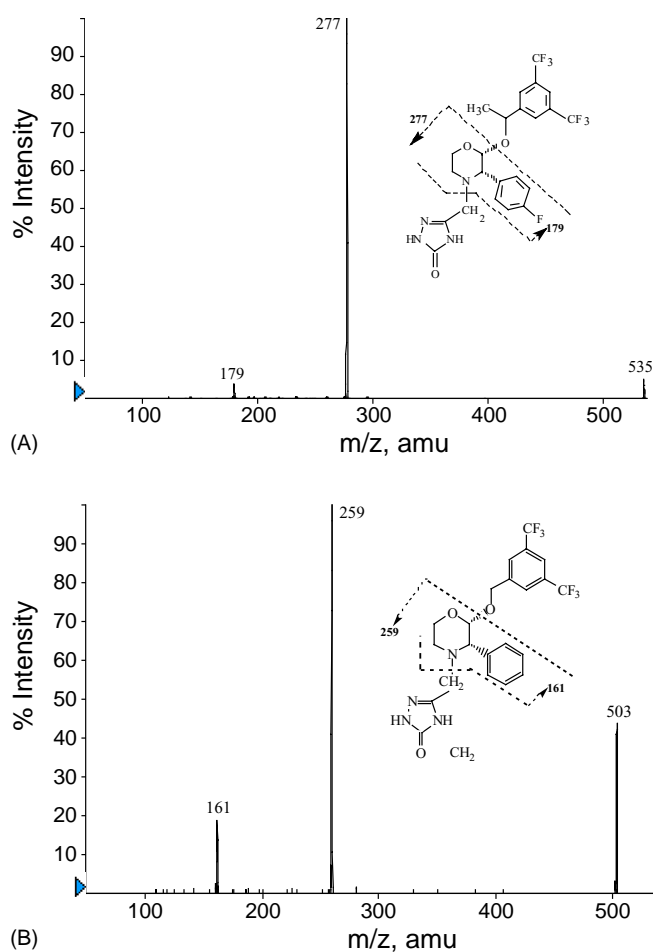

Fig. 2. The product ion mass spectra of the protonated molecules of Aprepitant (A) and **II** (B).

quantification and the potential for matrix effect was fully evaluated (vide infra). The chromatograms of blank control plasma extract shown in Fig. 4 confirmed assay selectivity under the HPLC conditions utilized. No detectable endogenous peaks were observed in all channels utilized for analytes quantification.

The major metabolic pathway of Aprepitant was fully characterized in absorption, metabolism and excretion studies using single IV and oral administration of ^{14}C-labeled prodrug of Aprepitant and ^{14}C-Aprepitant [23]. The major human metabolic pathways included *N*-dealkylation, oxidation followed by morpholine ring-opening, *O*-dealkylation, triazolone ring-opening, and glucuronidation. These metabolites were evaluated for "cross-talk" with the MS and MS/MS channels used for quantification of Aprepitant and **II**. It was confirmed that selectivity of the methods in the presence of metabolites was preserved.

The assay for Aprepitant was validated in human plasma in the concentration range of 10–5000 ng/ml using both single and triple quadrupole detection and in the concentration range of 1–500 ng/ml using the triple quadrupole instrument (detailed validation results in the lower concentration
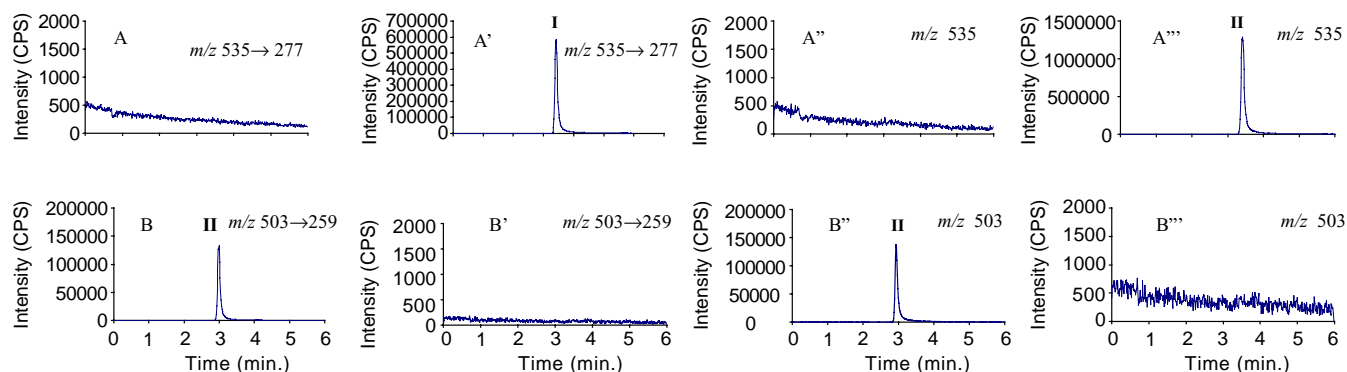
Fig. 3. Representative HPLC-MS/MS (MRM) chromatograms of plasma extracts obtained by multiple reaction monitoring at $m/z = 535 \rightarrow 277$ for **I** and $m/z = 503 \rightarrow 259$ for internal standard (**II**); (A and B)—blank control plasma spiked 1000 ng/ml of **II**; (A′ and B′) blank control plasma spiked 5000 ng/ml of **I**. Representative HPLC-MS (SIM) chromatograms of plasma extracts obtained by multiple reaction monitoring at $m/z = 535$ for **I** and $m/z = 503$ for internal standard (**II**); (A″ and B″) blank control plasma spiked 1000 ng/ml of **II**; (A‴ and B‴) blank control plasma spiked 5000 ng/ml of **I**.
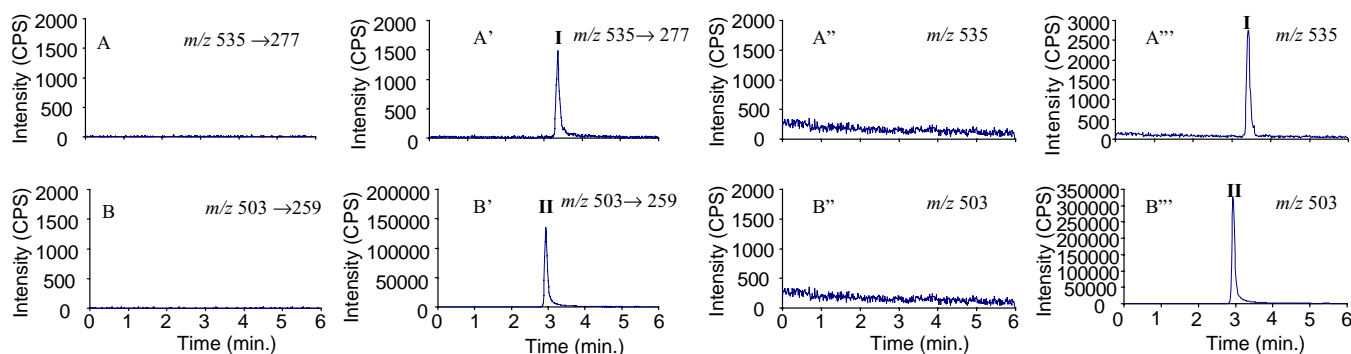

Fig. 4. Representative HPLC-MS/MS (MRM) chromatograms of plasma extracts obtained by multiple reaction monitoring at $m/z = 535 \rightarrow 277$ for **I** and $m/z = 503 \rightarrow 259$ for internal standard (**II**); (A and B) subject predose sample; (A′ and B′) subject 96 h post-dose plasma sample (calculated concentration of **I** was 10.9 ng/ml), spiked with 1000 ng/ml of **II**. Representative HPLC-MS (SIM) chromatograms of plasma extracts obtained by multiple reaction monitoring at $m/z = 535$ for **I** and $m/z = 503$ for internal standard (**II**); (A″ and B″) subject predose sample; (A‴ and B‴) subject's 24 h sample (calculated concentration of **I** was 14.3 ng/ml) spiked with 1000 ng/ml of **II**.

range are not presented, since this assay was used only to support the initial dose ranging clinical study). The difference between the nominal standard concentration and the back-calculated concentration from the weighted linear regression line was less than 8% for each point on the standard curve indicating that the linear regression analysis applied ($1/x^2$) provided an adequate fit of the data. Typical equations for the calibration curve for Aprepitant using the API 150 and API III$^+$ was $y = 0.001047x - 0.00052$ and $y = 0.000819x - 0.00042$, respectively. The correlation coefficients for the mean standard curve obtained in five different plasma lots (set 3) was greater than 0.999 using either single or triple quadrupole instruments, demonstrating linearity over the entire standard curve range.

The intra-day accuracy ranged from 95 to 103% with precision values of less than 8% indicating excellent accuracy and precision of the assay. The assay intra-day accuracy and precision data for the 10–5000 ng/ml range using both MS and MS/MS detectors are summarized in Table 1. Similar results (data not presented) were obtained in the 1–500 ng/ml range using MS/MS detection.

3.2. Recovery of analytes and assessment of the matrix effect

The matrix effect and the possibility for ionization suppression or enhancement for Aprepitant and **II** in different plasma samples (lots, subjects) were closely examined. An experimental procedure for the assessment of matrix effect described by us earlier [17] was followed. As seen in Table 2, the coefficients of variation (CVs, %) of the mean peak areas of Aprepitant and **II** (set 3 and set 2) at any given concentration in *five different* plasma lots were small (<10%) and comparable to directly injected standards (set 1), strongly indicating little or no difference in ionization efficiency and consistent recovery of the analytes between different plasma lots. In addition, by comparing peak areas of all analytes for samples spiked *after* extraction from plasma (set 2) with the analogous peak areas obtained by injecting *neat* standards directly (set 1), the extent of the "absolute" matrix effect over the entire concentration range of Aprepitant was determined to be 98 and 101% for Aprepitant and **II**, respectively. As expected, similar results (97 and 101% for

Table 1
Intra-day precision and accuracy of replicate analyses ($n = 5$) of Aprepitant in human control plasma analyzed by single (API 150) or Triple (API III[+]) quadrupole detectors

Nominal concentration (ng/ml)	Mean calculated concentration[a]		Precision (%)[b]		Accuracy (%)[c]	
	API 150	API III[+]	API 150	API III[+]	API 150	API III[+]
10	9.7	10.3	5.1	3.8	97	103
25	23.8	24.0	2.3	0.7	95	96
50	49.9	49.8	4.5	3.7	100	100
100	97.2	99.7	2.6	0.8	97	100
500	493.1	508.0	0.6	8.0	99	102
1000	1019.9	1003.8	6.2	1.5	102	100
2500	2529.8	2477.4	4.5	1.6	101	99
5000	4985.3	4844.6	3.1	2.6	100	97

[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 (CV%).
[c] Expressed as [(mean observed concentration)/(nominal concentration)] $\times$ 100 ($n = 5$).

Table 2
Precision (CV, %) of the determination of peak areas of analytes (Aprepitant and **II**) and peak area ratios of analytes Aprepitant to the internal standard (**II**) in five different sources of plasma for samples spiked before extraction (set 3)

Nominal concentration (ng/ml)	Peak area of Aprepitant[a]		Peak area of II[a]		Peak area ratios of Aprepitant/II, CV (%)	
	API 150	API III[+]	API 150	API III[+]	API 150	API III[+]
10	2.6	2.7	4.7	1.6	5.1	3.8
25	5.8	2.2	4.0	2.0	2.3	0.7
50	4.0	2.0	4.2	3.2	4.5	3.7
100	3.0	4.2	3.4	4.3	2.6	0.9
500	2.3	8.9	2.8	1.9	0.6	8.0
1000	4.5	2.4	3.2	1.3	6.2	1.5
2500	4.0	3.6	5.1	2.3	4.5	1.6
5000	3.5	2.3	4.3	2.3	3.1	2.6
Slope[b]					0.001027	0.000811

[a] $n = 5$.
[b] Mean ($n = 5$) slope (m) calculated from the equation $y = mx + b$, where x is the concentration of the analyte, y is the peak area ratio, and b is an intercept.

Aprepitant and **II**, respectively) were obtained when the single quadrupole MS system (API 150) was used. The values >100% indicate ionization enhancement in plasma versus neat standards, whereas values <100% indicate ionization suppression. Representative slopes of the five standard lines are presented in Table 3. The "relative" matrix effect [17],

Table 3
Representative slopes for Aprepitant spiked into five different lots of control plasma

Standard curve#	Slopes	
	API III[+]	API 150
1	0.001024	0.000814
2	0.001031	0.000813
3	0.001034	0.000820
4	0.001021	0.000802
5	0.001026	0.000806
Mean	0.001027	0.000811
S.D.[a]	5.26−E06	7.07−E06
CV (%)[b]	0.5	0.9

[a] Standard deviation.
[b] Coefficient of variation (CV, %).

based on peak area ratios and/or slopes of the standard lines in different plasma lots was not observed as indicated by small coefficient of variation (<1%) of the slopes of standard lines spiked into five different sources of plasma. All these data indicated that the effect of matrix on the ionization of Aprepitant and **II** was negligible and did not have any adverse effect on the quantitation of Aprepitant.

The extraction recovery (%) was calculated by comparing the mean peak areas of the analytes spiked after extraction (set 2) divided by the mean areas of the analytes of samples spiked before extraction (set 3) and multiplied by 100. The mean recovery was 98% for Aprepitant and **II**. In addition, the overall process efficiency [17] (%) was calculated by comparing the mean peak areas of samples spiked before extraction (set 3) divided by mean peak areas of neat standards and multiplied by 100. The mean process efficiency values were within the range of 97–99% for both detectors, indicating similar overall response from analytes after extraction from the matrix to the MS/MS response of standards injected directly. The extraction recovery, matrix effect, and process efficiency data clearly confirmed the reliability of the determination of Aprepitant using both detectors.

Table 4
Assessment of freeze-thaw (F/T) stability of Aprepitant in human plasma and inter-day assay precision and accuracy

Nominal Concentration (ng/ml)	One F/T cycle, mean calculated concentration (ng/ml, $n = 5$)[a]	Two F/T cycles, mean calculated concentration (ng/ml, $n = 3$)[a]	Three F/T cycles, mean calculated concentration (ng/ml, $n = 3$)[a]	Inter-day, mean calculated concentrations (ng/ml, $n = 16$)[a,b]
30	27.3 (3.1)	27.7 (3.7)	28.6 (5.9)	29.0 (7.6)
2500	2497.8 (2.3)	2505.6 (2.0)	2500.5 (1.2)	2518.6 (5.1)
4500	4571.7 (1.0)	4622.1 (0.8)	4582.6 (0.6)	4542.2 (4.4)

[a] Numbers in parentheses are coefficients of variation (CV, %).

[b] $n = 8$ days; interday assay accuracy was 96.7, 100.7, and 100.9% at QC concentrations of 30, 2500, and 4500 ng/ml, respectively. The accuracy was defined as the [(mean observed concentration)/(nominal concentration)] × 100.

3.3. Freeze-thaw stability and inter-day assay precision and accuracy

Freeze-thaw stability was examined by exposing quality controls (QC) samples to three freeze-thaw cycles (freezer nominal temperature of $-20\,^{\circ}$C). By comparing the initial mean values at three different concentrations of QC standards after one freeze thaw cycle to the similar mean values after subsequent freeze thaw cycles, the effect of freeze thawing on the stability of Aprepitant in plasma was determined. As can be seen from Table 4, there were no significant differences ($<5\%$) in the assay concentrations following multiple freeze-thaw cycles, thus indicating analyte/sample stability. The summary results of the analyses of QC standards during the assay of samples from a clinical trial are presented in Table 4. These data show good inter-assay accuracy and precision of the method. In addition these QC standards were analyzed after one year of storage at $-20\,^{\circ}$C. Mean values ($n = 4$) were 30.1 ± 2.9, 2500.3 ± 21.2, and 4687.8 ± 128.1 ng/ml versus initial concentrations of 27.3, 2497.8, and 4571.7 ng/ml, respectively, indicating excellent stability of Aprepitant in human plasma frozen at $-20\,^{\circ}$C.

3.4. Comparison of single and triple quadrupole MS detectors for the determination of Aprepitant

The single (MS) and triple quadrupole (MS/MS) detectors were fully evaluated for precision, accuracy, recovery, and matrix effect for the analysis of human plasma samples containing Aprepitant in the concentration range of 10–5000 ng/ml. Similar results were obtained using both instruments. A large number of clinical plasma samples were successfully analyzed on both instruments, indicating the ruggedness of the methods and instrumentation.

Comparing the absolute signal response generated by the injection of the low standard onto the single and triple quadrupole instruments, the single quadrupole instrument response was approximately two times more intense. Comparing the signal-to-noise ratios of the two instruments the triple quadrupole was two times more sensitive than the single quadrupole instrument, due to the higher noise levels incurred on the single quadrupole instrument.

Results of the cross validation of MS versus MS/MS methods for the determination of Aprepitant in selected human clinical samples are presented in Fig. 5. The correlation coefficient (0.9995) of the data obtained using two different detectors clearly indicated an excellent agreement of calculated concentrations from these two systems.

Initially, an attempt was made to reduce the limit of quantification of Aprepitant to 1 ng/ml using the single quadrupole instrument. The reconstitution volume after extraction was reduced by 66% (from 1 ml to 300 µl) and the injection volume was increased by 50% (from 15 to 30 µl). These volume adjustments allowed a larger amount of analyte to be injected onto the HPLC system, which was necessary to have a low limit of quantification of 1 ng/ml of Aprepitant. The single quadrupole instrument had sufficient sensitivity to detect 1 ng/ml of neat standards, but the presence of a coeluting peak that in one plasma lot was equivalent to 10% of the signal at the LOQ, was unacceptable for the reliable determination of Aprepitant. The API III[+] with its enhanced selectivity due to the triple quadrupole design was able to detect 1 ng/ml without interference from any endogenous background. Therefore the API III[+] was used for assay validation requiring LOQ of 1 ng/ml. The precision and accuracy values at the LOQ were 3 and 101%, respectively.
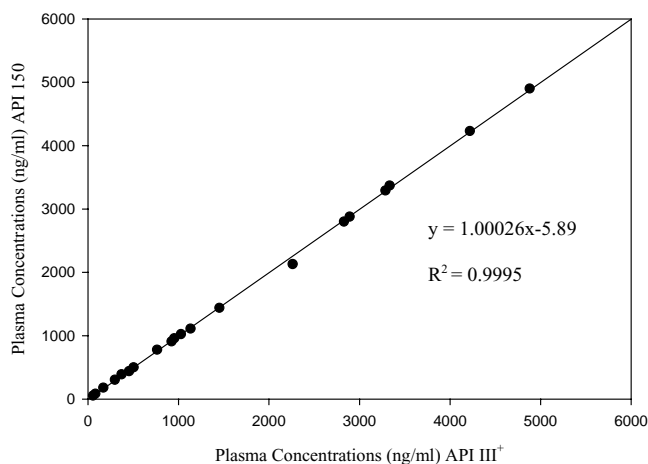

Fig. 5. Cross validation of methods for the determination of Aprepitant; plot of plasma concentrations of Aprepitant in selected human clinical samples assayed with the API III[+] (MS/MS) vs. API 150 (MS) systems.
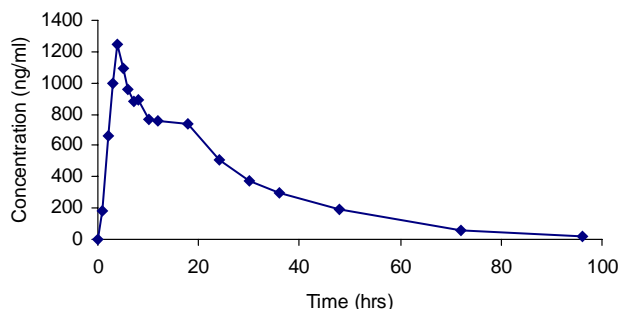
Fig. 6. A mean representative plasma concentration-time course of subjects receiving a 125 mg oral dose of Aprepitant.

3.5. Analyses of samples from clinical studies

The performance and ruggedness of the HPLC-MS/MS assay in the concentration range of 10–5000 ng/ml for Aprepitant was confirmed by analyzing plasma samples originating from over 50 clinical studies. The HPLC-MS assay was used in analyzing plasma samples from two large clinical studies. As an example, mean representative plasma concentration-time course is shown in Fig. 6 of subjects receiving a 125 mg dose of Aprepitant.

4. Conclusions

The analytical method for the determination of Aprepitant in plasma, based on liquid–liquid extraction of drug from basified biological matrix, was developed and validated. The limits of reliable quantification of Aprepitant were 1 and 10 ng/ml for the triple and single quadrupole instruments, respectively, when 1 ml of biological matrix was processed. The absence of matrix effect was demonstrated by analysis of neat standards and standards spiked into plasma extracts originating from five different sources. The methods were used to support a large-scale clinical program. The use of the single quadrupole instrument provided a lower cost alternative for the determination of Aprepitant at concentrations ≥ 10 ng/ml with similar accuracy and precision values as obtained using triple quadrupole (MS/MS) detector.

Acknowledgements

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