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A sensitive and rapid liquid chromatography-tandem mass spectrometry method for the quantification of the novel neurokinin-1 receptor antagonist aprepitant in rhesus macaque plasma, and cerebral spinal fluid, and human plasma with application in translational NeuroAIDs research

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ABSTRACT

A sensitive and rapid liquid chromatography-tandem mass spectrometry method has been developed for to assess therapeutic exposures of aprepitant in HIV-infected patients and rhesus macaques. The method utilized a simple sample-preparation procedure of protein precipitation with methanol. Chromatographic separation was performed on a reversed phase C_8 column (Hypersil Gold, 50 mm × 2.1 mm, 3 μm) using a mobile phase composed of acetonitrile and water in 0.5% formic acid through gradient elution. Electrospray ionization in positive mode was incorporated in the tandem mass spectrometric detection. The lower limit of quantitation of aprepitant in plasma of rhesus macaques and human and cerebral spinal fluid of rhesus macaques were 1, 1, and 0.1 ng/mL, respectively. The method has been successfully employed to measure aprepitant in preclinical and clinical samples collected from three SIV-infected rhesus macaques and ten patients with HIV infection. In conclusion, this liquid chromatography-tandem mass spectrometry method is suitable for preclinical–clinical translational research exploring exposure–response relationships with aprepitant as well as therapeutic drug monitoring of aprepitant.

1. Introduction

Substance P (SP) is the most abundant neurokinin in mammalian CNS and a potent modulator of neuroimmunoregulation [1]. It has been reported that SP enhances HIV infection by directly assisting virus replication in macrophages and CD4+ cells and/or indirectly influencing HIV proliferation by inducting of inflammatory cytokines (e.g., IL-1, IL-6, and TNF-α) [1]. One of the three human neurokinin receptors, neurokinin-1 receptor (NK-1R), is mainly responsible for mediating the biological responses of SP [1]. The NK-1R antagonist, aprepitant, was reported to down-regulate CCR5 receptor expression on monocyte-derived macrophages (MDM), and inhibit HIV R5 strain replication in MDM. NK-1R receptor antagonists might also reverse the impairment of NK cell function found in HIV infection via antagonism again substance P, whose effects are mediated through NK-1R receptor [2].

Aprepitant (Emend®), a neurokinin-1 receptor (NK-1R) antagonist, is licensed by the United States FDA as an antiemetic against chemotherapy-induced emesis and marketed by Merck & Co. in 2003. Currently, aprepitant is also being evaluated as a new therapy in NeuroAIDS patients from the Integrated Preclinical and Clinical Program (IPCP) grant mechanism supported by the NIH at the Children's Hospital of Philadelphia and University of Pennsylvania [2,3]. Developing sensitive bioanalytical methods to detect the exposure of aprepitant and its metabolites in biological fluids (e.g., plasma, cerebral spinal fluid [CSF]), is crucially important to facilitate pharmacokinetics and pharmacodynamics study in cell culture, simian immunodeficiency virus (SIV) infected rhesus macaques, and HIV-infected patients. Quantitation of aprepitant in human plasma has been reported using liquid–liquid extraction and HPLC-MS/MS with atmospheric-pressure chemical ionization (APCI) mass spectrometric detection [4,5]. In both of the published methods, the lower limit of quantitation (LLOQ) of aprepitant was reported as 10 ng/mL in human plasma [4,5]. In order to better characterize aprepitant in

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rhesus macaque CSF and human plasma, we incorporated protein precipitation and HPLC-MS/MS with electrospray ionization technique to develop a more sensitive and rapid bioanalytical method to quantify aprepitant in CSF and plasma. Our assay was validated in the concentration range of 0.1–10 ng/mL in CSF and 1–1000 ng/mL in plasma of rhesus macaque and human, respectively. Compared with traditional aprepitant sample-preparation procedures [4,5], including liquid–liquid extraction, nitrogen blowing-down, and reconstitution with mobile phase, time duration and efforts for sample preparation in our assays was dramatically reduced by using a simple protein precipitation procedure, which is more suitable for preparing infectious samples from HIV-infected patients in hospitals and other clinical research laboratories. This method can be applied to therapeutic drug monitoring of aprepitant in clinics.

The purpose of this research was to develop a sensitive, time- and cost-efficient HPLC-MS/MS method to determine concentrations of aprepitant in CSF and plasma of rhesus macaque and human. This is the first report describing bioanalytical methods for aprepitant quantification in CSF. The method has been applied to pharmacokinetics/pharmacodynamics (PK/PD) study of aprepitant in SIV-infected rhesus macaques and HIV-infected patients.

2. Materials and methods

2.1. Chemicals and reagents

Aprepitant was received from the Merck Research Laboratories (Rahway, NJ, USA). Internal standard (IS), quadrideuterated aprepitant (aprepitant-d4) was purchased from SynFine Research, Inc. (Ontario, Canada). HPLC grade acetonitrile and methanol (CHROMASOLV®) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (A.C.S. reagent grade, Rieldel-de Haën®) was obtained from Sigma–Aldrich Fluka (St. Louis, MO, USA). OmniSolv HPLC grade water was purchased from (EMD Chemicals, Inc., Gibbstown, NJ, USA). Rhesus macaque CSF and plasma were purchased from Bioreclamation, Inc. (East Meadow, NY, USA). Blank human plasma was obtained from blood bank at the Children's Hospital of Philadelphia (CHOP) (Philadelphia, PA, USA).

2.2. Apparatus and chromatographic-mass spectrometric conditions

Sample analysis was performed on an API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) coupled with a Shimadzu HPLC system (Shimadzu, Columbia, MD, USA) using electrospray ionization (ESI). The Shimadzu HPLC system consists of two LC-10ADVP delivery pumps, a DGU-14A vacuum degasser, and a SIL-HTC autosampler. The Shimadzu system is connected with API 3000 via a 6-port Valco valve (VICI Valco Instruments Co., Inc., Houston, TX, USA).

Chromatographic separation was conducted on a Hypersil Gold C_8 column (50 mm × 2.1 mm, 3 μm) with a Hypersil Gold C_8 guard column (10 mm × 2.1 mm, 3 μm) (Thermo Electron Corporation, Waltham, MA, USA). Compounds of interest were separated from interference using a gradient mobile phase comprised of 0.5% formic acid water (A) and acetonitrile with 0.5% formic acid (B) at a flow rate of 0.3 mL/min. The mobile phase was comprised of a 90:10 (v/v) mixture of components A and B for the first 1.5 min of each chromatographic run, increased to 98% of B in a linear gradient from 1.5 to 2.5 min, kept at 98% of B till 4.2 min, and then returned to 10% of B at 4.3 min. The equilibration time for the column with the initial mobile phase was 2.9 min. The Valco valve was programmed to divert HPLC flow to waste when data acquisition was not required.

Mass spectrometric detection was performed using an ESI source in positive mode under the following conditions: curtain gas, 12 units; nebulizer gas flow, 12 psi; turboIonSpray gas flow, 7–8 L/min; collision gas, 6 units; turboIonSpray (IS) voltage, 3500 V; entrance potential (EP), 10 V; collision energy (CE), 29 V; source temperature, 500 °C; and dwell time, 200 ms. The optimized declustering potential (DP) and collision cell exit potential (CXP), were set at 40 and 15 V, respectively. Multiple reaction monitoring (MRM) was used to detect aprepitant and IS at 535.3/277.1 and 539.3/281.1, respectively. Analytical data were acquired and integrated by Analyst software (version 1.4.2; Applied Biosystems/MDS Sciex, Toronto, Canada).

2.3. Preparation of working solutions of standards and quality control (QC) standards

Stock standard solutions of aprepitant and internal standard (1 mg/mL) were prepared in methanol. The stock standard solution of aprepitant was diluted with methanol to yield a 100 μg/mL stock solution. This solution was further diluted with solution of methanol to give a series of aprepitant working standards of 0.02 to 20 μg/mL and QC standards of 0.02, 0.05, 2, 8, and 16 μg/mL for plasma of rhesus macaque and human. Aprepitant CSF standards ranged from 2 to 200 ng/mL and QC standards contained 2, 5, 20, and 160 ng/mL for rhesus macaque. IS solution was with 1% formic acid in methanol made at 100 ng/mL for plasma samples and 10 ng/mL for CSF samples.

2.4. Preparation of standards for calibration curves and QC standards in biological matrix

Different concentrations of working solutions of standards for calibration curves and QC standards were added to blank plasma or CSF to give different sets of plasma or CSF samples for calibration curves and QC standards. The calibration curve for plasma was constructed with eight standards of aprepitant at 1, 2, 5, 25, 125, 375, 500, and 1000 ng/mL in plasma. QC standards for plasma contained four concentrations of aprepitant at 2.5, 100, 400, and 800 ng/mL in plasma. For CSF samples of rhesus macaque, calibration curves was built with six standards at 0.1, 0.2, 0.5, 1.25, 5, and 10 ng/mL; QC standards were made at 0.25, 1, and 8 ng/mL.

2.5. Sample collection

The rhesus macaque blood and CSF samples were collected from three SIV-infected rhesus macaques in Tulane National Primate Research Center (Covington, LA, USA). Blood samples were drawn at 0, 1, 2, 4, 8, and 12 h on days 1, 7, and 14, respectively, when macaques were orally administrated with 80 mg- or 125 mg-capsules of aprepitant (Emend®, Merck & Co., Inc., Whitehouse Station, NJ, USA) daily. CSF samples were collected at trough level on days 1, 7, and 14 before oral administration of aprepitant in rhesus macaques.

Human blood samples were collected from HIV-infected patients recruited with age no less than 18 years old at School of Medicine University of Pennsylvania (Philadelphia, PA, USA). Human blood samples were collected at 0, 0.5, 1, 2, 4, and 8 h, respectively, on days 1 and 14 following capsule doses of 125 mg- or 250 mg-aprepitant was orally administered to each HIV-infected patient daily. Trough level blood was obtained on days 3, 7, and 10 as well for all the patients. Generally, blood and CSF samples were stored in the tubes containing heparin as the anticoagulant, then centrifuged (within 2 h from collection) at 1800 g for 15 min at 4 °C.

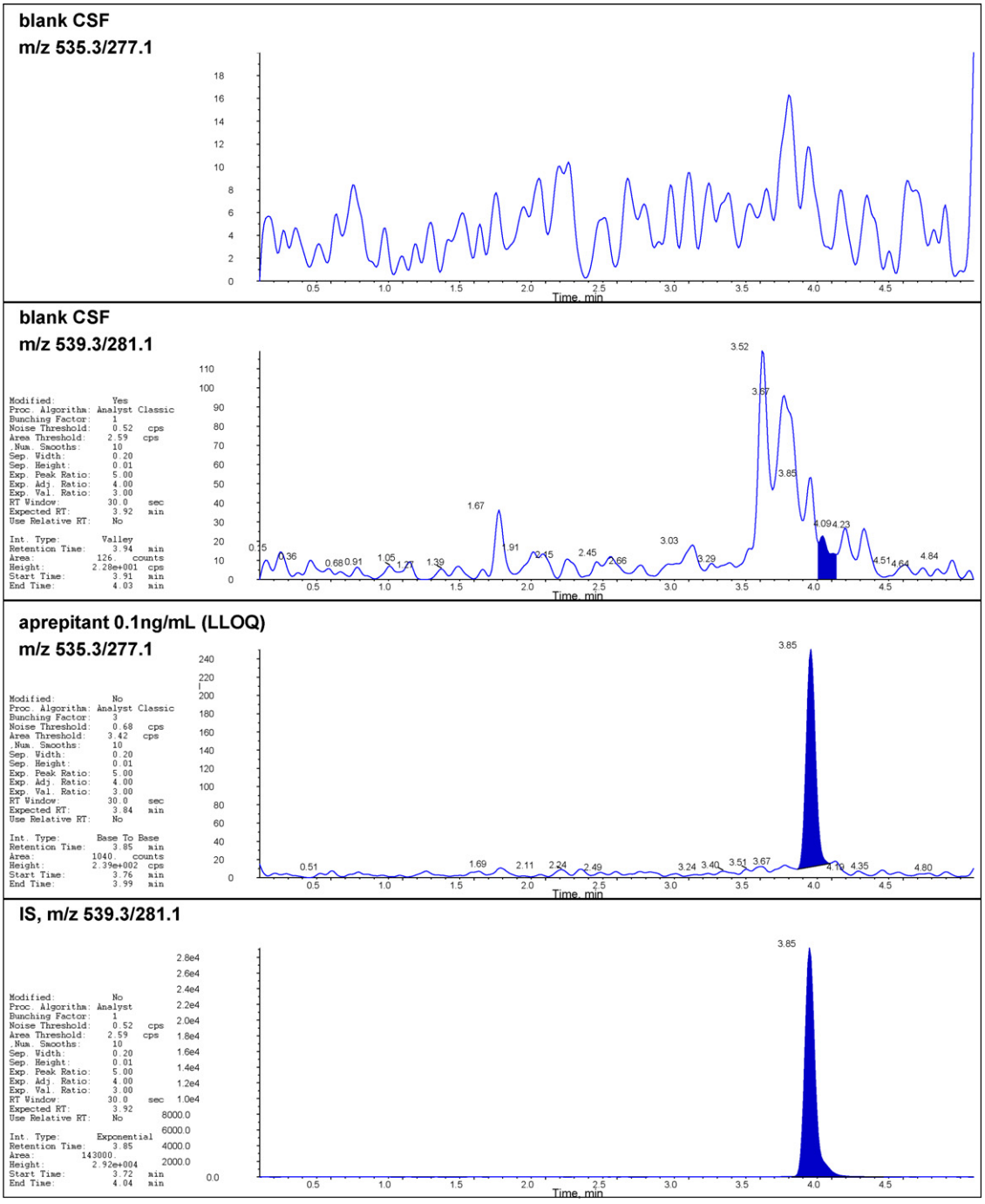

Fig. 1. Representative chromatogram of aprepitant in CSF.

The supernatant was then transferred to polypropylene tubes and stored at −70 °C until LC-MS/MS analysis.

2.6. Sample preparation

2.6.1. Plasma samples

One hundred microliters of plasma standards (standard curve), quality control standards, and/or rhesus macaque or human samples were added into individual 2-mL centrifuge tubes (VWR, West Chester, PA, USA). After 300 μL of IS solution was added into each tube except tubes containing blank plasma and methanol with 1% formic acid, tubes were capped and vortexed for 3 min, then centrifuged at 17,390 g for 10 min. Three hundred microliters of supernatant was transferred into an HPLC insert for LC-MS/MS analysis. Five microliters of supernatant was injected into the LC-MS/MS system.

2.6.2. CSF samples

Fifty microliters of CSF standards (standard curve), quality control standards, and/or rhesus macaque samples were added into individual 2-mL centrifuge tubes (VWR, West Chester, PA, USA). After 150 μL of IS solution was added into each tube except tubes containing blank plasma and methanol with 1% formic acid, the same procedures were followed as described in the plasma sample preparation. Finally, 150 μL of supernatant was transferred into an HPLC insert for LC-MS/MS analysis. Thirty microliters of supernatant was injected into the LC-MS/MS system.

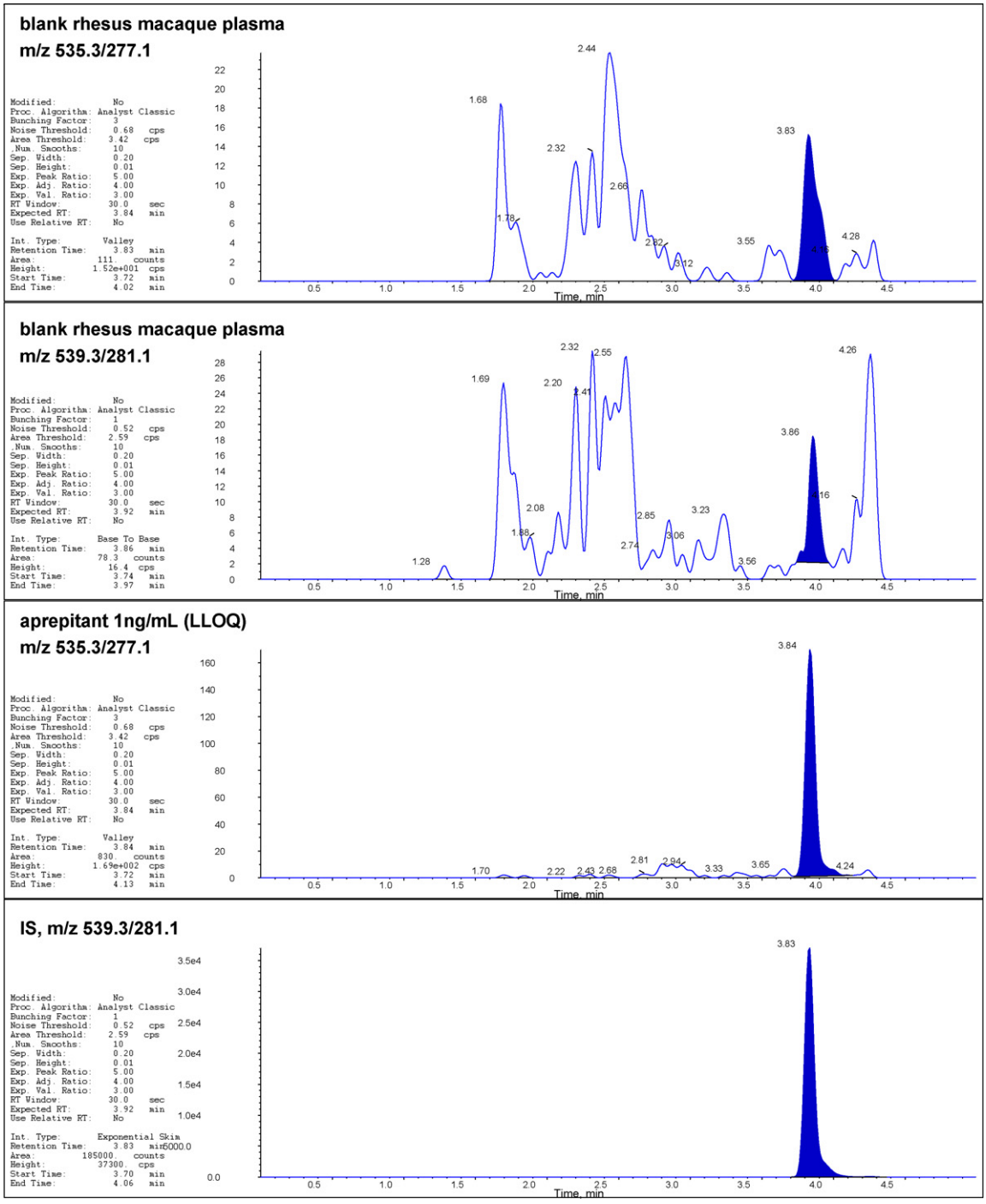

Fig. 2. Representative chromatogram of aprepitant in rhesus macaque plasma.

2.7. Method validation

Method validation was conducted in accordance with bioanalytical method validation guidelines for industry enacted by U.S. FDA [6]. Blank biological matrices were extracted without fortification of analyte and IS to determine the extent to which endogenous substances are comprised of the interference at the retention time and precursor/fragment ion values of the analyte and IS. Chromatograms were evaluated by a unique combination of retention time, precursor, and fragment ions for both the analyte and IS. The limit of detection (LOD) is typically determined as a signal to noise ratio of 5.

QC standards and LLOQ in both plasma and CSF, were subjected to preparation procedures described above, and injected into LC-MS/MS. The assays described above were repeated five times within the same day to obtain intra-day precision and over three different days to obtain inter-day precision, both expressed as a percentage of relative standard deviation (RSD) values.

2.8. Calibration and accuracy

Calibration curves were constructed with corresponding sets of standards in biological matrix (plasma or CSF) described in Preparation of Standards for Calibration Curves and QC Standards in

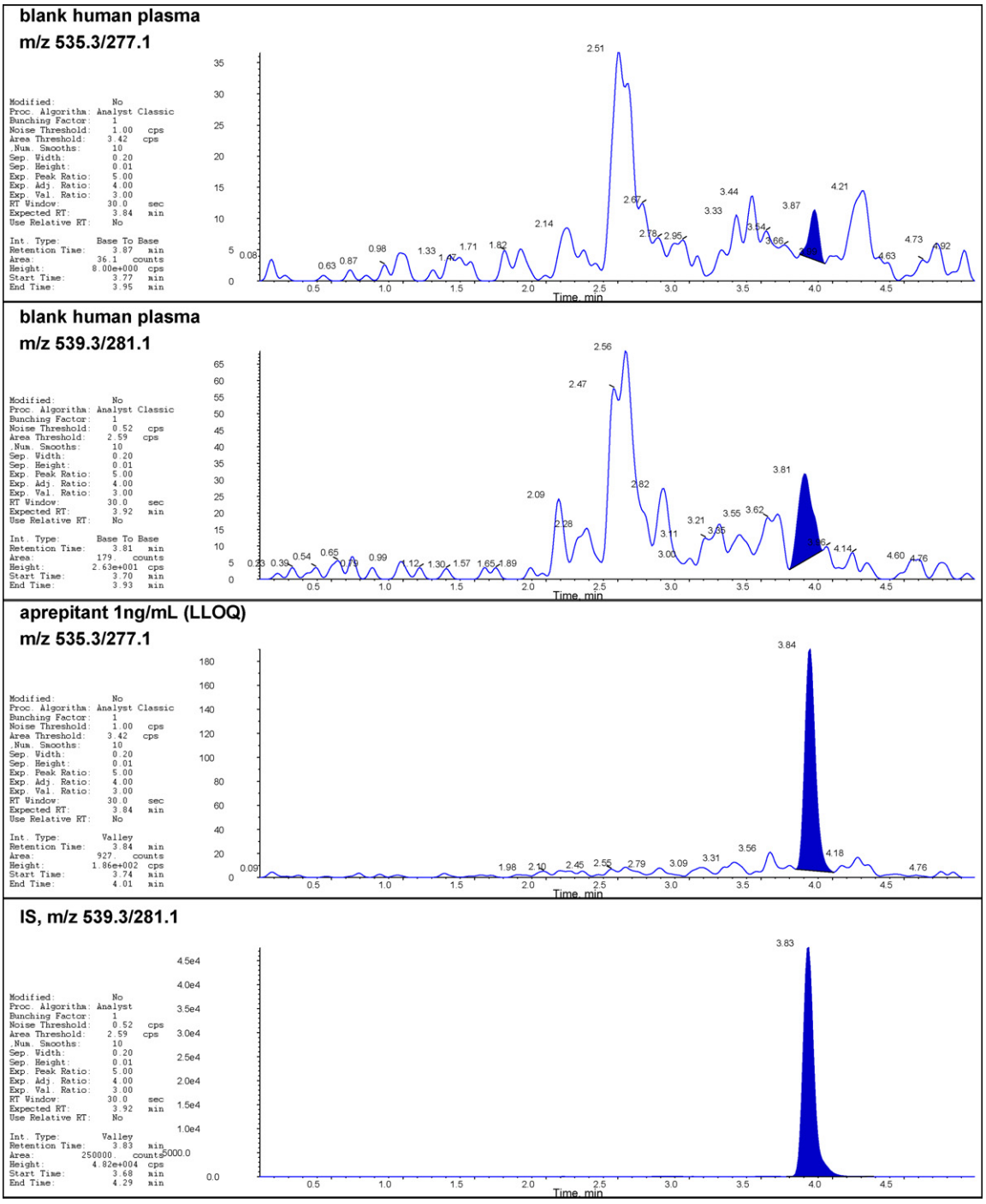

Fig. 3. Representative chromatogram of aprepitant in human plasma.

Biological Matrix. QC standards were run with calibration curves to ensure the quality of sample analysis. Sample preparation and LC-MS/MS analysis were performed in triplicate for each data point. The analyte/IS peak area ratios obtained were plotted against the corresponding concentrations of the analytes. Calibration curves were constructed using a weighted $1/x^2$ linear regression. The values of LLOQ were calculated, according to FDA guidelines of bio-analytical method validation for industry, as the analyte response should be at least five times the response compared to blank response [6]. QC standards in corresponding biological matrix were used to calculate accuracy according to the following equation: $100 \times$ [predicted concentration–nominal concentration]/nominal concentration.

3. Results and discussion

3.1. Choice of chromatographic and sample preparation conditions

Due to high lipophilicity of aprepitant, C_8 HPLC columns was chosen instead of commonly selected C_{18} columns where it takes much higher percentage of organic solution and longer time to elute aprepitant and thereby incurs coelution with endogenous substances. Additionally, symmetric peaks are displayed for the basic compound of aprepitant on Hypersil columns, compared with tailing peaks exhibited in several other brands of columns chosen during the method development.

Table 1
Accuracy and precision of aprepitant in biological LLOQ & QC Standards matrices.

LLOQ and QC standards (ng/mL)	Accuracy %	Intra-day precision (RSD%, $n=5$)	Inter-day precision (RSD%, $n=3$)
Rhesus macaque plasma			
1	106	4.21	13.2
2.5	95.2	4.56	8.88
100	91.0	1.83	2.43
400	96.1	2.32	1.94
800	95.3	2.01	1.87
Rhesus macaque CSF			
0.1	92.1	5.29	7.74
0.25	96.9	2.72	5.73
1	96.7	2.25	3.52
8	99.9	2.21	3.02
Human plasma			
1	98.1	6.71	9.61
2.5	99.7	8.66	10.4
100	96	1.57	2.71
400	95.1	2.17	2.4
800	93.9	2.34	4.38

Two functions are involved in IS solution: one is to add IS to correct errors occurred during the process of extraction and sample injection on LC-MS/MS; the other is to extract aprepitant out of biological matrix with maximum recovery and precipitate protein. Addition of formic acid in the IS solution facilitated the extraction of aprepitant and IS with high recovery rate from the biological matrix due to the basic characteristics of aprepitant. Percentage of formic acid added in IS solution has been tested and 1% formic acid served the best of extraction rate without compromising the integrity of aprepitant compound in the biological matrix and processed samples for LC-MS/MS analysis. Compared with liquid–liquid extraction procedures for aprepitant in human plasma samples published [4,5], the protein precipitation procedure we applied dramatically reduced time, equipments and materials, and human power in this otherwise traditionally time- and effort-consuming part of bioanalysis. In addition, this simple sample preparation procedure provides greater benefits in terms of safety and efficiency when preparing clinical samples from HIV-infected patients.

3.2. Method validation

The peak areas of aprepitant at the LLOQ were at least five times greater than those of interference substances in all three biological matrices examined. No interference of aprepitant was observed in blank rhesus macaque CSF. The LOD was 0.05, 0.5, and 0.5 ng/mL for aprepitant in rhesus macaque CSF, rhesus macaque plasma, and human plasma, respectively.

Calibration curves were set up in three biological matrices. Good linearity ($r^2 > 0.9962$) was found in calibration ranges of aprepitant in five different lots of all three biological matrices, demonstrating linearity over the entire standard curve range. The LLOQ was 0.1, 1, and 1 ng/mL for aprepitant in rhesus macaque CSF, rhesus macaque plasma, and human plasma, respectively (Figs. 1–3). Typical equations for the calibration curves for aprepitant was $y = 0.0455x + 0.000714$ (rhesus macaque CSF), $y = 0.00414x + 0.000645$ (rhesus macaque plasma), $y = 0.0041x + 0.00043$ (human plasma), respectively.

Accuracy and precision assays were carried out using LLOQ and QC standards. The results of these assays are given in Table 1. The RSD values of precision assays were lower than 13.2%.

Table 2
Representative standard curve slopes for aprepitant spiked into five different lots of biological Calibration matrices.

Calibration curve	Slope		
	Rhesus macaque CSF	Rhesus macaque plasma	Human plasma
1	0.0455	0.00408	0.00385
2	0.0439	0.00414	0.00388
3	0.0445	0.00429	0.00384
4	0.0446	0.00406	0.0041
5	0.0448	0.00405	0.0043
Mean	0.0447	0.00412	0.00399
Standard deviation	0.000577	0.0000991	0.000201
CV%	1.29	2.4	5.04

3.3. Carryover and matrix effects

The potential for carryover effect was investigated by injecting a sequence of the at least three successive aliquots of extracted plasma/CSF samples containing the highest calibration concentration (i.e., 1000 ng/mL for plasma; 100 ng/mL for CSF) of aprepitant in standard curves into LC-MS/MS system followed by at least three successive aliquots of extracted drug-free plasma/CSF sample. The residual concentration found in the first extracted drug-free plasma/CSF sample following an extracted sample at the highest concentration was used to calculate the carryover rate. The carryover effect was calculated as less than 0.1% of the highest calibration concentration in corresponding biological matrix.

The potential for matrix effects was tested by comparing the peak area of aprepitant from plasma/CSF samples spiked after the protein precipitation with the analogous peak areas obtained by directly injecting the neat standards among the different sources of plasma/CSF samples. Absolute matrix effect with the range of 96–102% was determined in plasma/CSF, when comparing difference between peak areas of both analyte and IS and/or peak area ratios of the samples spiked after the protein precipitation and those of the neat standards. In addition, the matrix effect was not observed as indicated by small coefficient of variation (<5.5%) of the slopes of the calibration curves in different lots of plasma and CSF (Table 2). As such, the protein precipitation procedure coupled with suitable chromatographic conditions ensured no matrix effect among different lots of plasma/CSF. The overall process recovery (%) was calculated by comparing the mean peak areas of aprepitant spiked before protein precipitation or mean peak area ratio with IS divided by mean peak areas of neat standards or mean peak area ratio with IS and then multiplied by 100. The mean values of overall process recovery were within the range of 94–103%.

3.4. Stability of aprepitant

There was no significant difference in assay concentrations for processed samples from an analytical run, including non-zero standards, a blank, and a control, and all QC standards, after stored in the HPLC autosampler set at 4 °C or in a refrigerator for at least 24 h. No significant difference in aprepitant concentrations was observed for the rhesus macaque plasma and CSF samples of aprepitant stored in −70 °C for two years and human plasma samples stored in −70 °C for one year.

3.5. Applications

The validated method has been utilized in a PK/PD studies in rhesus macaques and HIV-infected patients. The analysis of plasma and CSF samples from a rhesus macaque and a patient with HIV infection are given in Figs. 4 and 5 respectively. The method continues to provide reliable data in an ongoing Phase IB PK/PD/safety

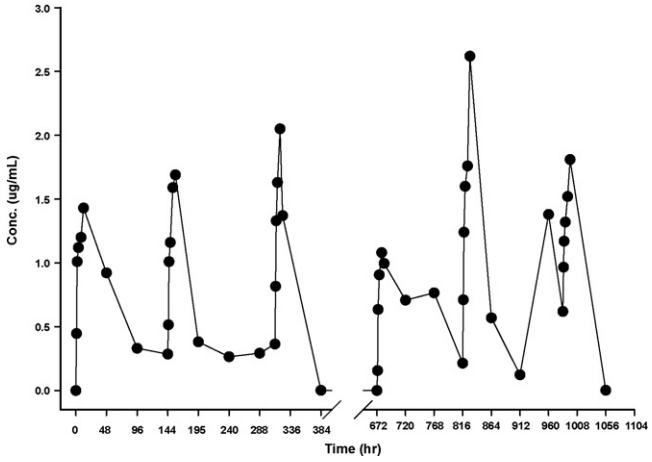

Fig. 4. Representative PK profile of aprepitant in a SIV-infected rhesus macaque following oral administration.

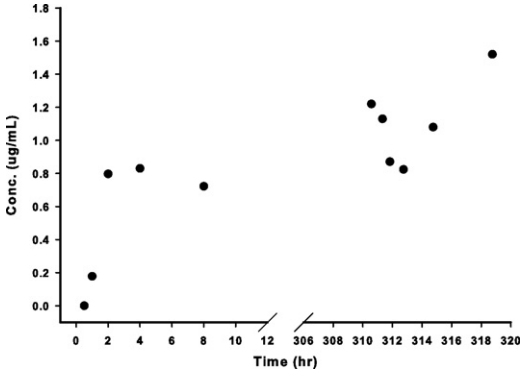

Fig. 5. Representative plasma concentration-time profile of aprepitant in an HIV-infected patient following oral administration.

trial in Neuro-AIDS patients. We expect to use these results to evaluate aprepitant exposure–response characteristics based on its immunologic, virologic, and CNS-mediated antipsychotic effects. Allometric scaling to facilitate preclinical-to-clinical bridging and the animal disease model and dose optimization modeling to guide future clinical investigation are ongoing efforts in our laboratory reliant on this data.

4. Conclusion

The LC-MS/MS method developed here is sensitive and rapid using a simple sample preparation method of protein precipitation. When compared with the results obtained from previous published LC-MS/MS analyses for aprepitant quantification, the present method allows the determination of aprepitant at lower concentrations (LLOQ = 1 ng/mL instead of LLOQ = 10 ng/mL in plasma; LLOQ = 0.1 ng/mL in CSF, first time reported). In addition, our simple sample preparation procedure of protein precipitation, if compared with published sample pretreatment procedure using liquid-liquid extraction procedure for aprepitant, demonstrated better results in terms of precision and extraction yield with lower consumption of organic solvent/materials and equipment, time, and human capital. Furthermore, a small volume of plasma (100 μL) or CSF (50 μL) was required for this method, thus reducing the amount of the blood and CSF needed for PK study and minimizing collection difficulties in HIV-infected patients and SIV-infected rhesus macaques, especially with CSF sample collection.

In conclusion, this method has been shown to have good precision, high accuracy, and satisfactory stability for aprepitant detection in biological matrices. It is well suited for cell culture, PK/PD and metabolism study of aprepitant in animals and humans. Due to its simplicity, accuracy, and efficiency, this method can be well applied to clinical settings where therapeutic drug monitoring in patients treated with aprepitant is warranted.

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