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Determination of Aprepitant in Human Plasma by Using LC-MS/MS with Electrospray Ionization

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Abstract

A precise, sensitive and high throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determination of Aprepitant (APT) in human plasma was developed and validated using Quetiapine (QTP) as internal standard. The analyte and internal standard were extracted from human plasma using liquid-liquid extraction. Chromatographic separation was performed on Discovery C18 10 cm×4.6 mm, 5 µm column with an isocratic mobile phase composed of 5 mM Ammonium Acetate (pH 4.00):Acetonitrile (10:90), at a flow-rate of 0.9 ml/min. The MS-MS detection was performed on a AB Sciex API 3200 tandem mass spectrometer operated in Multiple reaction monitoring (MRM) at positive mode at m/z 535.10/277.10 and 384.00/253.10 for APT and QTP respectively. A linear dynamic range of 10.004-5001.952 ng/ml for APT was evaluated with mean correlation coefficient (r) of 0.9991. The precision of the assay (expressed as coefficient of variation, CV) was less than 15% at concentrations of LQC, MQC, HQC and was less than 20% for LLOQQC. Percent recoveries for APT at high, middle and low quality control samples was found to be 71.9%, 68.0%, and 63.8% respectively and for internal standard 77.7%. The analyte was found to be stable throughout five freeze-thawing cycles, bench top, wet extract, dry extract, auto sampler and interim stability studies. Therefore, the proposed method was found to be suitable for the routine quality control analysis of Aprepitant in human plasma in bioequivalence studies.

Keywords: Aprepitant; Quetiapine; LC-MS/MS; Validation

Introduction

Aprepitant (APT) is chemically (5-[[2(R)-[1(R)-(3,5-bistrifluoromethylphenyl)ethoxy]-3(S)-(4-fluorophenyl) morpholin-4-yl]methyl]-2,4-dihydro-[1,2,4]triazol-3-one) and belongs to class of substance P antagonists (SPA). It acts as an anti-emetic by blocking the neurokinin 1 (NK_1) receptor. NK_1 is a G protein-coupled receptor which is located in the central and peripheral nervous system having a dominant ligand known as substance P (SP). SP is found in high concentrations in the vomiting centre of brain and results in vomiting when activated. APT crosses blood-brain barrier and blocks SP by occupying NK_1 receptors in brain neurons [1]. Thus, APT is used for the prevention of acute, delayed chemotherapy-induced and postoperative nausea and vomiting. It has little or no affinity towards 5-HT_3 receptors but it is shown to increase the activity of 5-HT_3 receptor antagonists such as ondansetron and the corticosteroid dexamethasone, which are also used to prevent nausea and vomiting caused by chemotherapy [2]. It has been recently demonstrated that substance P (SP) and neurokinin -1 (NK1) receptor antagonists induce cell proliferation and cell inhibition in human melanoma cells [3]. NK_1 receptor antagonists might also reverse the impairment of NK cell function found in HIV infection via antagonism against SP, whose effects are mediated through NK_1 receptor [4]. Currently, APT is also under evaluation as a new therapy in Neuro AIDS 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 [5].

Literature review reveals that very few analytical methods have been established for the estimation of APT. Estimation of impurities and Diastereomers in APT bulk drug substance [6], Characterization and Quantization of APT drug substance polymorphs by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy [7], stability of an extemporaneous oral liquid APT formulation [8], estimation of APT capsules by RP-HPLC [9], Stability-indicating HPLC method for quantitative analysis of APT [3,10] were reported. Metabolic disposition of Aprepitant in rats and dogs was also reported [11]. Estimation of APT in rhesus macaque plasma, cerebral spinal fluid and in human plasma by LC-MS method was reported [12]. Other liquid chromatography and tandem mass spectroscopy methods for determination of APT in human plasma were also recently reported [13,14]. The present study describes development and validation of a simple, specific, rapid and sensitive liquid chromatography - tandem mass spectrometry (LC-MS/MS) method for the determination of Aprepitant in human plasma with a limit of quantification (LOQ) of 10.004 ng/ml during a 2.5 min run time using QTP as internal standard. The structures of Aprepitant and Quetiapine are displayed in figure 1.

Materials and Methods

Reagents and chemicals

Aprepitant (APT) (99.21% purity), was obtained from Roorkee Research & Analytical Labs Pvt.Ltd. and Quetiapine (QTP) (99.56% purity) from Splendid Labs Pvt.Ltd., Pune, India. Methanol of HPLC grade obtained from Merck, Mumbai India. Acetonitrile and Tertiary Butyl Methyl Ether (TBME) of HPLC grade, Ammonium Acetate,

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Formic acid and Ammonia of GR/AR grade were purchased from Fisher scientific Pvt. Ltd., Mumbai, India. High purity water was prepared through a Milli-Q water purification system.

Instrumentation

LC-MS/MS analysis was performed using API 3200 triple quadrupole instrument (Applied Biosystems SCIEX, Toronto, Canada) coupled with Shimadzu HPLC system (Shimadzu SIL HTC, USA) in multiple reaction monitoring (MRM) mode. Electron spray ionization in positive mode was used for ionization. Data processing was performed on Analyst software version 1.5.1. A high-speed desk centrifuge Sorvall Legend XTR Thermo Scientific was used to centrifuge the samples. Ultra microbalance SE2 of Sartorius and Semi Microbalance CPA225D of Sartorius was used for weighing the samples.

MS/MS conditions

The APT and QTP had Multiple reaction monitoring (MRM) at m/z 535.10/277.10 and 384.00/253.10 respectively. The tuned MS/MS conditions of APT and QTP were represented in table 1. The mass spectrum of drug and IS are displayed in figures 2 and 3.

Chromatographic conditions

The separation of the compounds was made on Discovery C18 10 cm×4.6 mm, 5 µm column. A mixture of 5 mM Ammonium Acetate (pH 4.00): Acetonitrile (10:90) was used as mobile phase and was filtered through 0.45 µ membrane filters before use and degassed in an ultrasonic bath. All analysis was performed under isocratic condition at a flow rate of 0.9 ml/min at ambient temperature. The sample volume injected was 10 µl with run time of 2.5 min. Under the chromatographic conditions described above, both APT and QTP were eluted with retention times of 1.55 min and 1.70 min (Figure 4).

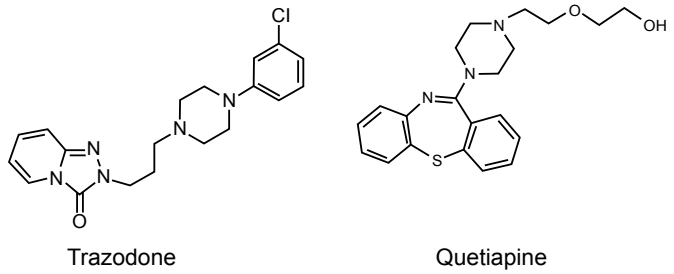

Figure 1: Structures of Trazodone and Quetiapine.

PARAMETER	APREPITANT	QUETIAPINE
Declustering potential (DP)	30	40
Entrance potential (EP)	10	10
Collision cell entrance potential (CEP)	26.37	21.38
Collision energy (CE)	55	
Collision cell exit potential (CXP)	5	
Curtain gas (CUR)	30	
Collision associated dissociation (CAD)	6	
Ion spray voltage (ISV)	4500	
Heater temperature (TEM)	475.00°C	
Nebulizer gas (GS1)	40	
Heater gas (GS2)	45	

Table 1: Tuned MS/MS conditions of APT and QTP.

Preparation of standards and quality control samples

The primary stock solutions of APT and QTP of 1000 µg/ml were prepared in methanol. The stock solution of internal standard was diluted to concentration of 400 ng/ml by using diluent 50% v/v methanol in water. The stock solution of APT was then serially diluted with 50% v/v methanol in water to provide working standard solution of desired concentration. Standard stock solutions of APT were added to drug free human plasma to obtain calibration curve concentration levels of 10.004, 20.008, 500.195, 1508.429, 2432.950, 3201.249, 4001.562 and 5001.952 ng/ml. In a similar way, spiking of aqueous quality control dilutions was done in human plasma to prepare the quality control samples of concentrations 10.576 ng/ml (LLOQ QC), 25.181 ng/ml (LQC), 2098.448 ng/ml (MQC) and 3605.580 ng/ml (HQC). Primary stock solutions were kept at 2-8°C when not in use. All matrix based samples shall be stored in the deep freezer at –70°C ± 15°C.

Sample preparation method

Required set of calibration curve standards and QC's were withdrawn from deep freezer and allowed them to thaw at room temperature. Thawed samples were vortexed to ensure complete mixing of contents. 50 µl of internal standard solution (400 ng/ml) was taken into a RIA vial tube and 300 µl of plasma sample was added to it and vortexed. 100 µl of 2.0% (v/v) Ammonia solution was added to the above RIA vial and vortexed. To it 2.5 ml of the Tertiary Butyl Methyl Ether (TBME) solution was added and vortexed at 2000 rpm for about 10 minutes. The samples were centrifuged for 10 min at 4000 rpm at 10°C. 2.0 ml of supernatant layer from the centrifuged samples was taken into separate RIA vial. The samples were evaporated until dryness under the Nitrogen evaporator with 50°C of temperature. The samples were reconstituted with 300 µl of mobile phase. Then the samples were transferred into auto injector and 10 µl of sample was injected.

By using above materials and methods validation parameter were performed (i.e Auto sample carryover, Matrix effect, Precision and Accuracy, recovery and stability in matrix etc.)

Results and Discussion

Method validation

Validation runs were conducted according to the guidance of USFDA [15], on six consecutive days. Each validation run consisted of a minimum of one set of calibration standards and six sets of QC plasma samples at four concentrations of LLOQ QC, LQC, MQC and HQC.

Auto sample carryover and Matrix effect with IS normalizing factor

There was no carryover was observed by using optimised above LC-MS/MS and sample processing condition. Matrix ion suppression or enhancement effects on the MRM LC-MS/MS sensitivity were evaluated by the post extraction sicked samples. Different plasma lot Ob blank was extracted according to our sample extraction procedure. And then spiked Th analyte and internal standard into these matrixes. From the tach lot, three samples at two concentration (HQC and LQC) level were prepared. All these extracted and post extract spicked were analyzed by LC-MS/MS. It was observing matrix effect and IS normalizing factor for both analyte and internal standard accuracy ± 15%. The results were represented in tables 2a-2d.

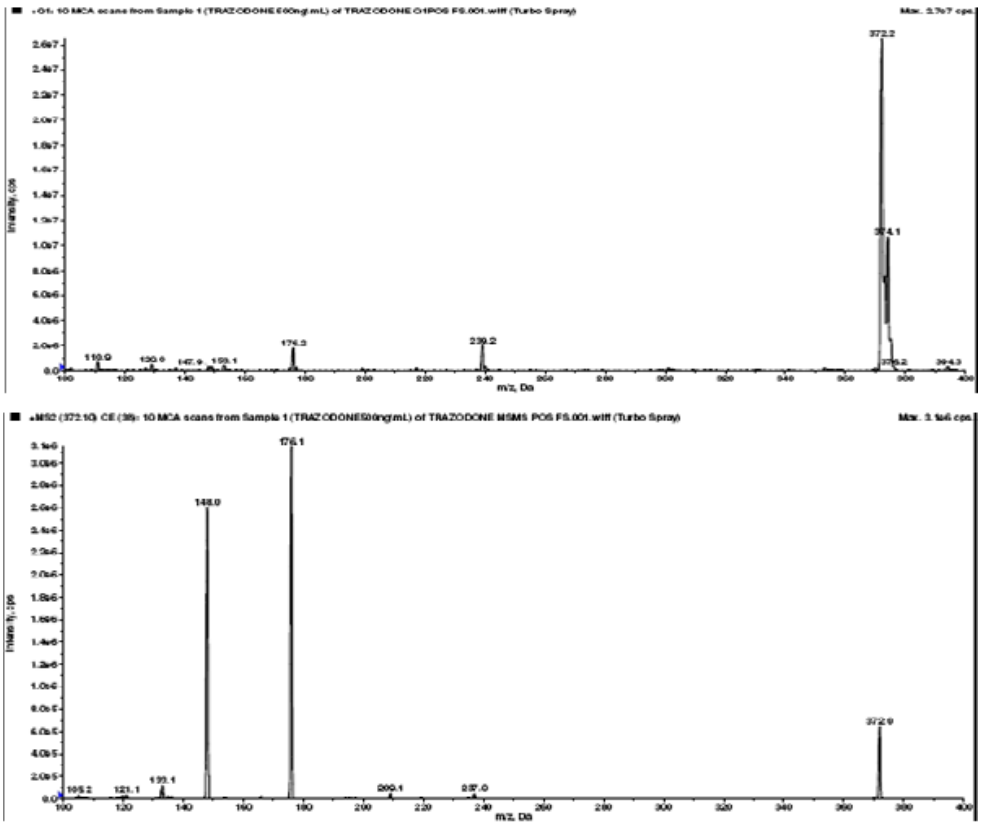

Figure 2: Full scan mass spectrums of Trazodone parent ion and product ion.

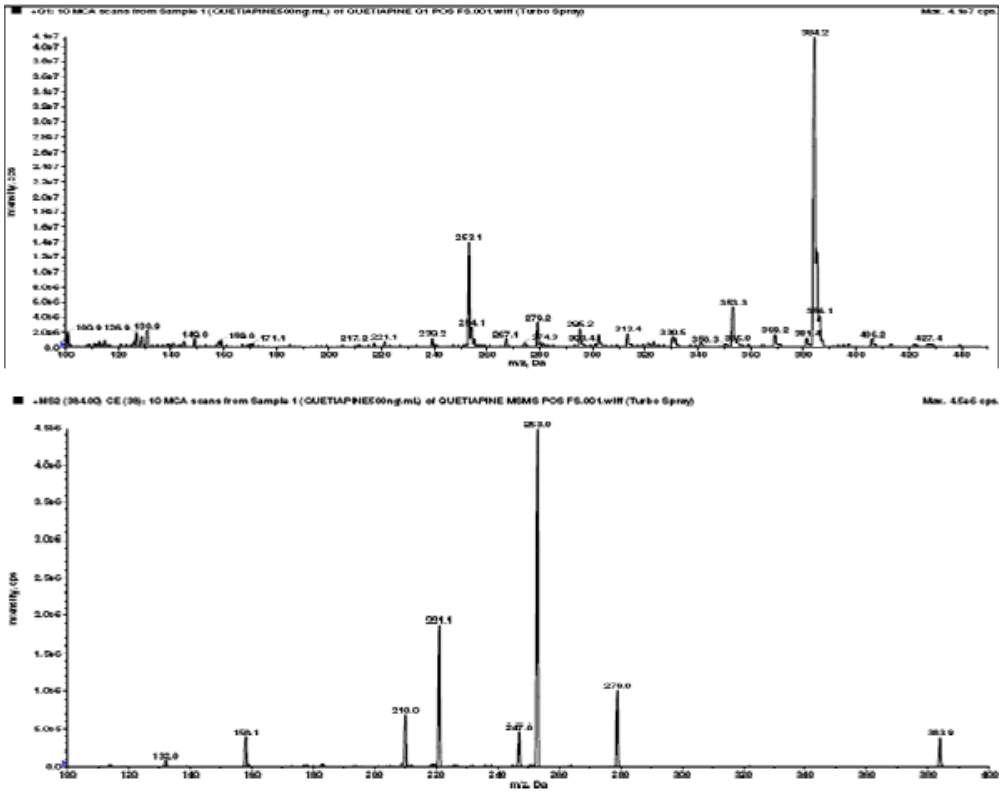

Figure 3: Full scan mass spectrums of Quetiapine parent ion and product ion.

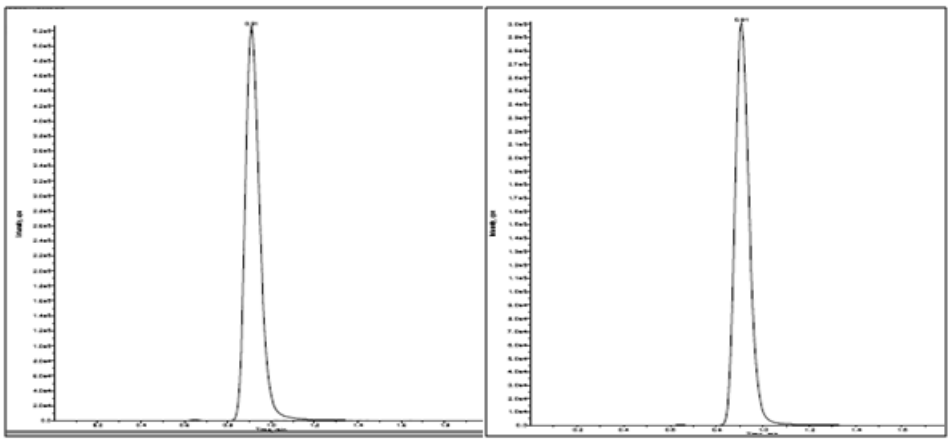

Figure 4: Retention time of Trazodone and Quetiapine.

Matrix ID	HPM/130/12		HPM/131/12		HPM/133/12		HPM/134/12		HPM/164/12		HPM/165/12	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
	614429	11790	621408	12122	619934	11708	616694	11386	605133	11253	583671	11043
	623405	11773	605263	11195	604992	10925	582814	11269	618123	11527	590071	10717
	608829	11491	615390	11573	603769	11461	621443	11642	594887	10677	603499	10866
N	3	3	3	3	3	3	3	3	3	3	3	3
Average	615554.3	11684.7	614020.3	11630	609565	11364.7	606983.7	11432.3	606047.7	11152.3	592413.7	10875.3
Standard Deviation	7352.87	167.94	8159.18	466.12	9000.61	400.29	21065.8	190.77	11644.97	433.85	10119.46	163.2
CV (%)	1.2	1.4	1.3	4.0	1.5	3.5	3.5	1.7	1.9	3.9	1.7	1.5

Table 2a: Results for matrix effect of Aprepitant.

Matrix ID	HPM/130/12		HPM/131/12		HPM/133/12		HPM/134/12		HPM/164/12		HPM/165/12	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
	157796	142794	154361	143363	157647	144131	156453	142050	150725	145502	155087	134696
	154518	141267	151610	138063	157484	136738	153487	137155	151886	138790	149717	132549
	154836	138796	157496	138351	155391	140020	155092	140002	153526	129473	152937	130175
N	3	3	3	3	3	3	3	3	3	3	3	3
Average	155716.7	140952.3	154489	139925.7	156840.7	140296.3	155010.7	139735.7	152045.7	137921.7	152580.3	132473.3
Standard Deviation	1807.76	2017.49	2945.09	2980.3	1258.09	3704.24	1484.67	2458.34	1407.31	8049.7	2702.71	2261.45
CV (%)	1.2	1.4	1.9	2.1	0.8	2.6	1	1.8	0.9	5.8	1.8	1.7

Table 2b: Results for matrix effect of Quetiapine.

PLASMA ID	Aprepitant			Quetiapine			IS Normalized Factor
	EXT	AQS	MF	EXT	AQS	MF	
HPM/130/12	615554.3	581308	1.06	155716.7	146851	1.06	1.01
HPM/131/12	614020.3	587921	1.06	154489	148046	1.05	1.01
HPM/133/12	609565.0	583943	1.05	156840.7	147853	1.07	1.00
HPM/134/12	606983.7	578207	1.05	155010.7	148541	1.05	1.00
HPM/164/12	606047.7	580871	1.04	152045.7	145224	1.03	0.99
HPM/165/12	592413.7	570523	1.02	152580.3	146949	1.04	0.97
Average	607430.78	580462.17	1.047	154447.18	147244	1.05	0.997
Standard deviation	8265.515	5873.769	0.0151	1839.145	1184.75	0.0141	0.0151
%CV	1.4	1.0	1.4	1.2	0.8	1.3	1.5

Table 2c: Results for IS Normalized factor at HQC level.

Linearity and Lower Limits of Quantification (LLOQ)

Calibration curves were prepared by assaying plasma samples at eight concentrations of APT ranging 10.004 - 5001.952 ng/ml with correlation coefficient (r) of 0.9991 (Figure 5). The linearity of each calibration curve was determined by plotting the peak area ratio(y) of APT to QTP versus the nominal concentration (x) of ATP. The calibration curves were constructed by weighed ($1/x^2$) least square linear regression. The limit of detection was defined (3.3 ng/ml), as analyte responses are at least five times the response compared to blank responses. The lowest standard on the calibration curve 10.004 ng/ml was defined as limit of quantification since the analyte peak was identifiable, discrete and reproducible with a precision of less than or equal to 20% and accuracy of 80-120% (Figure 6).

Precision and accuracy

The method precision and accuracy were evaluated by using replicate analysis of QC's at four concentrations of LLOQQC, LQC, MQC and HQC. Intra-day evaluation was done on the same day, whereas inter-day was done on consecutive days. Inter day batch accuracy ranged from 90.6% to 106.3%. Inter day batch precision ranged from 2.4% to 10.8%. Intraday batch accuracy ranged from 91.5% to 103.7%. Intraday batch precision ranged from 2.0% to 7.9%. The mean concentration, standard deviation (SD), coefficient of variation (%CV) was evaluated and their results were tabulated in tables 3a and 3b.

Extraction recovery

Recovery analysis was repeated for six replicates at three concentrations (LQC, MQC and HQC). The extraction recovery of APT from spiked samples were determined by comparing the peak areas of analytes or internal standard in extracted samples to the corresponding peak areas of analytes or internal standard in post extracted spiked samples (extracted blank samples followed by spiking analyte and internal standard at a concentration level equivalent to 100% recovery). The results were represented in table 4.

Re-injection reproducibility

The Re injection Reproducibility evaluation is done by comparing the results of re-injected set of samples with that of the original set and results were represented in table 5.

Stability studies

Stability studies were carried out for stock solutions, stock dilutions and spiking solutions by comparing fresh stock and stability stock. Six

PLASMA ID	Aprepitant			Quetiapine			IS Normalized Factor
	EXT	AQS	MF	EXT	AQS	MF	
HPM/130/12	615554.3	581308	1.06	155716.7	146851	1.06	1.01
HPM/131/12	614020.3	587921	1.06	154489	148046	1.05	1.01
HPM/133/12	609565.0	583943	1.05	156840.7	147853	1.07	1.00
HPM/134/12	606983.7	578207	1.05	155010.7	148541	1.05	1.00
HPM/164/12	606047.7	580871	1.04	152045.7	145224	1.03	0.99
HPM/165/12	592413.7	570523	1.02	152580.3	146949	1.04	0.97
Average	607430.78	580462.17	1.047	154447.18	147244	1.05	0.997
Standard deviation	8265.515	5873.769	0.0151	1839.145	1184.75	0.0141	0.0151
%CV	1.4	1	1.4	1.2	0.8	1.3	1.5

Table 2d: Results for IS Normalized factor at LQC level.

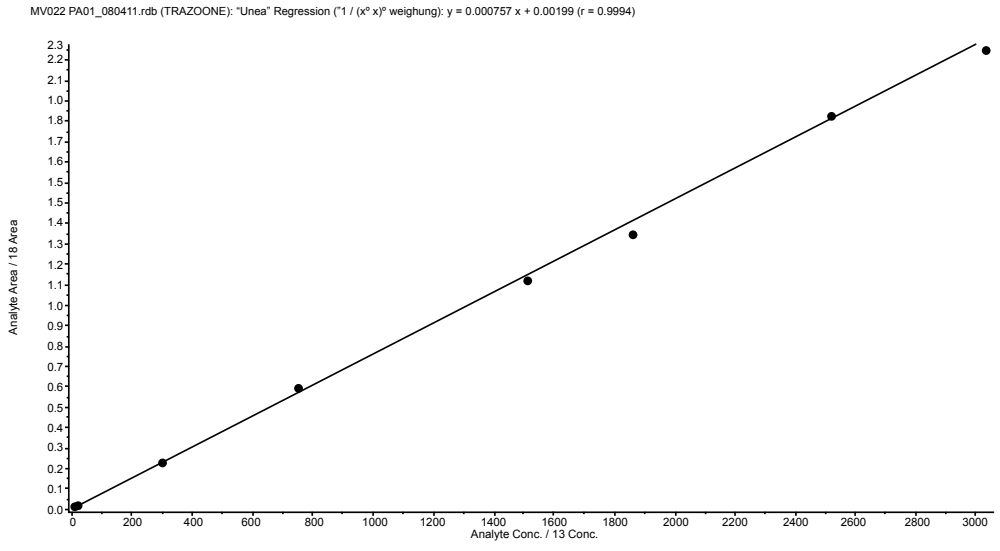

Figure 5: Calibration curve of Trazodone.

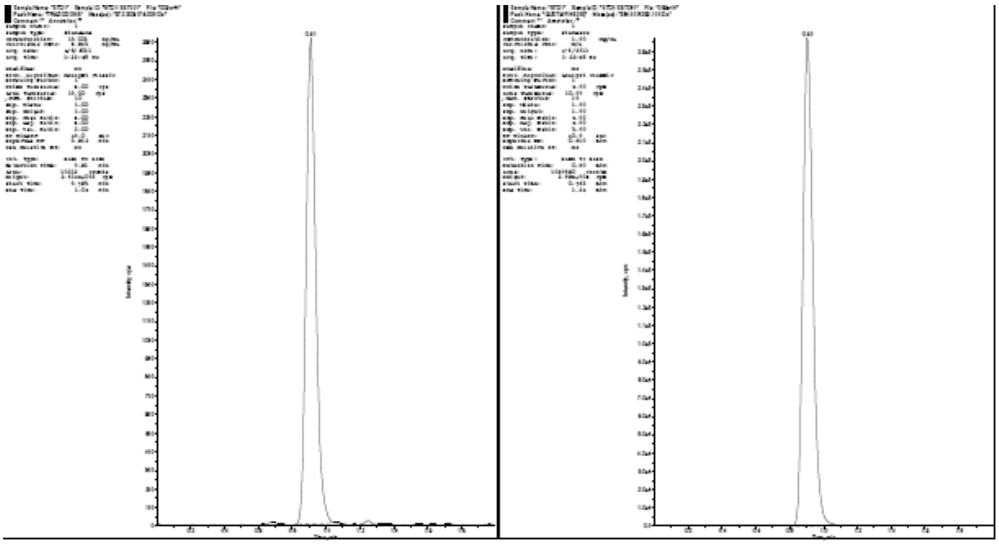

Figure 6: STD 1 of Trazodone and IS.

replicates of each Low quality control (LQC) and high quality control (HQC) samples were processed, analyzed and quantified against freshly prepared calibration curve. The precision and accuracy for the stability samples must be within ≤ 15 and ± 15% of their respective nominal concentrations. Stability in biological matrix was carried for the following and the results were represented in table 6.

Freeze-thaw stability was obtained by taking the samples from the deep freezer at −70°C ± 15°C over five freeze-thaw cycles. The bench top stability was evaluated by keeping replicates of the LQC and HQC samples were withdrawn from deep freezer, kept at room temperature approximately for 17 hours (hrs). Wet extract stability of the samples evaluated at room temperature and refrigerator approximately for 17 hrs and 41 hrs respectively. Dry extract stability of the samples evaluated at refrigerator conditions for 41 hrs. Auto injector stability was evaluated for 41 hrs. Samples were initially stored in -25°C and later retrieved after 03 days. The samples were then processed and analyzed.

Batch	Concentration in ng/ml	Mean Detected Concentration, ± SD (ng/ml)	Mean Accurracy (%)	%CV
Intraday P&A	3605.580	3586.306 ± 13.54	99.5	0.4
	2098.450	2084.240 ± 7.88	99.3	0.4
	25.181	24.276 ± 1.22	96.4	6.3
	10.576	9.187 ± 0.08	86.9	8.2
Interday P&A	3605.580	3592.114 ± 9.91	99.6	0.3
	2098.450	2082.542 ± 4.55	99.2	0.2
	25.181	22.651 ± 1.18	90.0	5.2
	10.576	9.137 ± 0.32	86.4	3.6

Table 3a: Intra- and Inter- day Precision and Accuracy of Aprepitant sipcked in human plasma.

PARAMETERS	HQC	MQC	LQC	LLOQ QC
Nominal Conc.	3605.58	2098.45	25.181	10.576
Mean	3589.69	2083.77	23.542	9.166
SD (±)	18.1303	8.6949	1.1647	0.9706
CV (%)	0.5	0.4	4.9	10.6
Accuracy	99.6	99.3	93.5	86.7

Table 3b: Precision and Accuracy studies of APT samples (ng/ml). LLOQQC – Lower limit of quantification Quality control samples, LQC - Lower Quality Control, MQC - Medium Quality Control, HQC - Higher Quality Control, SD - Standard Deviation and CV - Coefficient of variance.

Drug	Nominal conc.	% Recovery	Standard deviation	% CV
Aprepitant				
HQC	3605.580 ng/ml	71.9	4.661	6.5
MQC	2098.448 ng/ml	68.0	2.585	3.8
LQC	25.181 ng/ml	63.8	2.718	4.3
Quetiapine	400 ng/ml	77.7	4.333	5.6

Table 4: Extraction recovery data of analyte and internal standard.

Observed Concentration(ng/ml)		
Parameter	**HQC**	**LQC**
Average Conc.	3595.66	23.952
Standard Deviation	5.4635	0.7881
CV (Precision %)	0.2	3.3
Nominal Conc.	3605.58	25.181
Accuracy (%)	99.7	95.1

Table 5: Results for reinjection reproducibility.

STABILITIES	TIME	% STABILITY	
		HQC	LQC
Freeze-thaw	5 cycles	96.3	98.9
Bench top	17 h	92.3	94.3
Wet extract at refrigerator	41 h	95.9	96.1
Wet extract at bench top	17 h	95.3	100.5
Dry extract	41 h	94.9	92.8
Auto sampler	41 h	101.4	96.9
Interim	03 days	104.9	101.5

Table 6: Results for Stability Studies.

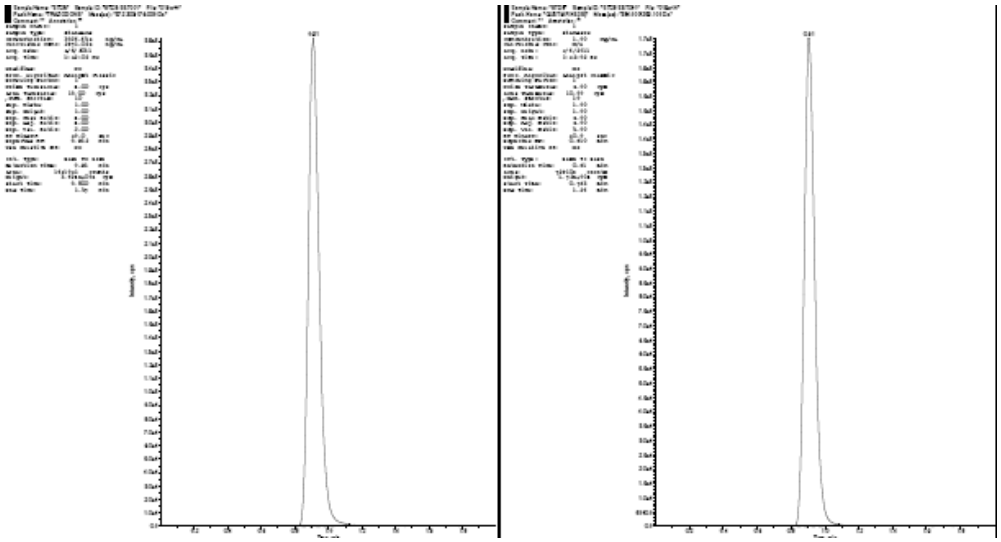

Figure 7: STD 8 of Trazodone and IS.

Conclusion

A highly selective and rapid LCMS/MS method employing liquid–liquid extraction for the determination of APT in human plasma has been developed and validated with a Lower limit of Quantification of 10.004 ng/ml. The validation data also demonstrates good precision, accuracy and high extraction efficiency. The validated method allows quantification of APT in the linear range of 10.004-5001.952 ng/ml. In conclusion, this paper describes a very simple and sensitive LCMS/MS method for the quantization of APT suitable to monitor plasma concentrations during clinical pharmacokinetic and bioequivalence studies in humans.

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