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Validated HPLC-MS/MS Method for Determination of Trazodone in Human Plasma

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Abstract

A precise, sensitive liquid chromatography - tandem mass spectrometry method has been developed and validated for the quantitative determination of Trazodone in human plasma. The analyte was extracted using liquid-liquid extraction. Chromatographic separation of drug was achieved by using a Inertsil C8 50×4.6 mm, 3 µm column, with isocratic mobile phase of 2 mM.

Ammonium Acetate (pH 4.00): Organic mixture (10:90) at a flow rate of 0.9 ml/min. Organic mixture is composed of acetonitrile: methanol (80:20). Quetiapine was used as internal standard. Detection was carried out by AB Sciex API 3200 tandem mass spectrometer using positive electro-spray ionization mode by multiple reactions monitoring method at m/z 372.20/176.00 and 384.00/253.10 for Trazodone and Quetiapine respectively. Calibration curve was linear in the tested range of 10.001 – 3036.634 ng/ml with correlation coefficient(r) of 0.9994. The coefficient of variance (%CV) of this method was <11% for intraday and inter-day assays. The extraction recoveries for Trazodone at high, middle and low quality control samples was found to be 39.8%, 36.1%, and 40.8% respectively and 65.8% for internal standard. The proposed method was found to be validated for its linearity, precision, accuracy, recovery, reinjection reproducibility and stability study.

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

Introduction

Trazodone is chemically 2-{3-[4-(3-chlorophenyl)piperazin-1-yl] propyl}-2H,3H-[1,2,4]triazolo[4,3-a]pyridin-3-one. It is a serotonin antagonist and reuptake inhibitor (SARI), which is a second generation antidepressant compound belonging to the class of phenylpiperazine. It acts as a serotonin agonist at high doses and low doses. The drug showing antidepressant activity is due to the blockage of serotonin reuptake by inhibiting serotonin reuptake pump at the presynaptic neuronal membrane. Trazodone shows its therapeutic actions through 5-HT_{2A} receptors. Trazodone also induces anti-anxiety and sleep-inducing effects [1]. It does not have similar properties to selective serotonin reuptake inhibitors (SSRIs) since its inhibitory effect on serotonin reuptake and 5-HT_{2C} receptors are relatively weak [2]. The result of alpha-adrenergic action blocking and modest histamine blockade at H_1 receptor due to sedative effect of trazodone. It weakly blocks presynaptic alpha2-adrenergic receptors and strongly inhibits postsynaptic alpha1 receptors. Trazodone does not show any action on the reuptake of norepinephrine or dopamine within the CNS. It has fewer anticholinergic side effects than most of the tricyclic antidepressants such as dry mouth, constipation and tachycardia. Trazodone metabolizes to its primary m-chlorophenyl piperazine (mCPP) which is a non selective serotonin receptor agonist which might outweigh the benefits of Trazodone [3-6].

The official methods for the determination of trazodone in pharmaceutical dosage forms includes potentiometric non-aqueous titration with perchloric acid [7] and HPLC using an octadecyl silane column and methanol–0.01 M ammonium phosphate buffer pH 6.0 (60:40) as the mobile phase [8]. Several analytical methods that have been reported for the determination of Trazodone in pharmaceutical formulations such as spectrophotometry [9-12], ion-selective electrode [13], voltammetry [14,15], colorimetry [16], instrumental TLC [17] and HPLC [18-20]. Various methods have been reported for the determination of Trazodone in biological fluids, including HPLC [21-25], capillary gas chromatography [26], GC-MS/MS [27] and LC-MS/MS [28]. A combination of spectrophotometric, spectrofluorimetric and LC determination of Trazodone has been also reported [29]. In this paper the main objective of the study was to develop a sensitive, rapid, precise, accurate method of determining trazodone in human plasma without interference from its metabolic products having Limit of Quantification 10.001 ng/ml using liquid-liquid extraction. The structures of Trazodone and Quetiapine are displayed in figure 1.

Materials and Methods

Reagents and chemicals

Trazodone (99.00% purity), Quetiapine (99.56% purity) were obtained 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 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,

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

MS/MS conditions

Detection of the ions was performed in MRM mode monitoring with positive polarity. The precursor to product ion transitions for Trazodone and Quetiapine were found to be m/z 372.20/176.00 and 384.00/253.10 respectively. The tuned MS/MS conditions of Declustering Potential (DP) were 50 V, 30 V; Collision energy (CE) were 65 V, 40 V; Collision Cell Entrance potential (CEP) were 20.99V, 21.38V; Collision Cell Exit Potential (CXP) were 5 V, 10 V for analyte and internal standard respectively. Conditions of Entrance potential (EP) 10V, Heater temperature 475°C, Curtain gas 30 psi, Collision associated dissociation (CAD) 6 psi, Nebulizer gas (GS1) 40 psi and Heater gas (GS2) 45 psi, Ion spray voltage (ISV) 5000 V were optimized for both analyte and internal standard. The mass spectrum of Trazodone and Quetiapine were displayed in figures 2 and 3.

Chromatographic conditions

The compounds were separated on a Inertsil C8 50×4.6 mm, 3 µm column analytical column. A mixture of 2 mM Ammonium Acetate (pH 4.00): Organic mixture (10:90) [Organic mixture is composed of acetonitrile: methanol (80:20)] was used as mobile phase Efficient and symmetrical peaks were obtained at ambient temperature at a flow rate of 0.9 ml/min with a sample injection volume 5 µl with run time of 2.0 min. Retention times of Trazodone and Quetiapine were found to be 0.96 min and 0.96 min (Figure 4).

Preparation of standards and quality control samples

Standard stock solutions of Trazodone and Quetiapine were prepared in methanol at a concentration of 1 mg/ml. These solutions were kept at 2-8°C. The stock solution of Quetiapine was diluted to concentration of 400 ng/ml using diluent 50% v/v methanol in water.

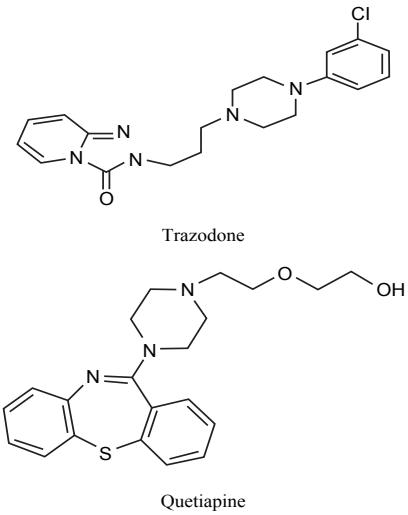

Figure 1: Structures of Trazodone and Quetiapine.

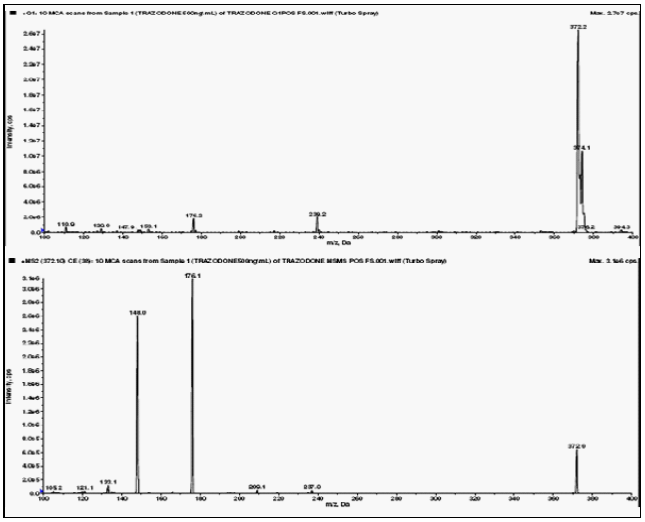

Figure 2: Full scan mass spectrum of Trazodone parent ion and product ion.

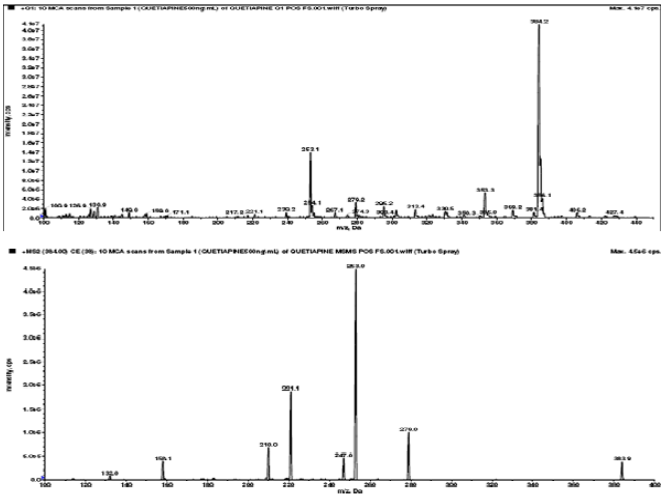

Figure 3: Full scan mass spectrum of Quetiapine parent ion and product ion.

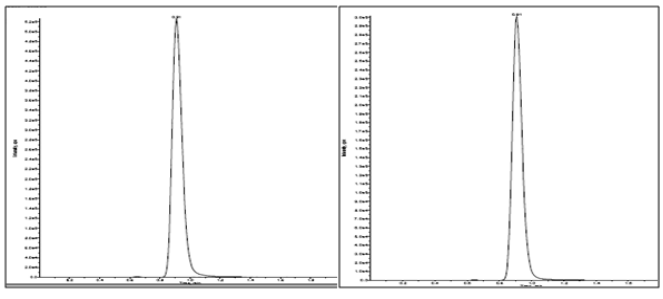

Figure 4: Retention time of Trazodone and Quetiapine.

Working standard solutions were prepared from stock solutions by using diluent 50% v/v methanol in water. Working standard solutions were prepared by spiking stock solution into drug-free human plasma to obtain concentrations of 10.001, 20.002, 302.146, 755.366, 1510.731, 1865.100, 2520.406 and 3036.634 ng/ml. The Quality Control (QC) samples were prepared in an analogous manner to the calibration

standards to give concentrations of Lower Limit of Quantification Quality Control (LLOQ QC) 10.610 ng/ml, Lower Quality Control (LQC) 25.690 ng/ml, Medium Quality Control (MQC) 1284.485 ng/ml and Higher Quality Control (HQC) 2177.092 ng/ml. The samples were stored at -70 ± 15°C for further processing.

Sample preparation

50 µl of internal standard solution (400 ng/ml) was added into labeled ria vial tubes and spiked with 300 µl of plasma sample (respective concentration) into each tube and vortexed briefly. 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. Then the samples were centrifuged at 4000 rpm for approximately 10 min at ambient temperature. The upper organic layer from each sample was transferred into pre-labeled auto sampler vials and was evaporated until dryness under the Nitrogen evaporator. Then the samples were reconstituted with 0.3 ml of mobile phase and analyzed.

Data processing

The MRM chromatographic peaks were integrated using Analyst software version 1.5.1 supplied by MDS technologies. Peak area ratios of Trazodone to Quetiapine were plotted versus concentration and a linear curve fit, weighted by $1/X2$ (where X = concentration) was used to produce the regression line.

Results and Discussion

Method validation

The validation parameters such as linearity, precision, accuracy, recovery, reinjection reproducibility and stability studies were conducted according to USFDA guidelines [30].

Linearity

Calibration curves were linear over the concentration range 10.001-3036.634 ng/ml for Trazodone. The best linear fit and least square residuals for the calibration curve could be achieved with the linear equation y=mx + c with a 1/x2 weighing factor, where y was the peak area ratio of Trazodone to Quetiapine and x was the concentration of Trazodone. The correlation coefficient(r) for Trazodone was above 0.9994 (Figure 5) over the concentration range.

Lower Limit of Quantification (LLOQ)

LLOQ, the lowest concentration in the standard curve, which can be measured with acceptable mean response for analyte peak at the assay sensitivity limit (10.001 ng/ml), was fivefold greater than the mean response for the peak in blank human plasma samples at the retention time of the analyte (Figures 6 and 7).

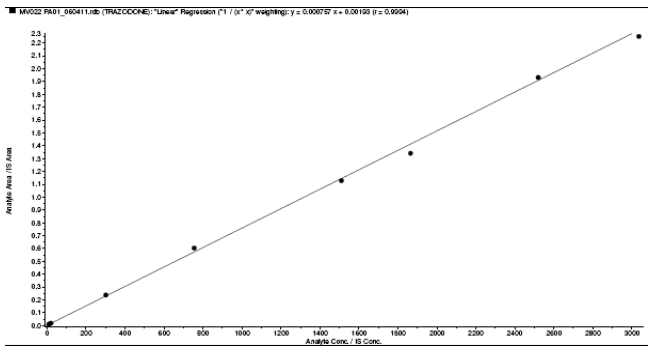

Figure 5: Calibration curve of Trazodone.

Precision and accuracy

Intra and inter batch accuracy and precision evaluations were performed by repeated analysis of Trazodone in human plasma. The run consisted of a six replicates of each LLOQQC, LQC, MQC and HQC samples. The overall precision of the method expressed as relative standard deviation and accuracy of the method. Inter day batch accuracy ranged from 89.6% to 105.8% and precision ranged from 1.8% to 10.5%. Intraday batch accuracy ranged from 91.5% to 104.3% and precision ranged from 2.2% to 7.2%. The mean concentration, standard deviation (SD), coefficient of variation (%CV) was evaluated and their results were tabulated in table 1.

Extraction recovery

Recovery of Trazodone was evaluated by comparing the mean peak areas of six extracted LQC, MQC and HQC samples to mean peak areas of six unprocessed reference solutions. Recovery of internal standard Quetiapine was evaluated by comparing the mean peak area of extracted samples to mean peak areas of unprocessed reference solutions of the same concentration. The results were represented in table 2.

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 3.

Stability studies

As a part of method validation, stabilities such as bench top stability, auto-sampler stability, freeze thaw stability, dry extract

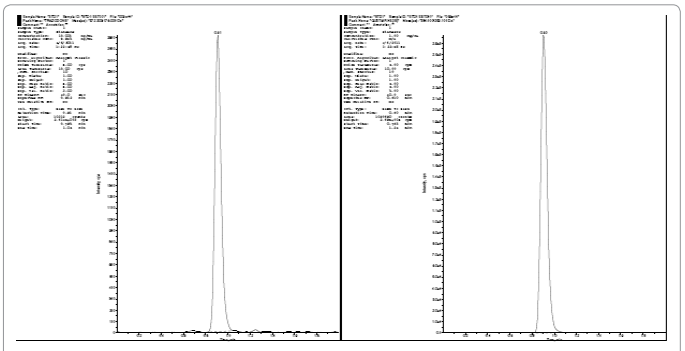

Figure 6: STD 1 of Trazodone and IS.

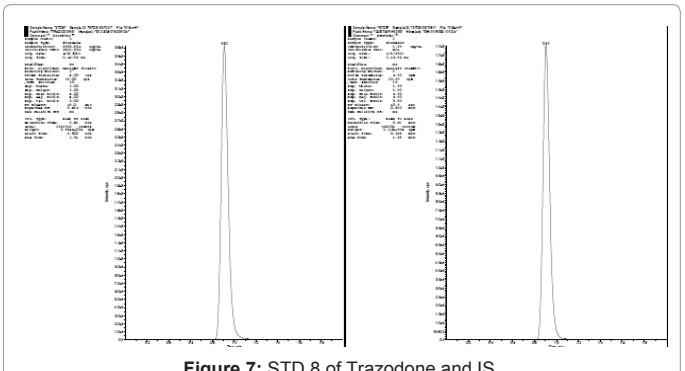

Figure 7: STD 8 of Trazodone and IS.

Parameters	HQC	MQC	LQC	LLOQ QC
Nominal Conc.	2177.09	1284.49	25.69	10.61
Mean	2167.86	1271.37	24.68	10.31
SD (±)	17.026	13.283	0.8002	0.529
CV (%)	0.8	1	3.2	5.1
Accuracy	99.6	99.0	96.1	97.2

LLOQQC – Lower limit of quantification Quality control samples, LQC - Lower Quality Control, MQC - Medium Quality Control, HQC - Higher Quality Control, SD – Standard Deviation, CV – Coefficient of variance.

Table 1: Precision and Accuracy studies of Trazodone (ng/ml).

Drug	Nominal conc.	% Recovery	Standard deviation	%CV
Trazodone				
HQC	2177.092 ng/ml	39.8	1.489	3.7
MQC	1284.485 ng/ml	36.1	1.793	5.0
LQC	25.690 ng/ml	40.8	0.832	2.0
Quetiapine	400 ng/ml	65.8	4.258	6.5

Table 2: Extraction recovery data of analyte and internal standard.

Observed Concentration(ng/ml)		
Parameter	**HQC**	**LQC**
Average Conc.	2171.29	22.945
Standard Deviation	4.7724	0.2752
CV (Precision %)	0.2	1.2
Nominal Conc.	2177.09	25.69
Accuracy (%)	99.7	89.3

Table 3: Results for reinjection reproducibility.

stability, wet extract stability (in refrigerator and on bench top) were validated. Six replicates were analyzed for each of LQC and HQC samples at each storage condition. The concentration of Trazodone after each storage period was compared to the initial concentration as determined for the samples that were freshly prepared and processed immediately. Accuracies of the QCs will be quantified against a freshly prepared calibration curve. The precision and accuracy for the stability samples must be within ≤ 15 and ± 15%, respectively, of their nominal concentrations. Results were represented in table 4.

Freeze-thaw Stability (FTS)

Samples were taken from the deep freezer at –70°C ± 15°C and allowed for unassisted thawing at room temperature. This process is continued for 05 cycles. At the completion of 05 freeze and thaw cycles the samples were processed and analyzed and the results are calculated from freshly prepared calibration curve.

Bench Top Stability (BTS)

The stability of analyte in human plasma stored at room temperature (bench-top stability) was determined by processing bench top stability quality control samples after keeping them at room temperature approximately for 18 hours (h) and quantifying them against the freshly prepared calibration curve.

Wet extract stability

Wet extract bench top and wet extract refrigerator stability of Trazodone was determined by processing and reconstituting quality control samples, keeping them at room temperature and refrigerator approximately for 19 h and 39 h respectively and quantifying them against freshly prepared calibration curve.

Dry extract stability

Dry extract stability of analyte was determined by processing HQC and LQC samples, keeping them in refrigerator for 39 h and quantifying them against freshly prepared calibration curve.

Stabilities	Time	%STABILITY	
		HQC	LQC
Freeze-thaw	5 cycles	100.8	99.8
Bench top	19 h	99.7	99.0
Wet extract at refrigerator	39 h	98.4	101.5
Wet extract at bench top	19 h	96.8	108.1
Dry extract	39 h	100.9	98.2
Auto sampler	44 h	98.1	108.1
Interim	03 days	101.9	100.4

Table 4: Results for stability studies.

Auto injector stability

To assess the auto- injector stability of Trazodone, quality control samples were stored into the auto-sampler for the stability period of 44 h. These samples were then quantified against freshly prepared calibration curve.

Interim stability

Samples were initially stored in -25°C and later retrieved after 03 days. The samples were then processed and quantified against freshly prepared calibration curve.

Conclusion

The results obtained from validation concludes that, the developed method is simple, linear, accurate, precise, less time consuming, economically useful, applicable for the routine analysis of pharmaceutical dosage forms, bioavailability- bioequivalence studies and pharmacokinetic studies to quantify Trazodone in human plasma by using LC-MS/MS.

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