ejpmr, 2023, 10(8), 422-427

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTIFICATION OF TRAZODONE IN HUMAN PLASMA BY HPLC-MS/MS

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Article Received on 10/06/2023 **Article Revised on 30/06/2023** **Article Accepted on 21/07/2023**

ABSTRACT

Objective: The development and validation of bioanalytical process approach for assessing the antidepressant medication Traz in human plasma is the ultimate goal of the current investigation. **Methods:** The method involves tandem-mass spectroscopy combined with HPLC SPE technique bioanalytic method development and validation conducted for the first time. Using Aceton-M: 5 mM ammonium formate buffer (90:10% V/V), at 1.00 mL/min flow rate, with 10µl of injection volume, allowed for excellent separation and elimination. This chromatographic analysis analyte and IS were started under isocratic conditions to develop an easier separation technique in a shorter run time. **Report:** According to the method, the range of calibration curve is from 25.2640-352.3060 ng/ml. By using numerous supporting data points, including batch recovery percentages of 85.0433% and 82.3800% over TRAZ and TRAZD6, a technique validation has been established. **Conclusion:** The simple, highly accurate, precise, sturdy, reproducible and reliable HPLC-MS/MS technique was formed that proves all the stability parameters and is suitable for the bioequivalence study in the future. This method may also be applicable for the combined formulations.

KEYWORDS: Trazodone, LC-MS/MS, Trazodone D6, LLOQ, USFDA.

INTRODUCTION

Trazodone is a trizolopyridine derivative [C19H22ClN5O]. "2-3-[4-(3-chlorophenyl) piperazin-1-yl]propyl[1, 2, 4]triazolo[4,3-a]pyridin-3(2H)-one" is the chemical formula.[1] with a molar mass of 371.87gmol-1 and a melting point of 87° Celsius (189° Fahrenheit).[2] Solubility is 0.29 mg/ml in water, with a partition coefficient value of 2.68.[3] It's a BCS class 2 drug.[4] TRZ is an FDA-approved antidepressant used in psychotherapy to treat depression disorders. approved under the brand name Oleptro in 2010.[5] Trazodone is a SARI drug (reuptake inhibitor and serotonin antagonist), which prevents both serotonin transporters as well as type-II receptors. It also prevents the serotonin reuptake, blocks the release of serotonin's histamine, and blocks serotonin's release into the brain.[6] In the US pharma market, it will be available in the form of orally administered tablets and capsules at dose levels of 50, 100, 150, and 300 mg.[7]

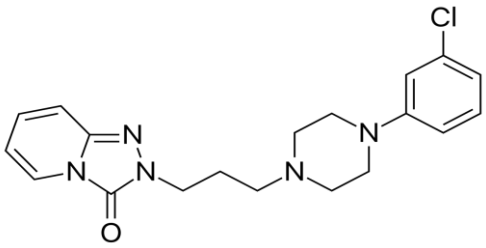

Figure 1: Structural veiw of Trazodone.

Pharmacokinetic profile[8]

Bioavailability- orally 65%; Binding potency with protein - 89 to 95%; Tmax - 1hr; Metabolism - in liver by CYP3A4C and CYP2D6; Metabolite- mCPP; T½- 4 to 5 hours (immediately release dosage form); Elimination - urine: 70 to 75% & feces: 21%.[15]

LC-MS is a novel hyphenation technique for the isolation and identification of pharmaceutical substances in biological substrates and is also used to study the pharmacokinetics of drug compounds. Hyphenation methods are combinations or Couplings of different analytical techniques.[10][13] Chromatography is mainly

combined with spectroscopic techniques. typically, separation is done by chromatography, after which the isolated compounds are detected spectrophotometrically. Hirschfield (1980) brings the terminology hyphenation to refer to an online combination of a separation technique and one or more spectral detection techniques (Tandem-MS).[11],[12],[14]

MATERIALS AND METHODS

Materials

Trazodone was obtained as a working standard from Simson Pharma Ltd., India. Trazodone D6 (internal standard) was obtained from Clearsynth Lab Ltd., Mumbai, India. Acetone M (LC-MS grade) was purchased from Burdick & Jackson Pvt. Ltd., India. AcN (LC-MS grade) was obtained from Merck in Mumbai, India. Ammonium formate (reagent grade) was purchased from Sigma Aldrich, India. Ammonium acetate (LC-MS grade) was purchased from Spectrum Chemicals Limited, India. HPLC-grade water ("milli-Q water purification system"). Blank plasma (K2 EDTA) was procured from Micro Therapeutic Research Labs Pvt. Ltd., Chennai, India.

Instrumentation

On a high-performance liquid chromatography system made by Waters Alliance using the 2695 separation model, tandem mass spectroscopy was carried out using a Quattro Micromass^{TM} API (HPLC-MS/MS) outfitted with a positive electron spray ionization interface source. Using a Hypurity C18 (100 mmx4.6 mm, 5m) column, chromatography was carried out. Masslynx V4.1 software was utilized to acquire the data.

Optimized Chromatographic conditions

On a Hypurity C18 (100mm x 4.6mm, 5m) column, samples were examined. As the mobile phase in the isocratic mode, a mixture of Aceton-M and a 5 mM buffer of ammonium formate (90:10% V/V) was utilized. 10µl of injection was used, and the autosampler's temperature was set at 10°c. Analyte and IS should retain information for 1.5 ± 0.30mins. The analysis took 2.50 minutes to complete in its entirety.

Optimized Mass spectroscopy conditions

Positive electron spray ionization technique is used to perform analysis with source temperature of 150ºC. The compound ionization and tuning were carried out by which the TRAZ parent ion (M.W 371.87 g/mol-1) and TRAZ D6 (M.W 330.47g/mol).

Table 1: Summarises The Common Mass Parameters.

Tuning	Q1mass (m/Z)	Q3 mass (m/Z)	Dwell	CE	Cone volts
Trazodone	372.08	176.15	0.200	20	30
Trazodone D6	378.12	182.05	0.200	20	30

Capillary (kV): 3.50; Cone gas flow (L/hr): 50; Source temperatures: 120°C; Desolvation gas (L/hr): 800; Desolvation temperature: 350°C

Preparation of CC and QCs Std

A master stock solution of Trazodone and Trazodone D6 (1000 l/mL) was formed utilizing acetone. The Traz D6 spiking solution (5 l/ml) has been formed by diluting the IS master stock with diluent.

CC standards were created by spiking an appropriate amount of the corresponding spiking solution into control plasma to attain 8 varied concentrations ranging from 25.2640-3502.3060 ng per ml Trazodone. The standards were kept in refrigerator. QC samples have been created by spiking an appropriate volume of respective spiking solution to control PLSMA to attain five varied content at the LOQQC, LQC, INTQC, MQC, and HQC levels of Trazodone.

METHOD VALIDATION

Method validation consists of a series of experiments that can be performed to get the quantitative measurements in a particular matrix type that shows the method is reliable and reproducible whenever conducted in another LC system and by an analyst that should be constant. Parameters may include selectivity, sensitivity, matrix factor, precision, accuracy, recovery, dilution effects, carryover, hemolysis effect, matrix stability, and ruggedness.

RESULTS AND DISCUSSION

Method Development & Optimization

The mass spectroscopy parameters for the analysis of the ion state of TRAZ and TRAZ D6 were developed. They were then monitored through multiple reactions. The results of the analysis revealed that the former's parent ion was at 372.08 amu, while the latter's was at 378.12. The MS/MS spectra of trazodone revealed that the latter's daughter ion intensity was at 182.05 amu.

For chromatography, the peaks of the analyte and IS were properly integrated so that adjacent peaks did not affect the TRAZ and TRAZ D6. They were also free from any overlap or closely eluting peaks. The chromatograms in Figures 2 to 5 are shown.

SELECTIVITY

As nine batches of human blank plasma with K2EDTA as an anticoagulant were examined for selectivity, none of the batches revealed any interference during the retention period or transition of the analyte and IS (6 normal, 1 hemolyzed, 1 lipemic, and 1 heparin batch).

SENSITIVE & LOD

The LLOQ "lower limit of quantification" for trazodone was 25.2640 ng per mL. The developed method's LOD was 26.1875 ng/ml.

MATRIX EFFECT

The total precision of matrix factor is represented using the CV% ("Coefficient Of Variation"). The required overall CV for an IS normalised matrix factor of 15% is the acceptance criterion. The outcomes are shown in Tables 2 and 3.

Table 2: IS normalised matrix factor for Traz –LQC.

"Parameter	Analyte peak area	IS peak area	IS Normalized MF"
Mean	357.8	10184.1	0.97667
SD	29.57	927.5	0.060828
%CV	8.26	9.10	6.23

Table 3: IS normalised matrix factor for Traz – HQC.

"Parameter	Analyte peak area	IS peak area	IS Normalized MF"
Mean	13647.1	9155.4	0.98889
SD	1007.65	850.57	0.036209
% CV	7.38	9.29	3.66

Precision, Accuracy & Recovery

The calibration curve has been observed to be 0.0008 slope and linear, 0.0013 intercepts, and 0.9975 r2 determination coefficient. The outcome demonstrates the repeatability and dependability of this method between 25.2640 and 3502.3060 ng/ml. Intra-assay was conducted for both PA and Non-PA batches. Blank samples were clear with no interference meets specifications. The standard curve and blank were represented in Figures 2 and 3. All the QC samples meet the specifications. The mean recovery for Traz and Traz D6 was calculated and was found to be 98.5508% and 97.67%. Refer to Tables 4,5,6 and 7.

Table 4: Back-calculated concentration of the calibration curve.

CC Concentration	Mean	S.D	%CV	%Nominal	%Deviation
STD A (25.2640 ng/mL)	26.1875	0.02	0.84	103.66	3.66
STD B (69.2180 ng/mL)	63.2949	0.04	0.92	92.89	-7. 11
STD C (134.4020 ng/mL)	127.0022	0.09	1.27	94.49	-5. 51
STD D (336.0040 ng/mL)	327.8264	0.24	4.04	97.57	-2. 43
STD E (672.0060 ng/mL)	676.6441	0.50	5.47	100.69	0.69
STD F (1341.0100 ng/mL)	1399.0230	1.05	2.8	104.09	4.09
STD G (2688.0200 ng/mL)	2784.6395	2.09	3.25	103.59	3.59
STD H (3502.3060 opng/mL)	3608.0298	2.71	3.95	103.02	3.02

Acceptance criteria

Except for the LLOQ standard (i.e., STD 1), where it should be within 20.0 percent of nominal value, mean accuracy (% Nominal) for calibration standards should be within 15.0 percent of nominal value. Except for the LLOQ standard (STD 1), that requires a precision (% CV) of 20.0%, all CC standards require a precision (% CV) of 15.0%.

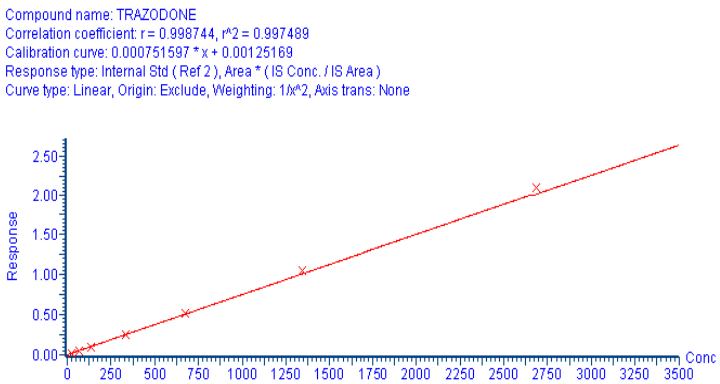

Figure 2: Respective chromatogram of linearity curve.

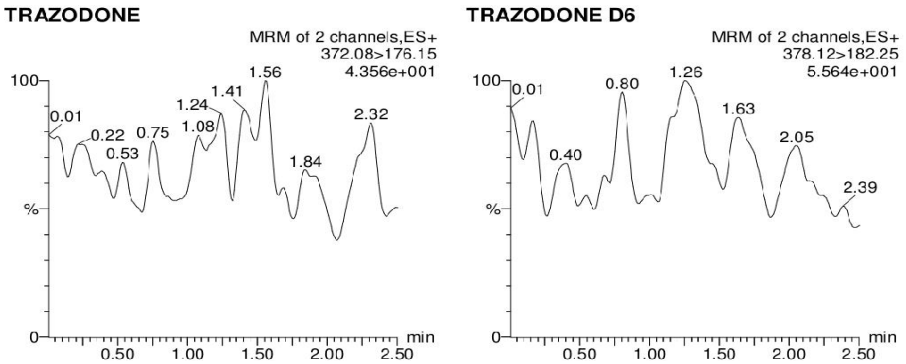

Figure 3: Respective chromatogram of Blank Human Plasma.

Table 5: Inter-Assay PA batch of Qc sample.

Type	Parameter	LLOQQC (25.4500 ng/mL)	LQC (69.5360 ng/mL)	INTQC (335.9240 ng/ml)	MQC (1343.6920 ng/mL)	HQC (2687.3820 ng/mL)
Inter-assay P&A (n=18)	Inter run mean	26.50045	63.10910	322.12018	1253.07902	2383.20022
	Inter run SD	0.61	0.33	2.06	25.44	42.69
	Inter run CV%	2.32	0.53	0.64	2.03	1.79
	Inter run % Nominal	104.13	90.76	95.89	93.26	88.68
	%Deviation	4.12	-9.24	-4.10	-6.74	-11.32

Acceptance criteria: For LLOQQC, the Mean Accuracy should be within 20.0%, and for other QCs, within 15.0%. Precision for LLOQQC is 20.0%, whereas for other QCs it is 15.0%.

Table 6: Recovery for Trazodone.

"Sample level	Recovery	Mean	SD	%CV"
HQC	98.07	98.55087	0.94166	0.96
MQC	97.95			
LQC	99.64			

Table 7: Recovery for Trazodone D6.

Sample level	Recovery	Mean	SD	%CV
MQC	82.24	82.3800	0.77949	0.95
HQC	83.22			
LQC	81.68			

Dilution Integrity

At least four out of six dilutions QC samples must be within specifications, and the precision (CV) and accuracy must be within 15.0 percent of the nominal value. For results, see Table 8; all DIS-150 and QC samples were in compliance with the requirements, and the Non-PA batch passed.

Table 8: Dilution integrity for Trazodone.

Actual concentration (µg/mL)	5351.8040	5351.8040
Dilution factor	2	4
Mean	4881.55397	4955.98290
SD	19.991356	40.109103
%CV	0.41	0.81
%Nominal	91.21	92.60

ASCO-Test

When the ASCOT was analyzed, no considerable carryover was found.

Hemolysis effect

At least four out of six haemolysed QC samples must be within requirements, and accuracy should be within 15.0% of nominal value. Every level's mean Haemolysed QC accuracy should fall within a 15.0% range. At each level of the haemolysed QC samples, precision (CV) is less than 15.0%. For findings, see Table 8 for information on whether the Low and High Haemolysed QC samples met the requirements.

Table 8: Hemolysis Effect- QC PA.

Qc Samples	Qc concentrations (ng/mL)	Mean	SD	%CV	%Nominal
Hemo-Low	69.5360	63.18985	0.704273	1.11	91.06
Hemo-High	2687.3820	2407.96803	50.230368	2.09	90.22

Matrix stability

When tested for freeze-thaw stability (At -70°C and -30°C) over 6 cycles, bench top stability for 0 hr & 08.75 hrs, working standard solution stability for 25.00 hrs at normal temperature, and long-term stability of matrix up to 8 days were proven, there has been no discernible variation in the content of trazodone in the matrix. The results are shown in Tables 9 and 10.

Table 9: Freeze/thaw and Bench top stability.

Stages	QC concentrations	Mean	SD	%CV	%Nominal
(a) Freeze/ thaw stability of Trazodone in plasma (n=6)					
At -70ºC±15ºC	LOW (69.5360 ng/mL)	71.29755	0.714404	1.00	102.53
	HIGH (2687.3820 ng/mL)	2447.14403	26.067603	1.07	91.06
At -30ºC±10ºC	LOW (69.5360 ng/mL)	70.82708	0.932615	1.32	101.86
	HIGH (2687.3820 ng/mL)	2447.47163	28.412765	1.16	91.07
(b) Bench Top Stability in plasma at RT(n=6)					
0 Hour	LOW (69.5740 ng/mL)	70.79830	1.212413	1.71	101.76
	HIGH (2675.9020 ng/mL)	2530.45043	102.509776	4.05	94.56
08.75 Hours	LOW (69.5360 ng/mL)	69.61398	1.877425	2.70	100.11
	HIGH (2687.3820 ng/mL)	2425.99142	32.124348	1.32	90.27

Acceptance Criteria: The accuracy for stability sample against comparison sample is within ± 15%

Table 10: Working standard and long-term stability.

QC concentration	Stage	Mean	SD	%CV	%Nominal
Working standard solution stability (n=6)					
LOW (69.5360 ng/mL)	0.00 hrs	0.04238	0.00280	6.60	94.10
	25.00 hrs	0.03988	0.00252	6.31	
HIGH (2687.3820 ng/mL)	0.00 hrs	1.63888	0.04785	2.92	95.04
	25.00 hrs	1.55752	0.05108	3.28	
Long-term stability (n=6)					
LOW (69.5360 ng/mL)	Fresh	0.03923	0.00042	1.07	100.50
	08 Days	0.03937	0.00020	0.51	
HIGH (2687.3820 ng/mL)	Fresh	1.73507	0.00628	0.36	100.41
	08 Days	1.73500	0.00362	0.21	

Acceptance Criteria: Mean Test/Mean Ref must be within 90-110 % range.

CONCLUSION

According to FDA requirements, a simple, highly accurate, precise, robust, repeatable, and reliable HPLC-MS/MS technique to measure Trazodone in human plasma has been created. The results of validation attest to accuracy and repeatability of assay as well as the dependability of analytical system. The assay is precise, and the findings show that there are no interferences that may be predicted. The measured analytes are demonstrated to be stable under stress situations that could arise during the implementation of this approach.

ACKNOWLEDGEMENT

The Department of Pharmaceutical Analysis, Annai JKK Sampoorani Ammal College of Pharmacy, JKKMMRF's, Komarapalayam, Namakkal, Tamil Nadu-638183, India, is acknowledged by the authors for providing the supportive environment needed to complete this work.

Conflict of interest: None.

ABBREVIATIONS

HPLC- High-Performance Liquid Chromatography, MS- Mass Spectrometry, Traz - Trazodone, TrazD6- Trazodone D6, K_2EDTA- Di Potassium Ethylene Diamine Tetra Acetic Acid, ZC- Zero Calibrator, LLOQ, LLOQQC- Lower Limit of Quantification Quality Control, HQC- High-Quality Control, FDA- Food and Drug Administration, %CV- Percentage Coefficient of Variation, LQC- Low-Quality Control, MQC- Mid Quality Control, SD- Standard Deviation, IS- Internal Standard, Pvt- Private, Ltd- Limited.

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