Arabian Journal of Chemistry (2019) **12**, 1615–1622

King Saud University

Arabian Journal of Chemistry

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Trace level quantification of 1-(3-chloropropyl)-4-(3-chlorophenyl)piperazine HCl genotoxic impurity in trazodone using LC–MS/MS

Nandigam Venugopal [a], Ambavaram Vijaya Bhaskar Reddy [a,b], Gajulapalle Madhavi [a,*], Jafariah Jaafar [b], Vemula Madhavi [a], Kalluru Gangadhara Reddy [a]

[a] *Department of Chemistry, Sri Venkateswara University, Tirupati 517502, A.P., India*
[b] *Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81300 Johor Bahru, Johor, Malaysia*

Received 12 March 2013; accepted 8 October 2014
Available online 17 October 2014

KEYWORDS

LC–MS/MS;
Linearity;
Method development;
Trazodone;
Validation

Abstract A selective and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the quantitative determination of 1-(3-chloropropyl)-4-(3-chlorophenyl)piperazine HCl (CCP HCl) a process related impurity in trazodone. The method provided excellent sensitivity at a typical target analyte level of <0.1 ppm, when the API samples were prepared at 5.0 mg/mL. The CCP HCl sample was analyzed on a C18 symmetry (100 mm × 4.6 mm, 3.5 μm) column interfaced with a triple quadruple tandem mass spectrometer operated in a multiple reaction monitoring (MRM) mode. Positive electro spray ionization (ESI) was employed as the ionization source and the mobile phase used was 5.0 mM ammonium acetate–acetonitrile (30:70, v/v). The injection precision of the lowest concentration standards was excellent with %RSD-1.42%. The calibration curve showed good linearity over the concentration range of 0.03–1.5 ppm with a correlation coefficient of >0.9996. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.01 and 0.03 ppm, respectively. The developed method was validated as per ICH guidelines in terms of LOD, LOQ, linearity, precision, accuracy, specificity and robustness.

1. Introduction

The presence of potential genotoxic impurities (GTIs) in active pharmaceutical ingredients (API) and drug products continues to receive considerable attention. These impurities cannot be reduced to zero but the amount of GTIs should be limited to a level that represents an insignificant risk to clinical trial subjects or patients (Kram and McGovern, 2007). Trazodone

* Corresponding author. Tel.: +91 9440096500; fax: +91 2289544.
E-mail address: gmchem01@gmail.com (G. Madhavi).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

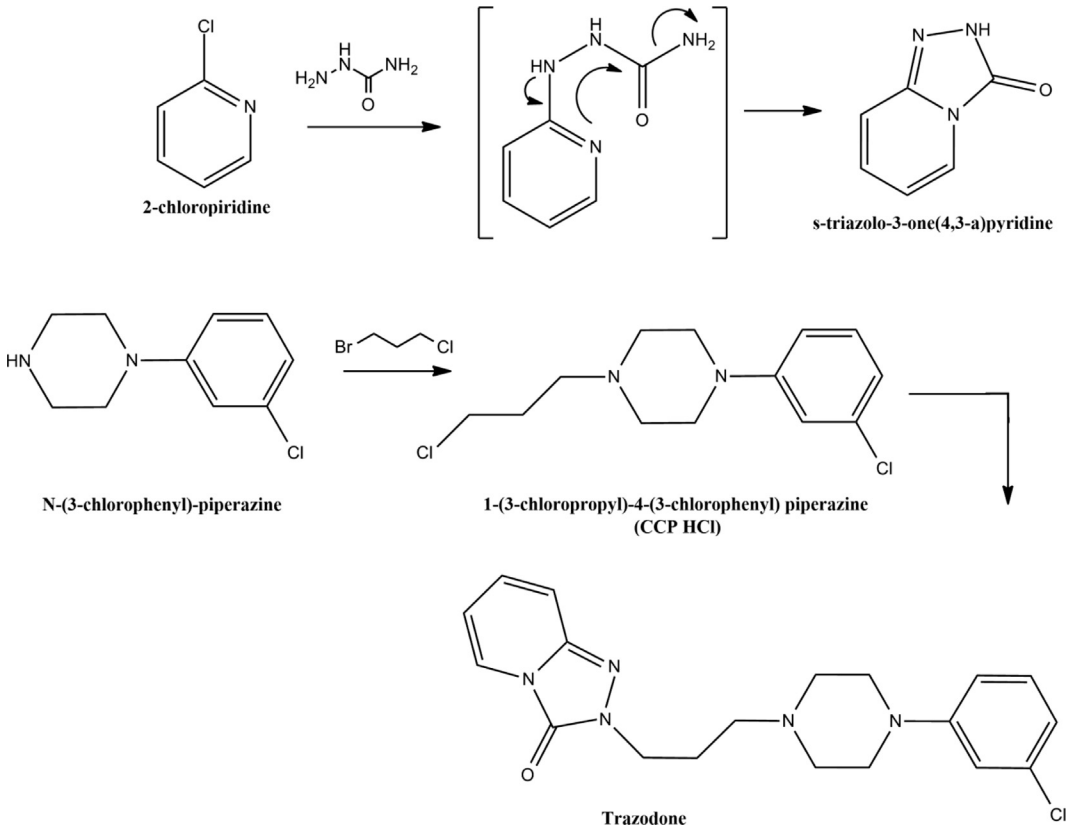
http://dx.doi.org/10.1016/j.arabjc.2014.10.006
1878-5352

hydrochloride is a triazolopyridine derivative designated as 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-1,2,4-triazolo[4,3-a]pyridin-3(2H)-one hydrochloride. It is an antidepressant agent and in clinical use the compound has been proven to be an antidepressant equivalent in effectiveness to imipramine (Prasad and Sastry, 2002), and it has often been prescribed for patients who have problems like sleep disturbance due to drug and/or alcohol consumption (Friedmann et al., 2003). Trazodone is popular among the substance abuse treatment providers because it is non-addictive, available as a generic agent, no restrictions on prescription duration, not associated with abuse liability, high overdose risk and/or life-threatening withdrawal syndromes (Maj et al., 1979; De Jonghe and Swinkels, 1992). 1-(3-Chloropropyl)-4-(3-chlorophenyl)piperazine HCl (CCP HCl) is the most important intermediate during the synthesis of trazodone (Scheme 1) (Acraf et al., 2009), and it is identified as GTI in trazodone (Ashby and Tennant, 1988; Muller et al., 2006). However, no methods have been reported for the determination of CCP HCl a process related impurity in trazodone. But several methods have been developed for the estimation of trazodone in biological samples employing HPLC-UV, HPLC-fluorescence, gas chromatography and mass spectrometric detection (Gupta and Lew, 1985; Carda-Broch et al., 2007; Andriollo et al., 1992; Belvedere et al., 1975; Gammans et al., 1985). According to the current regulatory guidance for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5 μg/day intake of individual impurity. Based on the TTC limit of 1.5 μg/day and on the maximum adult daily dose of trazodone of 400 mg/person, its GTIs are required to be controlled at a concentration limit of 3.75 μg/g (ppm) in the drug substance. Due to its higher sensitivity and selectivity, liquid chromatography–tandem mass spectrophotometry (LC–MS/MS) has been applied for the quantification of process related impurities in drug substances. In continuation of our previous publications on the determination potential of GTIs in pantoprazole, zolmitriptan and ritonavir drug substances (Venugopal et al., 2012, 2014; Vijaya Bhaskar Reddy et al., 2013), we have now developed a simple LC–MS/MS method that can quantify CCP HCl at permitted levels in trazodone. The developed method was fully validated as per ICH guidelines in terms of limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, specificity and robustness.

2. Experimental

2.1. Reagents and standards

HPLC grade acetonitrile and ammonium acetate were purchased from Merck (Mumbai, India). Formic acid, trifluoroacetic acid and methanol were obtained in their analytical grade from SD Fine Chemicals Limited (Mumbai, India). Purified water was collected through Milli-Q-Plus water purification system (Millipore, Milford, MA, USA). Reference substance of CCP HCl was obtained from Sigma–Aldrich (St. Louis, MA, USA). Nylon filter membranes (0.22 μm × 47 mm dia.) were purchased from Fisher Scientific Pvt. Ltd., (Mumbai, India).



Scheme 1 Schematic reaction mechanism showing the formation of CCP HCl during the preparation of trazodone.

2.2. Preparation of buffer solution

0.39 g of accurately weighed ammonium acetate was diluted to 1000 mL with Milli-Q-water, its pH was adjusted to 5.0 ± 0.05 with formic acid and filtered through a 0.22 µm nylon membrane filter paper. The mobile phase solution consisting of ammonium acetate and acetonitrile (30:70%, v/v at pH 5.0 ± 0.05) was prepared and filtered through a 0.22 µm nylon membrane sample filter paper and degassed. All the solutions were stored at ambient temperature.

2.3. Preparation of standard and sample solutions

A stock solution of trazodone (5.0 mg/mL) was prepared by dissolving an appropriate amount in methanol and a stock solution of CCP HCl at 0.1 mg/mL was also prepared in methanol. The diluted stock solution (0.001 mg/mL) was prepared by diluting 1.0 mL of the 0.1 mg/mL solution to 100 mL with methanol. The working standard solution was prepared by dissolving 50 mg of accurately weighed trazodone into a 10 mL volumetric flask and the solution was made up to the graduation mark after adding 5.0 µL of 1.0 µg/mL diluted stock solution to give 5.0 ng/mL and 5.0 mg/mL of PGIs with respect to trazodone which corresponds to 1.0 ppm of CCP HCl contamination relative to the drug substance. The GTI samples for validation at 0.4, 0.8, 1.0, 1.2 and 1.5 ppm concentrations relative to the drug substance were prepared in the same manner using 1.0 µg/mL of diluted stock solution. The concentration of the standard solutions and samples was optimized to achieve a desired signal-to-noise ratio (S/N) and good peak shape. All the standards were sonicated well and then filtered through 0.22 µm membrane filters before analysis.

2.4. Instrumentation

The MS system used was an Applied Biosystems Sciex API 4000 model (Switzerland) and is coupled with HPLC system consisting of LC-20AD binary gradient pump, a SPD-10AVP UV detector, SIL-10HTC auto sampler and a column oven CTO-10ASVP (Shimadzu Corporation, Kyoto, Japan). Data acquisition and processing were conducted using the Analyst 1.5.1 software on a Dell computer (Digital equipment Co.).

2.5. Operating conditions of LC–MS/MS

The analytical column used in LC–MS/MS was symmetry C18 (100 mm × 4.6 mm, 3.5 µm) column (Waters Co., USA) in isocratic mode using 5.0 mM ammonium acetate-acetonitrile in the ratio of 30:70 (v/v). The flow rate was 0.8 mL/min, which split down to 0.2 mL/min into the MS source. The column oven temperature was maintained at 40 °C, and the sample cooler temperature was set to 10 °C. The injection volume was 10 µL. Positive electro spray ionization (ESI) probe operated with MRM mode was used for the quantification of CCP HCl. In this method CCP HCl was monitored with its transition ion pair *m*/*z* 273.2/120.1 (protonated) and trazodone was monitored with its transition ion pair *m*/*z* 372.2/214.2 (protonated). The ion spray voltage (V), declustering potential (DP) and entrance potential (EP) were kept as 5500 V, 50 V and 10 V, respectively. The curtain gas flow, ion source gas 1 and ion source gas 2 nebulisation pressure (psi) were maintained as 25 psi, 28 psi and 30 psi, respectively. All the parameters of LC and MS were controlled by Analyst software version 1.5.1.

2.6. Validation study

The developed method was validated in terms of specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, robustness and solution stability.

A thorough and complete method validation for the analysis of CCP HCl was done by following the US FDA guidelines (Guidance for Industry, CVM., 2001). The method validation was started by injecting 1.0 ppm individual solutions of CCP HCl with respect to 5.0 mg/mL of trazodone and their S/N (signal to noise) ratios determined. Now, to determine LOD and LOQ values, CCP HCl concentration was reduced sequentially such that they yield S/N ratios as 3:1 and 10:1, respectively. The precision of the LOD and LOQ values was experimentally verified by injecting six standard solutions of the compounds at the determined concentrations. Linearity for CCP HCl was fixed in the range of LOQ to 150% (0.03–1.5 ppm) of the estimated permitted level (viz.1.0 ppm with respect to 5.0 mg/mL of trazodone solution). Hence, 40%, 80%, 100%, 120%, 150% solutions of CCP HCl were prepared and injected individually. The calibration curve was drawn between the peak areas versus concentration of CCP HCl. The slope, intercept and correlation coefficient values were derived from liner least-square regression analysis. The precision was evaluated at two levels viz. repeatability and intermediate precision. Repeatability was checked by calculating the relative standard deviation (%RSD) of six replicate determinations by injecting six freshly prepared solutions containing 1.0 ppm of CCP HCl the same day. The same experiments were done on six different days for evaluating intermediate precision. To determine the accuracy of the method, a known amount of sample was taken separately at different intervals and spiked with a known quantity of CCP HCl (LOQ to 100% level). A recovery study by the standard addition method was performed to evaluate accuracy and specificity. Accordingly, the accuracy of the method was determined by spiking 0.03 ppm, 0.5 ppm and 1.0 ppm of CCP HCl separately to three batches of pure sample and three batches of formulation samples of trazodone (5.0 mg/mL). Each determination was carried out three times. The specificity, defined as the ability of the method to measure the analyte specifically in the sample matrix, was determined by analyzing the tablets of trazodone. The robustness of the method was studied with deliberate modifications in the flow rate of the mobile phase and column temperature. The optimized flow rate of the mobile phase was 0.8 mL/min and the same was altered by 0.2 units i.e. from 0.8 mL/min to 0.6 mL/min. The effect of column temperature on resolution was studied at 38 and 42 °C instead of room temperature (28 °C). However, the mobile phase components are held constant as described above. Stability of CCP HCl in diluent was checked by keeping it in an auto sampler and observing the variations in its peak areas. Methanol was tried as diluent in sample preparation.

3. Results and discussion

3.1. Method development

The main aim of the present LC–MS/MS method was separation and quantification of CCP HCl in trazodone active drug substance. Sample preparation is an important step in the pharmaceutical impurity analysis to control matrix effects and to improve the sensitivity as well as to achieve better analyte recovery. Several diluents were evaluated with respect to chromatographic efficiency and finally we found that methanol was a suitable solvent which provided good response, recovery and proper peak shapes for CCP HCl and trazodone.

Several attempts were made with different stationary phases including Kromasil C18, Symmetry C18 and Zorbax Rx C8 (with different dimensions) to achieve proper separation of the analyte and drug substance. Kromasil C18 and Zorbax Rx C8 were not found suitable as the response of analyte was found less and it is not well resolved from the trazodone drug substance peak. Only symmetry C18 (100 mm × 4.6 mm, 3.5 μm) column provided superior peak shape, baseline separation, desired linearity and reproducibility. Different compositions of mobile phase using 5.0 mM ammonium acetate-acetonitrile (10:90, 20:80 and 30:70, v/v) were checked for peak separation and sensitivity. Good separation and responses were observed using a mixture of 5.0 mM ammonium acetate-acetonitrile (30:70, v/v). Both isocratic and gradient elution modes were evaluated, but the isocratic elution was observed to be more efficient in achieving optimum separation of analyte from the drug substance peak. The column was thermostated at 30 °C to avoid any shift in retention time. The signal intensity obtained for CCP HCl in positive mode was much higher than that in negative mode. Then, the possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources under positive ion detection mode was evaluated during the early stages of method development. ESI spectra revealed higher signals for the molecule compared to APCI source. Therefore the method development was further limited to ESI source. Retention times of CCP HCl and trazodone were observed to be about 3.87 and 1.75 min respectively. The reproducibility of retention times for the analytes was expressed as % CV which was found to be less than 2.0% for 100 injections on the same column.

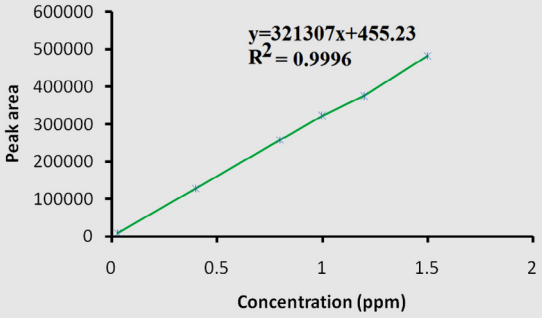
3.2. Method validation

The established method for the determination of CCP HCl in trazodone was validated according to US FDA and ICH guidelines.

3.2.1. Linearity

Linearity of the developed method was satisfactorily demonstrated with a six point calibration graph in the range of LOQ-150% of the estimated permitted level (viz. 1.0 ppm with respect to 5.0 mg/mL of trazodone solution). The calibration curve was drawn between the peak areas versus concentration of analyte. The slope, intercept and correlation coefficient values were derived from least squares linear regression analysis. The correlation coefficient obtained for CCP HCl was >0.9996 (Table 1). The linearity experiment revealed that the mass spectrometric responses were proportional to their concentration within the range of 0.03–1.5 ppm for CCP HCl impurity.

Table 1 Linearity plot of CCP HCl in the concentration range of 0.03–1.5 ppm.



Concentration (ppm)	Peak Area
0.03	9640
0.4	128533
0.8	257066
1.0	321332
1.2	385509
1.5	481995
Correlation	0.9997
Slope	321307
Intercept	455.230

3.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

To determine the LOD and LOQ values first we injected a 1.0 ppm solution of CCP HCl with respect to 5.0 mg/mL of trazodone, then the concentration was reduced sequentially to yield an S/N ratio of 3:1 and 10:1, respectively. Each predicted concentration was verified for its precision by preparing the solutions at about the predicted concentration and injecting each solution six times for analysis. The correlation coefficient obtained in each case was >0.9998. The predicted concentrations for LOD and LOQ are 0.01 and 0.03 ppm, respectively. The corresponding chromatograms are shown in Fig. 1 and further results are given in Table 2.

3.2.3. Precision

The precision of the method was evaluated at two levels viz. repeatability and intermediate precision. Repeatability was checked by calculating the % relative standard deviation (%RSD) of six replicate determinations by injecting six freshly prepared solutions containing 1.0 ppm of CCP HCl the same day. The same experiments were done on six different days to evaluate intermediate precision. The developed method was found to be precise as the %RSD values for intraday precision were less than 1.0%, similarly the %RSD values for interday precision were found to be less than 2.0% (Table 3).

3.2.4. Specificity

Specificity is the ability to assess the analyte unequivocally in the presence of the components which may be expected to be

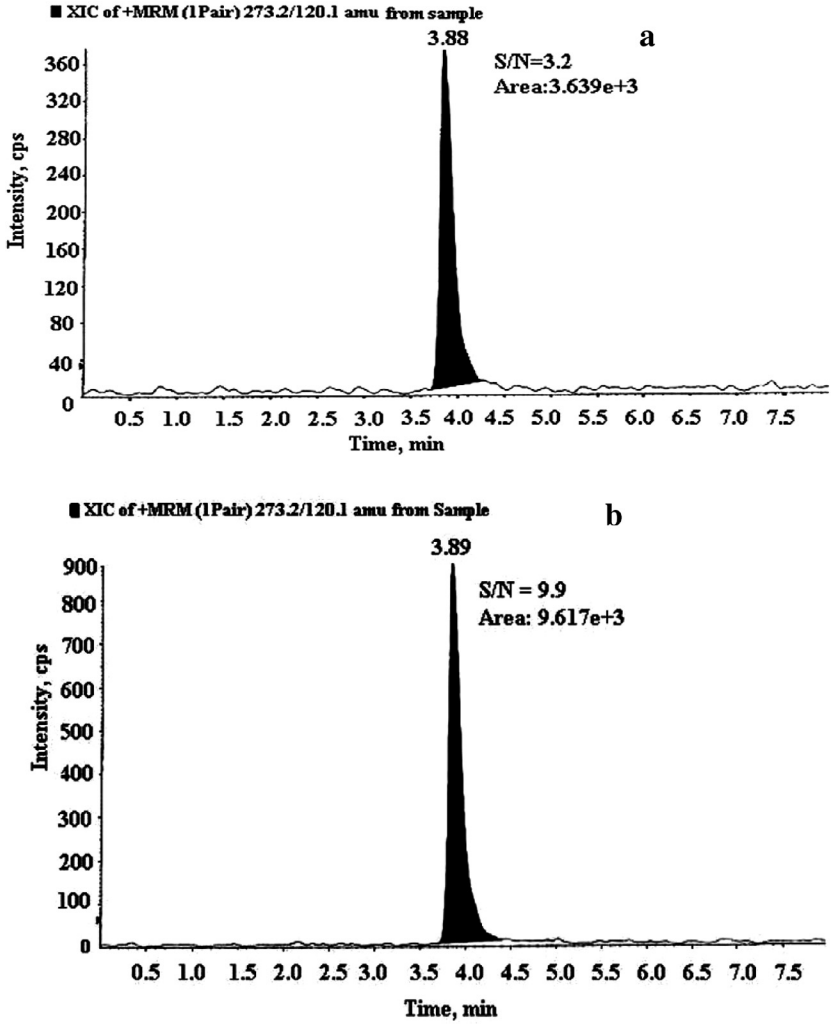


Figure 1 (a) LOD and (b) LOQ chromatograms of CCP HCl.

Table 2 LOD and LOQ data of CCP HCl at the concentrations of 0.01 ppm and 0.03 ppm, respectively.

Injection ID	LOD (peak area)	LOQ (peak area)
1	3639	9617
2	3784	9712
3	3529	9740
4	3648	9724
5	3456	9590
6	3722	9665
Mean	3663.00	9674.66
Std. dev.	54.6406	61.1282
%RSD	0.014	0.006
Concentration (ppm)	0.01	0.03

Table 3 Repeatability and intermediate precision of CCP HCl at a concentration of 1.0 ppm.

Injection ID	Repeatability	Intermediate precision
1	320500	320500
2	320100	319200
3	320200	318600
4	319100	318200
5	320800	318200
6	319200	317400
%RSD	0.22%	0.33%

present in sample matrix. The specificity of the method was best determined by analyzing the tablets of trazodone. The solutions of trazodone and CCP HCl were prepared individually at specification levels in the diluent and the solution of trazodone spiked with CCP HCl was also prepared and injected to LC–MS/MS. From the results we observed that the common excipients used in the tablets were not interfered at the retention times of CCP HCl impurity and trazodone. The corresponding blank and specificity chromatogram is shown in Fig. 2.

3.2.5. Recovery studies

The recovery of the method was determined in triplicate at LOQ, 0.5 ppm and 1.0 ppm concentrations in three batches

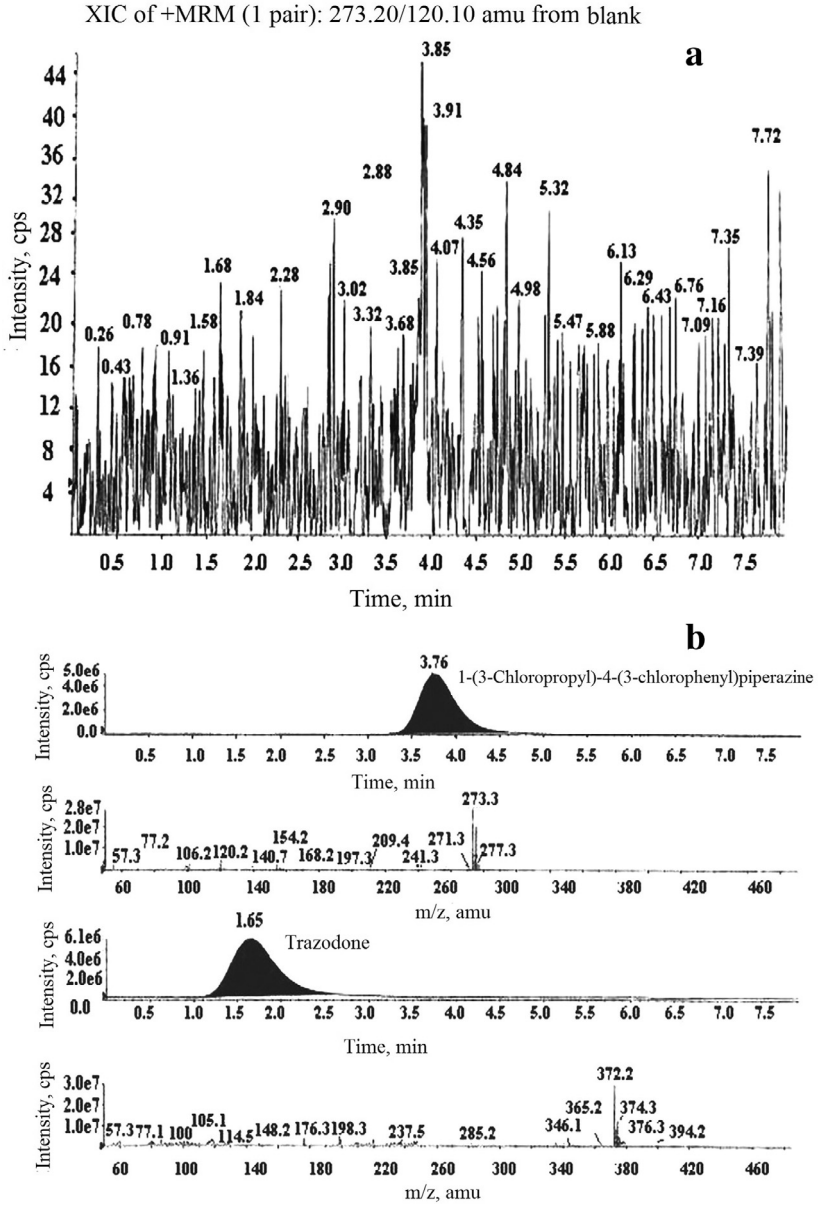


Figure 2 (a) Blank and (b) specificity chromatogram of CCP HCl.

of pure and formulation samples of trazodone. The recovery data are presented in Table 4. The results exhibited excellent recoveries of CCP HCl within the range of 96.69–101.94%. The recoveries at such lower concentrations were satisfactory with %RSD <2.0. Sample and accuracy chromatograms at LOQ levels are shown in Fig. 3. The results indicated that no extra amount of CCP HCl than spiked was found in pure and formulation batches of trazodone.

3.2.6. Robustness

Robustness of the method was determined by making deliberate changes in experimental conditions including flow rate and column oven temperature. The actual flow rate of mobile phase was 0.8 mL/min and the same was altered by 0.2 units i.e. 0.6 mL/min and 1.0 mL/min. The effect of temperature on chromatographic resolution was also studied at 38 °C and 42 °C (altered by 2 °C units). No significant change in the chromatographic performance was observed for all the above deliberately varied experimental conditions, which indicated the robustness of the method.

3.2.7. Stability and dilution integrity

The stability experiments were performed thoroughly to evaluate the stability of CCP HCl stock solutions at room temperature. The results demonstrated that the stock solution of CCP HCl was stable at room temperature for 48 h. The values for the percent change for the above stability experiments are compiled in Table 5. The percentage recoveries of stock solution at different time intervals were within the range from 96.0% to 102.0% of their nominal values. The results obtained

Table 4 Evaluation of accuracy and specificity of the method at LOQ, 0.5 ppm and 1.0 ppm concentrations.

Sample	% Recovery of CCP HCl[a] (mean ± %RSD)		
	0.03 ppm	0.5 ppm	1.0 ppm
Pure sample I	99.41 ± 1.39	100.15 ± 1.16	98.97 ± 1.22
Pure sample II	97.52 ± 1.42	101.30 ± 1.43	99.13 ± 0.86
Pure sample III	98.94 ± 1.06	98.97 ± 1.22	101.94 ± 1.45
Formulation sample I	100.24 ± 1.01	100.60 ± 1.44	100.18 ± 1.07
Formulation sample II	96.69 ± 1.61	99.52 ± 0.92	101.65 ± 0.59
Formulation sample III	101.27 ± 1.54	100.94 ± 1.48	100.72 ± 1.38

[a] Mean value of three determinations.

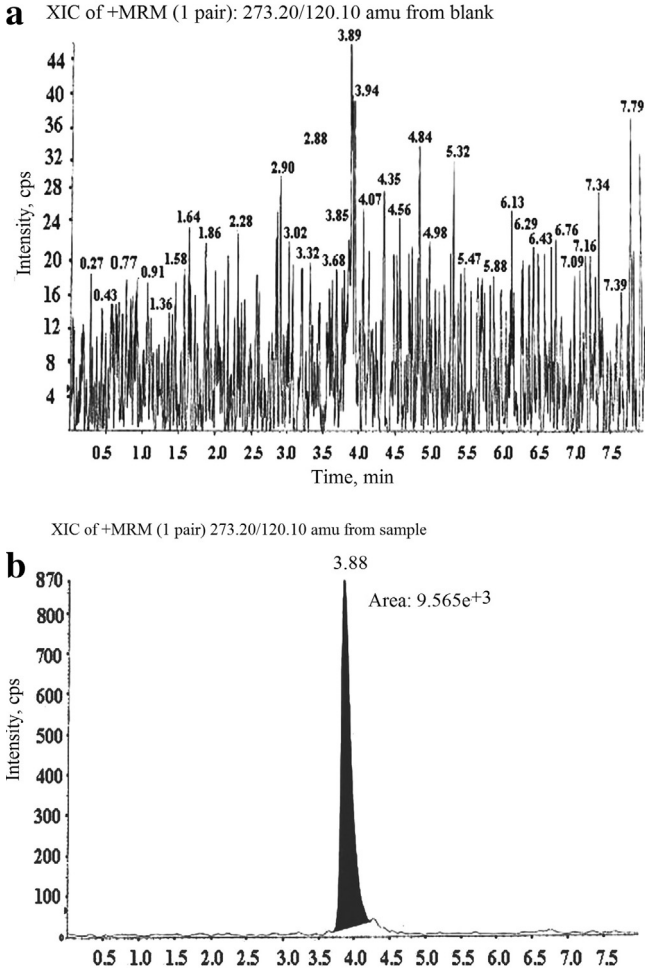


Figure 3 (a) Sample and (b) accuracy chromatogram of CCP HCl.

were then compared with the precision results of the method. The difference between recoveries at 0^{th} h and 48^{th} h was not more than 10%, which indicates that the sample prepared in diluent was stable for at least '48' h. Therefore, it is recommended to complete the analysis before 48 h of their preparation in methanol

4. Conclusions

The desired goal of the study is to develop a simple analytical method that is capable of quantifying CCP HCl in trazodone drug substance. Hence, a simple LC–MS/MS method which is capable to quantify the CCP HCl impurity at permitted levels is developed and validated. The method was fully validated and presents good linearity, specificity, accuracy, precision and robustness. The LOD and LOQ values for CCP HCl are very low as 0.01 and 0.03 ppm, respectively. The sample prepared in analytical solution is found to be stable for at least 48 h. Therefore the above mentioned LC–MS/MS method for the analysis of CCP HCl impurity is found to be simple, selective and sensitive. The method presented here could be very useful for monitoring of CCP HCl in trazodone during its manufacturing.

Acknowledgements

One of the authors Dr. A. Vijaya Bhaskar Reddy is highly grateful to the UGC (BSR), Government of India, New Delhi for financial assistance in the form of an award of Meritorious Research Fellowship and also thankful to the Universiti Teknologi Malaysia for providing Post Doctoral Financial Support through the Research University Grant.

Table 5 Solution stability data of CCP HCl at LOQ concentration.

Time	Sample area	Standard area	Spiked area	Theoretical conc. (ppm)	Measured conc. (ppm)	% Recovery
0 h	0	9885	9853	0.03	0.0299	99.67
	0	9885	9983	0.03	0.0302	100.99
12 h	0	9885	9810	0.03	0.0297	99.24
	0	9885	9942	0.03	0.0301	100.57
24 h	0	9885	9748	0.03	0.0295	98.61
	0	9885	9680	0.03	0.0293	97.92
48 h	0	9885	9780	0.03	0.0296	98.93
	0	9885	9890	0.03	0.0300	100.05

References

Acraf, Marcello, M., Tommaso, I., Giovanni, B.C., Giuseppe, B., 2009. WO2009019133 A1.

Andriollo, O., Lartigue-Mattei, C., Chabard, J.L., Bargnoux, H., Petit, J., Berger, J.A., 1992. J. Chromatogr. B 575, 301.

Ashby, J., Tennant, R.W., 1988. Mutat. Res. 204, 17.

Belvedere, G., Frigerio, A., Pantarotto, C., et al, 1975. J. Chromatogr. A 112, 631.

Carda-Broch, S., Gil Agusti, M.T., Monferrer Pons, L., Esteve Romero, J.S., 2007. J. Chromatogr. A 1156, 254.

De Jonghe, F., Swinkels, J.A., 1992. Drugs 43, 40.

Friedmann, P.D., Herman, D.S., Freedman, S., et al, 2003. J. Addict. Dis. 22, 91.

Gammans, R.E., Kerns, E.H., Bullen, W., et al, 1985. J. Chromatogr. B 339, 303.

Guidance for Industry: Bioanalytical Method Validation, Centre for Veterinary Medicine (CVM), May 2001.

Gupta, R.N., Lew, M., 1985. J. Chromatogr. B 342, 442.

Kram, D.J., McGovern, T., 2007. Adv. Drug Deliv. Rev. 59, 38.

Maj, J., Palider, W., Rawlow, A., 1979. J. Neural Transm. 44, 237.

Muller, L., Mauthe, R.J., Riley, C.M., Andino, M.M., De antonis, D., Beels, C., et al, 2006. Regul. Toxicol. Pharmacol. 44, 198.

Prasad, A.V.S.S., Sastry, C.S.P., 2002. J. Indian Chem. Soc. 79, 452.

Venugopal, N., Vijaya Bhaskar Reddy, A., Gangadhar Reddy, K., et al, 2012. J. Pharm. Biomed. Anal. 70, 592.

Venugopal, N., Vijaya Bhaskar Reddy, A., Madhavi, G., 2014. J. Pharm. Biomed. Anal. 90, 127.

Vijaya Bhaskar Reddy, A., Venugopal, N., Madhavi, G., et al, 2013. J. Pharm. Biomed. Anal. 84, 84.