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FORCED DEGRADATION STUDIES AND DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC CHROMATOGRAPHIC METHOD FOR TETRAZEPAM ASSAY AND RELATED SUBSTANCES

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ABSTRACT

Tetrazepam (TZP) is 7-Chloro-5-cyclohex-1-en-1-yl-1-methyl-1, 3-dihydro-2*H*-1,4-benzodiazepin-2-one, its available as muscle relaxant. In this study, degradation behavior of Tetrazepam was studied by subjecting the drug to various ICH stress conditions. Also a new, simple, sensitive and accurate stability-indicating methods were established for quantitative determination of Tetrazepam in the presence of various and related compounds (Impurity I, II and III). An expectable separation was achieved with ODS C18 column with flow rate 1.0 ml/min. UV Detection wavelength was used for estimation of Tetrazepam over a concentration range of 5 – 300 µg/ml with mean recovery of 99.23 – 101.78 %. Methods can analysis Tetrazepam related compound LOQ limit up to 0.016 µg/ml. Method can well resolve all degraded product as compare to Tetrazepam. Developed method can routinely used for the estimation of Tetrazepam related compounds from the dosage form and also for stability sample.

KEYWORDS: Tetrazepam, related compounds, stability indicating, HPLC, Tablet.

INTRODUCTION

Stability testing and forced degradation studies play a very crucial role during drug development. Stability is fundamental to all product characteristics and the term "stability indicating assay" has been used to describe "a procedure which affords specific determination of a drug substance in the presence of its degradation products". The prime goal of studying the stability of a drug is to determine the shelf-life of the drug. Identification of the degradation products, establishment of degradation pathways, determination of intrinsic stability of the drug molecules and validation of the analytical procedure are some of the goals achieved by stress testing.

The various conditions specified for forced degradation studies include thermal, acidic, alkaline and neutral hydrolysis conditions and oxidative and light stress.

Tetrazepam (TZP) is, 7-Chloro-5-cyclohex-1-en-1-yl-1-methyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one, occurs as light yellow or yellow crystalline powder, practically insoluble in water, freely soluble in methylene chloride and soluble in acetonitrile.[1]

Tetrazepam is used therapeutically as muscle relaxant.[2] Tetrazepam is an unusual benzodiazepine in its molecular structure as it has cyclohexenyl group which has substituted the typical 5-phenyl moiety.

Identification of Tetrazepam is carried out by infrared absorption spectrophotometry[1], also thin layer chromatography, gas chromatography, high performance liquid chromatography, and ultraviolet spectrum were reported as methods of determination.[3] So far very few liquid / gas chromatography procedures have been described for the determination of Tetrazepam.[4-13]

In the present study, during Alkali degradation, it was observed significant degradation behavior of TZP to form Impurity-I & II, while during Oxidative degradation potential degradation product has been observed as Impurity-III (reported as Impurity A in BP). The structure of possible related compounds/degradants is identified/characterized by the various characterization techniques such as UV, IR, NMR & Mass and chromatographically by HPLC spiking studies.

Also developed methods are precise, accurate, specific and sensitive stability indicating methods for estimation of TZP in presence of its degradation products.

Experimental

Apparatus: A Shimadzu HPLC, Model: LC-10ATvp (Shimadzu) with rheodyne injector, UV-Visible detector, Model: SPD-10 AVP (Shimadzu) and class VP software. HPLC Column, C18 (size-250 x 4.60 mm, I.D-5 µ) (Phenomenex). Nylon filter 0.45 µm. PH meter (Thermo

electro corporation). Drug was weighed on balance, Model ALC 210.4 (Acculab). Sonicator used was Ultra Sonicator (Fast Clean Ultrasonic Cleaner).

Reagents and Materials: Tetrazepam (TZP) was kindly supplied as gift samples by Torrent Research Center, Ahmadabad, India. HPLC grade Acetonitrile and sodium acetate was purchased from S.D. Fine Chemicals Ltd. (Mumbai). The water for HPLC was prepared by triple glass distillation and filtered through a nylon 0.45 µm – 47 mm membrane filter (Gelman Laboratory, Mumbia, India). Sodium hydroxide, hydrochloric acid and 30 % Hydrogen peroxide was purchased from Qualigens Fine Chemicals (Glaxo Ltd.). AR grade Acetonitrile was purchase form ACS chemicals (Ahmedabad). Impurity-I, II and Impurity-III is inhouse isolated by degradation process of Tetrazepam.

Chromatographic conditions: HPLC method. – The mobile phase has been used for separation consisting of Acetonitrile: water (50:50 v/v, pH 4.0, with phosphoric acid) using phenomenax C18 column with flow rate 1.0 ml/min. The elution was monitored by peak area at 228 nm, and the injection volume was 20 µL.

Stress studies: Acidic conditions. For acidic hydrolysis, Acid degradation study was performed by treating sample with 2 N hydrochloric acid, kept at about 60°C for 4 hours and analyzed as per method.

Alkaline conditions. Alkaline degradation studies were performed by keeping the drug content in 2 N NaOH, kept at about 60°C for 4 hours and mixture was neutralized.

Oxidation. Oxidative degradation study was performed by treating sample with 30%w/v Hydrogen peroxide , kept kept at about 60°C for 4 hours and analyzed as per method.

Photodegradation studies. Photodegradation studies were carried out by exposing the drug powder drug in a photostability chamber for 10 days. The powder was spread as a thin layer in a petri plate. The samples of both solution and powder were kept in parallel in darkness for the same period.

Thermal stress studies. The bulk drug, in a thin layer in a petri plate and drug solution (1 mg/mL) were exposed to thermal stress conditions in a hot air oven at 100ºC for 24 hours.

Preparation of standard stock solutions: TZP (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml acetonitrile properly and diluted up to mark with acetonitrile to obtain final concentration of 1000 µg/ml. 10 µg/ml solution was prepared for related compound.

Preparation of related compounds stock solutions: Separate stock solution of related compounds Impurity I, II and III of 10 µg/ml were prepared by dissolving 10 mg of each of related substance in 100 ml of acetonitrile. Further diluted 5 ml of resulted solution to 50 ml with Acetonitrile.

System Suitability Test: System suitability test of the chromatographic system was performed before each validation run using five replicate injections of a standard solution. Theoretical plates, and tailing factor were determined.

Method validation

Calibration curve: From the stock solution of TZP (1000 µg/ml) and TZP related compound (10 µg/ml), appropriate aliquots selected to prepared final concentrations of 10 to 300 µg/ml of TZP and 0.1 to 3 µg/ml of TZP related compound solution. All these solutions were injected into HPLC column and the peak area of each solution was measured at selected wavelength. Figure shows the resolution of TZP and its related substance.

Accuracy (% Recovery): To ensure the accuracy of method, recovery studies were performed by standard addition method at 80%, 100% and 120% levels of drug concentrations, to the pre-analyzed samples and they were re-analyzed.

Accuracy of the method for all the related substances was determined by analyzing TZP sample solutions spiked with all the related substances at three different concentration levels of 0.1 µg/ml, 1 µg/ml and 3 µg/ml and sample concentration of 1000 µg/ml each in triplicate.

Precision

Repeatability: Repeatability was performed by analyzing six separate TZP solutions of concentration 1000µg/mL that were prepared by spiking the related substances to give 1 µg/mL of each of Impurity I, II and III. The %R.S.D for each related substance was evaluated.

Intermediate Precision: The intermediate precision of the method for TZP and related substances was determined on three separate sample solutions prepared by spiking the related substances by two different analysts on two different days. The mean values of results for each day and for each analyst were compared.

Robustness: The robustness of the method was checked by repeatedly injecting (n = 5) standard solutions of 100 µg/ml in two C18 column one was made by phenomenex and one by hypersil for the HPLC method.

Limit of Detection and Limit of Quantification: The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following

equations as per International Conference on Harmonization (ICH) guideline17.

LOD = 3.3 x (X/ S)
LOQ=10 x (X/S)

Where X = the standard deviation of the response and S = the standard deviation of y-intercept of regression lines.

Isolation of TZP related compounds: Drug substance was kept under Alkaline medium for 24 hrs and impurity formation compound was filtered and isolated to check the retention time of degradation product formation. Formed product retention time is matching with the degradation product observed during alkali degradation. Further Impurity was purified by preparative TLC method. Alkali degradtion impurity was detonated as Impurity – I & II.

Drug substance was kept under with 30% H2O2 in alkaline medium for 24 hrs and impurity formation compound was filtered and isolated to check the retention time of degradation product formation. Retention time of product formed is matching with the degradation product observed during peroxide degradation. Further Impurity was purified by preparative TLC method. Alkali degradtion impurity was detonated as Impurity –III.

Analysis of TZP related compound in TZP tablet formulation: Full content was transferred into a 10 ml volumetric flask containing 5 ml ACN, sonicated for 15 min and further diluted to 10 ml with ACN. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 20 µl of this solution was injected into HPLC column for two times and peak area was measured at 228 nm and average was considered for HPLC method.

The amount of TZP in sample solution was determined by fitting the responses into the regression equation of HPLC.

RESULT AND DISCUSSION

Table 1: System suitability parameters of TZP.

Sr. No	System suitability parameters	Retention time (minutes)	RRT	Theoretical Plate	Tailing Factor
1	TZP	5.379	**1.0**	8695	1.02
2	Impurity I	7.932	**1.5**	5984	1.05
3	Impurity II	8.736	**1.6**	4628	1.12
4	Impurity III	1.325	**0.2**	6582	1.03

Table 2: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	TZP	Impurity I	Impurity II	Impurity III
2	Linearity range	1- 300µg/ml		0.1 – 3 µg/ml	
4	Correlation coefficient (r^2)	0.9999	0.9999	0.9999	1
5	Intercept	156325	56282	26852	6325
6	Slope	5623	325	4582	254
8	Precision				
	Intra day Average % RSD (n = 5)	0.26	0.65	0.45	0.55
	Inter day Average % RSD (n = 5)	1.21	0.89	1.25	1.24
	Reproducibility of measurements %RSD	0.21	0.56	0.21	0.65
	% Recovery	99.23-101.78	99.96-100.14	99.99-101.12	99.56-100.52
9	Limit of detection (µg/ml)	0.016	0.0012	0.0014	0.0081
10	Limit of quantification (µg/ml)	0.0528	0.0039	0.0046	0.0267

%RSD calculated form five replication of readings.

Table 3: Accuracy data of TZP by HPLC with UV detection

Initial conc. (µg/ml)(A)	Quantity of std. Added (µg/ml)(B)	Total Amount (A + B)	Peak Area	
			Total quantity Found*± S.D.	%Recovery ± S.D
100	150	250	250.65 ± 0.28	100.26 ± 0.26
100	300	400	399.56 ± 0.42	99.89 ± 0.27
100	450	550	549.82 ± 0.65	99.96 ± 0.21

***Average of five readings**

Table 4: Accuracy data of TZP related substance by HPLC with UV detection

Amount Added	Impurity I		Impurity II		Impurity III	
	Total quantity Found*	%Recovery ± S.D	Total quantity Found*	%Recovery ± S.D	Total quantity Found*	%Recovery ± S.D
0.1	0.099	99.00±0.98	0.101	101.05± 0.38	0.099	99.85±0.26
1	1.01	101.06±0.69	1.02	102.06±0.16	0.99	99.06±0.45
3	2.99	99.66±0.75	3.01	100.33±0.54	3.01	100.28±0.28

***Average of five readings**

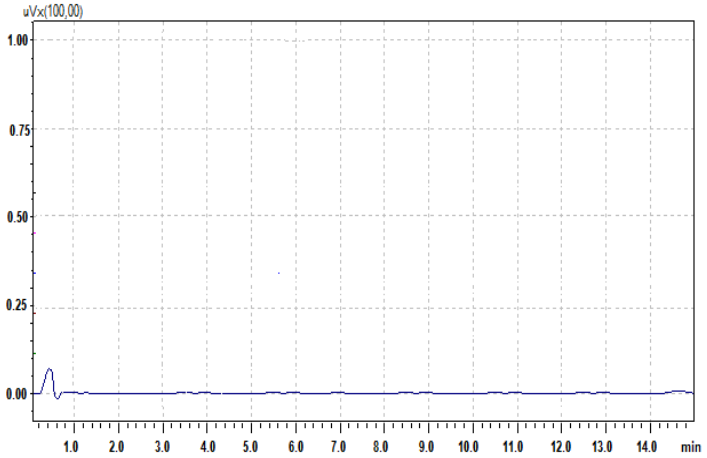

Figure 1: Blank solution by HPLC with UV detection

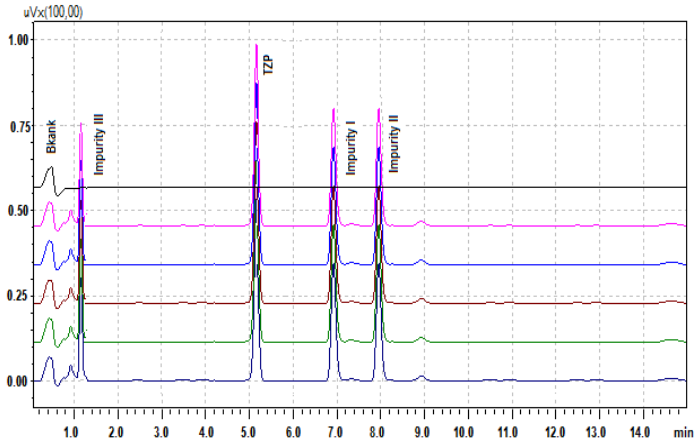

Figure 2. Linearity of Tetrazepam and related substances.

HPLC Method: To optimize the HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for TZP was obtained with a mobile phase consisting of buffer (Acetonitrile: Water (50: 50, v/v) pH adjusted to 4.0 with o-Phosphoric Acid. The elution was monitored by peak area at 228 nm and the injection volume was 20 µL using phenomenax C18 column with flow rate 1.0 ml/min. Detection wavelength was 228 nm. A complete resolution of the peaks with clear baseline separation was obtained (Figure 1).

Three related substances were detected and well resolved by the method. The retention data for TZP and related substances is indicated in Table 1.

Validation of the Proposed Method: Linearity. – Linear correlation was obtained between peak areas and concentrations of TZP in the range of 10 – 300 µg/ml and 0.1 to 3 µg/ml for TZP related compounds. The linearity of the calibration curves was validated by the high value of correlation coefficients of regression (Table 2).

Accuracy. – The recovery experiments were carried out by the standard addition method. The recoveries obtained were ranged between 99.23 – 101.76%. The values of % assay range 99-102% indicated there is no any interference form excipient present in formulation. Figure show that TZP can well separate form its all type

of degradation products so developed HPLC method is specific and selective for TZP.

Precision was expressed in terms of % R.S.D. All values for precision were within recommended limits.

The % RSD values for precision and LOD and LOQ were reported in table 2.

CONCLUSIONS

In this study, it was possible to develop a selective and validated stability indicating HPLC assay method for Tetrazepam on a C18 column, which could separate the drug and its degradation products formed under a variety of stress conditions. TZP was found to be sensitive to the alkali and oxidative condition, whereas it was comparatively stable in Acid, thermal and photolytic condition. TLC used to isolated and separate TZP form its degradation products. Based on NMR and Mass data three Impurities was isolated and characterized as Impurity I (5- chloro-2-(methylamino)pheny) (cyclohex-1-en-1-yl)Methanone, , Impurity II 7-chloro-10-methyl-3,4,4a,10-tetrahydroacridin-9(2H)-one, Known as Temazepam and Impurity III 7-Chloro-5-(cyclohex-1-enyl)-1-methyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (Identified as oxidative impurity, reported as Impurity A in BP).

The results of the analysis of pharmaceutical dosage forms by the proposed methods are highly reproducible and reliable and are in good agreement with the label claim of the drug. The method can be used for relates substance analysis of TZP in pharmaceutical preparation and also it is hoped that this report on stability indicating method and degradation of TZP would be helpful for the multiple generic manufacturers of the drug around the globe by saving them for unnecessary repetition of the same studies.

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