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Stability indicating HPLC method for the determination of tetrazepam in presence of its acidic degradation products

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ABSTRACT

High performance liquid chromatographic technique was proposed for the determination of tetrazepam (TP) in presence of its degradation products. The method was based on HPLC separation of TP from its acidic degradation products using ZORBAX Eclipse XDB-C18 column at ambient temperature with mobile phase consisting of acetonitrile : distilled water, pH was adjusted to 4.00 using phosphoric acid (50:50 v/v). Quantification was achieved with UV detection at 228 nm based on peak area. The drug was subjected to acid hydrolysis. Complete separation was achieved for the parent compound and all degradation products in an overall analytical run time of approximately 10 min with the parent compound TP eluting at approximately 4.31 min. The method was linear over the concentration range of 1–10 µg/ml (r^2 = 1.0000) with a limit of detection and quantitation 0.0349 and 0.1164 µg/ml, respectively. The method has the requisite accuracy, selectivity, sensitivity and precision to assay TP in tablets.

KEYWORDS

Tetrazepam;
HPLC determination;
Acidic degradation;
Stability indicating study.

INTRODUCTION

Tetrazepam, 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 seen in other benzodiazepine and has the following structural formula (Figure 1).

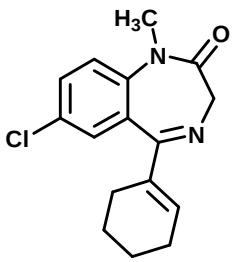

Figure 1 : Tetrazepam is an unusual benzodiazepine in its molecular structure

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].

EXPERIMENTAL

Chemicals and reagents

Pharmaceutical grade TP (Global Napi Pharmaceuticals, Egypt) was used and certified to contain 99.8%. Methanol (HPLC grade, SGMA, Germany), Acetonitrile (HPLC grade, SGMA, Germany), Phosphoric acid, Hydrochloric acid, Sodium Hydroxide used were analytical grade (ADWIC, El-Nasr Pharmaceuticals Chemicals Co. Egypt).

Myolastan Tablets (Batch No. 87208) labeled to contain 50 mg TP per tablet. Manufactured by Global Napi Pharmaceuticals, Egypt, under license of Sanofi Synthelabo – France.

HPLC instrumentation and conditions

Samples were loaded into Rheodyne 7725i injection valve, equipped with a 20-µL sample loop (Rheodyne, Berkeley, CA, USA). HPLC separation and quantitation were made on ZORBAX Eclipse XDB-C18 (4.6 X 15 mm i.d., 5 µm particle size, analytical column from Agilent Technologies, USA) with a mobile phase consisting of acetonitrile : distilled water, pH was adjusted to 4.00 using phosphoric acid (50:50 v/v). An isocratic pump was used to deliver the mobile phase at a flow rate of 1 ml/min (Agilent 1100 Series Iso pump G1310A, Agilent Technologies, USA). The samples were also filtered using 0.45 um disposable filters (Millipore). The UV-VIS detector (Agilent 1100 Series VWD G1314A) was set at 228 nm. Data acquisition was performed on Agilent LC ChemStation software. All determinations were performed at ambient temperature.

Preparation of mobile phase

The mobile phase was prepared by mixing acetonitrile and distilled water in a ratio of 65:35 (v/v) and pH was adjusted to 4.00 using phosphoric acid. The flow rate was 1 ml/min. Mobile phase was filtered through 0.45µm membrane filter (Millipore, Milford, MA, USA) and degassed before application by means of ultra-sonication for 5 min.

Preparation of solution

Preparation of intact TP standard stock solution

Intact TP standard solution (100 µg/ml) in methanol was prepared.

Preparation of the acidic degradation products solution of TP

An accurately weighed amount of intact TP (10 mg) was refluxed for 1 hour with 10 ml 2N HCl. The solution was then neutralized by adjusting the pH using NaOH. The volume was completed to 100 ml volumetric flask using methanol to produce concentration equivalent to 100 µg/ml. Five ml was further diluted into 100 ml volumetric flask and completed to the mark with methanol to produce a solution of concentration equivalent to 5 µg/ml.

Preparation of laboratory prepared mixture for stability indicating characterization of the method

Five ml aliquot from TP standard solution stock (100 µg/ml) was transferred into 100 ml volumetric flask. The volume was completed with methanol to produce a solution of 5 µg/ml of TP. Aliquot portions of the prepared stock (9, 8, 7, 6, 5, 4, 3, 2 ml) were further transferred into a serious of 10 ml volumetric flasks, volumes were completed to the mark by aliquots from the acidic degraded sample solution (5 µg/ml) (1, 2, 3, 4, 5, 6, 7, 8 ml respectively) to prepare different mixtures containing 10-80% of the degradation product.

Standard solutions and calibration

Ten ml aliquot of TP standard solution (100 µg/ml) were diluted with methanol in 100 ml volumetric flask to produce a stock solution containing 10 µg/ml of TP. Different aliquots of the prepared TP stock solution (1-10 ml), equivalent to 1-10 µg/ml, were transferred into a serious of 10 ml volumetric flasks. The volumes were adjusted with the mobile phase to prepare the standard solutions. An aliquot of 20 µL was injected into the chromatographic system and processed according to the method described in this work. Flow rate: 1 ml/min.

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Pharmaceutical sample preparation

An accurately weighed amount of the powdered tablets equivalent to10 mg TP was transferred into a 100 ml volumetric flask, the volume was completed to the mark using methanol. The solution (100 µg/ml) was shaken for 1 hour using ultrasonic bath. The solution was then filtered on a dry funnel and a dry filter paper; the first ten ml were rejected. Twenty five ml of the filtrate was further diluted to 100 ml using methanol to produce a diluted solution of concentration (25 µg/ml) TP. Different aliquots of the last diluted solution (3, 5, 7 ml) were transferred into a serious of 25ml volumetric flasks and the volumes were completed to the mark with the mobile phase to produce solutions of concentration equivalent to (3, 5, 7 µg respectively). The general procedure for HPLC method described in this work was followed and the concentration of the drug was calculated. The accuracy of the analytical method was also checked by applying the standard addition technique.

RESULTS AND DISCUSSION

In this part simple, precise, and selective HPLC method for the determination of oral skeletal muscle relaxant drug TP in pure and in presence of their degradation products was developed. The method was successfully applied for the determination of the drug in their pharmaceutical dosage forms.

Chromatographic conditions

To optimize HPLC assay parameters, the effect of the mobile phase composition was studied.

Effect of acetonitrile volume

The influence of acetonitrile concentration on the separation efficiency was investigated at constant pH. Increasing acetonitrile concentration to 70% led to decrease in retention time and decrease in peak symmetry. At lower acetonitrile concentration (50%) separation occurred by band broadening and excessive increase in retention time. Acetonitrile concentration of 65% was found to give acceptable separation between the target compounds.

Effect of pH

Retention time increases by the increase of pH over the range of 3-5, while it decreases again over a range of 6-7. It showed best resolution at pH 4.00.

Final conditions for which the method was validated were: ZORBAX Eclipse XDB-C18 analytical column and acetonitrile-distilled water, pH 4.00) (65:35 v/v) as a mobile phase and injection volume of 20µl for standard solutions and samples of tablets were applied. Under these conditions successful separation and sufficient retention of the target analyte as well as its acidic degradation products was achieved. Representative chromatograms showing successful separation of all compounds of interest is shown in figure 2. The average retention time ± SD for TP and its degradation products

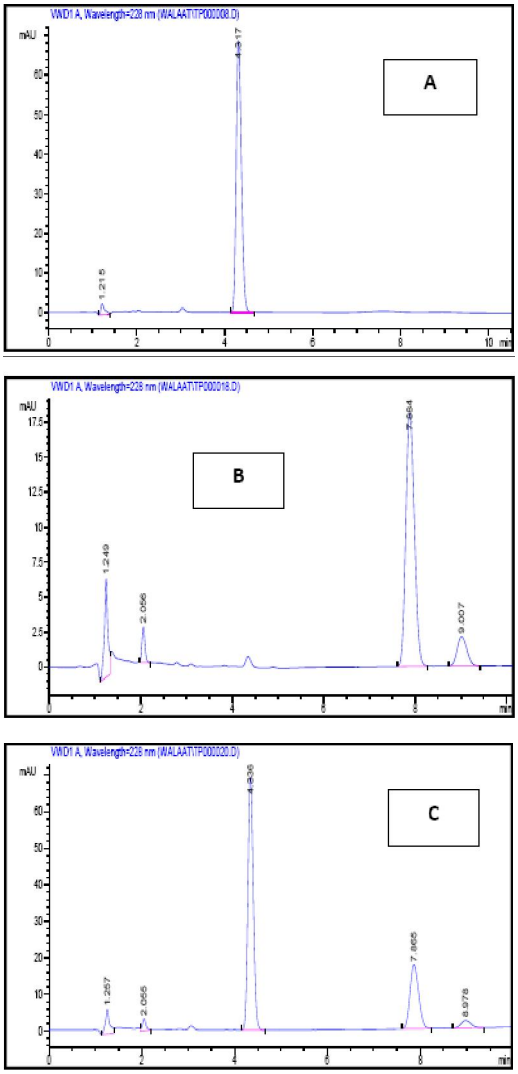

Figure 2 : Typical HPLC chromatograms of: (A) intact TP 4 µg/ml (t_r = 4.317 min): (B) acidic degradation products equivalent to 4 µg/ml (t_r =2.056, 7.884, 9.007 min): (C) intact TP 4 µg/ml (t_r = 4.336 min) in presence of its acidic degradation products equivalent to 4 µg/ml TP (t_r = 2.005, 7.865, 8.978 min)

were found to be 4.32 ± 0.02 min, 2.05 ± and 7.87 ± and 9.01 ± 0.01 min, respectively for ten replicates.

Validation of the methods

(1) Linearity

Peak areas of TP were plotted versus TP concentration and linear regression analysis performed on the resultant curve. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p < 0.05$) different from zero (TABLE 1). Characteristic parameters for regression equitation for the HPLC method obtained by least squares treatment of the results were given in TABLE 1. Typically, the regression equation for the calibration curve was found to be $Y = 137.4334\ C + 0.0940$, where C is the concentration in µg/ml, y is the peak area.

TABLE 1 : Characteristic parameters for the regression equation of the proposed HPLC method for the determination of TP

Parameters	Value
Calibration range (µg/ml)	1-10 µg/ml
Detection limit (µg/ml)	0.0349
Quantitation limit (µg/ml)	0.1164
Regression equation (Y)[a] : Slope (b)	137.4334
Standard deviation of the slope (S_b)	0.2582
Relative standard deviation of the slope (%)	0.1879
Confidence limit of the slope [b]	136.7165-138.1504
Intercept (a)	0.0940
Standard deviation of the intercept [b]	1.5672
Confidence limit of the intercept	-4.2572-4.4451
Regression coefficient	1.0000
Standard error of estimation	2.0140

[a]Y = a + bC, where C is the concentration of Tetrazepam in µg/ml and Y is the peak area; [b]95% confidence limit

(2) Intra- and Inter-day validations

TABLE 2 shows the precision of the analytical procedure for both intra- and inter-day variations expressed as the coefficient of variance (CV%). Repeatability (intra-day CV%, n = 5) was excellent being in range of 0.325-1.114. Reproducibility (inter-day CV%, n = 5) was in range of 0.277-0.780 (TABLE 2).

Determination in TP in myolastan tablet and in presence of its degradation products

The method was applied for the determination of the drug in laboratory prepares mixture with its acidic degradation products (TABLE 3) (10% up to 80%for

TABLE 2 : Intra- and inter- day validation for determination of TP by HPLC method

Concentration (µg/ml)	Intra-day assay	
	Recovery% ± SD[a]	CV%
2	99.81 ± 1.112	1.114
4	100.07 ± 0.959	0.958
8	99.64 ± 0.324	0.325
	Inter-day assay	
	Recovery% ± SD[a]	CV%
2	99.27 ± 0.564	0.568
6	100.64 ± 0.279	0.277
10	100.56 ± 0.784	0.780

[a]Mean and S.D. for five determinations

TABLE 3 : Determination of TP in presence of its acidic degradation products using HPLC method

Conc.Taken(µg/ml)		Peak area	Conc. Found of TP (µg/ml)	Recovery %
Intact TP	Degradate			
4.5	0.5	630.5	4.587	101.93
4	1	556.6	4.049	101.22
3.5	1.5	486.1	3.536	101.03
3	2	413.4	3.007	100.23
2.5	2.5	341.9	2.487	99.48
2	3	274.6	1.997	99.85
1.5	3.5	206.3	1.500	100.00
1	4	138.6	1.008	100.80
In presence of acidic degradation products Mean±SD = 100.57±0.818				

TABLE 4 : Application of standard addition technique for determination of TP in Myolastan tablet by HPLC method

Conc. Taken (µg/ml)		Peak area		Conc. Found (µg/ml)		Recovery %	
Myolastan	Added	Myolastan	Total	Myolastan	Added	Myolastan	Added
3	0	420.2	420.2	3.057	0	101.90	0
	2		697.6		2.018		100.90
	4		970.8		4.006		100.15
	6		1257.1		6.089		101.48
5	0	698.8	698.8	5.084	0	101.68	0
	1		838.3		1.015		101.50
	4		1248.4		3.999		99.97
	5		1396.3		5.075		101.50
7	0	974.4	974.4	7.089	0	101.27	0
	1		1112.9		1.008		100.80
	2		1251.0		2.013		100.65
	3		1389.3		3.019		100.63
For Myolastan tablet Mean±SD=101.62±0.319 For TP Mean±SD=100.84±0.569							

degradate), and in Myolastan tablet. The validity of the method was assessed by applying the standard addition technique and good recoveries have been obtained (TABLE 4).

The amounts of TP in Myolastan tablet was 50 mg per tablet. None of the tablet ingredients interfered with the analyte peak. The results of the presented HPLC method were compared with those of the reported HPLC method[3]. Statistical comparison between the results was performed with regards to accuracy and precision using Student's t-test and F-ratio at 95% confidence level (TABLE 5). There is no significant difference between the two methods.

TABLE 5 : Statistical comparison between the results obtained by applying HPLC method for the analysis of Tetrazepam and the reported method

Statistical item	HPLC method	Reported method
Mean ± SD	99.31 ± 1.050	99.42 ± 0.476
RSD	1.0573	0.4788
Variance	1.1022	0.2262
n	5	5
t-test	0.2134 (1.8595)*	
F-test	4.8730 (6.3882)*	

CONCLUSION

The suggested method is simple, accurate, selective and sensitive. Application of the proposed method to the analysis of TP in laboratory prepared mixtures and pharmaceutical formulation shows that neither the degradation products nor the excipients interfere with the determination, indicating that the proposed method could be applied as stability indicating methods for the determination of pure TP and in presence of its degradation products, either in bulk powder or in pharmaceutical formulations. Statistical analysis of the results obtained by the proposed method and by the reported method, revealed no significant difference within a probability of 95%.

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