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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF DIAZEPAM AND PROPRANOLOL HYDROCHLORIDE IN TABLETS

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ABSTRACT

A simple, specific, accurate and precise reverse phase high performance liquid chromatographic method was developed for simultaneous estimation of diazepam and propranolol hydrochloride in pharmaceutical tablet formulation. The separation was achieved on Phenomenex C_{18} column (250 mm i.d., 4.6 mm, 5 µm particle size) using methanol: acetonitrile: water (50 : 25 : 25, v/v/v, pH adjusted to 2.8 ± 0.05 with ortho- phosphoric acid) as the mobile phase at a flow rate of 1.0 ml min^{-1}. The quantification was achieved with PDA detector at 235 nm. The injection volume was 20 µl. The retention times of diazepam and propranolol hydrochloride were 5.38 ± 0.29 min and 3.80 ± 0.15 min, respectively. The method was validated for linearity, precision, specificity, robustness and recovery according to the ICH guidelines. The linearity was obtained in the concentration range of 0.1-5.0 µg/ml for both drugs with mean recovery of 100.3 ± 0.47 and 100.2 ± 0.78 % for diazepam and propranolol hydrochloride, respectively. The limit of detection and quantification for diazepam were 0.015 and 0.050 µg/ml, respectively and for propranolol hydrochloride were 0.014 and 0.045 µg/ml, respectively. The method was found to be simple and highly sensitive and can be useful in the routine quality control of diazepam and propranolol hydrochloride in bulk manufacturing and pharmaceutical dosage forms.

Key words: Diazepam, propranolol hydrochloride, RP-HPLC, validation, simultaneous, tablet

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INTRODUCTION

Diazepam (DZP) (Figure 1) is chemically 7-chloro-1, 3-dihydro-1-methyl-5-phenyl-1, 4-benzodiazepin-2-one; $C_{16}H_{13}ClN_2O$[1], used as an anxiolytic agent[2]. It is official in IP, USP and BP. IP[3] and BP[4] describes non-aqueous titration method and USP[5] describe liquid chromatography method for its estimation. Literature survey reveals spectrophotometric[6-9], spectrofluorimetric[10], GC[11], HPLC[12], HPTLC[13-14], LC/MS[15] and radioimmunoassay[16] methods for the estimation of DZP in single dosage form. Propranolol hydrochloride (PRO) (Figure 2) is chemically (*RS*)-1-isopropylamino-3- (1-naphthyloxy) propan-2-ol hydrochloride; $C_{16}H_{21}NO_2$, HCl[17], is beta-adrenoceptor antagonist[18]. The combination of DZP and PRO has been shown to be effective in the management of chronic anxiety. The combination was generally more effective than diazepam[18]. Propranolol hydrochloride is official in IP, USP and BP. IP[19] and BP[20] describes potentiometric titration method and USP[21] describe liquid chromatography method for its estimation. Literature survey reveals spectrophotometric[22-24], fluorimetric[25], HPLC[26-27] and chemiluminescence[28] methods for estimation of propranolol hydrochloride in single dosage form. This combination is not official in any pharmacopoeia, so no official method is available for the estimation of these two drugs in combined dosage forms. Literature survey reveals spectrophotometric[29] method for the simultaneous estimation of DZP and PRO in combined dosage form. The present manuscript describes simple, sensitive, accurate, precise, specific and robust HPLC method for simultaneous determination of diazepam and propranolol hydrochloride in pharmaceutical tablet dosage form.

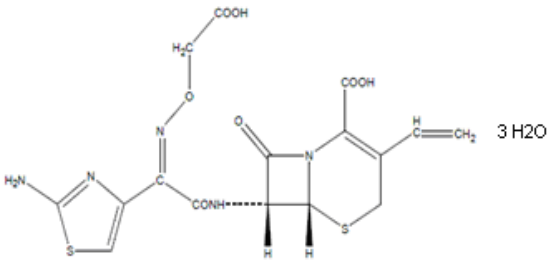

Figure 1: Chemical structure of Diazepam (DZP)

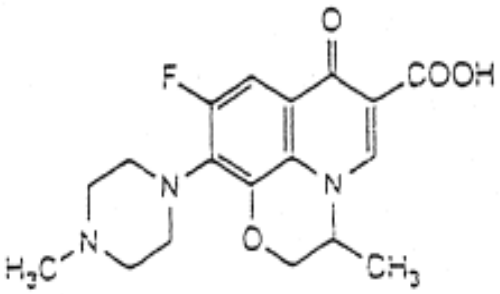

Figure 2: Chemical structure of Propranolol Hydrochloride (PRO)

MATERIALS AND METHODS

Reagents and Materials

DZP and PRO bulk powder was kindly gifted by Santham Pharmaceutical Ltd, Gandhinagar, Gujarat, India. The commercial fixed dose combination product containing 2 mg DZP and 10 mg

PRO was procured from the local pharmacy. HPLC grade acetonitrile and methanol was purchased from S.D. Fine Chemicals Ltd., Mumbai, India. The water for HPLC was prepared by triple glass distillation and filtered through nylon 0.45 µm-47 mm membrane filter (Gelman laboratory, Mumbai, India). Ortho-phosphoric acid and methanol was purchased from S.D. Fine Chemicals Ltd., Mumbai, India and were of analytical reagent grade.

Instrumentation

A Shimadzu (Columbia, MD) RP-HPLC instrument (LC-2010C_{HT}) equipped with a UV-Visible detector and a photodiode array detector, manual injector with 20 □ L loop, Phenomenex (Torrance, CA) C_{18} column (250 mm × 4.6 mm id, 5 □ m particle size), LC-solution software, Digital pH meter (LI 612 pH analyzer, Elico, Mumbai), analytical balance (Sartorius CP224S, Gottingen, Germany), ultra sonic cleaner (Frontline FS 4, Mumbai, India) were used in the study.

Preparation of mobile phase

The mobile phase comprised of methanol: acetonitrile: water (50:25:25, v/v/v, pH adjusted to 2.80 ± 0.05 with ortho-phosphoric acid.). The mobile phase was filtered through nylon 0.45 µm-47 mm membrane filter and was degassed before use.

Preparation of DZP and PRO standard stock solutions

Accurately weighed DZP (10 mg) and PRO (10 mg) was transferred to a separate 100 ml volumetric flask and dissolved and diluted to the mark with methanol to obtain a standard stock solutions having concentration DZP (100 µg/ml) and PRO (100 µg/ml).

Preparation of DZP and PRO mixed standard solution

DZP and PRO standard stock solution (10 ml) was transferred to 100 ml volumetric flask and volume was made up with mobile phase to obtain a mixed standard solution having concentration DZP (10 µg/ml) and PRO (10 µg/ml).

Preparation of sample solution

Twenty tablets were weighed and powdered. The quantity of the powder equivalent to 2 mg of DZP and 10 mg of PRO was transferred to a 100 ml volumetric flask. The content was mixed with methanol (50 ml), sonicated for 20 min to dissolve the drug as completely as possible and the volume was adjusted up to the mark with methanol. The solution was then filtering through Whatman filter paper no. 41. The above solution (1.0 ml) was transferred to a 10 ml volumetric flask and the volume was adjusted up to mark with mobile phase. An aliquot of this solution (1.0 ml) was taken in to a 10 ml volumetric flask and the volume was adjusted up to mark with mobile phase.

Determination of analytical wavelength

The standard solution of DZP and PRO were injected under the chromatographic condition described above. The elution showed reasonable good response at 235 nm with PDA detector. So both drugs were detected at that common analytical wavelength.

Validation of the proposed HPLC method

Calibration curve (linearity)

Calibration curves were constructed by plotting peak areas Vs concentrations of DZP and PRO, and the regression equations were calculated. The calibration curves were plotted over the concentration range 0.1–5 µg/ml for both DZP and PRO. Accurately measured standard working solutions of DZP and PRO (0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0, 4.0 and 5.0 ml) were transferred to a series of 10 mL of volumetric flasks and diluted to the mark with mobile phase. Aliquots (20 µl) of each solution were injected under the operating chromatographic conditions described above.

Accuracy (% recovery)

The accuracy of the method was determined by calculating recoveries of DZP and PRO by the standard addition method. Known amounts of standard solutions of DZP and PRO were added at 50, 100 and 150 % level to prequantified sample solutions of DZP and PRO (0.3 + 1.5 µg/ml). The amounts of DZP and PRO were estimated by applying obtained values to the regression equation of the calibration curve.

Method precision (% repeatability)

The precision of the instrument was checked by repeatedly injecting (n = 6) standard solutions of DZP and PRO (1.0 µg/ml) under the same chromatographic condition and peak area, retention time and tailing factor was measured. The results were reported in terms of percent relative standard deviation (% RSD).

Intermediate precision (reproducibility)

The intraday and interday precision of the proposed method was determined by analyzing the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of standard solutions of DZP and PRO (0.8, 1 and 2 µg/ml). The results were reported in terms of percent relative standard deviation (% RSD).

Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations as per International Conference on Harmonization (ICH) guidelines[30].

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where σ = the standard deviation of the response

S = Slope of calibration curve.

Robustness

The robustness of the method was established by introducing small changes in various parameters like mobile phase composition, pH of mobile phase, wavelength and flow rate. For the same, mobile phases having different compositions, like methanol: acetonitrile: water (51 + 24 + 25, v/v/v), (51 + 25 + 24, v/v/v), (50 + 26 + 24, v/v/v), (50 + 24 + 26, v/v/v) were tried and chromatograms were run. The changes made in wavelength, flow rate and pH were ± 1 nm, ± 0.05 ml/min and ± 0.1 units, respectively. Robustness of the method was evaluated by calculating the % RSD values. Robustness of the method was done at different concentration levels in the range of 0.1–5 µg/ml for both DZP and PRO.

Specificity

The specificity of the method was ascertained by analyzing standard drugs and sample of DZP and PRO. The peak purity of DZP and PRO were assessed by comparing the peak purity of sample with standard DZP and PRO.

Analysis of DZP and PRO in combined dosage form

The response of the sample solution was measured at 235 nm under the chromatographic condition mentioned above for the quantitation of DZP and PRO. The amounts of the DZP and PRO present in the sample solution were determined by fitting the responses into the regression equation for DZP and PRO.

RESULTS AND DISCUSSION

Method development

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for DZP and PRO was obtained with a mobile phase methanol: acetonitrile: water (50:25:25, v/v/v, pH adjusted to 2.80 ± 0.05 with ortho-phosphoric acid) at a flow rate of 1.0 ml/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection at 235 nm based on peak area. Complete resolution of the peaks with clear baseline was obtained (Figure 3). Peak purity of drugs was confirmed by comparing the spectra of standard and sample solutions. The overlain spectrum of

standard and sample shows good correlation (Figure 4). System suitability test parameters for DZP and PRO for the proposed method are reported in Table 1.

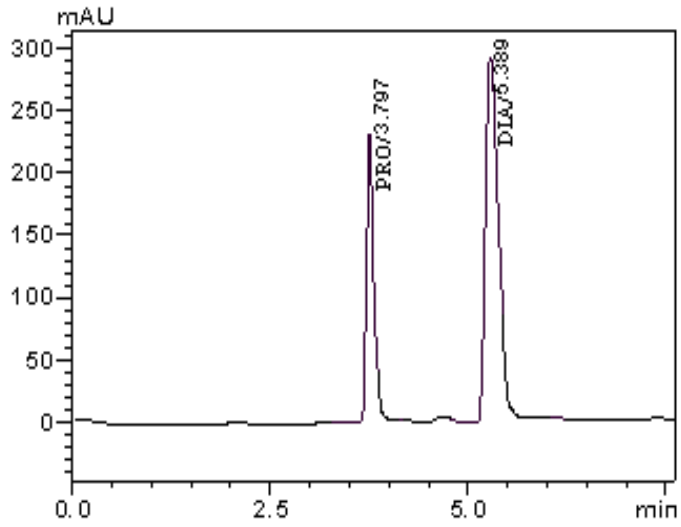

Figure 3: RP-HPLC chromatogram of standard DZP and PRO with corresponding retention time at 235 nm

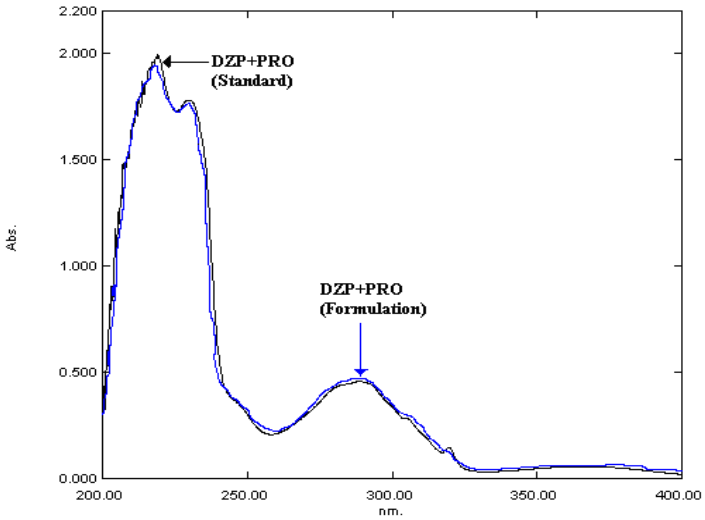

Figure 4: Overlain spectra of DZP and PRO for standard and marketed formulation

Table 1: System Suitability Test Parameters

Parameters	DZP ± RSD[a]	PRO ± RSD
Retention time (min)	5.38 ± 0.29	3.80 ± 0.15
Tailing factor	1.25 ± 0.26	1.19 ± 0.20
Theoretical plates	4085 ± 0.35	3548 ± 0.39
Resolution	4.72 ± 0.19	4.72 ± 0.19

[a]RSD = Relative standard deviation

Validation of the proposed method

Linearity

Linear correlation was obtained between peak area versus concentrations of DZP and PRO in the ranges of 0.1–5 µg/ml. The linearity of the calibration curve was validated by the high values of correlation coefficient of regression (Table 2).

Table 2: Regression Analysis Data and Summary of Validation Parameters

Parameters	HPLC Method	
	DZP	PRO
Detection wavelength (nm)	235	235
Concentration range (µg/ml)	0.1-5	0.1-5
Slope	14800	11832
Intercept	3648	3146
Correlation co-efficient (r^2)	0.9960	0.9990
LOD[a] (µg/ml)	0.015	0.014
LOQ[b] (µg/ml)	0.05	0.045
Accuracy (% recovery, n = 6)	100.3 ± 0.47	100.2 ± 0.78
Repeatability (% RSD[c], n = 6)	0.41	1.08
Precision (% RSD)		
Interday (n[d] = 6)	0.64 - 1.33	0.55 - 1.23
Intraday (n = 6)	0.39 - 0.78	0.30 - 1.07

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantitation.
[c] RSD = Relative standard deviation
[d] n = number of determinations

Method precision (% repeatability)

The RSD values for DZP and PRO were found to be 0.41 % and 1.08 %, respectively (Table 2). The RSD values were found to be <1 %, which indicates that the proposed method is repeatable.

Intermediate precision (reproducibility)

The low RSD values of interday (0.64 - 1.33 % and 0.55 - 1.23 %) and intraday (0.39 - 0.78 % and 0.30 - 1.07 %) variations for DZP and PRO, respectively, reveal that the proposed method is precise (Table 2).

LOD and LOQ

LOD values for DZP and PRO were found to be 0.015 µg/ml and 0.014 µg/ml, respectively and LOQ values for DZP and PRO were found to be 0.05 µg/ml and 0.045 µg/ml, respectively (Table 2). These data show that the proposed method is highly sensitive for the determination of DZP and PRO.

Accuracy

The recovery experiments were performed by the standard addition method. The recoveries obtained were 100.3 ± 0.47 % and 100.2 ± 0.78 % for DZP and PRO, respectively (Table 3). The low value of standard deviation indicates that the proposed method is accurate.

Table 3: Recovery data

Drug	Level	Amount of sample taken (µg/ml)	Amount of standard spiked (%)	Mean % recovery ± SD*
DZP	I	0.3	50	100.4 ± 0.67
	II	0.3	100	100.4 ± 0.42
	III	0.3	150	100.1 ± 0.34
PRO	I	1.5	50	99.90 ± 0.68
	II	1.5	100	100.5 ± 1.15
	III	1.5	150	100.3 ± 0.54

* Mean % Recovery ± SD of six observations.

Robustness

The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2 %. The low value of % RSD indicated robustness of the proposed method.

Specificity

The specificity of the method was ascertained by analyzing standard drugs and sample of DZP and PRO. The peak purity of standard DZP and PRO were 1.000 and 0.999, respectively. Peak purity for sample was found to be 0.999 for both drugs. The above results suggest that proposed method is specific for the simultaneous estimation of DZP and PRO

Assay of the pharmaceutical formulation

The proposed validated method was successfully applied to determine DZP and PRO in their tablet dosage form. The result obtained for DZP and PRO was comparable with the corresponding labeled amounts (Table 4). The RP-HPLC chromatogram for DZP and PRO in sample was recorded and is shown in Figure 5.

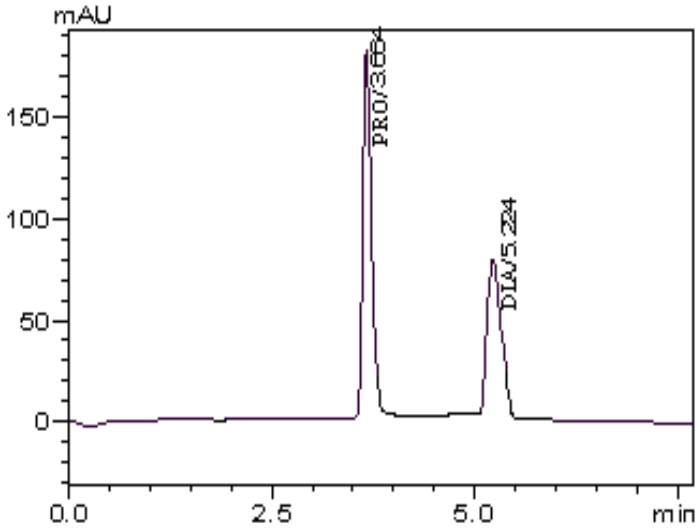

Figure 5: RP-HPLC chromatogram of sample DZP and PRO with corresponding retention time at 235 nm

Table 4: Assay results for the combined tablet dosage form (n = 6)

Tablet	Label claim (mg)		Amount found (mg)		% Label claim ± S. D. (n = 3)	
	DZP	PRO	DZP	PRO)	DZP	PRO
I	2	10	1.97	10.15	98.50 ± 1.29	101.5 ± 0.69
II	2	10	2.02	9.95	101.0 ± 0.65	99.50 ± 1.12

CONCLUSION

The proposed RP-HPLC method was found to be simple, sensitive, accurate, precise, specific and robust and can be used for the routine quality control analysis of DZP and PRO in combined dosage form without any interference of excipients.

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