International Journal of Drug Development & Research
| January-March 2013 | Vol. 5 | Issue 1 | ISSN 0975-9344 |
Available online http://www.ijddr.in
Covered in Official Product of Elsevier, The Netherlands
SJR Impact Value 0.13 & H index 2

Full Length Research Manuscript

Development and Validation of High Performance Thin-Layer Chromatography and Derivative Spectrophotometry methods for determination of Diazepam and Propranolol Hydrochloride in Combined Dosage Form

Bhadani Shweta*, Patel Paresh, Modi Hiral
*Saraswati Institute of Pharmaceutical Sciences, At & P Dhanap, Ghandhinagar, Gujarat, India.

Abstract

The manuscript describes validated high performance thin layer chromatography (HPTLC) and first derivative UV spectrophotometric methods for the estimation of diazepam (DZP) and propranolol hydrochloride (PRO) in combined dosage form. The HPTLC separation was achieved on an aluminium-backed layer of silica gel $60F_{254}$ using mobile phase ethylacetate-methanol-toluene-triethylamine (1.0 + 3.0 + 6.0 + 0.1, v/v/v/v). Quantification was achieved with UV detection at 235 nm over the concentration range 25 – 250 ng/spot and 200 – 2000 ng/spot for DZP and PRO respectively, with mean recovery of 100.3 ± 0.54 and 100.2 ± 0.35 % for DZP and PRO, respectively by HPTLC method. Derivative spectrophotometric method was based on the estimation of both the drugs at their respective zero crossing point (ZCP). The first-order derivative spectra were obtained at N = 1 (scaling factor), Δλ = 2.0 nm, and the determinations were made at 248 nm (ZCP of PRO) for DZP and 242 nm (ZCP of DZP) for PRO over the concentration range of 2.5–30 µg/mL for both DZP and PRO with mean recovery of 100.2 ± 0.49 and 100.1 ± 0.13 % for DZP and PRO, respectively by first derivative UV spectrophotometric method. These methods were found to be simple, sensitive, accurate, precise, reproducible and economical and applicable for the simultaneous determination of DZP and PRO in combined dosage form.

*Corresponding author, Mailing address:
Bhadani Shweta
E-Mail: shwetapatel308@yahoo.co.in

Key words:
Diazepam, Propranolol Hydrochloride, HPTLC, Derivative spectrophotometry, Zero crossing point.

How to Cite this Paper:
Bhadani Shweta*, Patel Paresh, Modi Hiral "Development and Validation of High Performance Thin-Layer Chromatography and Derivative Spectrophotometry methods for determination of Diazepam and Propranolol Hydrochloride in Combined Dosage Form" Int. J. Drug Dev. & Res., January-March 2013, 5(1): 91-98.

Article History:----------
Date of Submission: 21-11-2012
Date of Acceptance: 06-12-2012
Conflict of Interest: NIL
Source of Support: NONE

INTRODUCTION

Diazepam (DZP) is an anxiolytic agent and chemically it is 7-chloro-1, 3-dihydro-1-methyl-5-phenyl-1, 4- benzodiazepin-2-one; $C_{16}H_{13}ClN_2O$[1]. Propranolol Hydrochloride (PRO) is Beta-

adrenoceptor antagonist and is chemically (*RS*)-1-isopropylamino-3- (1-naphthyloxy) propan-2-ol hydrochloride; $C_{16}H_{21}NO_2$, HCl[1].

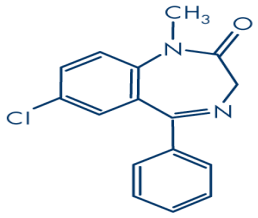

Figure 1: Structure of Diazepam

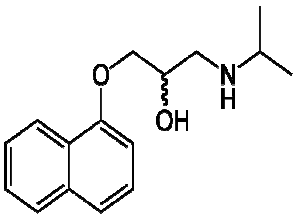

Figure 2: Structure of Propranolol HCL

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[2]. Literature survey reveals that various methods like Spectrophotometry[3], Gas liquid chromatography[4], Fluorimetry[5], First derivative spectroscopy[6], Capillary electrophoresis[7] and HPLC[8] are reported for the estimation of diazepam in single dosage form. Literature survey also reveals various methods like HPTLC[9], Chemiluminometry[10], Colorimetry[11], Polarogrphy[12], Spectrophotometry[13] and HPLC[14] are reported 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. The present manuscript describes simple, sensitive, accurate, precise, reproducible and economical HPTLC and derivative spectroscopic methods for the simultaneous estimation of DZP and PRO in combined dosage form.

MATERIALS AND METHODS

Apparatus

A Camag HPTLC system (Switzerland) with Linomat 5 automatic sample applicator and Camag TLC Scanner III, Camag (Muttenz, Switzerland) flat bottom and twin-trough flat-bottom TLC developing chamber (10 × 10 cm), Pre coated silica gel aluminum plate $60F_{254}$, (10 cm × 10 cm; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologist, Mumbai), UV cabinet with dual wavelength UV lamp, Camag win-CATS software, Hamilton syringe (100 µl), Ultrasonic bath (Frontline ultrasonic bath), a Shimadzu (UV-1700) double beam UV-visible Spectrophotometer, attached to a computer software UV probe 2.0, with a spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells, CP224S analytical balance (Sartorius, Gottingen, Germany), Ultrasonic cleaner (Frontline FS 4, Mumbai, India) and corning volumetric flasks were used during the study.

Reagents and Materials

Diazepam and Propranolol HCl bulk powder was kindly gifted by Santham Pharmaceutical Ltd, Gujarat (India), with 99.96% purity. The commercial fixed dose combination product containing 2 mg DZP and 10 mg PRO was procured from the local pharmacy. Toluene, Methanol, Ethyl acetate, Triethylamine and sulphuric acid were procured from S.D. Fine Chemical Ltd. (Mumbai, India) and were of analytical grade, nylon 0.45 µm – 47 mm membrane filter (Gelman Laboratory, Mumbai, India).

Analytical Conditions

HPTLC method — Solution of DZP and PRO were applied to silica gel $60F_{254}$ HPTLC plates (10 cm × 10 cm) by means of a Linomat V automatic spotter equipped with a 100µL syringe and operated with settings of band length, 6 mm; distance from the plate edge, 10 mm; and distance from the bottom of the plate, 10 mm. The plate was developed in a twin-trough chamber previously saturated for 20 min with the mobile phase, ethylacetate-methanol-toluene-triethylamine (1.0 + 3.0 + 6.0 + 0.1, v/v/v/v), for a distance of 8 cm. The spots on the air-dried plate

were scanned with the scanner III at 235 nm using the deuterium source.

First derivative spectrophotometric method – The standard solutions of DZP (10 µg/mL) and PRO (10 µg/mL) were scanned separately in the UV range of 200-400 nm. The zero order spectra thus obtained was then processed to obtain first derivative spectrum (N = 1, Δλ = 2.0 nm). The two spectra were overlain as shown in the Figure 5. It appeared that DZP showed zero crossing at 242 nm while PRO showed zero crossing at 248 nm. At the zero crossing point of DZP (242 nm), PRO showed a first derivative absorbance, whereas at the zero crossing point of PRO (248 nm), DZP showed a first derivative absorbance. Hence the wavelengths 248 nm and 242 nm were selected as analytical wavelengths for determination of DZP and PRO, respectively. These two wavelengths can be employed for the estimation of DZP and PRO without any interference from the other drug in their combined formulation.

Preparation of DZP and PRO Standard Stock Solutions

HPTLC method – A mixed standard stock solution of DZP (25 µg/mL) and PRO (200 µg/mL) were prepared by accurately weighing DZP (2.5 mg) and PRO (20 mg) and dissolving in methanol and diluted to 100 mL with methanol in the same volumetric flask.

First derivative spectrophotometric method - A standard stock solution of DZP (100 µg/mL) was prepared by accurately weighing DZP (10 mg) and dissolving in 0.05 M methanolic sulphuric acid and diluted to 100 mL with the same and a standard stock solution of PRO (100 µg/mL) was prepared by accurately weighing PRO (10 mg) and dissolving in 0.05 M methanolic sulphuric acid and diluted to 100 mL with the same.

Preparation of Sample Solutions

HPTLC method - Twenty tablets were weighed and powdered. The quantity of the powder equivalent to 20 mg of DZP and 100 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. The solution was then filtering through a nylon 0.45 µm membrane filter. The volume was adjusted up to the mark with methanol.

First derivative spectrophotometric method - 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 0.05M methanolic sulphuric acid (50 mL), sonicated for 20 min. to dissolve the drug as completely as possible. The solution was then filtering through a nylon 0.45 µm membrane filter. The volume was adjusted up to the mark with 0.05M methanolic sulphuric acid. An aliquot of this solution (1.5 mL) was taken in to a 10 mL volumetric flask and the volume was adjusted up to mark with 0.05M methanolic sulphuric acid.

Method Validation

(i) Calibration curve (linearity of the HPTLC method) — Calibration curves were plotted over the concentration range of 25 – 250 ng/spot and 200 – 2000 ng/spot for DZP and PRO respectively. Accurately prepared mixed standard solutions of DZP and PRO (1.0, 2.0, 4.0, 6.0, 8.0 and 1.0 µL) were applied to the plate. The calibration graphs were developed by plotting peak area vs concentrations (n = 6) with the help of winCATS software.

(*ii*) *Calibration curve (linearity of the first derivative spectrophotometric method).*—

Calibration curves were plotted over a concentration range of 2.5-30 µg/mL for DZP and PRO. Accurately measured standard working solutions of DZP and PRO (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL) were transferred to a series of 10 mL of volumetric flasks and diluted to the mark with 0.05M methanolic sulphuric acid, and first-derivative absorbances (D^1) were measured at 248 nm for DZP and 242 nm for PRO. The calibration curves were constructed by plotting absorbance's vs concentrations.

(iii) Accuracy (% Recovery)

The accuracy of the methods 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, 75 and 100 % level to prequantified sample solutions of DZP and PRO (200 + 1000 ng/spot and 3 + 15 µg/mL for HPTLC and first derivative spectrophotometric method, respectively). The amounts of DZP and PRO were estimated by applying obtained values to the regression equation of the calibration curve.

(iv)Method Precision (% Repeatability)

The precision of the instrument was checked by applying the same sample solution 6 times on a plate with automatic spotter using the same syringe and by taking 6 scans of the sample spot for both DZP (100 µg/mL) and PRO (800 µg/mL) without changing the position of the plate and by repeated scanning and measurement of absorbance of solution of (n = 6) of DZP and PRO (10 µg/mL) without changing the parameter for the first derivative spectrophotometric method.

(v) Intermediate Precision (Reproducibility)

The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 3 times on the same day and on 3 different days over a period of one week for 3 different concentration of standard solutions of DZP and PRO (50, 100 and 150 ng/spot and 400, 800 and 1200 ng/spot; respectively) for the HPTLC method and (5, 10 and 15 µg/mL) for the first derivative spectrophotometric method. The results were reported in terms of relative standard deviation (% RSD).

(vi) Method robustness

Robustness of the methods was studied by changing the composition of the mobile phase and determining the stability of the drug in methanol for 24 h at ambient temperature. Spot stability was observed by performing 2-dimentional HPTLC development using the same mobile phase.

(vii) 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. [15]

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where σ= the standard deviation of the response and S = Slope of calibration curve.

Analysis DZP and PRO in Combined Dosage Form

Pharmaceutical formulation of DZP and PRO was purchased from local pharmacy. The response of the sample solution was measured at 235 nm for the quatitation of DZP and PRO by using HPTLC method and 248 nm and 242 nm for quantitation of DZP and PRO, respectively by derivative spectrophotometric methods as described above. 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 in both the methods.

RESULTS AND DISCUSSION

HPTLC Method

Several mobile phases were tried to accomplish good separation of DZP and PRO. Using the mobile phase ethylacetate-methanol-toluene-triethylamine (1.0 + 3.0 + 6.0 + 0.1, v/v/v/v) and 10 × 10 cm HPTLC silica gel $60F_{254}$ aluminum-backed plates, good separation was attained with retardation factor (R_f) values of 0.78 for DZP and 0.37 for PRO. A wavelength of 235 nm was used for the quantitation of the drugs. Resolution of the peaks with clear baseline separation was found (Figure 3).

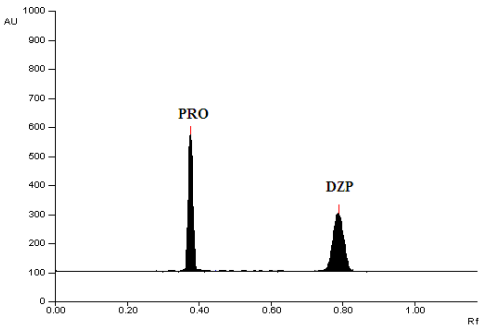

Figure 3 HPTLC densitogram of DZP (200 ng/spot) and PRO (1600 ng/spot) with scanning at 235nm

Stationary phase: 10 × 10 cm HPTLC silica gel $60F_{254}$ aluminum-backed plates, Mobile phase: ethylacetate-methanol-toluene-triethylamine (1.0 + 3.0 + 6.0 + 0.1, v/v/v/v), Detection: UV at 235 nm.

First derivative UV spectrophotometric method

The standard solution of DZP and PRO were scanned separately in the UV range and zero-order spectra thus obtained were then processed to obtain first-derivative spectra. The derivative spectra showed maximum absorbance at 248 nm (ZCP of PRO) for DZP and 242 nm (ZCP of DZP) for PRO. The first derivative absorbances were recorded at these wavelengths (Figure5). The overlain spectra for standard DZP and PRO were also recorded (Figure 4). First-derivative spectra give good quantitative determination of both drugs at their respective ZCPs without any interference. Second- and third-ordered spectra of the drug were not tested because the first-order spectra give satisfactory ZCPs and good quantitative determination of both drugs without any interference.

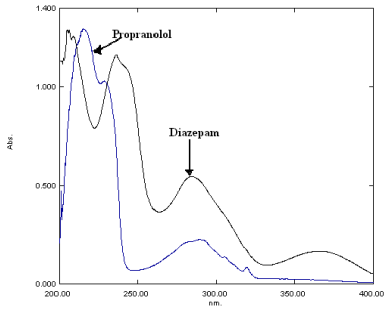

Figure 4 Overlain spectra of DZP (10 µg/mL) and PRO (10 µg/mL) from standard solution in 0.05M methanolic sulphuric acid

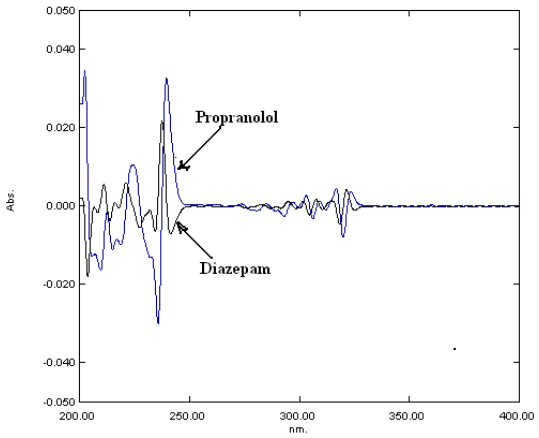

Figure 5 Overlain first derivative spectra of standard DZP (10 µg/mL) and PRO (10 µg/mL) in 0.05M methanolic sulphuric acid

Validation of the Proposed Method

Linearity - Linear correlation was obtained between peak areas and absorbance Vs concentrations of DZP and PRO in range of 25 – 250 ng/spot and 200 – 2000 ng/spot respectively for HPTLC and 2.5–30 µg/mL for derivative spectrophotometric method. The linearity of the calibration curves was validated by the high value of correlation coefficients of regression (Table 1).

Accuracy - The recovery experiments were carried out by the standard addition method. The mean recoveries obtained was 100.3 ± 0.54 and 100.2 ± 0.35 % for DZP and PRO, respectively by HPTLC method and 100.2 ± 0.49 and 100.1 ± 0.13 % for DZP and PRO, respectively by derivative spectrophotometric method (Table 1). The high values indicate that both methods are accurate.

Method precision - The % RSD values for DZP and PRO were found to be 0.24 and 0.14, respectively using HPTLC and 1.59 and 1.44, respectively for first derivative spectrophotometric method (Table 1). The low values of RSD indicate the proposed methods are repeatable.

Intermediate precision - The low RSD values of interday (0.58-1.76 and 0.18-1.02 %) and intraday (0.07-0.40 % and 0.11-1.81 %) variations for DZP and PRO, respectively by HPTLC and interday (0.84 – 1.96 % and 1.20 – 2.01%) and intraday (0.54 – 1.88 and 0.57 –1.96%) variations for DZP and PRO,

respectively by derivative spectrophotometric methods reveal that the proposed methods are precise (Table 1).

Method robustness - No significant change in peak area was observed during 24 hrs. No decomposition was observed in either the first or second direction of the 2-dimensional analysis for both drugs on the HPTLC plate. Hence, the method was found to be robust for the estimation of DZP and PRO.

LOD and LOQ - LOD values for DZP and PRO were found to be 0.23 and 7.93 ng/µL, respectively by HPTLC and 0.33 and 0.42 µg/mL, respectively by first derivative UV spectrophotometric method. LOQ values for DZP and PRO were found to be 0.69 and 24.03 ng/µL, respectively by HPTLC and 1.00 and 1.26 µg/mL, respectively by first derivative UV spectrophotometric method (Table 1).

Table 1: Regression analysis data and summary of validation parameters for the proposed HPTLC and first derivative UV spectrophotometric methods

Parameters	HPTLC method		First derivative UV spectrophotometric method	
	DZP	PRO	DZP	PRO
Concentration range	25-250 ng/spot	200-2000 ng/spot	2.5-30 µg/ml	2.5-30 µg/ml
Slope	13.875	1.3914	0.0045	0.0047
Intercept	223.1	955.12	0.0051	0.0031
Correlation coefficient	0.9976	0.9993	0.9978	0.9980
LOD[a]	0.23 ng/spot	7.93 ng/spot	0.33 µg/ml	0.42 µg/ml
LOQ[b]	0.69 ng/spot	24.03 ng/spot	1.00 µg/ml	1.26 µg/ml
Accuracy (% recovery, n = 6)	100.24 – 100.39	100.04 – 100.51	99.80 – 100.51	100.08 – 100.18
Repeatability (% RSD[c], *n* = 6)	0.24	0.14	1.59	1.44
Precision (%RSD)				
Interday (*n* = 6)	0.58-1.76	0.18-1.02	0.84-1.96	1.20-2.01
Intraday (*n* = 6)	0.07-0.40	0.11-1.81	0.54-1.88	0.57-1.96

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] % RSD = Percent relative standard deviation.

Assay of the pharmaceutical formulation

The proposed validated methods were successfully applied to determine DZP and PRO in their combined dosage form. The results obtained for DZP and PRO were comparable with the corresponding labelled amounts (Table 2). The first order derivative spectrum for DZP and PRO in sample was recorded and is shown in Figure 6.

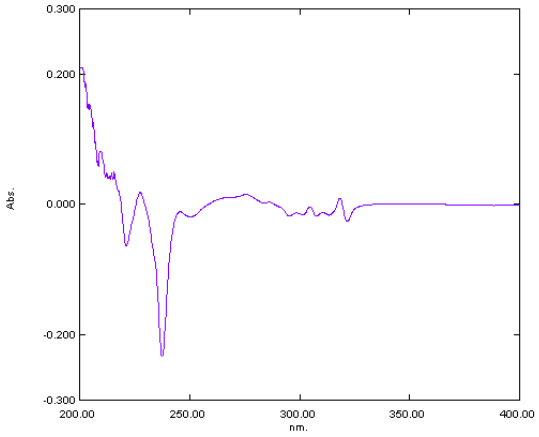

Figure 6 First order derivative spectra of DZP (3 µg/mL) and PRO (15µg/mL) in combined dosage form

Table 2: Assay results for the combined dosage form using the proposed HPTLC and first derivative UV spectrophotometric method.

Tablet	HPTLC method		First derivative UV spectrophotometric method	
	DZP ± S.D[a] (*n*[b] = 6)	PRO ± S.D[a] (*n*[b] = 6)	DZP ± S.D[a] (*n*[b] = 6)	PRO ± S.D[a] (*n*[b] = 6)
Brand A	99.65 ± 0.90	100.6 ± 0.37	100.3 ± 0.93	100.6 ± 1.11

[a] S.D = Standard deviation.
[b] *n* = Number of determinations.

Comparison of the proposed methods using t-test

The assay results for DZP and PRO in their combined dosage forms obtained using HPTLC and first derivative UV spectrophotometric method were compared with each other by applying paired t-test. The calculated t value 1.78 for DZP and 1.54 for PRO were less than the tabulated t-value (2.31) at 95 % confidence interval. Statistical comparison of the results obtained by proposed HPTLC method with the results obtained by proposed first derivative UV spectrophotometric method shows good agreement and indicates no significant difference in the content

of DZP and PRO by the proposed RP-HPLC and first derivative spectrophotometric method (Table 3).

Table 3: Comparison between results obtained by the proposed HPTLC methods and first derivative UV spectrophotometric method

Parameters	HPTLC method		First derivative UV spectrophotometric method	
	NEBI	AMLO	NEBI	AMLO
Assay results ± S.D[a]	99.65 ± 0.90	100.6 ± 0.37	100.3 ± 0.93	100.6 ± 1.11
n[b]	6	6	6	6
t-value (2.31)[c]	1.78	1.54	–	–

[a]S.D = Standard deviation
[b]n = number of determinations
[c]Figures in the parentheses represent corresponding to t-tabulated value at 95% confidence interval

CONCLUSION

The results of the analysis of pharmaceutical formulation by the proposed methods are highly reproducible and reliable and are in good agreement with the label claim of the drug. The additives usually present in the pharmaceutical formulations of the assayed samples did not interfere with determination of DZP and PRO. The methods can be used for the routine analysis of the DZP and PRO in combined dosage form.

Acknowledgements

The authors are greatly thankful to Santham Pharmaceutical Ltd, Intrad, India for providing gift sample of DZP and PRO and S. K. Patel College of Pharmaceutical Education and Research, Ganpat Vidyanagar, Kherva for providing all facilities to carry out the work.

REFERENCES

1) The Merck Index. An Encyclopedia of Chemicals, Drugs, and Biologicals, Merck & Co., Inc., Whitehouse Station, NJ, 2006, pp 509 & 1348.
2) Hallstrom C, Treasaden I, Edwards JG, Lader M. Diazepam, propranolol and their combination in the management of chronic anxiety. *The British Journal of Psychiatry* 1981; 139: 417-421.
3) El-Hawary WF, Issa YM. Spectrophotometric Determination of Diazepam in Pure form, Tablets and Ampoules. *International journal of Biomedical science* 2007; 3: 50-55.
4) Arthur J, De silva F, Morton A, Stefanovic V, Kaplan J. The Liquid Chromatography of Some Benzodiazepines. *Hoffmann-la Roche Inc.,* 1964; 36: 2099.
5) Salem AA, Barsoum BN, Izake EL. Spectrophotometric and fluorimetric determination of diazepam, bromazepam and clonazepam in pharmaceutical and urine samples. *Spectrochim Acta A Mol Biomol Spectrosc*. 2004; 60(4):771-80.
6) Mannucci C, Bertini J, Cocchini A, Perico A, Salvagnini F, Triolo A. Simultaneous determination of otilonium bromide and diazepam by first-derivative spectroscopy. *J Pharm Sci*. 1992; 81(12):1175-7.
7) Furlanetto S, Orlandini S, Massolini G, Faucci MT, La Porta E, Pinzauti S. Optimisation and validation of a capillary electrophoresis method for the simultaneous determination of diazepam and otilonium bromide. *Analyst* 2001; 126(10):1700-6.
8) Samia M, El-Gizawy M. Simultaneous Determination of Diazepam, Oxazepam and Temazepam in Spiked Urine By HPLC. *Analytical Letters* 2000; 33(4): 629 – 638.
9) Townshend A, Murillo Pulgarín JA, Alanon Pardo MT. Flow injection-chemiluminescence determination of propranolol in pharmaceutical preparations. *Analytica Chimica Acta* 2003; 488: 81-88.
10) Bhavar G, Chatpalliwar VA. Quantitative analysis of propranolol hydrochloride by high performance thin layer chromatography. *Indian J Pharm Sci*. 2008; 70(3):395-8.
11) Idowu OS, Adegoke OA, Olaniyi AA. Colorimetric assay of propranolol tablets by derivatization: novel application of diazotized 4-amino-3,5-dinitrobenzoic acid (ADBA). *J AOAC Int*. 2004; 87(3):573-8.
12) El-Ries MA, Abou-Sekkina MM, Wassel AA. Polarographic determination of propranolol in pharmaceutical formulation. *J Pharm Biomed Anal*. 2002; 30(3):837-42.

13) El-Emam AA, Belal FF, Moustafa MA, El-Ashry SM, El-Sherbiny DT, Hansen SH.Spectrophotometric determination of propranolol in formulations via oxidativecoupling with 3-methylbenzothiazoline-2-one hydrazone. *Farmaco*. 2003; 58(11):1179-86.

14) Abdel-Hamid M. E. Development and validation of analytical method for the estimation of propranolol in dosage form. *Journal of Clinical Pharmacy and Therapeutics*. 2008; 13: 183 – 189.

15) International Conference on Harmonisation (ICH), Topic Q2R1, Validation of Analytical Methods: Methodology, Yokohama, Japan, (2005).