INTERNATIONAL RESEARCH JOURNAL OF PHARMACY

www.irjponline.com ISSN 2230 – 8407

Research Article

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF PROPRANOLOL HCl AND CLONAZEPAM IN BULK AND PHARMACEUTICAL DOSAGE FORM

Tanikella Sai Annapurneswari[1,2], Sakinala Shilpa[2], Chodavarapu Bala Tripura Sundari[1]*, Vaidya Jayathirtha Rao[2], Anisetti Ravinder Nath[1]

[1]Department of Pharmacy & Biotechnology, University College of Technology, Osmania University, Hyderabad, India

[2]Crop Protection Chemicals Division, CSIR- Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad, India

Article Received on: 11/07/12 Revised on: 11/08/12 Approved for publication: 08/09/12

*Email: sundari_1@hotmail.com

ABSTRACT

The present work describes a reverse phase high performance liquid chromatographic method (RP-HPLC) for the simultaneous estimation of Propranolol HCl (PRH) and Clonazepam (CNZ) in bulk and in pharmaceutical dosage form. Chromatographic separation was performed on Agilent Eclipse xdb C18 (150 mm × 4.6 mm i.d., 5 µm) column, with a mobile phase comprising of a mixture of methanol, acetonitrile and 20 mM potassium dihydrogen phosphate buffer in the ratio of 27.5:27.5:45 v/v. The pH of buffer was adjusted to 3.0 with orthophosphoric acid. The flow rate was 1.0 ml/min with detection at 266 nm. Retention times of Propranolol HCl and Clonazepam were found to be 2.400 and 4.492 min respectively. As per International Conference on Harmonisation (ICH) guidelines the method was validated for linearity, accuracy, precision, limit of quantitation, limit of detection, and robustness. Linearity of PRH was found to be in the range of 20-120 µg/mL and that for CNZ was found to be 1-6 µg/mL. The correlation coefficients were 0.9994 and 0.9995 for PRH and CNZ respectively. The mean recoveries obtained for PRH and CNZ were 100.6% and 100.1%. This demonstrates that the developed method is simple, precise, accurate, reproducible and rapid for simultaneous estimation of these drugs in bulk and in tablet dosage forms.

Key words: Propranolol HCl, Clonazepam, RP-HPLC, Simultaneous determination, Validation.

INTRODUCTION

Propranolol hydrochloride (PRH): PRH **(Figure 1)** is chemically 1-naphthalen-1-yloxy-3-(propan-2-ylamino) propan-2-ol hydrochloride. It is a non-selective beta blocker mainly used in the treatment of hypertension. PRH is used in the treatment or prevention of many disorders including acute myocardial infarction, arrhythmias, angina pectoris, hypertension,hyperthyroidism, migraine, pheochromocytoma, menopause and anxiety.

Clonazepam (CNZ): CNZ **(Figure 2)** is 5-(o-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one. It is a Benzodiazepine drug which act, on the brain and central nervous system to produce a calming effect. It is used to treat the panic and anxiety symptoms associated with panic disorder. Further it is also used to treat seizures, anxiety, muscle spasms and insomnia.

The combination of PRH and CNZ is useful in the treatment of pulsatile tinnitus[1] and in management of chronic anxiety. Propranolol hydrochloride is official in IP[2], USP[3] and BP[4]. IP and BP describe potentiometric titration methods while USP describe liquid chromatographic method for its estimation. Clonazepam is official in IP[5], BP[6] and USP[7], all of which describes liquid chromatographic method for its estimation.

Their combination is not official in any pharmacopoeia, so no official method is available for the estimation of these two drugs in combination.

Literature survey reveals a few spectrophotometric[8-12], HPLC[13-16] and bioanalytical methods[17-24] for the estimation of both drugs as a single component and in combination with other drugs. However, thorough literature survey revealed that there are no analytical methods reported for the analysis of these drugs in combined dosage form. The objective of the present work is to develop a highly sensitive, simple, rapid, and precise RP HPLC method for the estimation of propranolol hydrochloride and clonazepam in combined tablet dosage form.

EXPERIMENTAL:

Instrumentation:

JASCO 2080 model chromatograph equipped with an Agilent eclipse xdb-reverse phase C18 column (150 x 4.6 mm I.D: particle size 5 µm) was employed for the study. Sample injection was done with a Rheodyne 7725 injection valve via a 20 µL loop. Detection of the drug was done by using a UV-2075 detector (JASCO) and the output signal was monitored and integrated by JASCO BORWIN software. Solubility of the compound was enhanced by sonication on an ultrasonicator. A JASCO V-550 UV-Visible spectrophotometer was used to record the UV spectra of Propranolol HCl and Clonazepam combination to select the working wavelength for detection of the drugs. A Digisun Electronic analytical balance (model DI 707) was used for preparation of all samples and buffer solutions required.

Chemicals and reagents:

The reference samples of Propranolol HCl and Clonazepam were obtained from Pellets Pharma Ltd and Suraksha Pharma Pvt Ltd, Hyderabad, India. Purified water was obtained by using 0.22µ Millipore Milli-Q water purification systems. HPLC grade acetonitrile and methanol (Merck, Mumbai) were used for preparing the mobile phase and the diluent. Potassium dihydrogen orthophosphate and orthophosphoric acid are of analytical grade obtained from Sigma Aldrich. Clotas Plus-H, a commercial tablet containing a combination of CNZ (0.5 mg) and PRH (10 mg) manufactured by Tas Med (I) Pvt. Ltd., Chandigarh was purchased from local firms.

Mobile Phase: Methanol, acetonitrile and 20 mM KH_2PO_4 buffer (adjusted to pH 3.0 with orthophosphoric acid) in the ratio of 27.5:27.5:45 v/v was used for separation of these drugs. Prior to use, the mobile phase was filtered through 0.22µ membrane filter after sonication of each solvent for 15 min.

Diluent: Mixture containing methanol, acetonitrile and 20 mM KH_2PO_4 buffer (adjusted to pH 3.0 with orthophosphoric acid) in the ratio of 25:25:50 v/v was used.

METHOD DEVELOPMENT

Selection of Wavelength

Wavelength was selected by scanning the standard solutions of both the drugs over 190 nm to 400 nm. Both the components show reasonably good response at 266 nm (isobestic point); therefore wavelength 266 nm was selected for further study.

Optimisation of Mobile Phase:

A number of eluting experiments were conducted for the optimization of separation of the drugs using mobile phase. A mixture of methanol, acetonitrile and phosphate buffer were screened as possible eluting systems in different proportions like 35:35:30, 30:30:40 and 25:25:50 v/v. A suitable optimised condition with a mixture of methanol, acetonitrile and KH_2PO_4 buffer (pH adjusted to 3.0 with OPA), in the ratio of 27.5:27.5:45 v/v provided an efficient separation of the drugs with good peak symmetry as well as retention times. A flow rate of 1.0 mL/min was found to be optimum in which the retention time was 2.400 min for PRH and 4.492 min for CNZ with baseline stability.

Preparation of standard stock solution

The stock solutions were prepared by dissolving a suitable quantity of CNZ and PRH to get the final concentration of 0.05 mg/mL (50 µg/mL) and 1 mg/mL (1000 µg/mL) in standard volumetric flask and volume was made up with methanol. Further dilutions were made from the stock solution with diluent in the required concentration range in 10 mL volumetric flasks for the calibration curve.

Preparation of sample solution

Twenty tablets (CLOTAS PLUS H tabs) each containing 10 mg of propranolol hydrochloride and 0.5 mg of clonazepam were weighed and powdered. Tablet powder equivalent to 5 mg CNZ and 100 mg PRH was extracted with small amount of methanol in a 100 ml volumetric flask. The solutions were shaken well and allowed to stand for 15 min with intermittent sonication to ensure complete solubility of drug. The contents were made up to the mark with methanol. This solution was centrifuged and supernatant was then filtered using Whatman filter paper No. 41. From the filtrate, dilution was made in a 10 ml volumetric flask using diluent to get 3 µg/ml clonazepam and 60 µg/ml propranolol HCl. A 20 µl injection of the above sample was performed and chromatographed.

METHOD VALIDATION

As per the International Conference on Harmonization (ICH) guidelines[25-27], the method validation parameters like linearity, precision, accuracy, limit of detection, limit of quantitation, robustness and specificity were experimentally determined and the method validated.

Linearity and range

Series of mixed standard solutions of Propranolol HCl and Clonazepam were prepared in 10 mL volumetric flasks using diluent to get final concentration of 20-120 µg/mL of propranolol HCl and 1-6 µg/mL of clonazepam. Each of these drug solutions (20 µL) were injected into the chromatographic system for three times. The peak area and retention time were recorded and the mean values of peak areas were plotted against concentrations.

Precision

The intra and inter day precision was determined by analyzing 60 µg/mL PRH and 3 µg/mL CNZ, six times each on same day (intra-day study). This was repeated on the second day (inter-day study).

Accuracy

The accuracy of the method was determined by recovery studies. The recovery studies were performed by standard addition method; at 50%, 100%, 150% level for both the drugs i.e; three different levels corresponding to 30.0, 60.0 and 90.0 µg/mL for PRH and 1.5, 3.0 and 4.5 µg/mL for CNZ. The analysis was conducted in triplicate. Percentage recovery was calculated by comparing the area before and after the addition of the working standard.

Limit of detection and limit of quantitation

The LOD and LOQ of PRH and CNZ by the proposed methods were determined on the basis of response and slope of the regression equation. LOD and LOQ values were calculated using the formula $3.3\times s/S$ and $10\times s/S$, respectively, where S is the slope of the calibration curve and s is the standard deviation of y-intercept of regression equation.

Robustness

The robustness of the developed method was determined according to ICH guidelines. Experimental conditions were deliberately altered one factor after the other. The effect of change in flow rate, buffer concentration, pH of buffer on the retention time, peak asymmetry and theoretical plate number were studied.

System suitability

For system suitability, six replicates of the working standard sample were injected and the parameters like plate number (N), retention time, resolution and peak asymmetry of samples were calculated.

Specificity and selectivity of the proposed method

Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix. Both these parameters were explored in the method development by using excipients.

Table 1: RESULTS OF LINEARITY STUDY FROM CALIBRATION CURVE

Drug	Conc.(µg/mL)	Equation of regression line	R^2
PRH	20-120	Y=11312x	0.9994
CNZ	1-6	Y=41387x	0.9995

Table 2: RESULTS OF PRECISION STUDY (n=6)

Drug	%RSD(Intraday)	%RSD(Interday)
Propranolol HCl	0.61	0.49
Clonazepam	0.77	0.1

RSD is Relative Standard Deviation and n is the number of replicates

Table 3: RESULTS OF ACCURACY STUDY (n=3)

Analyte	Amount(%) of drug added to analyte	Theoretical conc. (µg/mL)	Measured conc. (µg/mL)	% Recovery	%RSD
Propranolol HCl	50	30	30.06	100.16	0.44
	100	60	59.85	99.7	0.133
	150	90	91.7	101.8	0.272
Clonazepam	50	1.5	1.49	99.3	0.342
	100	3.0	2.97	99.2	0.474
	150	4.5	4.58	101.8	0.69

Table 4: SYSTEM SUITABILITY PARAMETERS

Parameters	PRH	CNZ
Retention time(min)	2.400	4.492
Resolution	-	9.66
Theoretical plates	2653	5248
Asymmetry	1.31	1.09
LOD (µg/mL)	1.21	0.075
LOQ (µg/mL)	3.68	0.22

Table 5: EVALUATION OF ROBUSTNESS STUDY FOR PROPRANOLOL HCl AND CLONAZEPAM

Parameter	Propranolol HCl			Clonazepam		
	RT*a	AF*b	TP*c	RT	AF	TP
Flow rate 0.9	2.66	1.36	2765	4.98	1.13	5662
Flow rate 1.1	2.17	1.36	2320	4.07	1.11	4421
pH 2.9	2.43	1.30	2397	4.57	1.11	4683
pH 3.1	2.40	1.32	2355	4.52	1.06	4553
Buffer conc.15mM	2.33	1.39	2379	4.39	1.15	4131
Buffer conc.25mM	2.39	1.35	2387	4.39	1.29	4447

*a: retention time(in min) , *b: asymmetry factor, *c: theoretical plates

Table 6: RESULTS OF ASSAY FROM TABLET DOSAGE FORM

Drug	Labelled Amount (mg)	Amount taken for assay (µg/mL)	Amount found (mg)	%Recovery
PRH	10	60	59.05	98.4
CNZ	0.5	3	3.05	101.6

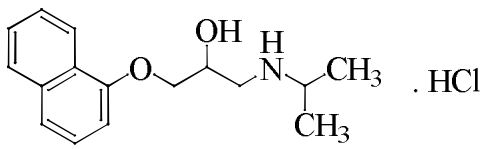

Figure 1: Structure of Propranolol HCl

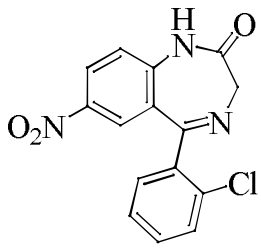

Figure 2: Structure of Clonazepam

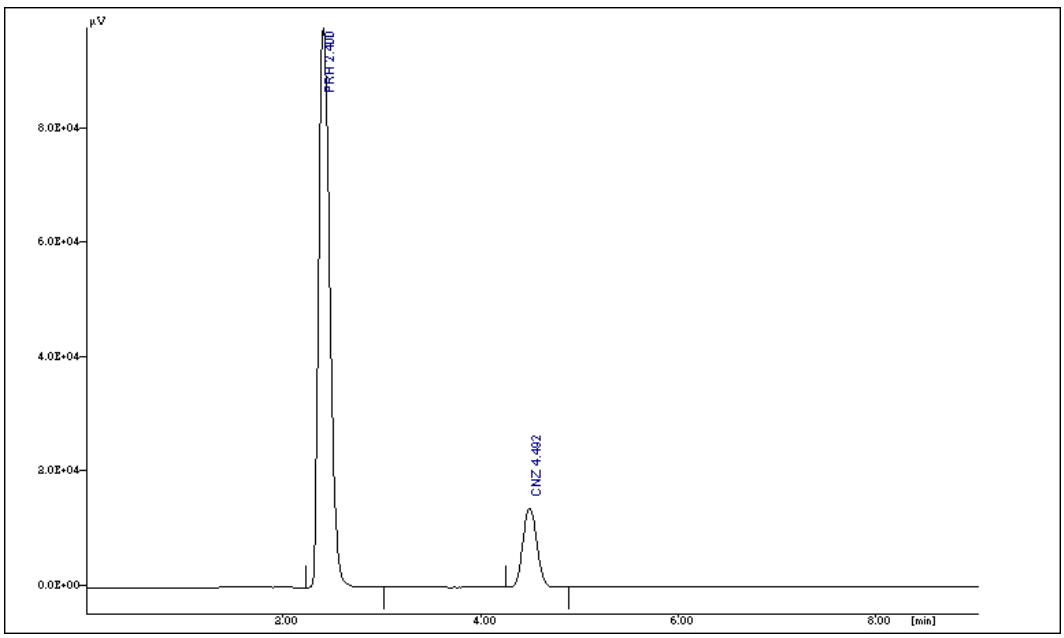

Figure 3: Chromatogram of standard solution of Propranolol HCl and Clonazepam

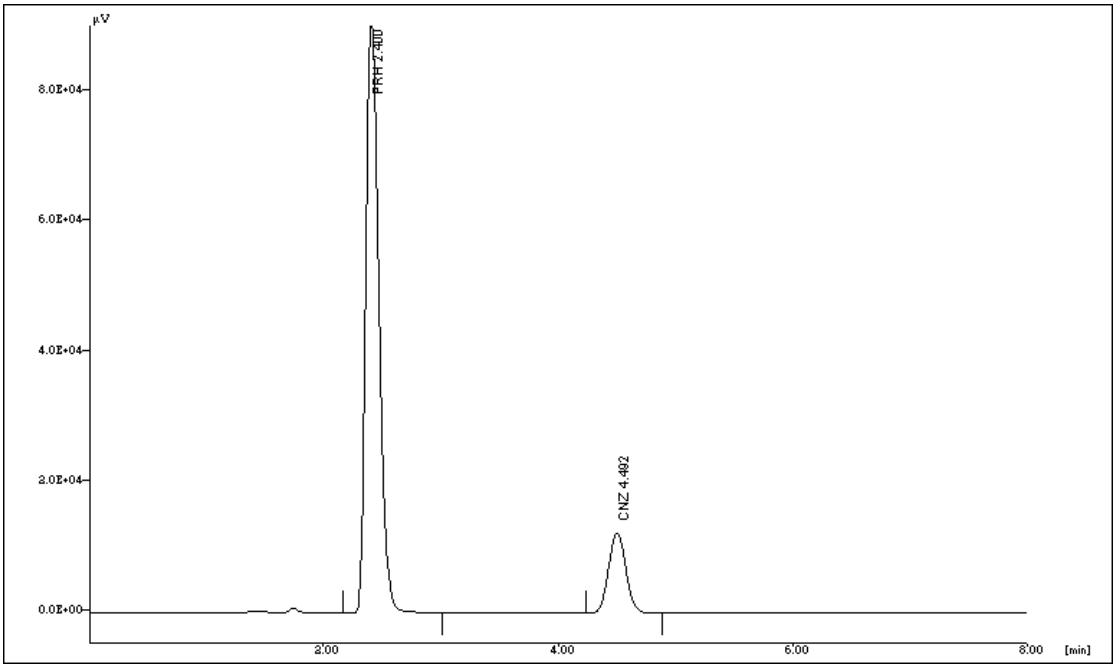

Figure 4: Typical Chromatogram of CLOTAS PLUS-H tablet formulation

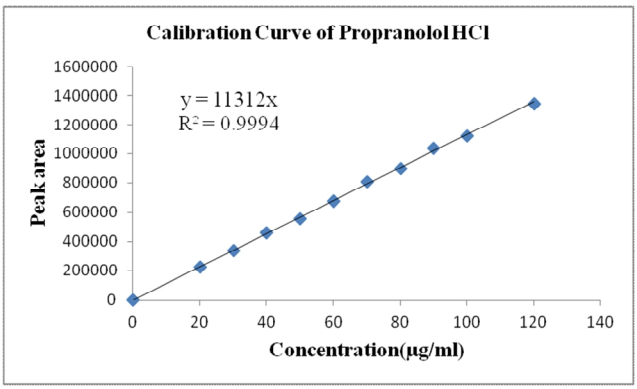

Figure 5: Linearity Curve of Propranolol HCl

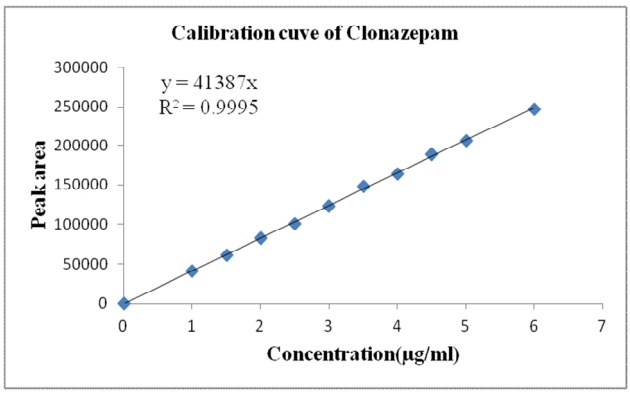

Figure 6: Linearity Curve of Clonazepam

RESULTS AND DISCUSSION

The goal of this present study was aimed at developing a sensitive, precise and accurate HPLC method for the analysis of Propranolol HCl and Clonazepam in its bulk and pharmaceutical combined dosage form. In order to achieve optimum separation of the component peaks, various proportions of buffer with acetonitrile and methanol were tested as mobile phase on an Agilent xdb C18 column. Mobile phase containing a mixture of methanol, acetonitrile and buffer in the ratio of 27.5:27.5:45 v/v was selected as it resulted in peaks with good symmetry and resolution. A flow rate of 1.0 mL/min was found to be optimum in the 0.5 to 1.0 mL/min range resulting in the short retention time, baseline stability and minimum noise. With the above optimised conditions, the retention times of PRH and CNZ were found to be 2.400 min and 4.492 min respectively showing the proposed method is time saving (Figure 3). The calibration curve showed linearity in the concentration range of 20-120 µg/mL for PRH and 1-6 µg/mL for CNZ (Figures 5 and 6). The regression equations of concentration over their peak areas were found to be y=11312x (R^2=0.9994) and y=41387x (R^2=0.9995) for PRH and CNZ respectively where y is the peak area and x is concentration of PRH and CNZ (µg/mL) (Table 1). The results of intraday and interday precision values are represented in (Table 2). The RSD % for assay of drugs during intra-day and inter-day were 0.61 and 0.77 for PRH and 0.49 and 0.1 for CNZ. Assay of the two drugs using the developed method showed acceptable relative error values that are less than 2 indicating that the method is highly precise. The percentage mean recovery at three different levels of study was 100.16, 99.7 and 101.8 for PRH and 99.3, 99.2 and 101.8 for CNZ (Table 3). The percentage mean recovery of individual analyte was high, satisfactory and indicates that the proposed method is accurate. The number of theoretical plates was determined to be 2653 and 5248 for PRH and CNZ respectively which indicate the efficient performance of the column. The LOD and LOQ were found to be 1.21 µg/mL and 0.075 µg/mL; 3.68 µg/mL and 0.22 µg/mL for PRH and CNZ respectively, which indicates the high sensitivity of the method (Table 4). The excipients used in formulation did not interfere with the drug peaks and thus the method is specific. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and excipients) showed almost no interfering peaks within retention time ranges. Figures 3, 4 show the representative chromatograms for standard and the formulation. The chromatograms show that the selected drugs were clearly separated and thus the proposed HPLC method is selective. In robustness study,

three factors (flow rate, pH and concentration of buffer) were deliberately altered. Under all the above conditions specified above, asymmetric factor was less than 2.0 and theoretical plates were more than 2300 for PRH and CNZ peaks, which illustrates good robustness of the developed method (Table 5). The amount of PRH and CNZ present in the sample solutions were determined by fitting the responses into the regression equations of the calibration curve for PRH and CNZ respectively and the results obtained were comparable with the corresponding label claim (Table 6).

CONCLUSION

Proposed study describes a new isocratic RP-HPLC method for the estimation of Propranolol HCl and Clonazepam in combination using simple mobile phase. The method gives good resolution between the compounds with a short analysis time. The method was validated and found to be simple, sensitive, accurate, precise and can be used for analysis of regular quality control samples.

ACKNOWLEDGEMENTS

The authors wish to thank Pellets pharma & Suraksha pharma, Hyderabad, for providing the gift samples of propranolol hydrochloride and clonazepam for this work. The authors are also thankful to the Director & Head, Crop Protection Chemicals Division, CSIR-IICT, Hyderabad for constant encouragement. We are grateful to Dr. B. China Raju and Mr. A.B.N Nageshwara Rao for their most valuable advice, guidance, support and keen interest.

REFERENCES

1. S. Albertino, A. R. Assuncao, and J. A. Souza, "Pulsatile tinnitus: Treatment with clonazepam and propranolol," Rev Bras Otorhinolaryngol (Engl Ed). 2005: 71(1): 111-113.
2. The Indian Pharmacopoeia, Vol. 3, Government of India, Delhi: The Controller of Publications, 2010:1987.
3. British Pharmacopoeia, Vol. 2, Stationary office, London: Medicines and Healthcare Products Regulatory Agency, 2010:1787.
4. The United States Pharmacopoeia, USP 32, NF 27, Vol. 3, Rockville, MD: The United States Pharmacopoeial Convention, Inc; 2009:3425.
5. The Indian Pharmacopoeia, Vol. 2, Indian Pharmacopoeia Commission (IPC), Ghaziabad: The Controller of Publications, 2007:334.
6. British Pharmacopoeia, Vol. 2, Stationary office, London: Medicines and Healthcare Products Regulatory Agency, 2009:1477.
7. The United States Pharmacopoeia, USP 30, NF 25, Vol. 2, Rockville, MD: The United States Pharmacopoeial Convention, Inc; 2007:1796.
8. Patel Satish A, Patel Paresh U, Patel Natavarlal U; Development and Validation of Spectrophotometric method for Simultaneous Estimation of Diazepam and Propranolol hydrochloride in Combined Dosage Form; International Research Journal of Pharmacy 2011; 2(8):119-123.
9. R. B. Kakde and D. D. Satone; Spectrophotometric Method for Simultaneous Estimation of Escitalopram Oxalate and Clonazepam in Tablet Dosage Form; Indian Journal Of Pharmaceutical Sciences; 2009 Nov-Dec;71(6):702–705.
10. Vinay B Patel, Jayant B Dave, Falguni M Patel, Chhaganbhai N Patel; UV Spectrophotometric Method for Identification and Estimation of Clonazepam in Tablet Dosage Form; International Journal of Pharmaceutical Research and Bio-Science; 2012; 1(2):62-70.
11. Amod S. Patil, Atul A. Shirkhedkar, Sanjay J. Surana, Prajakta S. Nawale; Q-Absorbance and Multicomponent UV Spectrophotometric Methods for Simultaneous Estimation of Propranolol Hydrochloride and Flunarizine Dihydrochloride in Capsules; Der Pharma Chemica, 2011;3(3):404-408.
12. Gandhi SV, Dhavale ND, Jadhav VY, Sabnis SS., Spectrophotometric and reversed-phase high-performance liquid chromatographic methods for simultaneous determination of escitalopram oxalate and clonazepam in combined tablet dosage form; J AOAC Int. 2008 Jan-Feb;91(1):33-38.
13. R. D. Chakole, M. S. Charde, N. Bhavsar, R. P. Marathe, Simultaneous Estimation Of Escitalopram and Clonazepam by RP-HPLC in Pharmaceutical Formulation; International Journal of Phytopharmacy Jan-Feb 2012; 2(1):25-29.
14. Tulja Rani G, Gowri shankar D, P.Kadgapathi, Satyanarayana B; Development of an RP-HPLC Method for the Simultaneous Estimation of Propranolol Hydrochloride and Diazepam in Combined Dosage form; Indian Journal of Pharmaceutical Education and Research Oct-Dec 2011; 45(4):296-300
15. Rakesh R. Prajapati, Dr. J.B. Dave, Dr. C.N. Patel; Development and Validation of Stability Indicating High-Performance Liquid Chromatographic Method For Simultaneous Determination Of Alprazolam and Propranolol in Combined Dosage Forms; International Journal Of Pharmacy & Technology April-June 2011; 3(2):2510-2523.
16. Tuljarani G.; D.Gowri Shankar, P.Kadgapathi, B.Satyanarayana; A Validated RP HPLC Method for Simultaneous Determination of Propranolol and Alprazolam in Bulk and in Pharmaceutical formulations; Journal of Pharmacy Research 2011; 4(2):358-360.
17. M V Srikanth, B Janaki Ram, S A Sunil, N Sreenivasa Rao and K V Ramana Murthy; Development and Validation of HPLC method for estimation of propranolol HCl in human plasma; Journal of Scientific & Industrial Research; February 2012; 71:120-123.
18. Zhang Yin ; Cheng Yi-Nong ; Zheng Xing-Zhong ; Simultaneous determination of diazepam and clonazepam in human serum by solid phase extraction RP-HPLC; Chinese Pharmaceutical Journal 2002; 22(6):337-339.
19. Bares IF, Pehourcq F, Jarry C.; Development of a rapid RP-HPLC method for the determination of clonazepam in human plasma. J Pharm Biomed Anal. 2004 Nov 19; 36(4):865-869.
20. Man-Wai Lo, Bernie Silber and Sidney Riegelman; An Automated HPLC Method for the Assay of Propranolol and Its Basic Meta bolites in Plasma and Urine; Journal of Chromatographic Science; 1982; 20(3):126-131.
21. Sallustio, Benedetta C.; Kassapidis, Chryssanthi; Morris,Raymond G.; High-Performance Liquid Chromatography Determination of Clonazepam in Plasma Using Solid-Phase Extraction; Therapeutic Drug Monitoring; April 1994; 16(2):174-172.
22. Elizabeth C. Kwong and Danny D. Shen; Versatile isocratic high-performance liquid chromatographic assay for propranolol and its basic, neutral and acidic metabolites in biological fluids; Journal of Chromatography B: Biomedical Sciences and Applications; 04/1987; 414(2):365-379.
23. William Shaw, Gerald Long and Jerry McHan; An HPLC Method for Analysis of Clonazepam in Serum; Oxford Journals-Journal of Analytical Toxicology; May-June 1983; 7(3):119-122.
24. Patel Satish, et al; Development and Validation of HPTLC Method for Simultaneous Determination of Diazepam and Propranolol Hydrochloride in Tablet Dosage Form; International journal of pharmaceutical frontier research; Oct-Dec 2011; 1(3):29-37
25. ICH, Q2B- Validation of Analytical Procedures: Methodology, International Conference on Harmonization, Nov, 1996.
26. International Conference on Harmonization, ICH Q1 A (R2); Stability Testing of New Drug Substances and Products 2003.
27. ICH Steering Committee (1996) International conference on harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use, validation of analytical procedure methodology, Geneva.

Source of support: Nil, Conflict of interest: None Declared