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Original Research Paper

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DIAZEPAM AND IMIPRAMINE IN BULK & PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Diazepam and Impiramine hydrochloride, using a ODS C-18 (HIQ SIL 4.6mm x 25cm, 10µm) column and a mobile phase composed of Methanol: Phosphate buffer (75:25 v/v). The retention times of Diazepam and Impiramine hydrochloride were found to be 2.85 min and 5.24 min, respectively. Linearity was established for Diazepam and Impiramine hydrochloride in the range of 10-500 µg/ml and 2-12 µg/ml, respectively. The percentage recoveries of Diazepam and Impiramine hydrochloride were found to be in the range of 99.83-99.91% and 98.81-99.96%, respectively. Both the drugs were subjected to variance of conditions like flow rate, difference in mobile phase and concluded that this method can be successfully employed for simultaneous quantitative analysis of Diazepam and Impiramine hydrochloride in bulk drugs and formulations.

Keywords: Diazepam, Impiramine hydrochloride, HPLC, Bulk drugs, Pharmaceutical formulations.

INTRODUCTION

Diazepam [7-chloro-1-methyl-5-phenyl-2, 3-dihydro-1H-1, 4-benzodiazepin-2-one] (figure 1), is a colorless to light yellow crystalline powder, almost odorless, freely soluble in water, methanol and solvent ether. Diazepam is anxiolytic, sedative & hypnotic, antiepeleptic and muscle relaxant. It is official in Indian Pharmacopoeia[1,2,3,4], which recommends a titrimetric method for its analysis. Impiramine hydrochloride, [3-(5, 6-dihydrobenzo[b] [1]benzazepin-11-yl)-N,N-dimethylpropan-1-amine HCl] (figure 2) is a white to off-white powder, odorless, crystalline powder, sparingly soluble in water and freely soluble in methanol. It is commonly used as an antidepressant and urinary incontinancy agent. Imipramine is official in British Pharmacopoeia[5,6], which recommends HPLC and HPTLC methods for its analysis. Diazepam and Imipramine combination suspension is combination in Indian market. This paper reports validated RP- HPLC method for simultaneous determination of Diazepam and Imipramine HCl in pharmaceutical formulation. The proposed method is simple, accurate, reproducible and suitable for routine determination of Diazepam and Imipramine in combined dosage form. The method was validated in compliance with ICH guidelines.[7] Literature survey reveals that many analytical methods are reported for determination of Diazepam and Imipramine.[8-35]

MATERIALS AND METHODS

Cipla Pharmaceuticals (Maharashtra, India) supplied pure drug sample of Diazepam and Impiramine hydrochloride procured from Umedica Laboratories Ltd. (Gujarat, India) and were certified to contain 99.32% (w/w) and 99.16% (w/w) respectively, on dried basis. Methanol and water used were of HPLC grade

and were purchased from Merck and CDH respectively. Potassium dihydrogen phosphate was purchased from Rankem. The suspension formulation (Parfil, Perron Pharmaceuticals, Karampura, New Delhi, India) containing 125 mg of Diazepam and 5 mg of Imipramine per 5 ml was procured from local market and used for analysis of marketed formulation. The liquid chromatographic system was of Younglin (ACME-9000), which consisted of following components: a gradient pump, variable wavelength programmable UV/Vis detector, a manual injection facility with 20 µl fixed loop. In addition, an electronic balance (Ohaus N-13123), a pH meter (Labtronics LT-11).

Selection of Analytical Wavelength

Stock solutions of both the drugs were prepared separately by dissolving 10 mg in 100 ml volumetric flask. UV spectrum showed maximum absorbance at 248 nm for Diazepam and 252 nm for Impiramine hydrochloride. The UV overlain spectra of both Diazepam and Imipramine showed that both drugs absorbed appreciably at 250 nm, so this wavelength was selected as the detection wavelength (figure 3).

Selection of Mobile Phase

The standard solution containing mixture of Diazepam and Impiramine was run and different individual solvents as well as combination of solvent have been tried to get a good separation and stable peak. Each mobile phase was filtered through 0.45 µ membrane filter. Initially methanol and water in different ratios were tried. It was found that methanol: water (Phosphate buffer) in ratio of 75: 25, v/v gave acceptable retention time (t_R = 2.853 min for Diazepam and t_R = 5.246 for Imipramine) at the flow rate of 1 ml/min and drugs showed typical peak nature and peaks were symmetrical at 250 nm (figure 9). Tailing factor for both Diazepam and Impiramine hydrochloride peak was less than 2 and the resolution was satisfactory. Ultimately mobile phase consisting of methanol, water (Phosphate buffer) (75: 25 v/v) was selected for validation purpose.

Preparation of Mobile Phase and Stock Solutions

Seven hundred and fifty ml of methanol and 250 ml of water were mixed and pH of mixture was adjusted to 6.6 with potassium hydroxide. This mixture was sonicated for 10 min and filtered through 0.22 µm membrane filter and used as mobile phase. Standard stock solution (1000 µg/ml) of Diazepam was prepared by dissolving 50 mg of Diazepam in 50 ml mixture of methanol and standard stock solution (100 µg/ml) of Impiramine hydrochloride was prepared by dissolving 5 mg of Impiramine hydrochloride in 50 ml mixture of methanol. The HPLC analysis was performed on reversed-phase high-performance liquid chromatographic system with isocratic elution mode using a mobile phase of methanol: water (75:25, v/v) pH 6.6 adjusted with potassium hydroxide on ODS C-18 column (250×4.6 mm, 5µm particle size) with 1 ml/min flow rate at 250 nm using UV detector.

Calibration Curves for Diazepam and Imipramine

- From Diazepam standard stock solution 10, 100, 200, 300, 400, 500µg/ml was prepared in mobile phase. Different concentrations of Impiramine hydrochloride 2, 4, 6, 8, 10, 12µg/ml were prepared in mobile phase.
- Procedure: The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. Then each dilution of both the drugs were injected and peak areas were recorded.

Analysis of Marketed Formulations

The peaks at t_R 2.868 min for Diazepam and at 5.247 min for Impiramine hydrochloride were observed in the chromatogram of the drug samples extracted from formulation. The drug content was found to be 99.48 % ± 0.62 (% RSD of 0.62) for Diazepam and 99.70 % ± 0.11(% RSD of 0.11) for Impiramine hydrochloride respectively.

Procedure for Analysis of Formulation

Each 5 ml (Perron Pharmaceuticals) was labeled to contain 25 mg of Diazepam and 1.25 mg of Impiramine hydrochloride. Volume equivalent to

10 mg of Diazepam and 0.5 mg of Impiramine hydrochloride was measured and transferred to 100 ml volumetric flask and dissolved in mixture of methanol and water (75: 25 v/v) to give concentration 100 µg/ml of Diazepam and 5 µg/ml of Imipramine and then solution was filtered through 0.45 µ membrane filter. From this solution, further dilutions were made using solvent to get a final concentration of 80 µg/ml of Diazepam and 4 µg/ml of Impiramine HCL respectively. Twenty micro liters of solution was injected into HPLC system to obtain chromatogram for standard drug solution.

Method Validation

The method of analysis was validated as per the recommendations of ICH[7] and USP[2] for the parameters like accuracy, linearity, precision and robustness. Diazepam showed good correlation coefficient in concentration range of 10-500 µg/ml (r^2 = 0.997 ± 0.98) and Impiramine hydrochloride showed good correlation coefficient in concentration range of 2-12 µg/ml (r^2 = 0.991 ± 0.96) for HPLC). For HPLC method the linearity of calibration graphs and adherence of the system to Beer's law was validated by high value of correlation coefficient and the S.D. for intercept value was less than 1.

The accuracy of the method was determined by calculating percentage recovery of Diazepam and Imipramine. For both the drugs, recovery studies were carried out by applying the method to drug sample to which known amount of Diazepam and Imipramine corresponding to 50, 100 and 150% of label claim had been added (standard addition method). At each levels of the amount six determinations were performed and the results obtained were compared. Intraday and interday precision study of Diazepam and Imipramine was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days for the concentration of 40, 80, 120 µg/ml and 2, 4, 6 µg/ml of Diazepam and Imipramine respectively.

For robustness evaluation of HPLC method a few parameters like flow rate, percentage of methanol in the mobile phase and pH of mobile phase were deliberately changed. One factor was changed at one time to estimate the effect. Each factor selected was changed at three levels (-1, 0, +1) with respect to optimized parameters. Robustness of the method was done at the concentration level 40, 80, 120 µg/ml and 2, 4, 6 µg/ml of Diazepam and Imipramine respectively.

System suitability tests are an integral part of chromatographic method which are used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 100 µg/ml and 4 µg/ml for Diazepam and Imipramine, respectively to check the reproducibility of the system.

RESULTS AND DISCUSSION

UV overlain spectra of both Diazepam and Imipramine showed that both drugs absorbed appreciably at 250 nm, so this wavelength was selected as the detection wavelength (figure 3). Details for selection of mobile phase are given in (table 1) and the chromatograms obtained are shown in (figure 4-9). The mobile phase consisting of Methanol: Phosphate buffer (75:25 v/v). The retention times of Diazepam and Impiramine hydrochloride were found to be 2.85 min and 5.24 min, respectively at 1ml/min flow rate was optimized which gave two sharp, well-resolved peaks. Tailing factor for both Diazepam and Impiramine hydrochloride peak was less than 2 and the resolution was satisfactory (figure 9). The calibration curve for Diazepam and Imipramine was found to be linear over the range of 10- 500 µg/ml and 2-12 µg/ml, respectively (figure 10-11). The data of regression analysis of the calibration curves is shown in (table 2-3). Experimental results of the amount of Diazepam and Impiramine hydrochloride in formulation, expressed as percentage of label claim were in good agreement with the label claims as stated in (table 4), thereby suggesting that there is no interference from any excipients, which are normally present in syrup. The proposed method was successfully applied to the determination of Diazepam and Imipramine in their combined suspension dosage form. The results for the combination were comparable with the

corresponding labeled amounts. The developed method was also found to be specific, since it was able to separate other excipients present in suspension from the two drugs. The linearity was evaluated by determining six standard working solutions containing 10-500 µg/ml in triplicate for Diazepam and 2-12 µg/ml for Impiramine hydrochloride (table 5). The recovery results were in the limit as stated in (table 6-7). Inter and intraday studies were also under limit, RSD was below 2% as shown in (table 8). Results for robustness evaluation for both the drugs are presented in (table 9). Insignificant differences in peak areas and less variability in retention times were observed.

In the proposed study, RP-HPLC method was developed for the simultaneous determination of Diazepam and Imipramine and validated as per ICH guidelines. Statistical analysis proved that method was accurate, precise, and repeatable. The developed method was found to be simple, sensitive and selective for analysis of Diazepam and Imipramine in combination without any interference from the excipients. The method was successfully used for determination of drugs in a pharmaceutical formulation. Assay results for combined dosage form using proposed method showed 99.65±0.40 % of Diazepam and 99.14±0.21 % of Imipramine. The results indicated the suitability of the method to study presence of Diazepam and Imipramine under various conditions *viz.* pH changes, change in mobile phase and change in flow rate.

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I thank to Suresh Kalawania (Drug Inspector) for providing gift sample of drugs from Sun Pharma Jammu Division.

Table 1: Selection of mobile phase

Sr. No.	Mobile Phase Composition	Retention Time (min)		Peak Area		Remarks
		Diazepam	Imipramine	Diazepam	Imipramine	
1.	Methanol	3.1	--	28889.37	--	No peak differentiation for both & Imipramine
2.	Methanol: Water (50 : 50)	2.9	--	28794.09	--	Asymmetry in peak of Diazepam
3.	Methanol: Phosphate buffer (pH 6.6) (90:10)	3.0	3.4	21860.18	1530.43	Tailing in peak of Diazepam. Short peak of Imipramine
4.	Methanol: Phosphate buffer (pH 6.6) (85:15)	2.2	3.1	20924.93	2728.74	Slight tailing in peak of Diazepam. Tailing and broadness in peak of Imipramine
5.	Methanol: Phosphate buffer (pH 6.6) (80:20)	2.8	3.5	23188.68	9127.94	Slight tailing in peak of Para & Imipramine.
6.	Methanol: Phosphate buffer (pH 6.6) (75:25)	2.8	5.2	23225.02	11689.06	Good resolution of both peaks.

Table 2: Calibration table for standard Diazepam (n=3)

Sr. No.	Conc. µg/ml	Area (µ AU)
1	10	11246.13
2	100	23225.02
3	200	35632.15
4	300	45542.42
5	400	61331.32
6	500	73753.83

Table 3: Calibration table for Impiramine hydrochloride (n= 3)

Sr. No	Conc. µg/ml	Area (µ AU)
1	2	8246.45
2	4	11689.19
3	6	14632.87
4	8	17542.69
5	10	19331.52
6	12	23753.14

Table 4: Analysis of marketed formulation

Sr. No.	Conc. µg/ml		Area (µ AU)		Conc. found µg/ml		% of labeled claim	
	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine
1	80	4	23227	11686	79.59	3.98	99.54	99.75
2	80	4	23225	11689	79.34	3.98	98.99	99.50
3	80	4	23227	11685	80.23	3.99	100.3	99.75
4	80	4	23227	11686	79.97	3.99	99.78	99.75
5	80	4	23227	11689	79.89	3.99	98.95	99.75

Table 5: Linear regression data for calibration curve (n = 3)

Parameters	Diazepam	Impiramine Hydrochloride
Linearity Range (µg/ml)	10 – 500	2 - 12
r^2 ± SD	0.997 ± 0.98	0.991 ± 0.96
Slope ± SD	9969 ± 0.85	2969 ± 0.97
Intercept ± SD	126.3 ± 0.87	5571 ± 0.93

Table 6: Statistical analysis for recovered Diazepam

Level of % Recovery	% Mean Recovery*		Standard Deviation		% R. S. D.		Standard Error	
	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine
50	99.83	99.16	0.025	0.862	0.013	0.689	0.012	0.276
100	99.89	99.10	0.036	0.657	0.034	0.613	0.019	0.315
150	99.91	99.18	0.049	0.586	0.057	0.589	0.028	0.287

* Mean of three readings

Table 7: Statistical analysis for recovered Impiramine HCl

Level of % recovery	% Mean Recovery*		Standard Deviation		% R. S. D.		Standard Error	
	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine
50	99.68	99.81	0.159	0.069	0.162	0.076	0.248	0.043
100	99.43	99.96	0.257	0.198	0.259	0.195	0.196	0.092
150	99.89	99.89	0.583	0.073	0.589	0.709	0.381	0.654

* Mean of three readings

Table 8: Observation of inter and intraday precision

Drug	Range (µg/ ml)	Inter day precision Found ± S.D. (µg/ml), R.S.D. (%)	Intraday Precision Found ± S.D. (µg/ml), R.S.D. (%)
Diazepam	10	10.2 + 0.1 ± 0.97	10.5 + 0.1 ± 0.99
	100	99.93+ 0.1 ± 0.29	100.0+ 0.15 ± 0.14
	200	199.6+ 0.4 ± 0.23	199.9+ 0.15 ± 0.07
	300	299.9+ 0.1 ± 0.97	300.1+ 0.18 ± 0.92
	400	400.3+ 0.7 ± 0.17	400.2+ 0.25 ± 0.06
	500	500.5+ 0.1 ± 0.21	500.1+ 0.11 ± 0.021
Impiramine HCL	2	2.12+ 0.1 ± 0.09	2.10 + 0.1 ± 4.14
	4	3.99+ 0.05 ± 1.31	4.11 + 0.05 ± 1.28
	6	6.12+ 0.15 ± 2.56	6.21 + 0.1 ± 0.26
	8	8.11+ 0.1 ± 1.32	8.14 + 0.05 ± 0.51
	10	10.1+ 0.1 ± 0.98	10.3 + 0.15 ± 1.44
	12	12.2+ 0.2 ± 1.52	12.2 + 0.3 ± 0.14

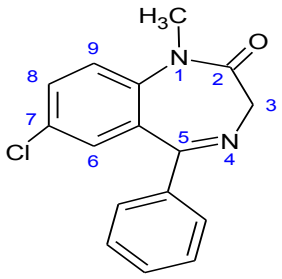
Table 9: Robustness testing HPLC method

Chromatographic Changes							
		Diazepam[a]			Imipramine[a]		
Factor[b]	Level	t_r[c]	k[d]	T[e]	t_r[c]	k[d]	T[e]
A: Flow rate(ml/min)							
0.90	-1	3.11	2.16	1.40	5.42	2.14	1.93
1.00	0	2.84	2.17	1.39	5.10	2.11	1.87
1.10	+1	2.23	2.12	1.26	4.83	2.09	1.86
Mean ±		2.83 ± 0.05	2.15 ± 0.10	1.39±0.34	5.41±0.44	2.14±0.39	1.87±0.45
B: %age of methanol in the mobile phase (v/v)							
89	-1	3.01	2.19	1.45	5.50	2.20	1.97
90	0	2.85	2.15	1.42	5.10	2.16	1.96
91	+1	2.81	2.18	1.46	4.86	2.17	1.94
Mean ±		2.85±0.07	2.16±0.10	1.44±0.14	5.10±0.46	2.21±0.56	1.95±0.07
C: Change in pH							
6.5		2.85	2.19	1.43	5.27	2.15	1.98
6.6		2.87	2.14	1.38	5.23	2.10	1.89
6.7		2.89	2.11	1.41	5.26	2.13	1.92
Mean ±		2.87±0.09	2.14±0.90	1.40±0.57	5.25±0.45	2.12±0.89	1.93±0.76

[A] average of three concentrations 40, 80, 120 µg/ml for Diazepam and 2, 4, 6 µg/ml for Impiramine hydrochloride respectively
[B] three factors were slightly changed at three levels (1, 0, -1); each time a factor was changed from level (0) the other factors remained at level (±1).
[C] retention time. [D] capacity factor. [e] tailing factor.

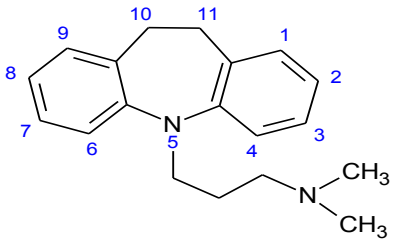
Table 10: System suitability test results

Sr. No.	Rt. (min)		Peak area		Tailing factor (T)		Resolution (R)	No. of Theoretical plates (N)		Capacity factor (K')		Selectivity (α)
	Diazepam	Imipramine	Diazepam	Imipramine	Diazepam	Imipramine		Diazepam	Imipramine	Diazepam	Imipramine	
1	3.10	5.50	23225.02	11689.19	1.42	2.06	2.02	7381.40	3132.45	2.15	2.10	2.01
2	2.23	4.86	23227.16	11687.80	1.38	1.91	1.98	7387.35	3128.16	2.18	2.09	2.02
3	2.85	5.10	23231.54	11685.23	1.26	1.89	2.00	7378.73	3139.77	2.12	2.08	2.03
4	2.83	5.40	23222.82	11690.06	1.43	1.97	1.97	7390.03	3135.48	2.15	2.11	2.01
5	2.87	4.85	23223.79	11692.31	1.45	1.89	1.99	7369.98	3130.62	2.14	2.13	2.02
Statistics												
Mean	2.776	5.142	23226.07	11688.92	1.388	1.944	1.992	7381.49	3133.29	2.148	2.102	2.018
±S.D	0.324	0.300	3.463	2.633	0.075	0.072	0.019	7.86	4.49	0.021	0.019	0.009
C.V.	0.23	0.65	0.096	0.0410	1.893	0.005	0.349	0.241	0.397	0.326	0.080	0.413



7-chloro-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one

Figure 1: Structure of Diazepam



3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine

Figure 2: Structure of Imipramine hydrochloride

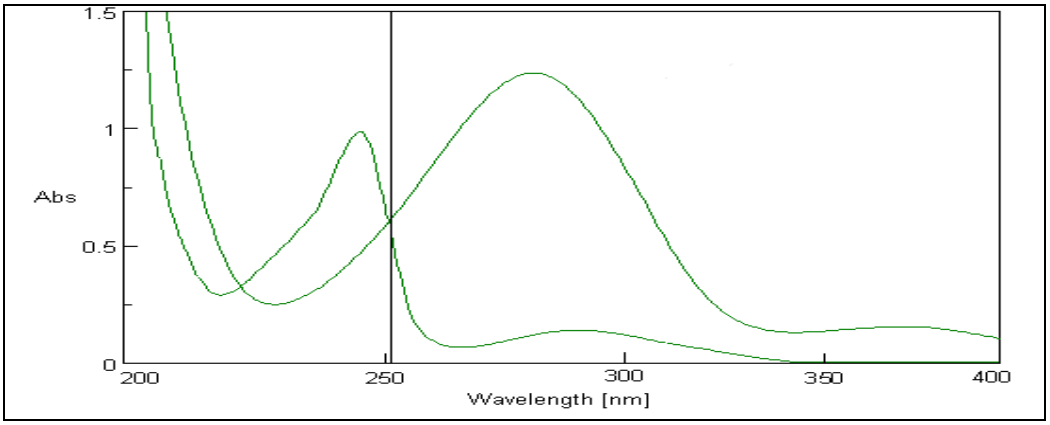


Figure 3: UV-Absorption overlay spectra of Diazepam & Impiramine HCl

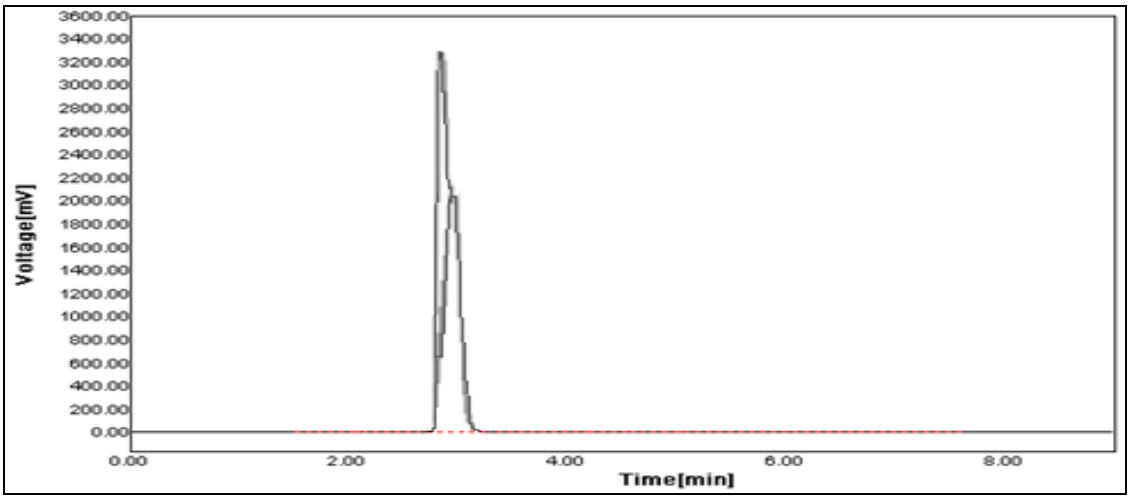


Figure 4: Chromatogram obtained by using mobile phase- Methanol

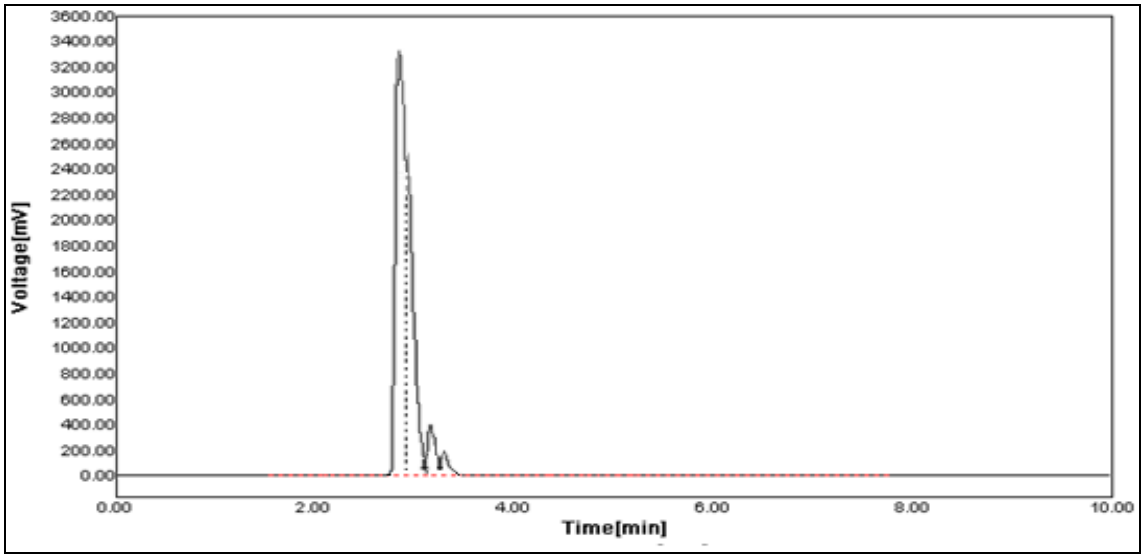


Figure 5: Chromatogram obtained by using mobile phase- Methanol: Water (50: 50)

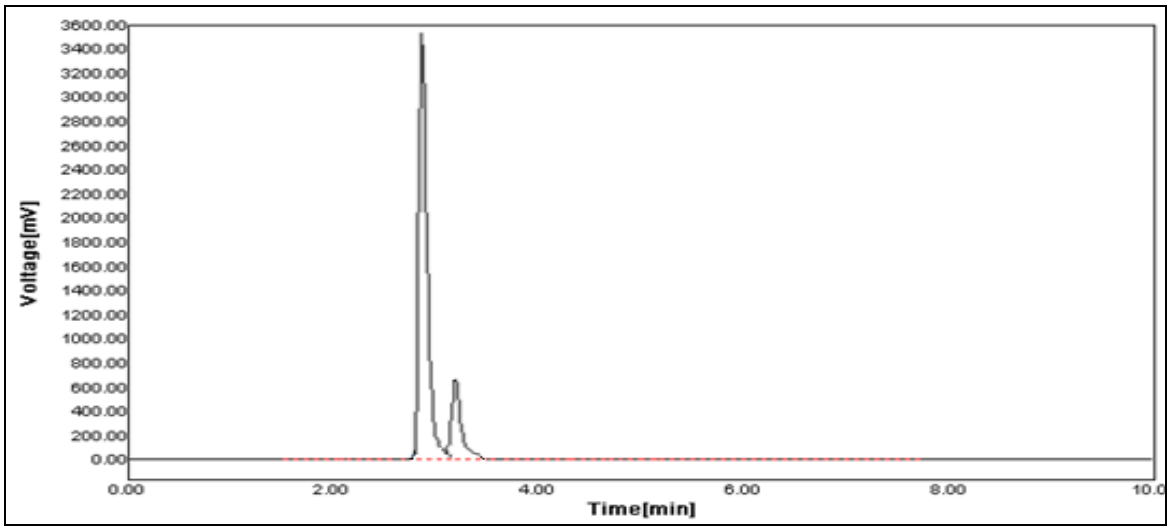


Figure 6: Chromatogram obtained by using mobile phase- Methanol: Phosphate buffer (pH 6.6) (90 : 10)

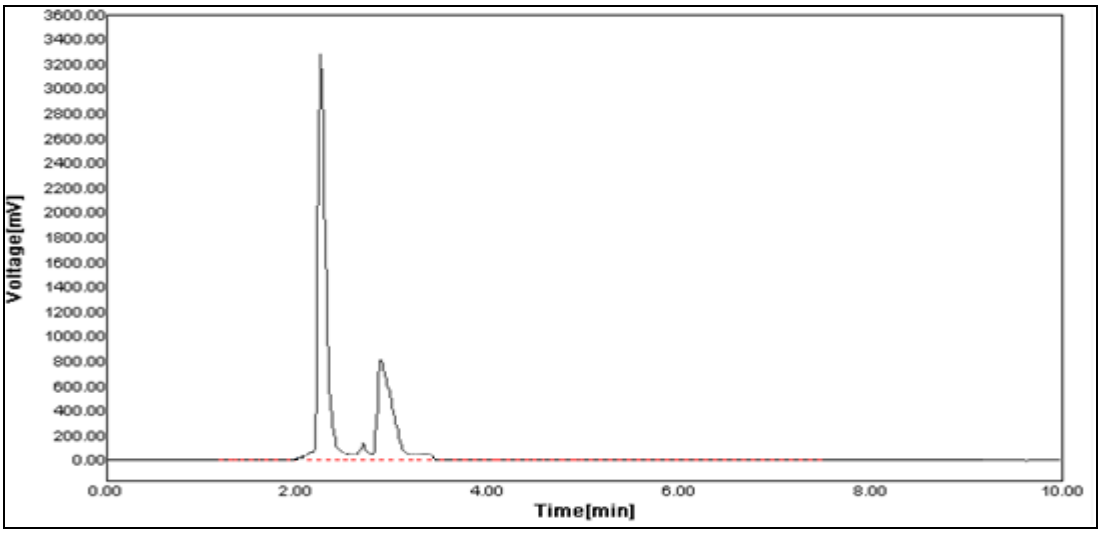


Figure 7: Chromatogram obtained by using mobile phase -Methanol: Phosphate buffer (pH 6.6) (85: 15)

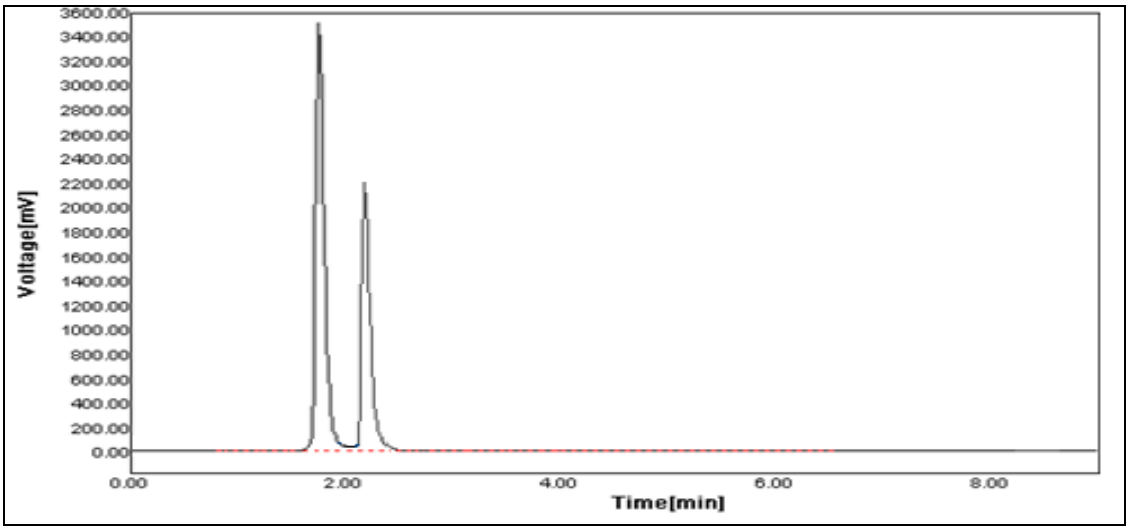


Figure 8: Chromatogram obtained by using mobile phase - Methanol: Phosphate buffer (pH 6.6) (80: 20)

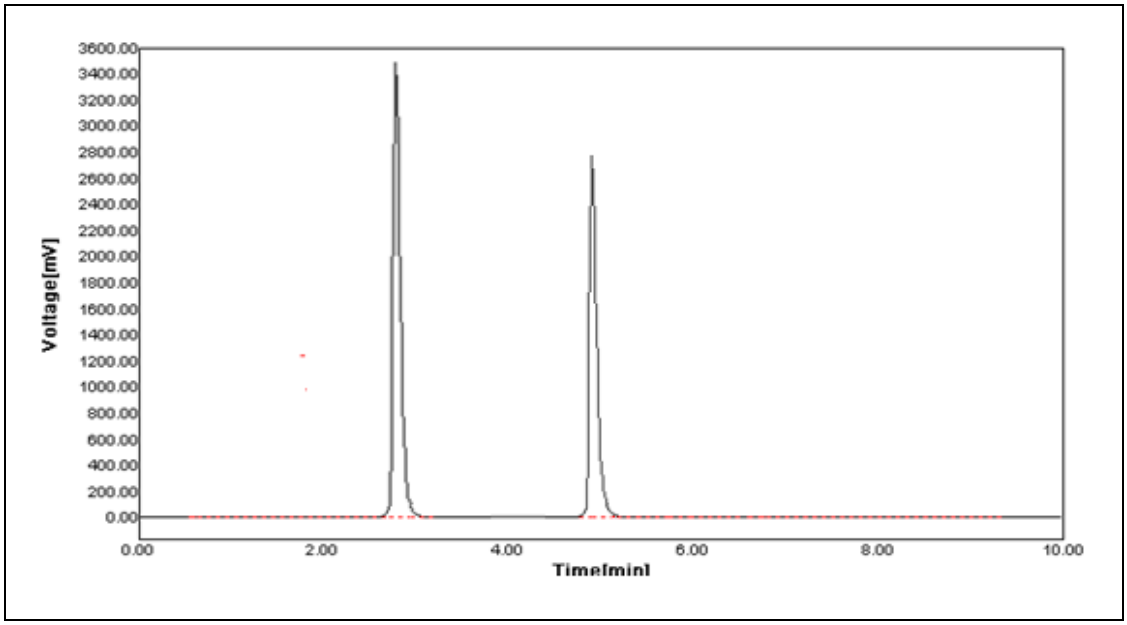


Figure 9: Chromatogram obtained by using mobile phase- Methanol: Phosphate buffer (pH 6.6) (75: 25)

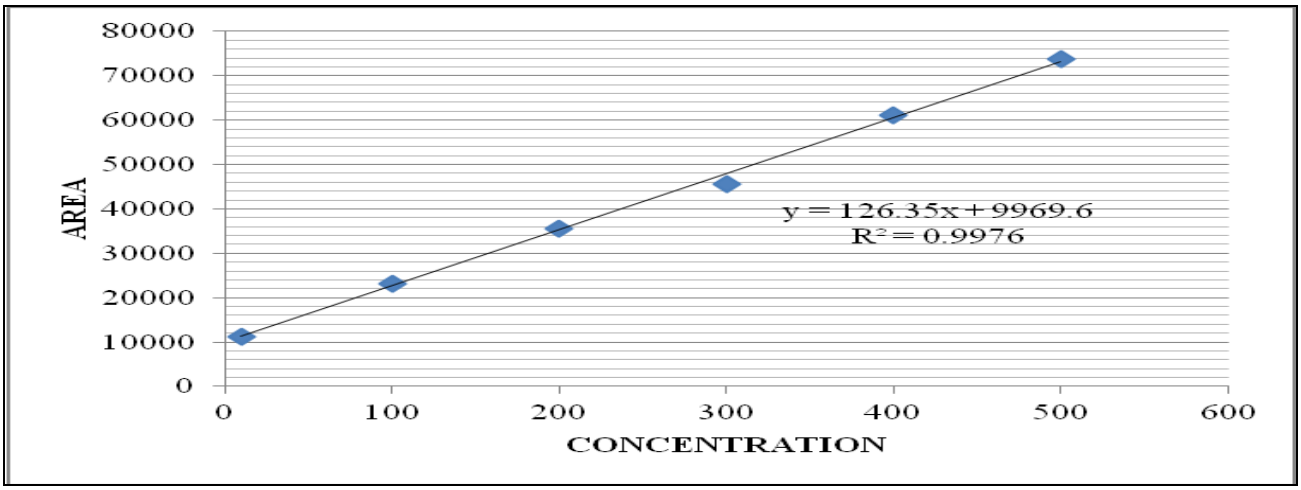


Figure 10: Calibration curve of Diazepam

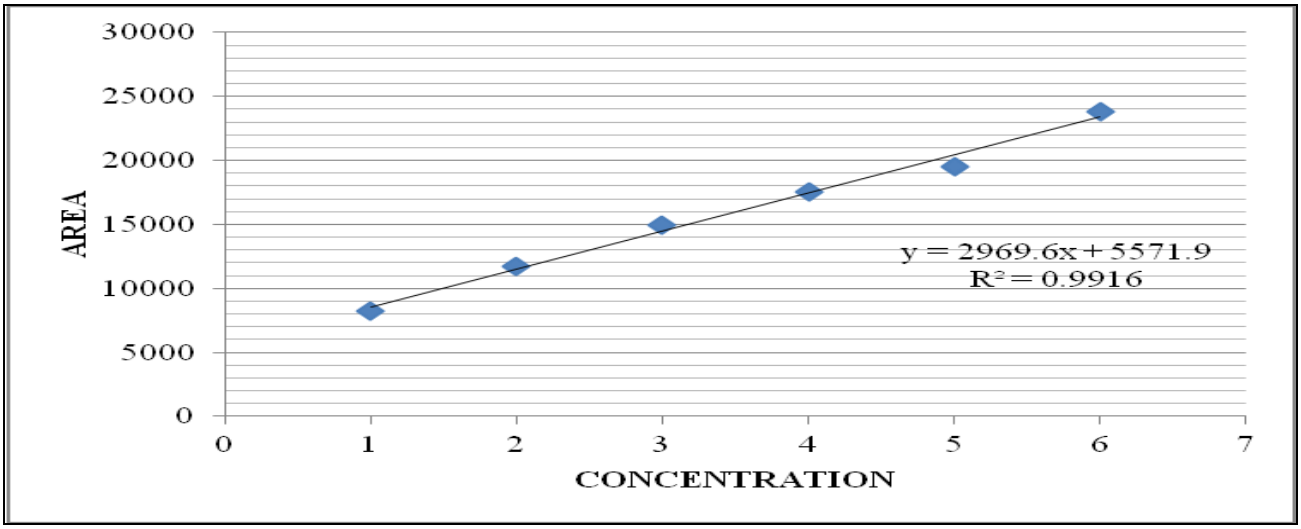


Figure 11: Calibration curve of Impiramine hydrochloride

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