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DEVLPOMENT AND VALIDATION OF RP-HPLC METHODS FOR SIMULTAINOUS ESTIMATION OF IMIPRAMINE HCL AND DIAZEPAM IN BULK AND 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 Imipramine hydrochloride, using a ODS C-18 (250 x 4.6mm, 5µm) column at temperature 30^0C and flow rate 1mL/min. A mobile phase compried of Methanol: water (Phosphate buffer (75:25 v/v, pH 6.6 with potassium hydroxide). The retention times of Diazepam and Imipramine hydrochloride were found to be 2.85 min and 5.24 min, respectively. Linearity was established for Diazepam and Imipramine hydrochloride in the range of 10-500 µg/ml and 2-12 µg/ml, respectively. The recovery was in the range of 97.67 – 99.59% for Imipramine hydrochloride (IMI) and 97.39 – 99.52% for diazepam (DIA), 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 Imipramine hydrochloride in bulk drugs and formulations.

KEYWORDS: Imipramine HCL, Diazepam hydrochloride, HPLC, Bulk drugs, Pharmaceutical formulations.

INTRODUCTION

Imipramine HCL, [3-(5, 6-dihydrobenzo[b] [1]benzazepin-11-yl)-N,N-dimethylpropan-1-amine HCl] (figure 1) 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 Pharmacopoeia5,6, which recommends HPLC and HPTLC methods for its analysis. **Diazepam** [7-chloro-1-methyl-5-phenyl-2, 3- dihydro-1H-1, 4-benzodiazepin-2-one] (figure 2), 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 Pharmacopoeia1,2,3,4, which recommends a titrimetric method 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. Literature survey reveals that many analytical methods are reported for determination of Diazepam and Imipramine.

MATERIALS AND METHODS

- **Instruments, Materials and Chemical with their sources**

Table 1: List of materials used and their sources.

Materials	**Sources**
Imipramine	Umedica Laboratories Ltd. (Gujarat, India)
Diazepam	Cipla Pharmaceuticals (Maharashtra, India)
Acetonitrile	Merks. Ltd., Mumbai, INDIA. (HPLC grade)
Methanol	
Water	
Potassium di-hydrogen phosphate	
Dichlromethanol (DMF)	
Buffers	
Deionized water	S. D. fine Chemicals,Mumbai INDIA
Solvents (Analytical Grade)	
Hydrochloric acid, Sodium hydroxide, and Hydrogen peroxide used for stress degradation studies were of analytical reagent grade	CDH Chemicals, Delhi, India
Lactose (Bulking agent), Micro crystalline solution (Binder), Aerosil (Gladient), Magnesium stearate (Lubricant)	Chemdyes corporation (Baroda)

- **List of instruments used and their sources**

Table 2: List of instruments used and their sources.

Instrument/Software	Sources
HPLC	Model and Make: Labtronics (Model 3201) Detector: UV-Visible detector Pump: 515 HPLC pump (Gradient) Column: ODS C_{18} column Particle size: 5 µm Length: 250 mm, Diameter: 4.6 m
FT-IR Spectrophotometer	Model: 8400 FTIR Made by: Shimadzu Scan Range: 15600-30 cm KBr press: Model M-15, Techno Search Instruments
Double- Beam UV-VIS spectrophotometre	Made by:Labtronics (Model 2802) Wavelength range: 200nm-800nm Software: UV Probe 2.34 Quartz Cell: 1cm
Sonicator	Shimadzu, Japan.
Zeta Potential	Zetatrac, Metrohm, India
Scanning Electron Microscope	Hitachi S-4700, Japan
Electronic digital balance	Shimadzu, Japan
Magnetic Stirrer	Remi Equipments, Mumbai, India
Sonicator	PCI, Mumbai, India.
Dialysis membrane Fifty	Himedia, India.
pHmeter.	SYSTRONIC, Chennai-INDIA.
Sartorious electronic balance	Model CP- 224 S, Labtronic instruments Ltd, INDIA.
STATISTICA 8.0	StatSoft Inc., USA
MELTING POINT APPARATUS	Model: VMP-D Made by: Veego Instruments Corporation

Selection of solvent for Imipramine Hydrochloride (IMI) and Diazepam (DIA)

Initially water was used to check out the solubility of both the drugs, where the Imipramine Hydrochloride (IMI) and Diazepam (DIA) was sparingly soluble in non polar solvent, solubility is more in ethanol and methanol. Therefore methanol has been selected as a common solvent for analysis.

Solvent	Imipramine Hydrochloride (IMI)	Diazepam (DIA)
Water	Freely soluble	Soluble
Methanol, Ethanol	Freely soluble	Slightly soluble
Ether	Insoluble	Slightly soluble
Acetone	Freely soluble	Freely soluble

Selection of Analytical Wavelength

The λ_{max} indicate the value of wavelength maxima for the drug that show highest maxima. From the overlain UV spectrum of Imipramine Hydrochloride (IMI) and Diazepam (DIA) it

was found that at 251 nm both the drug has considerable absorbance shown in fig. 3 Therefore 251 nm was selected as a common analytical wavelength for the analysis of both the drugs.

Selection of Mobile Phase

The mobile phase comprised of methanol: water (Phosphate buffer) (75:25 v/v, pH 6.6 with potassium hydroxide). The 250 ml of buffer solution was mixed with 750ml of methanol and the pH was adjusted to 6.6 ± 0.3 with potassium hydroxide 3. The solution was filtered using 0.45 µ membrane Whatman filter paper (No.1). The solution was sonicated for 10 min for degassing prior to use in an ultrasonic bath (Table: 1).

Preparation of Mobile Phase and Stock Solutions

The 50 mg of Imipramine Hydrochloride (IMI) were accurately weighed and transferred to 50 ml volumetric flask containing few ml (10 ml) of methanol. The flasks were sonicated for 2 minutes to dissolve the solids and volume was made up to the mark with diluent to obtain a standard solution containing 1000µg/ml Imipramine Hydrochloride (IMI).

The 50 mg of Diazepam (DIA) were accurately weighed and transferred to separate 50 ml volumetric flask containing few ml (10 ml) of methanol. The flasks were sonicated for 2 minutes to dissolve the solids and volume was made up to the mark with diluent to obtain a standard solution containing 1000µg/ml Diazepam (DIA).

Calibration Curves for Diazepam and Imipramine

Mobile phase to obtain 6 different solutions containing 2, 4, 6, 8, 10, 12µg/ml of Imipramine hydrochloride and 10, 20, 50, 100, 200, 400µg/ml of Diazepam respectively. The 20 µl of solutions were injected and chromatograms were obtained using 1.0 ml/min flow rate. The effluent was monitored at 251 nm. Calibration curves were constructed by plotting peak area versus concentrations and regression equations were computed. Appropriate aliquots of Imipramine hydrochloride working standard solution were taken in 6 different 10 ml volumetric flasks. The volume was adjusted to the mark with mobile phase to obtain 6 different solutions containing 2, 4, 6, 8, 10, 12µg/ml of Imipramine hydrochloride. (Figure 12).

Appropriate aliquots of Diazepam working standard solution were taken in 6 different 10 ml volumetric flasks. The volume was adjusted to the mark with mobile phase to obtain 6 different solutions containing 10, 20, 50, 100, 200, 400µg/ml of Diazepam (Figure 13).

Appropriate aliquots of Imipramine hydrochloride and Diazepam working standard solution were taken in 6 different 10 ml volumetric flasks. Appropriate aliquots of Imipramine hydrochloride and Diazepam working standard solution were added to the same flasks. The volume was adjusted to the mark with.

Analysis of Marketed Formulation

Twenty tablets weighed accurately and finely powdered. A powder quantity equivalent to 25mg of Imipramine hydrochloride and 5mg of Diazepam was accurately weighed and transferred to 100 ml volumetric flask containing few ml (60 ml) of methanol. Flask was sonicated for 10 min and volume was made up to the mark with methanol. The above solution was filtered in another 50 ml volumetric flask through Whatman filter paper (No. 41) and volume was made up to the mark with the same solvent. From the above solution aliquot of 1 ml was transferred to a 10 ml volumetric flask and the volume was made up to the mark with the mobile phase to obtain a solution containing 25 µg/ml of Imipramine hydrochloride and 50 µg/ml of Diazepam. The solution was sonicated for 10 min. This solution of Imipramine hydrochloride and Diazepam was injected as per the above chromatographic conditions and peak area was recorded. The amounts of both the drugs were calculated by keeping these values to the straight line equation of calibration curve (table 11).

Validation of Hplc Method

The method of analysis was validated as per the recommendations of ICH7 and USP2 for the parameters like accuracy, linearity, precision and robustness. Diazepam showed good correlation coefficient in concentration range of 10-500 µg/ml (r2 = 0.997 ± 0.98) and Imipramine hydrochloride showed good correlation coefficient in concentration range of 2-12 µg/ml (r2 = 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 6 different days for the concentration of 10, 20,50, 100, 200 µg/ml and 2, 4, 6, 8, 10,12 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 50µg/ml and 10 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 (Table: 9,10).

RESULTS AND DISCOSION

UV overlain spectra of both Diazepam and Imipramine showed that both drugs absorbed appreciably at 251 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 4-12). The mobile phase consisting of Methanol: Water (Phosphate Buffer) (75:25 V/V, pH 6.6 With Potassium Hydroxide). The retention times of Diazepam and Imipramine 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. The peak purity of imipramine and Diazepam shown in (figure 15-16). 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 13-14). The data of statistical analysis of the calibration curves is shown in (table 3). Experimental results of the amount of Diazepam and Imipramine hydrochloride in formulation, expressed as percentage of label claim were in good agreement with the label claims as stated in, thereby suggesting that there is no interference from any excipients, which are normally present in syrup. 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 10-200 µg/ml in triplicate for Diazepam and 2-12 µg/ml for Imipramine hydrochloride (table 3). The recovery results were in the limit as stated . Inter and intraday studies were also under limit, RSD was below 2% as shown in (table 5-6). Results for robustness evaluation for both the drugs are presented in (table 8). 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. The recovery was in the range of 97.67 – 99.59% for Imipramine hydrochloride (IMI) and 97.39 – 99.52% for diazepam (DIA), respectively (table 7). 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 (table 12-13).

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)	8.1	9.45	18060.18	530.43	A more retention time
3.	Methanol: Water (50 : 50)	2.9	--	28794.09	--	Asymmetry in peak of Diazepam
4.	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
5.	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
6.	Methanol:Phosphate buffer (pH 6.6) (80:20)	2.8	3.5	23188.68	9127.94	Slight tailing in peak of Para & Imipramine.
7	Methanol:Phosphate buffer (pH 6.6) (75:25)	2.8	5.2	23225.02	11689.06	Good resolution of both peaks.

Table 2: Optimized RP-HPLC method chromatographic condition.

Column	ODS C_{18} (250 x 4.6mm, 5µm)
Mobile Phase	Methanol and Water (Phosphate buffer) (75:25) v/v, pH 6.6 adjusted with Potassium Hydroxide
Flow rate	1 ml/min
Detection	251nm
Column Temperature	30ºC
Retention Time	2.85 min for DIAZEPAM and 5.25 for IMIPRAMINE
Run Time	10min
Injection volume (loop)	20 µl

Table 3: Statistical analysis data of calibration curve.

Parameters	**Imipramine**	**Diazepam**
Linear Range	2 – 12 µg/ml	10 – 200 µg/ml
Slope	2903	229.4
Intercept	208.2	119.1
Regression Coefficient (r^2)	0.998	0.998
Standard deviation of slope	0.153	0.138
Standard deviation of intercept	0.842	1.211
LOD (µg/ml)	0.16	3.1
LOQ (µg/ml)	0.53	9.3

Table 4: Repeatability study.

Concentration	**Imipramine**	**Diazepam**
Peak Area	11689.19	23123.79
	11609.12	23201.9
	11711.59	23031.54
	11691.91	22987.24
	11607.05	23621.91
	11719.1	22953.79
Mean	11671.33	23153.36
SD	50.29	247.00
RSD	0.004	0.01
% RSD	0.43	1.07

Table 5: Intraday and Interday Precision study for Imipramine.

Intraday Precision		
Conc. (µg/ml)	**(Conc. found ± S.D) (n=3)**	**% RSD**
2	2.10 ± 0.1	1.14
6	6.21 ± 0.1	0.26
12	12.2 ± 0.3	0.14
Inter day Precision		
2	2.12 ± 0.1	0.09
6	6.12 ±0.15	1.56
12	12.2 ± 0.2	1.53

n=Three determination

Table 6: Intraday and Interday Precision study for Diazepam.

Intraday Precision		
Conc. (µg/ml)	**(Conc. found ± S.D) (n=3)**	**%RSD**
10	10.5 ± 0.1	0.99
100	100.0 ± 0.15	0.14
200	199.9 ± 0.15	0.71
Interday Precision		
10	10.2 ± 0.1	0.97
100	99.93 ± 0.1	0.29
200	199.6 ± 0.4	0.23

n=Three determination

Table 7: Accuracy study.

Level	Drug added (µg/ml)	% Drug Recovered ± SD	% RSD
Imipramine			
50	2	99.81 ± 0.069	0.076
100	4	99.96 ± 1.13	0.195
150	6	101.89 ± 0.73	0.709
Diazepam			
80	25	99.68 ± 0.159	0.162
100	50	101.43 ± 0.257	0.259
150	75	99.89 ± 0.568	0.589

a=Average of Three determination

Table 8: Robustness study for Imipramine hydrochloride and Diazepam.

Parameters	**Change in condition**	**Imipramine hydrochloride**	**Diazepam**
		%RSD	**%RSD**
Flow rate Changed (1 ml/min)	0.9	0.92	0.85
	1.1	1.58	0.57
Column Temperature (30ºC)	25ºC	0.68	0.95
	35ºC	1.45	0.63
pH of mobile phase changed (pH=6.60)	6.55	0.71	1.57
	6.65	1.62	0.79
Mobile Proportion Changed Methanol: water (Phosphate buffer) (75:25 v/v)	Methanol: Water (Phosphate buffer) (70:30 v/v)	0.73	0.66
	Methanol: Water (Phosphate buffer) (80:20 v/v)	0.91	0.80
Detection wavelength (251nm)	246 nm	0.89	1.35
	256nm	1.05	0.78

Table 9: Various validation parameter and their acceptance criteria.

Validation Parameters	Acceptance Criteria
Correctness	Recovery 98- 102% (individual)
Reproducibity	Relative Standard Deviation < 2%
Repeatability	Rel. Std Dev. < 2%
Ruggedness	Rel. Std Dev. < 2%
Specificity/ Selectivity	No interference, the P. P. I/ > 0.999
Regression range of linearity	Correlation coefficient r^2 > 0.999 or 0.995
Solution Stability	> 12 hour
Detection Limit	Signal /Noise > 2 or 3
Quantitation Limit	Signal /Noise > 10

Table 10: Summary of validation parameters.

Parameters	Imipramine	Diazepam
Linear Range	2- 12 µg/ml	10 – 200 µg/ml
Regression Coefficient	0.998	0.998
Regression equation	y = 2903.x - 208.1	= 229.4x + 119.1
Recovery %	99.81 % - 101.89%	99.68 %-101.43%
Repeatability (RSD, n=6)	0.43	1.07
Precision (RSD) Intra - day (n=3) Inter - day (n=3)	 0.14–1.14% 0.09–1.56%	 0.14–0.99% 0.23–0.97%
Reproducibility	Reproducible	Reproducible
Limit of Detection (µg/ml)	0.16	3.1
Limit of Quantitation (µg/ml)	0.53	9.3
Robustness	Robust	Robust
Solvent stability	Stable for 48hrs	Stable for 48hrs
Specificity	Specific	Specific
Peak Purity	0.998	0.997

Table 11: Assay results of marketed formulation.

Formulation	Drug	Amount Taken (mg)	Amount Found[n] (mg)	%IMI ± SD	%DIA ± SD
Depranil Plus Tablet	IMI	25	24.58	98.33 ± 1.13	99.60 ± 1.85
	DIA	5	4.98		
Depsol Forte Tablet	IMI	25	25.02	100.08 ± 1.75	99.20 ± 1.54
	DIA	5	4.96		

n= Average of Three determination

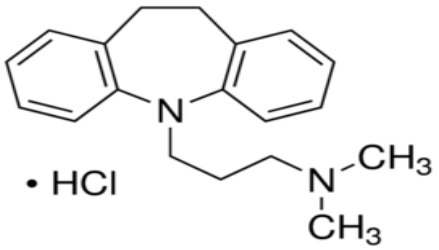
IMI=Imipramine, DIA=Diazepam

Table: 12 System Suitability Test Results of Diazepam (DIA).

Sr. No.	Rt. (min)	Peak area	Tailing factor (T)	Resolution (R)	No. of Theoretical plates (N)	Capacity factor (K')	Selectivity (α)
1	3.10	23225.02	1.42	2.02	7381.40	2.15	2.01
2	2.23	23227.16	1.38	1.98	7387.35	2.18	2.02
3	2.85	23231.54	1.26	2.00	7378.73	2.12	2.03
4	2.83	23222.82	1.43	1.97	7390.03	2.15	2.01
5	2.87	23223.79	1.45	1.99	7369.98	2.14	2.02
Mean	2.776	23226.07	1.388	1.992	7381.49	2.148	2.018
S.D	0.324	3.463	0.075	0.019	7.86	0.021	0.009
% RSD	0.23	0.096	1.893	0.349	0.241	0.326	0.413

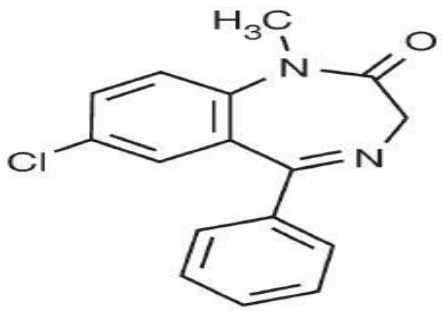
Table 13: System Suitability Test Results of Imipramine (IMI).

Sr. No.	Rt. (min)	Peak area	Tailing factor (T)	Resolution (R)	No. of Theoretical plates (N)	Capacity factor (K')	Selectivity (α)
1	5.50	11689.19	2.06	2.02	3132.45	2.10	2.01
2	4.86	11687.80	1.91	1.98	3128.16	2.09	2.02
3	5.10	11685.23	1.89	2.00	3139.77	2.08	2.03
4	5.40	11690.06	1.97	1.97	3135.48	2.11	2.01
5	4.85	11692.31	1.89	1.99	3130.62	2.13	2.02
Mean	5.142	11688.92	1.944	1.992	3133.29	2.102	2.018
S.D	0.300	2.633	0.072	0.019	4.49	0.019	0.009
% RSD	0.65	0.0410	0.005	0.349	0.397	0.080	0.413



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

Figure 1: Structure of Imipramine hydrochloride.



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

Figure 2: Structure of Diazepam.

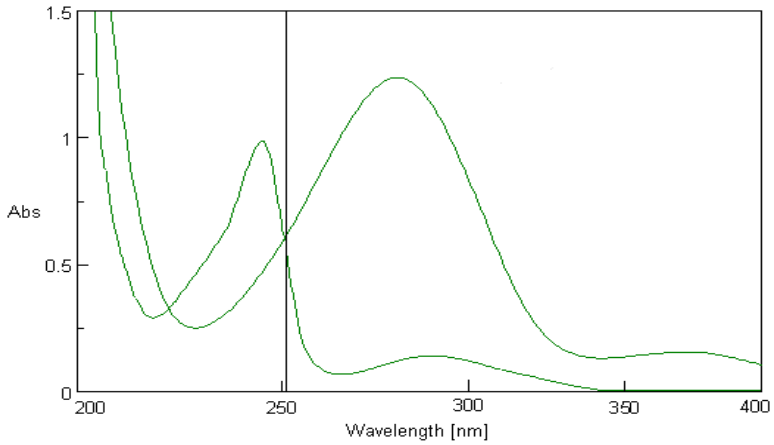


Figure 3: Overlain UV spectrum of Imipramine Hydrochloride (IMI) and Diazepam (DIA) in methanol in the range of 200nm-400nm.

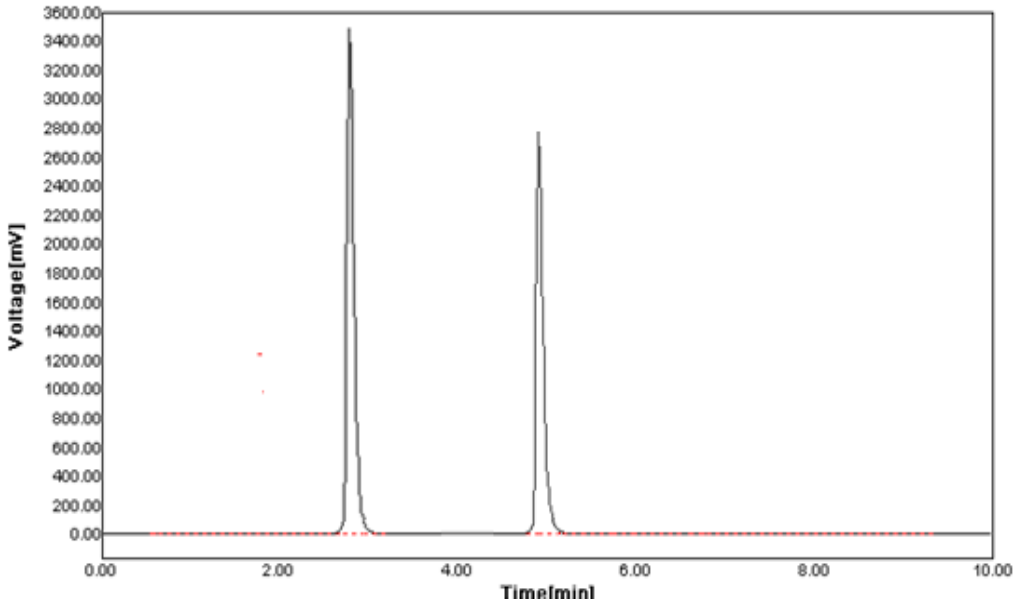


Figure 4: Standard HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml) in mix standard.

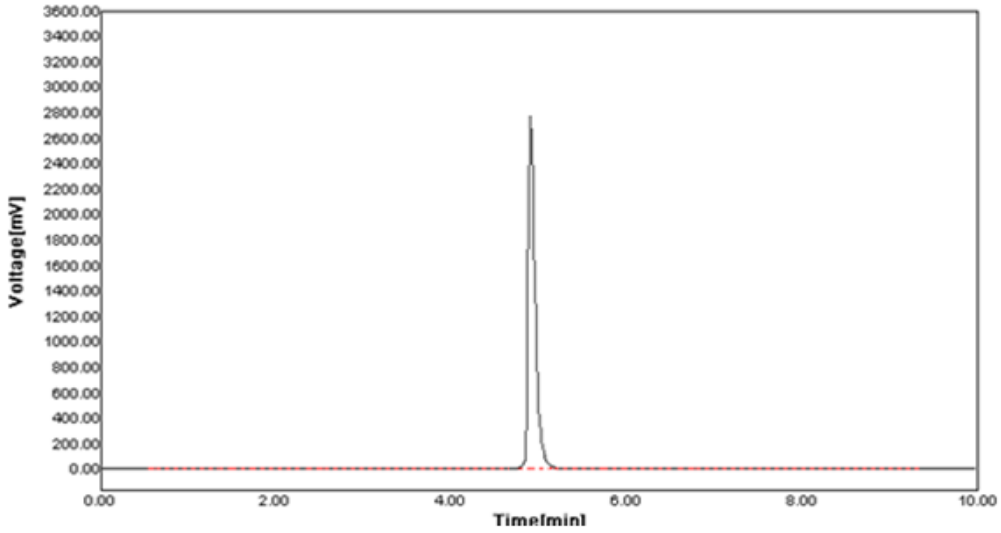


Figure 5: Standard HPLC Chromatogram of standard solution of IMI (10 µg/ml).

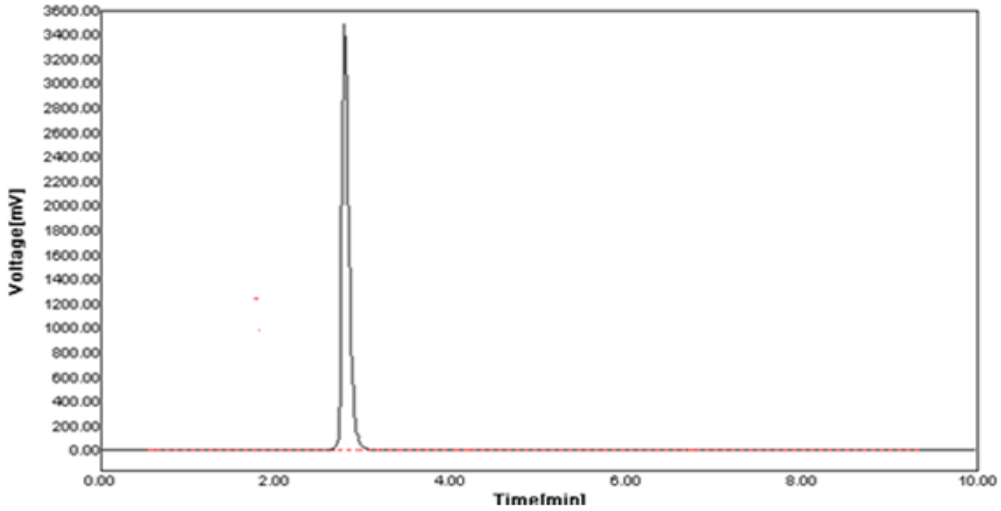


Figure 6: Standard HPLC Chromatogram of standard solution of DIA (100 µg/ml).

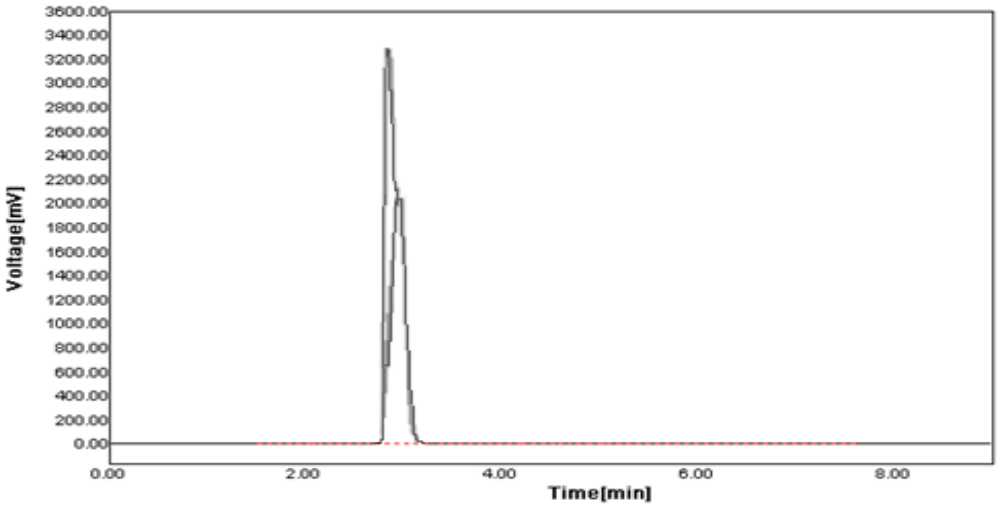


Figure 7: HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml)obtained by using mobile phase- Methanol.

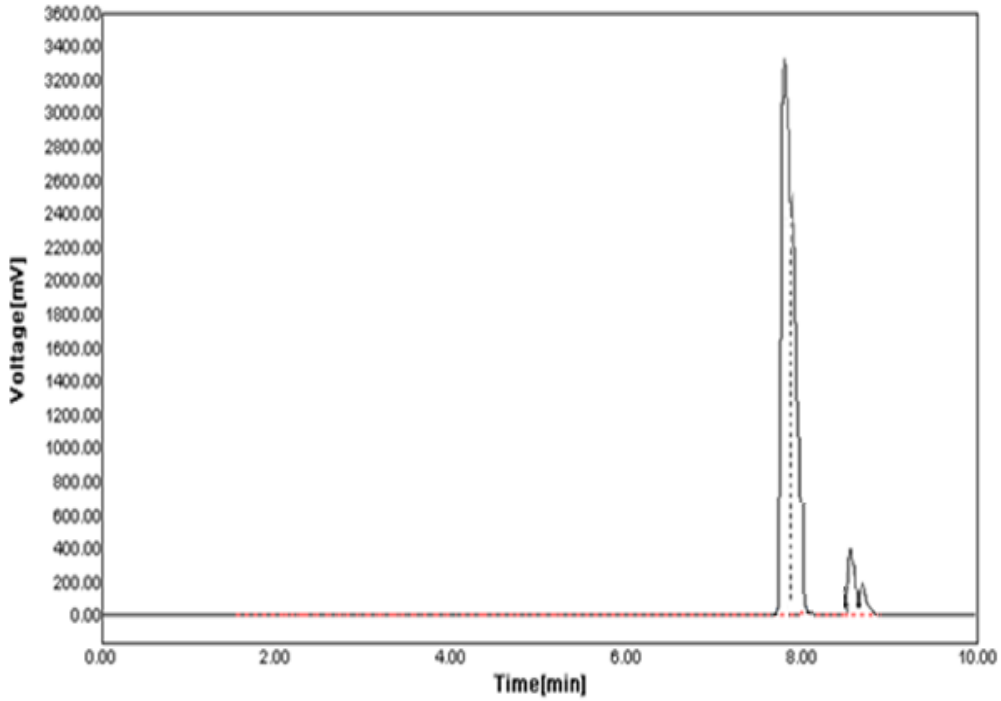


Figure 8: Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml)obtained by using mobile phase- Methanol: Water (10: 90)

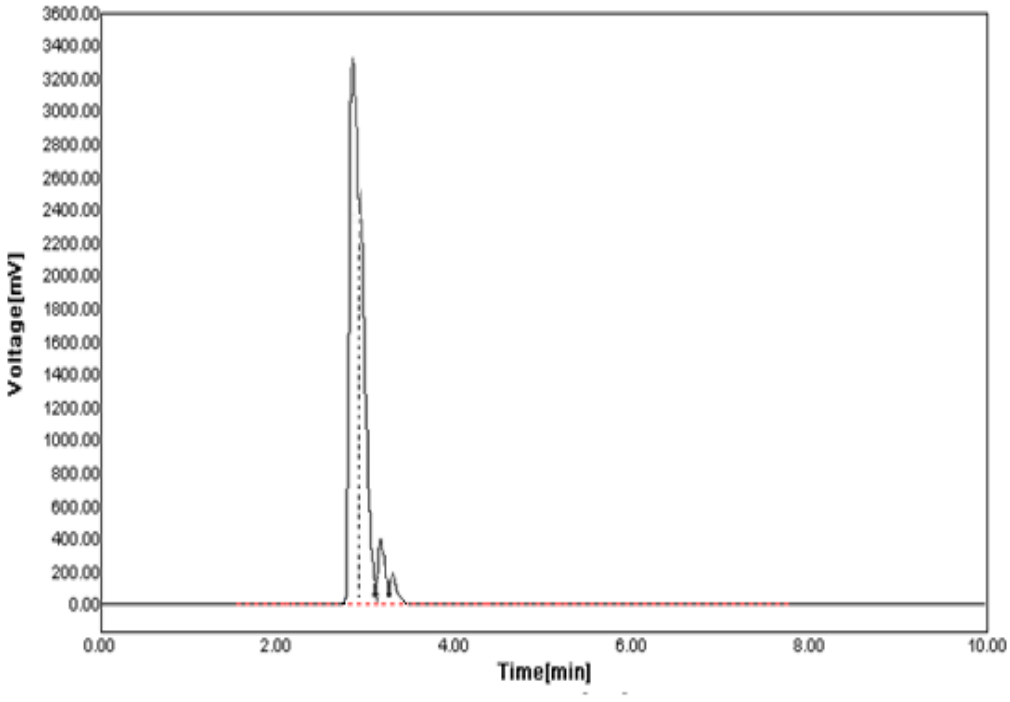


Figure 9: Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml)obtained by using mobile phase- Methanol: Water (50: 50).

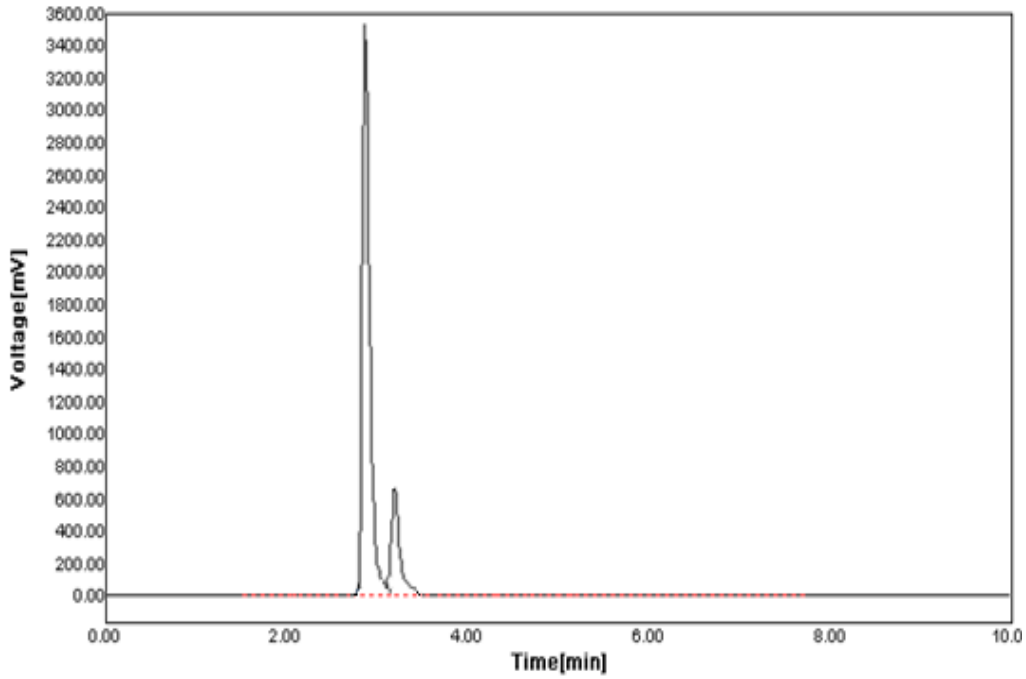


Figure 10: HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml) obtained by using mobile phase- Methanol: Phosphate buffer (pH 6.6) (90: 10)

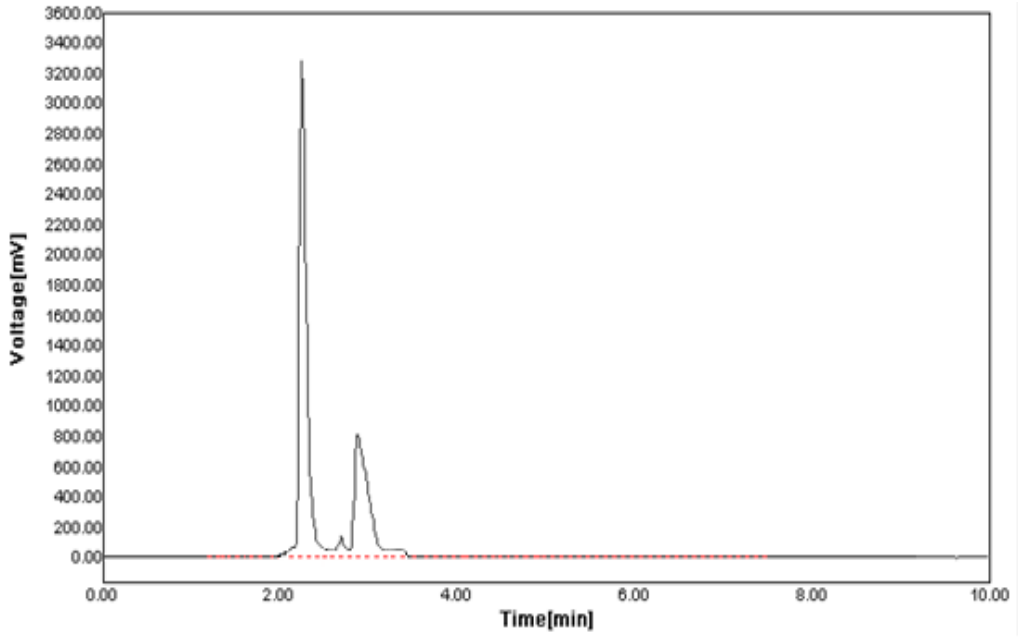


Figure 11: HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml)obtained by using mobile phase - Methanol: Phosphate buffer (pH 6.6) (85: 15).

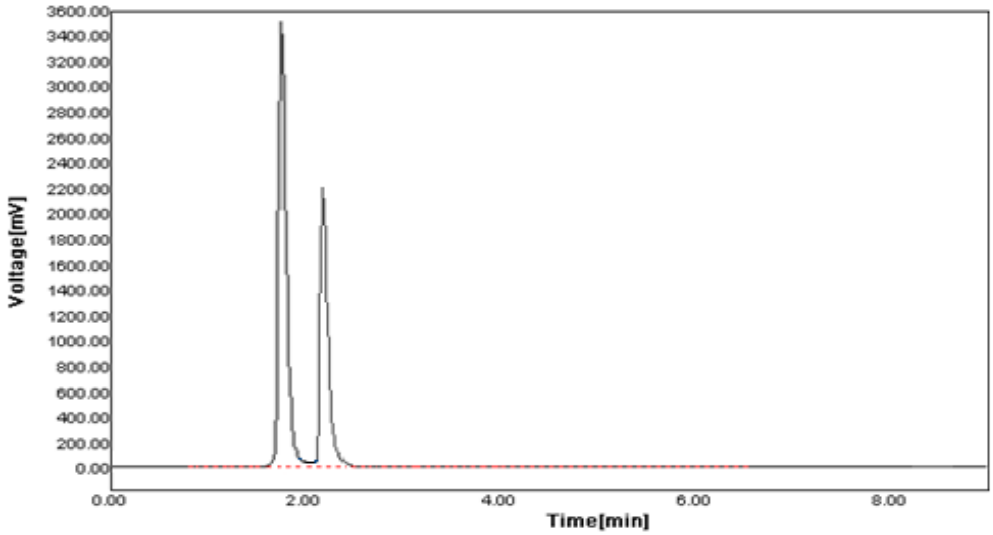


Figure 12: HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml) obtained by using mobile phase - Methanol: Phosphate buffer (pH 6.6) (80: 20).

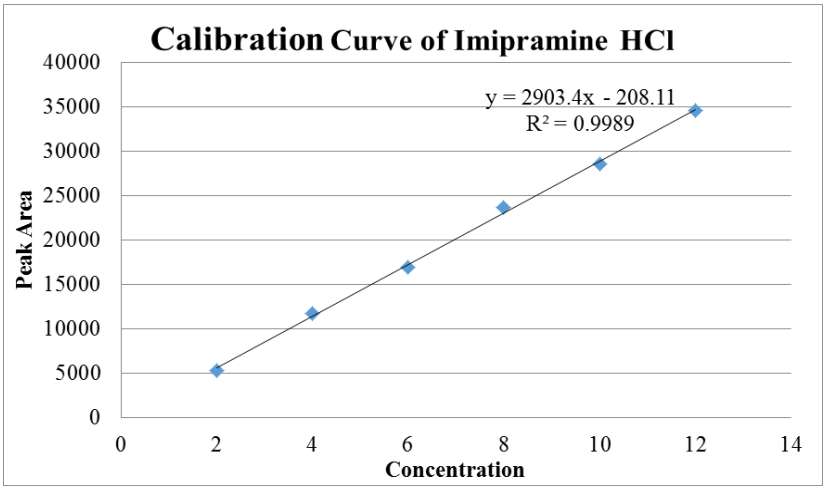


Figure 13: Calibration curve of Imipramine HCl standard.

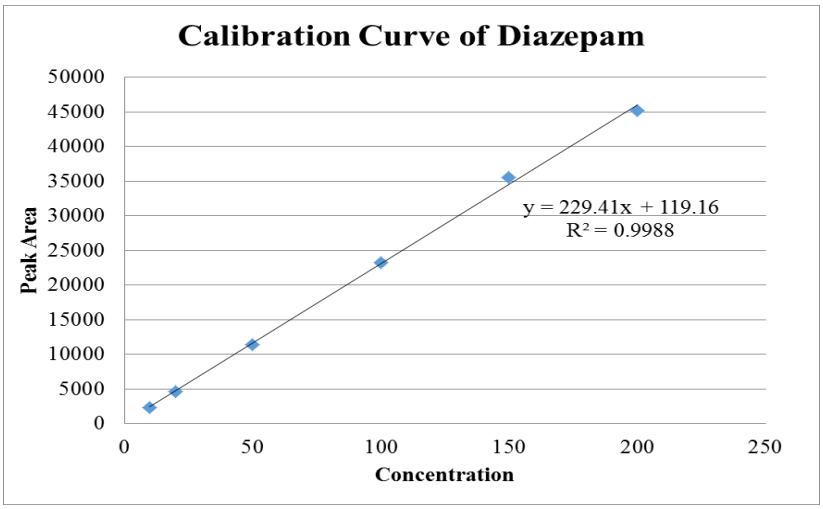


Figure 14: Calibration curve of Diazepam standard.

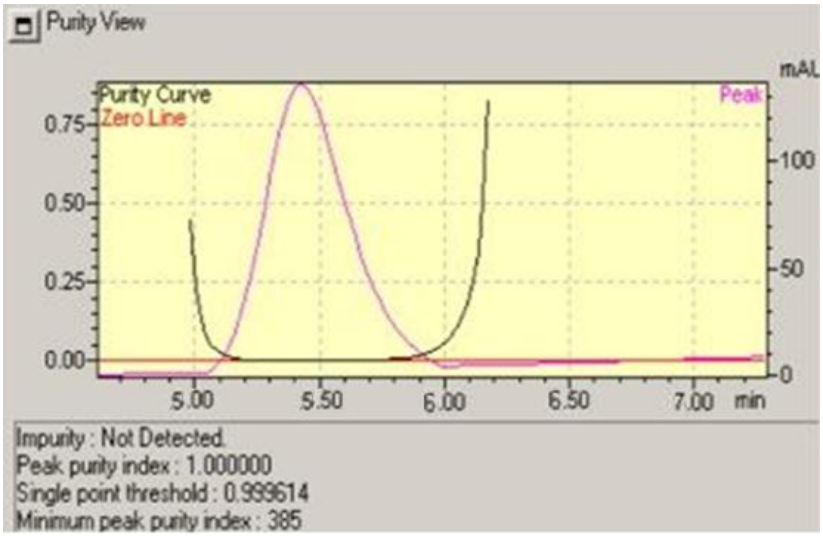


Figure 15: Peak Purity of Imipramine.

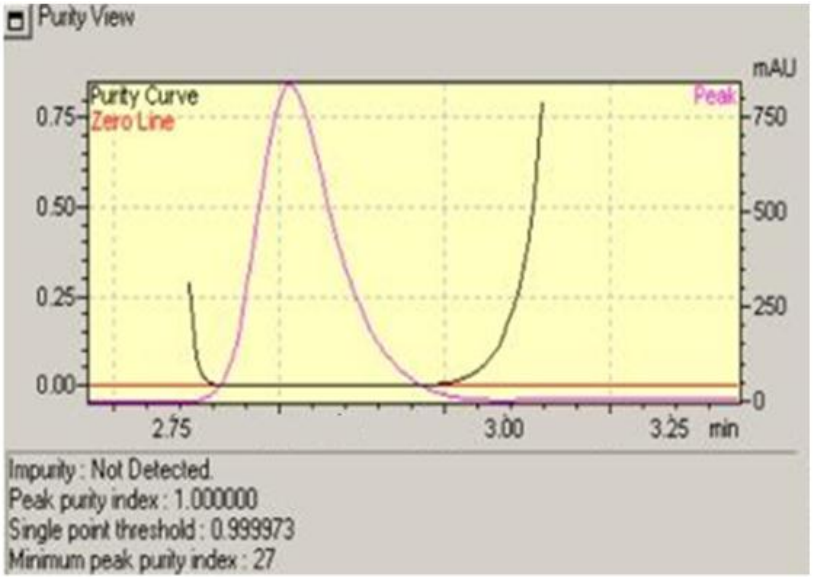


Figure 16: Peak Purity of Diazepam.

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