International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

Development of RP-HPLC Method and Stability Studies for Simultaneous Estimation of Imipramine HCL and Diazepam in Bulk and Pharmaceutical Formulations.

[1*]Darshana Yadav, [1] Mahima Dave [1]Archana Vasava, [1]Dhara Patel, [1]Jagruti Solanki, [1]Khusbu Patel, [2]Prof. Mitali Dalwadi, [2]Dr.Chainesh Shah and [3]Dr.Umesh Upadhyay

[1]Student, [2]professor, [3]Principal

Department of QA(Quality Assurance), Sigma Institute of Pharmacy, Bakrol, Ajwa Road, Vadodara, Gujarat, India,- 390019.

Date Of Submission: 15-05-2021 Date Of Acceptance: 26-05-2021

ABSTRACT: A new rapid, economical and isocratic reverse phase high performance liquid chromatograpy (RP-HPLC) method was developed for te determination of imipraine HCL aand diazepam in pharmaceutical formulation.The chromatographic sseperation was achieved isocratically on ODS C18 column (250 x 4.6mm, 5µm) , mobile phase at flow rate 1mL/min and Uv detection at 251nm. Linerity was measured in the range of 2-12µg/m for imipraine HCL and 10-200 µg/m for diazepam. The limit of detection and Quantitation were 0.16 and 0.53 µ g/ml for imipramine HCL and 3.1 and 9.3 µ g/m for diazepam. Satisfactory validtion was also obtained from recovery (99.81% -101.89% for imipramine HCL and 99.68% - 101.43% for diazepam) studies, intraday and interday precision (<2%) and robustness results.

Keywords:- Stability Studies, Imipramine HCL, Diazepam, Reverse Phase HighPerformance Liquid Chromatography (RP-HPLC).

I. INTRODUCTION

The preamble of research is to develop analytical methods in combination of imipramine and Diazepam by various analytical techniques like, UV Spectrophotometric, LC-MS. The drug Imipramine is used as antidepressants as longer time. The drug mainly inhibit the reuptake of neurotransmitters. The drug Diazepam is used as anti-anxiety, anti-convolsant, sedative hypnotic and centrally acting muscle relaxants. Diazepam is a benzodiazepine derivative drug which can be synthesize by substituted aniline and benzophenone in the presence of zinc chloride. Diazepam is considered to be best muscle relaxer. The drug has great abuse potential due to sedative nature.

Imipramine is also used in panic disorder that is form of intense, overwhelming & uncontrollable anxiety. Anxiety is not a voluntary, controllable emotion but condition that may be ignoring it or wishing it away. Attacks of panic disorder are with definite onset & spontaneously. These may occur at home or public and many times interrupting sleep. These are diagonised by three attacks in a 3 week period. Attacks are not stimulated by physical exertion, exposure to phobic stimulus or life threatening situations & following characteristic labored breathing, palpitations, chest discomfort, chocking sensation, dizziness, uncertaintity of feelings, tingling in feet or hand, cold or hot flashes, numbness, sweating & trembling.

This mainly located in the locus ceruleus, input signals are mainly disturbed depending on both the current stage of the bacteria & the type of incoming signal. If arriving signals are not properly increased to signal a life threating stress, the organism is aroused to fight or flight. The extra amplification of arriving signal gives rise to a state of extra exicitment, automatic discharges and amplified respiratory drive. If the incoming signal is calm & non threatening, stimuli is toned & locus ceruleus does not over react. Panic persons are very sensitive to stimulant effect of caffeine & Na lactate that when infused, change intracellular pH & amplifying impuse transfer by brain causing panic characteristics.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

Table:-1 Drug Profile

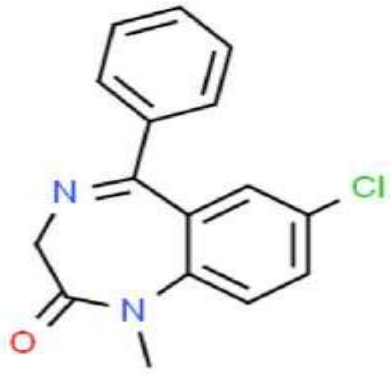
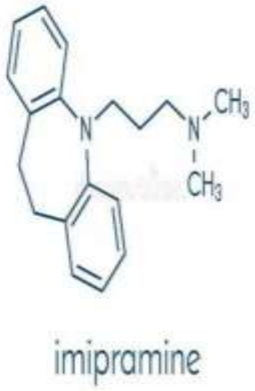
SR. NO. 1.	PARAMETER	DIAZEPAM	IMIPRAMINE
2.	Structure		
3.	Chemical formula	C16H13CIN20	C19 H24 N2
4.	IUPAC Name	9-chloro-2-methyl-6-phenyl-2,5-diazabicyclo.	3-(10,11-dihydro-5H-dibenzo [b, f] azepine – 5-yl)-N, N-dimethylpropan-1-amine

Table:-2 MATERIAL AND METHODOLOGY

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)	

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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)

Table:-3 List of Instruments 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

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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

EXPERIMENTAL WORK

- Selection of common solvent for Imipramine Hydrochloride (IMI) and Diazepam (DIA)
- Preparation of Mobile Phase
- Preparation of standard Stock solutions
- Selection of analytical wavelength
- Selection of chromatographic condition
- Validation of method
- Force degradation study

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.

TABLE:-4 Solubility Data of Imipramine Hydrochloride and Diazepam

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

Preparation 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 sonnicated for 10 min for degassing prior to use in an ultrasonic bath.

Diluent Preparation

There was mobile phase used as a diluent.

Preparation of standard stock solution (Standard stock solution (1000µg/ml)):

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).

Selection of analytical wavelength:

The λ_{max} indicate the value of wavelength maxima for the drug that show highest maxima.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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. 4.1.5. Therefore 251 nm was selected as a common analytical wavelength for the analysis of both the drugs.

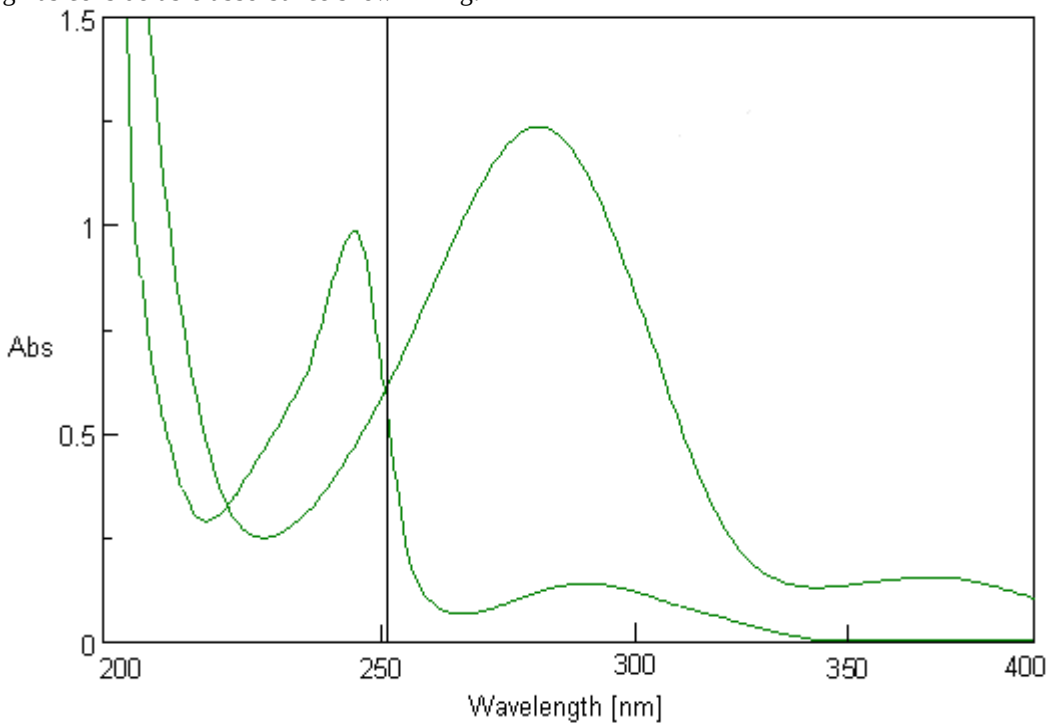


Figure:-1 Overlain UV spectrum of Imipramine Hydrochloride (IMI) and Diazepam (DIA)in methanol in the range of 200nm-400nm

Selection of chromatographic condition:

The experiments were performed on Labtronics (Model 3201) separation module with Labtronics UV-Visible detector. The chromatographic and the integrated data were recorded using empower 2 software using gradient HPLC pump (515). Chromatographic separations were performed on ODS C_{18} (250 x 4.6mm, 5µm) column at 30ºC usingmethanol: water (Phosphate buffer) (75:25 v/v, pH 6.6 with potassium hydroxide) as mobile phase with gradient mode. The flow rate was maintained at 1ml/ min. A 20µl of sample was injected using a fixed loop, and detection of all the components was carried out at 251 nm with adequate sensitivity.

VALIDATION OF HPLC METHOD

The validation tells how good the methods are, specifically whether it is good enough for the intended application. The US Food and Drug Administration (FDA) have edited draft guidelines with detailed recommendations for method validation of bio-analytical methods [Shah V.P., 2001] in the pharmaceutical industry. The International Conference on Harmonisation (ICH) has provided definitions of validation issues included in "analytical procedures" for the fields of bio-analytical methodology, pharmaceutical and biotechnological procedures [ICH, Q2A, Q2B, Q6B, 2002]. Likewise the US Pharmacopeia (USP) has published guidelines for method validation for analytical methods for pharmaceutical products [USP, 1995]. However the guidelines from ICH and USP are not as detailed as those from the FDA, and in the analytical biotechnology area there exists no detailed validation guidelines. Validation was done as per ICH guideline Q2 (R1). The most common validation parameters will be briefly described below.

Stress Study (Stability study)

Stress study was carried out under the condition of acid/base hydrolysis, oxidation, thermal, as mentioned in ICH Q1A (R2). The stability assay methods are gaining importance for the evaluation of active pharmaceutical substance.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

USFDA emphasize the stability indicating assay methods for the estimation of active ingredients in pharmaceutical dosage form. Validated, quantitative, analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product. These are specific so that the contents of active ingredient, degradation products and other components of interest can be accurately measured without interference. The term sufficient decomposition is taken in the broadest sense, meaning 80-100% decomposition, if the objective is isolation of the degradation products or between 20-80% decomposition when the objective is to establish the degradation pathways (Bakshi et.al, 2002).

Forced degradation study or stress study using acid hydrolysis, alkali hydrolysis, neutral hydrolysis, chemical oxidation, dry heat degradation and photo degradation studies were carried out as per ICH guidelines (ICH, 2002).

Stock solutions were prepared by accurately weighing 25 mg each of Impiramine hydrochloride and Diazepam and transferring to two separate 25 ml volumetric flasks containing few ml of methanol. The flasks were swirled to dissolve the solids and diluted up to the mark with methanol. These stock solutions were used for forced degradation studies.

- **Forced Degradation Study**
  - **Base Induced Degradation (Base hydrolysis)**
  - **Acid induced Degradation (Acid hydrolysis)**
  - **Neutral hydrolysis**
  - **Oxidative stress degradation**
  - **Thermal Degradation**

i. Dry heat degradation
ii. Wet heat degradation

- **Photo degradation study**
- **System suitability**

A system suitability test was used to verify that the resolution and repeatability of the system were adequate for the analysis intended. A system suitability test was an integral part of the method development to verify that the system is adequate for the analysis of Impiramine hydrochloride and Diazepam to be performed. System suitability test of the chromatography system was performed before each validation run. Five replicate injections of a system suitability standard preparation (4µg/ml for Impiramine hydrochloride and 100µg/ml for Diazepam) and one injection of a check standard were made. The parameters measured were retention time, peak area, theoretical plates, and asymmetry of Impiramine hydrochloride and Diazepam.

- **Linearity and Range**

Linearity was studied by preparing solutions of six different concentrations of 2, 4, 6, 8, 10, 12µg/ml of Impiramine hydrochloride and 10, 20, 50, 100, 150, 200µg/ml of Diazepam respectively. Each concentration was repeated six times. Linearity was assessed in terms of slope, intercept and correlation coefficient of Impiramine hydrochloride and Diazepam. The calibration curves were developed by plotting absorbance versus concentrations (n = 6). The linearity is statistical value that can be calculated from the graph. The standard curve making in excel sheet to calculate a regression data with help of coefficient of correlation, slop and intercept.

- **Precision**

The precision was studied as Repeatability, Intra and Interday precision and Reproducibility. Precision is checked for reproducibility of the system. To prove the method is precision, different observations and check the closeness of each others. If the values are near equal then the system is reproducible. The precision does not indicate the correctness but if the procedure is accurate then it should be precise.

- **Repeatability**

Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions (same reagents, equipments, settings and laboratory) over a short interval of time. The repeatability studies were carried out by estimating response of 4 µg/ml of Impiramine hydrochloride and 100 µg/ml of Diazepam six times and results are reported in terms of RSD.

- **Intra and Inter day precision**

The intraday and inter day precision study was carried out at three different concentration of Impiramine hydrochloride and Diazepam. Intraday precision was evaluated by estimating the corresponding responses three times on the same day and inter day precision was evaluated by estimating the corresponding responses three times on three different days (first, third, and fifth day) at different concentrations of Impiramine hydrochloride and Diazepam. Intraday precision of the developed RP-HPLC method were determined by analyzing sample solutions of Impiramine

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

hydrochloride (2, 6, 12 µg/ml) and Diazepam (10, 100, 200 µg/ml) at three levels covering low, medium and high concentrations of the calibration curve three times on the same day (n = 3). Interday precision was determined by analyzing sample solutions of Impiramine hydrochloride (2, 6, 12 µg/ml) and Diazepam (10, 100, 200 µg/ml) at three levels covering low, medium and high concentrations over a period of 3 days (n = 3). The peak areas of Impiramine hydrochloride and Diazepam obtained were used to calculate mean and RSD values.

- **Reproducibility**

The 4 µg/ml of Impiramine hydrochloride and 100 µg/ml of Diazepam solution were used for reproducibility studies. The areas were measured at different laboratory using same instrument by another analyst and the values obtained were evaluated using F-test to verify their reproducibility.

- **Accuracy**

Accuracy of stabilityindicating assay method for Impiramine hydrochloride and Diazepam was performed by recovery studies. Most widely used synthetic mixture of tablets excipients (i. e. lactose, starch, magnesium stearate and talc) were prepared (placebo) in the ratio of their permitted concentration in formulation of tablets. The accuracy of the method was studied by analysis of standard at three different levels, i.e. multiple level recovery studies (50%, 100% and 150%). Known amount of Impiramine hydrochloride (50%, 100% and 150%). and Diazepam (50%, 100% and 150%)were added to a pre quantified sample solution of Impiramine hydrochloride and Diazepam, and the amount of Impiramine hydrochloride and Diazepam were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

- **LOD and LOQ**

The sensitivity of the method was determined in terms to limit of detection (LOD) and limit of quantification (LOQ). The detection limit is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by using the following equations as per International Conference on Harmonization (ICH) guidelines which is based on the calibration curve.

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

Where, σ = Standard deviation of y-intercepts of regression lines

S = Slope of calibration curve.

- **Specificity and Selectivity**

Specificityis the ability of the developed method to assess unequivocally the analyte in the presence components which may be expected to be present for e.g. impurities, degradants or matrix components etc. it is measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due only to a single component; that is, that no co-elution exist. The specificity of the method was ascertained by analyzing Impiramine hydrochloride and Diazepam in the presence of excipients (Lactose, micro-crystalline cellulose, Aerosil, Magnesium stearate) by preparing synthetic mixture. The results obtained of PIO, MET and GLI were confirmed by comparing with results of standards.

II. RESULT AND DISCUSSION

The optimzation of RP-HPLC method defined the simulataneous influence of some important conditions, such as the mobile phase compostion, pH of the mobile phase and temprture ,on thr seperation and detrmiination. The RP-HPLC method was done forthe determination of Imipramine HCL and diazepam in combintional dosage form.

Table:-5 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

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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

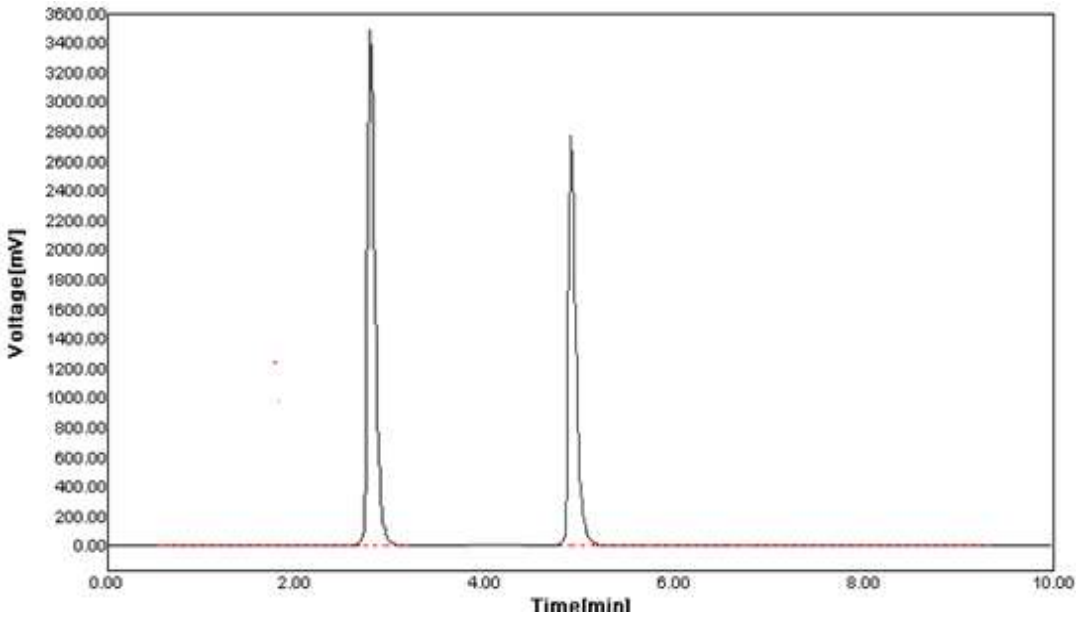


Figure:-2 Standard HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml) in mix standard

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

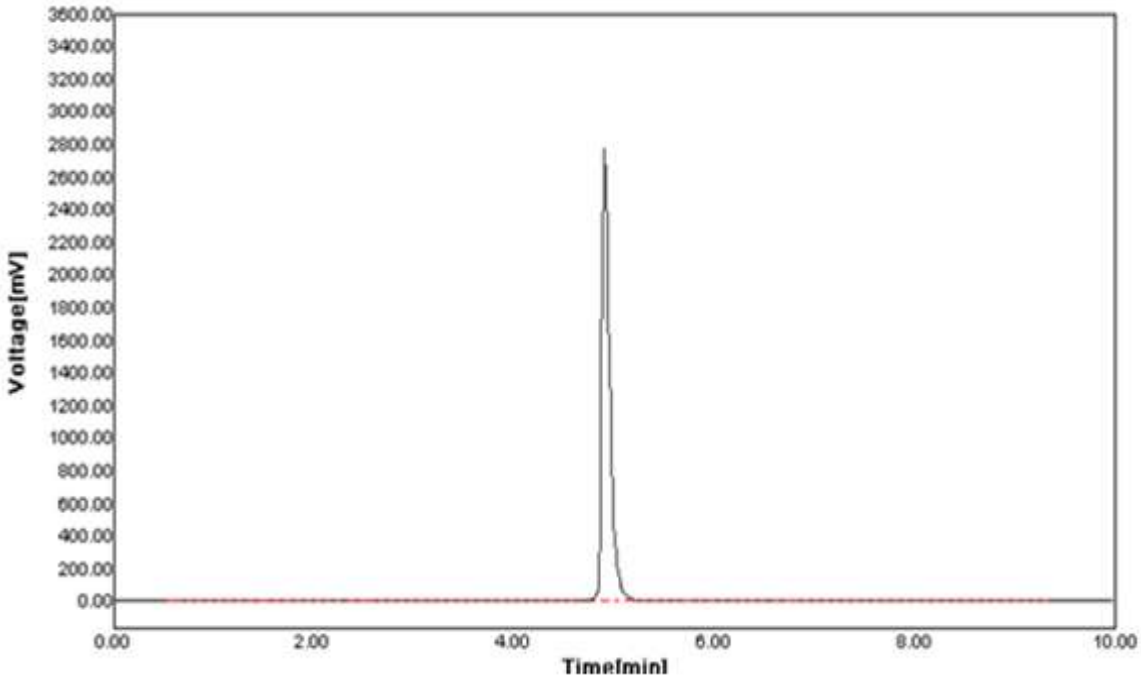


Figure:-3 Standard HPLC Chromatogram of standard solution of IMI (10 µg/ml)

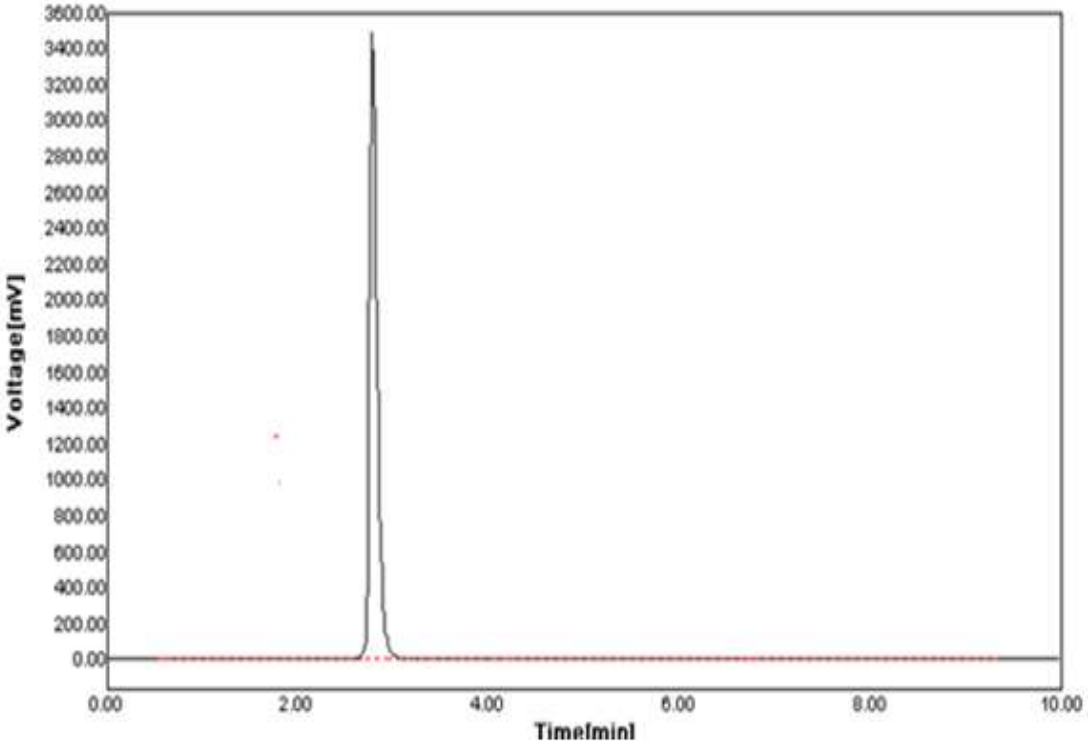


Figure:-4 Standard HPLC Chromatogram of standard solution of DIA (100 µg/ml)

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

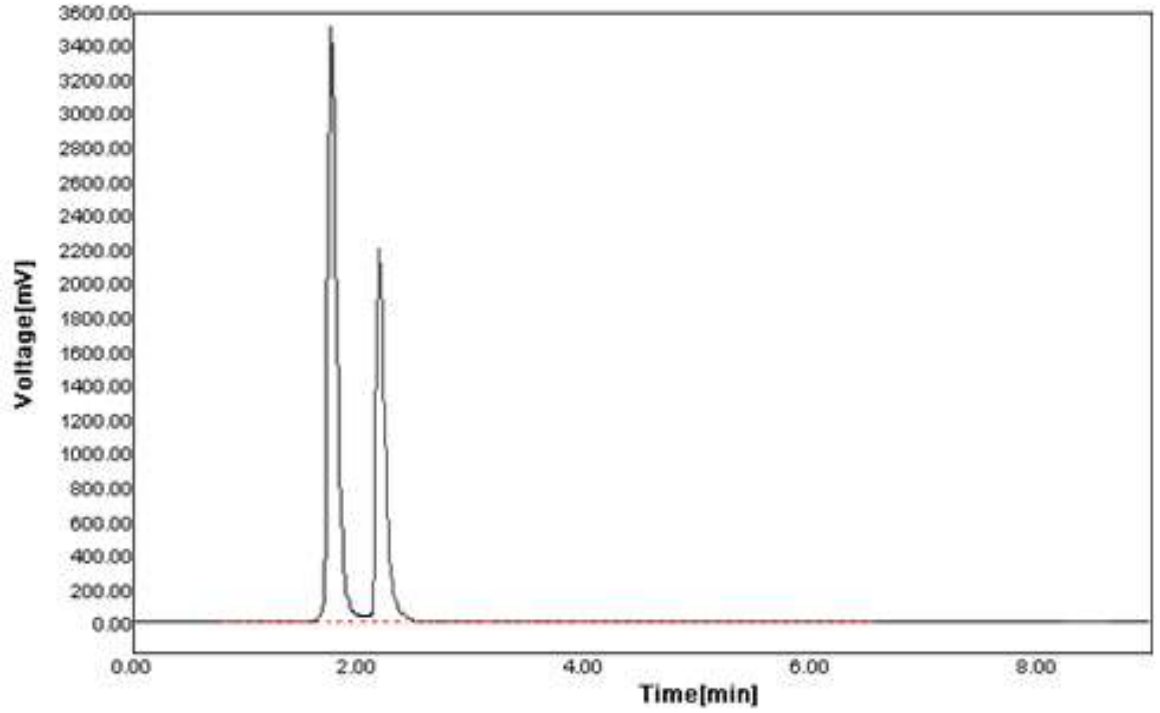


Figure:-5 HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml) obtained by using mobile phase - Methanol: Phosphate buffer (pH 6.6) (75: 25).

➢ **Selection of analytical wavelength:**

From the overlain UV spectrum of Imipramine Hydrochloride (IMI) and Diazepam (DIA) it was found that at 251 nm Imipramine Hydrochloride (IMI) and Diazepam (DIA) have considerable absorbance shown in fig. 6. Therefore 251 nm was selected as a common analytical wavelength for the analysis of both the drugs.

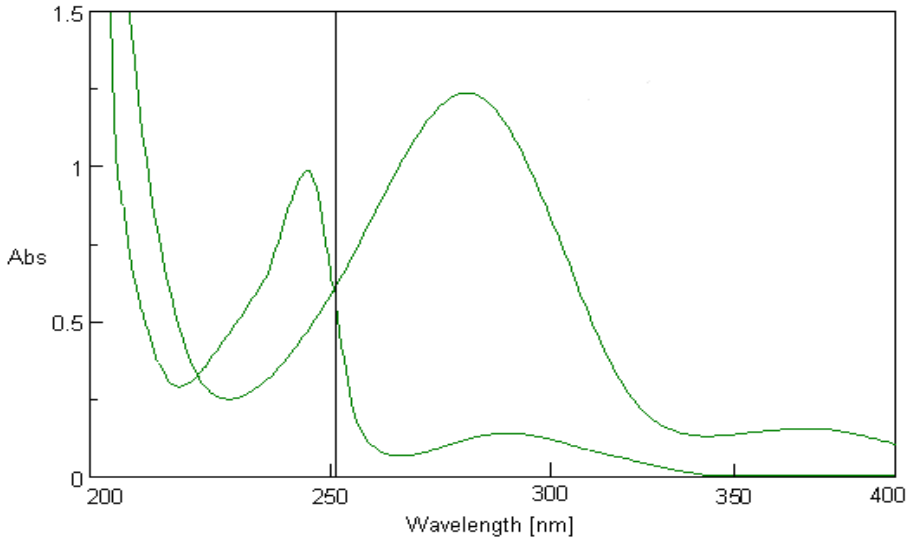


Figure:-6 Overlain UV spectrum of IMI and DIA in methanol in the range of 200nm-400nm

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

❖ **Method Validation**

When a method has been developed it is important to validate it to confirm that it is suitable for its intended purpose. The validation tells how good the methods are, specifically whether it is good enough for the intended application. The method Validation is today an essential concern in the activity of analytical chemistry laboratories. It is already well implemented in pharmaceutical industry. The US Food and Drug Administration (FDA) have edited draft guidelines with detailed recommendations for method validation of bio-analytical methods [Shah V.P., 2001] in the pharmaceutical industry. The International Conference on Harmonisation (ICH) has provided definitions of validation issues included in "analytical procedures" for the fields of bio-analytical methodology, pharmaceutical and biotechnological procedures [ICH, Q2A, Q2B, Q6B, 2002]. Likewise the US Pharmacopeia (USP) has published guidelines for method validation for analytical methods for pharmaceutical products [USP, 1995]. However the guidelines from ICH and USP are not as detailed as those from the FDA, and in the analytical biotechnology area there exists no detailed validation guidelines. The most common validation parameters will be briefly described below. The method was validated for linearity, precision, accuracy, limit of detection, limit of quantification, ruggedness.

➢ **Stress study**

To develop stability indicating RP-HPLC assay method, both the drugs were subjected to acid hydrolysis, alkali hydrolysis, neutral hydrolysis, chemical oxidation, and dry heat degradation and photo degradation conditions. HPLC was used to evaluate the percentage degradation of each drug under certain conditions. The stress studies were carried out by preparing Imipramine hydrochloride and Diazepam solution of 1 mg/mL in respective stressors, and were used for stress studies under optimized conditions as given in Table 6. Analyses revealed the degradation behaviour of the Imipramine hydrochloride and Diazepam, as summarized in Table 7.

Table:-6 Optimized Stress degradation studies conducted on Imipramine hydrochloride and Diazepam

Stress degradation condition	**Stressor**
Base Induced Degradation	0.5 N NaOH, reflux at 75°C for 2hrs
Acid induced Degradation	0.5 N HCl, reflux at 75 °C for 4 hrs
Neutral hydrolysis	Double distil Water, reflux at 75 °C for 4 hrs
Oxidative degradation	3 % Hydrogen Peroxide (H_2O_2)reflux at 75 °C for 4 hrs
Thermal degradation	Dry Heat: Drug powder kept in hot air oven at 75 °C for 4 hrs Wet Heat: Drug solution kept in boiling water bath for 4 hrs

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

Photolytic degradation	Drug powder was exposed to direct sunlight for 72 hrs Drug powder was exposed to UV light (254nm and 365nm) for 4 hrs

➢ **Base Induced Degradation**

Stress study or forced degradation of Imipramine hydrochloride and Diazepam in basic medium was performed in the solution of 0.5N NaOH heated at 75ºC for 2 hr. Base Induced Degradation were slightly to moderate degraded in 0.5N NaOH medium. The peaks of the degradation products were well resolved from the drug peaks. The recovery was found to be 94.85% of Imipramine hydrochloride and 89.85% of Diazepam. The chromatograms of base degraded sample showed degradation product peaks at retention time (RT) 6.11 min for DIA. For the alkaline hydrolysis, the formation of degradable product was confirmed by comparing to chromatogram of the solution kept under normal conditions

Tanble:-7Forced degradation studies data of Imipramine hydrochloride and Diazepam by Proposed RP-HPLC Method

Stress condition	**Time**	**Imipramine hydrochloride**		**Diazepam**	
		% Assay	**% Degrade**	**% Assay**	**% Degrade**
Alkaline hydrolysis (0.5N NaOH)	2 hr	94.85	5.15	89.85	11.15
Acidic hydrolysis (0.5N HCl)	4 hr	96.72	3.28	91.15	8.85
Neutral Hydrolysis	4 hr	98.72	2.08	98.15	1.85
Oxidative Degradation (3% H_2O_2)	4 hr	97.92	2.08	97.15	2.85
Dry heat (75 ℃)	4 hr	94.09	5.91	93.25	6.75
Wet Heat (Boiling Water bath)	4 hr	96.49	3.51	91.25	8.75
Sun light	72 hr	97.49	2.51	97.52	2.48
UV radiation (254nm)	4hr	98.92	1.08	96.67	3.33
UV radiation (365nm)	4hr	98.65	1.35	97.79	2.21

➢ **Acid induced degradation (Acid hydrolysis)**

Stress study or forced degradation of Imipramine hydrochloride and Diazepam in acid medium was performed in the solution of 0.5N HCl heated at 75ºC for 2 hr. Acid Induced Degradation were slightly to moderate degraded in 0.5N HCl medium. The peaks of the degradation products were well resolved from the drug peaks (fig 5.1.17). The recovery was found to be 94.85% of Imipramine hydrochloride and 89.85% of

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

Diazepam. For the alkaline hydrolysis, the formation of degradable product was confirmed by comparing to chromatogram of the solution kept under normal conditions. HPLC chromatogram of blank and sample from tablet are shown in figure 10 and 11 respectively.

- **Neutral hydrolysis**

Stress study or forced degradation of Imipramine hydrochloride and Diazepam in neutral medium was performed in the solution of in the double distill water heated at 75ºC for 2 hr for the neutral hydrolysis. Imipramine hydrochloride and Diazepam drugs were slightly neutral medium. The peaks of the degradation products were well resolved from the drug peaks.The recovery was found to be 98.72% of Imipramine hydrochloride and 98.64% of Diazepam. For the neutral hydrolysis, the formation of degradable product was confirmed by comparing to chromatogram of the solution kept under normal conditions.

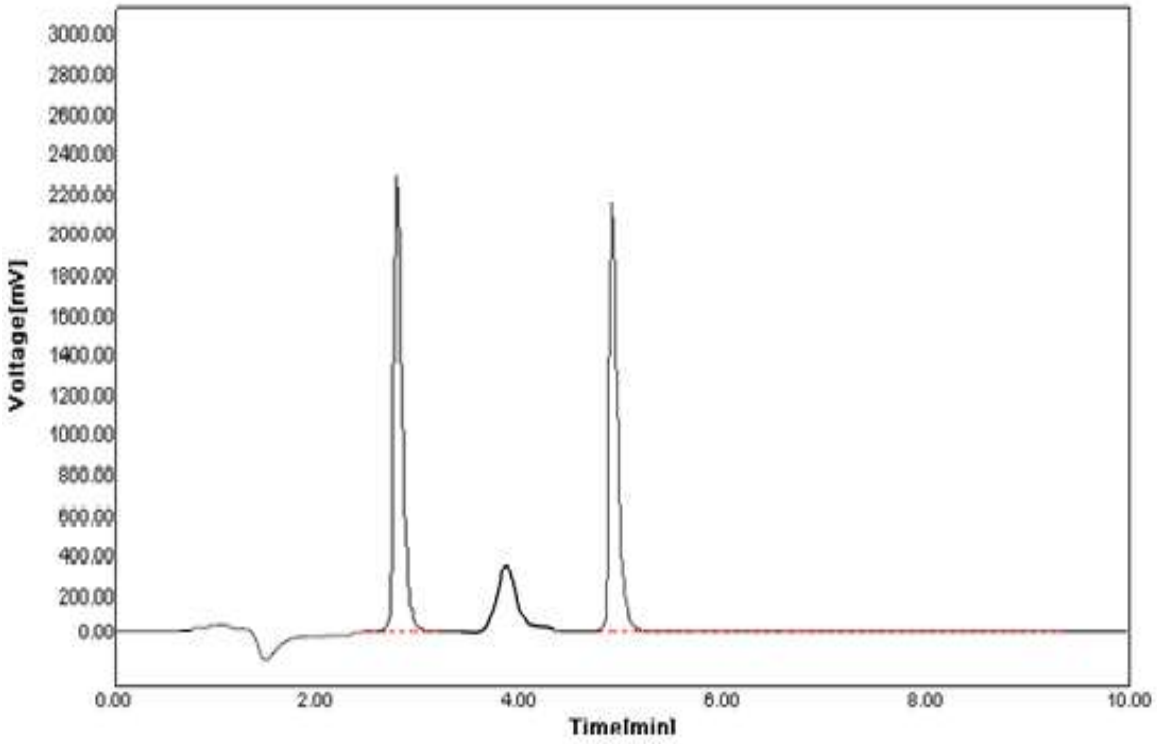


Figure:-7 RP-HPLC chromatogram of Imipramine hydrochloride and Diazepamstandard in acid medium at 75ºC for 2hrs.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

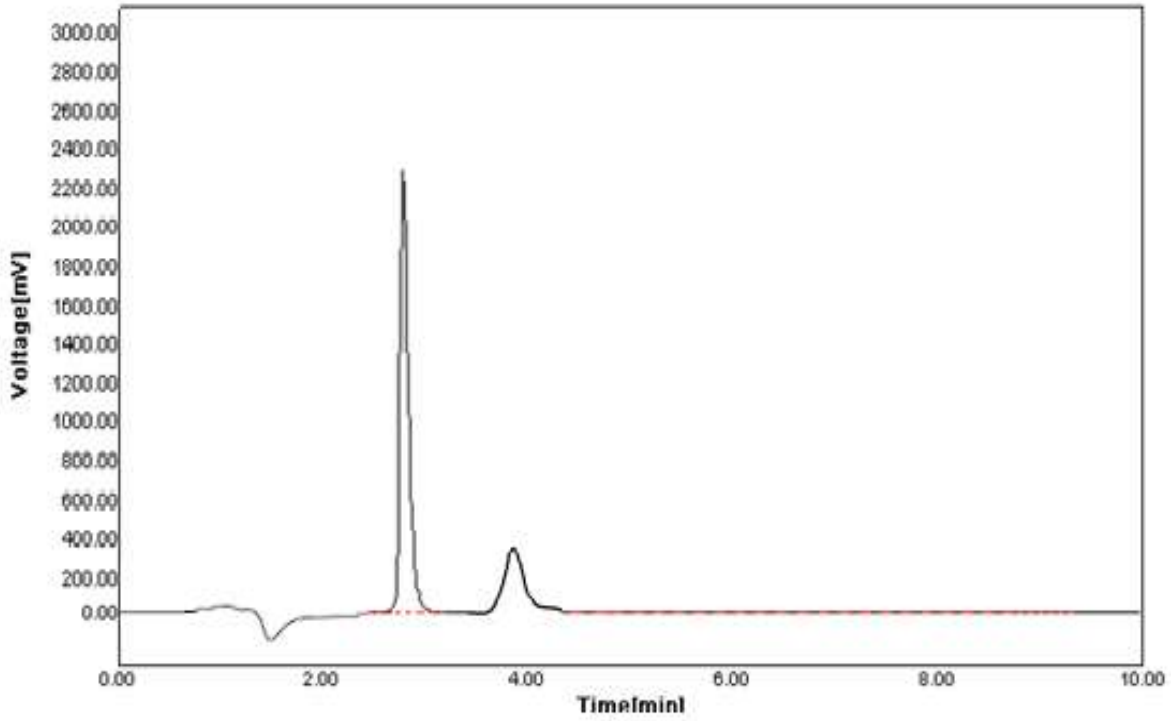


Figure:-8 RP-HPLC chromatogram of Imipramine hydrochloride standard in acid medium at 75°C for 2hrs.

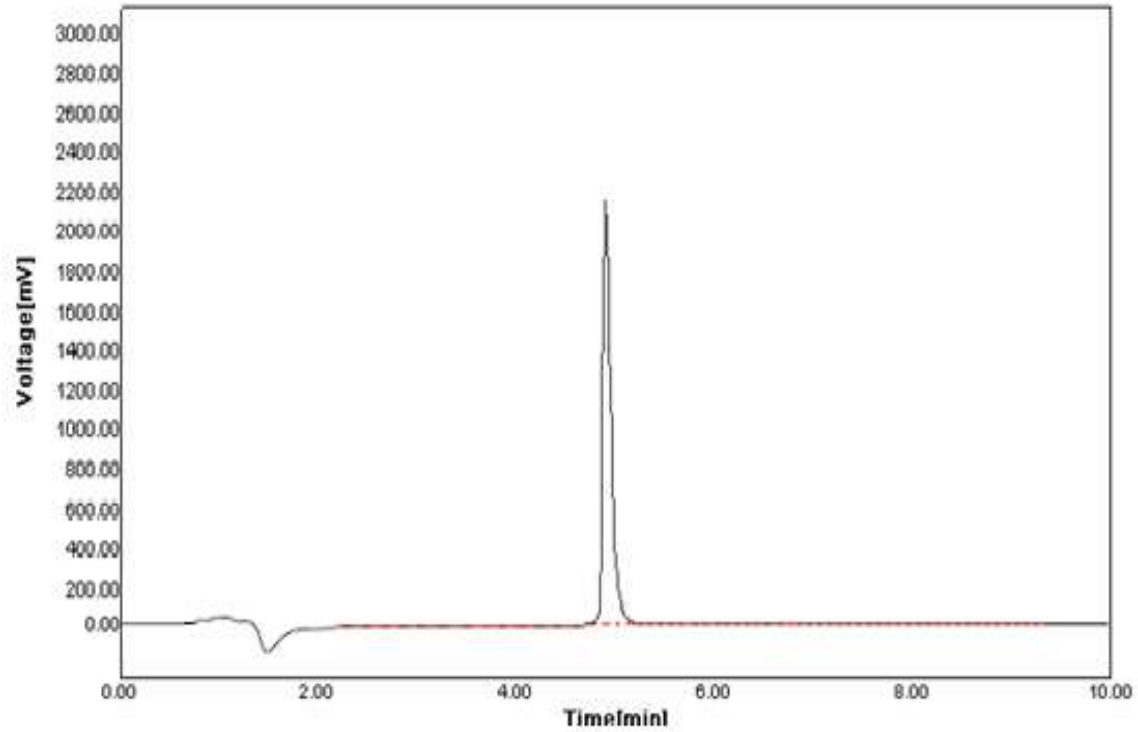


Figure:-9 RP-HPLC chromatogram of Diazepam standard in acid medium at 75°C for 2hrs.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

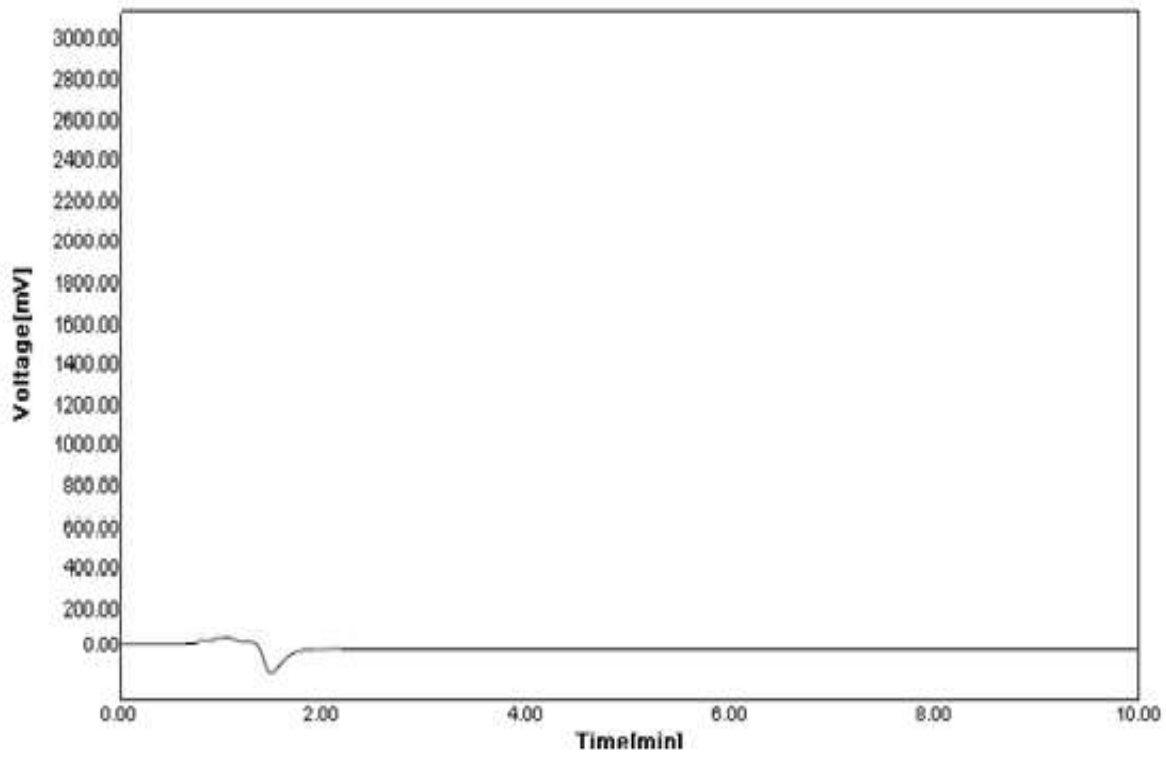


Figure:-10 RP-HPLC chromatogram of blank acid medium at 75ºC for 2hrs.

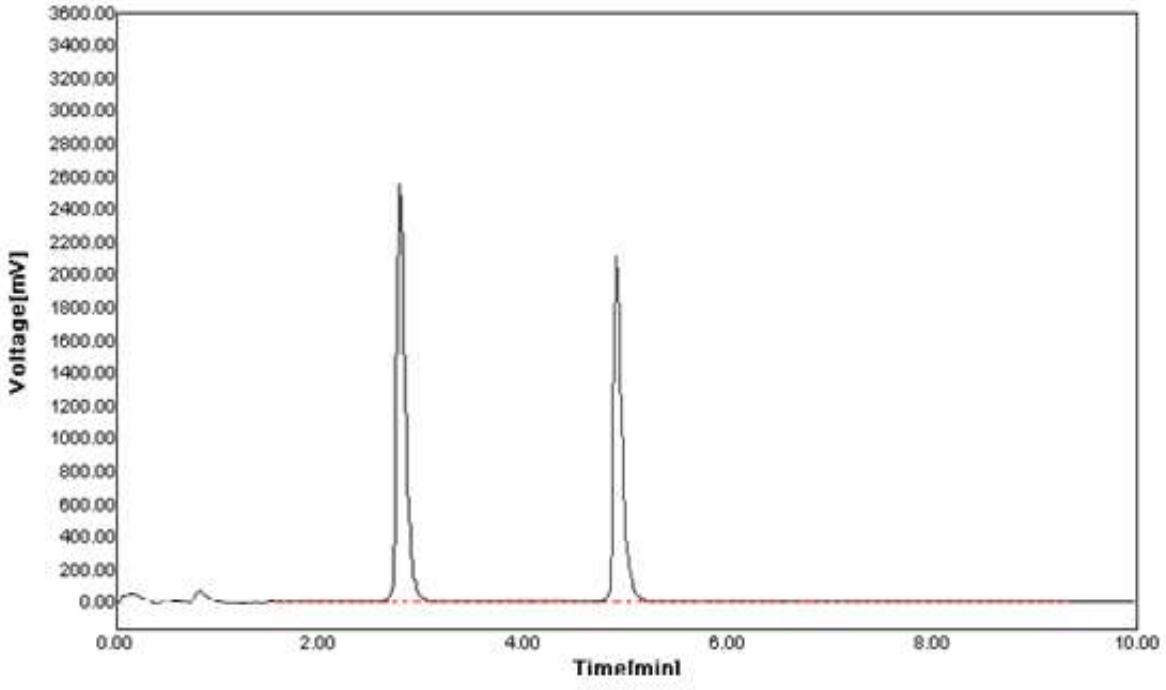


Figure:-11 RP-HPLC chromatogram of Imipramine hydrochloride and Diazepamstandard in neutral medium at 75ºC for 2hrs.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

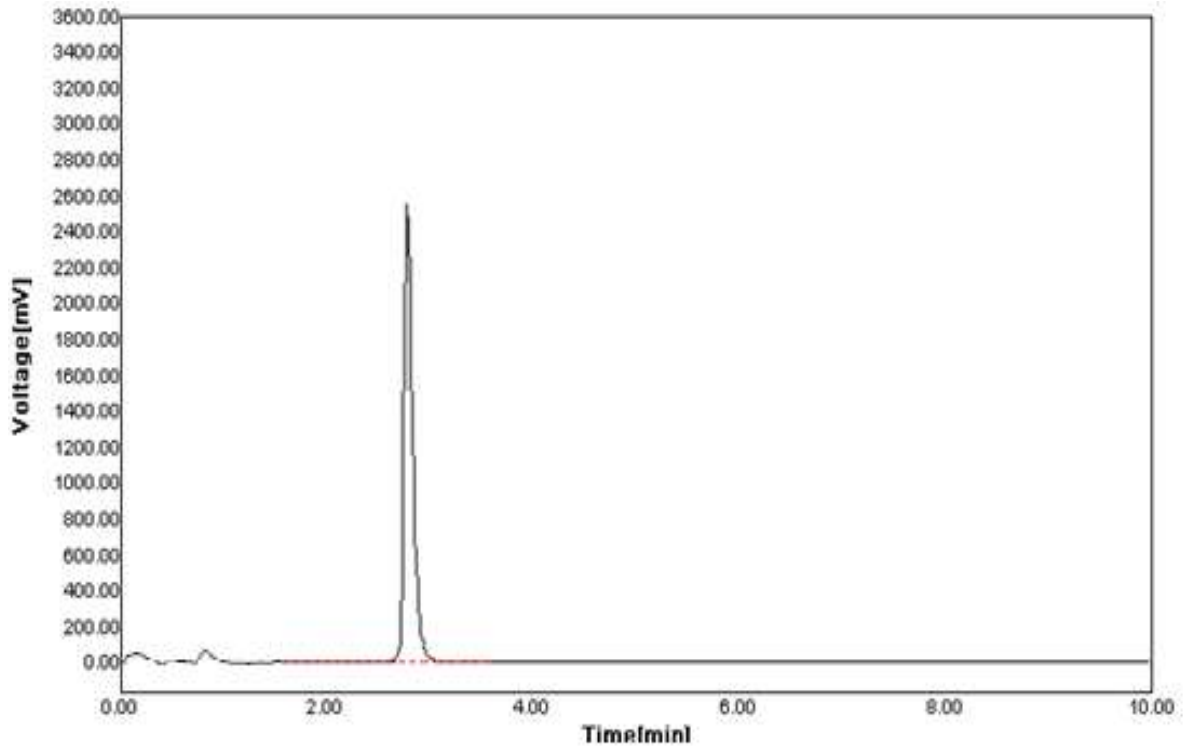


Figure:-12 RP-HPLC chromatogram of Imipramine hydrochloride standard in neutral medium at 75ºC for 2hrs.

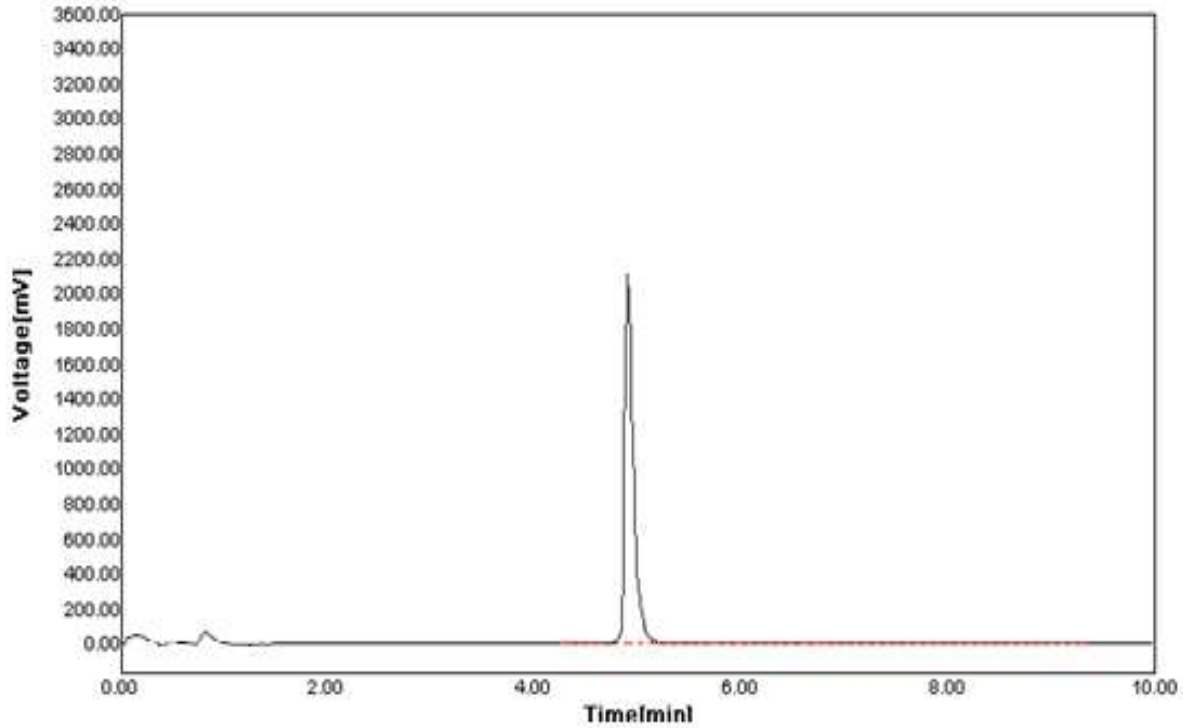


Figure:-13 RP-HPLC chromatogram of Diazepam standard in neutral medium at 75ºC for 2hrs.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

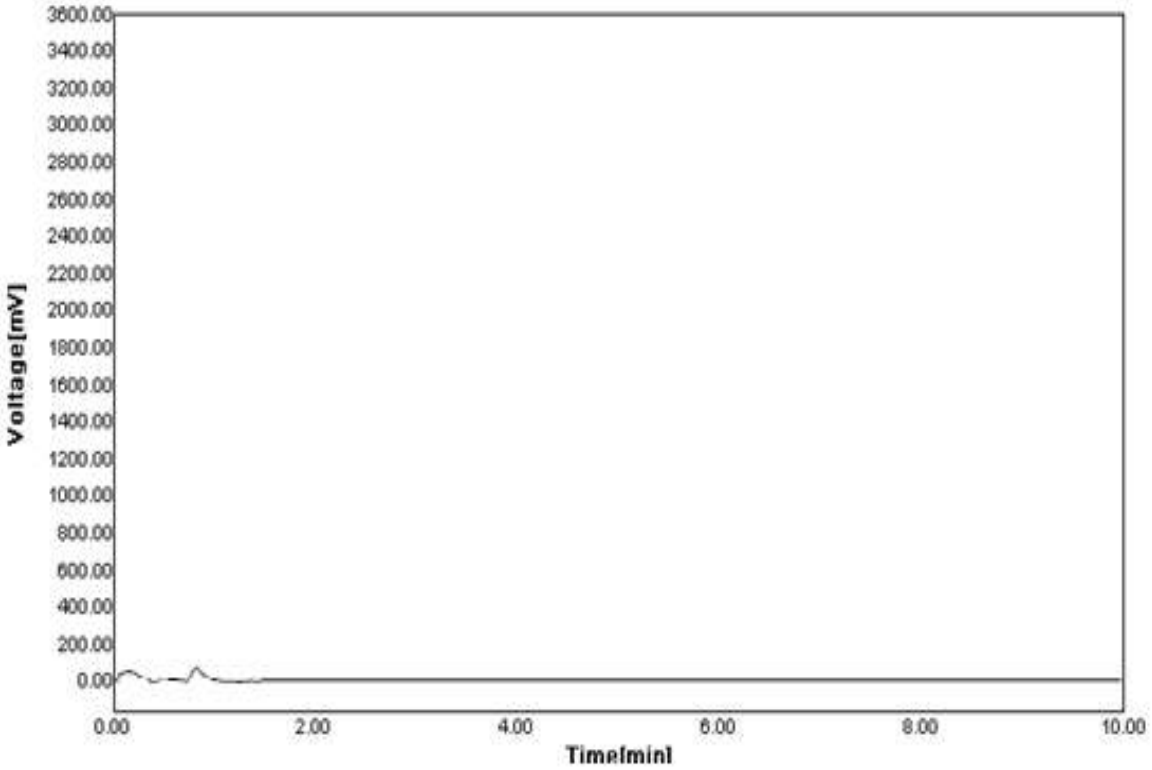


Figure:-14 RP-HPLC chromatogram of blank neutral medium at 75ºC for 2hrs.

➢ **Oxidative stress degradation**

Oxidative stress degradation of Imipramine hydrochloride and Diazepam was performed in the 3% H_2O_2 heated at 75ºC for 2 hr for the oxidative study. Imipramine hydrochloride and Diazepam drugs were slightly degraded H_2O_2 medium and not formation of any degradation peak only peak area were reduced. The Oxidative stress degradation of Imipramine hydrochloride and Diazepamchromatogram is shown in fig 12. The recovery was found to be 97.92% of Imipramine hydrochloride and 96.64% of Diazepam. HPLC chromatogram of blank and sample from tablet are shown in figure 13 and 14 respectively.

➢ **Thermal degradation**

i. Dry heat degradation

Dry heat degradation of Imipramine hydrochloride and Diazepam was studies in oven at 75^{o}C for 4 hrs. Dry heat degradation study showed that Imipramine hydrochloride and Diazepamis stable at 75^{o}C and no additional peak in HPLC chromatogram. The Dry heat degradation chromatogram of standard Imipramine hydrochloride and Diazepamis shown in figure 10. The recovery was found to be 94.09 % of Imipramine hydrochloride and 93.25% of Diazepam. The Dry heat degradation chromatogram of Imipramine hydrochloride and Diazepam extracted from tablet is shown in figure 11.

ii. Wet heat degradation

Wet heat degradation of Imipramine hydrochloride and Diazepam was studies at 75^{o}C for 4 hrs. Wet heat degradation study showed that Imipramine hydrochloride and Diazepam are stable at 70^{o}C and no additional peak in HPLC chromatogram. The Dry heat degradation chromatogram of Imipramine hydrochloride and Diazepamis shown in figure 11 The recovery was found to be 96.49 % of Imipramine hydrochloride and 95.25 % of Diazepam. The wet heat degradation chromatogram of Imipramine hydrochloride and Diazepam extracted from tablet is shown in figure 11

Both sample stored in the dark place to exclude the photo degradation effect on the Imipramine hydrochloride and Diazepam. In dry heat and wet heat condition Imipramine hydrochloride and Diazepamwas found to be stable.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

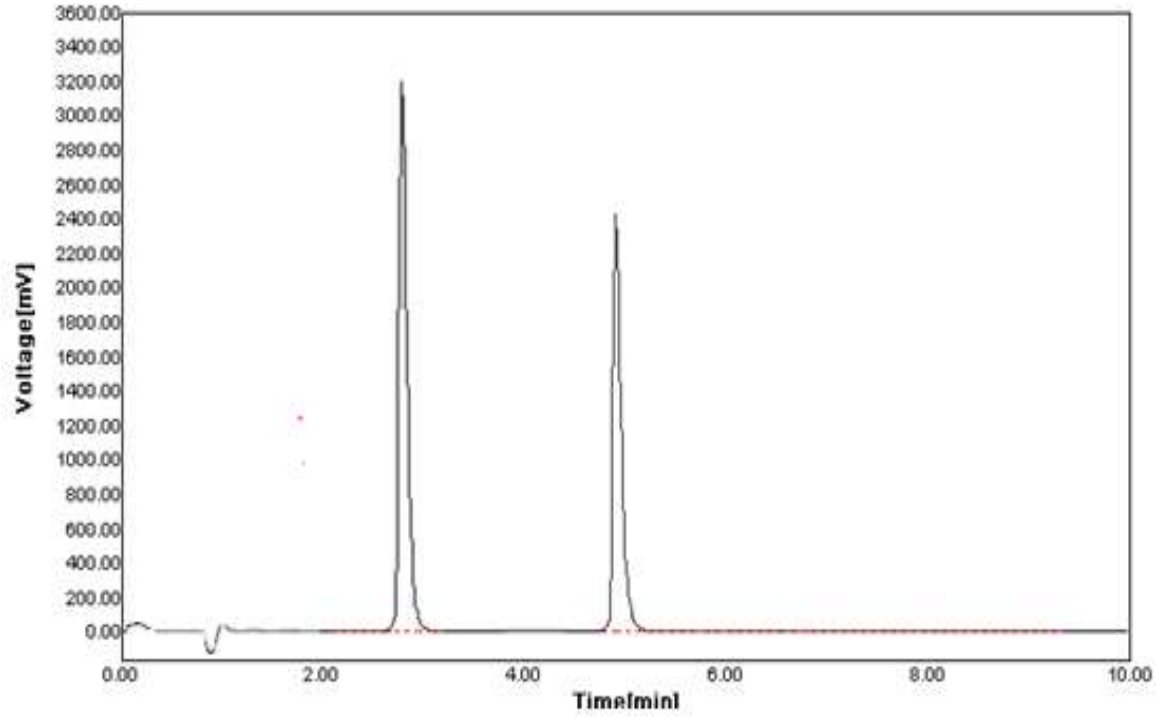


Figure:-15 RP-HPLC chromatogram of Imipramine hydrochloride and Diazepam in mix standard in oxidative medium (3% H_2O_2) at 75ºC for 2hrs.

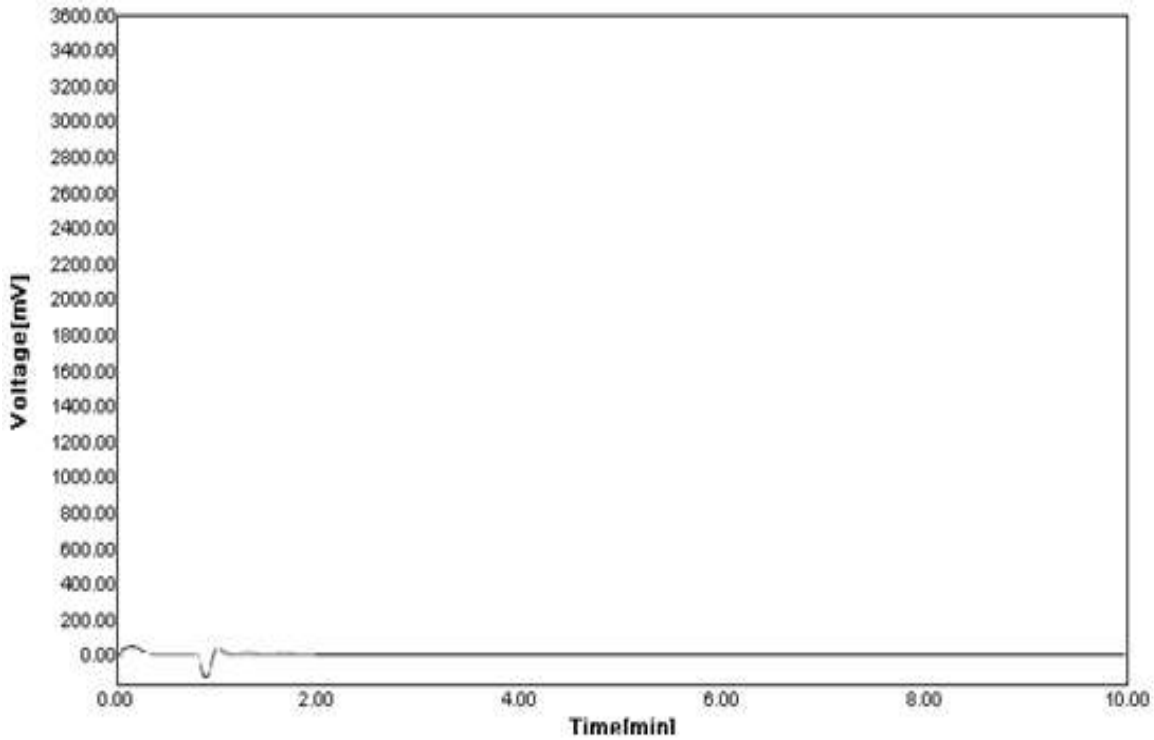


Figure:-16 RP-HPLC chromatogram of blank oxidative medium (3% H_2O_2) at 75ºC for 2hrs.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

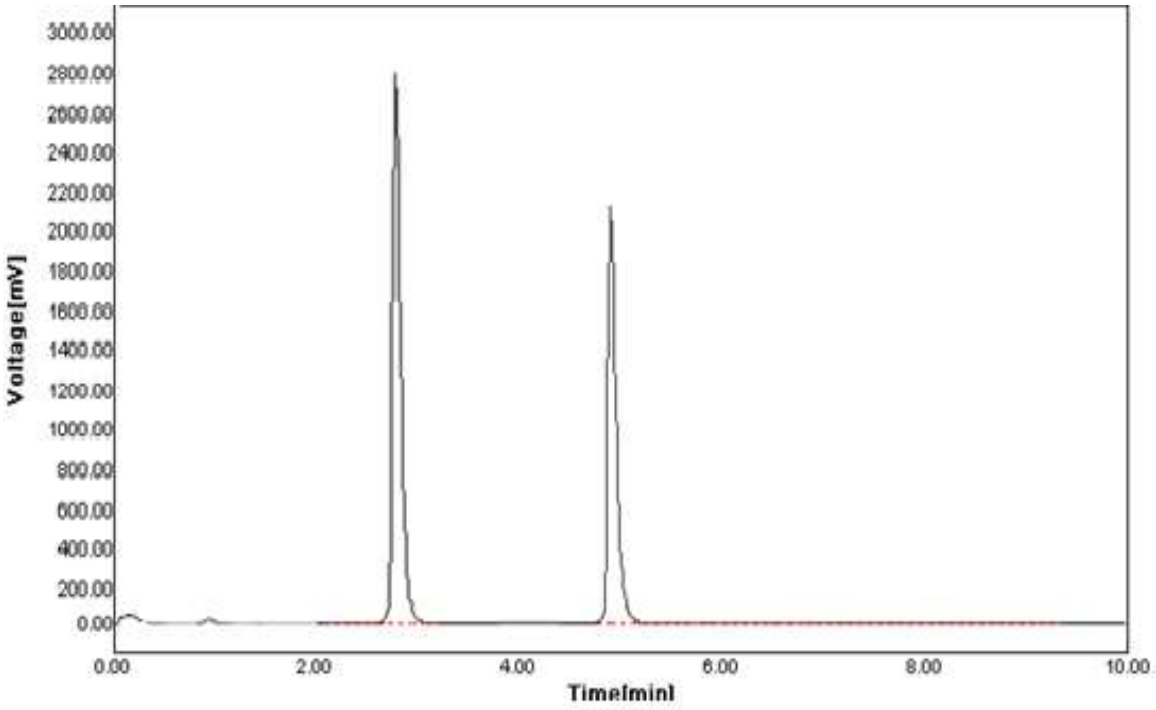


Figure:-17 RP-HPLC chromatogram of Imipramine hydrochloride and Diazepamin tablet formulation in dry heat at 75ºC for 2hrs.

- **Photo degradation study**

Photo degradation of Imipramine hydrochloride and Diazepam was studies in sunlight for 72 hrs to study the effect of light on the Imipramine hydrochloride and Diazepam.Photo degradation study showed that Imipramine hydrochloride and Diazepamwere stable in sunlight. The Photo degradation chromatogram of Imipramine hydrochloride and Diazepamis shown in figure 5.1.34. The recovery was found to be 97.49 % of Imipramine hydrochloride, and 97.52 % of Diazepam.

Furthermore, a stress degradation study in direct UV radiation was performed by exposing the solid drugs of Imipramine hydrochloride and diazepam and their mixture to UV radiation at 254 and 365 nm for 4 h at room temperature. The UV light degradation (254nm and 365nm) chromatogram of Imipramine hydrochloride and Diazepamis shown in figure 18 and figure 19. In photo degradation study and UV light degradation study of Imipramine hydrochloride and Diazepam no additional peak in HPLC chromatogram were found. The recovery was found to be 98.92 % of Imipramine hydrochloride and 96.67 % of diazepamfor UV light degradation at 254nm. The recovery was found to be 98.62 % of Imipramine hydrochloride, 97.79 % of diazepamfor UV light degradation at 356nm.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

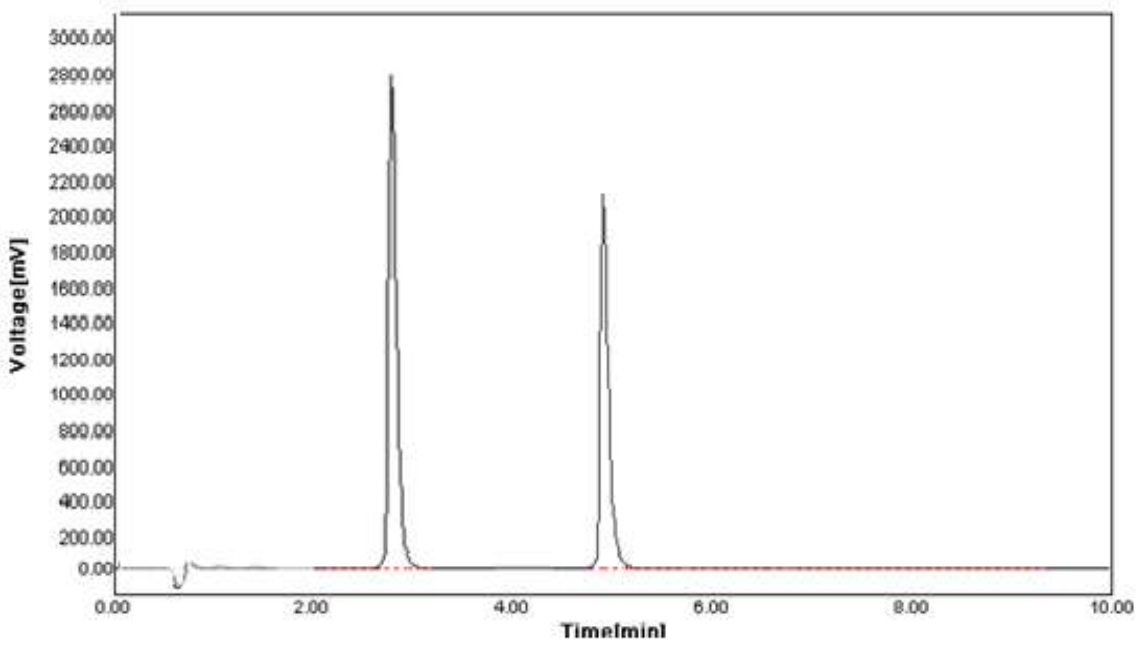


Figure:-18 RP-HPLC chromatogram of of Imipramine hydrochloride and Diazepamin mix standard in direct sun light for 72 hrs.

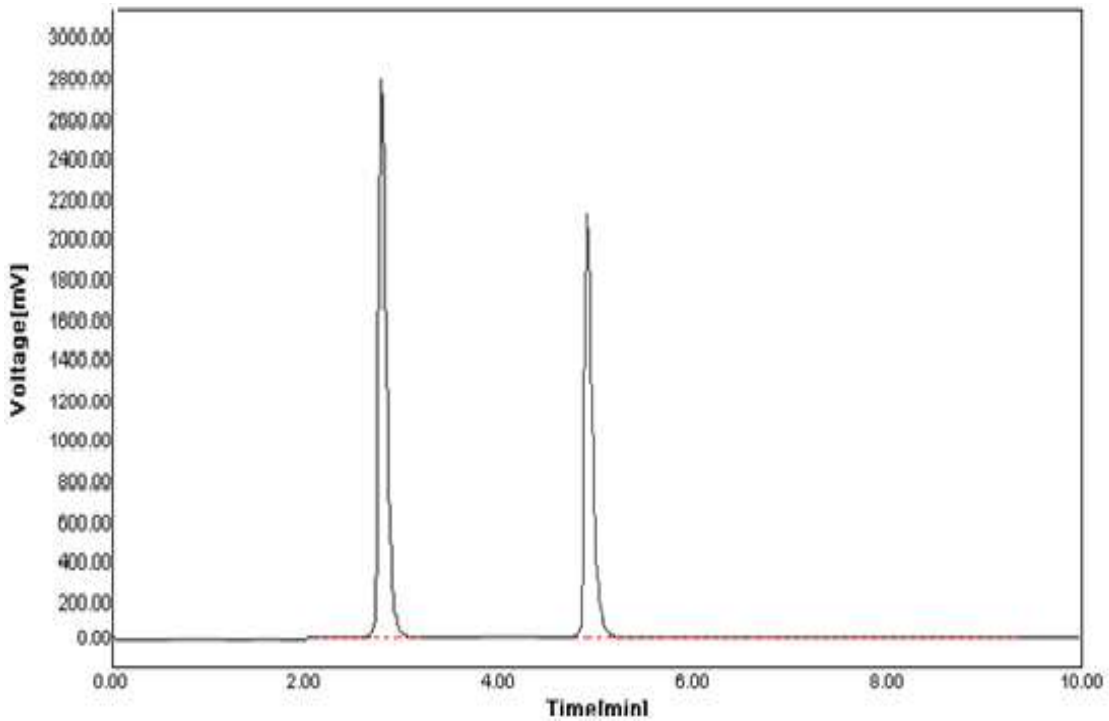


Figure:-19 RP-HPLC chromatogram of Imipramine hydrochloride and Diazepamin mix standard in UV-light (254nm) for 4 hrs.

- **System Suitability Tests**

The system-suitability tests are integral part of high performance liquid chromatography system. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The tests are

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

based on concept that the equipment, electronics, analytical operations, and sample to be analyzed constitute an integral system that can be evaluated as such. . To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 4µg/ml and 100µg/ml for Imipramine and Diazepam, respectively to check the reproducibility of the system and results obtained are summarized in Table 8 and Table 9. The standard mixture solution was used assay system suitability solution it was injected into HPLC. The retention time, tailing factor, resolution, and theoretical plates for each drug were observed. The percentage relative standard deviation (%RSD) of five consecutive injections for each parameter was calculated. The system suitability parameters of the present method were found to be within acceptable limits. The system suitability data are presented in Table 8 and Table 9 The acceptable limits of the resolution between two adjacent peaks should be ≥ 2 and tailing factor should be ≤ 2 and the %RSD of these values should be ≤ 2. System suitability tests confirmed that the chromatographic system was adequate for the analysis planned to be done.

Table:-8 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	3.1	23225.1	1.42	2.02	7381.4	2.15	2.01
2	2.23	23227.1	1.38	1.98	7387.35	2.18	2.02
3	2.85	23231.6	1.26	2	7378.73	2.12	2.03
4	2.83	23222.8	1.43	1.97	7390.03	2.15	2.01
5	2.87	23223.8	1.45	1.99	7369.98	2.14	2.02
Mean	2.776	23226.1	1.388	1.992	7381.49	2.148	2.018
S.D	0.324	3.0799	0.075	0.019	7.86	0.021	0.009
%RSD	0.23	0.1326	1.893	0.349	0.241	0.326	0.413

Table:-9 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	5.5	11689.2	2.06	2.02	3132.45	2.1	2.01
2	4.86	11687.8	1.91	1.98	3128.16	2.09	2.02
3	5.1	11685.2	1.89	2	3139.77	2.08	2.03
4	5.4	11690.1	1.97	1.97	3135.48	2.11	2.01
5	4.85	11692.3	1.89	1.99	3130.62	2.13	2.02
Mean	5.142	11688.9	1.944	1.992	3133.29	2.102	2.018

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

S.D	0.3	2.366	0.072	0.019	4.49	0.019	0.009
% RSD	0.65	0.2024	0.005	0.349	0.397	0.08	0.413

- **Linearity and Range**

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range. The linearity and range of the method was determined by plotting a calibration curve over the concentration range of 2, 4, 6, 8, 10, 12µg/ml of Imipramine hydrochloride and 10, 20, 50, 100, 150, 200µg/ml of Diazepam, respectively. The calibration curve was constructed by plotting peak areas versus concentrations of 2, 4, 6, 8, 10, 12µg/ml of Imipramine hydrochloride and 10, 20, 50, 100, 200, 400µg/ml of Diazepam respectively shown in figure 20 and figure 21

The regression equation was found to be y = 2903.x - 208.1 and correlation coefficient was found to be 0.998 for Imipramine . The regression equation was found to be y = 229.4x + 119.1 and correlation coefficient was found to be 0.998 for Diazepam. Each response was the average of three determinations. The Statistical analysis data of calibration curve intercept, slope, and regression equation are shown in Table 10

Table:-10 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

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

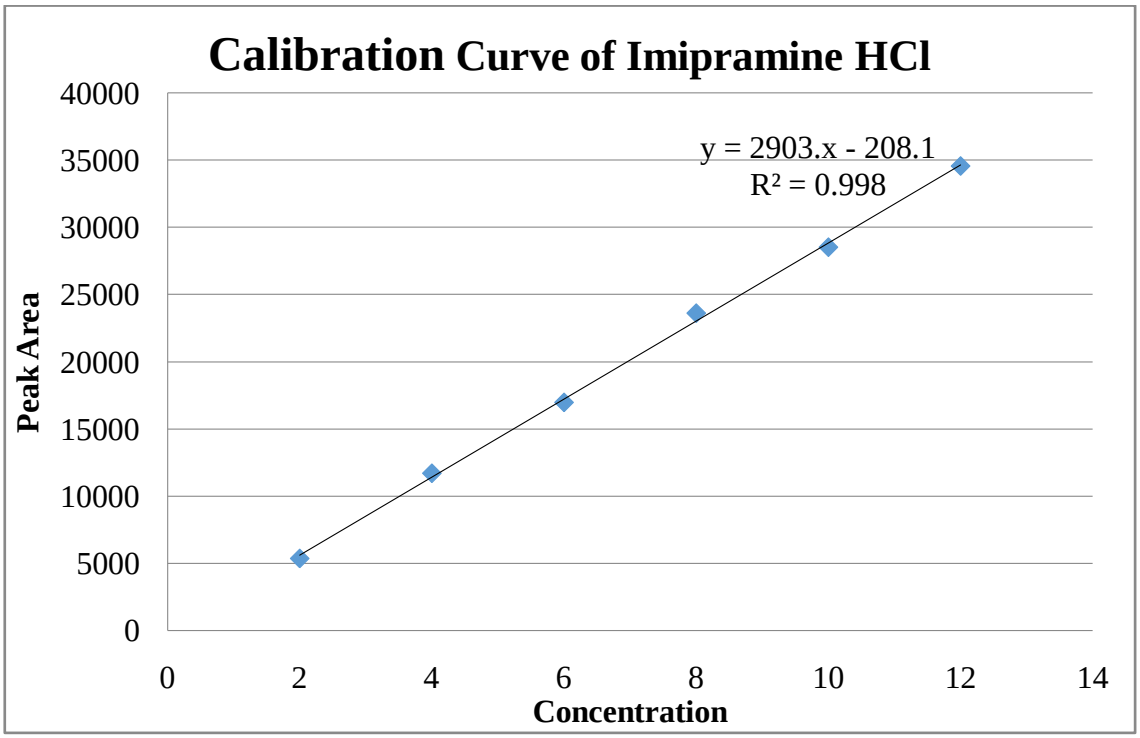


Figure:-20 Calibration curve of Imipramine HCl standard

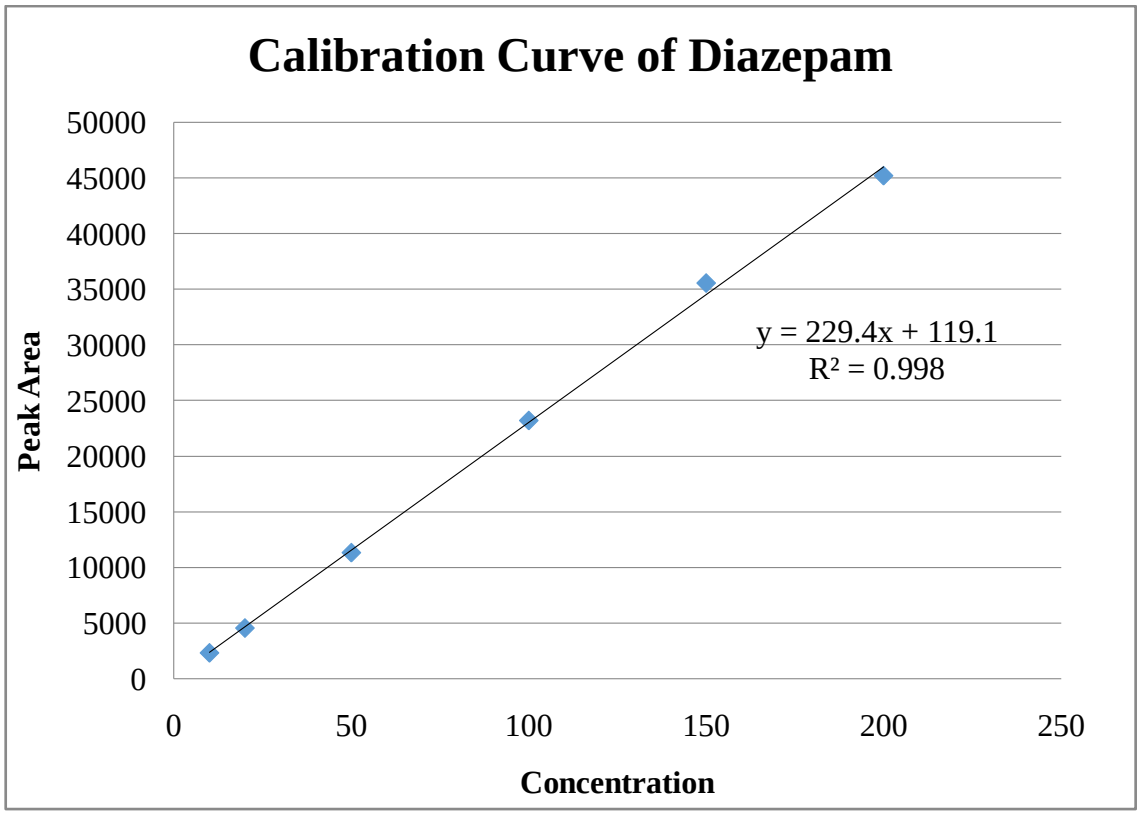


Figure:-21 Calibration curve of Diazepam standard

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

➢ **Precision**

The repeatability of developed method was determined by analyzing 4 µg/ml of Imipramine hydrochloride solution six times on the same day. The percentage RSD was found to be 0.43. The repeatability of developed method was determined by analyzing 5 100 µg/ml of Diazepam solution six times on the same day. The percentage RSD was found to be 1.07. The results of repeatability data are shown in Table 11

The results of the intermediate precision (Intraday precision and Interday precision) experiments are shown in Table 12 for Imipramine . Replicate analyses of three different concentrations 2, 6 and 12µg/ml of Imipramine solutions showed good reproducibility. The percentages RSD of intraday and interday studies were found to be 0.14–1.14% and 0.09–1.56%respectively for Imipramine .

The results of the intermediate precision (Intraday precision and Interday precision) experiments are shown in Table 13 for Diazepam. Replicate analyses of three different concentrations 10, 100 and 200µg/ml of Diazepam solutions showed good reproducibility. The percentages RSD of intraday and interday studies was found to be 0.14–0.99% and 0.23–0.97%respectively for Diazepam.

Table:-11 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

SD: Standard Deviation
RSD: Relative Standard Deviation

Table:-12 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		

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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:-13 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

Reproducibility of the developed method was determined by two different analysts under the same chromatographic condition and on same liquid chromatography instrument for the 4 µg/ml of Imipramine hydrochloride and 100 µg/ml of Diazepam concentration level respectively. The effect on the peak was evaluated by applying the F-test. There was no significant difference was found indicating that the developed method was reproducible. The reproducible results are shown in table 14 table 15 for Imipramine hydrochloride and Diazepam respectively.

Table:-14 Reproducibility data for Imipramine

Analyst 1 Conc. found ± S.D (n = 3)	**Analyst 2 Conc. found ± S.D (n = 3)**	**Result of F-test**	**Inference**

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

4.21 ± 0.12	4.12 ±0.15	0.91	No Significant Difference

*** At 95% confidence interval, (F-Tabulated = 9.28)**

Table:-15 Reproducibility data for Diazepam

Analyst 1 Conc. found ± S.D (n = 3)	Analyst 2 Conc. found ± S.D (n = 3)	Result of F-test	Inference
100.42 ± 0.25	99.73 ± 0.15	1.82	No Significant Difference

*** At 95% confidence interval, (F-Tabulated = 9.28)**

The developed method was found to be precise and repeatable on the basis of the mean CV values for the repeatability and intermediate precision studies which were < 2 for, Imipramine hydrochloride and Diazepam respectively. The separations of the drug and various degradation products in a mixture of stressed samples were found to be similar when the analyses were performed with an LC system on different days.

- **Accuracy**

The recovery of the method was carried out by the standard addition to the preanalysed test sample at three different concentration levels 50%, 100% and 150%. Triplicate determinations were made at each concentration level. The accuracy of the method was determined by calculating recoveries of 2, 4, 6 µg/ml of Imipramine and 25, 50 and 75 µg/ml of diazepam in the preanalysed concentration 4 µg/ml of Imipramine and 50µg/ml diazepam by method of standard addition. The recoveries of Imipramine and diazepamwere calculated by putting the peak area of the added concentration of Imipramine and diazepamin the regression equation of calibration curve respectively. The recoveries found to be 99.81 % - 101.89% for Imipramine and 99.68 %-101.43% for MET, respectively. The result of the method is indicating good accuracy for chromatographic method. The accuracy result shown in Table 16

Table:-16 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

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

➢ **Limit of detection and limit of quantitation**

According to ICH, the approach based on the standard deviation of the response and mean of slope was used for determining the Limit of detection (LOD) and limit of quantitation (LOQ). The detection limits for Imipramine and diazepamwere found to be 0.16µg/ml, 3.1µg/ml, respectively while quantitation limits were found to be 0.53 µg/ml 9.3µg/ml respectively. The above data shows that a microgram quantity of Imipramine and diazepamthe drugs can be accurately and precisely determined. The values of LOD and LOQ of Imipramine and diazepam respectively indicate the sensitivity of proposed method.

➢ **Specificity and Selectivity**

Selectivity of a method refers to the extent to which it can determine particular analytes under given conditions in mixtures or matrices, simple or complex, without interferences from other components. The specificity study was carried out to check the interference from the excipients used in the formulations by preparing synthetic mixture containing both the drugs and excipients. The peak purity index and HPLC chromatogram showed peaks of the drugs (Imipramine and diazepam) without any interfering peak and the estimation of both the drugs were found to be satisfactory. Test solution is prepared by mixing of Imipramine and diazepam with the tablet powder excipients. Specificity is proven by comparing the chromatogram of Diluent, standard solution and test preparation solution and by peak purity index to show that there was no any interference of excipients with the peak of Imipramine and diazepam, as shown in figure 22,23,24,25, and 26

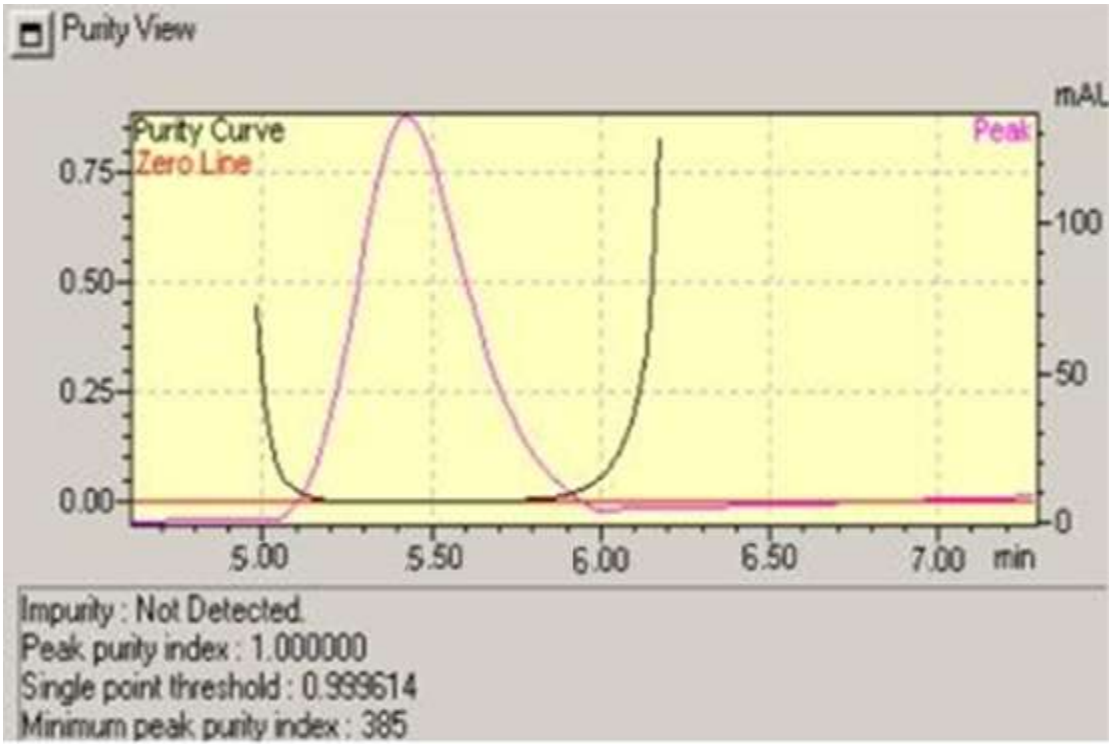


Figure:-22 Peak Purity of Imipramine

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

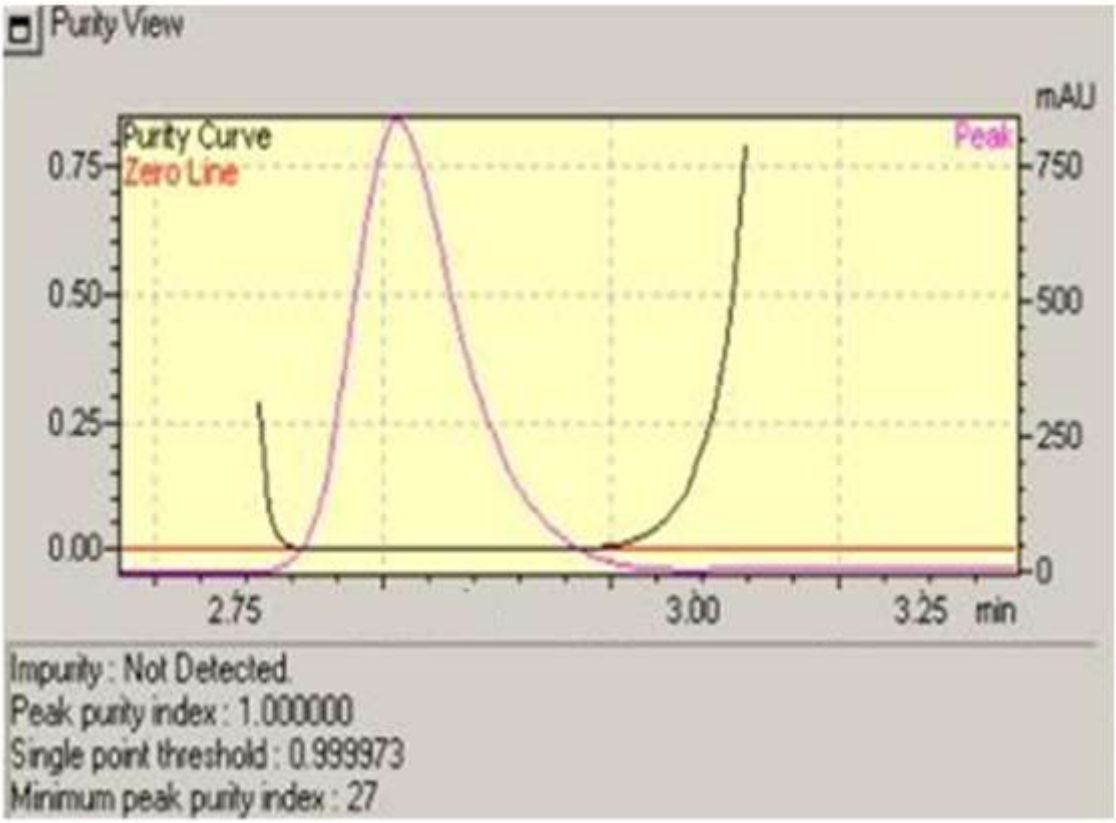


Figure:-23 Peak Purity of Diazepam

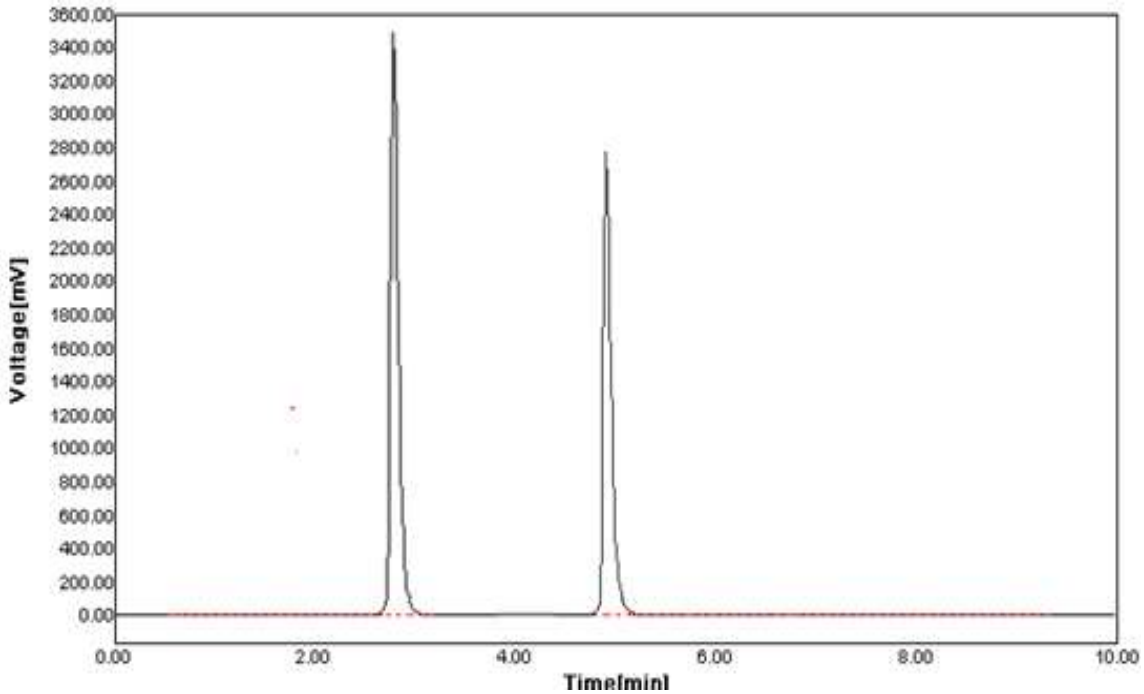


Figure:-24 Standard HPLC Chromatogram of standard solution of IMI (10 µg/ml), and DIA (100 µg/ml) in test preparation

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

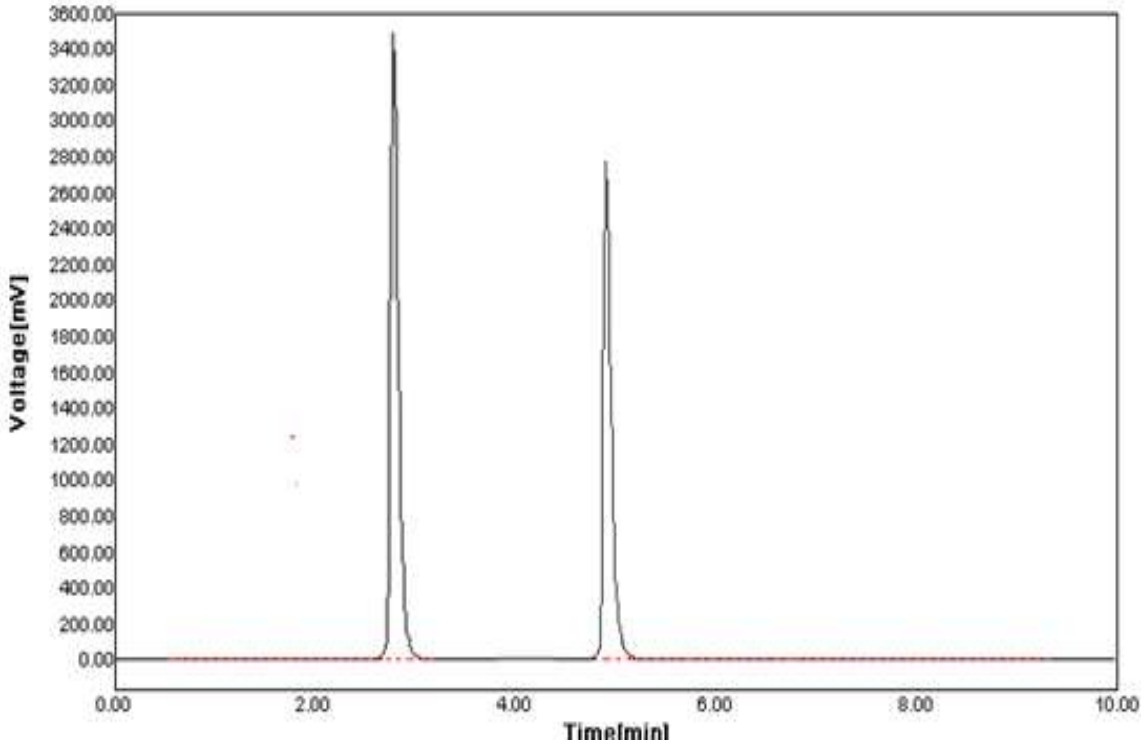


Figure:-25 Standard HPLC Chromatogram of IMI (10 µg/ml), and DIA (100 µg/ml) in tablet powder

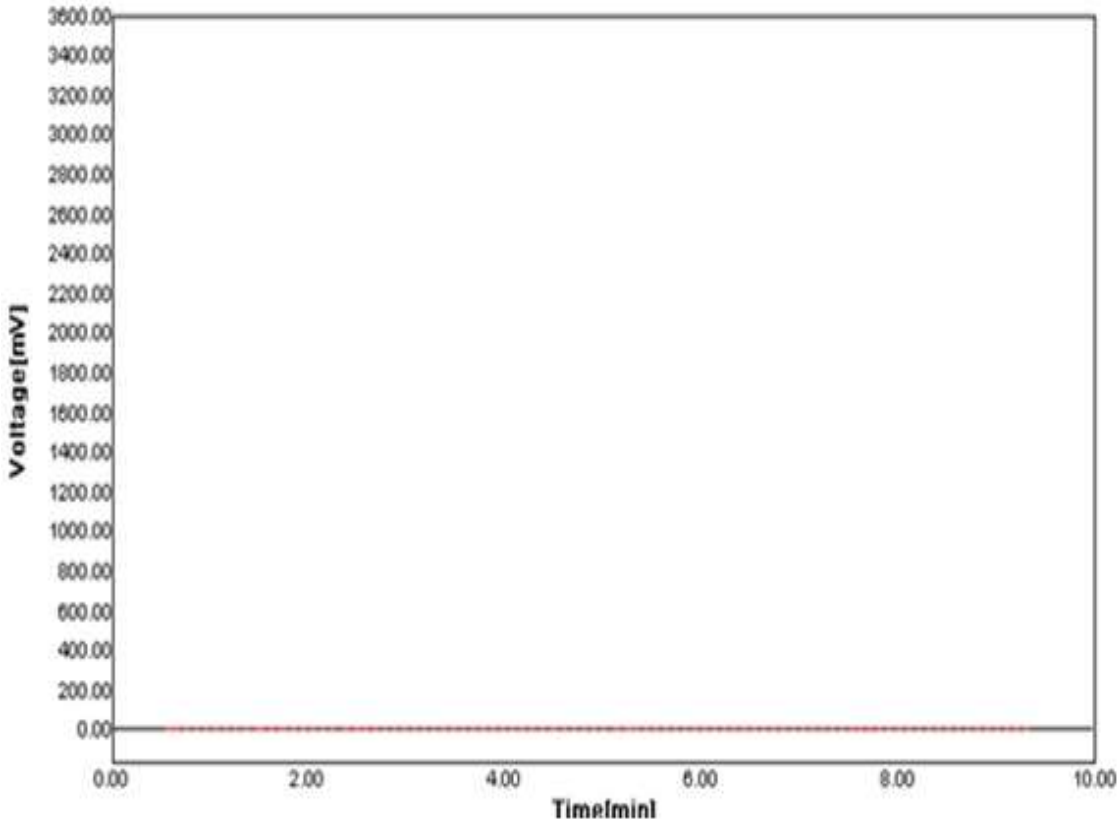


Figure:-26 HPLC Chromatogram of blank

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

➢ **Robustness**

Robustness is the measure of the capacity of a method to remain unaffected by small variations in the method parameters. Robustness of the method was determined in triplicate at a concentration level of 10 µg/ml of Imipramine hydrochloride and 50 µg/ml of Diazepam. After small changes in this parameter effect peak areas were determined and mean and RSD of peak areas calculated. Deliberate changes in the following parameters which affects % assay of 10 µg/ml of Imipramine hydrochloride and 50µg/ml of Diazepam and system suitability parameters were studied.

a) Change in % organic phase of mobile phase by ± 5.0 %
b) Change in pH of buffer of mobile phase by ± 0.5 of set pH
c) Change in the flow rate of the mobile phase by ± 10 % of the original flow rate.
d) Change in detection wavelength by ± 5.0 nm
e) Change in column temperature by ± 5.0 ºC

The method was found to be robust, as small but deliberate changes in method parameters have no detrimental effect on the method performance. The low value of percentage relative standard deviation indicates that the method is robust.

➢ **Solution stability**

The solutions at analytical concentration 10 µg/ml of Imipramine hydrochloride and 50 µg/ml of Diazepam were prepared and stored at room temperature for 48 h and analyzed at interval of 0, 4, 8 and 24 and 48 hr to check the stability of Imipramine hydrochloride and Diazepam when stored at ambient temperature in laboratory and the results were simultaneously compared with the freshly prepared Imipramine hydrochloride and Diazepam standard solution of the same concentration in the form of change in RSD of the response obtained. The percentage amount of both the drugs was found to be satisfactory and within the acceptable limit.

The acceptance criteria for various method validation parameters and their result are shown in table:-17 and compare with the obtained result. The summary of method validation parameters and their result obtained after method validation are shown in Table:-17 indicating that the developed is validated as per ICH guidelines and result are within the ICH guidelines values.

Table:-17 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

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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:-18 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:-19 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%

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 558-592 www.ijprajournal.com ISSN: 2249-7781

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

III. SUMMARY, CONCLUSION, MAJOR FINDINGS, RECOMMENDATIONS, FUTURE SCOPE AND LIMITATIONS OF RESEARCH WORK

SUMMARY

- Simple, sensitive and stability indicating chromatographic methods such as, RP-HPLC were developed for estimation of Imipramine hydrochloride (IMI) and diazepam (DIA) in their combined pharmaceutical dosage form.
- Stability Indicating RP-HPLC method was developed for the estimation of Imipramine hydrochloride (IMI) and diazepam (DIA). In RP-HPLC method, correlation coefficient was found to be 0.998 for Imipramine hydrochloride (IMI) and 0.998 for diazepam (DIA). The recovery was in the range of 99.81% -101.89% for Imipramine hydrochloride (IMI) and 99.68 %-101.43% for diazepam (DIA), respectively. The method was found to be accurate, precise, specific, selective, repeatable, robust and reproducible with different instruments, conditions and analysts. The limit of detection for Imipramine hydrochloride (IMI) and diazepam (DIA) were found to be 0.16µg/ml and 3.1µg/ml respectively, while limit of quantitation were found to be 0.53 µg/ml and 9.3 µg/ml respectively.
- The developed stability indicating RP-HPLC method was validated for linearity, accuracy, method precision, selectivity, sensitivity and robustness. It was found to be simple, sensitive, accurate, precise and robust
- The stability indicating nature of the proposed HPLC method was assessed by acid hydrolysis, alkali hydrolysis, oxidative, thermal and photolytic stress degradation studies.
- All the developed HPLC method can be used for routine analysis of Imipramine hydrochloride (IMI) and diazepam (DIA) in bulk and their pharmaceutical formulations.

CONCLUSION:

Simple, sensitive and stability indicating two different chromatographic methods such as, RP-HPLC was developed for estimation of Imipramine hydrochloride (IMI) and diazepam (DIA) in their combined pharmaceutical dosage form.

Simple, sensitive and Stability indicating RP-HPLC method was developed using C_{18} column as a stationary phase and Methanol and Water (Phosphate buffer) (75:25) v/v, pH 6.6 adjusted with Potassium Hydroxide as mobile phase. The flow rate was maintained at 1 ml/ min and detection was carried out at 251 nm where Imipramine hydrochloride (IMI) and diazepam (DIA) have significant absorbance. The retention times of Imipramine hydrochloride (IMI) and diazepam (DIA)were 2.85 min and 5.25 min. Forced degradation studies were carried out and degradation product peaks were well resolved from drug peaks. The method was validated and found to be sensitive, accurate and precise and stability indicating.

REFERENCE:-

[1]. Abhishek P & Vivek singh B (2011), Formulation-development & optimizations of pioglitazone HCl microspheres using ionotropic gelation techniques, Pharmacia, 1(2); 67-72.

[2]. Durkheimer W, Adam F., Fischer G, & Kirrstetter, in B. Testa (1998), Ed., Advances in drug research, academic Press, 17,61-234.
[3]. Okamouto Y, Kiryama K, Naimki Y, Matushita J, Fujiuka M & Yakuda T. (1996), Degradation kinetics & isomerization of cefdinir, a new oral cephalosporin, in aqueous solution, J Pharm Sci. Sep;85(9):976-83.
[4]. Okemoto Y. Itoeh K, Naumki Y, Masushita J, Fujeoka M. Yasuoda T. (1996), Methodudevelopment for the detrmination of cefedinir & its relateddsubstances by high-pressure liquiddchromatography, J. Pharma. Biomed. Anal. 1996 Apr;14 (6): 739 – 48 .
[5]. Kees F, Wellenhofer M, Grobecker H, (1996), Bioavailability of cefpodoxime proxetil with co-administered acetylcysteine. Arzneimittelforschang. 46(4), 435-438.
[6]. Caimus F, Desl&es A, Harcoueit L, Fariniotii R, (1994), High Performancerliquid chrometographic methodufor the detrmination of cefpdoxime levels in plasma & sinusmucosa. J. Chrometogr B Biorned. Application, 656 (2), 383 – 388 .
[7]. N. H. Vaidea, N. B. Dobaira & V. B. Patal, (2009), Convantional & Advanceid Spectrofotometric Methods for estemation of Cefitamet Pivoxel HCl in Bulk & in PharmaceuticalqFormulation, InternationaluJournal of Chem-Tech Reserch,Vol[m], Num. 02, pp 286 – 290 , AprilоJune 2009
[8]. Madhusudana Reddy T, Shreedhar M & Jayarama Reddy S, (2003), Voltametric behaviour of cefexime & cefpdoxime proxitel & detrmination in pharmaceuticalyformulations & urine. J. Pharm. Biomed.sAnal., 31(4), 811-818.
[9]. Silber Michael B, &rew J. Falkowski, Zee M.Look, Hideyo Noguchi.Detrmination of cefexime in biologicallsamples by Reverse-Phase High Performancegliquid Chrometography .Journaluof chrometograph.1987; 422: 145 - 152.
[10]. Castillo M, V.Girona, C.Pacareu, A.Riera, R.Pouplana, J.Bolos. Spectrophotometric determination of the stability of an ampicillin-dicloxacillin suspension. Jaurnal ofqPharmaceutical & Biomeidical Analyses. 1988; 6 (1): 23-28.
[11]. Erc-Jovenovic S. D.Agabba, D. Zivenov-Stakic, S.Valadimirov. High Pressure T.L.C. determination of ceftriazone, cefexime & cefotaxeme in dosageoforms.Journalgof Pharma. & BiomedicalsAnalysis. 1998;18: 893-898
[12]. Gehad G Mohamed, F.A.Nour EL-Dien, Eman U Farag. Spectrophotometric study of the mechanism between DDQ as π-acceptor & potassium iodate & flucloxacillin & dicloxacillin drugs & their determination in pure & in dosage forms. ScienceDirect Spectrochimica Acta Part A. 2006; 65: 11-19.
[13]. N&a RK, Gaikvad J, Prakash A. Simultenious spectrofotometric estemation of cefexime & ornidezole in tabletudosage form. Inter. Jaurnal of PharmaTech Reaserch. 2009; 1(3): 488-491.
[14]. Shahnaz Gauhar, Hafiz Muhammad Arshad, Raheela Bano, Iyad Naeem Muhammad. Devlopment of HPLC-UV Method for Analysis of cefexime in Raw Metarials & indCapsules. JordangJournal of Pharmaceut. Scinces. 2009; 2 (1): 53-65.
[15]. Madhura V. Dhoka. V&ana T. Gaw&e, Pranav P. Joshi. Simultenous estemation of Cefexime 3 H_2O & Erdostein in Pharma. dosage form by using RP-H.P.L.C. Interntional Jaurnal of Chem Tech Reseac. 2010 ; 2 (1): 79-87.
[16]. M.K Kumudavalli, S&eep Sahu, K. Abiteja, Dr.B. Jayakar. Developmnt & velidation of RP-H.P.L.C Method for Simultenious Detrmination of cefexime & potasium Clevulanate in tabletwdosage form. Internat. Journ. of Pharmaceutical Recent Res.. 2010; 2(2): 57-60.
[17]. A. R.Wankhde, Prasant Y. Mali, Bikram Karni, Anuba R. Khaile, C.S. Magidum. Developed & validated RP-HPLC method for simulteinous quantification of cefexime & cloxacilin in tabletgdosage form. Intern. Journa. of Pharmaceiutical & Biological Archives. 2010; 1(2): 317-320.
[18]. Yost RL, Derendorf H., (1985), fast chrometographic determinetion of cefotaxime & its metebolite in biologicaltfluids, J.Chromat., 341(1), 131-138.
[19]. Ling SS, Yuen KH., (2003), liquid chrometographic method for the detrmination of cefatoxime in human &

ratdplasma. J. Chromat. B. Analyt. Technol. Biomed. Life Sci., 783(1), 297-301.

[20]. Martinez LG, Campins- Falco P, (1998), spectrofotometric methods for dtermining cefotaxim based on derivatization with 1,.2-naphtoquinone- 4- sulfonate into solid phasedextration cartridges. Application to pharmaceuticl & urine samples. J. Chromat. B. Biomed. Sci. Appi/. 718(1), 143-151.

[21]. Nuevas L, Gonzaiez R, (1998), Derivative spectrophotomatric quantification of the triethylamonium salt of cefataxime in presence of relatedfcompounds from the syntheses. J. Pharm. & Biomed. Anal., 18(4-5), 579-583.

[22]. Scanes I, Hundt Af, Swart KJ, Jundt HK, (2001), simueltaneous determination of cefitaxime & desacitylcefotaxime in human plasma & cerebrospinalhfluid by HPLC. J.Chromat. B Biomed Sci. Appl., 750(1), 171-176.

[23]. DellD, Chambirlain J. Coppen F.J, (1981), Quantification of ceftaxime & desacitylcefotaxime in plasma & urine by high-Pressure liquid chrometography, J Chromatogr., Dec 11;226(2):431-40.

[24]. Dokladlova J, Queircia G T, Stankwich J.P, (1983), High-pressure liquid chrometographic quantification of cefperazone in humanuserum & urine, JwChromatogr., Aug 12;276(1):129-37.

[25]. Li FS, Xu ZX, Xiao HB, Liang XM, (2000), Simueltaneous quantification of sulbactam sodium & cefperazone sod. in sulperazion by high performance liquid chrometography, Se Pu., Nov;18(6):525-6.

[26]. Cazes J & Scott PW.,(2002) Chromatography Theory, 3rd edition., Marcel Dekker Incorporate New York.,4-5.

[27]. Skoog DA, Leqary JJ., (1992), Principle of Instrumental Analysis, 54th edition., Thomson asia Private Limited., 580.

[28]. Cazes J, Raymond PW., (2002), Chromatography Theory, Marcel Dekker Incorporate New York., 443-454.

[29]. Scott PW., (2001) Encyclopedia of Chromatography, 10th edition, Marcel Dekker Incorporate New York., 252-254.

[30]. Giddings JC., (1965) Dynamices of Chrometography, Part-1: Principles & theory (Chrometographic Science Series V. 1)., Marcele Dekkar Incorporate New York., 61-65.

[31]. Remington., (1990) Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Pensylvania., 529-554.

[32]. Scott PW., (1992) Liquid Chromatography Column Theory, John Willey & Sons, Chi Chaester., 1-13.

[33]. Conors KA., (1982) Liquid Chrometography- A Textbook of Pharma. Analysis, 3rd edition., Willeyrnterscience., 373-438.

[34]. Burlingame AL, Boyd RK., (1998) Analytical Chemistry, 70th edition., 647.

[35]. Bhatti M M., Hansone G D., Schultze L.,(1998), Instantaneous determenation of phenytoine, cefoperazone, & 10, 11-cefoperazone epoxide in hominoid plasmauby high-pressure liquid chrometography with ultra-violet identification, Jourinal of Pharmaceutical & Biomedical Analysise.,**16,(7)**, 1233-1240.

[36]. Mowafy HA., Alanazi FK., Maghraby Gamal M.EI., (2012), Developmant & validation of an High pressure liquid chrometography-Ultra violate process for the quantifecation of cefoperazone in rabbit plasmia, Saudi Pharmaceutical Journal., **20,** 29-34.

[37]. DEMİRKAYA F., KAFIOĞLU° Y.,(2005), Determinetion of Cefoperazone Consuming RP-High pressure liquid chromatography Method in Pharmaceutical Groundwork, Fabad Journal of pharmeceutical science., **30,**78-82.

[38]. Ambrusio AF., Silvia P. Sd., Carvlho C M., Carvalhu A P.,(2002), Mechenisms of action of Carbemazepine & Its Derivetives, Oxcarbazepine, BIA 2-093, & BIA 2-024.,Neurochemical Research., **27(1/2),**121-130.

[39]. Mathieu O., Picot M C., Gelisse P., Braton H., Demole P., Hilleire-Bues D.,(2011), Effects of cefoperazone & metabolites on I L-02, I L-5, I L-10 & IFN-Ɣ secration in epileptic patiants: the influance of co-medication. Phamacological reports.,**63,** 86-94.

[40]. Patil S R., Kumar L., Kohli G., Bansal A K., (2012),Valiadated H.P.L.C assay for Con-current Quantification of Aminopyrine, Carbamezapine, Furossemide & Phenytoine & its utility in testing of Ingradients Permebility through Caco-2 CellwMonolayers, Scientia Pharmaceutieca.,**80,** 89-100.

[41]. Barakat N S., Omare S A., Ahmede A A E., (2006) Carbamazepin uptake into rat brain following intra-olfactory transport. Journal of Pharmacy & Pharmacologi.,**58,** 63-72.

[42]. Patel R B., Patil M R., Bhatt K K., Patel B G.,(2011), method validation & development of HP thin layer chromatography Process for Estimate of Cefoperazone in Preparations & Its In Vitro Release Study. Chromatography Research International. **2011(Article ID 684369),** 1-8.

[43]. Rao Srinivasa K., Belorkar N.,(2010), developed method & validation of a explicit ability representing liqiud chrometographic method for cefoperazone in bulks & pharm. dosage form, Journal of Advanced Pharma. Research.,**1,**36-47.

[44]. Manoj Babu MK., (2004), Instantaneous seperation & quantitetion of 4 antiepileptic drugs- a study with possible for use in patient drug level observing. Journal of Pharmaceutical & Biomedical Analysise.,**34(2),** 315-324.

[45]. Chen K, Khayeam - Bashii H., (1991), Proportional analysis of antiepileptic drugs by gas chromatographi using capillery or filled columns & by fluorescence divergence immunoassay, Journal of Analytical Toxicology,**15(2),** 82-85.

[46]. Miller RB, Vr&erick M.,(1993), A Validatedwhigh pressure liquid chrometography process for the resolve of cefoperazone & cefoperazone 10, 11-epoxide in human plasma, Journal of Liqiud chrometography, **16(6),** 1249-1261.

[47]. Mennickent S, Mario V, Gloria C.,(2003), Development & validation of a process using influential planar chromatography for measureable analysis of cefoperazone in saliva, Journal of the Chilean Chemical Society, **48(3),** 1-6.

[48]. Owen A, Tetty J N, Morgan P, Pirmohamad M, Park B K.,(2001), liquid chromatography resolve of cefoperazone in murin brain, Journal of Pharmaceutical & Biomedical Analysis, **26(4),** 573-577.

[49]. ULU Tatar S.,(2006), Determinetion of cefoperazone in pharmaceuticale preperations using high pressure liquid chromatography & derivative spectrophotomatry, Turkish journal pharmaceutical science, **3(3),** 123-139

[50]. Goyal N, Bhandari A, Jain S and Patel R, (2011), Method Development and Validation of Etoricoxib and THC in combined pharmaceutical solid dosage form by RP-HPLC method, Int. J. Pharm. Studies Res., 2(1), 106-109.

[51]. Kumar S, Natraj D, Khan A, Kalyan Kumar B and Rao V J, (2011), Development and validation of RP-HPLC method for simultaneous estimation of Etoricoxib and THC in Pharmaceutical dosage forms, Int. J. Res. Pharm. & Chem, 1(3), 649-653.