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Optimization of Stability Indicating RP-HPLC method for The Estimation of an Antidepressant Agents Alprazolam and Imipramine in Pure & Pharmaceutical Dosage Form

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A Simple, Specific and Precise stability indicating Reverse Phase high performance liquid Chromatography method has been developed and validated for the estimation of Alprazolam and Imipramine in tablet dosage form using ODS-BP Hyperchrome C_{18} column (250 mm × 4.6 mm id, 5 µm particle size) as a stationary phase, Water (pH -6): Methanol:Triethylamine (70:30:0.1 % v/v/v) as mobile phase, flow rate of 1.0 mL/min and detection was carried out at 216 nm. The retention time of Alprazolam was 3.181 minute and Imipramine was 5.045 minute. RP-HPLC method was developed with linearity range of 0.5 – 1.5 µg/mLAlprazolam and 50 – 150 µg/mL Imipramine. The co-relation coefficient was found to be 0.9999 for Alprazolam and 0.9998 for Imipramine. The assay results obtained in good agreement with the corresponding labeled amount by developed method within range of 99.58% – 101.45% and 98.84% – 99.14% for Alprazolam and Imipramine respectively. Accuracy, Precision, LOD, LOQ, Specificity, Robustness were met all the acceptance criteria for the validation of analytical method as per ICH Q2 (R1) guideline. This method can be conveniently used to detect the possible degradation product in the combined dosage form of Alprazolam and Imipramine during stability studies (acidic, alkaline, oxidative, photolytic and thermal). The method proved to be affective on application to a stressed marketed tablet formulation.

Keywords: analytical method development, validation, simultaneous estimation, alprazolam, imipramine, stability indicating

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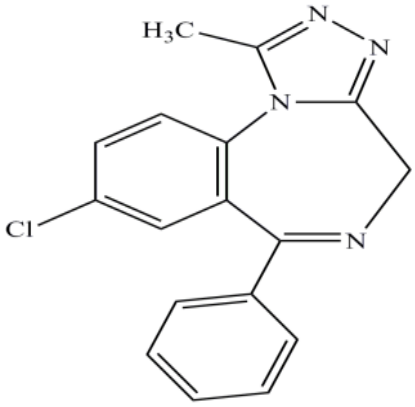
INTRODUCTION

Alprazolam is an anxiolytic drug. It is belonging to class of a benzodiazepine. It is chemically 8-Chloro- 1-methyl-6-phenyl-4H - [1,2,4]-triazol [4,3-a] [1,4] benzo-diazepine. It is used in the treatment of anxiety disorder, or the short-term relief of symptoms of anxiety and for the treatment of panic disorder, with or without agoraphobia and panic disorder. It is binding with inhibitory neurotransmitter GABA to the site opens the chloride channel, resulting in a hyperpolarized cell membrane that prevents further excitation of the cell. Some spectrophotometric and HPLC methods are reported in literature for estimation of Alprazolam alone and in combination with other drugs. Imipramine is a tricyclic antidepressant. It is chemically 10, 14, Dihydro-5H-Dibenz (b,f) azepine -5-9 dimethylaminopropyl. It is used in the treatment of depression and nocturnal enuresis in children. TCAs are potent inhibitors of serotonin and nor epinephrine reuptake. TCAs also block histamine H_1 receptors, α_1-adrenergic receptors and muscarinic receptors, which accounts for their sedative, hypotensive and anticholinergic effects [1,2]. In the literature, there are methods described for the individual estimation of Alprazolam and Imipramine by spectroscopy and liquid chromatography. A few methods have also been described for the simultaneous determination of Alprazolam with other drugs such as Propranolol, Fluoxetine HCl, Sertraline, Melatonin [3-14]. A few methods were also described for simultaneous determination of Imipramine with other drugs such as Chlordiazepoxide, Diazepam [15-25]. So, need to develop fast, Accurate, Precise, Specific stability indicating RP- HPLC Method for the simultaneous estimation of both the drugs and to validate the developed method as per ICH Guideline Q2(R1) [26-28].



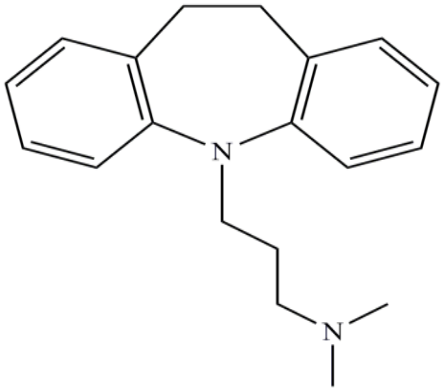


Figure 1. Alprazolam **Figure 2.** Imipramine

EXPERIMENTAL

Instrument & Apparatus

A high Performance Liquid Chromatography system S 1122 HPLC (Analytical Technologies) comprised of S 1122 Pump, 2203 UV -Visible detector, ODS-BP hyperchrome C_{18} (250mm × 4.6mm, 5µm), Rheodyne injector (20 µl) was used for analysis.

Table 1. Optimized chromatographic condition

Parameter	Optimized Condition
Stationary phase	ODS-BP Hyperchrome C_{18} column (250 mm × 4.6 mm, 5 µm).
Mobile phase	Water (pH 6 adjusted with 1% Ortho-Phosphoric Acid): Methanol: Triethylamine (70 : 30 : 0.1 %v/v/v)
Diluent	Same as Mobile Phase
Flow rate	1.0 mL/min
Wavelength	216 nm.
Injection volume	20 µl.

Chemicals & Reagents

Standard Alprazolam was procured as a gift sample from Aril Pharmaceuticals, Mumbai, Gujarat, India & Standard Imipramine procured as a gift sample from Sati Chemicals, Ahmedabad, India. The reagents utilised for analysis are HPLC grade Methanol, HPLC grade water, HPLC grade Acetonitrile, AR grade Ortho Phosphoric acid and AR grade Triethylamine.

Solubility Determination

Solubility of Alprazolam and Imipramine was determined in different solvents like Water, Methanol and Acetone.

Wavelength selection

Standard stock solutions of Alprazolam 1 µg/mL and Imipramine 100 µg/mL were prepared for the selection of wavelength and it was found that both drugs showed reasonably good response at 216 nm. So, 216 nm was selected as a wavelength for estimation. (Figure3).

Preparation of Stock Solution

Preparation of ALP Standard Stock Solution (10 µg/mL)

Accurately weighed 10 mg of Alprazolam was transferred to 100 mL volumetric flask, dissolved and diluted up to mark with mobile phase to obtain final concentration of 100 µg/mL Alprazolam. Solution was further diluted with mobile phase to obtain standard stock solutions of 10 µg/mL of Alprazolam. This solution was filtered through 0.45 µm membrane filter paper and sonicated for 10 min and used as standard stock solution.

Preparation of IMI Standard Stock Solution (1000 µg/mL)

Accurately weighed 100 mg of Imipramine was transferred to 100 mL volumetric flask, dissolved and diluted up to mark with mobile Phase to obtain final concentration of 1000 µg/mL Imipramine. This solution was filtered through 0.45 µm membrane filter paper, sonicated for 10 min and used as standard stock solution.

Preparation of ALP and IMI. Working Standard Solutions

From the Standard Stock Solution of Alprazolam (10 µg/mL) and Imipramine (1000 µg/mL). take 1 mL solution in 10 mL of volumetric flask separately for each and make up with mobile phase. This solution was containing 1 µg/mL Alprazolam and 100 µg/mL of Imipramine.

Preparation of combined standard solution

Accurately weighed quantities of Alprazolam (10 mg) and Imipramine (1gm) were transferred into100mL volumetric flask. They were dissolved and diluted up to the mark with mobile phase to give a combined stock solution (100 μg/mL) of Alprazolam and (10,000μg/mL) of Imipramine. Stock solution (10 mL) was transferred in 100mL volumetric flask and diluted up to mark with mobile phase to obtain combined working standard solution of Alprazolam 10 μg/mL and

Imipramine 1000 μg/mL. This solution was used to prepare standard solution for linearity.

Calibration Curve for ALP and IMI.

To establish the linearity of analytical method, a series of dilution ranging from 0.5 – 1.5 µg/mL for Alprazolam and 50 – 150 µg/mL for Imipramine were prepared. The combined solution of Alprazolam and Imipramine ranging from 0.5 to 1.5 μg/mL and 50 to 150 μg/mL were prepared by pipetting out 0.5, 0.75, 1, 1.25 and 1.5 mL from the combined working standard solution of Alprazolam (10 μg/mL) and Imipramine (1000 μg/mL) into series of 10mL volumetric flasks and the volume was adjusted to mark with mobile phase. All of these solutions were filtered and injected, Chromatogram of each solution was recorded and it was repeated for five times. A calibration graph was plotted between the mean peak area vs. respective concentration and the regression equation was derived.

METHOD VALIDATION

Linearity

Linearity study was carried at five different concentration levels. Linearity range of Alprazolam and Imipramine was found to be 0.5-1.5 µg/mL and 50-150µg/mL, respectively.

Accuracy

Accuracy was calculated by addition of standard drugs to preanalyzed sample at 3 different concentration levels and computing percentage recoveries. Standard limit of % recovery study is 98-102% as per ICH guideline. From the studies it was concluded that % recovery study of Alprazolam and Imipramine complies with standard limit of ICH guideline.

Precision

Repeatability

Solution containing mixture 1 µg/mL Alprazolam and 100µg/mL Imipramine was prepared. Prepared solution was analyzed six times in same day as per the proposed method.

Intermediate precision

Intraday Precision: Solution containing mixture of 0.5, 1, 1.5 µg/mL Alprazolam and 50, 100, 150 µg/mL Imipramine was prepared from their respective standard stock solution. Analysis was replicated for 3 different times within same day.
Intraday Precision: Solution containing mixture of 0.5, 1, 1.5 µg/mL Alprazolam and 50, 100, 150 µg/mL Imipramine was prepared from their respective standard stock solution. Analysis was replicated for 3 different days.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limits of Detection and Quantification of the developed method were calculated from the standard deviation of the y-intercepts and slope of the calibration curves of Alprazolam and Imipramine using the formulae as given below.

Limit of Detection=3.3 α / S

Limit of Quantitation=10α / S

Where α is the standard deviation of the y-intercepts and S is the slope of the calibration curve.

Robustness

As per ICH, the prepared solution was analyzed as per proposed method with small but deliberate change in chromatographic conditions as listed below:

- Change in flow rate: 0.8 mL/min; 1.2 mL/min;
- Change in mobile phase composition: Water : Methanol (± 2% of Mobile phase composition)
- Change in mobile phase pH: 6.2, 5.8.

System Suitability Parameters

System suitability tests were carried out on standard stock solution of ALP (1 µg/mL) and IMI (100 µg/mL) and these solutions were injected under optimized chromatographic condition. Various parameters like Resolution, Selectivity, Asymmetry and Theoretical plates were checked.

Analysis of Pharmaceutical Formulation

Twenty tablets were weighed accurately and their average weight was determined. The tablets were crushed to fine powder and from the triturate; tablet powder equivalent to 100 mg of Imipramine was weighed and transferred to 100 mL volumetric flask. To this flask, 50 mL mobile phase was added and the flask was sonicated for 15 min. The volume was adjusted up to the mark with mobile phase. The solution was then filtered through 0.45 µm membrane filter paper. Filtrate contained mixture of 10 µg/mL Alprazolam and 1000 µg/mL Imipramine. The filtrated solution was suitably diluted with mobile phase to get a final concentration of 1 µg/mL of Alprazolam and 100 µg/mL of Imipramine and sonicated for 5 min. Prepared solution was injected to system. The chromatogram was stopped after separation achieved completely. Concentration of Alprazolam and Imipramine were computed by putting value of their peak areas in respective standard regression equation obtained from calibration curve.

Forced Degradation study

Forced degradation studies were performed to evaluate the stability indicating properties (Specificity) of the proposed method. ALP (API) and IMI (API) individually and its standard mixtures were subjected to Acid, Base, Oxidation, Thermal & Photo Degradation to ensure the effective separation of degradation peaks and main peaks.

Forced degradation Standard stock preparation

Alprazolam (15 mg) and Imipramine (150 mg) were accurately weighed and transferred to a 100 mL volumetric flask, dissolved in sufficient quantity of Mobile Phase (Water : Methanol : Triethylamine) (70:30: 0.1% v/v/v) and then diluted up to the mark with Mobile phase. (The solution contains ALP 150 µg/mL & IMI 1500 µg/mL) And Further Dilute the 10mL of Alprazolam Solution in 100mL Volumetric Flask and make up to the mark. (The solution contains 15 µg/mL of ALP). The final solution was labeled as Standard Stock Solution.

Forced degradation working standard preparation

From Standard Stock Solution take 1 mL of ALP and 1 mL of IMI in 10 mL Volumetric Flask and make up to the mark with Mobile Phase.(1.5 µg/mL ALP and 150 µg/mL IMI) This solution was labeled a Working Standard Solution which is use for force degradation study and chromatographed.

Acid degradation

1 mL of standard stock solution was transferred in to 10 mL of volumetric flask. 2 mL of 0.1 M HCl solution was added and mixed well. The volumetric flask was heat in water bath at 70°C for 2 hrs. After time period the content was cooled to ambient temperature. The above solution was neutralized with 2 mL of 0.1 M NaOH solution and then diluted the volume with Mobile Phase and chromatographed.

Base degradation

1 mL of standard stock solution was transferred in to 10 mL of volumetric flask. 2 mL of 0.1 M NaOH solution was added and mixed well. The volumetric flask was heat in water bath at 70°C for 2 hrs. After time period the content was cooled to ambient temperature. The above solution was neutralized with 2 mL of 0.1 M HCl solution and then diluted the volume with Mobile Phase and chromatographed.

Oxidation Degradation

1 mL of standard stock solution was transferred in to 10 mL of volumetric flask. 2 mL 3% H_2O_2 was added and heat the volumetric flask for 2 hrs at 70°C, after time period content was cooled to ambient temperature and diluted to volume with Mobile Phase and chromatographed.

Photo Degradation

1 mL of standard stock solution was transferred in to 10 mL of volumetric flask. And place the volumetric flask into the sunlight for 4 hrs, after time period content was cooled to ambient temperature and diluted to volume with Mobile Phase and chromatographed.

Thermal Degradation

200 mg powder of Alprazolam and Imipramine were heated in oven at 105ºC for 4 hrs. After time period the content was cooled to ambient temperature and prepared the solution as per working standard solution and chromatographed.

All these solutions were filtered through the 0.45 µ membrane filter before injecting.

RESULT AND DISCUSSION

Method development

For the selection of analytical wavelength for the quantification of the drugs, the standard solution of Alprazolam 1 µg/mL and Imipramine100 µg/mL were scanned in UV-Visible spectrophotometer and their overlain spectra were shown strong absorbance at about 216 nm which was selected as the analytical wavelength for further analysis (Figure 3). For the effective separation of Alprazolam and

Imipramine, attempts were made by using mobile phases containing solvents of varying polarity, at different concentration level with implicating ODS-BP Hyperchrome C_{18} column (250 mm × 4.6 mm id, 5 µm particle size) as a stationary phase. Various mobile phase systems like water: methanol, water: Acetonitrile, Water: Methanol: 0.1% Triethylamine at different concentration levels with different pH was tried. Among the different mobile phase combinations employed, best resolution with sharp well defined peaks obtained with mobile phase composed of Water (pH:6): Methanol : 0.1% Triethylamine in the ratio of 70:30:0.1 v/v/v (Table1). The typical retention time of Alprazolam and Imipramine Were found to be about 3.207 min and 5.073 min. A representative chromatogram of standard and test was shown in Figure 4(a) and (b).

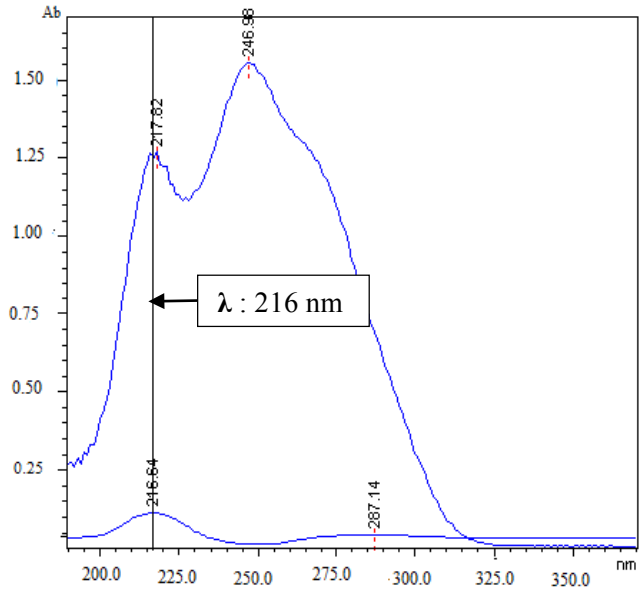


Figure 3. Overlaid Spectra of Alprazolam (1µg/mL) and Imipramine (100 µg/mL) in Methanol

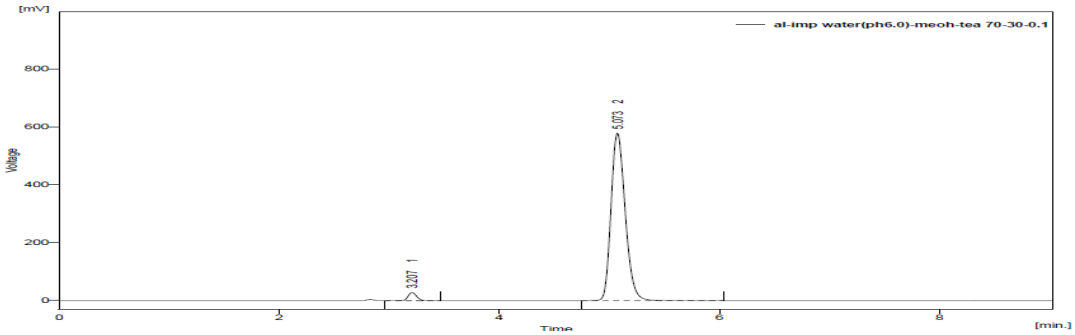


Figure 4(a). Chromatogram of Alprazolam (1 µg/mL) and Imipramine (1 µg/mL) standard in Optimized Condition {Water (pH6): Methanol: Triethylamine: 70: 30: 0.1 % v/v/v}

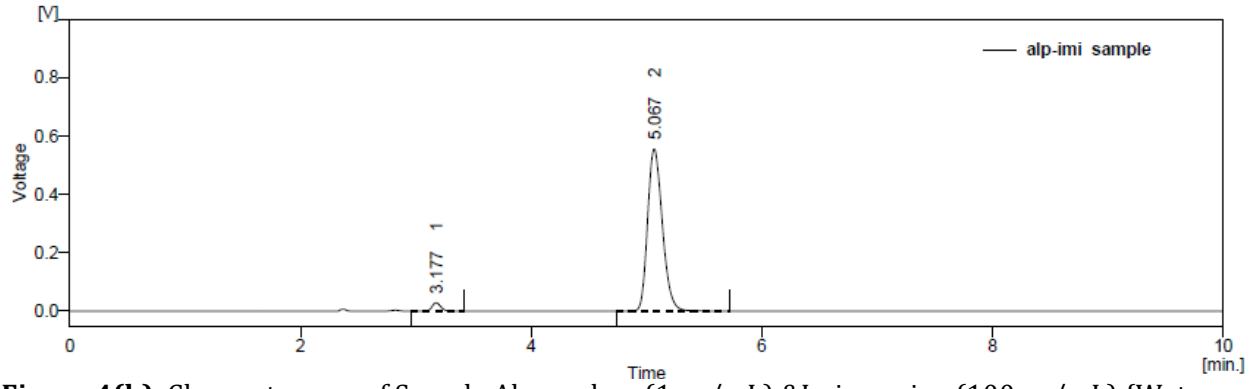


Figure 4(b). Chromatogram of Sample Alprazolam (1 µg/mL) &Imipramine (100 µg/mL) {Water (pH6): Methanol: Triethylamine: 70: 30: 0.1 % v/v/v}

Table 1. Optimized mobile phase condition

Mobile Phase	Flow Rate	R_t Alprazolam	R_t Imipramine	Resolution
Water(pH-6) : Methanol : Triethylamine (70 : 30 : 0.1% v/v/v)	1 mL/ min	3.207	5.073	9.692

Method Validation

The proposed RP – HPLC method was validated as per ICH Guidelines.

Linearity and Range

The linear relationship was existed between concentration and area for ALP and IMI in the concentration range of 0.5-1.5 µg/mL and 50-150µg/mL, respectively. The correlation coefficient for Alprazolam was 0.9999 and for Imipramine was 0.9998. A representative data of linearity is shown in Table 2.

Table 2. Result of Linearity for RP-HPLC Method (n=5)

Parameter		Alprazolam	Imipramine
Range		0.5 – 1.5	50 – 150
Linearity	**Equation**	y = 153.75x - 1.9981	y = 50.922x - 80.285
	R^2	0.9999	0.9998
	%RSD	0.23 – 0.50	0.26 – 1.84
LOD(µg/mL)		0.010	2.202
LOQ(µg/mL)		0.031	6.673

Accuracy (Standard Addition Method)

Result obtained reveals that % recovery of ALP and IMI was found to be 100.10 – 100.94 and 100.39 – 100.74 respectively (Table 3).

Table 3. Result of accuracy for RP-HPLC Method (n=3)

Drug	% of Std Added	% Recovery (Mean ± SD)	%RSD
Alprazolam	80%	100.94 ± 0.82	0.81
	100%	100.10 ± 1.05	1.05
	120%	100.27 ± 1.27	1.26
Imipramine	80%	100.39 ± 1.53	1.53
	100%	100.66 ± 0.66	0.65
	120%	100.74 ± 1.06	1.05

Precision

For repeatability, % RSD was found to be 0.41 and 1.40 for ALP and IMI, respectively. For intraday precision, % RSD was found to be 0.91–1.29% and 0.72–1.07% for ALP and IMI, respectively. For intraday precision, % RSD was found to be 0.25 – 0.52 % and 1.17 – 0.27 % for ALP and IMI, respectively (Table 4,5,6).

Table 4: Result of Repeatability for RP-HPLC Method

Drug	Area (Mean, n = 6)	SD (n =6)	% RSD
Alprazolam	152.4613	0.62	0.41
Imipramine	5072.426	71.17	1.40

Table 5: Result of Intraday Precision for RP-HPLC Method (n=3)

Drug	Conc. in µg/mL	Area (Mean, n = 3)	SD (n =3)	% RSD
Alprazolam	0.5	73.955	0.453	0.613
	1	151.7733	0.304	0.200
	1.5	227.582	0.456	0.200
Imipramine	0.5	2438.4366	34.975	1.435
	1	5028.7466	7.485	0.148
	1.5	7547.5916	12.828	0.169

Table 6: Result of Interday Precision for RP-HPLC Method (n = 3)

Drug	Conc. in µg/mL	AREA (Mean, n = 3)	SD (n =3)	% RSD
Alprazolam	0.5	73.9626	0.39	0.52
	1	152.069	0.39	0.25
	1.5	228.206	0.81	0.35
Imipramine	0.5	2397.355	6.48	0.27
	1	5003.295	58.69	1.17
	1.5	7554.571	11.99	0.15

Robustness

Variation in the flow rate, mobile phase, and pH has been made to the analytical method in order to evaluate and measure the capacity of the method to remain unaffected by such variations. The % RSD was found to be less than 2. (Tables7).

Table 7: Result of Robustness for RP-HPLC Method (n=3)

Variation and Level		Conc. in µg/mL		Mean Area ± SD		% RSD	
		ALP	IMI	ALP	IMI	ALP	IMI
Change in	0.8 mL/min	1	100	158.35 ±0.92	5250.79 ± 28.77	0.58	0.54
Flow Rate	1.2 mL/min	1	100	149.02 ± 0.91	4935.34 ± 24.96	0.61	0.50
Change in pH	5.8 pH	1	100	156.61±0.92	519279± 27.55	0.58	0.53
	6.2 pH	1	100	149.07 ±1.05	4936.99± 31.11	0.72	0.64
Change in	+2%	1	100	150.66 ±1.42	5021.72± 98.39	0.94	1.95
Mobile phase Composition	-2%	1	100	149.59 ±1.54	4946.80± 59.46	1.03	1.20

LOD and LOQ

LOD were found to be 0.010 and 2.202 μg/mL for ALP and IMI respectively. LOQ were found to be 0.031 and 6.673 μg/mL ALP and IMI respectively.

System suitability Parameters

System suitability was established to determine the adequate resolution and reproducibility of the proposed method. Parameters including Retention time, Tailing factor, Resolution and No. of theoretical plates were calculated. (Table 8).

Table 8: Results of System suitability parameters for RP-HPLC Method

System suitability parameters	Drug	
	Alprazolam	Imipramine
Resolution (R_s)	9.7068	
Relative Retention or Selectivity Factor (α)	3.1812	5.045
Tailing factor or Symmetry factor (T)	1.278	1.380
Column efficiency or Theoretical Plates (N)	7450.6 ± 25.55	7193 ± 34.14

Assay of marketed Formulation

Percentage purity of ALP and IMI was found to be 100.51% and 99. % for ALP and IMI, respectively (Table 9).

Table 9: Assay of Alprazolam and Imipramine in marketed formulation (ALZOSET) (n= 3)

Drug	% Assay Mean	SD	% RSD
Alprazolam	100.51	0.94	0.93
Imipramine	99.00	0.16	0.16

Forced degradation studies

From the degradation of these solutions under the stress condition gives us an idea about the origin of degrading products. Degradants did not show any interference with the elution of drug peaks. Hence, the method is stability indicating.

Table 10. Summary of force degradation studies

Stress Condition	Alprazolam			Imipramine		
	Area	R.T	% Degradation	Area	R.T	% Degradation
As such	294.427	3.210	---	7796.094	5.150	---
Acid	258.821	3.187	12.1	7043.264	5.143	9.65
Base	263.779	3.230	10.40	6357.070	5.163	18.46
Oxidation	261.202	3.187	11.28	6927.428	5.087	11.14
Photolytic Condition	264.828	3.193	10.06	6601.144	5.073	15.33
Thermal Condition	247.657	3.213	15.89	7042.034	5.083	9.67

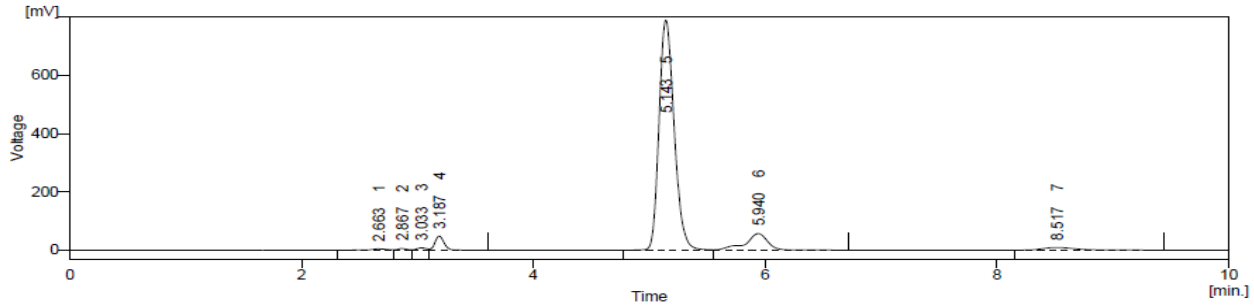


Figure 5. Degradation peaks of sample solution in acid condition

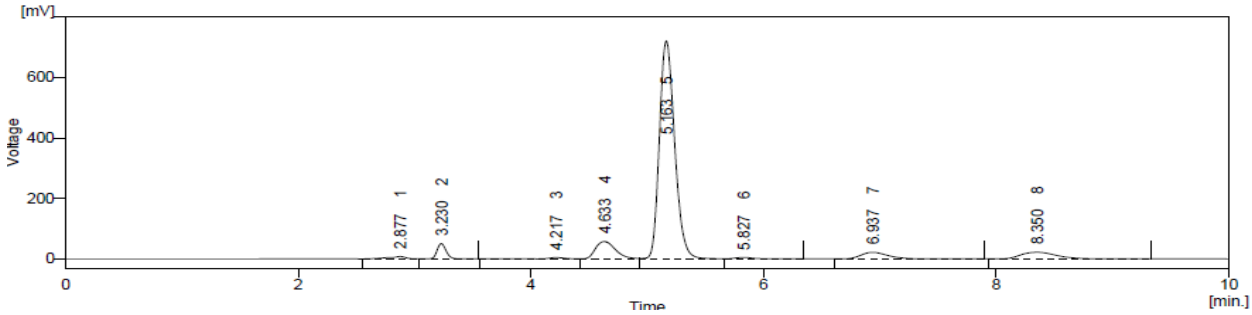


Figure 6. Degradation peaks of sample solution in base condition

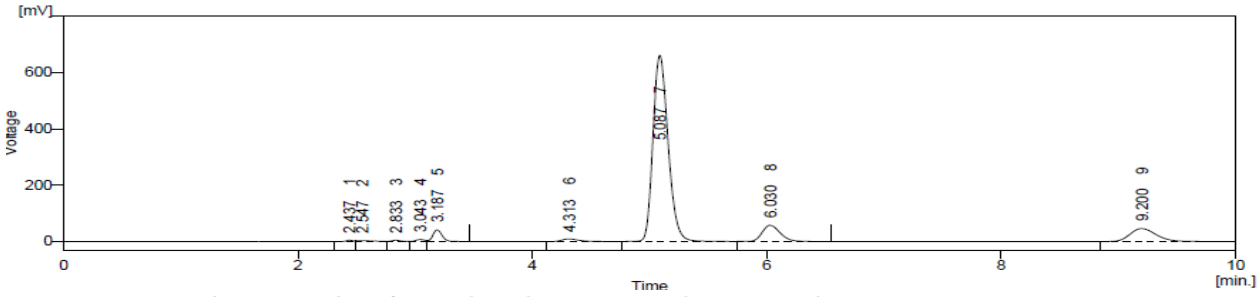


Figure 7. Degradation peaks of sample solution in oxidation condition

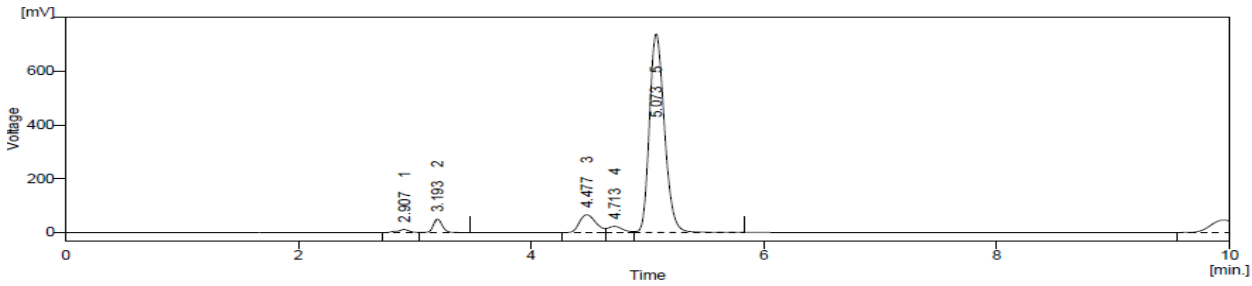


Figure 8. Degradation peaks of sample solution in photolytic condition

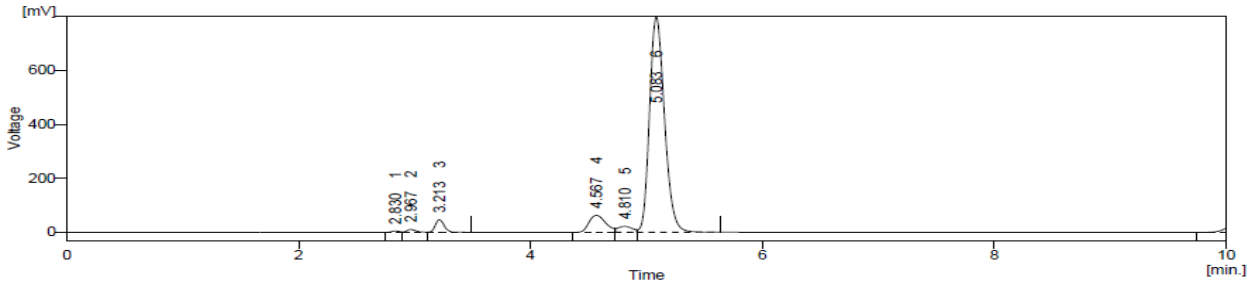


Figure 9. Degradation peaks of sample solution in thermal condition

CONCLUSION

A simple, Accurate and Precise stability indicating HPLC method has been developed for estimation of Imipramine and Alprazolam in tablet dosage form. The RP-HPLC Method for Imipramine and Alprazolam is validated for various parameter like specificity, Linearity, Range, LOD, LOQ, Robustness, Precision Accuracy. Linearity of the developed method was near to 1, range was found 0.5-1.5 µg/mL for Alprazolam and 50 – 150 µg/mL for Imipramine. %RSD was found to be less than 2 for repeatability, intraday precision and intermediate precision, Robustness. Force degradation study of drugs in combined dosage form was carried out according to ICH guideline Q1A (R2). In degradation study it was found that the Alprazolam was

more susceptible under stress condition in comparison with Imipramine except in thermal condition. So, the Degradation study by the RP-HPLC method can be successfully applied for the simultaneous estimation of these drugs in combined dosage form. The peaks of the degradants in each condition were well resolved from main peaks. There is no interference of any degradants at the retention time of the main peaks indicates that the developed method is stability indicating. The proposed method can be used as an alternative method for the analysis of Alprazolam and Imipramine in its Formulation.

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