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RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND IMIPRAMINE AS PER ICH GUIDELINES

Tayyaba Mahtab[1*] and S. Ramya Sri.[2]

[1]Sultan –ul- uloom college of Pharmacy, Jawaharlal Nehru Technological University, Hyderabad, Telangana-500034

[2]University College of Technology, Osmania University, Hyderabad, Telangana-500007.

Abstract:

A rapid and precise Reverse Phase High Performance Liquid Chromatographic method has been developed for the validated of Alprazolam and Imipramine, in its pure form as well as in tablet dosage form. Chromatography was carried out on an Inertsil column, C18(150x4.6 ID) 5µm column using a mixture of Phosphate buffer : ACN (30:70) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 238nm. The Retention Time of the 2.397min for Alprazolam and 3.493 min for Imipramine. The method produce linear responses in the concentration range of 0.5-1.5µg /ml for Alprazolam 50-150µg /ml for Imipramine. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Imipramine; Alprazolam, Method validation ICH guidelines.*

Corresponding author:

Tayyaba Mahtab,

Sultan –ul- uloom college of Pharmacy,
Jawaharlal Nehru Technological University,
Hyderabad, Telangana-500034

QR code

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INTRODUCTION:

a) Alprazolam is a Anti-Anxiety Agents, Hypnotics and Sedatives**,** Benzodiazepines bind nonspecifically to benzodiazepine receptors BNZ1, which mediates sleep, and BNZ2, which affects muscle relaxation, anticonvulsant activity, motor coordination, and memory. As benzodiazepine receptors are thought to be coupled to gamma-aminobutyric acid-A ($GABA_A$) receptors, this enhances the effects of GABA by increasing GABA affinity for the GABA receptor. Binding of the inhibitory neurotransmitter GABA to the site opens the chloride channel, resulting in a hyperpolarized cell membrane that prevents further excitation of the cell [1-4].

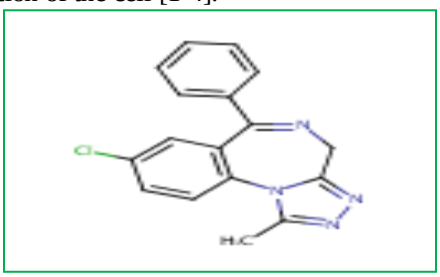


Fig. 1: Chemical structure of Alprazolam

b) Imipramine

Imipramine Adrenergic Uptake Inhibitors, Antidepressive Agents, Tricyclic. Imipramine works by inhibiting the neuronal reuptake of the neurotransmitters norepinephrine and serotonin. It binds the sodium-dependent serotonin transporter and sodium-dependent norepinephrine transporter preventing or reducing the reuptake of norepinephrine and serotonin by nerve cells. Depression has been linked to a lack of stimulation of the post-synaptic neuron by norepinephrine and serotonin [5-8].

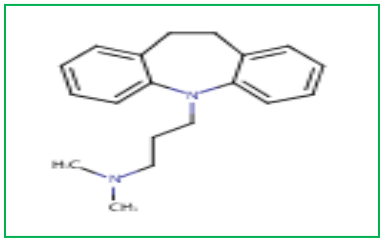


Fig .2: Chemical structure of Imipramine

The aim of the present investigation is to develop and validate a sensitive, precise and accurate RP-HPLC method for the simultaneous quantification of Imipramine and Alprazolam in bulk and in its combined pharmaceutical formulation. The proposed method is validated as per ICH guidelines [9-11].

MATERIALS AND METHOD:

Pure standards and Chemicals

Alprazolam and Imipramine were a procured gift sample from Sura Labs, Hyderabad. Methanol, Acetonitrile of HPLC grade was purchased from Merck (India) Ltd., Mumbai and HPLC grade water from milli Q water.

Instrumentation

Analysis was carried out using Shimadzu (LC 20 AT VP) HPLC system.

Chromatographic conditions

The HPLC separation and quantification of the Imipramine and Alprazolam were made on the Inertsil column, C18 (150x4.6 ID) 5µm. An isocratic mobile phase consisting of Phosphate buffer: ACN (30:70) was the optimized mobile composition and column temperature. The elution was monitored at 238nm. The mobile was pumped into the column at a flow rate of 1.0mL/min and the run time was 5min. The volume of injection loop was 20µL. Prior to injection of the drug solution the column was equilibrated for at least 15min with the mobile phase flowing through the system.

Preparation of standard solution:

Weigh accurately 1mg of Alprazolam and 100mg of Imipramine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From above stock solution 1µg/ml of Alprazolam and 100µg/ml of Imipramine is prepared by diluting 1ml of to 10ml with mobile phase. This solution is used for recording chromatogram.

Preparation of mobile phase:

Mobile Phase:

A mixture of 30 volumes of mixed Phosphate Buffer pH4.5: 70 volumes of Acetonitrile were prepared. The mobile phase was sonicated for 10min to remove gases.

Preparation of Mixed Phosphate buffer:

1.625gm of potassium di hydrogen phosphate (KH2PO4) and 0.3gms of di potassium hydrogen phosphate was weighed and dissolved in 100ml of water and volume was made up to 550ml with water. Adjust the pH to 4.5 using ortho phosphoric acid. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

Diluent Preparation:

The Mobile phase was used as the diluent.

System Suitability Studies

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system in order to show

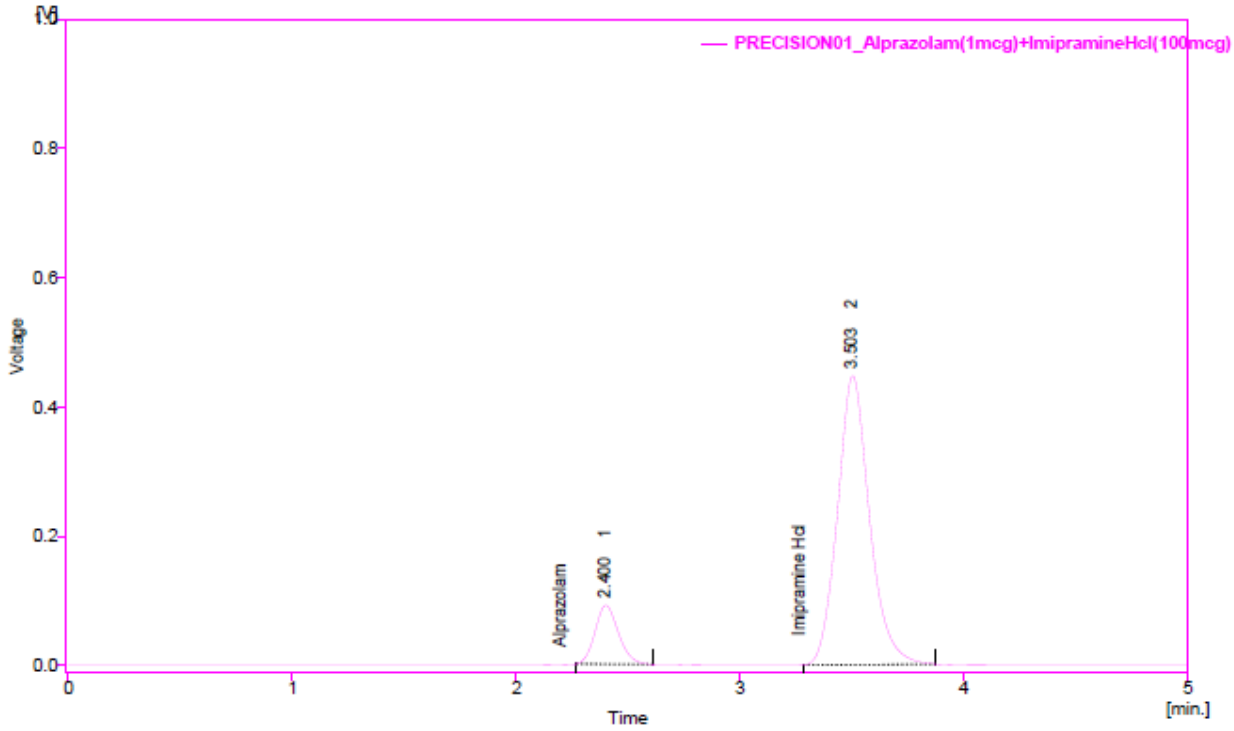
that the performance of the system meet the standards required by the method. Mixed standard solution of Imipramine and Alprazolam solution was injected in replicates and system suitability parameters were determined for the developed method include number of theoretical plates, resolution and tailing factor.

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Analytical method was tested for specificity to measure accurately quantities Alprazolam and Imipramine in drug product.

Sample Info:

Sample ID	: Precision	Amount	: 0
Sample	: Alprazolam(1mcg)+ImipramineHcl(100mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - PRECISION01_Alprazolam(1mcg)+ImipramineHcl(100mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.400	658.975	90.967	12.89	16.9	0.11
2	3.503	4455.055	446.977	87.11	83.1	0.15
	Total	5114.029	537.944	100.00	100.0	

Column Performance Table (From 50% - PRECISION01_Alprazolam(1mcg)+ImipramineHcl(100mcg))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.400	0.113	1.321	0.00	2484	24844	-
2	3.503	0.153	1.220	0.00	2892	28920	4.869

Fig.3: Typical chromatogram of mixed standard solution

Linearity and Range

The linearity was established by least squares linear regression analysis of the calibration curve for Imipramine and Alprazolam standard solutions by plotting the concentrations of the compound versus peak area response.

Accuracy and Precision

The accuracy of the method was determined by recovery experiments. The recovery studies were carried out 3 times. The percentage recovery and standard deviation of the percentage recovery were calculated. From the data obtained, added recoveries of standard drugs were found to be accurate. The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intraday studies, six repeated injections of standard and sample solutions were made and the response factor of drug peaks and percentage RSD were calculated. In the inter-day variation studies, six repeated injections of standard and sample solutions were made for three consecutive days and response factor of drugs peaks and percentage RSD were calculated.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms which demonstrated that the RP-HPLC method developed is robust. The results are shown in below table.

Table 1: Results for Robustness

Parameter	Alprazolam		Imipramine	
	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor
Flow				
0.8ml/min	3.057	1.278	4.470	1.226
1.0 ml/min	2.397	1.300	3.493	1.186
Wavelength				
236nm	2.390	1.200	3.487	1.214
238nm	2.397	1.300	3.493	1.186

Observation

From the observation it was found that the system suitability parameters were within limit at all variable conditions.

RESULTS AND DISCUSSION:

System Suitability Studies

The column efficiency, resolution and tailing factor were calculated for the standard solutions (Table 1).The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall within ± 2%Relative standard deviation range during routine performance of the method.

Table 2: Results for Ruggedness

Alprazolam	%Assay	Imipramine	%Assay
Analyst 01	98.36	**Analyst 01**	99.59
Analyst 02	99.78	**Analyst 02**	99.93
%RSD	**1.00%**	**%RSD**	**0.24%**

Observation

From the observation the %RSD between two analysts Assay values not greater than 2.0%, hence the method was rugged.

Linearity and range

Weigh accurately 1mg of Alprazolam and 100mg of Imipramine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 10µg/ml of Alprazolam and 1000µg/ml of Imipramine.

Table 3.Linearity Data of Alprazolam and Imipramine

Preparations	Volume from standard stock transferred in ml	Volume made up in ml (with mobile phase)	Concentration of solution(µg /ml)	
			Alprazolam	Imipramine
Preparation 1	0.5	10	0.5	50
Preparation 2	0.75	10	0.75	75
Preparation 3	1	10	1	100
Preparation 4	1.25	10	1.25	125
Preparation 5	1.5	10	1.5	150

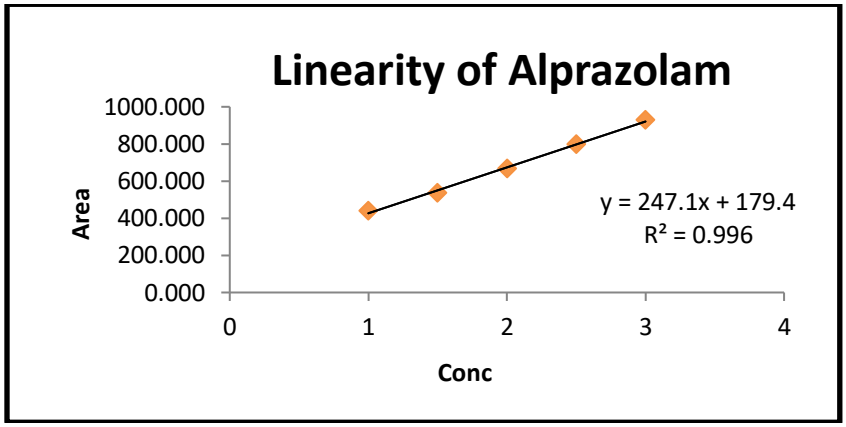


Fig.4: Calibration graph of Alprazolam

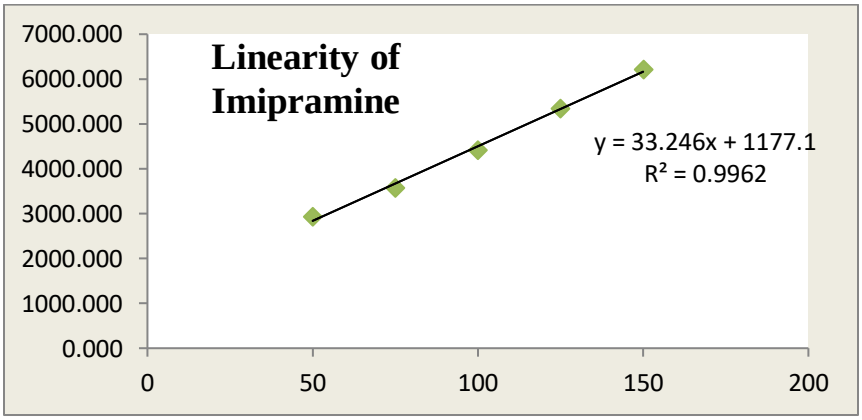


Fig.5: Calibration graph of Imipramine

Accuracy and Precision

The results of accuracy of the method were determined by recovery experiments. The percentage recovery and standard deviation of the percentage recovery were calculated. From the data obtained, added recoveries of standard drugs were found to be accurate.

The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intraday studies, six repeated injections of standard and sample solutions were made and the response factor of drug and percentage RSD were calculated. In the inter-day variation studies, six repeated injections of standard and sample solutions were made for three consecutive days and response factor of drug and percentage RSD were calculated From the results, the developed RP-HPLC method was considered to be precise.

Table 4: The accuracy results for Imipramine

%Concentration (at specification Level)	Peak Area	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	Mean Recovery
50%	2945.380	50	49.89	99.7%	99.7%
100%	4450.041	100	99.93	99%	
150%	6230.615	150	149.42	99.61	

Table 5: The accuracy results for Alprazolam

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	446.245	50	0.50	100.74	99.5%
100%	673.414	100	0.98	98.36	
150%	952.879	150	1.49	99.59	

Table 6: Results of Ruggedness for Alprazolam and Imipramine

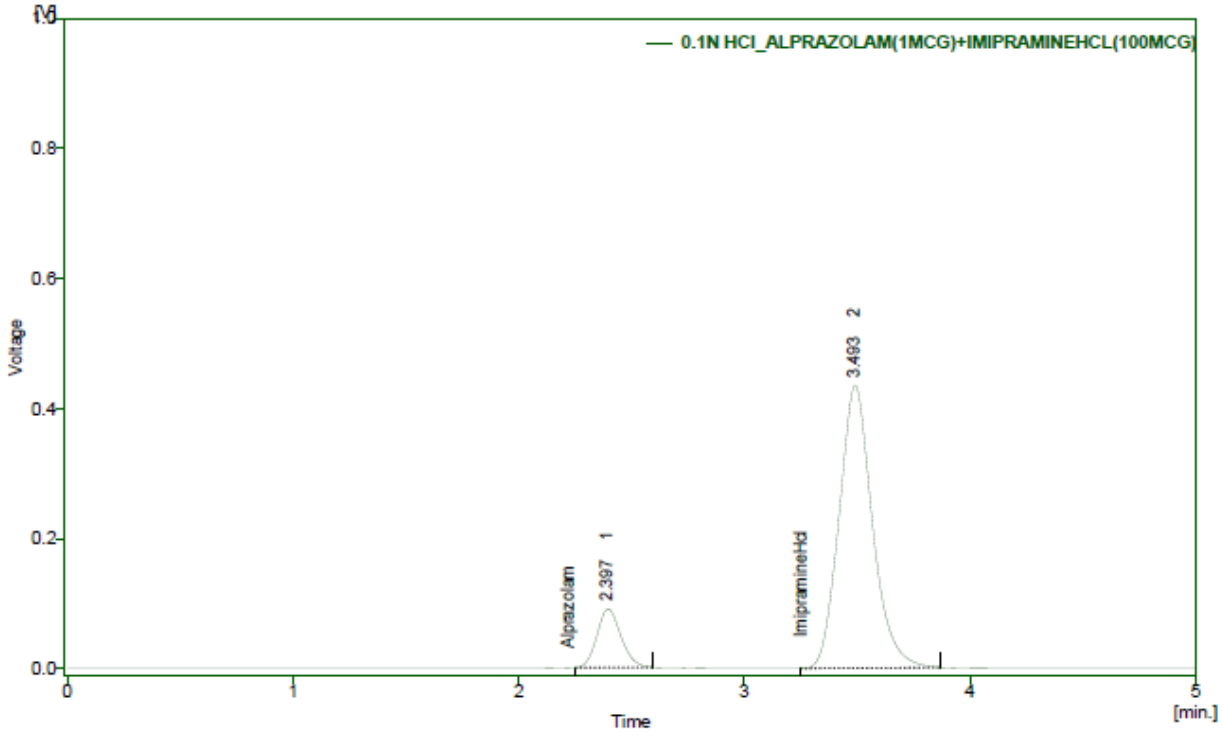
Alprazolam	%Assay	Imipramine	%Assay
Analyst 01	98.36	**Analyst 01**	99.59
Analyst 02	99.78	**Analyst 02**	99.93
%RSD	**1.00%**	**%RSD**	**0.24%**

Acceptance criteria:
From the observation the %RSD between two analysts Assay values not greater than 2.0%, hence the method was rugged.

FORCED DEGRADATION STUDIES
Acid degradation
Degradation was observed by the additon of 0.5N HCl

Sample Info:

Sample ID	:0.1N HCl	Amount	: 0
Sample	: Alprazolam(1mcg)+ImipramineHcl(100mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - 0.1N HCl_ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.397	665.044	90.259	12.96	17.2	0.12
2	3.493	4466.112	434.854	87.04	82.8	0.16
	Total	5131.156	525.113	100.00	100.0	

Column Performance Table (0.1N HCl _ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.397	0.117	1.276	0.00	2338	23379	-
2	3.493	0.160	1.186	0.00	2641	26409	4.665

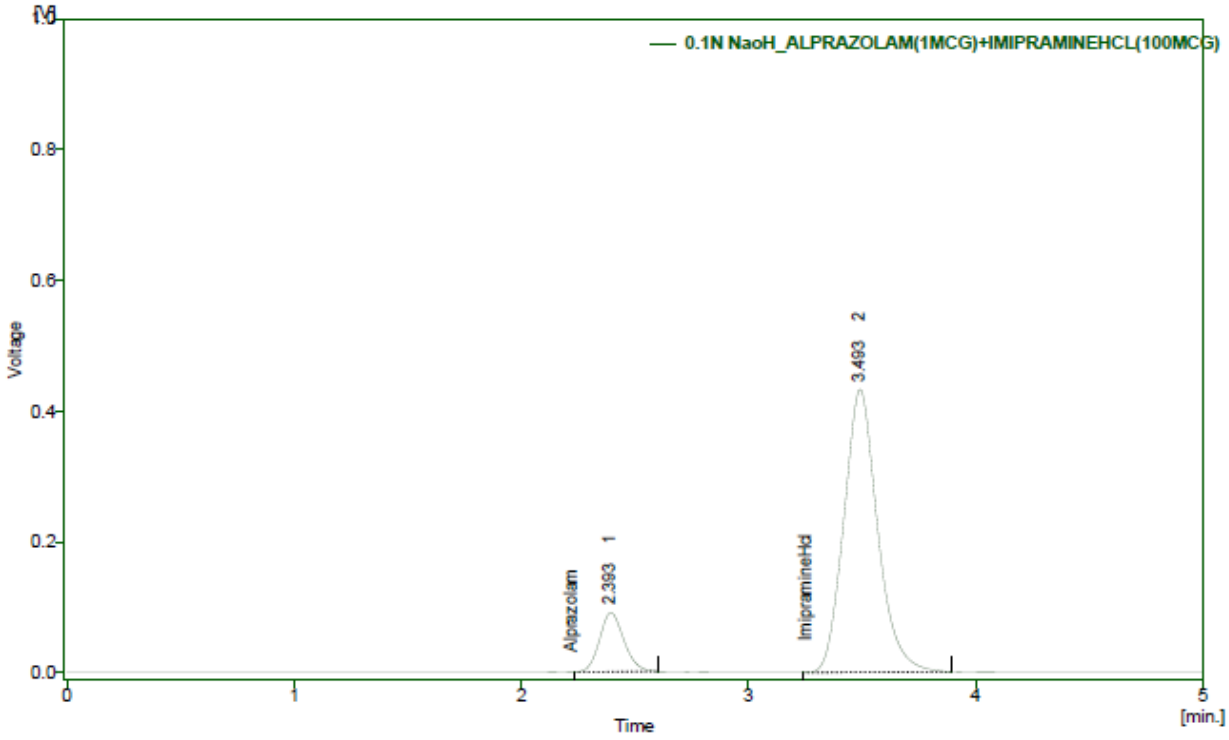
Fig.6: Showing acid degradation

Alkaline degradation

Degradation was observed by the additon of 0.5N NaOH

Sample Info:

Sample ID	:0.1N NaoH	Amount	: 0
Sample	: Alprazolam(1mcg)+ImipramineHcl(100mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - 0.1N NaoH_ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.393	671.359	90.437	13.05	17.3	0.12
2	3.493	4471.811	432.542	86.95	82.7	0.16
	Total	5143.170	522.979	100.00	100.0	

Column Performance Table (0.1N NaoH _ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.393	0.117	1.310	0.00	2331	23314	-
2	3.493	0.160	1.186	0.00	2641	26409	4.679

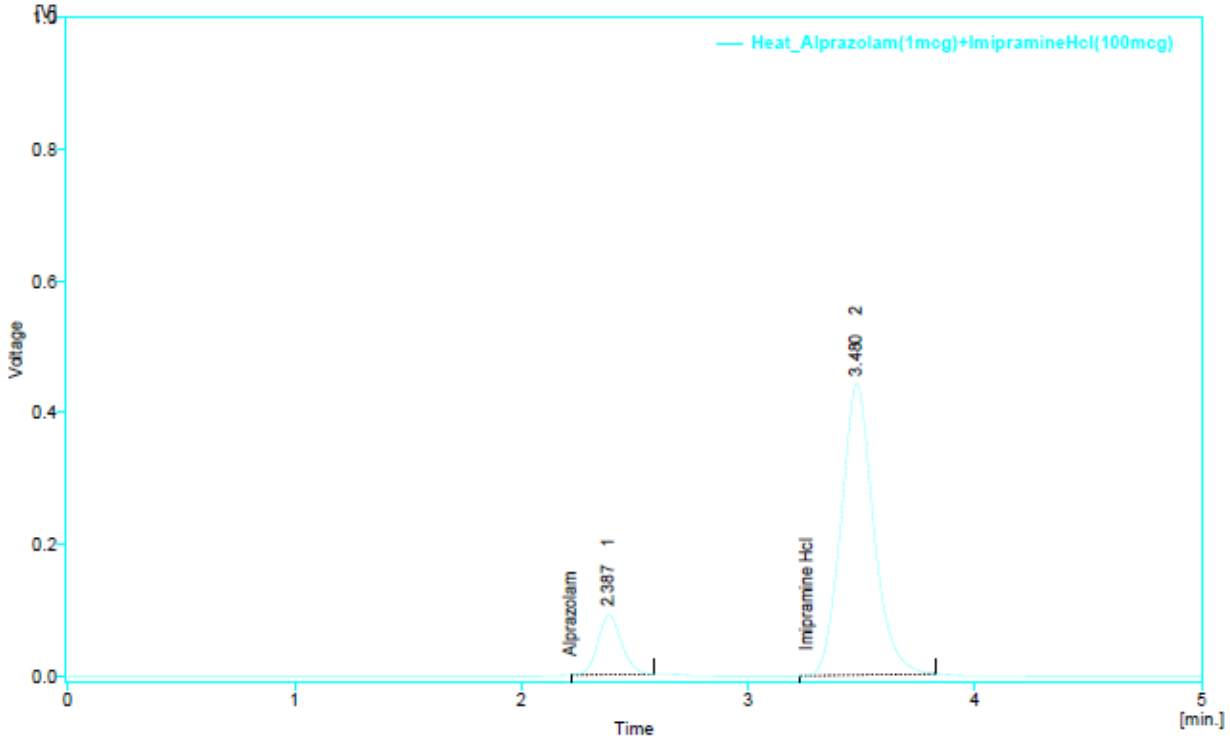
Fig.7: Showing Alkaline degradation

Thermal degradation

Degradation was observed when the sample solution was kept under heat at 60-80^0 C for 3hours.

Sample Info:

Sample ID	:Heat	Amount	: 0
Sample	: Alprazolam(1mcg)+ImipramineHcl(100mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - Heat_Alprazolam(1mcg)+ImipramineHcl(100mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.387	665.088	91.189	13.07	17.1	0.11
2	3.480	4423.534	443.362	86.93	82.9	0.15
	Total	5088.621	534.552	100.00	100.0	

Column Performance Table (Heat _Alprazolam(1mcg)+ImipramineHcl(100mcg))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.387	0.113	1.200	0.00	2457	24568	-
2	3.480	0.153	1.167	0.00	2854	28536	4.825

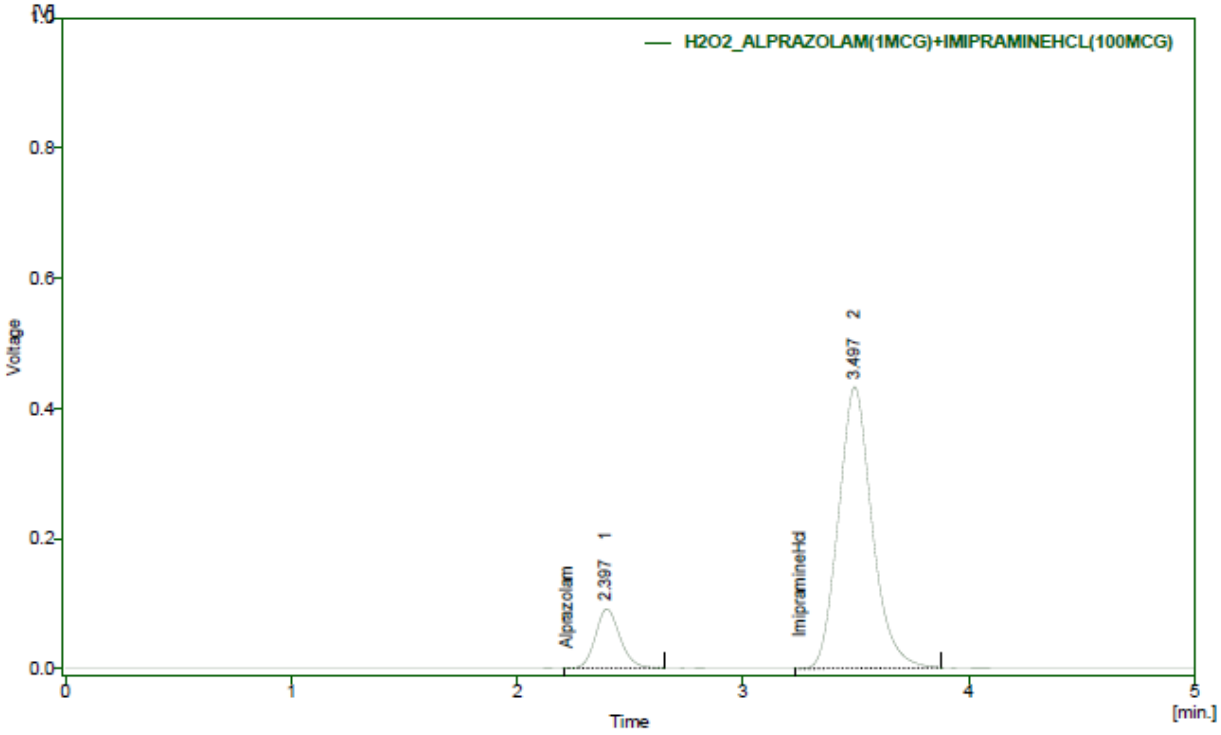
Fig.8: Showing Thermal degradation

Peroxide degradation

Degradation was observed by the additon of 3% H_2O_2

Sample Info:

Sample ID	: H2O2	Amount	: 0
Sample	: Alprazolam(1mcg)+ImipramineHcl(100mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - H2O2_ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.397	679.956	90.947	13.20	17.4	0.12
2	3.497	4470.463	432.328	86.80	82.6	0.16
	Total	5150.419	523.275	100.00	100.0	

Column Performance Table (From 50% - H2O2)_ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.397	0.117	1.310	0.00	2338	23379	-
2	3.497	0.160	1.186	0.00	2646	26459	4.679

Fig.9: Showing peroxide degradation

Photolytic degradation

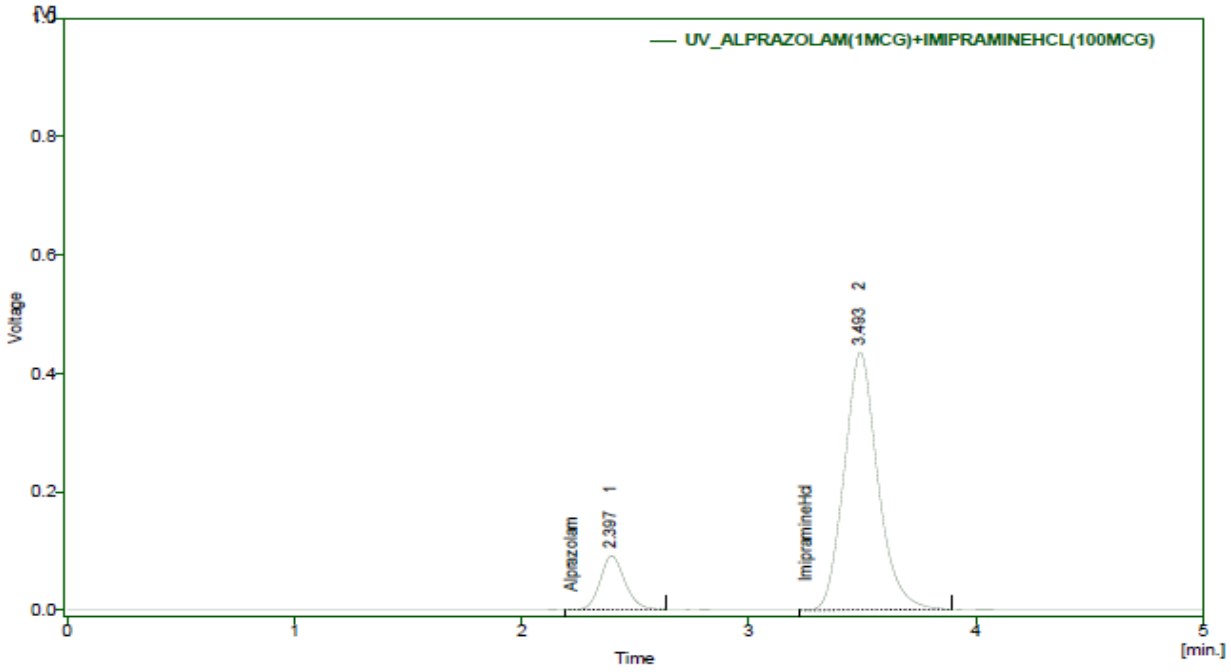
Degradation was observed by sunlight exposre.

Sample Info:

Sample ID :UV Amount : 0

Sample : Alprazolam(1mcg)+ImipramineHcl(100mcg) ISTD Amount : 0

Inj. Volume [ml] : 0.02 Dilution : 1



Result Table (Uncal - UV_ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.397	681.378	90.997	13.20	17.3	0.12
2	3.493	4481.001	435.177	86.80	82.7	0.16
	Total	5162.378	526.175	100.00	100.0	

Column Performance Table (UV _ALPRAZOLAM(1MCG)+IMIPRAMINEHCL(100MCG))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.397	0.117	1.267	0.00	2338	23379	-
2	3.493	0.160	1.186	0.00	2641	26409	4.665

Fig.10: Showing Photolytic degradation

Table 7: Results of degradation studies of Alprazolam

Conditions	Sample weight(mg)	Peak Area	% claim	%Degradation
Sample Control	120.72	720.429	98.14	-
Alkali Degradation	121.49	671.359	92.41	5.73
Acid Degradation	120.59	665.044	92.22	5.92
Thermal Degradation	121.59	665.088	91.54	6.6
Peroxide Degradation	120.98	679.959	93.99	4.15
UV Degradation	121.59	681.378	93.71	4.43

Table 8: Results for Degradation studies of Imipramine

Conditions	Sample weight(mg)	Peak Area	% claim	%Degradation
Sample Control	120.72	4498.38	99.46	-
Alkali Degradation	121.75	4471.840	98.371	1.09
Acid Degradation	123.57	4466.112	96.79	2.67
Thermal Degradation	122.94	4423.534	92.26	7.2
Peroxide Degradation	121.59	4470.463	98.47	0.99
UV Degradation	121.49	4481.001	98.78	0.68

DISCUSSION:

A simple and selective LC method is described for the determination of Alprazolam and Imipramine dosage forms. Chromatographic separation was achieved on a c18 column using mobile phase consisting of a mixture of mixed Phosphate buffer pH: 4.5 Acetonitrile (30:70v/v), with detection of nm. Linearity was observed in the range 0.5-1.5µg /ml for Alprazolam (r2 =0.996) & 50-150µg /ml for Imipramine (r2 =0.996) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim. The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

CONCLUSION:

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Alprazolam and Imipramine was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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