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Analytical method development and validation for the estimation of clomipramine HCL in API form and marketed pharmaceutical dosage form by reverse phase- high performance liquid chromatography

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Abstract
Objective: The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-HPLC method for the Quantitative Determination of Clomipramine HCL in active pharmaceutical ingredient and Marketed Pharmaceutical Dosage form.
Methods: A simple, selective, validated and well-defined stability that shows isocratic RP-HPLC methodology for the quantitative determination of Clomipramine HCL. The chromatographic strategy utilized Symmetry C18, 250 mm x 4.6 mm i.d.5 µm particle size, using isocratic elution with a mobile phase consists of Methanol and Phosphate Buffer (0.02M) (pH-3.8) was taken in the ratio of 70: 30% v/v. A flow rate of 1.0 ml/min and a detector wavelength of 245nm utilizing the UV detector were given in the instrumental settings. Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines.
Results: LOD and LOQ for the active ingredients were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of R2>0.999, means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, ruggedness were determined as a part of method validation and the results were found to be within the acceptable range.
Conclusion: The proposed method to be fast, simple, feasible and affordable in assay condition. During stability tests, it can be used for routine analysis of the selected drugs.

Keywords: Clomipramine HCL, RP-HPLC, method development, validation, accuracy, precision

1. Introduction

Clomipramine [1] is a Tricyclic Antidepressant. Clomipramine [2] is a dibenzoazepine that is 10, 11-dihydro-5H-dibenzo [b, f]azepine which is substituted by chlorine at position 3 and in which the hydrogen attached to the nitrogen is replaced by a 3-(dimethylamino) propyl group. One of the more sedating tricyclic antidepressants [3], it is used as the hydrochloride salt for the treatment of depression as well as obsessive-compulsive disorder and phobias. It has a role as a serotonergic antagonist, a serotonergic drug, a serotonin uptake inhibitor, an EC 1.8.1.12 (trypanothione-disulfide reductase) inhibitor, an antidepressant and an anticoronaviral agent. It is functionally related to an imipramine. It is a conjugate base of a clomipramine [4] (1+).Clomipramine is a tricyclic antidepressant used in the treatment of obsessive-compulsive disorder and disorders with an obsessive-compulsive component, such as depression, schizophrenia, and Tourette's disorder. May be used to treat obsessive-compulsive disorder and disorders with an obsessive-compulsive component (e.g. depression, schizophrenia, Tourette's disorder).

Unlabeled indications include: depression, panic disorder, chronic pain (e.g. central pain, idiopathic pain disorder, tension headache, diabetic peripheral neuropathy, and neuropathic pain), cataplexy and associated narcolepsy (limited evidence), autistic disorder (limited evidence), trichotillomania (limited evidence), onchophagia (limited evidence), stuttering (limited evidence), premature ejaculation, and premenstrual syndrome. The IUPAC Name of ClomipramineHCL[5]is (3-{14-chloro-2-azatricyclo [9.4.0.0^{3,8}]pentadeca-1(11),3,5,7,12,14-hexaen-2-yl}propyl)dimethylamine hydrochloride. The Clomipramine HCL is as following

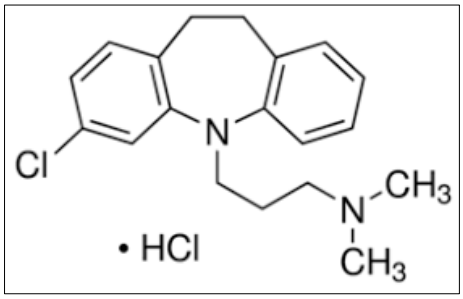

Fig 1: IUPAC Name of Clomipramine HCL

Experimental

Materials and Instruments

The following are the list of instruments/Equipments, chemicals/reagents and standards to perform the HPLC Analysis[6]of the drug Clomipramine HCL.

Equipments

Table 1: List of Equipments

S. No.	Instruments/Equipments/Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C_{18} Column, 250 mm x 4.6 mm and 5µm particle size
7.	P^H Analyser (ELICO)
8.	Vaccum Filtration Kit (Labindia)

Chemicals and Reagents

Table 2: List of Chemicals used

S. No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

Working Standard: Working Standard[7]of Clomipramine HCL: 10 ppm

HPLC Instrumentation & Conditions: The HPLC system employed was HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.

Standard preparation for UV-spectrophotometer Analysis

The Standard Stock Solutions– 10 mg of Clomipramine HCL standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10 ml volumetric flask and make up to volume with methanol to get 10 ppm concentration.

Itscanned in the UV spectrum[8] in the range of 200 to 400nm. This has been performed to know the maxima of Clomipramine HCL, so that the same wave number can be utilized in HPLC UV detector for estimating the Clomipramine HCL.

Different trials for chromatographic conditions

Table 3: Different Chromatographic Conditions

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Develosil C_{18}, 250 mm x 4.6 mm and 5 µm Column	Acetonitrile : Water = 65 : 35	0.8 ml/min	245 nm	Base line noise is high	Method rejected
Develosil C_{18}, 250 mm x 4.6 mm and 5 µm Column	Acetonitrile : Water = 55 : 45	0.8 ml/min	245 nm	Tailing is more	Method rejected
Zorbax C_{18}, 250 mm x 4.6 mm and 5 µm Column	Methanol : Acetonitrile = 30 : 70	0.9 ml/min	245 nm	Extra peaks	Method rejected
Phenomenex C_{18}, 250 mm x 4.6 mm and 5 µm Column	Methanol : Acetonitrile = 60 : 40	1.0 ml/min	245 nm	Good sharp peak	Method accepted

Symmetry C_{18}, 250 mm x 4.6 mm and 5 µm Column	Methanol : Acetonitrile = 50 : 50	1.0 ml/min	245 nm	Improper peak separation	Method rejected
Symmetry C_{18}, 250 mm x 4.6 mm and 5 µm Column	Methanol : Phosphate Buffer (0.01M) (pH-2.8) = 40 : 60	1.0 ml/min	245 nm	Tailing peaks	Method rejected
Symmetry C_{18}, 250 mm x 4.6 mm and 5 µm Column	Methanol : Phosphate Buffer (0.02M) (pH-3.2) = 60 : 40	1.0 ml/min	245 nm	Tailing peaks	Method rejected
Symmetry C_{18}, 250 mm x 4.6 mm and 5 µm Column	Methanol : Phosphate Buffer (0.02M) (pH-3.8) = 70 : 30	1.0 ml/min	245 nm	Proper Peak	Method Accepted

Preparation of 0.02M Phosphate Buffer (pH-3.8)
Prepare 800 mL of distilled water in a suitable container. Add 2.72172 g of Potassium dihydrogen Phosphate to the solution to the solution. Adjust solution to final desired pH 3.8 using diluted solution of orthophosphoric acid and add distilled water until volume is 1 Litre.

Preparation of Mobile Phase
Mix a mixture of 0.02M Phosphate Buffer (pH-3.8) 700 ml (70%) and 300 ml Methanol HPLC (30%) and degas in ultrasonic water bath for 15 minutes. Filter[9] through 4.5 µ filter under vacuum filtration.

Preparation of Standard Solution
Accurately weigh and transfer 10 mg of Clomipramine HCL working standard into a 10 ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette out 0.1 ml of Clomipramine HCL from the above stock solutions [10] into a 10ml volumetric flask and dilute up to the mark with Diluent.

Results and Discussion
Method Development
Its canned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Clomipramine HCL, so that the same wave number can be utilized in HPLC UV detector[11] for estimating the Clomipramine HCL.

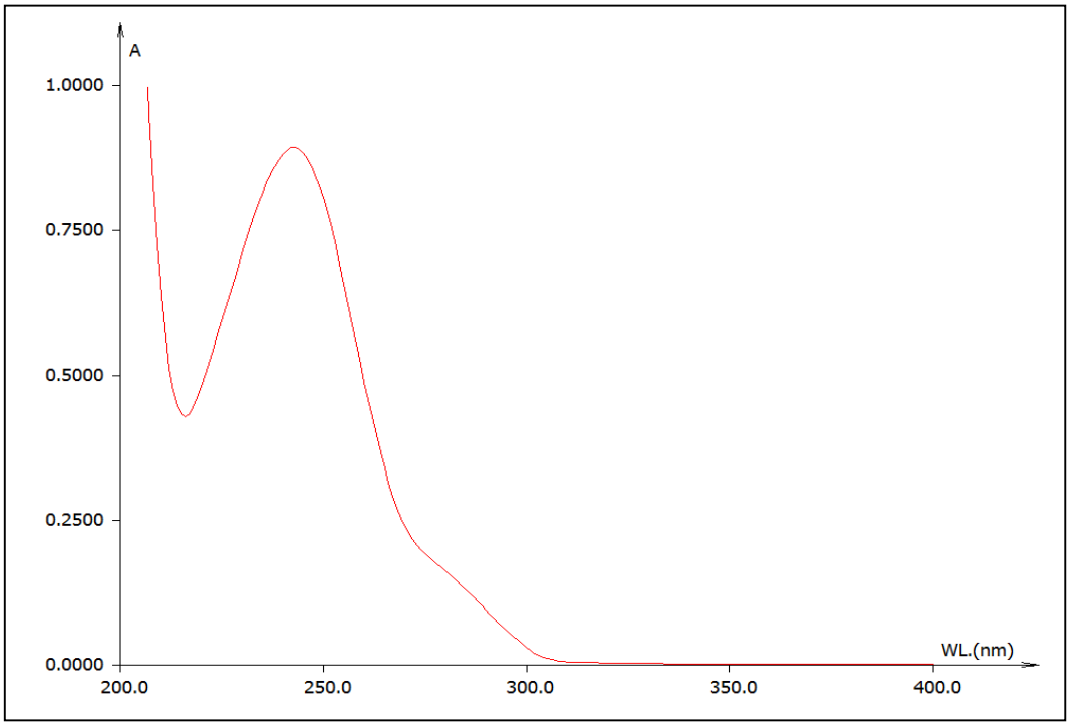

Fig 2: UV-Spectrum for Clomipramine HCL

Observation: While scanning the Clomipramine HCL solution we observed the maxima at 245nm.
Optimized Chromatographic Conditions
Column: Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size
Mobile Phase: Methanol: Phosphate Buffer (0.02M) (pH-3.8) (70: 30% v/v)
Flow Rate: 1.0 ml/minute
Wave length: 245 nm
Injection volume: 10 µl
Run time: 7 minutes
Column temperature: Ambient

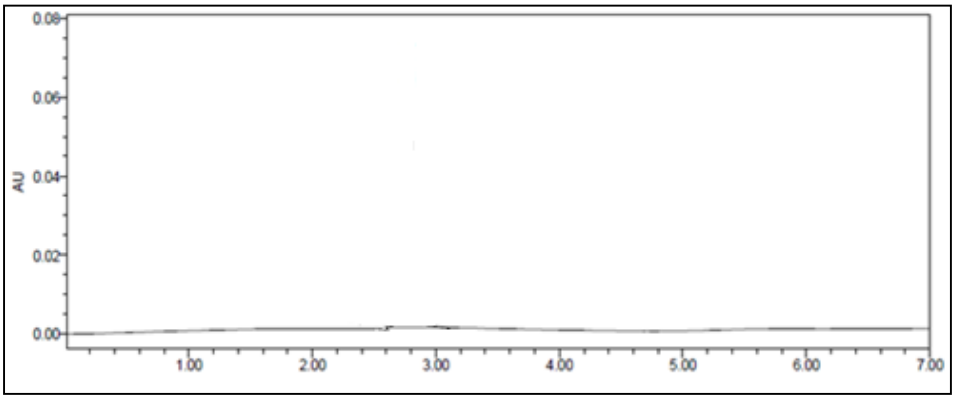

Fig 3: Chromatogram for Blank Solution

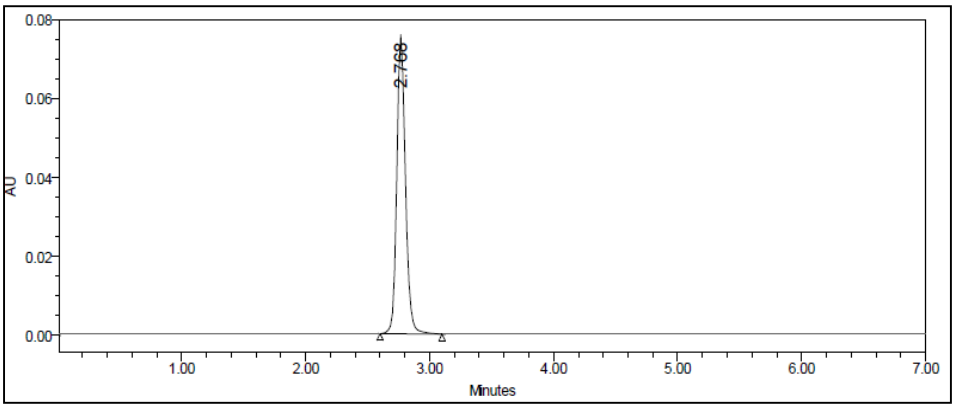

Fig 4: Optimized Chromatogram for Clomipramine HCL

Preparation of 0.02M Phosphate Buffer (pH-3.8): Prepare 800 mL of distilled water in a suitable container. Add 2.72172 g of Potassium dihydrogen Phosphate to the solution to the solution. Adjust solution to final desired pH 3.8 using diluted solution of orthophosphoric acid and add distilled water until volume is 1 Litre.

Preparation of Mobile Phase: Mix a mixture of 0.02M Phosphate Buffer (pH-3.8) 700 ml (70%) and 300 ml Methanol HPLC (30%) and degas [12] in ultrasonic water bath for 15 minutes. Filter through 4.5 µ filter under vacuum filtration [13].

Preparation of standard solution

Accurately weigh and transfer 10 mg of Clomipramine HCL working standard into a 10ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.1ml of Clomipramine HCL from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent [14].

Observation: The selected and optimized mobile phase was Methanol: Phosphate Buffer (70: 30% v/v) and conditions optimized were flow rate (1.0 ml/minute), wavelength (245nm), Run time was 07 mins. Here the peak has shown better theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the Clomipramine HCL drug.

Validation of analytical method

The developed method was validated as per ICH guidelines[32] in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ) and system suitability.

System suitability test

System suitability testing [15-17] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-4 & 5.

Table 4: Data of System Suitability Test

S. No.	Injection No.	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.786	715268	47844	5857	1.36
2	Injection 2	2.784	716584	46985	5986	1.38
3	Injection 3	2.768	715364	47258	5784	1.35
4	Injection 4	2.789	714895	47152	5896	1.34
5	Injection 5	2.784	716587	47258	5749	1.36
6	Injection 6	2.781	718549	47985	5657	1.39
Mean			716207.8		5821.5	1.36
S.D			1347.976			
%RSD			0.18821			

Table 5: Acceptance Criteria and Results

S. No.	Parameter	Limit	Result
1	Tailing factor	T ≤ 2	1.36
2	Theoretical plate	N > 2000	5821.5

Accuracy

Recovery Study

To determine the accuracy[18] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Clomipramine HCLwere taken and 3 replications of each has been injected to HPLC system[19]. From that percentage recovery values were calculated from the linearity equation y = 74143x + 7294.9. The results were shown in table-6.

Table 6: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	%Recovery of Pure drug	Mean %Recovery	
	Amount Injected	Amount Recovered				
S_1 : 80%	8	8.013	601425	100.162	Mean = 100.195%	% Mean Recovery = 100.364%
S_2 : 80%	8	8.012	601396	100.150		
S_3 : 80%	8	8.022	602123	100.275		
S_4 : 100%	10	10.038	751584	100.380	Mean = 100.356	
S_5 : 100%	10	10.039	751642	100.390		
S_6 : 100%	10	10.030	750969	100.300		
S_7 : 120%	12	12.057	901253	100.475	Mean = 100.541	
S_8 : 120%	12	12.073	902431	100.608		
S_9 : 120%	12	12.065	901864	100.541		

Observation: From the Accuracy Method, we observed that the mean % Recovery [20] of the drug is 99.686 which are within the range of 98-102%.

Precision

Repeatability

The precision [21] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Clomipramine HCL (API). The percent relative standard deviation [22] was calculated for Clomipramine HCL.

Table 7: Results of Repeatability readings

HPLC Injection Replicates of Clomipramine HCL	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	716984	5986	1.36
Replicate – 2	2.795	715698	5897	1.37
Replicate – 3	2.789	716859	5869	1.39
Replicate – 4	2.797	718548	5967	1.37
Replicate – 5	2.797	714895	5984	1.35
Replicate – 6	2.799	715986	5879	1.38
Average		716495	5930.333	1.37
Standard Deviation		1268.126		
% RSD		0.17699		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.176 which are within the acceptable range as per ICH guidelines [23].

Intermediate Precision

The Intermediate Precision [24] consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Intra-Day

Table 8: Peak results for Intra-Day Precision

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Clomipramine HCL	2.784	716587	48685	1.38	5954	1
2	Clomipramine HCL	2.768	717845	48698	1.39	5935	2
3	Clomipramine HCL	2.786	716857	46989	1.36	5798	3
4	Average		717096.3	48124	1.376	5895.66	
5	S.D		662.2698				
6	% RSD		0.092354				

Table 9: Peak results for Inter-Day Precision

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Clomipramine HCL	2.780	716987	49867	1.34	5968	1
2	Clomipramine HCL	2.794	718695	48574	1.33	5998	2
3	Clomipramine HCL	2.775	718542	48569	1.39	5859	3
4	Average		718074.7	49003.33	1.353333	5941.667	
5	S.D		945.0483				
6	% RSD		0.131609				

Observations: The intra & inter day variation [25] of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Clomipramine HCL revealed that the proposed method is precise.

Linearity & Range

To evaluate the linearity [26], serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve [27] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table 10: Linearity Concentrations of Clomipramine HCL

S. No.	Concentration (in ppm)	Peak Area
1	0	0
2	6	457896
3	8	607574
4	10	752268
5	12	896587
6	14	1036579

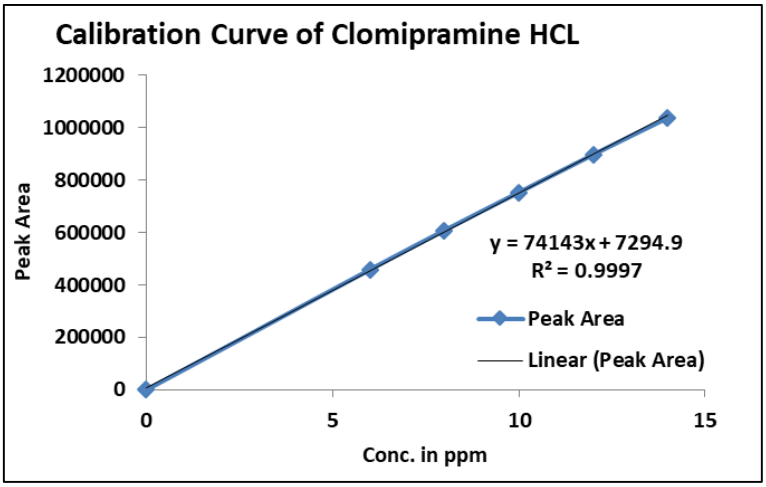

Fig 5: Calibration Curve of Clomipramine HCL

Observation: We observed that the calibration curve showed good linearity in the range of 6-14 µg/ml, for Clomipramine HCL with correlation coefficient (R^2) of 0.9997. A typical calibration curve has the regression equation of y = 74143x + 7294.9 for Clomipramine HCL.

Specificity: Specificity [28] of the pharmaceutical analysis is the ability to measure accurately and specifically the concentration of API, without interference from other active ingredients, diluents, mobile phase. Solutions of mobile phase, sample solution, standard solution were injected into liquid chromatography. Retention times of samples and standard were compared.

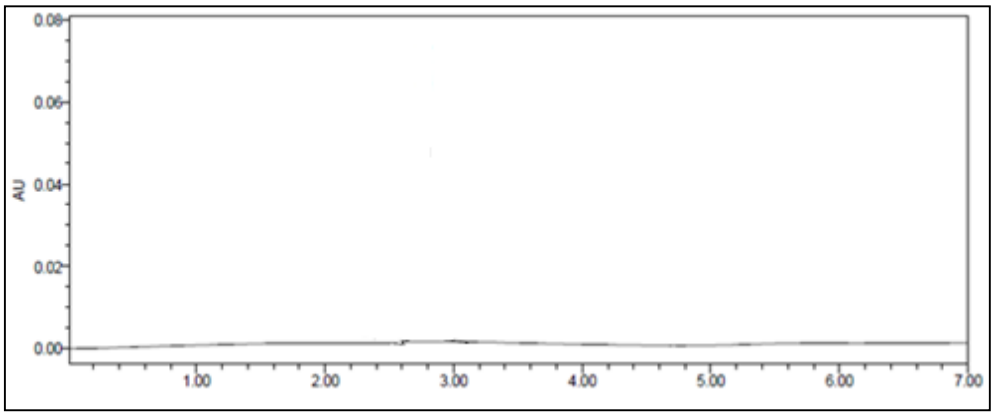

Fig 6: Chromatogram for Blank Solution

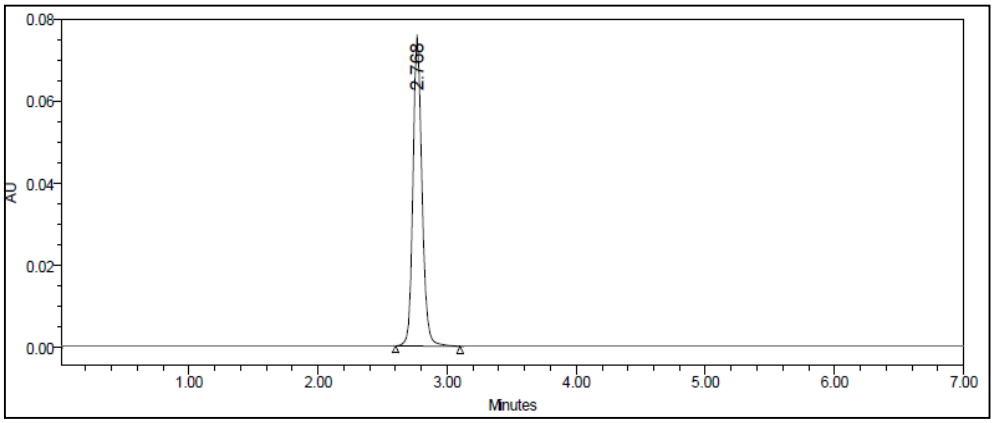

Fig 7: Optimized Chromatogram for Clomipramine HCL Standard

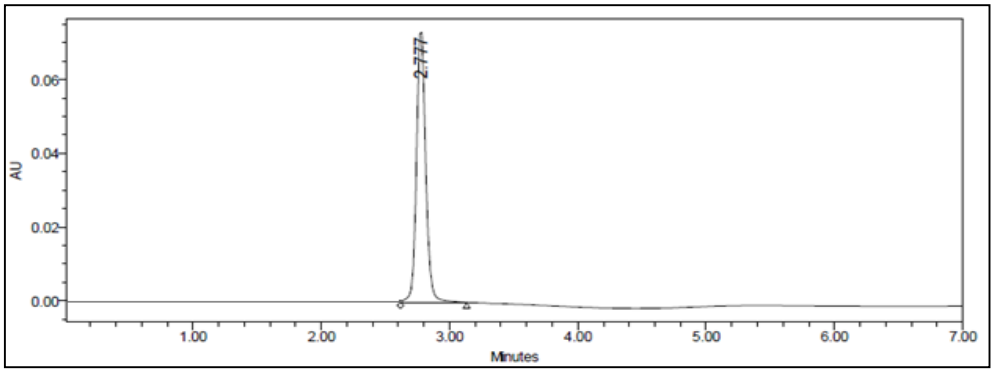

Fig 8: Optimized Chromatogram for Clomipramine HCL Sample

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate 1 ml (± 0.1 ml/min), Wavelength of detection 245 nm (±2 nm) & organic phase content in mobile phase 60 (±5%) studied to determine the robustness [29] of the method are also in favour of (Table-11, % RSD <2%) the developed RP-HPLC method for the analysis of Clomipramine HCL (API).

Table 11: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors
Flow (1.1 ml/min)	5954	1.35
Flow (0.8 ml/min)	6188	1.39
More Organic (70+5)	5748	1.41
Less Organic (70-5)	6185	1.48
Wavelength of Detection (250 nm)	6184	1.69
Wavelength of detection (240 nm)	6247	1.47
Temperature (30 ^{0}C)	6324	1.34
Temperature (20 ^{0}C)	6985	1.32

LOD & LOQ [30]**:** The detection limit (LOD) and quantization limit (LOQ) may be expressed as:
L.O.D. = 3.3(SD/S)
L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response
S = Slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte. The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.507 & 1.539 µg/ml respectively.

Estimation of Clomipramine HCL in Pharmaceutical Dosage Form
Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.
The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.
A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-12.

ASSAY

% Assay=AT/AS×WS/DS×DT/WT×P/100×AW/LC×100

Where
AT = Peak Area of Clomipramine HCL obtained with test preparation
AS = Peak Area of Clomipramine HCL obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg

DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard
Results obtained are tabulated below:

Table 12: Assay[31] of Clomipramine HCL in Clonil-50 Tablets

Brand name of Tablets/Capsules	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Clonil-50 Tablets (Intas Pharmaceuticals Ltd.)	50mg	49.486 (± 0.354)	99.247% (± 0.398)

Result & Discussion: The %Purity of Clonil-50 Tablets containing Clomipramine HCL was found to be 99.247% (± 0.398).

Summary and Discussion

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 245nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: Phosphate Buffer (0.02M) (pH-3.8) (70: 30% v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 7min because analyze gave peak around 2.768min and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 6-14ppm of the Clomipramine HCL target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Clomipramine HCL in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatization or purification steps. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method and validation was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Clomipramine HCL in bulk drug and in Pharmaceutical dosage forms.

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