

## Research Article

# Validation and Peak Purity Assessment of RP-HPLC Method for Simultaneous Estimation of Amitriptyline HCl, Chlordiazepoxide and Comparison with Spectrophotometry

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### ABSTRACT

**Purpose:** The study was performed with the aim to design a Simple, precise and economical RP-HPLC method have been developed for simultaneous estimation of amitriptyline HCl and chlordiazepoxide in pharmaceutical formulation

**Approach:** RP-HPLC was developed using WATERS C18 250 mm × 4.6 mm (5 μm) column, utilizing a mobile phase of methanol: acetonitrile: 0.2 M orthophosphoric acid, pH 4.5 (adjusted with triethylamine) 56:24:20 ml (with a flow rate of 1ml/min) and U.V detection was carried out at 240nm. Optimization of this method was carried out as a function of mobile phase composition, pH and flow rate. Peak purity was assessed by spectral comparison method.

**Findings:** RP-HPLC method shows linearity in the range of for 0.05-1.6 μg/ml and 0.01-0.32 μg/ml amitriptyline HCl and chlordiazepoxide respectively. The LOD and LOQ values for amitriptyline HCl were 0.0250 μg/ml and 0.0759 μg/ml and 0.0045 μg/ml and 0.0135 μg/ml for chlordiazepoxide. The results of % purity was found to be 99.97 ± 0.58 % w/w and 99.29 ± 0.59 % w/w for HPLC method and 98.87 ± 0.03 % w/w and 98.81 ± 1.04 % w/w for first derivative spectrophotometric method for amitriptyline HCl and chlordiazepoxide respectively.

**Conclusion:** Results of descriptive statistics studies conclude that RP-HPLC method found to be more sensitive and convenient than first derivative spectrophotometric method.

**Keywords:** Amitriptyline HCl; Chlordiazepoxide; First Derivative Spectrophotometry; Reverse Phase High Performance Liquid Chromatography.

### INTRODUCTION

Amitriptyline HCl (AMI) is chemically, 3-(10,11-Dihydro-5H-dibenzo [a, d] cyclohepten-5-ylidene)-N,N- dimethyl-1-propanamine is used as an antidepressant. It acts by inhibiting active uptake of biogenic amines noradrenaline, 5-hydroxytryptamine and dopamine into their respective neurons.<sup>1</sup> Chlordiazepoxide (CHLOR) (Figure 1) 7-chloro-N-methyl-5-phenyl-3H-1,4-benzodiazepin-2-amine 4-oxide is used as anxiolytics.<sup>2</sup> AMI and CHLOR are official in IP, BP and USP and these pharmacopeias describes HPLC and titrimetric methods for the estimation of AMI and CHLOR.<sup>3,4,5</sup> Various techniques such as UV method; HPLC with FTIR and UV detection; difference spectrophotometric; micellar liquid chromatography; derivative spectrophotometry; simultaneous equation method have been reported for the individual or simultaneous estimation of AMI and CHLOR.<sup>6,7,8,9</sup> Further, it was found in the literature that simultaneous determination of AMI and CHLOR by using first derivative

spectrophotometry and HPLC are available in combination.<sup>10,11</sup> The above described methods in particular, UV method; derivative spectrophotometry; first derivative and simultaneous equation method and micellar liquid chromatographytime consuming and lacks the required sensitivity.<sup>12,13,14,15</sup> Further, micellar liquid chromatographytechniquerequires specific instrumentation techniques which are not easily available for routine analysis. Therefore, in the present study, method is optimized for effect of mobile phase composition (which influences peaks separation), mobile phase pH (which influences the ionization state of compounds) and flow rate (which influences peaks shape, retention time, and resolution, etc.) by analyzing various chromatographic parameters such as resolution, plates, height equivalent to theoretical plates, symmetry factor, retention time, peak areas, etc. Proposed method utilizes LC technique with UV detection which is the most common and cost effective method. For statistical comparison first derivative spectrophotometer method has been developed and validated.

## MATERIAL AND METHODS

### Instruments

This study was performed on RP-HPLC system of WATERS (Milford, USA) composed of 515 series pumps combined with Waters 2707 auto sampler along with Waters PDA 2998 series photodiode array detector set at wavelength range 190-800 nm with column Spherisorb® C18 bonded with 5 µm (ID: 4.6 mmx 250 mm) coupled with EMPOWER-2 software recording and processing of chromatographic data. Spectrophotometric studies were performed using Perkin Elmer Lambda 35 UV/Visible spectrophotometer coupled with computer loaded with UVWinLab PC software version 6.0.3.

### Chemicals and Reagents

Working standard of AMI and CHLOR were procured as gift sample from Sun Pharmaceuticals (Vadodara, India) with percent purity of 99.56 % and 99.45 % respectively. Acetonitrile, methanol, water, orthophosphoric acid (OPA 85%) and triethylamine

(TEA) were purchased from Rankem, (New Delhi, India). All these chemicals and solvents were of HPLC and spectroscopy grade and were used without further purification. All required solutions were prepared in HPLC grade water. HPLC water was obtained using water purification systems ELIX 03 (MILLIPORE, USA). Unless otherwise specified, all solutions were filtered through a 0.2 µm Ultipor® N66® Nylon 6.6 membrane filter (Pall Life Sciences, USA) prior to use. Marketed formulation of Libotryp-10 (Batch Number-JK10448, Wockhardt Ltd., Solan, India) was procured from local drug store, Moga.

### Preparation of Solutions

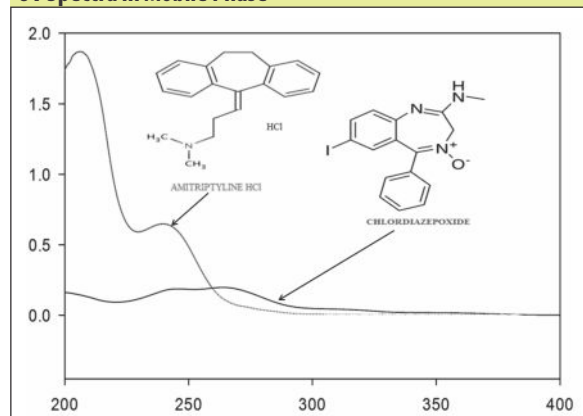
Stock solutions of 1 mg/ml of pure AMI and CHLOR were freshly prepared in methanol for spectrophotometric and chromatographic method. Aliquots of these solutions were diluted stepwise with the mobile phase to obtain 10 µg/ml of AMI and CHLOR. These solutions were used for the optimization of the proposed method.

### RP-HPLC Method Development and Validation

#### Chromatographic Conditions

Analysis was carried out on isocratic at 1.0 ml/min flow rate with mobile phase MeOH: ACN: 0.2M Orthophosphoric acid buffer, pH 4.5 (adjusted with triethylamine) (56:24:20). Mobile phase was prepared freshly every day and premixed, filtered and degassed by using sonicator before use. Absorbance was found to be maximum at 240 nm compare to the other

**Fig. 1: Chemical Structure of AMI and CHLOR along with their UV Spectra in Mobile Phase**



wavelengths so the eluted peaks were measured at 240 nm (Figure 1).

### **Optimization of Chromatographic Conditions**

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase composition varied at MeOH: ACN: 0.2M orthophosphoric acid buffer, pH 4.5 (adjusted with triethylamine) 49:21:30, 56:24:20 and 63:27:10 with flow rate of 1.0 ml/min, respectively. (ii) Change in pH from 4.0, 4.5, and 5.0 with mobile phase composition 56:24:20 respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition 56:24:20 respectively. Moreover, the effects of different level of all these factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry and HETP etc.

### **Validation Parameters**

Once the optimized chromatographic method has been developed it must be validated as per ICH guidelines<sup>15</sup> for linearity, precision, accuracy, sensitivity, robustness and recovery.

### **Linearity**

Linearity was determined by five different concentrations of AMI and CHLOR in triplicate and calibration curves were plotted in specified range of 0.05-1.6 µg/ml and 0.01-0.32 µg/ml for AMI and CHLOR respectively. Calibration curve was plotted by replicate analysis at all concentration levels and linear relationship was evaluated using the least square method within Microsoft Excel<sup>®</sup> program.

### **Precision and Accuracy**

Intra-day precision and inter-day precision was measured in terms of % RSD The experiments were repeated three times a day for intra-day precision and on six different days for inter-day precision. For the developed method recovery studies were also carried out for intra-day and inter-day precision for comparative purpose with the first derivative spectrophotometric method.

### **Sensitivity**

Sensitivity of method was determined by calculating LOB, LOD and LOQ using following equation:

$$\text{LOB} = 1.645 \sigma/S$$

$$\text{LOD} = 3.3 \sigma/S$$

$$\text{LOQ} = 10 \sigma/S$$

Where  $\sigma$  = Standard deviation of the response; S = Slope of the calibration curve

### **Robustness Studies**

Robustness was determined in form of % R.S.D by small deliberate changes in flow rate, mobile phase ratio, pH of mobile phase for HPLC method.

### **Percentage Purity**

Twenty tablets were accurately powdered and weighed equivalent to 12.5 mg and 5 mg of AMI and CHLOR respectively and diluted up to 25 ml with mobile phase. Working dilutions were prepared with mobile phase and used for analysis.

### **Peak Purity by Spectral Analysis**

Peak purity in RP-HPLC method was determined by recording spectrum of peak at minimum of 15 points with Empower-2 software and PDA detector.

### **System Suitability Parameters**

System suitability parameters were studied to verify that the resolution and reproducibility of the system are adequate for the analysis. Parameters such as selectivity, theoretical plates, tailing factor, resolution, capacity factor and asymmetric factor are determined.

### **Method Comparison**

#### **First Derivative Zero Crossing Spectrophotometry**

Absorption spectra of the samples were recorded between 200-400 nm against a reagent blank using a 1.0 cm quartz cell. The zero-order spectra of pure drugs and standard laboratory mixtures (12.5:5 ratios of AMI and CHLOR) and marketed formulation (with same ratio) were recorded and derivatized in first order using scaling factor 13. First derivative amplitudes were recorded at 219 nm and 239 nm for determination of AMI and CHLOR respectively.

## Statistical Analysis

Both of the developed methods were subjected to statistical analysis on the different parameters such as accuracy, precision and calibration curve. Descriptive analysis was also applied on both the methods to know the significance level in terms of accuracy.

## RESULTS AND DISCUSSION

### Optimized Chromatographic Conditions of RP-HPLC Method

#### Effect of Mobile Phase pH

With the aim of the optimization of mobile phase pH (4.0, 4.5, 5.0), the remaining two factors were kept constant, i.e. mobile phase composition (methanol: acetonitrile:0.2M orthophosphoric acid 56:24:20) and flow rate of 1 ml/min. Observed chromatographic responses were plotted against respective pH (Figure 2A and 3A) for AMI and CHLOR respectively. The number of theoretical plate as well as resolution between AMI and CHLOR were highest at pH 4.5. Thus, the best chromatographic separation was achieved at pH 4.5, and hence was considered to be optimum.

#### Effect of Mobile Phase Composition

Mobile phase composition varied at MeOH: ACN:0.2M orthophosphoric acid buffer 49:21:30, 56:24:20 and 63:27:10 with keeping pH 4.5 and flow rate of 1.0 ml/min (Figures 2B and 3B) generally, increasing the organic solvent concentration in the mobile phase induces a decrease in the distance between the solute molecule and the terminal carbon atoms (C18) in the ODS ligand and it results in lower retention time. This may be explained by elution power of mobile phase decreased at 49:21:30 v/v because relative amount of organic phase decreased and AMI and CHLOR were eluted at higher retention time. In contrast to this at 63:27:10, v/v composition, the drug affinity to stationary phase increased due to relatively lower buffer content in mobile phase and resulted in delayed elution. However, at 56:24:20, v/v composition, proper balance was attained between these two situations and resulted in least retention time. Plate number increased with increase in organic composition in mobile phase. However, the

asymmetry value at 63:27:10 v/v was higher than that of at 56:24:20 v/v. These suggest that the increased plates at 63:27:10 v/v was due to higher retention time value (even though it had greater peak width due to tailing). Further, acceptable resolution >2 was achieved at 56:24:20, v/v composition and was considered to be optimum.

#### Effect of Mobile Phase Flow Rate

It can be observed that theoretical plates were highest at flow rate of 1 ml/min with asymmetry of less than 1.5 (Figure 2C and 3C). The change in flow rate has insignificant effect on resolution while retention time decreased as the flow rate increased.

#### Validation Parameters

##### Linearity

Methods were found to be linear over concentration range of 0.05-1.6 µg/ml and 0.01-0.032 µg/ml with equation  $Y=196.3x+3542$  and  $Y=956.3x-6963$  for AMI and CHLOR respectively and Correlation coefficient (r) was found to be 0.9999 for both drugs. The linear regression equations were calculated by the least squares method using Microsoft Excel® program. Overlay of chromatograms of AMI and CHLOR was shown in Figure 4.

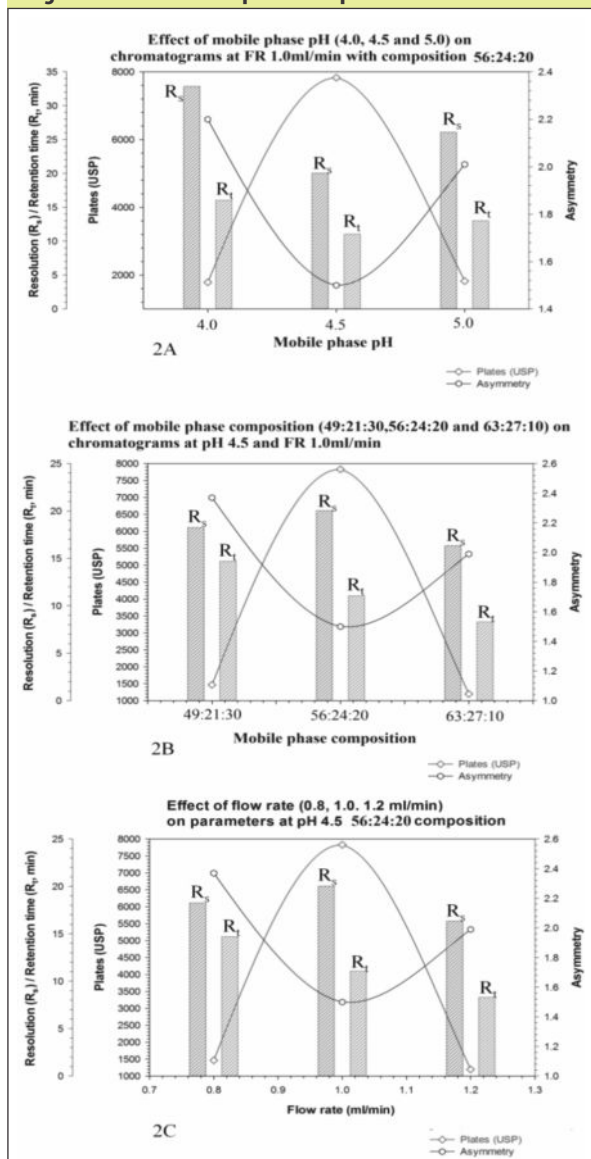
##### Precision and Accuracy

The results of intra-day and inter-day precision were found to be with < 2% R.S.D for AMI and CHLOR. These results of inter-day and intra-day precision suggest that these developed HPLC method was precise and reproducible and to examine the accuracies of method, recovery studies were carried out by standard addition method and results was evaluated by using t- test at three concentration level. Precision and recovery results are explained in (Table 1).

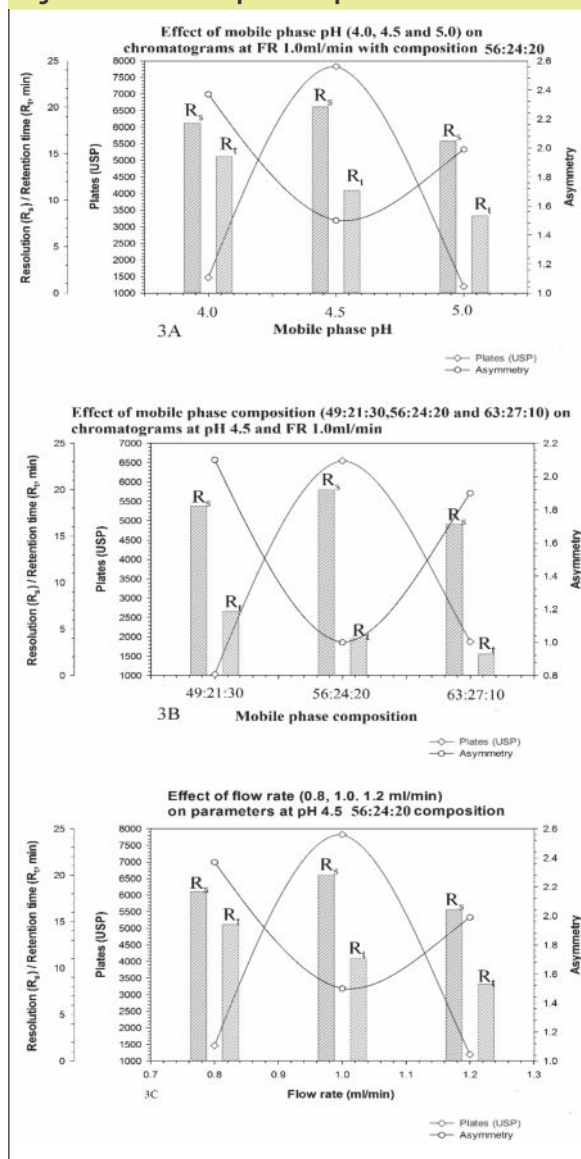
##### Sensitivity

Results of LOB, LOD and LOQ for AMI and CHLOR show that method was very sensitive and specific. This can further help in estimation of these compounds simultaneously at very low concentrations. LOB was found to be 0.0125 and 0.0022, LOD was 0.0250 and 0.0045 and LOQ was 0.0759 and 0.0135 for AMI and CHLOR in µg/ml respectively.

**Fig. 2: Effect of mobile phase composition**



**Fig. 3: Effect of mobile phase composition**



**Peak Purity Assessment**

Peak purity of samples in RP-HPLC method were determined by recording spectra of peak at 18 different points from peak start, peak apex and peak end positions. Figure 5 and 6 shows spectrum comparison of AMI and CHLOR with peak respectively.

**System Suitability Parameters**

System suitability parameters were studied to verify the resolution and reproducibility of the

chromatographic system are adequate for the analysis. A system suitability test according to USP was performed on the chromatograms obtained from standard and test solutions to check different above mentioned parameters and the results obtained from six replicate injection of the standard solution are summarized in (Table 2).

**Robustness Studies**

Robustness of method was determined in form of % R.S.D by small deliberate changes in flow rate, mobile

**Table 1: Results of Recovery Study of AMI and CHLOR by RP-HPLC Method**

Amount Spike (ng/ml)		Amount found (ng/ml)		SD		%RSD		Recovery		$t_{cal}$		CI	
AMI	CHLOR	AMI	CHLOR	AMI	CHLOR	AMI	CHLOR	AMI	CHLOR	AMI	CHLOR	AMI	CHLOR
<b>Intra-day (n=3)</b>													
400	160	399.81	159.74	0.5001	1.0944	0.13	0.69	99.95	99.84	0.95	0.57	0.74	1.63
500	200	499.97	199.33	0.4279	1.5275	0.09	0.77	99.99	99.67	0.17	1.07	0.64	2.27
600	240	599.33	239.67	1.5275	1.1547	0.25	0.48	99.89	99.86	1.07	0.71	2.27	1.72
<b>Inter-day (n=6)</b>													
400	160	398.59	158.41	2.9705	2.3716	0.75	1.50	99.65	99.01	1.16	1.63	4.42	3.53
500	200	498.15	197.98	3.6198	3.1095	0.73	1.57	99.63	98.99	1.25	1.58	5.38	4.62
600	240	597.622	37.38	3.2613	3.6334	0.55	1.53	99.60	98.91	1.78	1.75	4.85	5.40

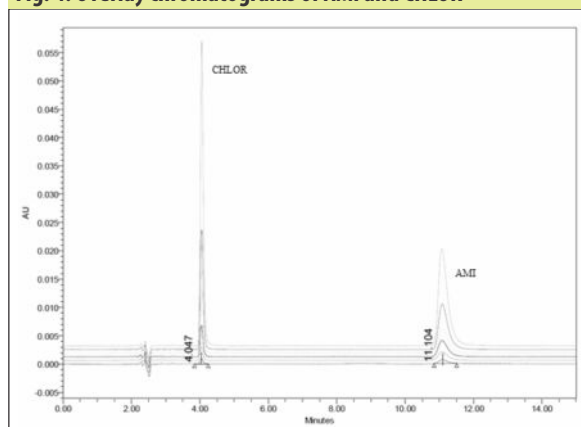
phase ratio, pH of mobile phase. The results of robustness studies are shown in (Table 3) and Figure 7 and 8.

### Comparison of Method

For comparison of the developed method first derivative spectrophotometric method was used. The 219 nm for AMI and 239 nm for CHLOR was the lambda max. Linearity  $Y=0.045X + 0.181$  and  $Y=0.028X - 0.00$  with r value is 0.9999 was obtained for both drugs. The results of % purity was found to be  $99.97 \pm 0.58$  % w/w and  $99.29 \pm 0.59$  % w/w for HPLC method and  $98.87 \pm 0.03$  % w/w and  $98.81 \pm 1.04$  % w/w for first derivative spectrophotometric method for AMI and CHLOR respectively.

### Statistical Analysis

These two methods were compared by applying the F-test. The calculated F-value of 12.79 for AMI and 1.35 for CHLOR are less than the tabulated F-value at the 95% confidence interval, which shows that there is insignificant difference with respect to accuracy and precision of the proposed method. Results of descriptive statistical analysis are shown in (Table 4).

**Fig. 4: Overlay chromatograms of AMI and CHLOR**

**Table 2: System Suitability Parameters Validated RP-HPLC Method**

SST Parameters	AMI	CHLOR
Capacity factor (k)	21.12	19.23
Separation factor ( $\alpha$ )	12.23	-
Theoretical plates (USP)	7828.71	6548.05
HETP (mm)	0.0032	0.0099
Resolution ( $R_s$ )	22.50	-
Asymmetry ( $A_s$ )	1.5	1.1
Tailing factor ( $T_s$ )	1.25	1



Fig. 7: Robustness of RP-HPLC Method for AMI

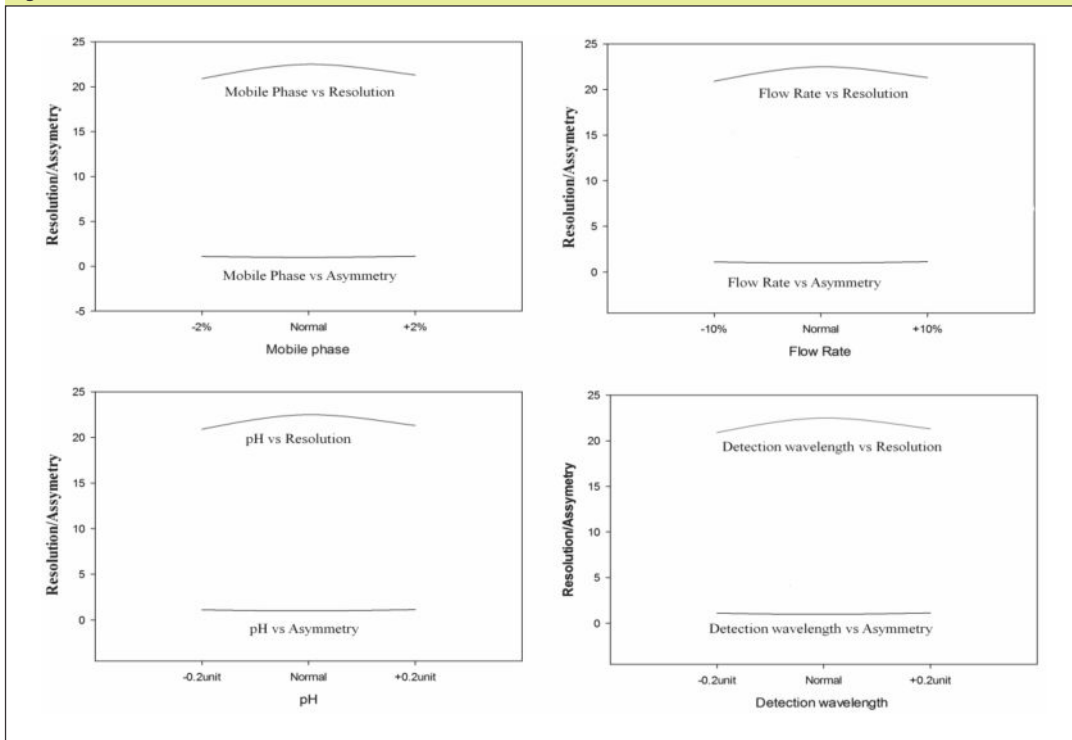
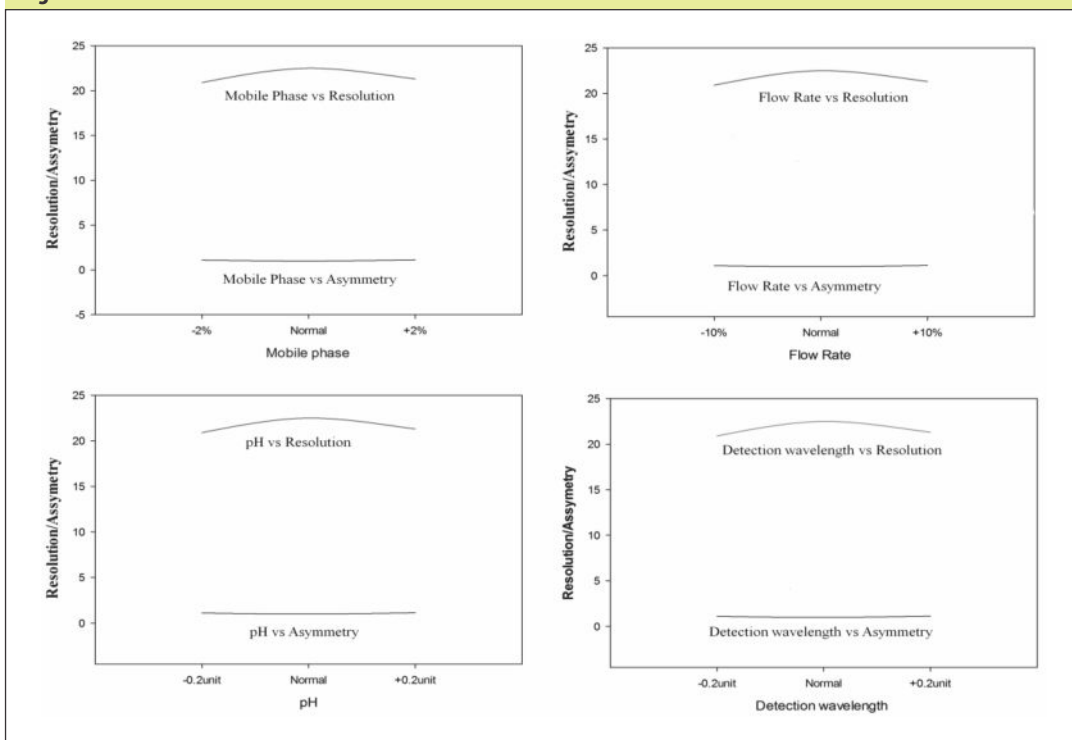


Fig. 8: Robustness of RP-HPLC Method for CHLOR



**Table 3: Results of robustness studies**

Parameters	Alterations	% RSD for Retention time (n=6)		% RSD for Area (n=6)		% RSD for Assay (n=6)	
		AMI	CHLOR	AMI	CHLOR	AMI	CHLOR
Mobile phase	-2%	0.3144	0.2813	0.2935	0.3984	0.4871	0.3971
	Normal	0.2456	0.1568	0.21	0.3871	0.4813	0.4103
	+2%	0.3467	0.1782	0.2974	0.3910	0.4319	0.4209
Flow rate	-10%	0.4117	0.3962	0.1298	0.5401	0.3042	0.5012
	Normal	0.3891	0.5781	0.1123	0.6017	0.1892	0.5148
	+10%	0.4219	0.6109	0.1492	0.3912	0.2791	0.6016
pH	-0.2unit	0.4024	0.4592	0.2017	0.4309	0.3921	0.7109
	Normal	0.3876	0.4813	0.2003	0.4812	0.4506	0.6710
	+0.2unit	0.3917	0.4791	0.1982	0.5406	0.5106	0.4156
Detection wavelength	-0.2unit	0.3187	0.5491	0.3176	0.6079	0.2984	0.3901
	Normal	0.3209	0.59120	.2457	0.6182	0.2135	0.3802
	+0.2unit	0.3396	0.5871	0.2789	0.6254	0.2007	0.3981

**Table 4: Descriptive Statistical Analysis Parameters of AMI and CHLOR on First Derivative Spectrophotometry and RP- HPLC Accuracy Results**

Parameters	UV		HPLC	
	AMI	CHLOR	AMI	CHLOR
Maximum	99.780	99.380	99.990	99.860
Minimum	99.210	98.890	99.600	98.910
Median	99.505	99.065	99.770	99.340
Mean	99.503	99.082	99.785	99.380
S.D.	0.195	0.591	0.177	0.255
S.E.	0.080	0.278	0.072	0.086
CI of mean	0.205	0.400	0.186	0.278
Skewness	-0.149	0.641	0.095	0.059
Kurtosis	0.267	-0.828	-2.896	-3.061
K-S distribution	0.168	0.238	0.277	0.292
K-S probability	0.697	0.331	0.160	0.114
Swilk W	0.974	0.894	0.835	0.7955
Wilk Probability	0.916	0.342	0.118	0.053

## CONCLUSION

In the present work, a new rapid, simple and sensitive RP-HPLC method has been developed, optimized and validated for the estimation of API and CHLOR

simultaneously using UV detector at isocratic elution. Optimization showed that the mobile phase pH and composition are crucial parameters to be controlled than flow rate for reproducible and quantitative estimation of the API and CHLOR. Method was found to be linear over an analytical range of 0.05–1.6 and 0.01–0.32 µg/ml with LOD 0.0250 and 0.0045 and LOQ 0.0759 and 0.0135 µg/ml, for AMI and CHLOR respectively. The results of descriptive statistics studies conclude that RP-HPLC method found to be more sensitive and convenient than first derivative spectrophotometric method. This method can be used for routine quantitative estimation of both components in tablet dosage form.

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