Chapter 4

Analytical Methods

4.1 INTRODUCTION

The present study reveals UV spectrophotometric methods for estimation of amoxicillin and levofloxacin and colorimetric method for clarithromycin. These methods were utilized for estimating drug release during dissolution studies. High Performance Liquid Chromatography (HPLC) methods were developed for estimation of amoxicillin, levofloxacin and clarithromycin and these methods were utilized for estimating drug content (assay) in the formulations.

4.2 AMOXICILLIN TRIHYDRATE

4.2.1 Materials

Amoxicillin trihydrate was received as a gift sample from Aristo Pharmaceuticals Ltd. (Mumbai, India). Hydrochloric acid, acetonitrile, monobasic potassium phosphate and potassium hydroxide were purchased from Qualigens Fine Chemicals (Mumbai, India).

4.2.2 Methods

4.2.2.1 UV Spectrophotometric Method

4.2.2.1.1 Linearity

It is ability of the method to obtain test results which are proportional to concentration of analyte (Hadjicostas, 2003). Stock solution was prepared by dissolving 57.385 mg of amoxicillin trihydrate (equivalent to 50.0 mg of amoxicillin) in upto 100 ml of 0.1N HCl. Suitable aliquots of stock solution (0.5-3.5 ml) were pipetted into 50 ml volumetric flask and volume was made up with 0.1N HCl. Absorbance was measured at λmax 229 nm (UV-1700, Shimadzu, Japan).

4.2.2.1.2 Specificity

Specificity is ability of a test method to determine accurately the analyte in presence of other components in sample matrix under stated conditions of test (Hadjicostas, 2003). Amoxicillin was estimated in presence of various excipients used in minimatrices as well as softgel formulation. Placebo blend was dispersed in 100 ml of 0.1 N HCl and sonicated for 10 -20 min. Filtered portion was suitably diluted and absorbance was measured at 229 nm.

4.2.2.1.3 Solution Stability

Solutions of linearity test were used for this purpose. Stability of the solutions was ascertained by observing changes in absorbance values upto 12 h.

4.2.2.1.4 Accuracy and precision

Accuracy indicates closeness of the result to the true value while precision is closeness of agreement between independent test results obtained under stipulated conditions (Hadjicostas, 2003). In order to determine accuracy and precision, known amount of drug was subjected to recovery study in triplicate and the variation in recovered amount was studied.

4.2.2.2 High Performance Liquid Chromatography (HPLC) Method

4.2.2.2.1 Method Parameters

HPLC method as per United States Pharmacopeia (USP) was implemented for estimation of drug content. Mobile phase contained buffer solution and acetonitrile. Buffer solution was prepared by dissolving 13.6 gm of monobasic potassium phosphate in 2000 ml of distilled water. pH was adjusted to 5.0±0.1 with potassium hydroxide solution. Buffer solution and acetonitrile were mixed in proportion 96:4 and the mixture was filtered through 0.2 micron nylon filter paper (Pall Life Sciences). Separation was carried out on C18 column having dimensions 250 mm x 4.6 mm with 5 µ particle size. Mobile phase was run at flow rate of 1.0 ml/min and detection was carried at 230 nm. Injection volume was 20 µl. The HPLC system was Shimadzu SPD-20A / LC-20AT.

4.2.2.2.2 Linearity

Standard stock solution was prepared by dissolving 57.385 mg of amoxicillin trihydrate (equiv to 50 mg of amoxicillin) in upto 50 ml of mobile phase. This solution (5 ml) was further diluted to 50 ml to obtain a standard stock solution having concentration of 100 µg/ml. Further dilutions were prepared by diluting 0.5,1,1.5,2,2.5,3 and 3.5 ml of standard stock solution to 10 ml with mobile phase. Individual solutions were injected and peak area for the principal peak was noted. Area obtained for individual solution was determined from the chromatogram and linearity curve was obtained by plotting concentration versus area. Experimental set was run in triplicate.

4.2.2.2.3 Specificity

Specificity of the method in estimating amoxicillin in presence of the excipients used in minimatrices and softgel was carried out. Placebo blend was dispersed in mobile phase, sonicated and filtered and suitable dilutions were injected to detect presence of any peak at the retention time corresponding to that of amoxicillin.

4.2.2.2.4 Solution Stability

Calibration curve solutions (three different concentrations) were utilized for checking stability. The prepared solutions were injected at specific interval upto 6 h and peak area for corresponding solution was measured and compared with initial one.

4.2.2.2.5 Accuracy and precision

In order to determine accuracy and precision, known amount of drug was subjected to recovery study in triplicate and the variation in recovered amount was studied.

4.2.3 Results and Discussion

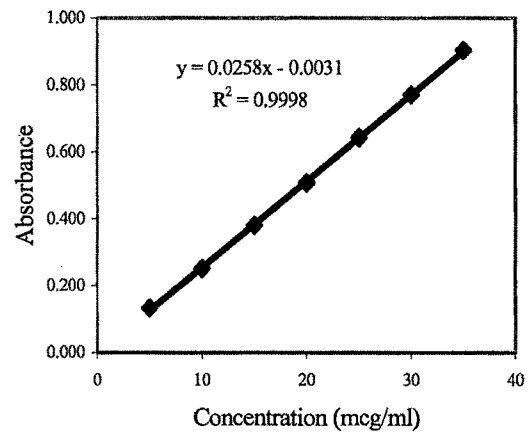
4.2.3.1 UV Spectrophotometric Method

Experimental set was run in triplicate and mean absorbance values were considered for plotting linearity curve (Table 4.1). Linearity was plotted in the range 5 to 35 mcg/ml (Fig 4.1a). Maximum absorbance was observed at 229 nm (Fig 4.1b) and hence further estimations were carried out at this wavelength. Regression analysis was carried out on the experimental data and the equation obtained was y = 0.0258x-0.0031. Good linearity was observed as R^2 value was 0.9998.

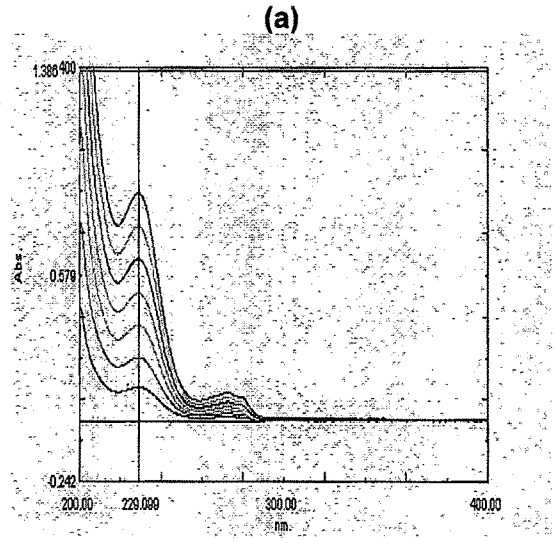
The method was specific because excipients were found non-interfering as there was no any absorbance for placebo sample at 229 nm which is λmax for amoxicillin. Calibration curve solutions were used for determining solution stability. Initial absorbance values and the values observed after 12 h did not differ much indicating that the solutions were stable upto that period of time. The method was also accurate and precise as the results of recovery studies were close to the actual value and difference in the results of different samples was minor.

Table 4.1 Calibration curve of amoxicillin by UV method

Concentration (mcg/ml)	Absorbance				Std. Deviation	%RSD
	Set I	Set II	Set II	Mean		
5	0.133	0.131	0.135	0.133	0.0020	1.5038
10	0.248	0.255	0.251	0.251	0.0035	1.3973
15	0.382	0.377	0.383	0.381	0.0032	0.8445
20	0.507	0.514	0.502	0.508	0.0060	1.1873
25	0.635	0.644	0.651	0.643	0.0080	1.2468
30	0.767	0.771	0.775	0.771	0.0040	0.5188
35	0.909	0.897	0.904	0.903	0.0060	0.6673



(a)



(b)

Fig 4.1 (a) Calibration curve, and (b) UV Spectra for calibration curve of amoxicillin

4.2.3.2 HPLC Method

Retention time for amoxicillin peak was 3.9 min and tailing factor was 1.19. Beer's law was obeyed in the range 5 to 35 mcg/ml. Regression equation obtained was y = 15.539x+8.6 (Fig 4.2a). Coefficient of determination (R^2) was 0.9993 indicating good linearity. HPLC chromatograms for the calibration plot are shown in Fig 4.2b.

Table 4.2 Calibration curve by HPLC

Concentration (mcg/ml)	Area				Std. Deviation	%RSD
	Set I	Set II	Set II	Mean		
5	90.1	92.8	93.3	92.1	1.7214	1.8698
10	162.8	160.3	164.2	162.4	1.9757	1.2163
15	235.9	230.3	236.1	234.1	3.2924	1.4064
20	315.7	318.8	320.7	318.4	2.5239	0.7927
25	397.2	400.4	406.8	401.5	4.8881	1.2176
30	471.2	469.3	476.8	472.4	3.8991	0.8253
35	553.3	549.4	561.6	554.8	6.2308	1.1231

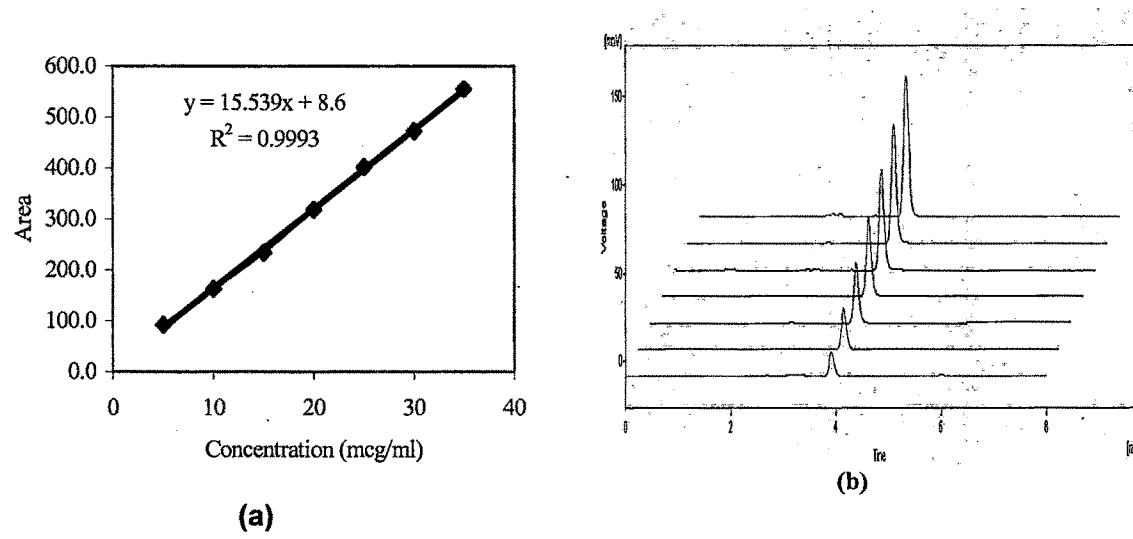
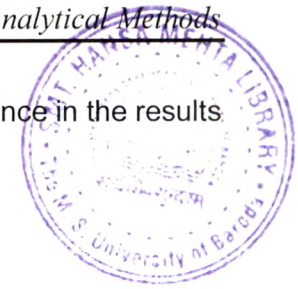


Fig 4.2 (a) Calibration curve (b) HPLC chromatogram of calibration curve of amoxicillin

Excipients were found to be non-interfering as the placebo solution did not show any peak at 3.9 min which was retention time for amoxicillin. Calibration curve solutions were used for determining solution stability. Peak area for freshly prepared samples and the samples stored for 6 h did not differ much indicating that the solutions were stable upto that period of time. The method was also accurate and precise as the



results of recovery studies were close to the actual value and difference in the results of different samples was minor.

4.3 LEVOFLOXACIN HEMIHYDRATE

4.3.1 Materials

Levofloxacin was received as a gift sample from Blue Cross Labs Ltd, (Nashik, India). Acetonitrile, triethylamine and hydrochloric acid were purchased from Qualigens Fine Chemicals (Mumbai, India).

4.3.2 Method

4.3.2.1 UV Spectrophotometric Method

4.3.2.1.1 Linearity

Levofloxacin stock solution was prepared by dissolving 51.245 mg of Levofloxacin hemihydrate (equivalent of 50.0 mg of levofloxacin) in upto 100 ml of 0.1N HCl and further diluting 40 ml of this solution to 100 ml. Suitable aliquots of stock solution (1 – 6 ml) were pipetted into 100 ml volumetric flask and volume was made up with 0.1N HCl. Absorbance was measured at λmax 293.6 nm.

4.3.2.1.2 Specificity

Ability of the method to specifically estimate levofloxacin in presence of minimatrix and softgel excipients was investigated. Placebo blend was dispersed in 0.1 N HCl and suitably diluted samples were subjected for absorbance measurement at 293.6 nm.

4.3.2.1.3 Solution Stability

Solution stability of the calibration curve solutions was ascertained by observing changes in absorbance values, at 293.6 nm, upto 12 h.

4.3.2.1.4 Accuracy and precision

It was determined as described in section 4.2.2.2.5

4.3.2.2 High Performance Liquid Chromatography (HPLC) Method

4.3.2.2.1 Method Parameters

HPLC method was developed for estimation of levofloxacin. Mobile phase was prepared by mixing water, acetonitrile and triethylamine in 50:50:0.1 proportion. pH was adjusted to 3.0±0.1 with orthophosphoric acid and mobile phase was filtered

through 0.2 micron nylon filter paper (Pall Life Sciences). Octadecylsilane (C18) column having dimensions 250 mm x 4.6 mm with 5µ particle size was used for carrying out separation. Mobile phase flow rate was 1.0 ml/min and detection was carried at 293.6 nm. Injection volume was 20 µl.

4.3.2.2.2 Linearity

Standard stock solution was prepared by dissolving 25.62 mg of levofloxacin hemihydrate (equivalent of 25.0 mg of levofloxacin) in upto 25 ml of mobile phase and diluting 1 ml of this solution to 50 ml with mobile phase. For plotting calibration curve, solutions having concentration 2,4,6,8,10,12 and 14 µg/ml were prepared by diluting 1 to 7 ml of standard stock solution to 10 ml with mobile phase. These solutions were individually injected and peak area obtained for the respective solution was determined from the chromatogram. Linearity curve was obtained by plotting concentration versus area. Experimental set was run in triplicate.

4.3.2.2.3 Specificity

Ability of the method to specifically estimate levofloxacin in presence of minimatrix and softgel excipients was investigated. Placebo blend was dispersed in mobile phase and suitably diluted samples were injected to ascertain presence of any peak at retention time corresponding to levofloxacin peak.

4.3.2.2.4 Solution Stability

Calibration curve solutions were utilized for checking stability. The prepared solutions were injected at specific interval upto 6 h and peak area for corresponding solution was measured and compared with initial one.

4.3.2.2.5 Accuracy and precision

It was determined as described in section 4.2.2.2.5

4.3.3 Results and Discussion

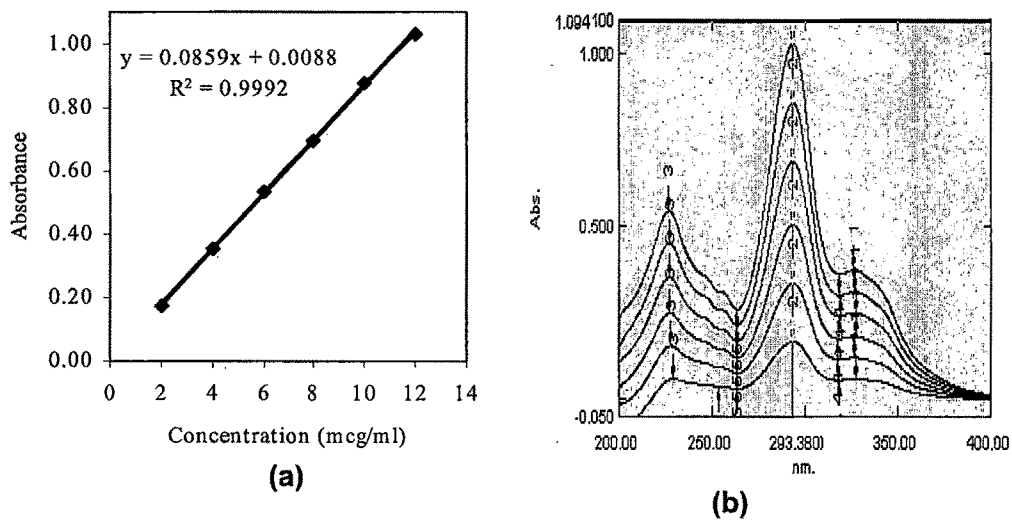
4.3.3.1 UV Spectrophotometric Method

Experimental set was run in triplicate and mean absorbance values were considered for plotting calibration curve (Table 4.3). Linearity was plotted in the range 2 to 12 mcg/ml. Regression equation obtained was y = 0.0859x+0.0088. Coefficient of determination (R^2) value was 0.9992 indicating good linearity. An absorbance

spectrum of levofloxacin in 0.1N HCl (Fig. 4.3b) shows 293.6 nm as wavelength of maximum absorbance i.e. λmax.

Table 4.3 Calibration curve of levofloxacin in 0.1N HCl

Concentration (mcg/ml)	Absorbance				Std. Deviation	%RSD
	Set I	Set II	Set II	Mean		
2	0.172	0.175	0.171	0.173	0.0021	1.2056
4	0.352	0.347	0.355	0.351	0.0040	1.1503
6	0.535	0.541	0.532	0.536	0.0046	0.8550
8	0.691	0.698	0.702	0.697	0.0056	0.7988
10	0.870	0.889	0.872	0.877	0.0104	1.1905
12	1.035	1.012	1.038	1.028	0.0142	1.3832



(a) (b)

Fig 4.3 (a) Calibration curve and (b) UV Spectra for calibration curve of levofloxacin

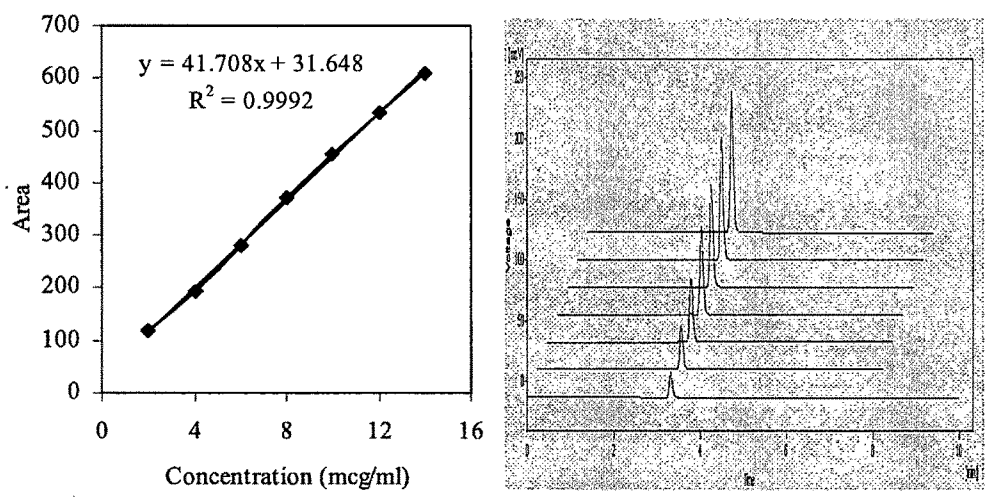
The excipients were found non-interfering as there was no any absorbance for placebo sample at 293.6 nm which is λmax for levofloxacin. Calibration curve solutions were used for determining solution stability. Initial absorbance values and the values observed after 12 h did not differ much indicating that the solutions were stable upto that period of time. The method was also accurate and precise as the results of recovery studies were close to the actual value and difference in the results of different samples was minor.

4.3.3.2 HPLC Method

Linearity was plotted in the range 2 to 14 mcg/ml. Regression equation obtained for this plot was y = 41.708x+31.648 (Fig 4.4a). R^2 value was 0.9992 indicating good linearity. Retention time for levofloxacin peak was 3.3 min and tailing factor was 1.33. HPLC chromatograms for the calibration plot are shown in (Fig 4.4b).

Table 4.4 Calibration curve by HPLC

Concentration (mcg/ml)	Area				Std. Deviation	%RSD
	Set I	Set II	Set II	Mean		
2	116.5	119.2	115.1	116.9	2.084	1.782
4	190.5	193.2	192.2	192.0	1.365	0.711
6	280.4	275.6	282.6	279.5	3.580	1.281
8	367.5	375.9	372.8	372.1	4.248	1.142
10	450.3	460.4	451.3	454.0	5.565	1.226
12	534.3	521.3	540.7	532.1	9.885	1.858
14	616.2	610.2	605.3	610.6	5.459	0.894



(a) (b)

Fig 4.4 (a) Calibration curve (b) HPLC chromatogram of calibration curve of levofloxacin

Excipients were found to be non-interfering as the placebo solution did not show any peak at 3.3 min which was retention time for levofloxacin. Calibration curve solutions were used for determining solution stability. Peak area for freshly prepared samples and the samples stored for 6 h did not differ much indicating that the solutions were stable upto that period of time. The method was also accurate and precise as the

results of recovery studies were close to the actual value and difference in the results of different samples was minor.

4.4 CLARITHROMYCIN

Colorimetric method (Kuchekar et al., 2003) was used for estimation of clarithromycin during dissolution study. HPLC method was used estimating drug content (assay)

4.4.1 Materials

Clarithromycin was received as a gift sample from Gujarat Liqui Pharma Caps Pvt Ltd., (Baroda, India). Acetonitrile, orthophosphoric acid, sodium dihydrogen orthophosphate, Folin ciocalteu reagent, sodium bicarbonate and hydrochloric acid were purchased from Qualigens Fine Chemicals (Mumbai, India).

4.4.2 Method

4.4.2.1 Colorimetric Method

4.4.2.1.1 Linearity

Stock solution of clarithromycin was prepared by dissolving 50.0 mg of Clarithromycin in upto 50 ml of 0.1N HCl. Suitable aliquots of stock solution (0.1 – 0.6 ml) were pipetted into 10 ml volumetric flask. In each flask, 2 ml of Folin Ciocalteu reagent (1 part diluted with 2 parts of distilled water) and 2 ml of 20% sodium carbonate solution was added. Volume was made up with 0.1N HCl. Solutions were mixed properly and allowed to stand for 20 minutes for proper colour development. Absorbance was measured at λmax 760 nm.

4.4.2.1.2 Specificity

This test was performed to ascertain that the Folin reagent formed coloured complex with only clarithromycin and not with any of the excipients used in minimatrices and softgel. Excipients were dispersed / dissolved in 0.1N HCl and individually treated with folin ciocalteu reagent in presence of sodium bicarbonate solution.

4.4.2.1.3 Solution stability

Stability of the coloured complex was determined till 2 h by determining absorbance at 760 nm.

4.4.2.1.4 Accuracy and Precision

It was performed as described in section 4.2.2.1.4

4.4.2.2 HPLC Method

4.4.2.2.1 Method Parameters

HPLC method was developed for estimation of clarithromycin. Buffer was prepared by dissolving 0.975 gm of sodium dihydrogen orthophosphate in upto 250 ml of water. Mobile phase was prepared by mixing buffer and acetonitrile in 30:70 proportion and pH was adjusted to 4.0±0.1 with orthophosphoric. The mixture was filtered through 0.2 micron nylon filter paper (Pall Life Sciences) using vacuum pump. Octadecylsilane (C18) column having dimensions 250 mm x 4.6 mm with 5µ particle size was used for carrying out separation. Mobile phase was flow rate of 1.0 ml/min and detection wavelength was 205 nm. Injection volume was 20 µl.

4.4.2.2.2 Linearity

Standard stock solution was prepared by dissolving 25 mg of clarithromycin in upto 25 ml of mobile phase. For plotting calibration curve, 0.2 to 1.2 ml of standard stock solution was diluted to 10 ml with mobile phase to obtain solutions having concentration 20,40,60,80,100 and 120 µg/ml. These solutions were individually injected and peak area obtained for the respective solution was determined from the chromatogram. Linearity curve was obtained by plotting concentration versus area. Experimental set was run in triplicate.

4.4.2.2.3 Specificity

Ability of the method to estimate clarithromycin in presence of the excipients, used in minimatrices and softgel, was carried out. Placebo blend was dispersed in mobile phase, sonicated and filtered and suitable solutions were injected to detect presence of any peak at the retention time corresponding to that of amoxicillin.

4.4.2.2.4 Solution Stability

Calibration curve solutions were utilized for checking stability. The prepared solutions were injected at specific interval upto 6 h and peak area for corresponding solution was measured and compared with initial one.

4.4.2.2.5 Accuracy and precision

It was determined as described in section 4.2.2.2.5

4.4.3 Results and Discussion

4.4.3.1 Colorimetric Method

Experimental set was run in triplicate and mean absorbance values were considered for plotting calibration curve (Table 4.5). Calibration curve was plotted in the concentration range 10 to 60 µg/ml. Regression equation obtained for the calibration plot was y = 0.0079x+0.0121. Coefficient of determination (R^2) value was 0.9992 indicating good linearity. Absorbance spectra of clarithromycin solutions prepared for calibration plot are shown Fig.4.5b. It shows 760 nm as wavelength of maximum absorbance.

Table 4.5 Calibration curve of clarithromycin by colorimetric method

Concentration (mcg/ml)	Absorbance				Std. Deviation	%RSD
	Set I	Set II	Set II	Mean		
10	0.089	0.087	0.086	0.087	0.0015	1.75
20	0.171	0.176	0.177	0.175	0.0032	1.84
30	0.247	0.249	0.242	0.246	0.0036	1.47
40	0.332	0.332	0.329	0.331	0.0017	0.52
50	0.408	0.395	0.398	0.400	0.0068	1.70
60	0.484	0.492	0.481	0.486	0.0057	1.17

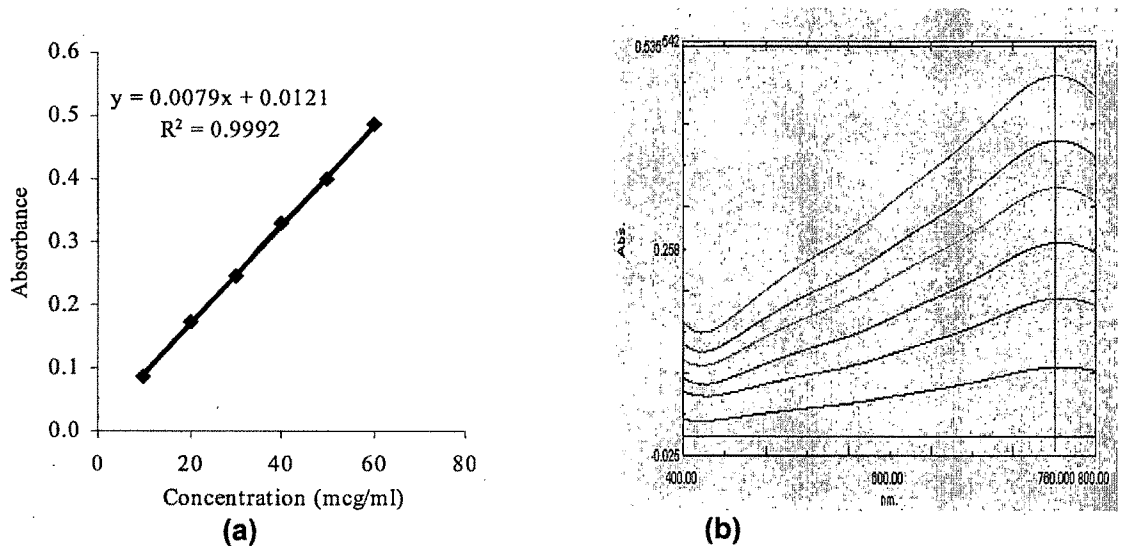


Fig 4.5 (a) Calibration curve and (b) Spectra for calibration curve of clarithromycin

The method was found to be specific as excipients did not show any colour formation after treatment with folin ciocalteu reagent. Calibration curve solutions were used for determining coloured complex stability. Initial absorbance values and the values observed after 2 h did not differ much indicating that the coloured complex was stable upto that period of time. The method was also accurate and precise as the results of recovery studies were close to the actual value and difference in the results of different samples was minor.

4.4.3.2 HPLC Method

Calibration curve was plotted in the range 20 to 120 mcg/ml. Regression equation obtained for this plot was y = 0.9642x+0.3622 (Fig 4.6a) and R^2 value was 0.9998 indicating good linearity. Clarithromycin peak was obtained at 3.5 min and tailing factor for this peak was 1.3. HPLC chromatograms for the calibration plot are shown in (Fig 4.6b). Excipients were found to be non-interfering as these did not show any peak at retention time corresponding to clarithromycin peak.

Table 4.6 Calibration curve by HPLC

Concentration (mcg/ml)	Area				Std. Deviation	%RSD
	Set I	Set II	Set II	Mean		
20	19.4	19.2	19.8	19.5	0.306	1.569
40	39.1	40.2	39.2	39.5	0.608	1.540
60	58.3	56.2	57.9	57.5	1.115	1.940
80	77.6	76.3	78.9	77.6	1.300	1.675
100	98.8	97.3	96.2	97.4	1.305	1.339
120	116.9	115.8	114.3	115.7	1.305	1.128

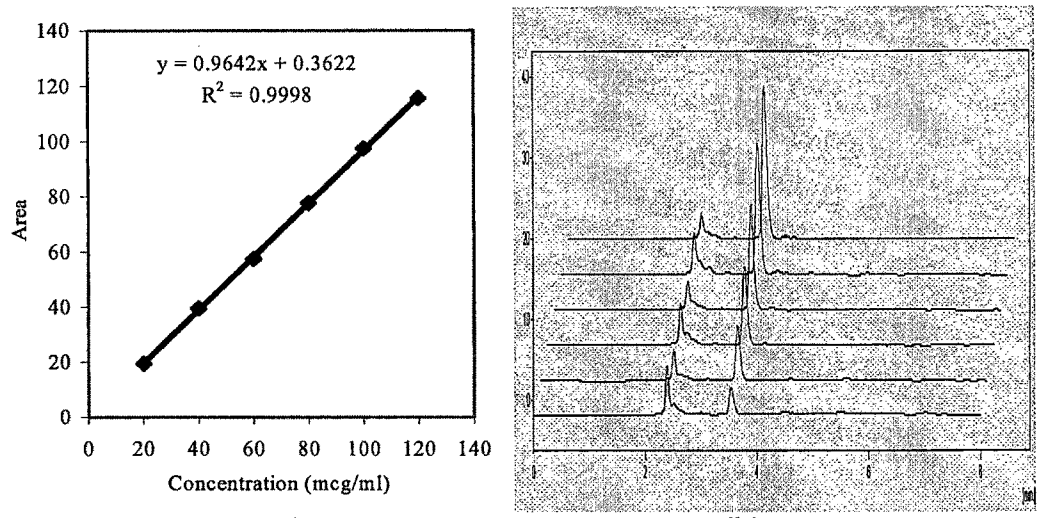


Fig 4.6 (a) Calibration curve (b) HPLC chromatogram of calibration curve of clarithromycin

Excipients were found to be non-interfering as the placebo solution did not show any peak at 3.5 min which was retention time for clarithromycin. Calibration curve solutions were used for determining solution stability. Peak area for freshly prepared samples and the samples stored for 6 h did not differ much indicating that the solutions were stable upto that period of time. The method was also accurate and precise as the results of recovery studies were close to the actual value and difference in the results of different samples was minor.