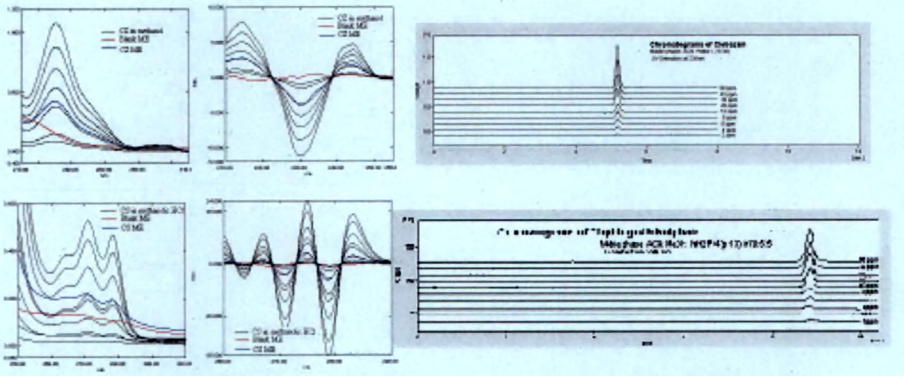
Chapter 3



Analytical Methods

3.1 INTRODUCTION

Analytical methods are important tools to estimate the drug content in the formulations and assess the stability of the drugs in the formulations over the period of time. The analytical methods are of volumetric methods and instrumental methods. Instrumental methods have advantages over volumetric methods because of their sensitivity, low sample requirement and accuracy. UV spectrophotometric method is the simplest instrumentation method capable of drug estimation in micrograms. In the presence of interfering components, derivative spectroscopy is used for drug estimation. Derivative spectroscopy finds application in resolving the interferences contributed by the excipients while estimating the drug content. This is done by selecting the zero-order crossing point of the excipient on the derivative spectra of drug. The concept of derivatizing spectral data was first introduced in 1950s, when it was shown to have many advantages. (1) Precise determination of the wavelength of peak maxima can be obtained from the zero crossing of the first derivative. (2) Improved spectral resolution is obtained, especially with the second derivative. Spectral features which appear as barely noticeable shoulders in the original spectrum become much more prominent. (3) Quantitative analysis can be performed in the presence of turbidity. HPLC method is more sophisticated method used for the estimation of samples with very low quantity of the drug, especially used for estimation of drugs in the biological samples.

3.2 MATERIALS AND INSTRUMENTS

3.2.1 Instrument and software for UV spectrophotometric measurement

Spectrophotometric measurements were carried out on a Shimadzu 1700 double beam UV Visible spectrophotometer with a fix slit width of 1nm coupled HP7540 computer loaded with UV PC software of version 2.10. The spectral bandwidth was 1 nm and the wavelength scanning speed was 2800 nm/min. Matched quartz cuvettes (1cm) were used for all the spectral measurements.

3.2.2 Instrument and software for HPLC measurement

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of Shimadzu LC-20 At Prominence solvent delivery module, a manual Rheodyne injector with a 20µl fixed loop and SPD-20A Prominence UV-Visible detector. The separation was performed on a Phenomenex C18 column (particle size 5µm, length 250mm X ID

4.6mm; Phenomenex Torrance, USA). Chromatographic data were recorded and processed using Spinchrome Chromatographic Station® CFR Version 2.4.0.193 (Spinchrome Pvt.Ltd., Chennai, India).

3.3 METHODS

3.3.1 UV spectroscopic method

3.3.1.1 Methodology

Preparation of stock solution of drugs:

Stock solution containing 1mg/ml was prepared by dissolving drug in the solvent.

Preparation of standard solution of drugs:

Standard solutions were prepared by pipetting out required volume of stock solution in 10 ml volumetric flasks and making the volume up to the mark with solvent to obtain known final concentrations in µg/ml. The spectras of the standard solutions were recorded using UV Visible spectrophotometer for 200nm to 400nm range against solvent as blank. The observations were recorded in triplicate.

Estimation of drugs in excipients and formulations:

A definite volume of the sample to be estimated like supernatant of the saturated excipients and formulations like solution, microemulsion and mucoadhesive microemulsion was taken in a 10ml volumetric flask and diluted up to the mark with solvent. The resultant solution was then sonicated for 2 min at ambient temperature and the spectra of the standard solutions was recorded using UV Visible spectrophotometer for 200nm to 400nm range against solvent as blank. The observations were recorded in triplicate.

3.3.1.2 Method validation

1. Linearity and Range:

Linearity of an analytical method is the ability to elicit the test results that are directly or by well defined transformation proportional to the concentration of the analyte in the samples within the given range.

2. Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined by calculating the recoveries of the analyte by the method of standard additions.

Known amounts of standard drug (80%, 100% and120%) were added to the preanalysed samples and the absorbances were measured.

3. Precision/ Repeatability/stability:

The precision of an analytical method is the degree of agreement among the individual test results when the procedure is applied repeatedly to multiple sampling of homogeneous sample. The precision of an analytical method is usually expressed as the SD (Standard Deviation) or RSD (% Relative Standard Deviation)

4. Limit of detection (LOD) and Limit of quantification (LOQ):

The limit of detection is quantitative parameter and can be defined as the lowest concentration of the analyte in a sample that can be detected with acceptable precision and accuracy under stated experimental conditions, but not necessarily quantities as an exact value (The United States Pharmacopoeia 27 NF 22, 2004). It is expressed as the concentration of analyte in the sample. Anything that changes the sensitivity of a method, including instrument and sample preparation will change the detection limit.

Limit of quantification is the lowest concentration of an analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished products. The LOQ is almost 10 times higher than that of the blank.

LOD (or) LOQ = k. S_B/ S

Where k - Constant (3 for LOD, 10 for LOQ)

S_B - Standard deviation of the analytical blank

S - Slope of the concentration/response graph.

3.3.2 HPLC method

3.3.2.1 Methodology

Preparation of stock solution of drugs:

Stock solution containing 1mg/ml was prepared by dissolving the drug in the mobile phase.

Preparation of Standard Solutions of drugs:

Appropriate and accurate aliquots of the stock solutions were transferred to 10ml calibrated flasks and diluted up to the volume with the mobile phase in order to get a series of known final concentrations in µg/ml.

Estimation of drugs in diffusion study samples, excipients and formulations:

A definite volume of sample to be estimated like supernant of the saturated excipients, diffusion study samples and formulations like solution, microemulsion and mucoadhesive microemulsion was taken in a 10ml volumetric flask and diluted up to the mark with methanol. The resultant solution was then sonicated for 2 min at ambient temperature and further dilutions were made up with the mobile phase and the samples were injected in triplicate.

Analytical conditions:

Analysis was isocratic with fixed flow rate of the mobile phase. The mobile phase was prepared freshly every day. The mobile phase was filtered through a 0.2 μm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The absorbance of drugs at the required wavelength was checked for any interference. Prior to injecting solutions, the column was equilibrated for 60 minutes with the mobile phase flowing through the system. Each solution was injected in triplicate and relative standard deviation was required to be below 2% on peak area basis.

3.3.2.2 Method Validation

Apart from the parameters mentioned under UV spectroscopic method (Linearity and Range, Accuracy, Precision / Repeatability, Limit of detection (LOD) and Limit of quantification (LOQ), additionally system suitability test was performed for HPLC method.

System suitability:

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates (HETP) asymmetry of the peaks and resolution between the two consecutive peaks. Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure was used.

3.4 EXPERIMENTAL CONDITIONS

3.4.1 CLOBAZAM

3.4.1.1 UV spectroscopic method

The Spectrophotometric method described in British Pharmacopoeia was used for the estimation of clobazam in excipients and formulations.

Materials:

Clobazam, Methanol

Table 3.1 Experimental conditions for clobazam by UVmethod

1.	Solvent	Methanol
2.	Stock solution conc.	1mg/ml
3.	Serial Conc. range	0.5 – 6 µg/ml
4.	Spectrum range	200nm to 400nm
5.	Spectrum Blank	Methanol
6.	Zero order peak	at 230nm

3.4.1.2 HPLC method

Estimation of clobazam and its metabolite N-desmethyl clobazam in biological fluids/tissues have been reported by many researchers (Knapp et al 1999; Kunicki et al 2001; Bolner et al 2001; Rouini et al 2005). With slight modification, the method mentioned by Rouini et al 2005 was used for estimation of clobazam in formulations, diffusion medium and excipients.

Chemicals and Reagents:

Acetonitrile and Methanol were of HPLC grade and purchased from Merck chemicals, India. Triple distilled water was used throughout the study. All the other solvents and reagents used were of analytical grade were filtered through a 0.2µm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

Table 3.2 Experimental conditions for clobazam by HPLC method

1.	Mobile phase	Acetonitrile: Water (70:30)
2.	Stock solution conc.	1mg/ml
3.	Serial Conc. range	250 ng/ml -50 µg/ml
4.	Flow rate	1 ml/min
5.	UV detection	at 230nm

3.4.2 CLOPIDOGREL BISULPHATE

3.4.2.1 UV spectroscopic method

A simple UV spectrophotometric method was developed from the monograph given in British Pharmacopoeia. The method was validated and was used for the estimation of clopidogrel bisulphate in excipients and formulations.

Materials:

Clopidogrel bisulphate, 0.1N methanolic hydrochloric acid

Table 3.3 Experimental conditions for clopidogrel bisulphate by UV method

1.	Solvent	0.1N methanolic HCl
2.	Stock solution conc.	1mg/ml
3.	Serial Conc. range	5 - 250 µg/ml
4.	Spectrum range	200nm to 400nm
5.	Spectrum Blank	0.1N methanolic HCl
6.	Zero order peak	at 270nm and 278nm

3.4.2.2 HPLC method

Many literatures were found for the estimation of clopidogrel bisulphate by various methods like gas chromatography, HPTLC from bulk powder, formulations and biological samples (Agrawal H et al 2003; Koradia et al 2004; Kamble et al 2005 and Robinson et al 2007). Estimation of clopidogrel bisulphate and its carboxylic acid metabolite in biological fluids/tissues have been reported by many researchers (Ramakrishna et al 2006; Sonu et al 2005) Estimation of clopidogrel in the bulk and in the pharmaceutical products were also published in the literatures (Patel et al. 2006; Anandkumar et al 2007) With slight modification, the method mentioned by Anandkumar et al 2007 was used for estimation of clopidogrel bisulphate in formulations, diffusion medium and excipients.

Chemicals and Reagents:

Acetonitrile and Methanol were of HPLC grade purchased from Merck chemicals, India. Sodium dihydrogen phosphate (NaH_2PO_4), Tri ethylamine and 85% ortho phosphoric acid (H_3PO4) of analytical grade were purchased from Sd fine Chemicals. Triple distilled water was used throughout the study. All the other

solvents and reagents used were of analytical grade were filtered through a 0.2µm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

Sodium dihydrogen Phosphate buffer: 20mM sodium dihydrogen phosphate buffer pH 3 was prepared by dissolving 3.12gm of sodium dihydrogen phosphate dihydrate in 1000ml of distilled water and the pH was adjusted.

Table 3.4 Experimental conditions for clopidogrel bisulphate by HPLC method

1.	Mobile phase	Acetonitrile: Methanol: Sodium dihydrogen Phosphate buffer (pH 3) (70: 5: 25)
2.	Stock solution conc.	1mg/ml
3.	Serial Conc. range	1 – 50 µg/ml
4.	Flow rate	1.5 ml/min
5.	UV detection	at 240nm

3.5 RESULTS

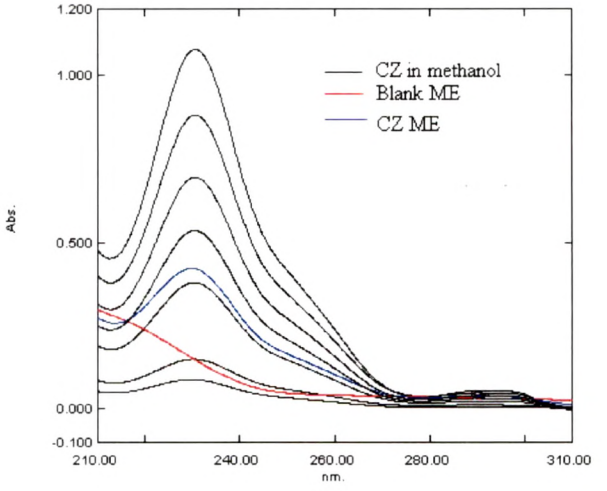
3.5.1 CLOBAZAM

3.5.1.1 UV spectroscopic method

Table 3.5 Absorbance of clobazam (zero order) at 230nm

S.No.	Conc. (µg/ml)	Absorbance at 230 nm	SD	%RSD	Variance	Std. Error
1	0.5	0.089	0.0014	1.5890	0.000002	0.0020
2	1	0.169	0.0014	0.8368	0.000002	0.0037
3	2	0.375	0.0057	1.5085	3.2E-05	0.0057
4	3	0.539	0.0049	0.9175	2.45E-05	0.0221
5	4	0.738	0.0042	0.5749	0.000018	0.0089
6	5	0.874	0.0085	0.9708	7.2E-05	0.0347
7	6	1.094	0.0205	1.8752	0.000421	0.0045

Fig.3.1 Zero order curve of clobazam at 230nm



Graph 3.1 Std plot of clobazam in methanol at 230nm (zero order curve)

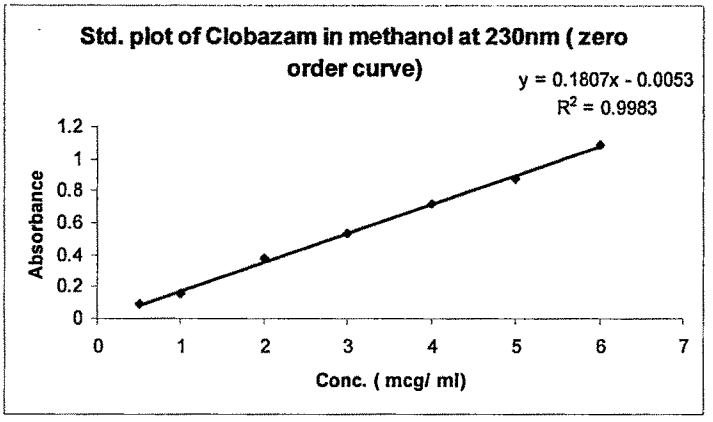


Table 3.6 Recovery study for accuracy of clobazam estimation by UV method

Quantity of Clobazam added %	Expected Concentration (µg/ml)	Obtained concentration (µg/ml)	% Recovery	%RSD
80	5.4	5.6347	104.3448	0.5268
100	6.0	6.1112	101.8547	0.5308
120	6.6	6.5887	99.8295	0.2415

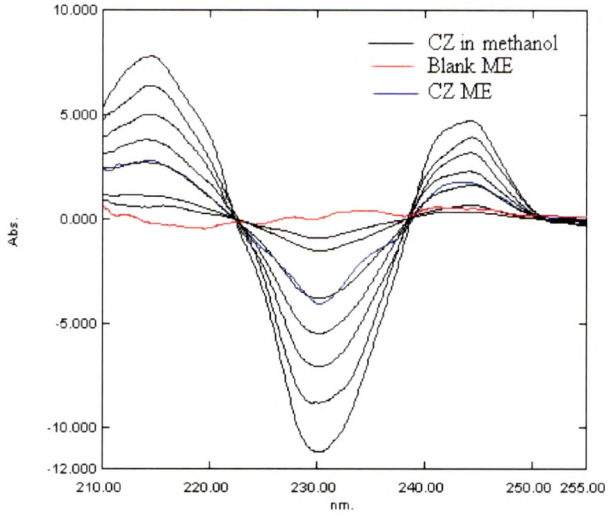
Table 3.7 Intra-day precision & Inter-day precision of Inter-day precision of clobazam estimation by UV method

Amount	Intra-day precision			Inter-day precision		
	Mean absorbance	SD	%RSD	Mean absorbance	SD	%RSD
1	0.169	0.0014	0.8368	0.167	0.0014	0.8468
3	0.539	0.0049	0.9175	0.541	0.0028	0.5228
6	1.094	0.0205	1.8753	1.067	0.0110	1.0336

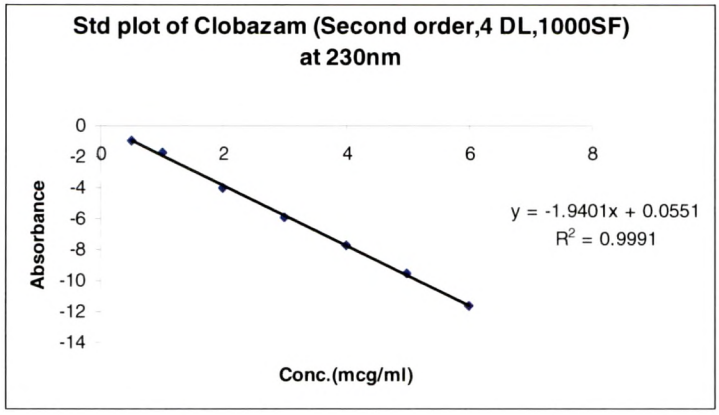
Table 3.8 Absorbance of clobazam (second derivative curve) at 230nm

S.No.	Conc. (µg/ml)	Absorbance at 230 nm	SD	%RSD	Variance	Std. Error
1	0.5	-0.933	0.0085	0.9095	7.2E-05	0.0049
2	1	-1.729	0.0255	1.4723	0.000648	0.0147
3	2	-3.966	0.0219	0.5528	0.00048	0.0127
4	3	-5.863	0.0184	0.3136	0.000338	0.0106
5	4	-7.696	0.0785	1.0199	0.006161	0.0454
6	5	-9.49	0.0283	0.2980	0.0008	0.0164
7	6	-11.65	0.0566	0.4856	0.0032	0.0327

Fig.3.2 Second order curve of clobazam (4DL, 1000SF) at 230nm



Graph 3.2 Std plot of clobazam (second order, 4DL, 1000SF) at 230nm

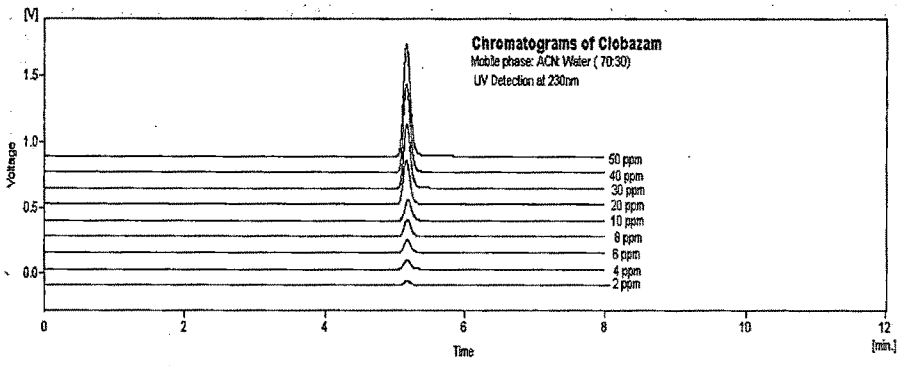


3.5.1.2 HPLC method

Table 3.9 Calibration of clobazam by HPLC method

S.No.	Conc. (µg/ml)	Area	SD	%RSD	Variance	Std. Error
1	0.25	46.04	0.4805	1.0438	0.2309	0.2778
2	0.5	89.13	0.5303	0.5950	0.2813	0.3066
3	0.75	127.13	1.0013	0.7876	1.0025	0.5788
4	1	187.35	0.2015	0.1076	0.0406	0.1165
5	2	211.96	0.5091	0.2402	0.2592	0.2943
6	4	455.54	0.3606	0.0792	0.1301	0.2085
7	6	661.72	1.8314	0.2768	3.3541	1.0587
8	8	907.42	0.7142	0.0787	0.5101	0.4128
9	10	1077.84	1.1526	0.1069	1.3285	0.6662
10	20	2340.77	0.3253	0.0139	0.1058	0.1880
11	30	3320.43	0.7919	0.0239	0.6272	0.4578
12	40	4611.51	1.6405	0.0356	2.6912	0.9483
13	50	5854.27	1.5981	0.0273	2.5538	0.9237

Fig. 3.3 Chromatograms of clobazam



Graph 3.3 Std plot of clobazam by HPLC at 230nm detection

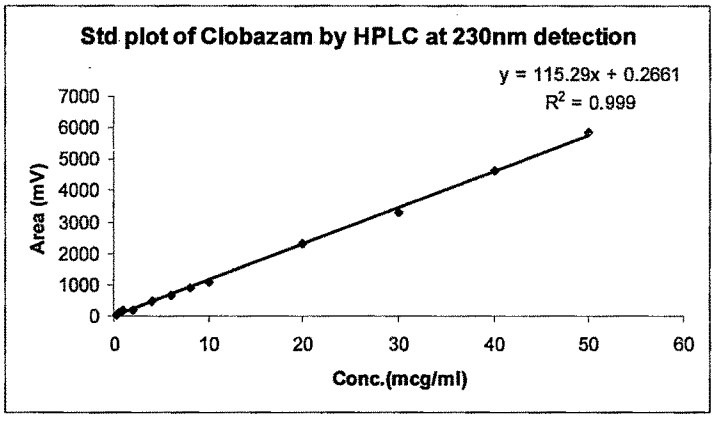


Table 3.10 Recovery study for accuracy of clobazam estimation by HPLC method

Quantity of Clobazam added %	Expected Concentration (µg/ml)	Obtained concentration (µg/ml)	% Recovery	%RSD
80	18	18.3727	102.0705	0.0051
100	20	20.4368	102.1839	0.0139
120	22	21.4664	97.5747	0.0497

Table 3.11 Intra-day precision & Inter-day precision of Inter-day precision of clobazam estimation by HPLC method

Amount	Intra-day precision			Inter-day precision		
	Mean area	SD	%RSD	Mean area	SD	%RSD
6	661.72	1.8314	0.2768	668.05	3.1183	0.4667
10	1077.84	1.1526	0.1069	1085.5	0.0205	1.8890
20	2340.77	0.3253	0.0139	2350.4	20.6475	0.8785

Table 3.12 System suitability for clobazam estimation

S.No	Parameters	Clobazam(10µg/ml)
1.	Retention time	5.18 ± 0.012
2.	Asymmetry factor	1.346
3.	Efficiency(Th.pl)	12293.5 ± 55.86
4.	Efficiency/L (tp/m)	245864 ± 111.86
5.	Capacity factor	1.2620
6.	LOD	0.0096µg/ml
7.	LOQ	0.0319µg/ml

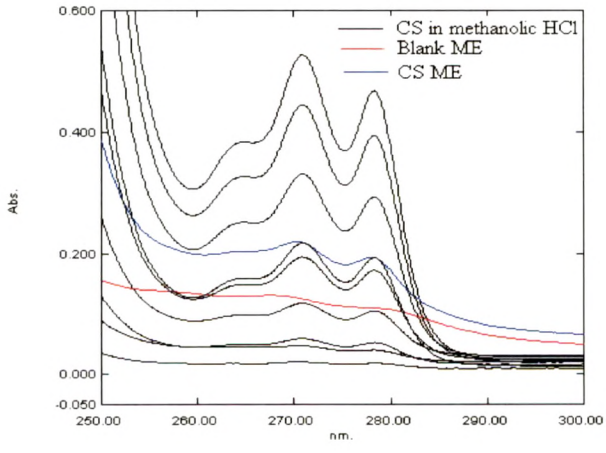
3.5.2 CLOPIDOGREL BISULPHATE

3.5.2.1 UV spectroscopic method

Table 3.13 Absorbance of clopidogrel bisulphate (zero order curve) at 270nm

S.No.	Conc. (µg/ml)	Absorbance at 270 nm	SD	%RSD	Variance	Std. Error
1	5	0.0208	0.00035	1.7039	1.25E-07	0.00021
2	10	0.0476	0.00078	1.6358	6.05E-07	0.00045
3	20	0.0579	0.00042	0.7328	1.8E-07	0.00025
4	40	0.1084	0.00424	1.9139	0.000018	0.00245
5	80	0.1793	0.00191	1.0651	3.65E-06	0.00111
6	100	0.2196	0.00085	0.3864	7.2E-07	0.00049
7	150	0.3208	0.00064	0.1984	4.05E-07	0.00037
8	200	0.4197	0.00163	0.3876	2.65E-06	0.00094
9	250	0.5302	0.00057	0.1066	3.2E-07	0.00033

Fig. 3.4 Zero order curve of clopidogrel bisulphate at 270nm



Graph 3.4 Std plot of cloidogrel bisulphate in 0.1N methanolic HCl at 270nm (zero order curve)

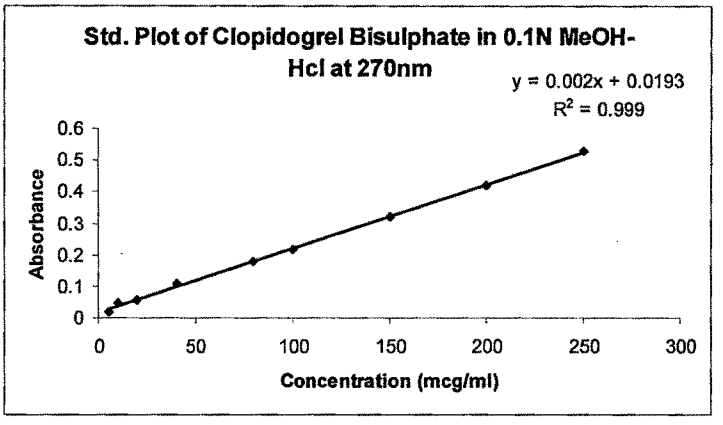


Table 3.14 Recovery study for accuracy of clopidogrel bisulphate estimation by UV method

Quantity of Clopidogrel added %	Expected Concentration (µg/ml)	Obtained concentration (µg/ml)	% Recovery	%RSD
80	90	92.8	103.1111	2.4382
100	100	100.15	100.15	0.3864
120	110	112.85	101.6818	1.2531

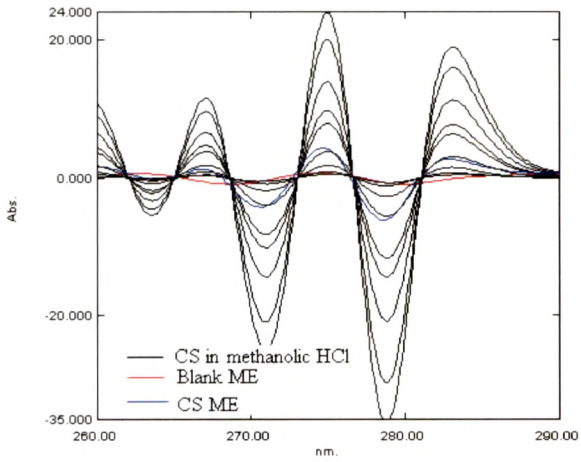
Table 3.15 Intra-day precision & Inter-day precision of Inter-day precision of clopidogrel bisulphate estimation by UV method

Amount	Intra-day precision			Inter-day precision		
	Mean absorbance	SD	%RSD	Mean absorbance	SD	%RSD
20	0.0579	0.00043	0.7328	0.0562	0.00156	2.768
40	0.1084	0.00424	1.9139	0.108	0.00212	1.9641
80	0.1793	0.0019	1.0651	0.1805	0.0028	1.5316

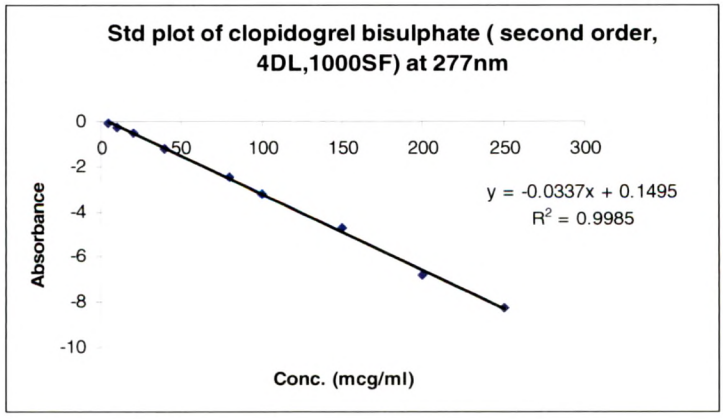
Table 3.16 Absorbance of clopidogrel bisulphate (second derivative curve) at 277nm

S.No.	Conc. (µg/ml)	Absorbance at 277 nm	SD	%RSD	Variance	Std. Error
1	5	-0.046	0.004243	-9.2231	0.000018	0.00245
2	10	-0.239	0.012021	-5.0297	0.000145	0.00695
3	20	-0.53	0.007778	-1.4676	6.05E-05	0.00449
4	40	-1.218	0.019799	-1.6255	0.000392	0.01145
5	80	-2.481	0.132936	-1.3582	0.017672	0.07684
6	100	-3.211	0.129401	-4.0299	0.016744	0.07479
7	150	-4.697	0.079903	-1.7012	0.006385	0.04619
8	200	-6.823	0.014849	-0.2176	0.000221	0.00858
9	250	-8.254	0.185969	-2.2531	0.034585	0.10749

Fig. 3.5 Second derivative curve of Clopidogrel bisulphate (4DL, 1000SF) at 277nm



Graph 3.5 Std plot of clopidogrel bisulphate (second order, 4DL, 1000SF) at 277nm

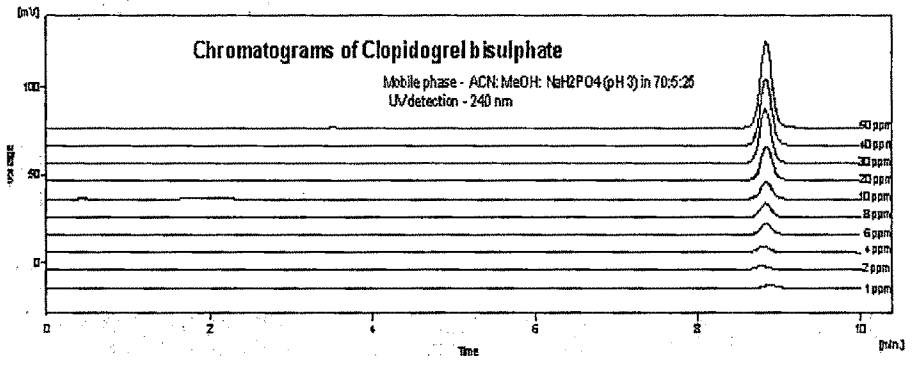


3.5.2.2 HPLC method

Table 3.17 Calibration of clopidogrel bisulphate by HPLC method

S No.	Conc. (µg/ml)	Area	SD	%RSD	Variance	Std. Error
1	1	11.681	0.0057	0.0484	3.2E-05	0.0033
2	2	22.037	0.1099	0.4987	0.2487	0.1884
3	4	38.738	0.0028	0.0072	8E-06	0.0016
4	6	58.8365	0.4659	0.7919	0.2171	0.2694
5	8	78.0975	0.1336	0.1711	0.0179	0.0773
6	10	94.9975	0.1124	0.1182	0.0126	0.0649
7	20	199.324	0.2135	0.1071	0.0456	0.1234
8	30	303.504	0.5996	0.1976	0.3596	0.3466
9	40	393.459	0.3041	0.0773	0.0925	0.1757
10	50	503.3695	0.3500	0.0695	0.1225	0.2023

Fig.3.6 Chromatograms of clopidogrel bisulphate



Graph 3.6 Std plot of clopidogrel bisulphate by HPLC at 240nm detection

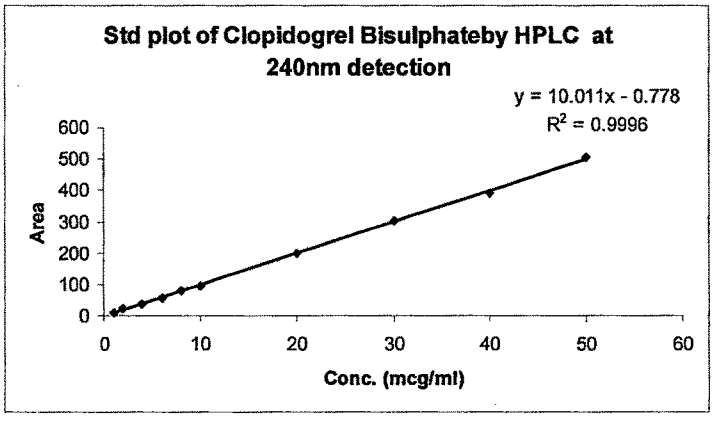


Table 3.18 Recovery Test for accuracy of clopidogrel bisulphate estimation by HPLC method

Quantity of Clopidogrel added %	Expected Concentration (µg/ml)	Obtained concentration (µg/ml)	% Recovery	%RSD
80	24	24.6295	102.6229	0.0843
100	30	29.5117	98.3723	0.1639
120	36	35.9052	99.7367	0.1235

Table 3.19 Intra-day precision & Inter-day precision of Inter-day precision of clopidogrel bisulphate estimation by HPLC method

Amount	Intra-day precision			Inter-day precision		
	Mean area	SD	%RSD	Mean area	SD	%RSD
8	78.0975	0.1336	0.1711	77.242	0.3846	0.498
20	199.324	0.2136	0.1071	193.87	5.5189	2.8647
40	393.459	0.3041	0.0773	392.87	6.9331	1.7647

Table 3.20 System suitability for clopidogrel bisulphate

S.No	Parameters	Clopidogrel bisulphate (10µg/ml)
1.	Retention time	8.84 ± 0.002
2.	Asymmetry factor	1.093
3.	Efficiency(Th.pl)	16904.5 ± 9.19
4.	Efficiency/L (tp/m)	338096.5 ± 180.31
5.	Capacity factor	2.8602
6.	LOD	0.0216µg/ml
7.	LOQ	0.0720µg/ml

3.6 DISCUSSION

CLOBAZAM

The UV spectroscopic method described in the USP was used for the clobazam estimation. On screening of 5µg /ml solution, two absorption maxima were obtained at 230nm and 290nm. Because of low sensitivity at 290nm for a given concentration, λ_{max} of 230nm was used (Fig. 3.1). The method was validated for linearity, accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found to be less than 5% (Table 3.5). The absorbance was found to be linear in the range of 1-6 µg /ml with r^2 value of 0.9983 (Graph 3.1). The % recovery of 99.83% to 104.34% (Table 3.6) showed that the method was accurate to estimate clobazam in that 1-6 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of clobazam at 3 different concentration levels were shown in Table 3.7.

Since some of the excipients were found to be interfering while drug estimation in the solubility study, derivative spectroscopy was used for estimating clobazam in the presence of the interfering excipients. The zero-order absorption spectra were derivatized and derivative spectra from first and second order were recorded. The zero crossing point of the clobazam formulation was found to be 230nm (Fig. 3.2) and the derivatised spectras were evaluated for their linearity (Table 3.8) and the regression value for the range of 1-6 µg/ml range was found to be 0.9991 (Graph 3.2). The LOD and LOQ of the UV spectroscopic method were found to be 0.0096 and 0.03195 µg/ml.

HPLC method was found to be sensitive enough to detect the lowest quantity of clobazam (250ng/ml). The elution was done with the flow rate of 1ml/min of acetonitrile : water (70:30) and the retention time of clobazam was found to be 5.18 minutes at 230nm of detection.(Fig. 3.3). The linearity of the estimation was found to be 0.9991 (Graph 3.3) in the range of 250 ng/ml - 50 µg/ml (Table 3.9). The % recovery of 97.57% to 102.18% (Table 3.10) showed that the method was accurate to estimate clobazam in that 250ng -50 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of clobazam at 3 different concentration levels were shown in Table 3.11 and found to be less than 2%. The system suitability parameters for clobazam estimation by HPLC were shown in Table 3.12. No interference was observed in the HPLC estimation, since the drug was extracted from excipients and the components of the diffusion media while elution.

CLOPIDOGREL BISULPHATE

A simple UV spectroscopic method was developed from the monographs of clopidogrel bisulphate from British Pharmacopoeia. The arbitrary concentrated solution (50μg/ml) of clopidogrel bisulphate in 0.1N methanolic hydrochloric acid was screened. Two absorption maxima were obtained at 270nm and 278nm. Because of low sensitivity at 278nm for a given concentration, λ_{max} of 270nm was used (Fig 3.4). The method was validated for linearity, accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found less than 2% (Table 3.13). The absorbance was found to be linear in the range of 5-250 μg /ml with r^2 value of 0.999 (Graph 3.4). The % recovery of 100.15% to 103.11% (Table 3.14) showed the method was accurate to estimate clobazam in that 5-250 μg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of clopidogrel bisulphate at 3 different concentration levels were shown in Table 3.15.

Since the excipients were found to be interfering while drug estimation in the solubility study, derivative spectroscopy was used for estimation of clopidogrel bisulphate in the presence of the interfering excipients. The zero-order absorption spectra were derivatized and derivative spectra from first and second order were recorded. The zero crossing point of the clopidogrel bisulphate formulation was found to be 277nm (Fig. 3.5) and the derivatised spectras were evaluated for their linearity (Table 3.16) and the regression value for the range of 5-250 μg /ml range was found to be 0.9985 (Graph 3.5). The LOD and LOQ of the UV spectroscopic method were found to be 0.8660 and 2.8867 μg/ml.

HPLC method was found to be sensitive enough to detect the lowest quantity of clopidogrel bisulphate (1 μg/ml). The elution was done with the flow rate of 1.2ml/min of Acetonitrile: Methanol: Sodium dihydrogen Phosphate buffer (pH 3) (70: 5: 25) and the retention time of clopidogrel bisulphate was found to be 8.84 minutes at 240nm of detection (Fig. 3.6). The linearity of the estimation was found to be 0.9996 (Graph 3.6) in the range of 1 - 50 μg/ml (Table 3.17) The % recovery of 98.37% to 102.62% (Table 3.18) showed the method was accurate to estimate clopidogrel bisulphate in that 1-50 μg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of clopidogrel bisulphate at 3 different concentration levels were shown in Table 3.19 and found to be less than 3%. The system suitability parameters for clopidogrel bisulphate estimation by HPLC were shown in Table 3.20. No interference was observed in the HPLC estimation, since the drug was extracted from excipients and the components of the diffusion media while elution.

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