Chapter 3

Analytical methods

3. 1.Introduction:

The analytical methods used in studies of drug containing microemulsion and solid lipid nanoparticles should, in addition to possessing the desired characteristics of accuracy, precision, reproducibility, ruggedness, robustness etc. also posses the ability to be used in conjunction with techniques common the formulation studies. These techniques minimum interference from the other components of the formulation variables. The methods used should, preferably, by stability indicating, which would, when used, draw attention to any potential incompatibility between various components of microemulsion and solid lipid nanoparticles.

3.2. Experimental

3.2.1. Drugs

Acyclovir was received as a gift sample from Alembic Ltd, Vadodara, India. Efavirenz was received as a gift sample from Ranbaxy Labs, Indore, India.

3.2.2. Reagents:

Labrasol (Caprylocapryl macrogol-8-glyseride), Plurol Olique (Polyglycerol 6-dioleate), Labrafac (Medium chain triglycerides), Labrafil M 1944 CS (oleeoyl macrogol-6-glycerides), labrafac Hydro (mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethylene glycol), Labrafac Lipo (Medium chain triglycerides EP), transcutol (diethylene glycol monoethylether) Glyceryl palmitostearate (Precirol ATO 5) was received from Colorcon Asia Pvt. Ltd. (mfg: Gattefoss, France) , Cremophor RH 40 (polyethoxylated hydrogenated castor oil), Polaxomer 188 (poly(oxyethylene), poly(oxypropylene) block polymers) was purchased from BASF corporation, Tween 80 (Polyoxyethylene sorbitan monooleate), Propylene glycol, Oleic acid was purchased from SD fine chemicals, India. Sunflower oil, Stearic acid, Glyceryl monotostearate, Glyceryl tristearate was purchased from National chemical Ltd, India. Methanol, perchloric acid, Acetonitrile, Glycerin, Potassium dihydrogen phosphate, disodium hydrogen phosphate (ExcelR grade) was purchased from Qualigens fine chemicals, Mumbai, India. Octane sulphonic acid sodium salt was purchased from (Spectrum Lab, India). Ammonium acetate was purchased from Sigma, USA.

3.2.3 Apparatus:

Cyclomixer and centrifuge (Remi Equipments, Mumbai), Ultracentrifuge (Sigma, USA), electrically heated thermostatically controlled water bath (superfit equipments, Mumbai), Shimadju UV-1601 UV-visible spectrophotometer (Shimadju Corporation, japan), HPLC Hewlett-Packard (Aglient) 1100 series components including a quaternary pump, auto sampler and variable wavelength UV detector (Palo Alto, CA, USA). HPLC (Dionex). Vibromixer (SPINIX, India), Biofuge Pico Micro centrifuge (Heraeus Instruments, Hanau, Germany).

3.4. Acyclovir:

3.4.1. Estimation of acyclovir in 0.1N HCl

Acyclovir in 0.1 N HCl shows absorbance in the ultraviolet region

3.4.1.1. Solution:

Stock solution of acyclovir was prepared by dissolving 10mg of acyclovir in 100ml of 0.1N HCl. 10ml of this solution was taken in 50ml volumetric flask and diluted to 50 ml by 0.1N HCl. This solution (20µg/ml) was preserved and used as stock solution.

3.4.1.2. Procedure for calibration curve:

Suitable aliquots (1ml - 8ml) of the stock solution were pipetted into 10ml volumetric flasks and the volume was made upto 10ml with 0.1N HCl to give final concentrations of 2, 4, 6 8, 10 and 20µg/ml. The solution was shaken well and their absorbance was measured at 252nm using 0.1N HCl as blank on a Shimadju 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.1 tabulated the raw and regressed data (method of least squares) so obtained. Absorptivity scan over the UV wavelength range between 200 and 400nm for10µg/ml solution of acyclovir in 0.1N HCl and the calibration curves obtained are shown in Figure 3.1 and 3.2.

Table 3.1: Calibration curve for acyclovir in 0.1N HCl

Concentration (µg/ml)	Mean absorbance* ± S.D	Regressed value
2	0.118 ± 0.003	0.1062
4	0.231 ± 0.008	0.2192
6	0.318 ±0.005	0.3322
8	0.438 ± 0.009	0.4452
10	0.547 ± 0.015	0.5582
20	1.133 ± 0.024	1.1232

Regression equation; Y= 0.0565 x – 0.0068

Correlation coefficient= 0.9989

* data are expressed as mean ± standard deviation (S.D.) and mean of 6 values

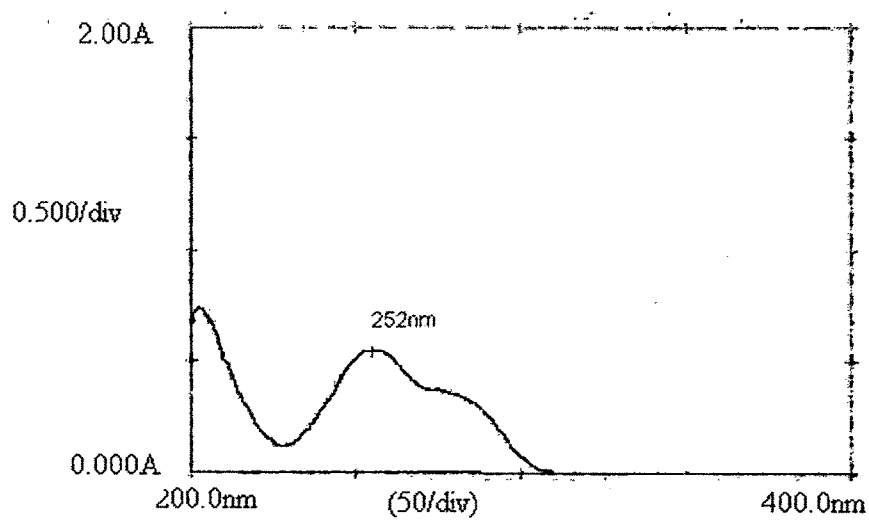

Figure 3.1. Absorptivity scans of acyclovir in 0.1 N HCl

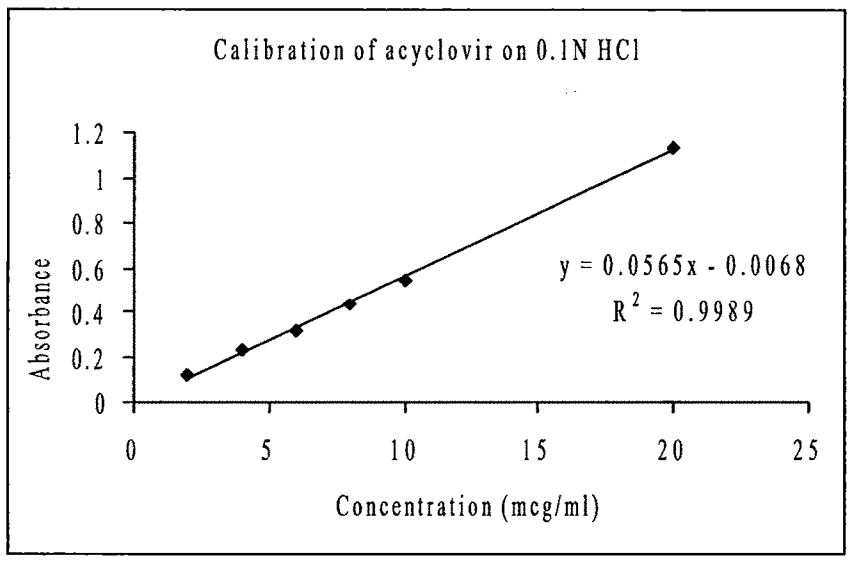

Figure 3.2. Calibration curve of acyclovir in 0.1N HCl.

3.4.1.3. Stability of the solution:

Stability of the solutions, prepared above for the calibration of acyclovir, was ascertained by changing the absorbance of the solutions over a period of 48 hours at room temperature.

3.4.2. Estimation of acyclovir in ethanol

Acyclovir in ethanol shows absorbance in the ultraviolet region

3.4.2.1. Solution:

Stock solution of acyclovir was prepared by dissolving 10mg of acyclovir in 100ml of ethanol. 10ml of this solution was taken in 50ml volumetric flask and diluted to 50 ml with ethanol. This solution (20µg/ml) was preserved and used as stock solution.

3.4.2.2. Procedure for calibration curve:

Suitable aliquots (1ml - 8ml) of the stock solution were pipetted into 10ml volumetric flasks and the volume was made upto 10ml with ethanol to give final concentrations of 2, 4, 8, 12, 16 and 20µg/ml. The solution was shaken well and their absorbance was measured as 236.5nm using ethanol as blank on a Shimadju 1601 UV-Visible

spectrophotometer. The above procedure was repeated six times. Table 3.2 tabulated the raw and regressed data (method of least squares) so obtained. Absorptivity scans over the UV wavelength range between 200 and 400nm for 4µg/ml solution of acyclovir in ethanol and the calibration curves obtained are shown in Figure 3.3 and 3.4.

3.4.2.3. Stability and selectivity:

Stability of the solutions of acyclovir in ethanol, used for the preparing calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature. The selectivity of the method for acyclovir was investigated by carrying out the procedure detailed above in the presence of potential interferences such as labrasol, tween 80, plurol olique, propylene glycol, labrafac etc., at the levels at which these materials were included in the formulation.

3.4.2.4. Estimation of acyclovir from drug-oil mixture, microemulsion and SLN or its supernatant

To 25µl of oil-drug mixture/ microemulsion / SLN or supernatant of SLN in a 25ml volumetric flask, ethanol was added to dissolve the drug and other components and the volume was made up to the mark with ethanol. The resulted solution was further diluted with ethanol, if necessary, and the absorbance was measured at 236.5 nm, against a blank comprising oil/empty microemulsion or empty SLN diluted with ethanol in the similar manner. Estimations were made in triplicate and the mean absorbance was determined. The amount of acyclovir in the oil-drug mixture/ microemulsion / SLN or in supernatant of SLN was then obtained using the regression value.

Table 3.2: Calibration curve for acyclovir in ethanol

Concentration (µg/ml)	Mean absorbance* ± S.D.	Regressed value
2	0.1830 ± 0.0038	0.2018
4	0.3618 ± 0.0087	0.3506
8	0.6565 ± 0.0050	0.6482
12	0.9601 ± 0.0096	0.9458
16	1.2283 ± 0.0150	1.2434
20	1.5400 ± 0.0240	1.541

Regression equation; Y= 0.0744 x + 0.053

Correlation coefficient= 0.9993

* data are expressed as mean ± standard deviation (S.D.) and mean of 6 values

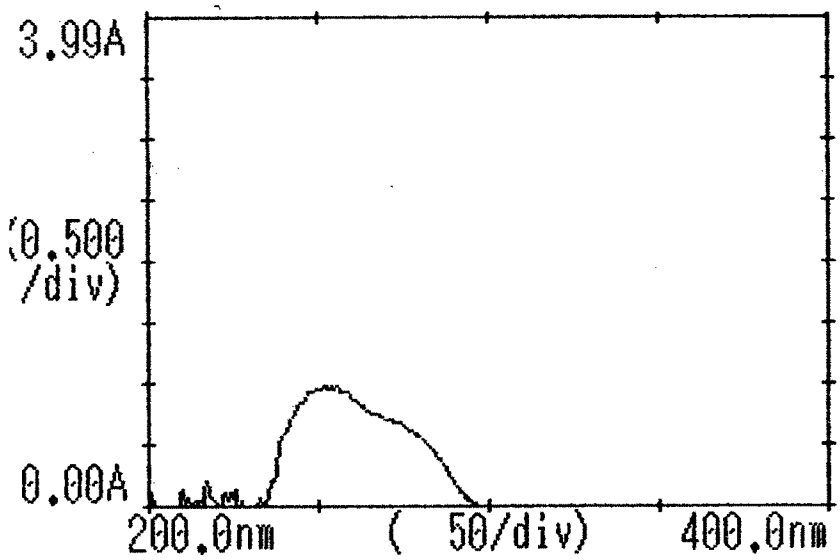

Figure 3.3: Absorptivity scan of acyclovir in ethanol

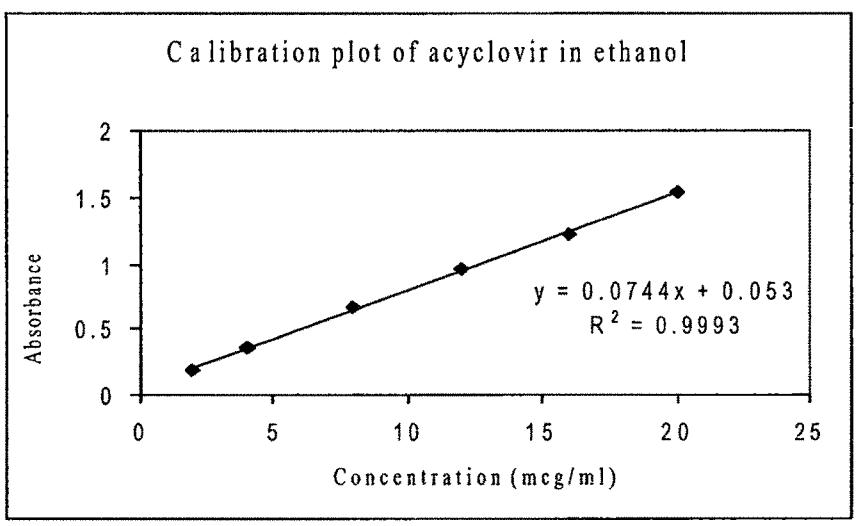

Figure 3.4: Calibration curve for the estimation of acyclovir

3.4.3. Estimation of acyclovir in phosphate buffer saline (PBS) pH 7.4:

Preparation of PBS pH 7.4: Phosphate buffer saline pH 7.4 was prepares as per IP. Dissolved 2.38gm of disodium hydrogen phosphate, 0.19gm of potassium dihydrogen phosphate and 8.0gm of sodium chloride in 1litre-distilled water. pH of the solution was then adjusted to 7.4 by adding either 0.1N sodium hydroxide or by 0.1N hydrochloric acid. Acyclovir in phosphate buffer saline pH 7.4 shows absorbance in the ultraviolet region

3.4.3.1. Solution:

Stock solution of acyclovir was prepared by dissolving 10mg of acyclovir in 100ml of PBS pH 7.4. 10ml of this solution was taken in 50ml volumetric flask and diluted to 50 ml by PBS pH 7.4. This solution (20µg/ml) was preserved and used as stock solution.

3.4.3.2. Procedure for calibration curve:

Suitable aliquots (1ml - 8ml) of the stock solution were pipettes into 10ml volumetric flasks and the volume was made upto 10ml with PBS pH 7.4 to give final concentrations of 2, 4, 8, 12, 16 and 20µg/ml. The solution was shaken well and their absorbance was measured as 252nm using PBS pH 7.4 as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.3 tabulated the raw and regressed data (method of least squares) so obtained. Absorptivity scans over the UV wavelength range between 200 and 400nm for 8µg/ml solution of acyclovir in PBS pH 7.4 and the calibration curves obtained are shown in Figure 3.5 and Figure 3.6.

Table 3.3: Calibration curve for acyclovir in PBS pH 7.4

Concentration (µg/ml)	Mean absorbance* ± S.D.	Regressed value
2	0.166 ± 0.0034	0.1524
4	0.2545 ± 0.0041	0.2618
8	0.4643 ± 0.0043	0.4806
12	0.6895 ±0.0055	0.6994
16	0.9573 ± 0.0086	0.9182
20	1.1195 ± 0.0051	1.137

Regression equation; Y= 0.0547 x + 0.043

Correlation coefficient= 0.9967

* data are expressed as mean ± standard deviation (S.D.) and mean of 6 values

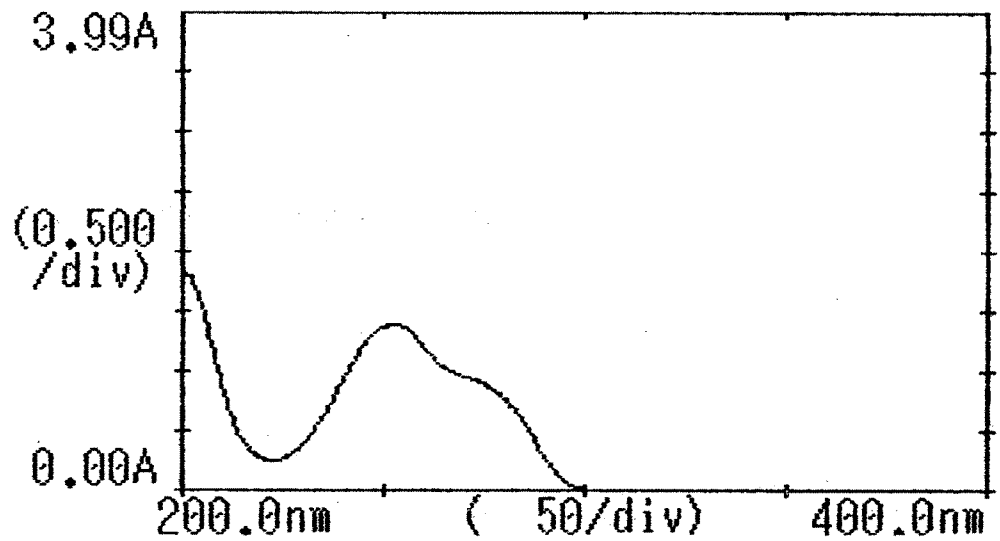

Figure 3.5: Absorptivity scan of acyclovir in PBS pH 7.4

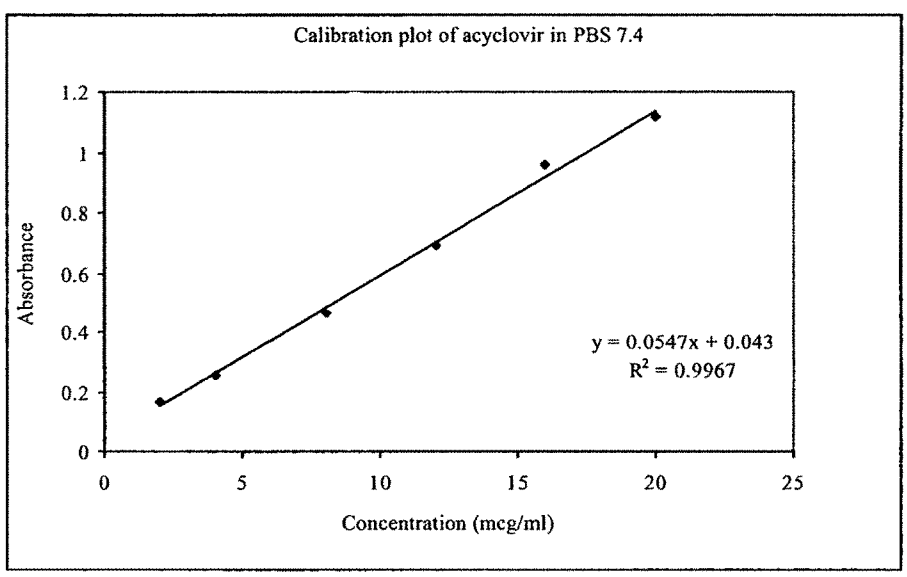

Figure 3.6: Calibration curve for the estimation of acyclovir.

3.4.3.3. Stability of the solution:

Stability of the solutions of acyclovir in ethanol, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature

3.4.4. Estimation of acyclovir in rat plasma:

Several high-performance liquid chromatography (HPLC) methods exist for the quantification of ACV in plasma, serum and urine. Some of these methods require more specialized equipment like flurometric detector, (H. Mascher et al1992) (K.K. Peh, 1997) (J.O. Svensson, 1997) or extremely large volume of samples (G. Land, 1981)(A.M. Molikhia, (1990) (P. Nebinger, 1993)(R. Boulieu, (1997). Radioimmunoassay (RIAs) (J. Lycke et al 1989) and enzyme-linked immunosorbent assays are also reported for acyclovir (R.P. Quinn, 1979) (S.M. Tadepalli,1986). Other technique used for the estimation involves thermostatized column, permitting a chromatographic process at a controlled temperature. (Mascher et al1992,) (J. Salomoum et al 1987) (K. Swart, et al 1994). Solid phase extraction is commonly used for sample clean up technique, but may not be always necessary for relatively simple matrices. (Hung C. Vo et al. 2002) (Marcos Fernandez et al 2003)

For the pretreatment of the samples, some studies have used plasma centrifugation and direct injection of the supernatant in the chromatographic column (J. Salomoum et al 1987) Other studies used deproteinizing agents such as trichloroacetic acid [K.K. Peh 1997) H. Mascher 1992) (Ch. Pham-Huy et 1996) (C. McMullin 1996) (R. Bangaru 2000), trifluoroacetic acid (R. Smith, 1985) and aluminum sulphate solution (G. Land 1981) followed by centrifugation and injection of the supernatant. However, these techniques do not permit an adequate purification of the extracts. HPLC with UV detection (K. Swart et al 1994) (R. Smith et al 1985) (Ch. Pham-Huy et al 1999) (C. McMullin et al 1996) (R. Bangaru et al 2000)(J.M. Poirier et al 1999) is a very common method used for the determination of ACV; however, some of these methods present a detection limit higher than 100 ng/ml (R.Smith et al 1995)(C. McMullin et al 1996) (

J.M. Porier et al 1999), which is sensitive enough for pharmacokinetic studies (K. K. Peh et al 1997)

3.4.4.1. Chromatographic system:

The HPLC system consisted of Hewlett-Packard (Aglient) 1100 series components including a quaternary pump, auto sampler and variable wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Innertsil ODS-3V column (250 x 4.6mm, 5µm) (GL science, Japan).

3.4.4.2. Chromatographic conditions:

The mobile phase used for the plasma and tablet sample was 20mM Ammonium acetate with 5mM Octane sulphonic acid sodium salt in water (pH adjusted to 3 by acetic acid) - methanol (98:2, v/v). Filtration of the buffer was done using 0.2µ nylon 6.6 membrane filters and degassing by sonication.

3.4.4.3. Calibration curve in rat plasma:

Blank plasma was collected from untreated anaesthetized rat and kept at -20^0C until analysis. 200µl of the thawed plasma samples mixed with 100µl of water (for blank plasma) and the standard stock solution of different concentration (form 50ng/ml to 5µg/ml) of acyclovir and vortexed for one minute using vibromixer (SPINIX, India). To this add 20µl of 35% perchloric acid and mixed for 1minute for protein precipitation. Then this mixture was centrifuged at 10,000rpm for ten minute using a Biofuge Pico Micro centrifuge (Heraeus Instruments, Hanau, Germany). After centrifugation, 100µl of supernatant solution was recovered for injection into HPLC.

3.4.4.4. Validation of the HPLC method:

The chromatographic method was validated on two different days, to determine the sensitivity, linearity, precision and accuracy of the present HPLC method.

Sensitivity of the method was tested in plasma of 5 different rats and in water.

Linearity was determined by a calibration curve with standard acyclovir solution in the range 10ng/ml to 5µg/ml, with n=5 where the slope was calculated using the acyclovir concentration.

Precision was determined by the elaboration of three standard calibration curves, two from the same day (intra-day precision) and third one from a different day (inter-day precision).

Accuracy of the assay was defined as a percentage of the systemic error, which was calculated as the agreement between the measured value of the validation samples and the true value. Accuracy values were determined over two days and were always within acceptable limits (<15%) at all validation concentrations.

Accuracy = (True value- measured value) x 100/ True value .....(Equ.1)

3.5. Efavirenz:

3.5.1: Estimation of Efavirenz in methanol:

Efavirenz in methanol shows absorbance in the ultraviolet region

3.5.1.1: Solution:

Stock solution of efavirenz was prepared by dissolving 10mg of efavirenz in 100ml of methanol. 10ml of this solution was taken in 50ml volumetric flask and diluted to 50 ml by methanol. This solution (20µg/ml) was preserved and used as stock solution.

3.5.1.2: Procedure for calibration curve:

Suitable aliquots (0.5ml - 5ml) of the stock solution were pipetted into 10ml volumetric flasks and the volume was made upto 10ml with methanol to give final concentrations of 1, 2, 4, 8, 10 and 20µg/ml. The solution was shaken well and their absorbance was measured as 247nm using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.6 tabulated the raw and regressed data (method of least squares) so obtained. Absorptivity scans over the UV wavelength range between 200 and 400nm for 10µg/ml solution of efavirenz in methanol and the calibration curves obtained are shown in Figure 3.11 and Figure 3.12.

3.5.1.3: Stability and selectivity:

Stability of the solutions of efavirenz in methanol, used for the preparing of calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature.

The above method for estimating efavirenz was carried out in presence of labrasol, cremophor RH 40, propylene glycol, plurol olique, labrafac and other components of microemulsion or SLN to ascertain the selectivity of the method.

3.5.2: Estimation of efavirenz from drug-oil mixture, microemulsion and SLN or its supernatant

To 25µl of oil-drug mixture/ microemulsion in a 25ml volumetric flask, methanol was added to dissolve the drug and other components and the volume was made up to the mark by with methanol. The resulted solution was further diluted with methanol, if necessary and the absorbance was measured at 247 nm, against a blank comprising oil/empty microemulsion diluted with methanol in the similar manner. Estimations were made in triplicate and the mean absorbance was determined. The amount of efavirenz in the oil-drug mixture/ microemulsion was then obtained using the regression value.

3.5.2.1: Estimation of efavirenz from SLN / supernatant

The un-entrapped drug was removed from the SLN suspension by adding 100 µl of the 0.02 M protamine sulphate in 3ml of SLN suspension followed by centrifugation at 20,000 rpm for 20min. 0.5mlof the supernatant was taken in a 25ml volumetric flask, methanol was added to dissolve the drug and the volume was made up to the mark with methanol. The resulted solution was further diluted with methanol, if necessary and the absorbance was measured at 247 nm, against methanol blank.

The entrapped drug was estimated using the reported method. The SLN suspension was passed through the cephadrex G25 column and collected. From this passed and collected sample, suitable aliquot was taken and dissolved in methanol and absorbance taken by UV-VIS spectrophotometer at 247nm against the solvent blank.

Table 3.4: Calibration curve for efavirenz in methanol

Concentration (µg/ml)	Mean absorbance* ± S.D	Regressed value
1	0.0741 ± 0.0016	0.0565
2	0.1147 ± 0.0034	0.1096
4	0.209 ± 0.0046	0.2158
8	0.408 ± 0.0075	0.4282
10 .	0.5263 ± 0.0037	0.5344
20	1.0768 ±0.0047	1.0654

Regression equation; Y= 0.0531 x +0.0034

Correlation coefficient= 0.9986

* data are expressed as mean ± standard deviation (S.D.) and mean of 6 values

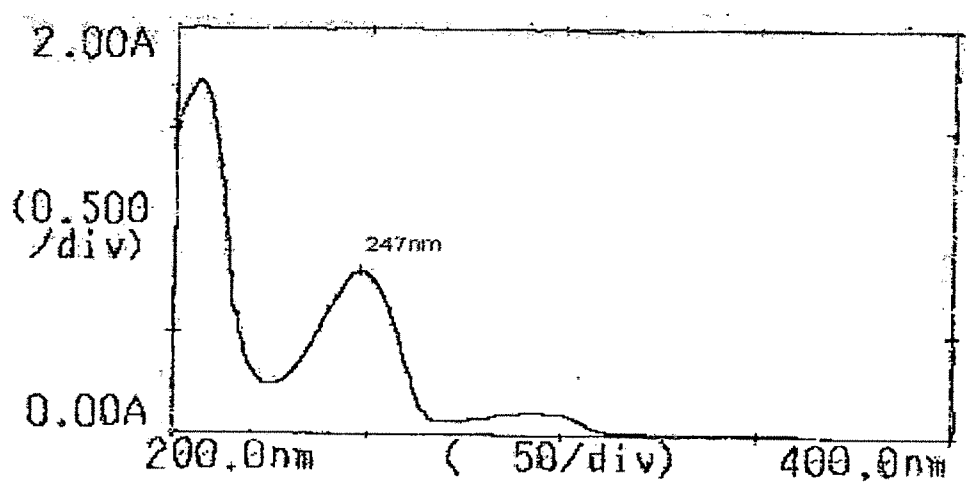

Figure 3.7: Absorptivity scan of efavirenz in methanol

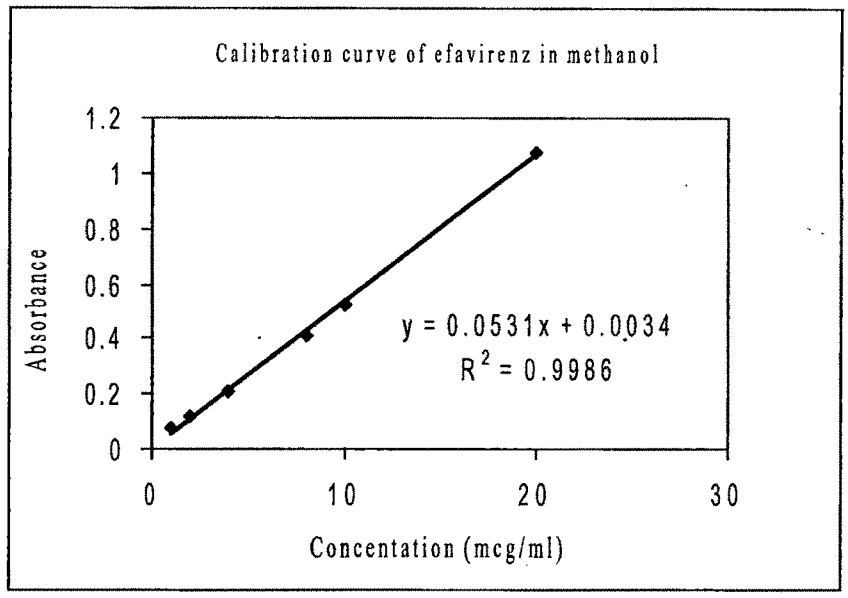

Figure 3.8: Calibration curve for the estimation of efavirenz.

3.5.3: Estimation of Efavirenz in 1% SLS

Efavirenz in 1% SLS (sodium lauryl sulphate) shows absorbance in the ultraviolet region

3.5.3.1: Solution

Stock solution of efavirenz was prepared by dissolving 10mg of efavirenz in 100ml of 1% SLS. This solution was used as stock solution A 5ml of this solution was taken in 100ml volumetric flask and diluted to 100 ml by 1% SLS. This solution (5µg/ml) was preserved and used as stock solution B.

3.5.3.2: Procedure for calibration curve:

Suitable aliquots (0.5ml -1 ml) of the stock solution A and suitable aliquots (0.5ml - 8ml) of the stock solution B were pipetted into 10ml volumetric flasks separately and the volume was made upto 10ml with methanol to give final concentrations of 1 - 20µg/ml. The solution was shaken well and their absorbance was measured as 247nm using 1% SLS as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.5 tabulated the raw and regressed data (method of least

squares) so obtained. Absorptivity scan over the UV wavelength range between 200 and 400nm for 10µg/ml solution of efavirenz in 1% SLS and the calibration curves obtained are shown in Figure 3.9 and Figure 3.10.

Table 3.5: Calibration curve for efavirenz in 1% SLS

Concentration (µg/ml	Mean absorbance* ± S.D	Regressed value
1	0.0653 ± 0.0019	0.0555
2	1.0433 ± 0.004	0.0994
4	0.2033 ± 0.0071	0.1872
5	0.2231 ± 0.031	0.2311
10	0.439 ± 0.002	0.4506
20	0.8948 ± 0.0088	0.8896

Regression equation; Y= 0.0436 x +0.0167

Correlation coefficient= 0.9989

* data are expressed as mean ± standard deviation (S.D.) and mean of 6 values

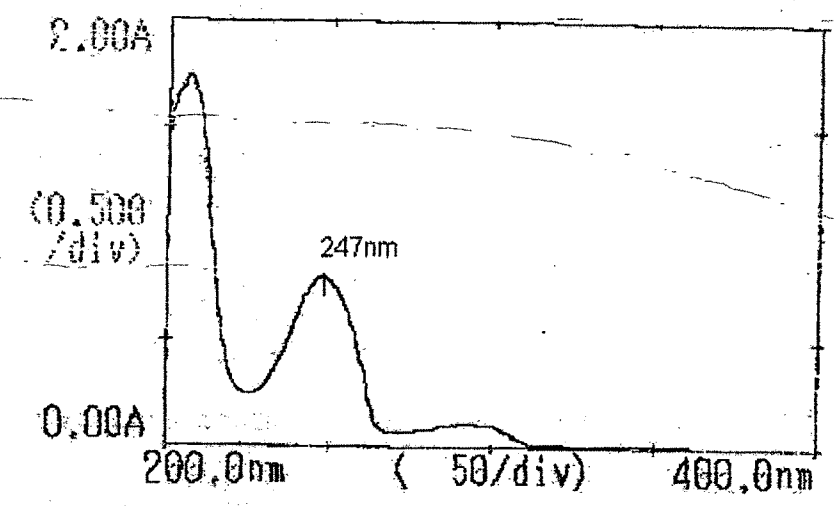

Figure 3.9: Absorptivity scan of efavirenz in 1% SLS

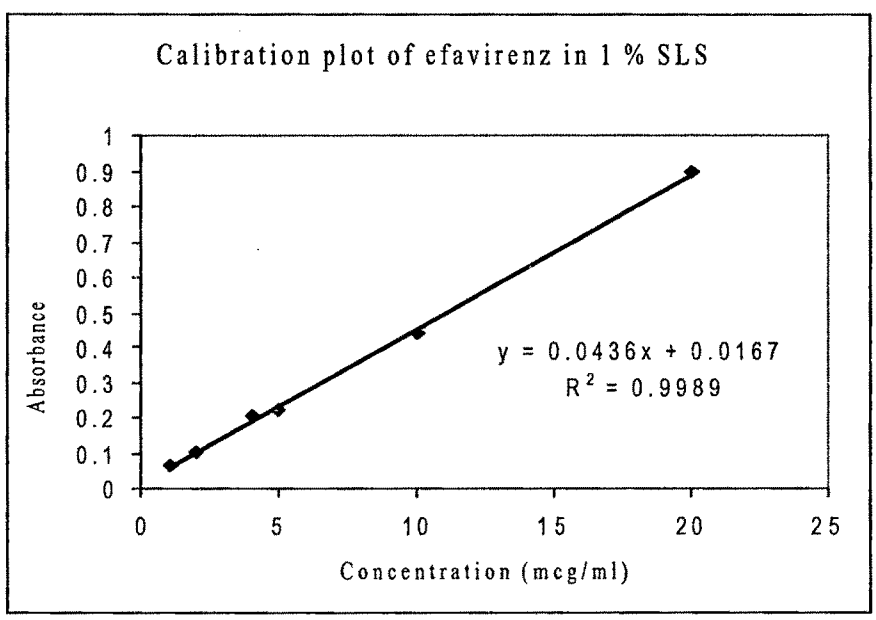

Figure 3.10: Calibration curve for the estimation of efavirenz.

3.5.4: Estimation of efavirenz in rat plasma:

For the estimation of efavirenz the reported method (Suresh K. Balani, et al 1998) was used.

3.5.4.1: Chromatographic system:

The HPLC system consisted of Dionex 1100 series components including a quaternary pump, auto sampler and variable wavelength UV detector. Chromatographic separations were achieved using an Hypersil ODS column (250 x 4.6mm, 10µm) (Thermo Electron Corporation)

3.5.4.2: Chromatographic conditions:

The mobile phase used for the plasma sample was 58% acetonitrile in 0.1% ammonium bicarbonate (NH_4HCO_3), pH 7.4 in water (pH adjusted to 7.4 by 0.1N HCl or by 0.1N NaOH) - Filtration of the buffer was done using 0.2µ nylon 6.6 membrane filters and degassing by sonication. Flow rate was adjusted to 1 ml/min. The HPLC effluent was monitored at 247 nm. The column was washed with 90% acetonitrile in NH_4HCO_3 for 3 min at the end of each run. Overall run time was 15 min per injection. Retention times of efavirenz were approximately 8 minutes.

3.5.4.3: Calibration curve in standard solution:

Appropriate amounts of efavirenz were weighed and added to the mobile phase to yield final stock solution concentration of 1mg/ml. Standard solution of 0.10, 0.20, 0.40, 0.80, 1, 2, 5µg/ml of efavirenz was prepared by appropriately diluting the stock solution with mobile phase. Stock solution was kept in refrigerator when not in use and replaced on weekly basis. The stock solutions were assumed to be stable over a period of 1 week due to the low degree of variability (< 5% RSD) during that time. Fresh standard solutions were prepared for each day of analysis. Peak area of different concentration of efavirenz was shown in Table 3.6. Calibration curve was shown in Figure 3.11

Table 3.6: Peak area for different concentration of efavirenz in standard solution

Concentration (µg/ml)	Peak area* ± S.D	Regressed value
0.1	0.2191 (0.0054)	0.2514
0.2	0.427 (0.0024)	0.3812
0.4	0.6854 (0.001)	0.6407
0.8	1.1076 (0.0209)	1.1596
1	1.4568 (0.0056)	1.4191
2	2.6903 (0.0051)	2.7165
5	6.5674 (0.0129)	6.6087
10	13.1198 (0.0021)	13.0957

Regression equation; Y= 1.2974 x + 0.1217

Correlation coefficient= 0.9999

* data are expressed as mean ± standard deviation (S.D.) and mean of 3 values

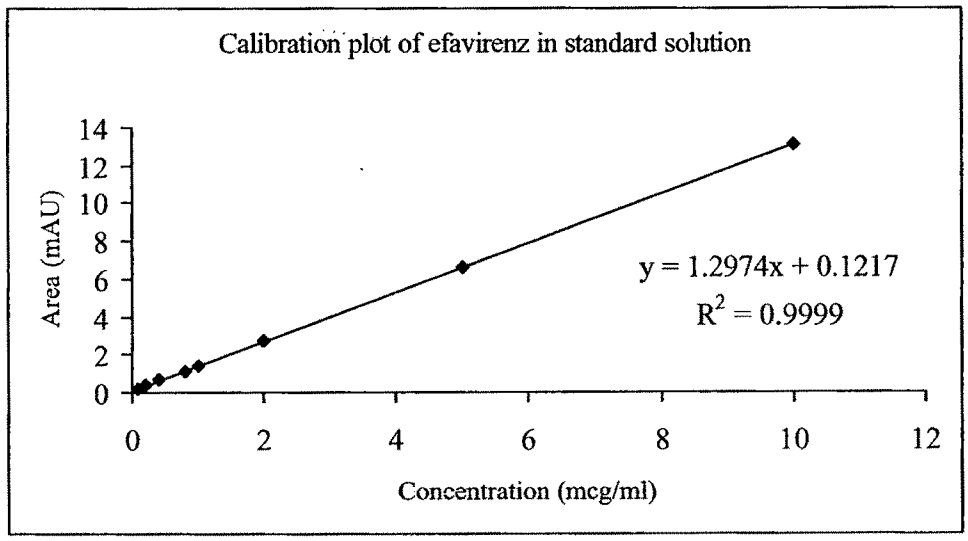

Figure 3.11: Calibration curve of efavirenz in standard solution

3.5.4.4: Calibration curve in rat plasma:

Blank plasma was collected from untreated anaesthetized rat and kept at -20^0C until analysis. 200µl of the thawed plasma samples mixed with 100µl of mobile phase (for blank plasma) and the standard stock solution of different concentration (form 100ng/ml to 5µg/ml) of efavirenz in mobile phase and vortexes for one minute using vibromixer. Then the mixture was basified by the addition 20µl of 0.1N sodium hydroxide ((NaOH) and mixed for 1minute. Then the mixture was extracted with 5 ml of methylene chloride. The resultant mixture was centrifuged at 10,000rpm for 10minute at 0^0C temperature. The organic layer was separated and the solvent was evaporated under a stream of nitrogen. The residues were reconstituted in a 4:1mixture of acetonitrile and 0.1% NH_4HCO_3, pH 7.4. 20µl of the reconstituted solution was injected into HPLC. The area obtained after injecting different known concentration of efavirenz was represented in Table 3.7 and calibration curve was presented in Figure 3.12

Table 3.7: Peak area for different concentration of efavirenz in rat plasma

Concentration (µg/ml)	Peak area* ± S.D	Regressed value
0.05	0.1107 (0.0046)	0.1146
0.1	0.2187 (0.0049)	0.2213
0.2	0.4394 (0.0078)	0.4346
0.4	0.8293 (0.0074)	0.8614
0.8	1.6905 (0.0071)	1.7149
1	2.1343 (0.0084)	2.1416
1.2	2.6897 (0.0056)	2.5684
1.4	2.9669 (0.0073)	2.9950
1.8	3.8348 (0.0114)	3.8407
3	6.3948 (0.0104)	6.409

Regression equation; Y= 2.1337 x + 0.0079

Correlation coefficient= 0.9995

* data are expressed as mean ± standard deviation (S.D.) and mean of 3 values

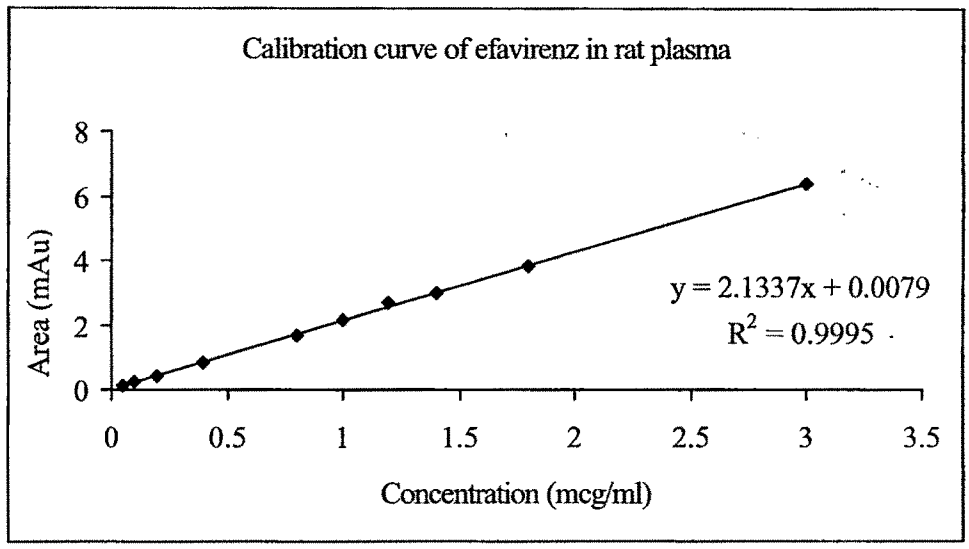

Figure 3.12: Calibration curve of efavirenz in rat plasma

3.6. Results and discussion:

3.6.1. Estimation of acyclovir in 0.1N HCl:

Acyclovir in 0.1N HCl yields a characteristics curve when scanned under UV wavelength range between 200-400nm. The absorptivity scan (Figure 3.1) shows absorption maxima at 252nm Table 3.1 indicated a linear relationship between absorbance and concentration of acyclovir. Beer's law was found to be obeyed between 2 - 20µg/ml. The data of regression analysis on the collected data along with the raw data are presented in Table 3.1. The regression equation obtained was Y= 0.0565x – 0.0068 and regression coefficient found was 0.9989 (Figure 3.2).

The variance of response variables, S^2yx was obtained 1.8 x 10^{-4} .This lower values denoted that the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also seen from the low value of the standard error of the mean absorbance of the solutions used for obtaining calibration curve. (Table 3.1). The variance of the slope, S^2b was calculated as 9.237 x 10^{-7}. The value of the slope (0.0565, Figure 3.2) indicated low sensitivity of the method.

The variance of the intercept, S^2a, calculated was 9.545 x 10^{-5} .The significance of the intercept was examined using the null hypothesis. The value of 't' was obtained as 0.238, whereas the value of t required for significance is 2.78 at 4 degree of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements.

The stability of drug in the solution was ascertained by measurements of absorbance of the solutions, used for preparing of calibration curve, at regular intervals of time. It was observed that the stability of drug was retained as its original intensity for 48hours. ANOVA studies of the mean absorbance value of solution at different time revels no significance difference between the readings. Thus the method was found to be satisfactory for estimation of acyclovir.

3.6.2. Estimation of acyclovir in ethanol:

Acyclovir in ethanol yields a characteristics curve when scanned under UV wavelength range between 200-400nm. The absorptivity scan (Figure 3.3) shows absorption maxima at 236.5nm Table 3.2 indicated a linear relationship between absorbance and

concentration of acyclovir. Beer's law was found to be obeyed between 2 - 20µg/ml. The data of regression analysis on the collected data along with the raw data are presented in table 3.2. The regression equation obtained was Y= 0.0744X + 0.053 and regression coefficient found was 0.9993 (Figure 3.4).

The variance of response variables, S^2yx obtained was 2.4 X 10^{-4} .This lower value denoted that the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also seen from the low value of the standard error of the mean absorbance of the solutions used for obtaining calibration curve (Table 3.2). The variance of the slope, S^2b was calculated as 1.009 X 10^{-6}. The value of the slope (0.0744, Figure 3.4) indicated low sensitivity of the method.

The variance of the intercept, S^2a, calculated was 1.48 X 10^{-4}.The significance of the intercept was examined using the null hypothesis. The value of 't' was obtained as 0.240, whereas the value of 't' required for significance is 2.78 at 4 degree of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of drug in the ethanol was ascertained by measurements of absorbance of the solutions, used for the preparing of calibration curve, at regular intervals of time. The drug was observed to be stable as the solution retained its original intensity for 48hours. ANOVA studies of the mean absorbance value of solution at different time revels no significance difference between the readings. The presence of the other constituents of the formulations such as tween 80, labrasol, labrafac, propylene glycol, plurol olique etc at the levels at which these materials were included in the formulation, did not affect the estimation of acyclovir. Thus the method was found to be satisfactory for estimation of acyclovir in formulation also.

3.6.3. Estimation of acyclovir in phosphate buffer saline (PBS) pH 7.4:

Acyclovir in PBS (pH 7.4 yields a characteristics curve when scanned under UV wavelength range between 200-400nm. The absorptivity scan (Figure 3.5) shows absorption maxima at 252nm Table 3.3 indicated a linear relationship between absorbance and concentration of acyclovir. Beer's law was found to be obeyed between 2 - 16µg/ml. The regression analysis on the collected data along with the raw data are

presented in table 3.3. The regression equation obtained was Y= 0.0547 x + 0.043 and regression coefficient found was 0.9967 (Figure 3.6).

The variance of response variables, S^2yx obtained was 4.7 X 10^{-4} .This lower value denoted that the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also seen from the low value of the standard error of the mean absorbance of the solutions used for obtaining calibration curve (Table 3.3). The variance of the slope, S^2b was calculated as 1.934 X 10^{-6}. The value of the slope (0.0547, Figure 3.6) indicated low sensitivity of the method.

The variance of the intercept, S^2a, calculated was 2.8 X 10^{-4} .The significance of the intercept was examined using the null hypothesis. The value of't' was obtained as 1.23, whereas the value of 't' required for significance is 2.78 at 4 degree of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of drug in the PBS (pH7.4) was ascertained by measurements of absorbance of the solutions, used for preparing calibration curve, at regular intervals of time. The drug was observed to be stable as the solution retained its original intensity for 48hours. ANOVA studies of the mean absorbance value of solution at different time revels no significance difference between the readings. Thus the method was found to be satisfactory for the estimation of acyclovir during *in-vitro* diffusion study through rat intestine

3.6.4. Estimation of acyclovir in rat plasma:

Various reported method was tried for the estimation of acyclovir in rat plasma. Though these methods are suitable for the estimation of acyclovir in water and some in human plasma, they were not found suitable for estimation in rat plasma. Drug peak was merged or not properly separated from the blank plasma peak (interfering peak). To achieve baseline resolution of acyclovir from the interfering peaks, the level of octane sulphonic acid sodium salt and the concentration of buffer and flow rate were altered until the desired separation was achieved. Figure 3.13 to Figure 3.15 shows the blank plasma peak, acyclovir peak in water, acyclovir peak in rat plasma respectively.

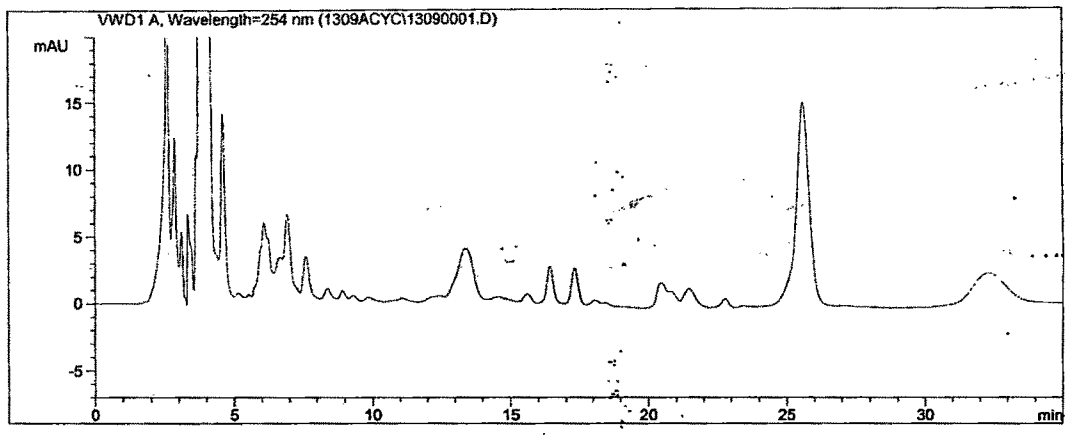

Figure 3.13: Blank rat plasma peak for estimation of acyclovir in rat plasma

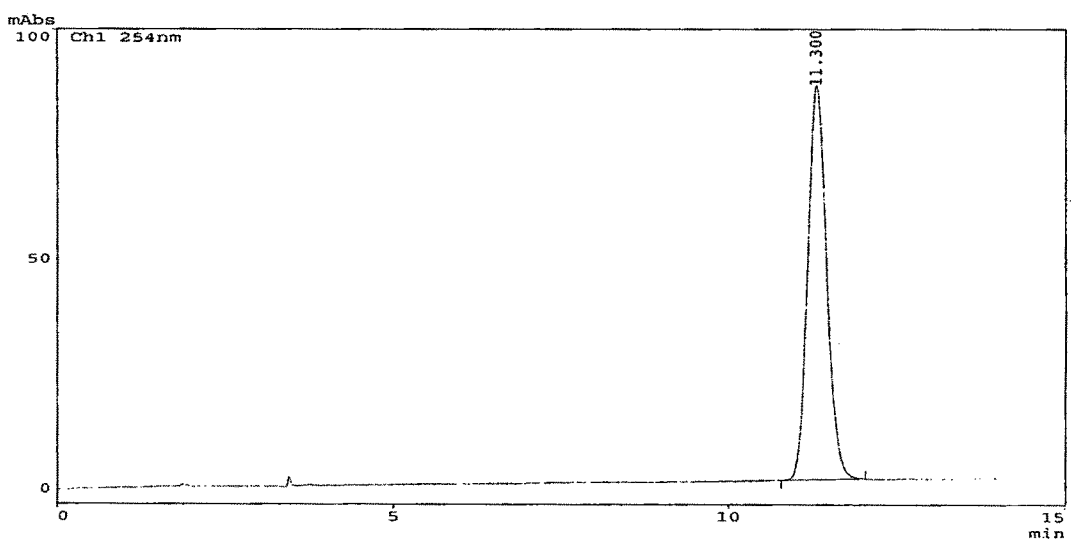

Figure 3.14: Acyclovir peak in water.

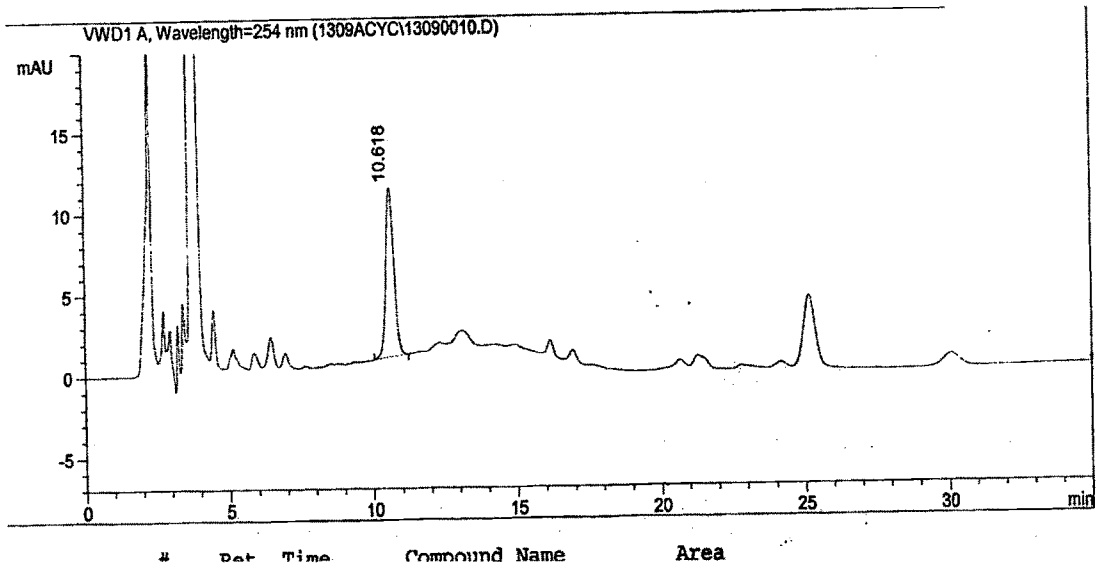

Figure 3.15: Acyclovir peak in rat blood plasma.

Flow rate was adjusted to as follows: 1ml/min (0-14min), 2ml/min (16-30 min) and then again 1ml/min (30-50min). Detection wavelength fixed at 254nm. Under these conditions acyclovir eluted at ~10.6minute. Linear relationship was observed in the range between 10ng/ml to 5µg/ml in water and 100ng/ml to 5µg/ml in plasma and correlation coefficient value was 0.9995 and 1 for water and plasma sample respectively. % Bias of all the sample was <10%, proves the accuracy of the method.

It was also found that the analytical peaks of acyclovir were well resolved from one another and in rat plasma from 5 rats. There was clear separation between acyclovir and the nearest plasma peak.

3. 6.4.1 Calibration curve in rat plasma:

Table 3.8 show the area obtained after injecting the known concentration of drug. Figure 3.16 represents the calibration curve of acyclovir in rat blood plasma.

Table 3.8: Peak area for different concentration of acyclovir

Concentration (µg/ml)	Peak area* ± S.D	Regressed value
0.05	10.58 ±0.44	10.372
0.1	15.72 ± 0.455	15.54
0.2	25.756 ± 0.561	25.875
0.4	46.43 ± 1.154	46.545
0.6	67.65 ± 1.766	67.215
0.8	87.736 ± 1.646	87.885
1	107.873 ± 1.292	108.555
3	315.313 ± 1.637	315.255
5	521.89 ± 1.5734	521.955

Regression equation; Y= 103.35 x + 5.2051

Correlation coefficient= 1

* data are expressed as mean ± standard deviation (S.D.) and mean of 3 values

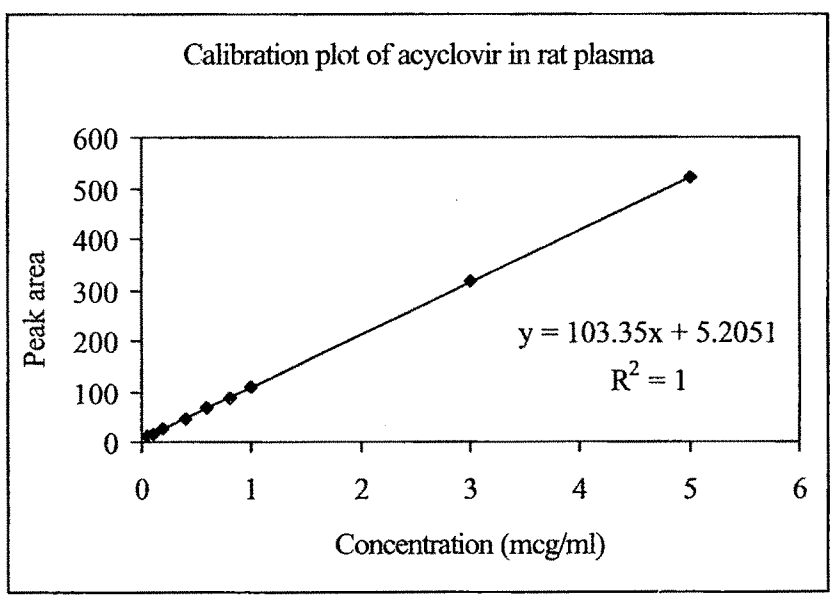

Figure 3.16 Calibration of acyclovir in rat plasma

3.6.4.2. Validation of the HPLC method:

Intra-day precision and accuracy of method were assessed by acyclovir in water and analyzing spiking plasma samples at low (100ng/ml), medium (1 µg/ml) and high (5 µg/ml) concentration. Five replicate of each concentration were analyzed and results are given in Table 9 and Table 10. To evaluate precision, the mean values and the %-RSD values were calculated for each concentration. The intra-day precision was found to be acceptable, with the % RSD values ranging from 2.38 to 5.27% spiked plasma. Moreover, the accuracy was calculated, which ranged from 98.56 -100.92% for spiked plasma. The inter-day data for precision and accuracy was also determined by analyzing three sample batches of spiked plasma at low, medium and high concentration on five separate days. The inter-day % RSD and % accuracy values are ranged from 1.23 to 3.79 and 96.6 to 98.83% respectively for spiked plasma, respectively. Results are also presented in Table 3.9 and 3.10.

Table 3.9: Precision and accuracy of intra-day analysis for determination of acyclovir in rat plasma by HPLC

Concentration (µg/ml)	Mean (± S.D)*	% R.S.D	% accuracy
0.1	0.985 (0.005)	5.27	98.56
1	1.009 (0.033)	2.38	100.92
5	4.949 (0.005)	2.96	98.99

* data are expressed as mean ± standard deviation (S.D.) and n=6

Table 3.10: Precision and accuracy of inter-day analysis for determination of acyclovir in rat plasma by HPLC

Concentration (µg/ml)	Mean (SD)*	% RSD	% Accuracy
0.1	0.096 (0.003)	3.79	96.6
1	0.988 (0.027)	7.08	98.83
5	4.915 (0.111)	1.23	98.3

* data are expressed as mean ± standard deviation (S.D.) and n=6

3.6.5. Estimation of efavirenz in methanol:

Efavirenz in methanol yields a characteristics curve when scanned under UV wavelength range between 200-400nm. The absorptivity scan (Figure 3.11) shows absorption maxima at 247nm Table 3.6 indicated a linear relationship between absorbance and concentration of efavirenz. Beer's law was found to be obeyed between 1 - 20µg/ml. The regression analysis on the collected data along with the raw data are presented in Table 3.6. The regression equation obtained was Y= 0.0531 x + 0.0034 and regression coefficient found was 0.9986 (Figure 3.12).

The variance of response variables, S^2yx was obtained 2.46 X 10^{-4}. This lower value denoted that the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also seen from the low value of the standard error of the mean absorbance of the solutions used for obtaining calibration curve (Table 3.6). The variance of the slope, S^2b was calculated as 9.94 X 10^{-7}. The value of the slope (0.0531, Figure 3.12) indicated low sensitivity of the method.

The variance of the intercept, S^2a, calculated was 9.69 X 10^{-5}. The significance of the intercept was examined using the null hypothesis. The value of't' was obtained as 0.238, whereas the value of't' required for significance is 2.78 at 4 degree of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of efavirenz in methanol was ascertained by measurements of absorbance of the solutions, used for preparing calibration curve, at regular intervals of time. The drug was observed to be stable as the solution retained its original intensity for 48hours. ANOVA studies of the mean absorbance value of solution at different time revels no significance difference between the readings. The presence of the other constituents of the formulations such as labrasol, transcutol, labrafil M 1944CS, cremophor RH 40, propylene glycol etc., at the levels at which these materials were included in the formulation, did not affect the estimation of efavirenz. Thus the method was found to be satisfactory for the estimation of efavirenz in formulation.

3.6.6. Estimation of efavirenz in 1% SLS:

Efavirenz in 1% SLS yields a characteristics curve when scanned under UV wavelength range between 200-400nm. The absorptivity scan (Figure 3.13) shows absorption maxima at 247nm Table 3.7 indicated a linear relationship between absorbance and concentration of efavirenz. Beer's law was found to be obeyed between 1 - 20µg/ml. The regression analyses on the collected data along with the raw data are presented in Table 3.7. The regression equation obtained was Y= 0.0436 x + 0.0167 and regression coefficient found was 0.9989 (Figure 3.14).

The variance of response variables, S^2yx obtained was 1.33 X 10^{-4} .This lower value denoted that the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also seen from the low value of the standard error of the mean absorbance of the solutions used for obtaining calibration curve (Table 3.7). The variance of the slope, S^2b was calculated as 4.81 X 10^{-5}. The value of the slope (0.0436, Figure 3.14) indicated low sensitivity of the method.

The variance of the intercept, S^2a, calculated was 5.29 X 10^{-7} .The significance of the intercept was examined using the null hypothesis. The value of 't' was obtained as 0.242, whereas the value of 't' required for significance is 2.78 at 4 degree of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of efavirenz in the 1% SLS was ascertained by measurements of absorbance of the solutions, used for preparing calibration curve, at regular intervals of time. The drug was observed to be stable as the solution retained its original intensity for 48hours ANOVA studies of the mean absorbance value of solution at different time revels no significance difference between the readings. Thus the method was found to be satisfactory for the estimation of efavirenz during in vitro diffusion study either by using rat intestine or dialysis bag.

3.6.7. Estimation of efavirenz in rat plasma:

Figure 3.17 to Figure 3.19 shows the chromatogram of blank rat plasma, efavirenz in mobile phase and efavirenz in rat plasma respectively.

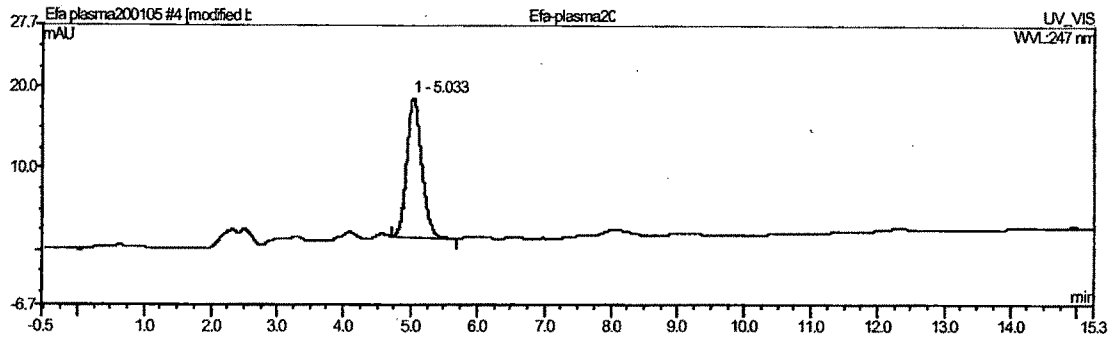

Figure 3.17: Blank rat plasma peak for estimation of efavirenz in plasma

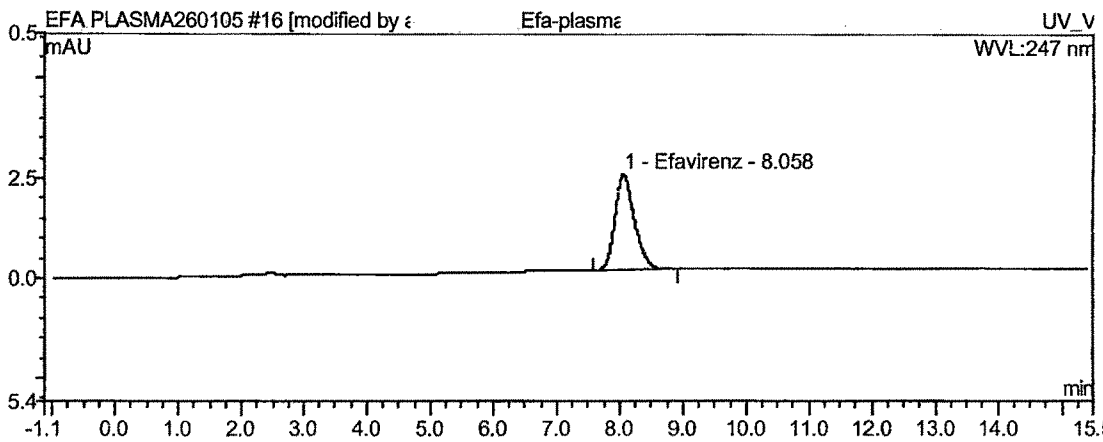

Figure 3.18: Efavirenz peak in mobile phase

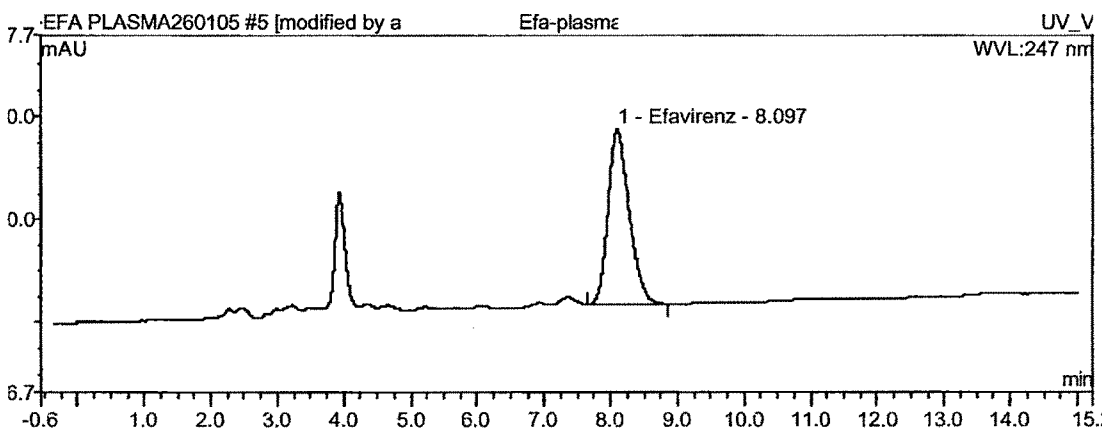

Figure 3.19: Efavirenz peak in rat blood plasma.

The retention time for the efavirenz in mobile phase was found at approx 8.0min. Linear relationship was observed in the range between 100ng/ml to 5µg/ml in mobile phase and in plasma and corrlation coefficient value was 0.9999 and 0.9995 for water and plasma sample respectively. % Bias of all the sample was <10%, proves the accuracy of the method.

It was also found that the analytical peaks of efavirenz were well resolved from one another and in rat plasma from 5 rats. There was clear separation between efavirenz and the nearest plasma peak. From the Figure 3.18, it was shown that retention time for efavirenz in mobile phase was approx 8.05miniute. But there was no peak near 8.0minute in blank rat plasma.

Accuracy and precision of the method was not determined since the method was reported earlier. The method was used to estimate the amount of efavirenz in rat plasma after administered the efavirenz formulations by oral route.

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