Chapter 3
Analytical Method

Analytical Method

3.1 Materials:

Saquinavir (Aurbindo Pharma Ltd, Mumbai) and Adefovir dipivoxil (Cipla Ltd, Mumbai) were received as gift samples. Ammonium bicarbonate, Potassium dihydrogen phospate, Disodium hydrogen phosphate, Sodium hydroxide, Hydrochloric acid were procured from SD Fine chemicals, Mumbai. Double distilled water (DDW) was purified by passing through 0.45μ Millipore filters (Millipore, Bangalore).

3.2 Estimation of Saquinavir by Ultraviolet Spectrophotometry (UV)

Analytical method for estimation of Saquinavir for dissolution of Saquinavir capsule in citrate buffer is reported in USP/NF 25. As there is no analytical method reported for UV spectrophotometric analysis of Saquinavir in methanol and different media (0.1 N HCl, pH 4.5 buffer and pH 7.2 buffer), the method was developed for the same.

3.2.1 Calibration Curve

Stock solution of Saquinavir (SQ) was prepared by dissolving 10 mg of drug in 100 ml of methanol (Stock solution - 100μg/ml).

Calibration plot in Methanol:

Suitable aliquots of the stock solution of SQ were pipetted out into 100 ml volumetric flasks and the volume was made upto 100 ml with methanol to give final concentrations ranging from 1-16 μg/ml (Table 3.1). The solutions were mixed using vortex mixture and their absorbances were measured using methanol as blank on UV-Visible Spectrophotometer (Shimadzu 1601, Japan) and calibration curve was plotted (Fig 3.1). The above procedure was repeated three times.

Calibration plot in Distilled water:

Suitable aliquots of the stock solution of SQ were pipetted out into 100 ml volumetric flasks and the volume was made upto 100 ml with Distilled water to give final concentrations ranging from 1-16 μg/ml. Distilled water was used as lank. Absorbance was measured at 239 nm on Shimadzu 1601 UV-Visible spectrophotometer and calibration curve was plotted (Table 3.2. Fig 3.2). The above procedure was repeated three times

Calibration plot in 0.1 N HCl:

The procedure was same as that of Calibration in Distilled water. 0.1 N Hcl was used as blank against different concentration (1- 16 µg/ml) of SQ in 0.1 N Hcl (Table 3.3. Fig 3.3). The procedure was repeated three times.

Calibration plot in pH 4.5 Buffer:

The procedure was same as that of Calibration in Distilled water. pH 4.5 buffer was used as blank against different test solutions of SQ in pH 4.5 buffer (1- 16 µg/ml) . The procedure was repeated three times (Table 3.4. Fig 3.4).

Calibration plot in pH 7.2 Buffer:

The procedure was same as that of Calibration in Distilled water. Different test solutions of SQ in pH 7.2 buffer were prepared and studied against pH 7.2 Buffer as blank. The procedure was repeated three times (Table 3.5. Fig 3.5).

3.2.2 Analytical method validation:

The method was validated for accuracy, precision and linearity.

3.2.2.1 Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample (Hubert et al., 1999; Hubert et al., 2003). The calibration curve was built by plotting the drug concentrations versus their respective absorbance.

3.2.2.2 Accuracy

Accuracy refers to the closeness of an individual observation or mean to true value (Bolton, 1990). The "true" value is the result which would be observed in absence of error. Accuracy of the assay is defined as the percentage of the agreement between the measured value and the true value as follows (Merodia et al, 2000):

$$Accuracy = \frac{True \quad value \ - Measured \quad value}{True \quad value} x \quad 100 \qquad \text{---- (3.1)}$$

Procedure: To confirm the accuracy of proposed method, recovery study was performed by the standard addition technique. Recovery greater than 96 % with low SD justifies the accuracy of the method.

3.2.2.3 Precision

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) or CV (Coefficient of variation) of inter-day and intra-day determinations. One of the common way of expressing the variability, which takes into account its relative magnitude is the ratio of the standard deviation to the mean, SD/Mean. This ratio, often expressed as a percentage, is called the *Coefficient of Variation* abbreviated as CV or RSD, the *relative standard deviation.* The variability in chemical and instrumental analysis of drugs is usually relatively small. Thus, it is not unusual to find a CV of less than 1% for some analytical procedures. (Bolton, 1990).

Procedure:

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels (1, 5 and 15µg /ml) of Saquinavir within linearity range. The experiments were repeated three times a day for *intra-day precision* and on three different days for *inter-day precision.*

3.2.3 Results and Discussion

SQ yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. Beer's law was obeyed between 1 and 16 µg /ml. Correlation coefficient for developed method was found to be 0.9978 (methanol), 0.9999 (water), 0.9993 (0.1 N HCl), 0.9998 (pH 4.5 buffer) and 0.9999 (pH 7.2 Buffer) signifying that a linear relationship existed between absorbance and concentration of the drug.

Table 3.1 and 3.2 show calibration value of SQ in methanol and water respectively. Fig 3.1 shows calibration curve of SQ in methanol. Table 3.3 and 3.4 show intraday and interday precision and accuracy for the SQ assay by UV spectroscopy. The low % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Boulangeret al., 2003; Guidance for industry, 2001).

Table 3.1 Calibration data of SQ in methanol

Sr.No.	Concentration (μg/ml)	Mean Absorbance* ± S.D.
1	1	0.076± 0.009
2	2	0.141±0.004
3	4	0.287±0.019
4	8	0.573±0.008
5	16	1.089±0.024

*= Average of 3 determinations

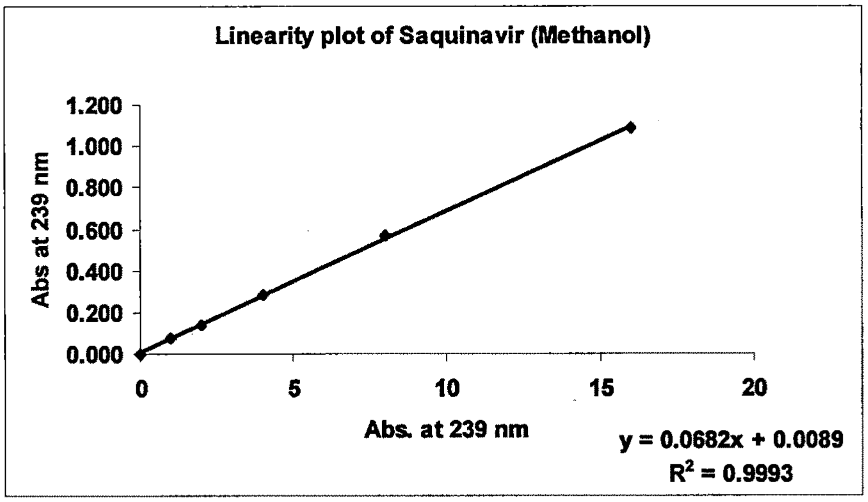


Fig .3.1 Calibration Curve of SQ in Methanol

Table 3.2 Calibration data of SQ in Water

Sr.No.	Concentration (µg/ml)	Mean Absorbance* ± S.D.
1	1	0.070± 0.006
2	2	0.138±0.001
3	4	0.259±0.012
4	8	0.526±0.035
5	16	1.035±0.085

*= Average of 3 determinations

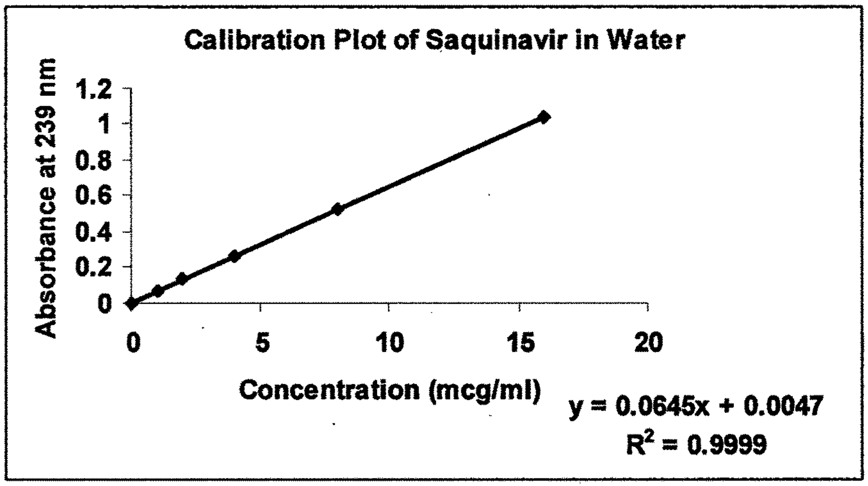


Fig. 3.2 Calibration Curve of SQ in Water

Table 3.3 Calibration data of SQ in 0.1 N HCl

Sr.No.	Concentration (µg/ml)	Mean Absorbance* ± S.D.
1	1	0.072± 0.006
2	2	0.148±0.007
3	4	0.258±0.012
4	8	0.534±0.035
5	16	1.01±0.085

*= Average of 3 determinations

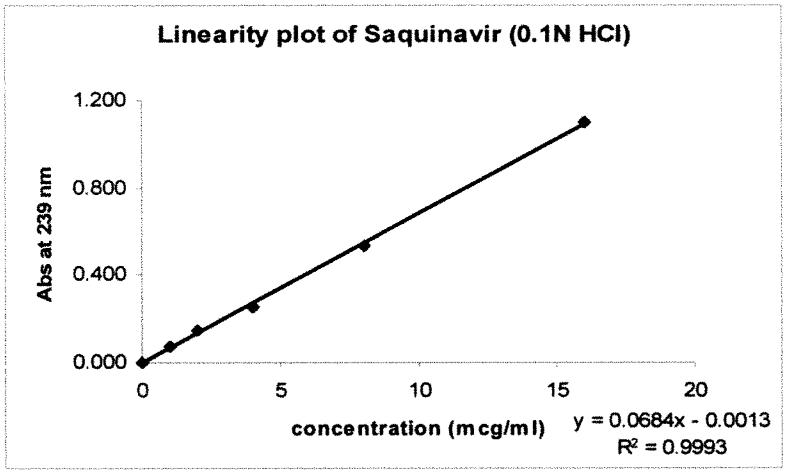


Fig.3.3 Calibration Curve of SQ in 0.1 N HCl

Table 3.4 Calibration data of SQ in pH 4.5 Buffer

Sr.No.	Concentration (µg/ml)	Mean Absorbance* ± S.D.
1	1	0.073± 0.0068
2	2	0.148±0.00156
3	4	0.258±0.0162
4	8	0.534±0.0150
5	16	1.01±0.009

*= Average of 3 determinations

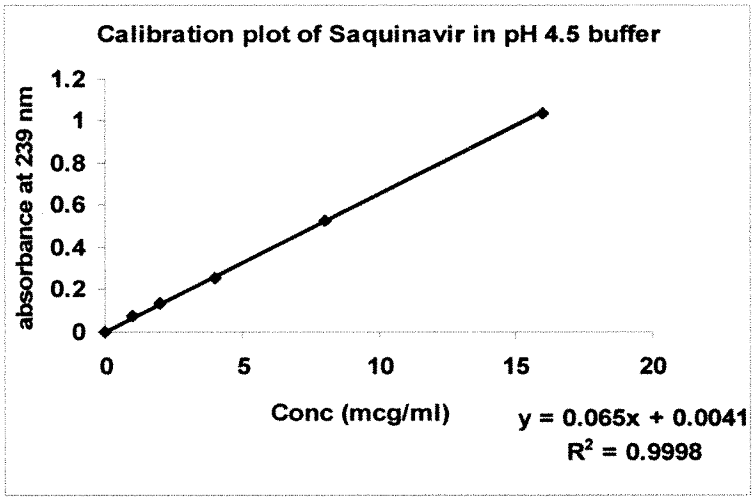


Fig. 3.4 Calibration Curve of SQ in pH 4.5 Buffer

Table 3.5 Calibration data of SQ in pH 7.2 Phosphate Buffer

Sr.No.	Concentration (μg/ml)	Mean Absorbance* ± S.D.
1	1	0.060± 0.015
2	2	0.148±0.01
3	4	0.258±0.002
4	8	0.534±0.005
5	16	1.01±0.003

*= Average of 3 determinations

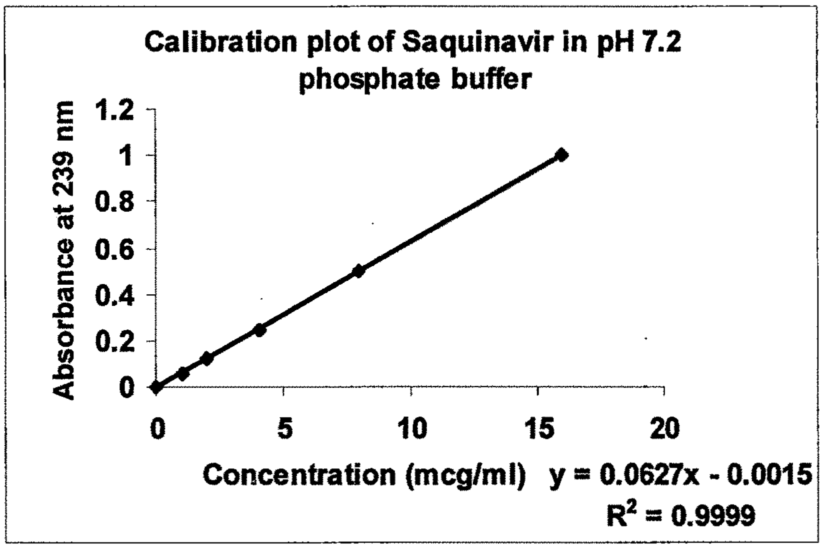


Fig. 3.5 Calibration curve of SQ in pH 7.2 Buffer

Linearity: Saquinavir was found to be linear in the range of 1 – 16 μg/ml as good correlation was observed between drug absorbance and concentration of analyte.

Accuracy:

Table 3.6 Accuracy study of Saquinavir in methanol and water

Solvent	Added conc. (mcg/ml)	Accuracy (%) $	RSD (%)*	S.E.**
Methanol				
	4	96.92	1.97	0.05
	5	94.73	1.08	0.04
	6	97.81	0.31	0.01
Water				
	4	97.68	1.37	0.04
	5	95.61	0.13	0.00
	6	97.51	0.98	0.04

$ - As per equation 3.1
* [RSD] = (standard deviation/mean concentration) x 100
** Standard Error (S.E.) = (standard deviation/ SQRT (degree of freedom)

Precision:

Table 3.7 and Table 3.8 represent the results measured in terms of % RSD for within-day and between-day variability studies. As evident the % RSD values of the data obtained were well below 2% indicates that the method was sufficiently precise.

Table 3.7 Intraday and Interday precision study of SQ in Methanol

Parameter	Actual Conc (µg/ml)	Measured (µg/ml)	RSD* (%)
Intraday	**1**	0.97	1.726
	5	4.95	1.196
	15	14.87	0.817
Interday	**1**	0.970	0.119
	5	4.923	0.244
	15	14.836	0.289

* Expressed as relative standard deviation,
[RSD] = (standard deviation/mean concentration) x 100

Table 3.8 Intraday and Interday precision study of SQ in Water

Parameter	Actual Conc (µg/ml)	Measured (µg/ml)	RSD * (%)
Intraday	1.00	0.99	1.40
	5.00	4.92	0.90
	15.00	14.88	0.38
Interday	1.00	0.91	0.94
	5.00	4.90	0.16
	15.00	14.67	1.28

* Expressed as relative standard deviation,
[RSD] = (standard deviation/mean concentration) x 100

3.3 Estimation of Adefovir Dipivoxil by Ultraviolet Spectrophotometry (UV)

3.3.1 Calibration Curve

Stock solution of Adefovir (ADF) was prepared by dissolving 10 mg of drug in 100 ml of distilled water (Stock solution - 100µg/ml). Scanning of standard solution of ADF was performed (Scanning range; 200-400nm) to determine its $\lambda_{max.}$

Calibration plot in Distilled water:

Suitable aliquots of the stock solution of ADF were pipetted out into 100 ml volumetric flasks and the volume was made upto 100 ml with Distilled water to give final concentrations ranging from 8-24 µg/ml (Table 3.9). Distilled water was used as blank. Study was carried out by measuring absorbance at 261 nm on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted (Fig 3.6). The above procedure was repeated three times

Calibration plot in IPA:

Suitable aliquots of the stock solution of ADF were pipetted out into 100 ml volumetric flasks and the volume was made upto 100 ml with IPA to give final concentrations ranging from 8-24 µg/ml (Table 3.10). The solutions were mixed using vortex mixture and their absorbances measured at 261 nm using IPA as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted (Fig 3.7). The above procedure was repeated three times.

Calibration plot in DCM: IPA (1:4):

The procedure was same as that of Calibration in *IPA*. DCM: IPA (1:4) was used as blank (Table 3.11, Fig.3.8). The procedure was repeated three times.

Calibration plot in 0.1 N HCl:

The procedure was same as that of Calibration in Distilled water. 0.1 N HCL was used as blank (Table 3.12, Fig.3.9). The procedure was repeated three times.

Calibration plot in pH 4.5 Buffer:

The procedure was same as that of Calibration in Distilled water. pH 4.5 Buffer was used as blank. The procedure was repeated three times (Table 3.13, Fig.3.10).

Calibration plot in pH 7.2 Buffer:

The procedure was same as that of Calibration in Distilled water. pH 7.2 Buffer was used as blank. The procedure was repeated three times (Table 3.13, Fig.3.10).

3.3.2 Analytical method validation:

The method was validated for accuracy, precision and linearity as per procedure in

Linearity

The concentrations for unknown samples and validation samples were obtained by using linear regression of the calibration curves.

Accuracy

The results of mean percentage recoveries of proposed method are shown in Table 3.14. Recovery greater than 96 % with low SD justifies the accuracy of the method.

Precision

The results were measured in terms of % RSD for within-day and between-day variability studies (Table 3.15 - 3.17). As evident the % RSD values of the data obtained were well below 2% indicates that the method was sufficiently precise.

3.3.3 Results and Discussion

ADF yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The calibration plot of ADF showed absorption maximum at 261 nm and this wavelength was chosen as the analytical wavelength. Beer's law was obeyed between 1 and 16 µg /ml. Correlation coefficient for developed method was found to be 0.9988 (water), 0.9967 (IPA), 0.9986 (0.1 N Hcl), 0.9988 (pH 4.5 buffer) and

0.998 (pH 7.2 Buffer) signifying that a linear relationship existed between absorbance and concentration of the drug.

Table 3.15 – 3.17 shows Intraday and Interday precision for the ADF assay by UV spectroscopy. The low % RSD values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Boulangeret al., 2003; Guidance for industry, 2001.).

Table 3.9. Calibration data of ADF in Distilled water

Sr.No.	Concentration (µg/ml)	Mean Absorbance* ± S.D.
1	8	0.226 ± 0.002
2	12	0.332 ± 0.001
3	16	0.435 ± 0.005
4	20	0.542 ± 0.00
5	24	0.676 ± 0.001

*= Average of 3 determinations

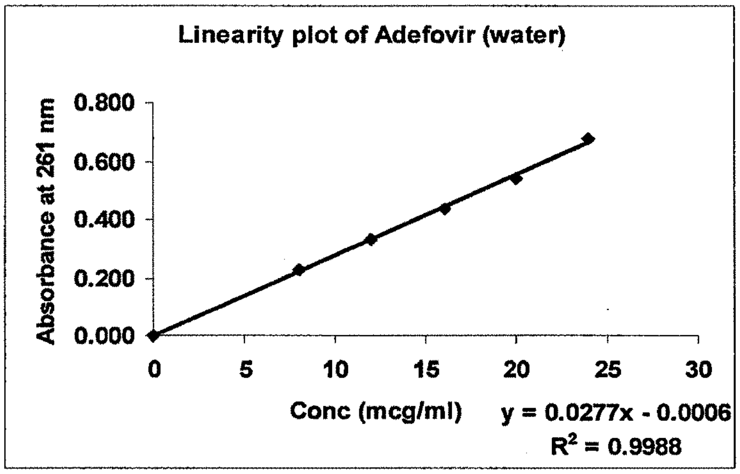


Fig. 3.6 Calibration Curve of ADF in water

Table. 3.10 Calibration data of ADF in IPA

Sr.No.	Conc (µg/ml)	Mean Absorbance ± S.D.
1	8	0.22 ± 0.07
2	12	0.368 ± 0.02
3	16	0.456 ± 0.01
4	20	0.581± 0.003
5	24	0.671± 0.01

*= Average of 3 determinations

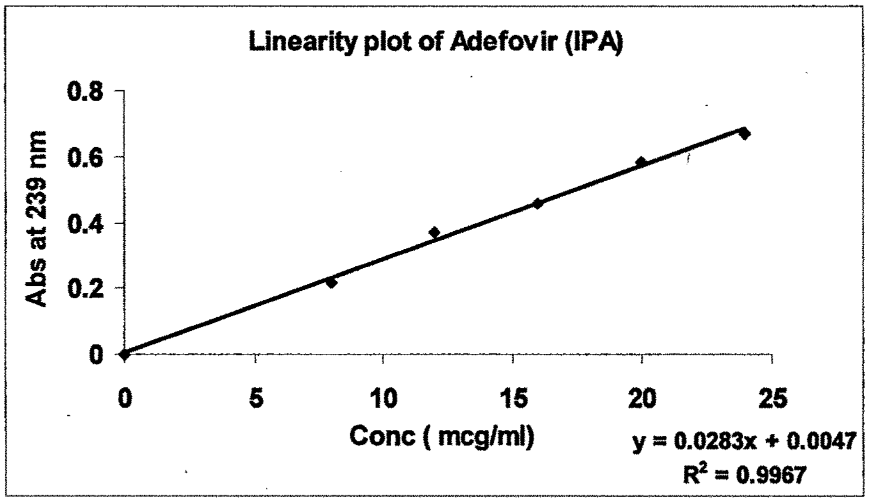


Fig. 3.7 Calibration Curve of ADF in IPA

Table. 3.11 Calibration data of ADF in DCM: IPA

Sr.No.	Conc (µg/ml)	Mean Absorbance* ± S.D.
1	8	0.245 ± 0.005
2	12	0.409 ± 0.03
3	16	0.564 ± 0.004
4	20	0.71± 0.045
5	24	0.867± 0.002

*= Average of 3 determinations

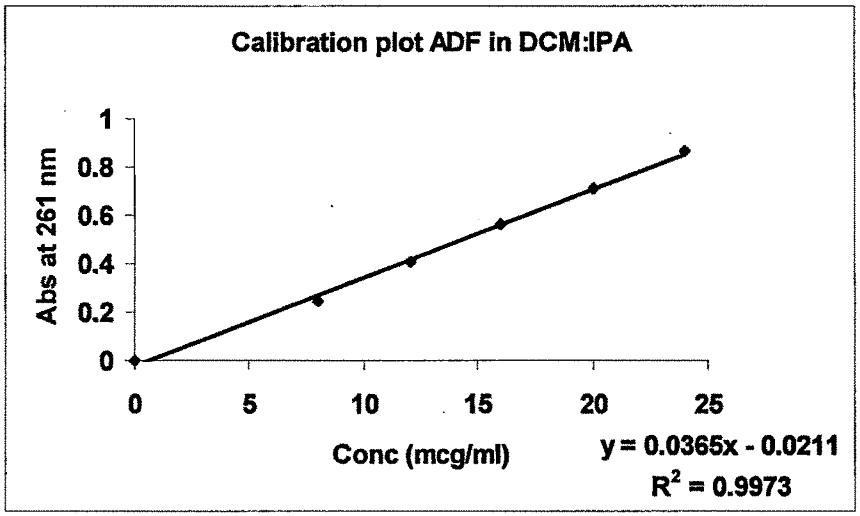


Fig. 3.8 Calibration Curve of ADF in DCM: IPA

Table. 3.12 Calibration data of ADF in 0.1 N HCl

Sr.No.	Conc (µg/ml)	Mean Absorbance ± S.D.
1	8	0.22 ± 0.17
2	12	0.344 ± 0.005
3	16	0.428 ± 0.008
4	20	0.541± 0.001
5	24	0.661± 0.029

*= Average of 3 determinations

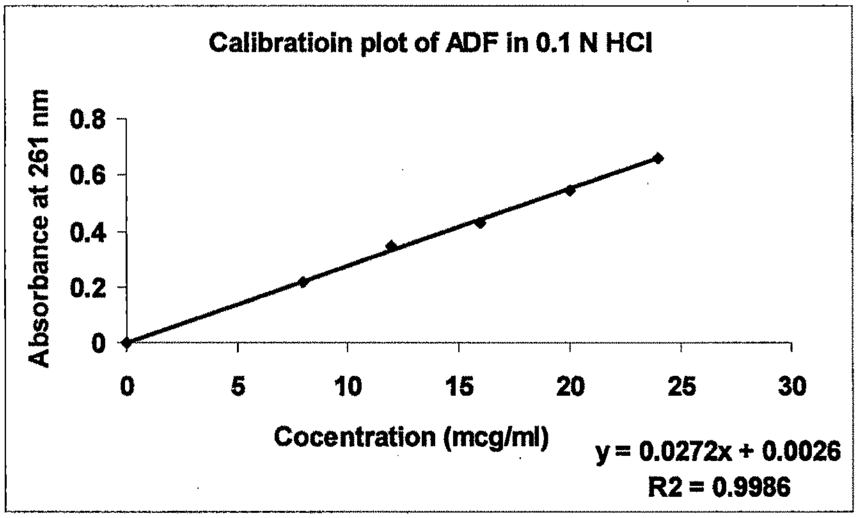


Fig. 3.9 Calibration Curve of ADF in 0.1 N HCl

Table. 3.13 Calibration data of ADF in pH 4.5 Buffer

Sr.No.	Conc (µg/ml)	Mean Absorbance ± S.D.
1	8	0.221 ± 0.01
2	12	0.325 ± 0.01
3	16	0.441 ± 0.01
4	20	0.547± 0.002
5	24	0.682± 0.01

*= Average of 3 determinations

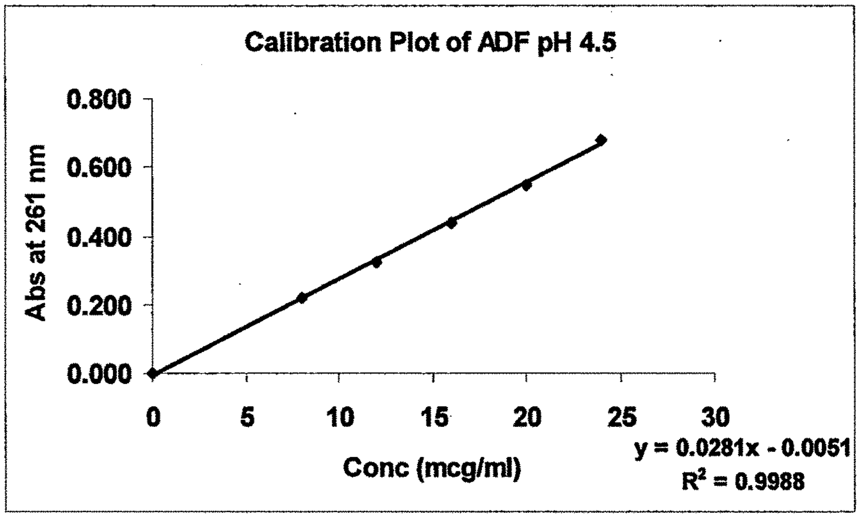
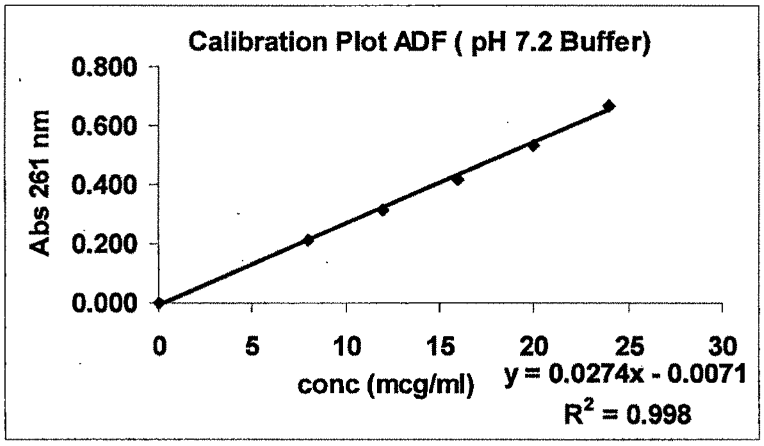


Fig. 3.10 Calibration Curve of ADF in pH 4.5 Buffer

Table. 3.13 Calibration data of ADF in pH 7.2 Buffer

Sr.No.	Conc (μg/ml)	Mean Absorbance ± S.D.
1	8	0.215 ± 0.005
2	12	0.315 ± 0.01
3	16	0.42 ± 0.004
4	20	0.535± 0.01
5	24	0.668± 0.008



*= Average of 3 determinations

Fig.3.11 Calibration curve of ADF in pH 7.2 Phosphate Buffer

Linearity: Adefovir Dipivoxil was found to be linear in the range of 8 – 24 μg/ml as good correlation was observed between drug absorbance and concentration of analyte.

Accuracy:

Table 3.14 Accuracy study of Adefovir Dipivoxil

Solvent	Added Conc. (mcg/ml)	Accuracy (%) $	RSD *(%)	S.E.**
Water	4	97.66	1.18	0.03
	5	95.40	0.36	0.02
	6	98.20	0.77	0.05
DCM:IPA				
	4	97.68	1.37	0.05
	5	94.99	1.15	0.05
	6	97.51	0.98	0.06
IPA				
	4	97.50	1.35	0.04
	5	94.73	1.08	0.05
	6	97.81	0.31	0.02

$ - As per equation 3.1
* [RSD] = (standard deviation/mean concentration) x 100
** Standard Error (S.E.) = (standard deviation/ SQRT (degree of freedom)

Precision:

Table 3.15 Intraday and Interday precision study in Water

Parameter	Actual conc (µg/ml)	Measured conc (µg/ml)	RSD (%) *
Intraday	1.00	0.98	1.42
	5.00	4.94	1.10
	15.00	14.85	0.79
Interday	1.00	0.97	0.06
	5.00	4.92	0.14
	15.00	14.81	0.35

* Expressed as relative standard deviation,
[RSD] = (standard deviation/mean concentration) x 100

Table 3.16 Intraday and Interday precision study in IPA

Parameter	Actual conc (µg/ml)	Measured conc (µg/ml)	RSD (%)
Intraday	1.00	0.98	1.16
	5.00	4.94	1.12
	15.00	14.89	0.88
Interday	1.00	0.97	0.12
	5.00	4.92	0.24
	15.00	14.84	0.29

* Expressed as relative standard deviation,
[RSD] = (standard deviation/mean concentration) x 100

Table 3.17 Intraday and Interday precision study in DCM: IPA

Parameter	Actual conc (µg/ml)	Measured conc (µg/ml)	RSD (%)
Intraday	1.00	0.94	5.23
	5.00	4.92	0.90
	15.00	14.88	0.38
Interday	1.00	0.91	0.94
	5.00	4.90	0.16
	15.00	14.67	1.28

* Expressed as relative standard deviation,
[RSD] = (standard deviation/mean concentration) x 100

Conclusion:

Calibration of SQ and ADF in different medias and solvents was carried out. The regression values obtained indicate that linear relationship was existed between absorbance and concentration of the drug. Accuracy and precision study was carried out in methanol and water in case of SQ and in water, DCM: IPA and IPA in case of ADF. There was no significant difference observed between actual and observed concentration which demonstrates accuracy of the method. The Relative Standard Deviation (%) value indicated that method was adequately precise for SQ and ADF.

References:

- Bolton S, Swarbrick J. (ed.), Basic definitions and concepts In: Pharmaceutical statistics: Practical and clinical applications, 3rd ed., Marcel Dekker, New York, 1990, pp 24.
- Boulanger B, Dewe W, Chiap P, Crommen J, Hubert PH. An analysis of the SFSTP guide on validation of bioanalytical methods: progress and limitations. J. Pharm. Biomed. Anal. 2003; 32: 753-765.
- Chamberlin, A. C., Andrew, P. K., Cheung, P., Florey, K. (ed), Analytical profiles of drug substances, vol. 5, Academic press Inc, New York, 1976, pp 283.
- Connors KA, Amidon GL, Stella VJ, Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists. John Wiley and Sons Inc., New York, 1986, pp 561-576.
- Guidance for industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), May 2001.
- Hubert PH, Chiap P, Crommen J, et al. The SFSTP Guide on the validation of chromatographic methods for drug bioanalysis : from the Washington Conference to the laboratory. Anal. Chim. Acta. 1999; 391:135-148.
- Hubert PH, Chiap P, Crommen J, Boulanger B, et al. Validation of the quantitative analytical procedures. Harmonization of the steps. S.T.P. Pharma practice. 2003; 13: 101-138.
- ICH Topic Q2B, Pharmaeuropa, 8. (1996) 108-111.
- Merodia M, Mirshahi T, Mirshahi M. et al. Development of a sensitive method for the determination of ganciclovir by reversed phase high-performance liquid chromatography. J.Chromatogr. 2000; 870:159-167.
- Moghbel A, Moghaddam A, Rezaeea S, et al. High-Performance Liquid Chromatography Determination of Methotrexate in Plasma. Iranian J. Pharm. Res. 2003: 149-152.
- Subramanian. N. et al. Radiolabeling, Biodistribution and Tumor Imaging of Stealth Liposomes Containing Methotrexate. Alasbimn Journal, 2003; 22 (6): 22-26.

- Nassim MA, Shirazi F, Cripps CM, et al. An HPLC method for the measurement of 5-fluorouracil in human plasma with a low detection limit and a high extraction yield. Int. J. Mol. Medicine. 2002; 10: 513-516.
- Winefordner JD, Long GL. Limit of detection. A closer look at the IUPAC definition. Anal. Chem. 1983; 55:712A-724A.