1 ANALYTICAL METHODS

1.1 MATERIALS

Ganciclovir was received as a gift sample from Ranbaxy (super speciality) Pvt. Ltd., Gurgaon, India and Triamcinolone acetonide was purchased from Sigma (St. Louis, EUA). Methanol and chloroform (A. R. grade) were purchased from Fischer Scientific, Mumbai, India. Chloroform (HPLC Grade), Methanol (HPLC Grade) and Acetonitrile (HPLC Grade) were purchased from Merck, Mumbai, India. Potassium dihydrogen phosphate, sodium hydroxide and all other analytical reagents were obtained from S.D. fine-chem limited, Mumbai, India. Distilled water used in the study was filtered using 0.22-µm nylon filter (Nylon N66 membrane filters 47 mm, Rankem, India).

1.2 ANALYTICAL METHODS

Analytical methods are important tools to estimate the drug content in the formulations and to assess the stability of the drugs in the formulations over a period of time. UV spectrophotometric method is the simplest instrumentation method capable of drug estimation in micrograms. HPLC method is more sophisticated method used for the estimation of samples with very low quantity of the drug, especially in the biological samples. Both UV and HPLC methods were developed in the project work, for chemical characterization of drugs (Ganciclovir and Triamcinolone acetonide). These methods were also employed for determining % drug entrapment, during the stability studies, *in-vitro*, e*x- vivo* and *in- vivo* studies.

1.3 INSTRUMENTS

1.3.1 Instrument specification for UV spectrophotometric measurement

Spectrophotometric measurements were carried out on a Shimadzu 1601 double beam UV Visible spectrophotometer coupled with UV Probe software, version 2.10, Shimadzu. 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.

1.3.2 Instrument specification 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 137 and SPD-20A Prominence UV-Visible detector. The separation was performed on a Vydac C18 column (particle size 5µm, length 250mm X ID 4.6mm; Vydac). Chromatographic data were recorded and processed using Spinchrome Chromatographic StationR CFR Version 2.4.0.193 (Spinchrome Pvt.Ltd., Chennai, India).

1.4 METHOD DEVELOPMENT

1.4.1 Estimation of Ganciclovir by Ultraviolet Spectroscopy (UV)

For estimation of ganciclovir in formulations, calibration curve was prepared in Chloroform: Methanol (2:8) and for determining the % drug release, calibration curve was prepared in PBS 7.4 buffer.

1.4.1.1 Calibration plot of ganciclovir in chloroform: methanol (2:8)

- **Preparation of stock solutions**

  Standard stock solution (1000µg/ml) was prepared by dissolving 10 mg ganciclovir in 10 ml of chloroform: methanol (2:8).

  A working sub stock (100 µg/mL) solution was prepared by accurately measuring and transferring 5ml of standard stock solution in 50 ml volumetric flask and making the volume upto the mark with chloroform: methanol (2:8).

- **Preparation of standard solutions**

  Standard solutions were prepared by pipetting out required volume of sub stock solution in 10ml volumetric flasks and making the volume up to the mark with solvent, to obtain final concentrations of 3,6, 9, 12 and 21 µg/ml. For determining the λmax of drug, the analysis was performed by scanning (10 µg/ml) solution of Ganciclovir in the ultraviolet range between 200 and 400 nm. The absorbances of all standard solutions were measured at λmax using chloroform: methanol as blank on Shimadzu 1601 UV Visible Spectrophotometer and calibration curve was plotted. Three standard samples (3, 9, and 15 µg/ml) were then subjected to intra-day and inter-day precison and accuracy studies. The absorbances were recorded in triplicate.

1.4.1.2 Calibration plot of ganciclovir in phosphate buffer saline (PBS, pH 7.4)

- **Preparation of *phosphate buffer saline (PBS, pH 7.4)***

  2.38 gm of di-sodium hydrogen phosphate, 0.19 gm of potassium di hydrogen phosphate and 8.0 gm of sodium chloride were dissolved in 1000ml of distilled water and the pH was adjusted to 7.4, if necessary, with NaOH.

- **Preparation of stock solutions**

  Standard stock solution (1000µg/ml) was prepared by dissolving 10 mg ganciclovir in 10ml PBS 7.4.

  A working sub stock (100 µg/mL) solution was prepared by accurately measuring and transferring 5ml of standard stock solution in 50 ml volumetric flask and making the volume upto the mark with PBS 7.4.

- **Preparation of standard solutions**

  Standard solutions were prepared by pipetting out required volume of sub stock solution in 10ml volumetric flasks and making the volume up to the mark with solvent, to obtain final concentrations of 3, 6, 9, 12 and 21 µg/ml. For determining the λmax of drug, the analysis was performed by scanning (10 µg/ml) solution of Ganciclovir in the ultraviolet range between 200 and 400 nm. The absorbances of all standard solutions were measured at λmax using PBS, pH 7.4 as blank on Shimadzu 1700 UV Visible Spectrophotometer and calibration curve was plotted. Three standard samples (3, 9, and 15 µg/ml) were then subjected to intra-day and inter-day precison and accuracy. The absorbances were recorded in triplicate.

1.4.2 Estimation of ganciclovir by High Performance Liquid Chromatography (HPLC)

Estimation of ganciclovir was carried out as per reported method of Shen et al., 2007 (Shen et al., 2007). The separation was performed on a reversed phase C-18 column, Vydac™ (250 mm × 4.6 mm, 5 µ) which was thermostated at 30°C throughout the analysis. A filtered and degassed mixture of water and acetonitrile (99.6:0.4 % v/v) was used as mobile phase. The mobile phase was delivered at a flow rate of 1 ml/min and the injection volume was 20 µl. The analysis of was performed at 254 nm. Data processing was done using Spinchrom CFR (Spinchotech, Japan).

- **Preparation of stock solutions**

  Stock solution (100 µg/ml) was prepared by dissolving 5mg of accurately weighed GCV in 50 ml of acetonitrile: water (0.4:99.6, v/v).

  5ml aliquot of standard stock solution was accurately measured and transferred to the 10 ml of volumetric flask to prepare a working stock solution of GCV (50 µg/ml).

- **Preparation of standard solutions**

  Standard solutions were prepared by pipetting out required volume of sub stock solution in 1ml of labelled Eppendorf tubes and making the volume up to the mark (1 ml) with solvent. Serial dilutions were performed to obtain final concentrations of 25000, 12500, 6250, 3125, 1560, 780 and 390 ng/ml. Standards were analysed by RP HPLC at UV detection wavelength 254nm and mobile phase flow rate 1 ml/min. After 10 min run time, results were processed using data processing software Spinchrom CFR. Calibration plot was constructed for the measured area against drug concentration. Three standard samples (390, 3125, and 25000 ng/ml) were then subjected to intra-day and inter-day precison and accuracy studies. The absorbances were recorded in triplicate.

1.4.3 Estimation of Triamcinolone acetonide by Ultraviolet Spectroscopy (UV)

For determining the entrapment efficiency of triamcinolone acetonide in emulsomes, calibration curve was prepared in chloroform: methanol (1:9) and for determining the % drug release, calibration curve was prepared in 0.1 M PBS, pH 7.4 buffer.

1.4.3.1 Calibration plot of triamcinolone acetonide in chloroform: methanol (1:9)

- **Preparation of stock solutions**

  Standard stock solution (1000µg/ml) was prepared by dissolving 10 mg triamcinolone acetonide in 10 ml of chloroform: methanol (1:9).

  A working sub stock (100 µg/mL) solution was prepared by accurately measuring and transferring 5ml of standard stock solution in 50 ml volumetric flask and making the volume upto the mark with chloroform: methanol (2:9).

- **Preparation of standard solutions**

Standard solutions were prepared by pipetting out required volume of sub stock solution in 10ml volumetric flasks and making the volume up to the mark with solvent, to obtain final concentrations of 3, 6, 9, 12, 15, 18 and 21 µg/ml. For determining the λmax of drug, the analysis was performed by scanning (10 µg/ml) solution of triamcinolone acetonide in the ultraviolet range between 200 and 400 nm. The absorbances of all standard solutions were measured at λmax using chloroform: methanol as blank on Shimadzu 1700 UV Visible Spectrophotometer and calibration curve was plotted. The absorbance was recorded in triplicate. Three standard samples (3, 12, and 21 µg/ml) were then subjected to intra-day and inter-day precison and accuracy studies. The absorbances were recorded in triplicate.

1.4.3.2 Calibration plot of triamcinolone acetonide in 0.1 M, phosphate buffer saline pH 7.4 (0.1 M PBS, pH 7.4)

- **Preparation of stock solutions**

Standard stock solution (1000µg/ml) was prepared by dissolving 10 mg triamcinolone acetonide in 10ml PBS 7.4.

A working sub stock (100 µg/mL) solution was prepared by accurately measuring and transferring 5ml of standard stock solution in 50 ml volumetric flask and making the volume upto the mark with 0.1 M PBS, pH 7.4.

- **Preparation of standard solutions**

Standard solutions were prepared by pipetting out required volume of sub stock solution in 10ml volumetric flasks and making the volume up to the mark with solvent, to obtain final concentrations of 3, 6, 9, 12 and 15 µg/ml. For determining the λmax of drug, the analysis was performed by scanning (10 µg/ml) solution of triamcinolone acetonide in the ultraviolet range between 200 and 400 nm. The absorbances of all standard solutions were measured at λmax using PBS, pH 7.4 as blank on Shimadzu 1700 UV Visible Spectrophotometer and calibration curve was plotted. Three standard samples (3, 12, and 18 µg/ml) were then subjected to intra-day and inter-day precison and accuracy studies. The absorbances were recorded in triplicate.

1.4.4 Estimation of triamcinolone acetonide by High Performance Liquid Chromatography (HPLC)

Estimation of triamcinolone acetonide was done by reported method of Araujo et al., 2011 with slight modifications. The separation was performed on a reversed phase C-18 column, Vydac™ (250 mm × 4.6 mm, 5 µ). A filtered and degassed mixture of acetonitrile, methanol and water (30:60:10 %, v/v) was used as mobile phase. The mobile phase was delivered at a flow rate of 1.5 ml/min and the injection volume was 20 µl. The analysis of was performed at 254 nm. Data processing was done using Spinchrom CFR (Spinchotech, Japan).

- **Preparation of stock solutions**

  Stock solution (100µg/ml) was prepared by dissolving 5mg of accurately weighed triamcinolone acetonide in 50 ml of acetonitrile: methanol: water (30: 10: 60, v/v).

  5ml aliquot of standard stock solution was accurately measured and transferred to the 10 ml of volumetric flask to prepare a working stock solution of TA (50µg/ml).

- **Preparation of standard solutions**

  Standard solutions were prepared by pipetting out required volume of sub stock solution in 1ml of labelled Eppendorf tubes and making the volume up to the mark (1 ml) with solvent. Serial dilutions was performed to obtain final concentrations of 25000, 12500, 6250, 3125, 1560, 780, 390 and 195 ng/ml. Standards were analysis by RP HPLC at UV detection wavelength 254nm and mobile phase flow rate 1 ml/min. After 8 min run time, results were processed using data processing software Spinchrom CFR. Calibration plot was constructed for the measured area against drug concentration. Three standard samples (195, 3125 and 25000 ng/ml) were then subjected to intra-day and inter-day precison and accuracy studies. The absorbances were recorded in triplicate.

1.5 ANALYTICAL METHOD VALIDATION

The UV and HPLC methods were validated for linearity, accuracy and precision.

1.5.1 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentrations (amount) of the analyte in the sample. (ICH Q2A guidelines)

1.5.2 Accuracy

Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (measured value). This is sometimes termed trueness (ICH Q2A guidelines). 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 is calculated by using following formula:

$$Accuracy = \frac{True\ value - Measured\ Value}{True\ Value}$$

1.5.3 Precision

The Precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability refers to variation under short intervals of time under same operating conditions. Intermediate precision expresses within laboratory variations: different days, different analyst, different equipment etc. The word reproducibility expresses the precision between laboratories (ICH Q2A guidelines).

Repeatability and reproducibility were determined by calculating RSD (Relative standard deviation) or CV (Coefficient of variation) of inter-day and intraday determinations. One of the common ways of expressing the variability, which takes into account its relative magnitude, is the ratio of the standard deviation (SD) 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* (Bolton, 1990).

$$Relative\ Standard\ Deviation = \frac{Standard\ Deviation}{= Mean\ Concentration}\ X\ 100$$

1.6 RESULTS AND DISCUSSION

1.6.1 Estimation of Ganciclovir by Ultraviolet Spectroscopy (UV)

1.6.1.1 Calibration plot of ganciclovir in chloroform: methanol (2:8)

In chloroform: methanol (2:8), ganciclovir showed λmax at 254.2 nm and thus this wavelength was chosen for analysis. Overlay UV spectra and calibration curve of various solutions in concentration range of 3- 15 µg/ml in chloroform: methanol (2:8) is shown in Fig. 3.1 and Fig. 3.2, respectively. For evaluation of the linearity of the UV method of ganciclovir, the standard solutions were prepared in various concentrations (n = 3) and analysis was carried out at 254 nm. The method was said to be linear for estimation of ganciclovir if its R^2 was near to 1. Least square regression method was used to determine the regression coefficient, r and the equation for the best fitting line.

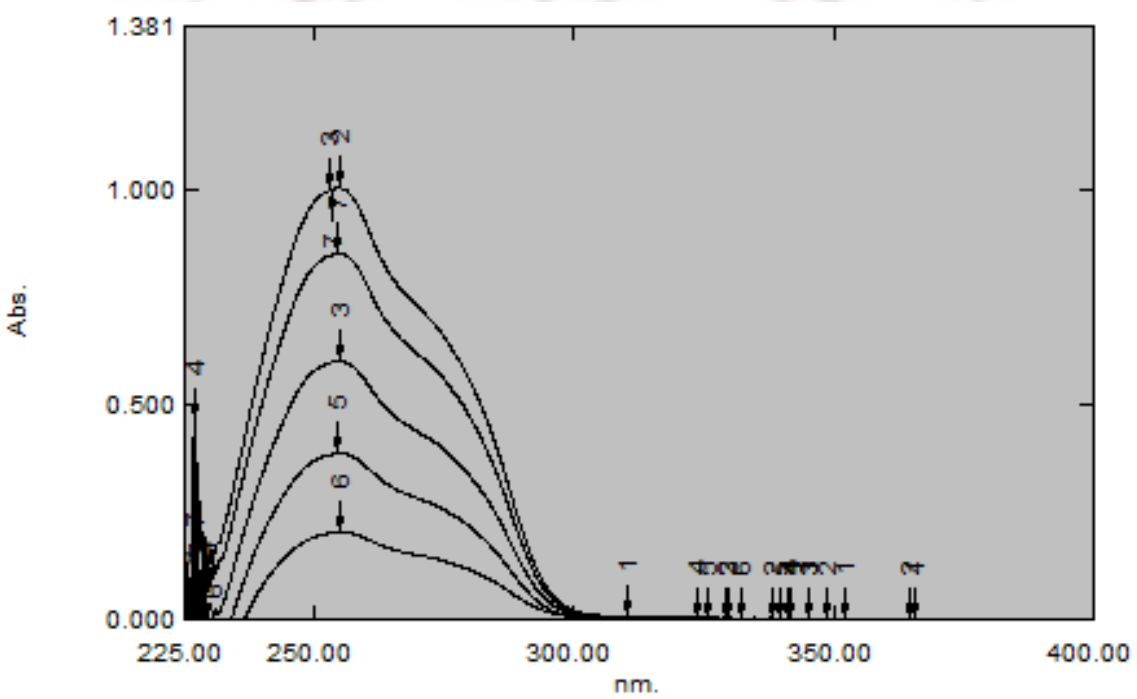

Fig 1.1 Overlay UV spectra of ganciclovir in chloroform: methanol (2:8)

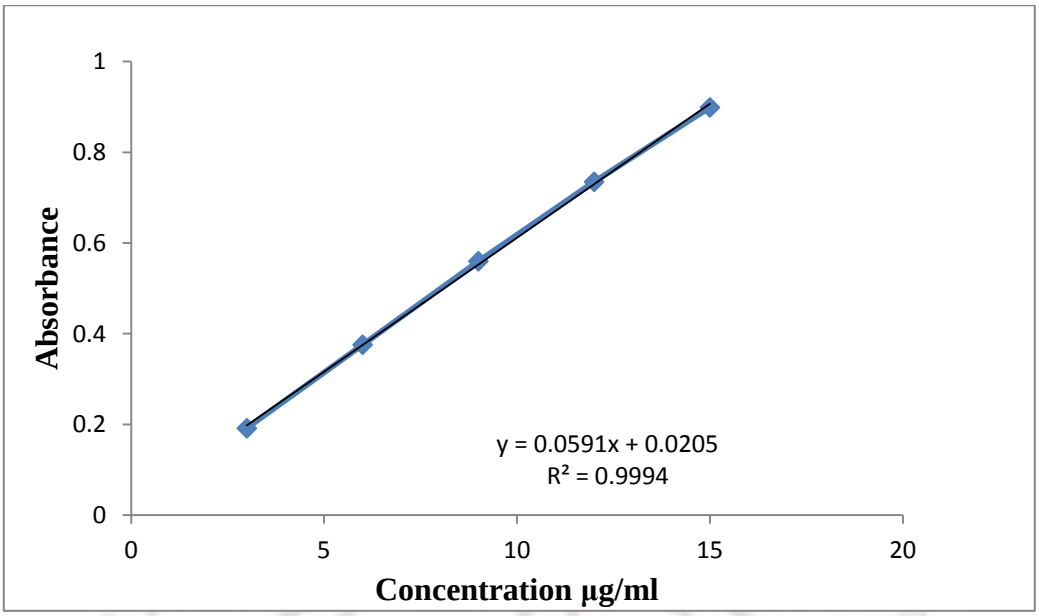

Fig 1.2 Standard calibration curve of ganciclovir in chloroform: methanol (2:8)

A regression equation was obtained from the plot and the correlation coefficient for the developed method was found to be 0.9994, signifying that a linear relationship existed between absorbance and concentration of the drug. Parameters indicating linearity for the used UV spectrometric method of analysis for ganciclovir are shown in Table 3.1.

Table 3.1 Linearity parameters of UV spectrometric method for ganciclovir in chloroform: methanol (2:8)

Parameters	Results
Λmax	254 nm
Linearity range	3-15µg/ml
Regression equation	y = 0.0591x + 0.0205
Correlation coefficient	0.9994

Interday and intraday precision and accuracy of ganciclovir by UV spectroscopy are shown in Table 3.2.

Table 3.2 Intraday and Interday precision and accuracy of ganciclovir in chloroform: methanol (2:8) by UV spectroscopy

Intraday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.90±0.050	1.72	96.66
9	9.17±0.035	0.38	101.88
15	14.86±0.059	0.39	99.06
Interday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.85±0.050	1.75	96.72
9	9.17±0.035	0.38	101.88
15	14.87±0.076	0.51	99.13

The results of inter-day and intraday precision show lower % RSD values (lower than 0.2%) which indicate excellent reproducibility of the method. No significant difference was noticed between the amount of drug added (actual) and observed concentration indicating accuracy of the method (Guidance for industry, 2001; Boulangeret al., 2003).

1.6.1.2 Calibration plot of ganciclovir in PBS 7.4

In PBS, pH 7.4, ganciclovir showed absorption maxima at 252 nm and thus this wavelength was chosen for analysis. Overlay UV spectra and calibration curve of various solutions in concentration range of 3- 15 µg/ml in PBS 7.4 are shown in Fig. 3.3 and Fig. 3.4, respectively. A regression equation was obtained from the plot and the correlation coefficient for the developed method was found to be 0.9997, signifying that a linear relationship existed between absorbance and concentration of the drug.

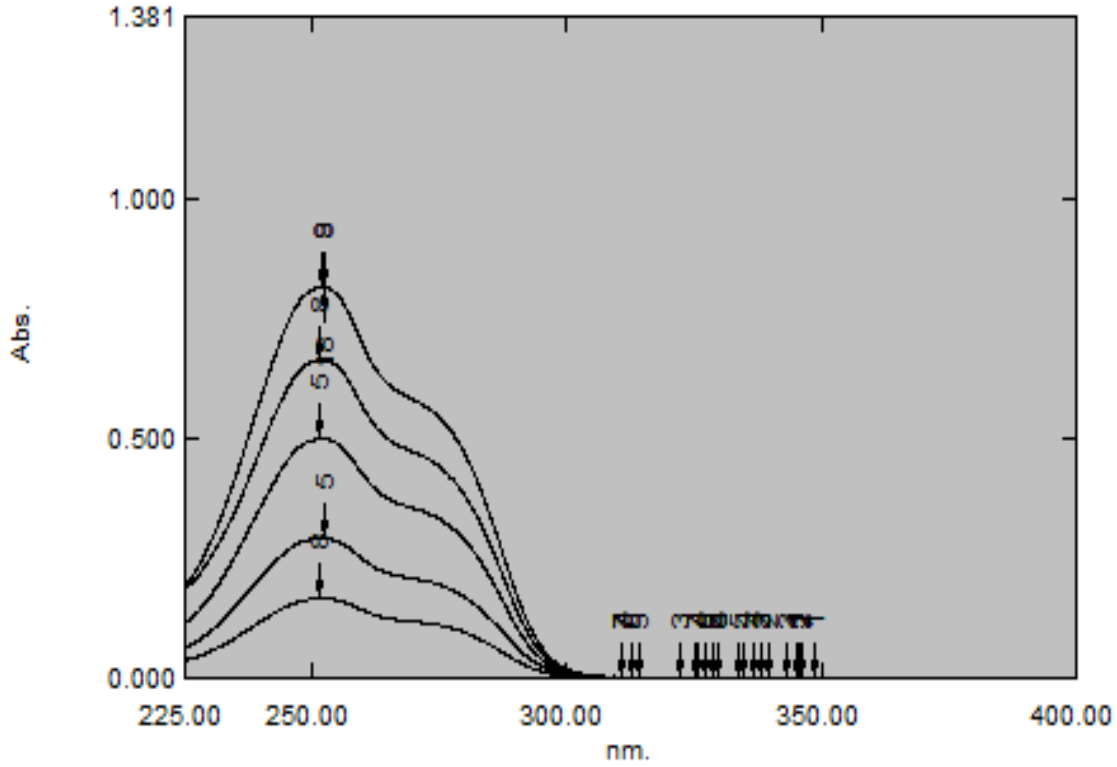

Fig 1.3 Overlay UV spectra of ganciclovir in PBS, pH 7.4

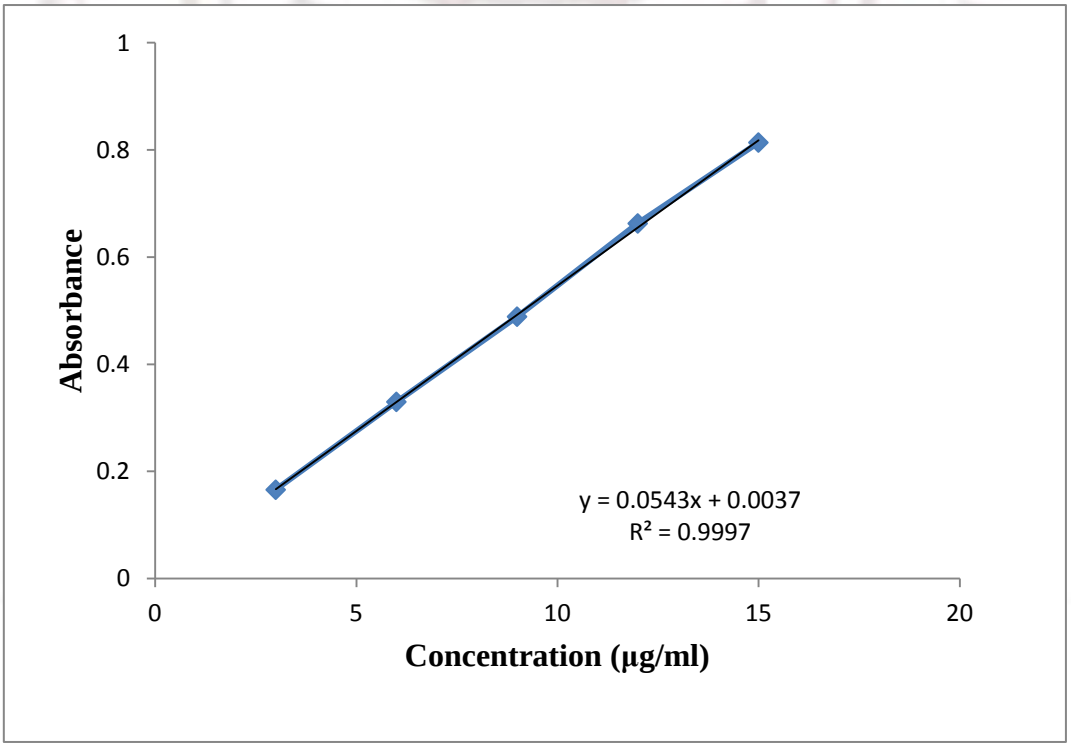

Fig 1.4 Standard calibration curve of ganciclovir in PBS, pH 7.4

Parameters indicating linearity for the used UV spectrometric method of analysis for ganciclovir are shown in Table 3.3.

Table 3.3 Linearity parameters of UV spectrometric method for ganciclovir in PBS, pH 7.4

Parameters	Results
λmax	254 nm
Linearity range	3-15µg/ml
Regression equation	Y= 0.0543x + 0.0037
Correlation coefficient	0.9997

Interday and intraday precision and accuracy of ganciclovir by UV spectroscopy are shown in Table 3.4.

Table 3.4 Intraday and Interday precision and accuracy of ganciclovir in PBS, pH 7.4

Intraday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.97±0.038	1.28	99.00
9	8.93±0.064	0.93	99.22
15	14.91±0.083	0.43	99.40
Interday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.99±0.039	1.30	99.66
9	8.95±0.076	0.85	99.44
15	14.96±0.094	0.63	99.73

1.6.2 Estimation of ganciclovir by High Performance Liquid Chromatography (HPLC)

Overlay HPLC spectra and calibration curve of various solutions in concentration range of 390- 25000 ng/ml in acetonitrile: water (0.4: 99.6, v/v) are shown in Fig. 3.5 and Fig. 3.6, respectively. A regression equation was obtained from the plot and the correlation coefficient for the developed method was found to be 0.9999, signifying that a linear relationship existed between absorbance and concentration of the drug. The retention time for ganciclovir was found to be 5.917 mins (Fig. 3.5).

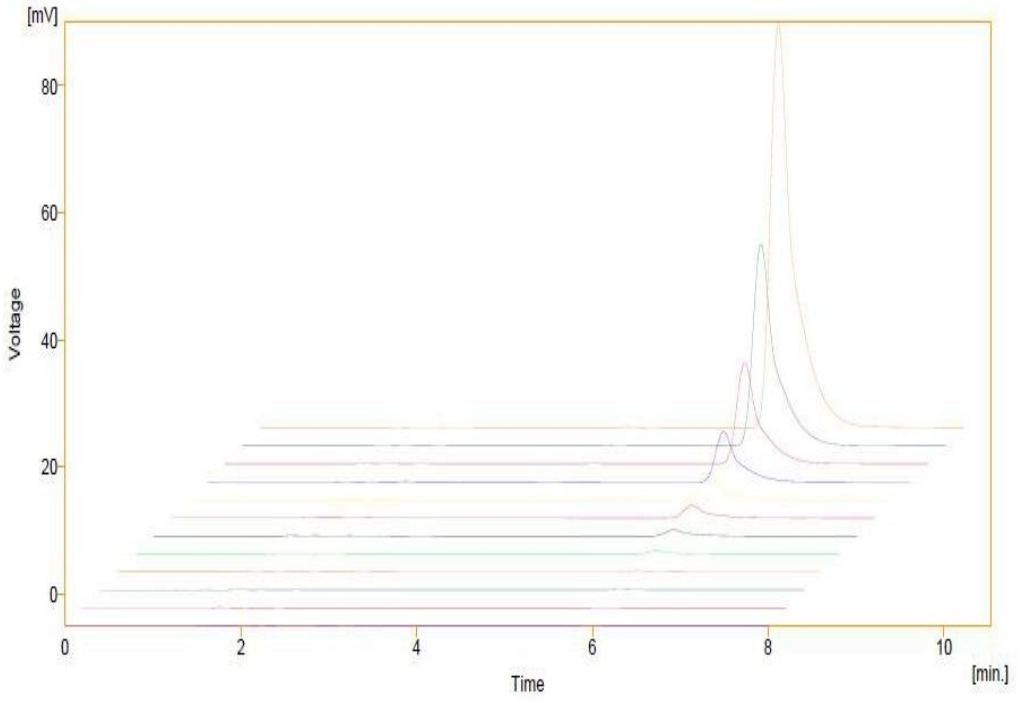

Fig 1.5 Overlay spectra of ganciclovir in acetonitrile: water (0.4:99.6, v/v) by HPLC

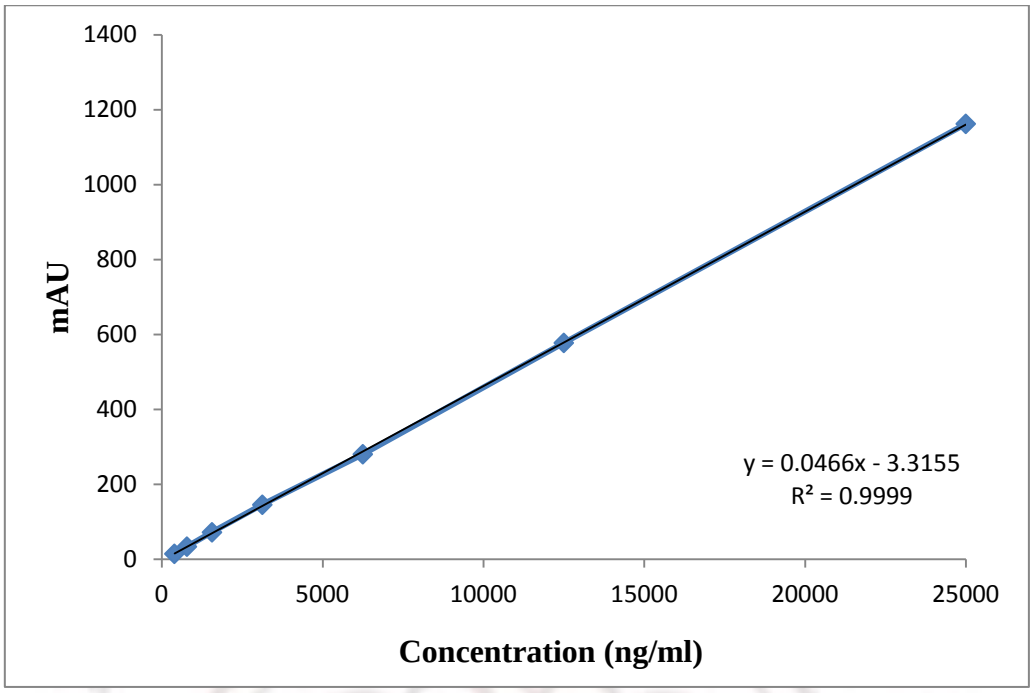

Fig 1.6 Calibration Curve of ganciclovir in acetonitrile: water (0.4:99.6, v/v) by HPLC

Parameters indicating linearity for the used HPLC method for ganciclovir analysis are shown in Table 3.5.

Table 3.5 Linearity parameters of HPLC method for ganciclovir in acetonitrile: water (0.4:99.6,v/v)

Parameters	Results
Λmax	254 nm
Linearity range	390-25000 ng/ml
Regression equation	Y= 0.0466x – 3.3155
Correlation coefficient	0.9999
Retention Time	5.917 Mins

Interday and intraday precision and accuracy of ganciclovir by UV spectroscopy are shown in Table 3.6.

Table 3.6 Intraday and Interday precision and accuracy of ganciclovir in acetonitrile: water (0.4:99.6, v/v) by HPLC

Intraday Analysis			
Standard Concentration (ng/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
390	395 ± 4.25	1.07	101.28
3125	3122± 9.61	0.31	99.90
25000	24870 ± 32.42	0.13	99.48
Inetrday Analysis			
Standard Concentration (ng/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
390	394 ± 4.47	1.13	101.02
3125	3125 ± 9.67	0.31	99.93
25000	24820± 33.37	0.13	99.68

Results of intraday and interday accuracy and precision indicated excellent within day and between day reproducibility of the method (RSD less than 0.2%).

1.6.3 Estimation of triamcinolone acetonide by Ultraviolet Spectroscopy (UV)

1.6.3.1 Calibration plot of triamcinolone acetonide in chloroform: methanol (1:9)

In chloroform: methanol (1:9), triamcinolone acetonide showed absorbtion maximum at 239 nm and thus this wavelength was chosen for analysis. Overlay UV spectra and calibration curve of various solutions in concentration range of 3- 15 µg/ml are shown in Fig. 3.7 and Fig. 3.8, respectively. A regression equation was obtained from the plot and the correlation coefficient for the developed method was found to be 0.9998, signifying that a linear relationship existed between absorbance and concentration of the drug.

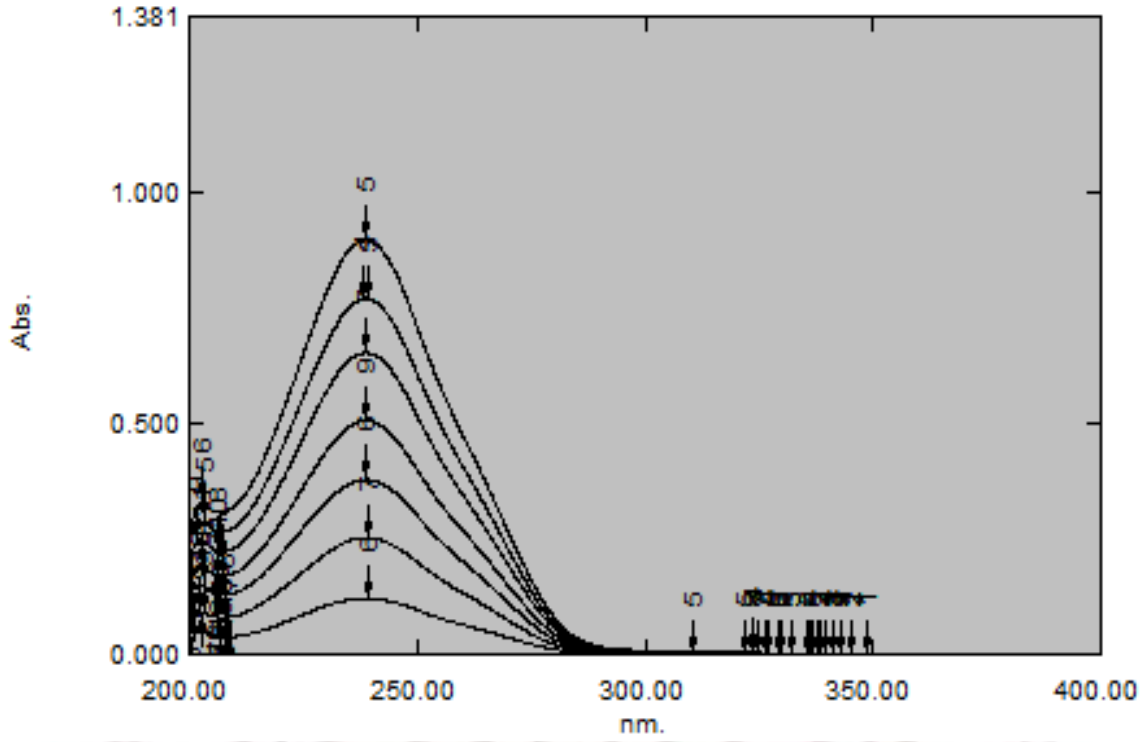

Fig 1.7 Overlay UV spectra of triamcinolone acetonide in chloroform: methanol (1:9)

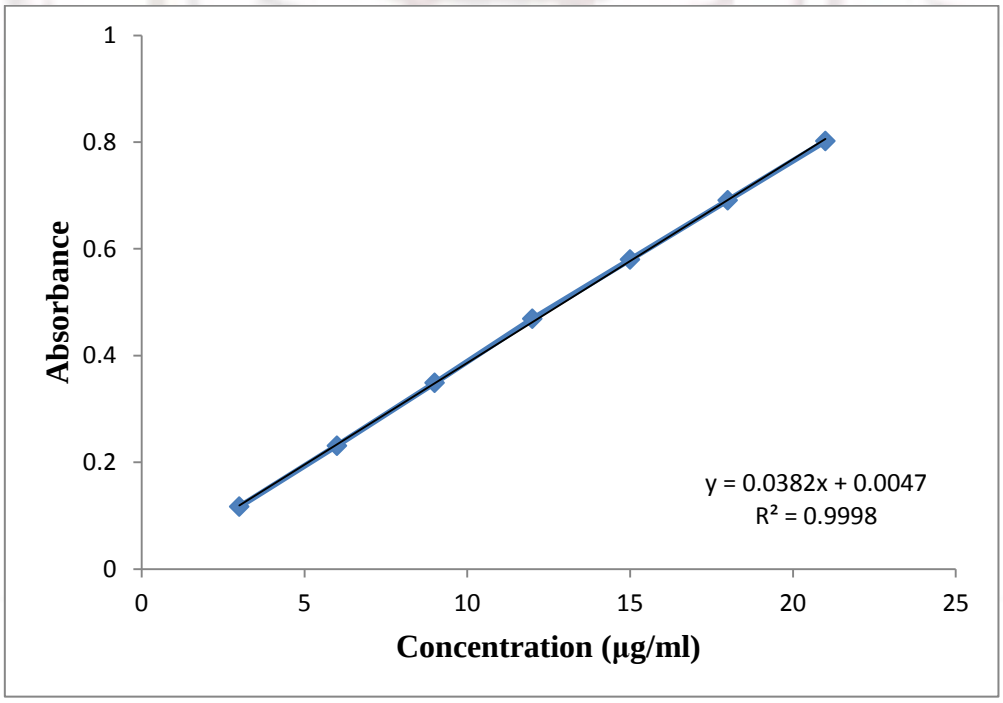

Fig 1.8 Standard calibration curve of triamcinolone acetonide in chloroform: methanol (1:9)

Parameters indicating linearity for the used UV spectrometric method of analysis for triamcinolone acetonide are shown in Table 3.7

Table 3.7 Linearity parameters of UV spectrometric method for triamcinolone acetonide in chloroform: methanol (1:9)

Parameters	Results
Λmax	239 nm
Linearity range	3-21µg/ml
Regression equation	y= 0.0382x + 0.0047
Correlation coefficient	0.9998

Intraday and interday precision and accuracy of triamcinolone acetonide by UV spectroscopy are shown in Table 3.8.

Table 3.8 Intraday and interday precision and accuracy of triamcinolone acetonide in chloroform: methanol (1:9) by UV spectroscopy

Intraday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.94±0.039	1.35	98,28
12	12.13±0.079	0.65	101.14
21	21.098±0.203	0.963	100.46
Interday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.95±0.054	1.84	98.57
12	12.14±0.091	0.75	101.21
21	21.098±0.203	0.963	100.46

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 (Guidance for industry, 2001; Boulangeret al., 2003).

1.6.3.2 Calibration plot of triamcinolone acetonide in 0.1M, Phosphate Buffer Saline , pH 7.4

In 0.1 M PBS, pH 7.4, triamcinolone acetonide showed absorbtion maxima at 241 nm and thus this wavelength is chosen for analysis Overlay UV spectra of various solutions in concentration range of 3- 18 µg/ml and calibration curve in 0.1 M PBS 7.4 are shown in Fig. 3.9 and Fig. 3.10, respectively.. A regression equation was obtained from the plot and the correlation coefficient for the developed method was found to be 0.9999, signifying that a linear relationship existed between absorbance and concentration of the drug.

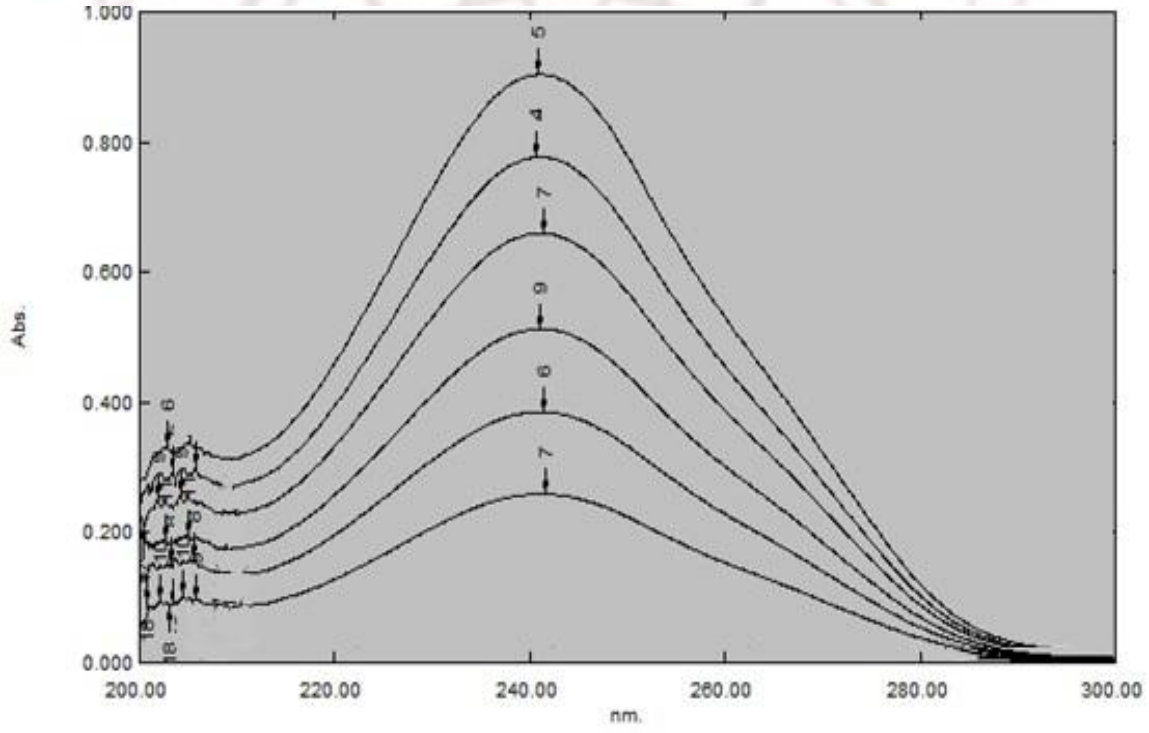

Fig 1.9 Overlay spectra of triamcinolone acetonide in 0.1M, Phosphate buffer saline (PBS, pH 7.4)

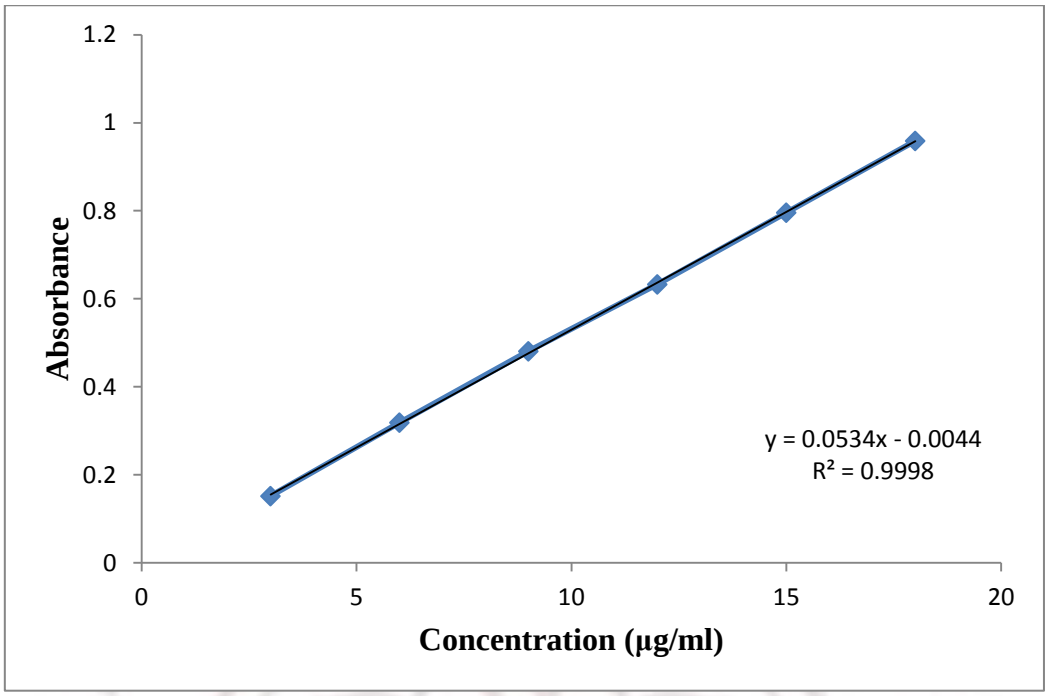

Fig 1.10 Standard calibration curve of triamcinolone acetonide in 0.1 M PBS, pH 7.4

Parameters indicating linearity for the used UV spectrometric method of analysis for triamcinolone acetonide are shown in Table 3.9.

Table 3.9 Linearity parameters of UV spectrometric method for triamcinolone acetonide in 0.1 M, PBS, pH 7.4

Parameters	Results
Λmax	239 nm
Linearity range	3-21µg/ml
Regression equation	y = 0.0534x – 0.0044
Correlation coefficient	0.9998

Intraday and interday precision and accuracy of triamcinolone acetonide by HPLC spectroscopy are shown in Table 3.10.

Table 3.10 Intraday and Interday precision and accuracy of triamcinolone acetonide in 0.1 M PBS, pH 7.4 by UV spectroscopy

Intraday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.88±0.047	1.62	96.22
12	11.93±0.094	0.78	99.49
18	18.01±0.158	0.88	100.07
Interday Analysis			
Standard Concentration (µg/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
3	2.88±0.047	1.62	96.22
12	11.94±0.104	0.87	99.54
18	18.03±0.187	1.03	100.18

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 (Guidance for industry, 2001; Boulangeret al., 2003).

1.6.4 Estimation of triamcinolone acetonide by High Performance Liquid Chromatography (HPLC)

Overlay HPLC spectra and calibration curve of various solutions in concentration range of 195- 25000 ng/ml in acetonitrile: water (0.4: 99.6, v/v) are shown in Fig. 3.11 and Fig. 3. 12, respectively. A regression equation was obtained from the plot and the correlation coefficient for the developed method was found to be 0.9999, signifying that a linear relationship existed between absorbance and concentration of the drug. The retention time for triamcinolone acetonide was found to be 4.547 mins (Fig. 3.11).

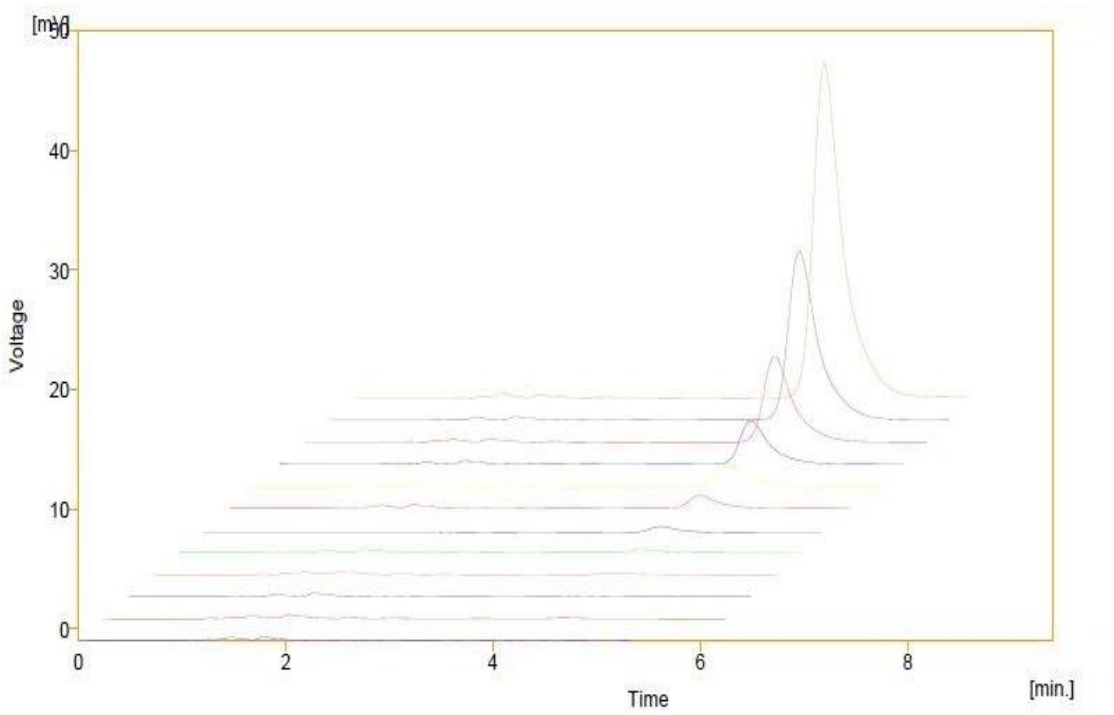

Fig 1.11 Overlay spectra of triamcinolone acetonide in acetonitrile: methanol: water (30:10:60, v/v) at λmax=254nm.

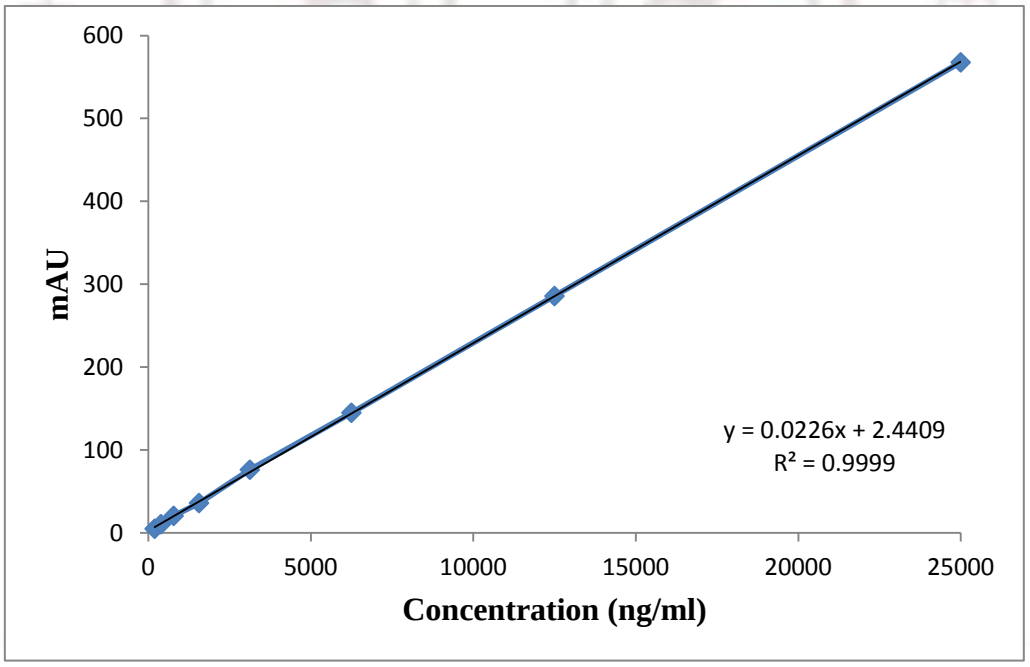

Fig 1.12 Calibration Curve of triamcinolone acetonide in acetonitrile: methanol: water (30:10:60, v/v) by HPLC

Parameters indicating linearity for the used HPLC method of analysis for triamcinolone acetonide are shown in Table 3.11.

Table 3.11 Linearity parameters of triamcinolone acetonide in acetonitrile: methanol: water (30:10:60, v/v)

Parameters	Results
Λmax	254 nm
Linearity range	195-25000 ng/ml
Regression equation	y = 0.0226x + 2.4409
Correlation coefficient	0.9999
Retention time	4.547 Mins

Intraday and interday precision and accuracy of triamcinolone acetonide by HPLC spectroscopy are shown in Table 3.12.

Table 3.12 Intraday and Interday precision and accuracy of triamcinolone acetonide in acetonitrile: methanol: water (30:10:60, v/v) by HPLC

Intraday Analysis			
Standard Concentration (ng/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
195	198 ± 3.78	1.90	101.53
3125	3121 ± 8.95	0.28	99.87
25000	24940 ± 35.91	0.14	99.76
Interday Analysis			
Standard Concentration (ng/ml)		**Precision (%)**	**Accuaracy (%)**
Actual (True)	Observed (Measured)		
195	199 ± 3.52	1.76	102.05
3125	3124 ± 8.98	0.28	99.96
25000	24820 ± 36.95	0.14	99.28

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 (Guidance for industry, 2001; Boulangeret al., 2003).

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