Chapter 3
Analytics

3.1 Introduction

Analytical methods are important tools to estimate the drug content in the formulations and assess the stability of the drugs in the formulations over the period of time. The analytical methods include volumetric methods and instrumental methods. Instrumental methods have advantages over volumetric methods because of their sensitivity, low sample requirement and accuracy. UV spectrophotometric method is the simplest instrumentation method capable of drug estimation in micrograms. In the presence of interfering components, derivative spectroscopy is used for drug estimation. HPLC method is more sophisticated method used for the estimation of samples with very low quantity of the drug.

3.2 Materials and Instruments

Instrument and Software for UV Spectrophotometric Measurement

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

Instrument and Software for HPLC Measurement

The chromatographic system (Shimadzu, Kyoto, Japan) was Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20µl fixed loop and SPD-20A Prominence UV-Visible detector. The separation was performed on a Phenomenex C18 column (particle size 5µm, length 250mm X 4.6mm; Phenomenex Torrance, USA). Chromatographic data were recorded and processed using Spinchrome Chromatographic Station® CFR Version 2.4.0.193 (Spinchrome Pvt.Ltd., Chennai, India).

Materials and Reagents

Halobetasol propionate, Tacrolimus, Methanol (HPLC grade), Water (HPLC grade), Tetrazolimu Blue, Tetramethyl ammonium hydroxide in MeOH/Toluene, Glacial acetic acid, Acetontrile (HPLC Grade). All the reagents were AR Grade

3.3 Methods

3.3.1 Method Validation Parameters for UV Spectroscopic Method

Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined by calculating the recoveries of the analyte by the method of standard additions. Known amounts of standard drug (80%, 100% and 120%) were added to the preanalysed samples and the absorbances were measured.

Precision/ Repeatability/Stability:

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

Linearity and Range:

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

3.3.2 Method Validation Parameters for HPLC Method

Apart from the parameters mentioned under UV spectroscopic method (Linearity and Range, Accuracy, Precision / Repeatability, additionally system suitability test was performed for HPLC method.

System Suitability:

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

3.3.3 Direct Estimation of Halobetasol Propionate by UV Spectrometry

Most of the drugs being organic in nature and absorb in the UV range. Hence, can be estimated by a direct UV spectroscopic method.

Preparation of Stock Solution of Drugs: Stock solution containing 1mg/ml was prepared by dissolving drug in Methanol.

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

Table 3.1: Experimental conditions for halobetasol propionate by UV method

Solvent	Methanol
Stock solution conc.	1mg/ml
Serial Conc. range	5-45 µg/ml
Spectrum range	200nm to 400nm
Spectrum Blank	Methanol
Zero order peak	at 239 nm

3.3.4 Colorimetric Estimation of Corticosteroid by Tetrazolium Blue Method

There are reports of some colorimetric methods of estimation for corticosteroids (Singh and Verma, 2008, Mostafa et al, 2002). The following procedure is applicable for determination of those pharmacopeial steroids that possess reducing functional groups such as α ketols.

Tetrazolium Blue Reagent 0.5%w/v of blue tetrazolium in methanol.

Standard Preparation: Dissolve in alcohol a suitable quantity of accurately weighed Halobetasol propionate, and dilute quantitatively and stepwise with alcohol to obtain a solution having varying concentrations.

Dosage Form: Take appropriate quantity of formulation and sonicate in 10 ml of methanol for 2min, filter the solution and use for analysis as for standard preparation.

Assay Procedure: to 1 ml of stock solution, add 2.0 ml of terazolium blue reagent and 6.5 ml of terabytl ammonium hydroxide was added. Heat it in

water bath at 90 C for 45 min in dark and allowed to cool to room temperature. The volume was adjusted to 10 ml with glacial acetic acid and intensity was recorded at 525 nm on UV Visible spectrophotometer against reagent blank. A calibration curve of concentration versus absorbance was plotted and regression calculated.

Assay Procedure Optimization: The assay conditions were optimized for tetrazolium blue reagent volume, Heating time and temperature using a stock solution 20 μg/ml.

Table 3.2: Optimization conditions for tetrazolium blue method

Solvent	Methanol
Stock solution conc.	1mg/ml
Spectrum range	400nm to 600nm
Spectrum Blank	Reagent Blank
Zero order peak	at 525 nm
Concentration of Solution for optimization trials	20 μg/ml
Volume of Tetrazolium Blue reagent	0.5 -2.5 ml
Heating Temperature	70- 90°C
Heating Time	15 - 60 min

3.3.5 Estimation of Halobetasol Propionate and Tacrolimus by HPLC

Preparation of Stock Solution of Drugs: Stock solution containing 1mg/ml was prepared by dissolving HP / Tac in methanol.

Preparation of Standard Solutions of Drugs: Appropriate and accurate aliquots of the stock solutions were transferred to 10ml calibrated flasks and

diluted up to the volume with methanol in order to get a series of known final concentrations in μg/ml.

Estimation of Drugs in Diffusion Study Samples, Excipients and Formulations: A definite volume of sample to be estimated like supernatant of the saturated excipients, diffusion study samples and formulations like solution, microemulsion and cream was taken in a 10ml volumetric flask and diluted up to the mark with methanol. The resultant solution was then sonicated for 2 min at ambient temperature, filtered through 0.22 μm membrane filter. Further dilutions were made up with methanol and the samples were injected in triplicate.

Analytical Conditions:

Analysis was isocratic with fixed flow rate of the mobile phase. The mobile phase was prepared freshly every day. The mobile phase was filtered through a 0.22 μm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The absorbance of drugs at the required wavelength was checked for any interference. Prior to injecting solutions, the column was equilibrated for 60 minutes with the mobile phase flowing through the system. Each solution was injected in triplicate and relative standard deviation was required to be below 2% on peak area basis (USP).

Chemicals and Reagents:

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

Estimation of Halobetasol Propionate by HPLC Method

Estimation of HP by HPLC has been reported (PCT/US2006/013044). With slight modification, the method was used for estimation of Halobetasol propionate in formulations, diffusion medium and excipients.

Table 3.3: Experimental conditions for halobetasol propionate by HPLC method

Column	Phenomenex C18 (250 × 4.6mm, 5μ)
Mobile phase	Acetonitrile: Water (55:45)
Stock solution conc.	1mg/ml
Serial Conc. range	0.5 -20 mcg/ml
Flow rate	1 ml/min
UV detection	at 239 nm
Injection Volume	20 μL
Retention time	~17.3 min

Estimation of Tacrolimus by HPLC Method

Many HPLC methods and LC-MS were described for the estimation of tacrolimus in formulations and diffusion media (Moyana et al, 2006, Lamprecht et al, 2004, Park et al, 2009, Goebel et al, 2009). With slight modification, the method mentioned by Moyano et al, 2006 was used for estimation of tacrolimus in formulations, diffusion medium and excipients.

Table 3.4: Experimental conditions for tacrolimus by HPLC method (Methanol: water)

Column	Phenomenex C18 (250 × 4.6mm, 5μ)
Mobile phase	Methanol: Water (90:10)
Stock solution conc.	1mg/ml
Serial Conc. range	10-250 mcg/ml
Flow rate	0.8 ml/min
UV detection	at 210 nm
Injection Volume	20 μL
Retention Time	~ 7.0 min

Table 3.5: Experimental conditions for tacrolimus by HPLC method (Acetonitrile: water)

Column	Phenomenex C18 (250 × 4.6mm, 5μ)
Mobile phase	Acetonitrile: Water (55:45)
Stock solution conc.	1mg/ml
Serial Conc. range	10-250 mcg/ml
Flow rate	1.0 ml/min
UV detection	at 210 nm
Injection Volume	20 μL
Retention Time	~ 13.3 min

3.3.6 Measurement of IgE in biological samples of mice by ELISA

For separation of serum, collected blood sample is left for 0.5 hour to coagulate at 37°C and then centrifuged at 5000 rpm for 20 min at 4°C. Separated serum is stored at -20°C for further use.

The serum IgE levels are measured according to the manufacturer's instruction (IC Labs, USA). They are given in brief below.

- 5 µL of serum is diluted with 245µL diluent and mixed thoroughly.
- 100 µL of standard (serially diluted) or sample is pipetted (duplicate) into pre designated wells.
- Micro titer plate is incubated at room temperature for 30 min.
- The contents of the plate are aspirated out and then washed with wash solution three times.
- 100 µL of diluted enzyme antibody conjugate is added to each well and incubated for 30 min in dark at room temperature.
- Again plate is washed three times.
- 100 µL of TMB substrate solution is pipetted in each well and incubated for 10 min.
- 100 µL of stop solution is added to each well and the absorbance is read at 450 nm for the contents of each well.
- Plate reader is calibrated to air.

3.4 Results

3.4.1 Estimation of Halobetasol Propionate by UV Spectroscopic Method

Table 3.6 Absorbance of halobetasol propionate (zero order) at 239nm

Concentration (mcg/ml)	Mean Absorbance at 239 nm (n=3)	SD	%RSD
5	0.177	0.004	1.937
10	0.363	0.008	1.871
15	0.552	0.006	0.998
20	0.747	0.006	0.861
25	0.902	0.008	0.837
30	1.137	0.007	0.598
35	1.291	0.006	0.467
40	1.426	0.007	0.497
45	1.616	0.017	1.043

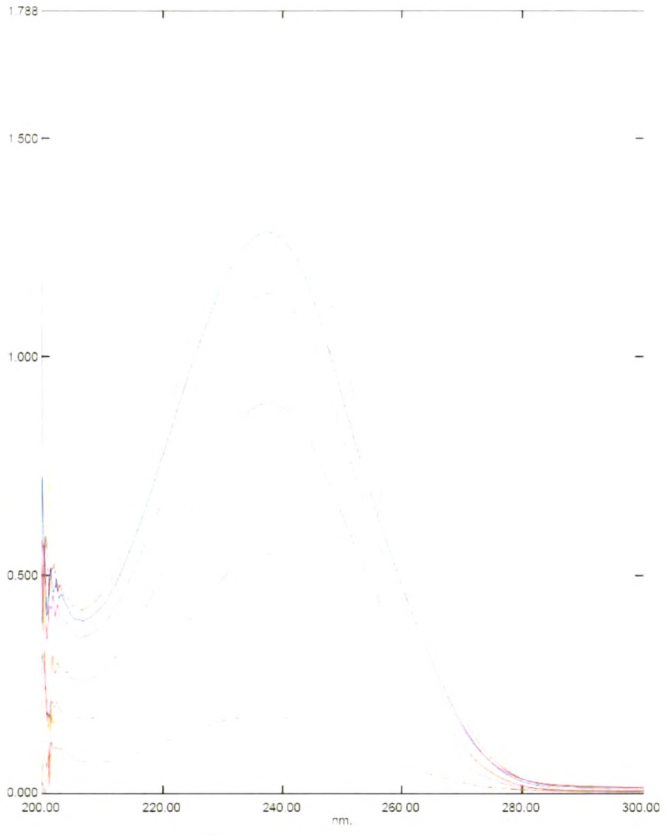

Fig 3.1: UV spectra of halobetasol propionate (5-45 mcg/ml) in methanol

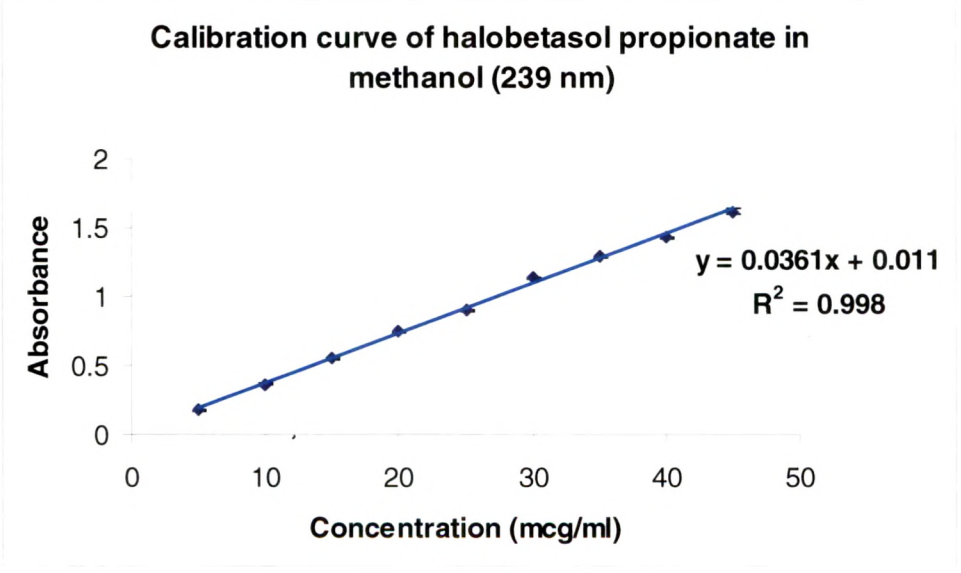

Fig 3.2: Calibration curve of halobetasol propionate in methanol at 239nm

Method validation parameters for estimation of halobetasol propionate by UV spectroscopic method

Table 3.7: Accuracy and precision of halobetasol propionate estimation by UV method

Analyte Concentration (mcg/ml)	Obtained concentration ± SD (mcg/ml) (n=3)	% Recovery ± SD (n=3)	%RSD
5.0	5.04 ± 0.07	100.8 ± 1.40	1.389
20.0	20.406 ± 0.167	102.031 ± 0.835	0.818
45.0	44.863 ± 0.863	99.92 ± 2.28	1.924

3.4.2 Colorimetric estimation of corticosteroid by tetrazolium blue method (525 nm)

Optimization of colorimetric assay

Table 3.8: Optimization of tetrazolium blue reagent volume

Volume of tetrazolium blue reagent (ml)	Absorbance ± SD (n=3)
0.5	1.008 ± 0.176
1.0	1.242 ± 0.091
1.5	1.380 ± 0.087
2.0	**1.651 ± 0.043**
2.5	1.621 ± 0.169

Table 3.9: Optimization of heating temperature

Heating temperature (°C)	Absorbance ± SD (n=3)
70	0.702 ± 0.072
80	0.929 ± 0.059
90	**1.222 ± 0.062**

Table 3.10: Optimization of heating time at 90°C

Heating time (min)	Absorbance ± SD (n=3)
15	1.180 ± 0.095
30	1.542 ± 0.104
45	**1.645 ± 0.038**
60	1.643 ± 0.055

Estimation of halobetasol propionate by colorimetric method

Table 3.11: Calibartion curve of halobetasol propionate by tetrazaolium blue method

Concentration (mcg/ml)	Mean absorbance at 525 nm (n=3)	SD	%RSD
2.5	0.293	0.005	1.706
5	0.554	0.011	1.985
7.5	0.743	0.021	2.841
10	1.056	0.019	1.799
12.5	1.273	0.017	1.335
15	1.489	0.016	1.075

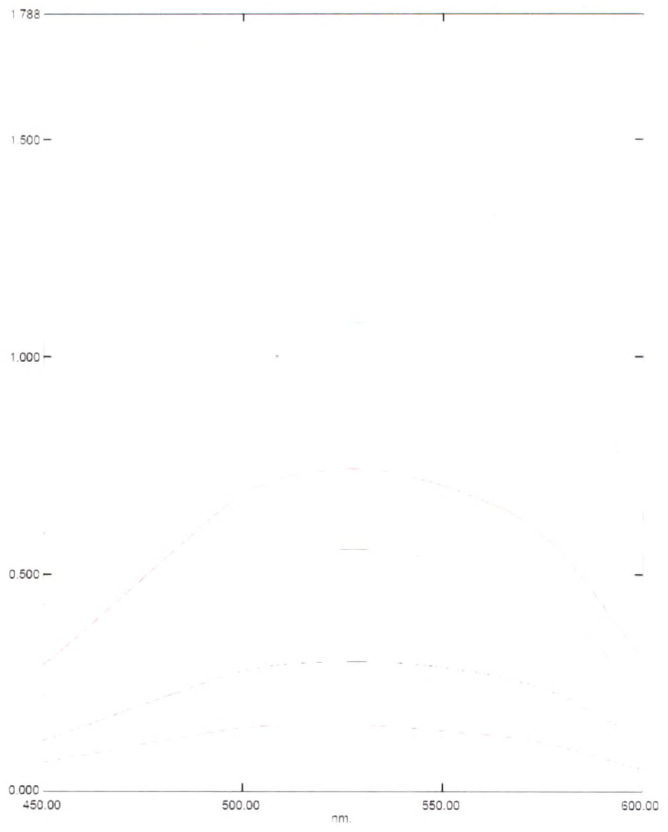

Fig 3.3: Visible spectra of halobetasol propionate (2.5-12.5 mcg/ml) by tetrazolium blue colormietric method

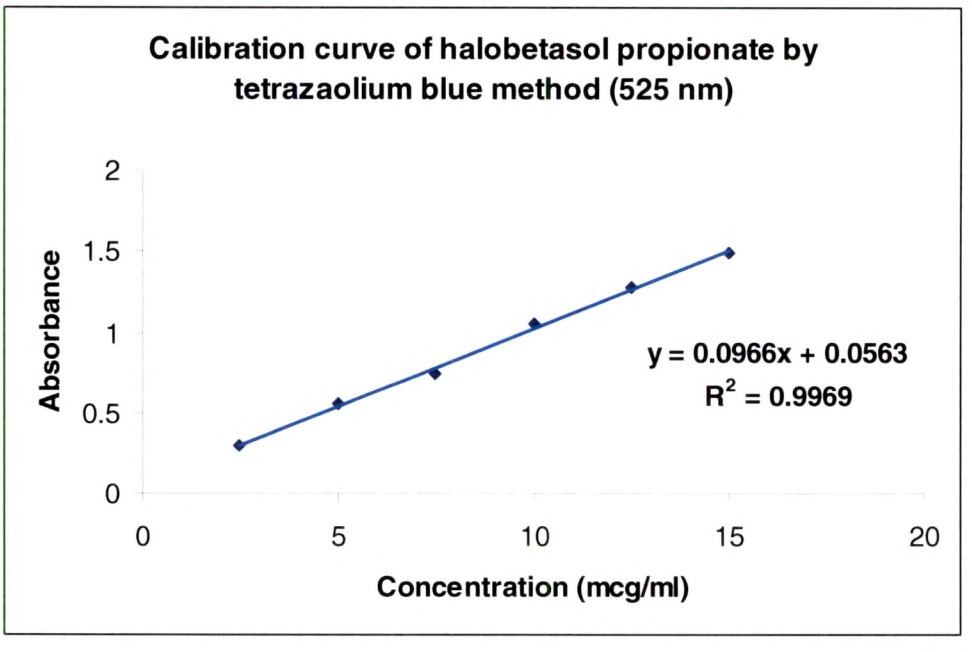

Fig 3.4: Calibration plot of halobetasol propionate by tetrazaolium blue method (525 nm)

Method validation parameters for estimation of halobetasol propionate by colorimetric method

Table 3.12: Accuracy and precision of Halobetasol propionate estimation by tetrazolium blue method

Analyte Concentration (µg/ml)	Obtained concentration ± SD (µg/ml) (n=3)	% Recovery ± SD (n=3)	%RSD
2.5	2.53 ± 0.105	101.07 ± 4.23	4.194
10.0	10.165 ± 0.417	101.65 ± 4.17	4.103
15.0	15.189 ± 0.476	101. 24 ± 3.17	3.137

3.4.3 Estimation of halobetasol propionate by HPLC method

Table 3.13: Calibration of halobetasol propionate by HPLC method

Concentration (mcg/ml)	Mean Area (n=3)	SD	%RSD
0.1	5.25	0.6	11.38
0.5	30.15	0.84	2.18
1	57.6	2.19	2.80
5	114.67	2.52	2.19
10	257.33	8.5	2.30
15	362.67	3.21	0.88
20	514.67	4.16	0.80

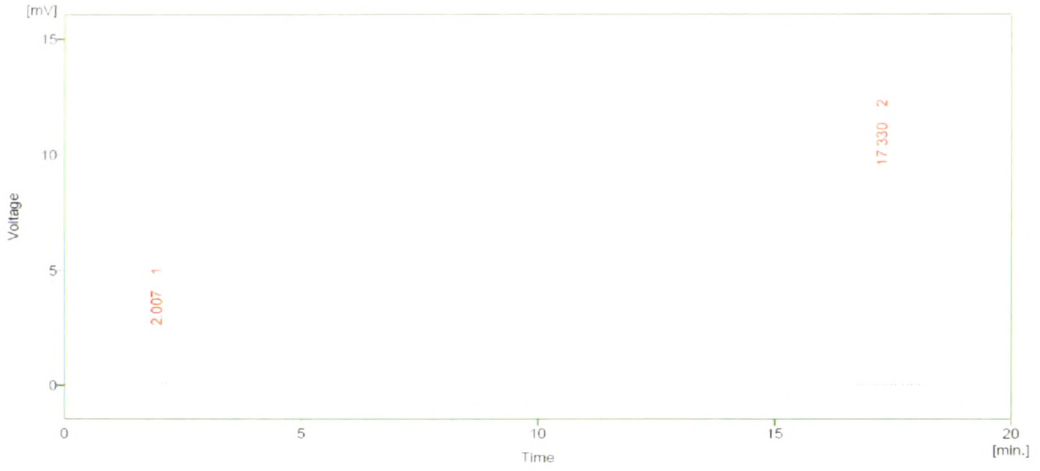

Fig. 3.5: Chromatogram of halobetasol propionate 10 mcg/ml

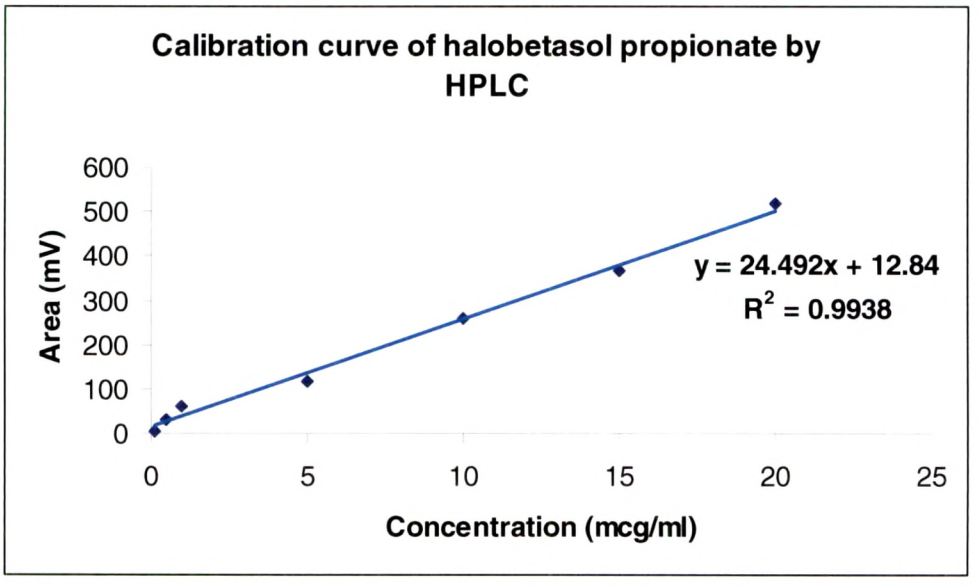

Fig 3.6: Calibration curve of halobetasol propionate by HPLC at 239 nm

Table 3.14 System suitability for halobetasol propionate estimation

Parameters	**Halobetasol propionate (10µg/ml)**
Retention time	~17.3 min
Asymmetry factor	0.846
Efficiency(Th.pl)	10293.5 ± 55.86
Capacity factor	2.345

Table 3.15: Accuracy and precision of halobetasol propionate estimation by HPLC

Analyte Concentration (mcg/ml)	**Obtained concentration ± SD (mcg/ml) (n=3)**	**% Recovery ± SD (n=3)**	**%RSD**
0.1	0.1002 ± 0.002	100.2 ± 1.601	1.606
5.0	5.033 ± 0.06	100.67 ± 1.20	1.197
20.0	19.97 ± 0.171	99.55 ± 0.85	0.858

3.4.4 Estimation of tacrolimus by HPLC method (Methanol: water)

Table 3.16: Calibration of tacrolimus by HPLC method

Concentration (mcg/ml)	**Mean Area (n=3)**	**SD**	**%RSD**
10	193.6	11.3	5.837
25	610.1	12	1.967
50	850.2	17.6	2.07
100	1550.4	33.1	2.135
150	1917.9	54.3	2.831
200	2824	63.4	2.245
250	3514	101.7	2.894

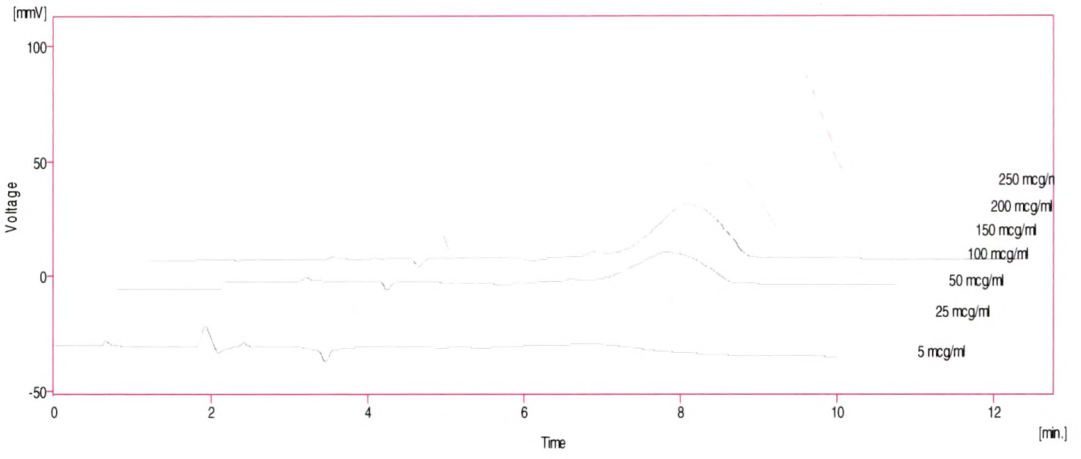

Fig. 3.7: Chromatograms of tacrolimus

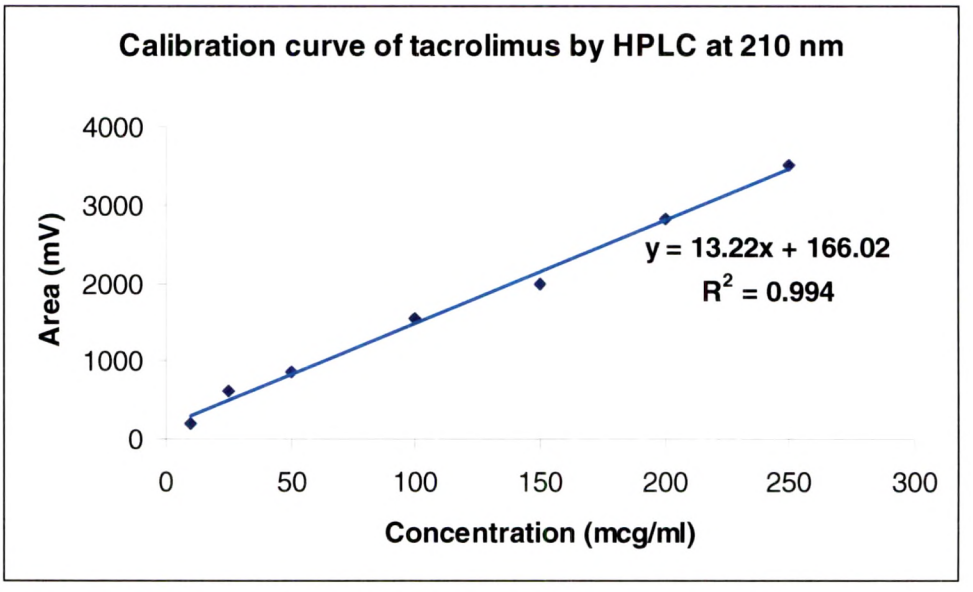

Fig 3.8: Calibration curve of tacrolimus by HPLC at 210 nm

Table 3.17 System suitability for halobetasol propionate estimation

Parameters	**Tacrolimus (50µg/ml)**
Retention time	~7.5 min
Asymmetry factor	1.894
Efficiency(Th.pl)	7293.5 ± 45.86
Capacity factor	1.856

Table 3.18: Accuracy and precision of Tacrolimus estimation by HPLC

Analyte Concentration (mcg/ml)	**Obtained concentration ± SD (mcg/ml) (n=3)**	**% Recovery ± SD (n=3)**	**%RSD**
10	9.97 ± 0.01	99.67 ± 1.00	1.004
100	101.8 ± 1.51	101.8 ± 1.51	1.483
250	250.3 ± 4.58	100.12 ± 1.83	1.830

3.4.5 Estimation of tacrolimus by HPLC method (Acetonitrile : water)

Table 3.19: Calibration of tacrolimus by HPLC method

Concentration (mcg/ml)	**Mean Area (n=3)**	**SD (n=3)**	**%RSD**
10	92.67	7.51	8.09
25	185	11.78	5.37
50	361	14.42	3.99
100	661.33	28.45	4.30
150	878.33	16.92	1.92
200	1030.33	65.04	5.31
250	1204	134.58	11.17

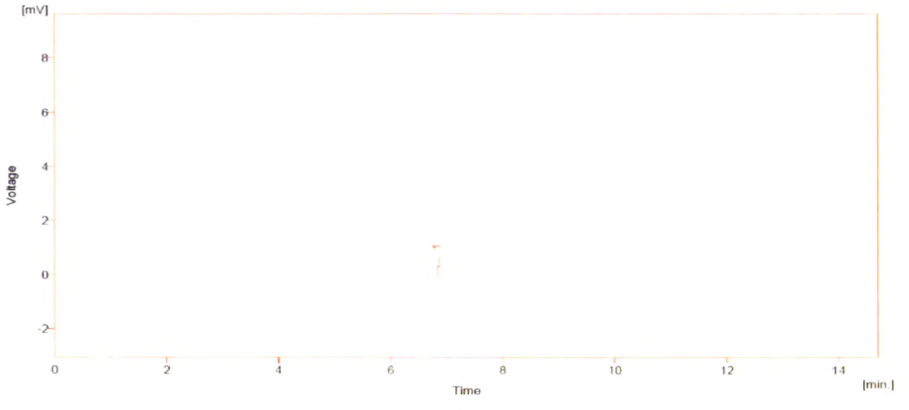

Fig. 3.9: Chromatogram of tacrolimus (50 mcg/ml)

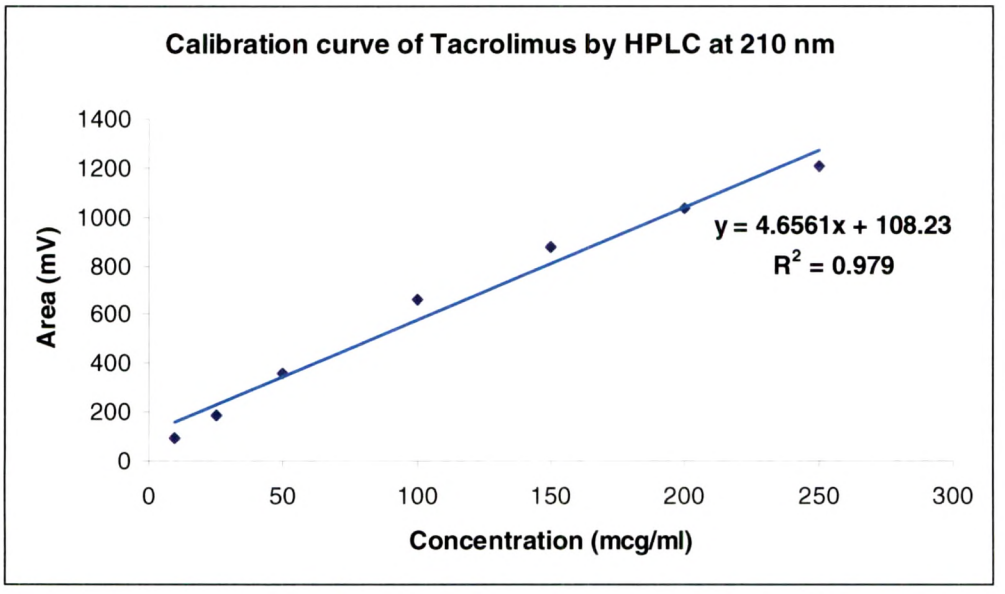

Fig 3.10: Calibration curve of tacrolimus by HPLC at 210 nm

Table 3.20 System suitability for tacrolimus estimation

Parameters	**Tacrolimus (50µg/ml)**
Retention time	~13.3 min
Asymmetry factor	1.422
Efficiency(Th.pl)	6253.5 ± 105.6
Capacity factor	1.56

Table 3.21: Accuracy and precision of Tacrolimus estimation by HPLC

Analyte Concentration (mcg/ml)	**Obtained concentration ± SD (mcg/ml) (n=3)**	**% Recovery ± SD (n=3)**	**%RSD**
10	10.04 ± 0.22	100.43 ± 2.21	2.195
100	98.56 ± 0.96	98.56 ± 0.96	0.975
250	246.8 ± 3.966	98.72 ± 1.58	1.607

3.4.6: Measurement of IgE in Biological Samples of Mice by ELISA

Table 3.22: Calibration plot of serum IgE levels in mice by ELISA

Concentration (ng/ml)	Mean Absorbance at 450 nm (n=3)	SD
0	0	0
6.25	0.1105	0.02
12.5	0.1955	0.035
25	0.333	0.084
50	0.5385	0.097
100	0.8095	0.102
200	1.066	0.104
400	1.2815	0.114

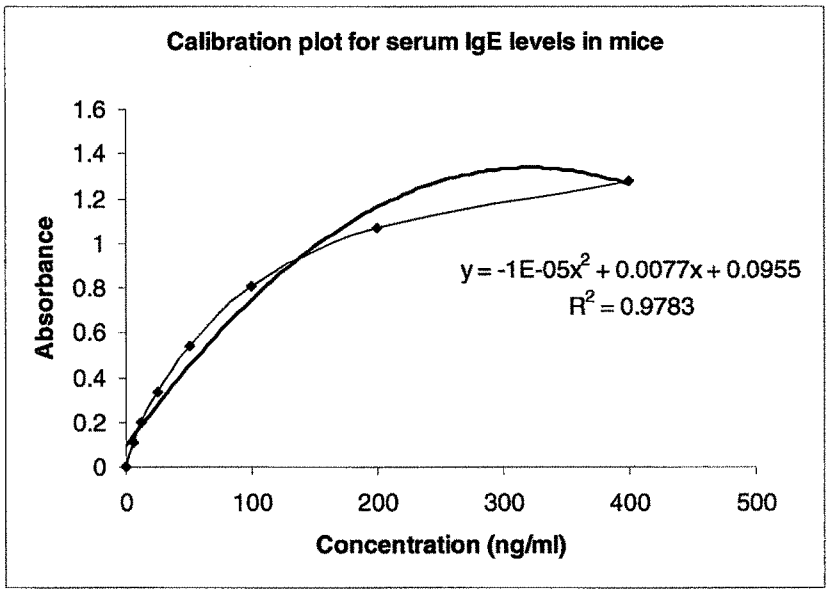

Fig. 3.11: Calibration plot of serum IgE levels in mice by ELISA

3.5 Discussion

Halobetasol Propionate

The UV spectroscopic method for halobetasol propionate utilized its absorption maxima at 239 nm (Fig. 3.1). The method was validated for linearity, accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found to be less than 2% (Table 3.7). The absorbance was found to be linear in the range of 5-45 μg /ml with R^2 value of 0.998 (Fig. 3.2). The % recovery of 97.5% to 103% (Table 3.7) showed that the method was accurate to estimate halobetasol in that 5-45 μg/ml range. The repeatability of the measurement was expressed in terms of %RSD which was below 2% (Table 3.6).

Some of the excipients were found to be interfering while drug estimation in the solubility study and drug assay in formulations. Hence, a colorimetric method was used for estimating halobetasol propionate. A colorimetric method based on tetrazolium blue dye has been described in USP for assay of steroids having an α ketol group. Several other researchers (Graham et al, 1978, Gorog, 1981) have also modified the method and used for estimation of corticosteroids. Colorimetric assay was optimized for heating time temperature and volume of reagent and optimized parameters were used thereafter. It was found have an absorption maxima at 525 nm (Fig 3.3) and linear in the range of 2.5 -12.5 mcg/ml with an R^2 value of 0.997 (Fig. 3.4). The method was validated for accuracy, precision and linearity (Table 3.12). Colorimetric analysis was used for routine analysis during formulation optimization.

HPLC method (PCT/US2006/013044) was based on reverse phase chromatography on a C18 column (Table 3.3). The elution was done with the

flow rate of 1ml/min of acetonitrile:water (55:45) and the retention time of halobetasol propionate was found to be ~17.3 minutes at 239 nm of detection.(Fig. 3.5). The linearity of the estimation was found to be 0.994 (Fig. 3.6) in the range of 0.5-20 μg/ml (Table 3.13). The % recovery of 98.5% to 101.6% (Table 3.15) showed that the method was accurate to estimate halobetasol propionate in that 500 ng -20 μg/ml range. The repeatability of the measurement was expressed in terms of %RSD close to 2%. The system suitability parameters for halobetasol estimation by HPLC were shown in Table 3.14. No interference was observed in the HPLC estimation, since the drug was extracted from excipients and the components of the diffusion media while elution.

Tacrolimus

Tacrolimus shows a peak close 210-220 nm in UV spectra. Hence, it was difficult to estimate tacrolimus by UV spectroscopic method. The common solvents like methanol are known to give noise in that region. This required the use of an HPLC method for the estimation of tacrolimus. Several researchers have reported HPLC methods for the estimation of tacrolimus in formulations (Moyana et al, 2006, Lamprecht et al, 2004, Park et al, 2009). A simple method described by Moyana et al was used for routine estimation of tacrolimus in formulation and diffusion media.

HPLC method was based on reverse phase chromatography on a C18 column (Table3.4). The elution was done with the flow rate of 0.8ml/min of Methanol:water (90:10) and the retention time of tacrolimus was found to be ~7 minutes at 210 nm of detection (Fig. 3.7). The linearity of the estimation was found to be 0.994 (Fig. 3.8) in the range of 10-250 μg/ml (Table 3.16). The % recovery of 98.6% to 103.3% (Table 3.18) showed that the method was accurate to estimate halobetasol propionate in that 10-250 μg/ml range. The

repeatability of the measurement was expressed in terms of %RSD which was below 2%. The system suitability parameters for tacrolimus estimation by HPLC were shown in Table 3.17. No interference was observed in the HPLC estimation, since the drug was extracted from excipients and the components of the diffusion media while elution.

Another HPLC method was developed using the same mobile phase as used in HP detection. This was done to develop a method for analysis in fixed dose combination cream. HPLC method was based on reverse phase chromatography on a C18 column (Table3.5). The elution was done with the flow rate of 1.0 ml/min of Acetonitrile :water (55:45) and the retention time of tacrolimus was found to be ~13.3 minutes at 210 nm of detection (Fig. 3.9). The linearity of the estimation was found to be 0.979 (Fig. 3.10) in the range of 10-250 μg/ml (Table 3.19). The % recovery of 98.6% to 103.3% (Table 3.20) showed that the method was accurate to estimate halobetasol propionate in that 10-250 μg/ml range. The repeatability of the measurement was expressed in terms of %RSD. The system suitability parameters for tacrolimus estimation by HPLC were shown in Table 3.21. No interference was observed in the HPLC estimation, since the drug was extracted from excipients and the components of the diffusion media while elution.

Serum IgE levels are measured by double antibody sandwich ELISA method. The calibration plot and sample analysis is conducted according to manufacturer's instruction (IC Labs, USA). The calibration plot was found to be following a second –order (quadratic) equation (fig. 3.11) as expected.

3.6 References

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