Chapter 4
Analytical Method

Management of Dyslexia and ADHD

4.1 Introduction

Analytical methods are important tools to estimate the drug content in the formulations, assess the stability of the drugs in the formulations over the period of time, evaluation of various parameters, like % Entrapment Efficiency, Diffusion Studies and *In vivo* pharmacokinetics have been developed. The analytical methods are of volumetric methods and instrumental methods. Instrumental methods have advantages over volumetric methods because of their sensitivity, accuracy and low sample requirement. UV spectrophotometric method is the simplest instrumentation method capable of drug estimation in micrograms. In the presence of interfering components, derivative spectroscopy is used for drug estimation. Derivative spectroscopy finds application in resolving the interferences contributed by the excipients while estimating the drug content. This is done by selecting a zero-order crossing point of the excipients on the derivative spectra of drug. HPLC method is more sophisticate method used for the estimation of samples with very low quantity of the drug. Analytical methods used for the estimation of drug content in the developed formulation, and for the estimation of drug *in vitro, ex vivo, in vivo* and stability studies were based on the spectrophotometric and HPLC methods.

4.2 Analytical Method Development: Modafinil

List of Material and Instruments

Table 4.1 List of Material

Material	Manufacturer/Supplier
Modafinil	Gift Sample: Alembic Pharmaceutical, Baroda
Methanol (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
Acetonitrile (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
Acetic Acid (HPLC Grade)	Rankem, Vadodara
Double Distilled Water	Prepared in the laboratory

Table 4.2 List of Instruments

Equipment /Instrument	Manufacturer
pH meter	Lab India Pvt. Ltd., Ambala, India
Digital analytical balance	Precisa 205 ASCS, Switzerland
UV-Visible spectrophotometer-1700	Shimadzu Corporation, Kyoto, Japan
HPLC with UV Detector	Shimadzu Corporation, Kyoto, Japan
Centrifuge	Remi instruments, Mumbai, India

4.2.1 UV Spectrophotometric Instrument and Software

Spectrophotometric measurements were carried out on a shimadzu 1700 double beam UV visible spectrophotometer with a fix slit width of 1.0 nm coupled HP7540 computer loaded with UV PC software of version 2.10. The spectral bandwidth was 1.0 nm and the wavelength scanning speed was 2800 nm/min. Matched quartz cuvettes (1cm) were used for all the spectral measurements.

4.2.2 HPLC Instrumentation and Conditions

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µl. The chromatographic separation was performed using a Supelco C18 (250 mm × 4.6 mm i.d., 5 µm particle size) column. Separation was achieved using a mobile phase consisting of Methanol: Water: Acetic acid in the ratio 500:500:1 (v/v), pumped at a flow rate of 1 ml/min. The eluent was monitored using UV detector at a wavelength of 220 nm. The column was maintained at 25°C and an injection volume of 20 µl was used. Water used in the mobile phase was doubled distilled passed by the vacuum filtration through 0.45 µm nylon membrane filter and degassed in an ultrasonic bath prior to use. Data acquisition and integration was performed using LC Solution software.

Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.45 µm pore size and 47 mm diameter purchased from Pall Life sciences, Mumbai, India.

4.3 Estimation of Modafinil in UV Visible Spectroscopy

Preparation of Calibration Plot of Modafinil in UV Visible Spectroscopy

4.3.1 Preparation of Stock Solution

Accurately weighed (25 mg) Modafinil was transferred to 25 ml volumetric flask. Small quantity of acetonitrile (ACN) was added to ensure complete dissolution of Modafinil and finally volume was made up to the mark with ACN (1 mg/ml).From the prepared solution (1 mg/ml) of Modafinil, aliquot of 1 ml was transferred to 10 ml volumetric flask. (1) Finally volume of stock solution was made up to the mark with ACN (100 µg/ml).

4.3.2 Determination of λ_{max}

1 ml of the stock solution was transferred to a 10 ml volumetric flask and diluted with ACN: Water (35:65) to make up the volume. (1) The solution thus prepared (10 µg/ml) of Modafinil was scanned in the range of 200 nm-400 nm using ACN: Water (35:65) as blank.

4.3.3 First Order Derivative in UV Spectrophotometer

First order derivative convert the zero order spectra in to derivative of dA/dλ which may give sharp and characteristic peak shape at somewhat different wavelength compared to its primitive spectral wavelength. Modafinil doesn't produce any characteristic peak shape shown in fig. 4.1therefore as shown in fig. 4.2, it is converted into first order derivative using statistical software. (2)

4.3.4 Preparation of Calibration Plot using First Order Derivative UV-Spectroscopy (2, 3)

From the stock solution, aliquots of 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.0 ml were accurately withdrawn with the help of pipette and transferred to each 10ml volumetric flasks and the volume was made up to the mark with ACN: Water (35:65) to give final concentration of 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0 µg/ml. The absorbance of all the prepared solutions was measured at the absorption maxima, using solvent mixture as a blank. The readings were recorded in triplicate. Results are shown in Table 4.4 and Fig. 4.3 show linearity range.

4.4 Analytical Method Validation for the Estimation of Modafinil Using UV-Spectrophotometer

For method validation Accuracy, Intraday & Interday Precision and Stability studies were performed. (3-5) Accuracy, also referred to as recovery is a tool for analysis of the trueness of test measurements with standard. The mean % recovery values, close to 100% represent high accuracy of the analytical methods. Precision is a measure of the consistency and reproducibility of a method. A precise method gives very close values for

repeated measurements of same sample under identical experimental conditions. Stability study indicates the stability of drug in solvent over a period of 24 hrs.

4.4.1 Accuracy of an Analytical Method

It expresses the closeness of test result between the value which is considered either as a conventional true value or an accepted reference value and the value determined. To determine the accuracy of the proposed methods, different levels of drug concentrations: lower concentration (LC), intermediate concentration (IC) and higher concentration (HC) (80 %, 100 %and 120 % respectively) were prepared from independent stock solutions and analyzed. Accuracy was assessed as the mean % recovery. The % recovery of the added pure drug was calculated as,

$\%Recovery = [(Ct - Cs)/Cs] \times 100$,

Where, Ct is the total concentration of drug determined from the method; Cs, known or expected drug concentration.

The results are shown in table 4.6 & 4.12 for UV method and HPLC method respectively. Accuracy was calculated by analysis of three triplicate samples for the above described methods.

4.4.2 Precision of an Analytical Method

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample. Precision may be measure of either the degree of reproducibility or the repeatability of the analytical method under normal operating conditions.

Intraday deviation and Interday deviation was checked for different concentrations. To study the intraday deviation, the solution of all concentration were prepared and analyzed at three different time slot during the day. To study the interday deviation the solution of different concentration were prepared and analyzed at three consecutive days. The experiments were performed in triplicates and the mean and % RSD were calculated to assess the suitability of the method.

The precision of an analytical method is usually expressed as the standard deviation (SD) or relative standard deviation (RSD). The standard deviation is calculated from following formula:

$$SD = [\Sigma (X - \bar{X})/n-1]^{1/2}$$

Where,

X = an individual measurement in a set.

$\bar{X}$ = arithmetic mean of the set.

n = total number of replicated measurements taken in set.

Results are tabulated in table 4.7 & 4.8 for UV spectrometric method and in table 4.13 and 4.14 for HPLC method.

4.4.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection is quantitative parameter and can be defined as the lowest concentration of the analyte in a sample that can be detected with acceptable precision and accuracy under stated experimental conditions, but not necessarily quantities as an exact value(The United States Pharmacopoeia 27 NF22,2004). It is express as the concentration of analyte in the sample. Anything that changes the sensitivity of a method, including instrument and sample preparation will change the detection limit.

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

$$LOD \text{ or } LOQ = k. \sigma / S$$

Where,

k - Constant (3.3 for LOD, 10 for LOQ)

σ - Standard deviation of the analytical blank

S -Slope of the concentration/response graph

4.4.4. 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. Result of linearity of Modafinil estimation are shown in Table 4.5 & 4.11 by UV spectroscopy and HPLC method respectively and Table 4.17 for

Modafinil estimation in plasma by HPLC. Within therange the regression coefficient values are near to 1.

4.4.5 Stability of an Analytical Method

To ascertain the accuracy of the proposed method of each known concentration of drug i.e concentration 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0µg/mL solutions were prepared in ACN: Water (35:65). The absorbance of each solution was measured on day 0. The same solutions were analyzed after 24 hrs and the changes in the absorbance were reported to evaluate the stability of the analytical method. The results were recorded in triplicate and are stated in Table 4.9.

4.5 Interference Study

In order to ascertain the non-interference of the excipients in estimation of Modafinil, solutions containing known concentration of each excipient were prepared in ACN: Water(35:65). The prepared solutions were scanned in the UV region between 200-400 nm using the respective blank. Also, to study the effect in presence of drug, Modafinil solution (10 µg/ml) was spiked with known concentrations of each excipient (Clove oil, Capmul MCM C8, Tween 80, PEG-400) and scanned in the UV region between 200 nm-400 nm.(6)

4.6 HPLC Method for Determination of Modafinil

The HPLC method of Modafinil has to be performed because Clove oil, Tween-80 and Capmul MCM C8 shows interference with Modafinil in UV spectroscopy method. In HPLC method the oil component and the drug component were separated in column and did not show interference during analysis of Modafinil. HPLC used for analysis have an isocratic pump (Model LC-20 AT, Shimadzu, Japan), an ultra violet variable wavelength detector (Model SPD-20A,Shimadzu, Japan), a rheodyne injector (Model P/N 7725i, Made in USA) and the parameters used in the analysis are as follows:(7-10)

Table 4.3 Parameters for RP-HPLC method

Sr. No.	Parameters	Information
1	Mode	RP-HPLC
2	Column	C18(Octadecylsilane-ODS), SUPELCO-516-C-18B (250 mm × 4.6 mm i.d., 5 µm particle size)
3	Detector	UV Spectrophotometer
4	UV detectionat λmax	220nm
5	Mobile Phase	Methanol : Water : Acetic Acid (500 : 500 : 1)
6	Flow Rate	1.0 ml/min
7	Injection Size	20µl
8	Retention time	8.56
9	Stock solution conc.	1mg/ml
10	Diluent	Methanol
11	Serial conc. Range	2.5µg/ml - 20µg/ml

The other supporting instruments used for analysis are HPLC Cartridge column, USA, Centrifuge make by REMI, COMPUFUGE, Model CPR 30, Vortex mixer: SPINEX and Bath sonicator make Sartorius, India.

4.6.1 Chemicals and Reagents

Methanol was of HPLC grade and purchased from Spectrochem Pvt. Ltd., Mumbai, India. Double distilled water used throughout the study. All other solvents and reagents used were analytical grade were filter through a 0.22 µm Ultipor® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

4.6.2 Mobile Phase Preparation

Added Methanol: Water: Acetic acid in the ratio of 500:500:1 in a reagent bottle and sonicated the solution using bath sonicator for 5 minutes with 3 repetitive cycles to remove air bubbles prior to use.

4.6.3 Preparation of Stock Solution

Accurately weighed (25 mg) Modafinil was transferred to 25 ml volumetric flask. Small quantity of methanol (HPLC grade) was added to ensure complete solution of Modafinil and finally volume was made up to the mark with methanol (1 mg/ml). From the prepared

solution (1 mg/ml) of Modafinil, aliquot of 1 ml was transferred to 10 ml volumetric flask. Finally volume was made up to the mark with methanol (100 µg/ml).

4.6.4 Preparation of Calibration Plot of Modafinil by HPLC Method:

From the stock solution, aliquots of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 ml were accurately withdrawn with the help of pipette and transferred to separate 10ml volumetric flasks and the volume was made up to the mark with methanol to give final concentration of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0 µg/ml. The area of all the prepared solutions was then measured in triplicate in HPLC as per above analytical condition.

4.7 Analytical Method Validation for the Estimation of Modafinil by HPLC

Apart from the parameters mentioned under UV spectroscopic method(Linearity and Range, Accuracy, Precision/ Repeatability, Limit of detection (LOD) and Limit of quantification (LOQ), additionally stability test was performed for HPLC method.The results were recorded in triplicate and are stated in Table 4.15.

4.8 Calibration Plot for Modafinil in Plasma:(9, 10)

4.8.1 Estimation of Modafinil in Plasma

Human Plasma was obtained from Suraktam Blood Bank, Vadodara, India. The blank plasma samples were spiked with stock solution prepared in methanol (1 mg/ml) to get concentration in desirable range. The protein precipitation was carried outby addition of methanol. For 0.4 ml of plasma sample, 600 µl of methanol was used. The separation of precipitate from organic phase was achieved by centrifugation (4000 rpm X 10 min) and calibration plot was developed by performing respective dilutions in the stock solution with methanol to achieve desirable concentration range of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 µg/ml. The chromatographic condition was maintained as discussed in 4.6. Calibration plots were drawn by plotting peak area vs. drug concentration. The column was equilibrated by passing at least 150-200 ml of mobile phase. 20µl of sample was loaded using syringe through rheodyne auto injector.

4.9 Results and Discussion

4.9.1 Determination of λmax and Calibration Plot for Modafinil

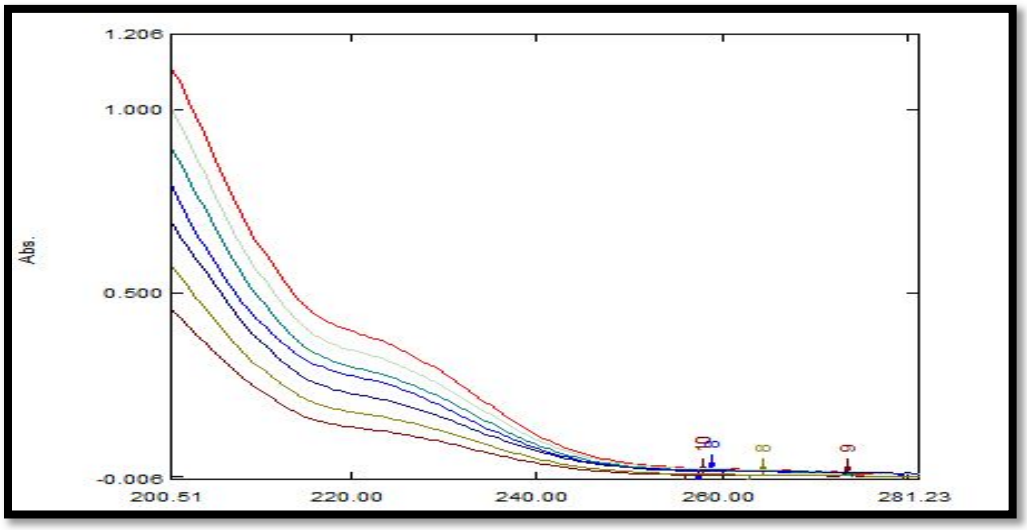

Fig. 4.1: Calibration Plot of Modafinil in ACN: Water (35:65) by UV Spectroscopy

For any identical analysis, peak shape is a characteristic factor to assess the maximum wavelength. As shown inFig. 4.1 Modafinil did not produce any characteristic peak when scanned in the ultraviolet range between 200 and 400 nm. Beer's lawcannot applied for a non-characteristic peak. Therefore, thisUV spectroscopic method of analysis will not be suitable for further study.A mathematical calculation can be used for conversion of these peaks to first order derivative for identical analytical method.

4.9.2 Calibration Plot using First Order Derivative UV Spectroscopy

Modafinil in ACN: Water(35:65) shows a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm and converted to first order derivative spectra using UV-Probe software v.2.10. Modafinil shows absorption maxima (λ max) at 232 nm shown in Fig. 4.2 and this wavelength was selected as the analytical wavelength. Beer's law was obeyed between 5 and 20 µg/ml (Table 4.4). Regression analysis was performed on the experimental data. Regression equation for standard plotand correlation coefficient is shown in Table 4.5 signifying that a linear relationship between concentration and absorbance. Lower values of standard deviation also indicate the reproducibility of the analytical method.

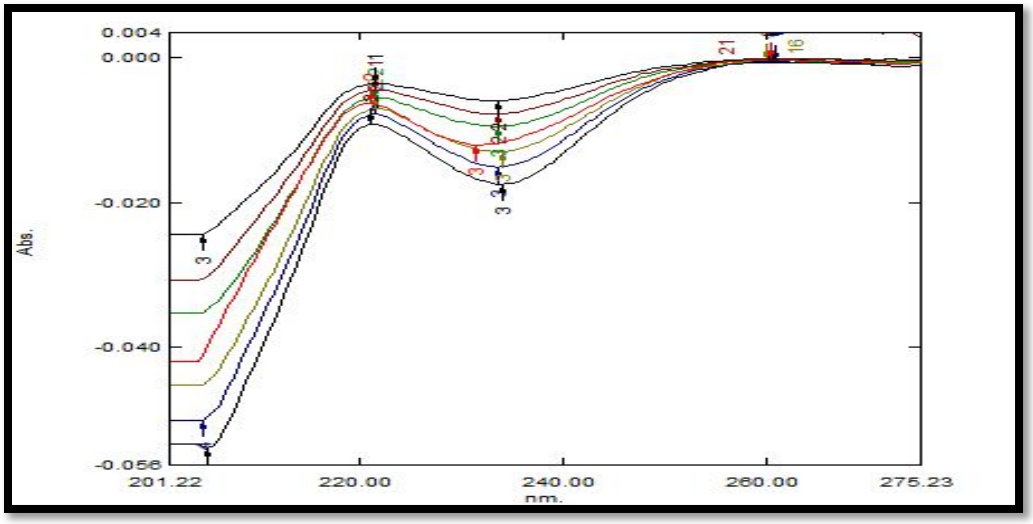

Fig. 4.2 First Order Derivative of Modafinil by UV Spectroscopy

Table 4.4 Absorbance for the Calibration Plot of Modafinil in ACN: Water (35:65) by (1st order derivative) UV Spectrometer

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D.*
1	05.0	-0.01230 ± 0.000365
2	07.5	-0.01902 ± 0.000213
3	10.0	-0.02475 ± 0.000457
4	12.5	-0.03178 ± 0.000444
5	15.0	-0.03658 ± 0.000499
6	17.5	-0.04242 ± 0.000184
7	20.0	-0.04940 ± 0.000233

* Mean ± S.D (n=3)

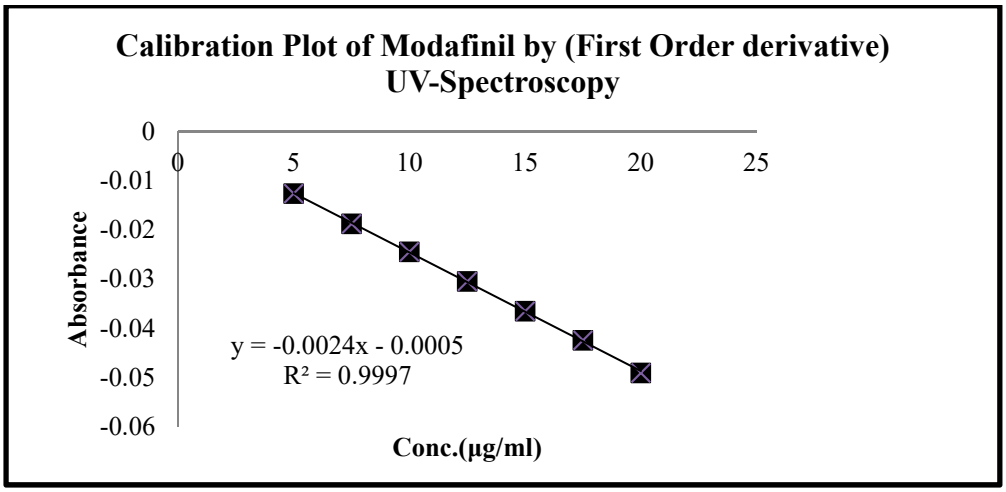

Fig. 4.3 Calibration Plot of Modafinil in ACN: Water (35:65) by UV Spectroscopy

Table 4.5 Linearity of Method of Analysis of Modafinil in ACN: Water (35:65) by UV Spectrometer

Parameters	λmax	Linearity Range	Regression equation	Regression coefficient
Result	232 nm	5 - 20 (µg/ml)	Y = -0.002x – 0.000	R^2=0.999

4.9.3 Validation of Analytical Method

Accuracy: As shown in table 4.6, mean % recovery for lower, intermediate and higher concentrations were found to be 101.7%, 99.60% and 100.1% respectively. Result show that UV Spectroscopy can accurately estimate any minor change in drug concentration.

Table 4.6 Accuracy of the UV Method for Modafinil

Sr. No.	Quantity of Modafinil added%	Expected Concentration (µg/ml)	Recovered Concentration (µg/ml)	% Recovery
1.	80(Lower)	10	10.17	101.7 %
2.	100(Intermediate)	15	14.94	99.60 %
3.	120(Higher)	20	20.02	100.1%

Precision

Intraday Precision

Table 4.7 Intraday Precision Analysis of Modafinil Estimated by UV Method (1)

Concentration (µg/ml)	Absorbance at different time slot			Mean Absorbance	SD	%RSD
	Slot 1	Slot 2	Slot 3			
5	-0.01214	-0.01247	-0.01229	-0.0123	0.000165	-1.34330
10	-0.02455	-0.02444	-0.02528	-0.0245	0.000457	-1.84412
15	-0.03617	-0.03645	-0.03714	-0.0365	0.000499	-1.36452
20	-0.04886	-0.04951	-0.04985	-0.0494	0.000503	-1.01813

Interday Precision

Table 4.8 Interday Precision Analysis of UV Method for Modafinil

Concentration (µg/ml)	Absorbance at different days			Mean Absorbance	SD	%RSD
	Day 1	Day 2	Day 3			
5	-0.01214	-0.01227	-0.01237	-0.01226	0.000115	-0.94067
10	-0.02455	-0.02514	-0.02511	-0.02493	0.000332	-1.33281
15	-0.03617	-0.03629	-0.03694	-0.03647	0.000414	-1.13607
20	-0.04886	-0.04851	-0.04984	-0.04907	0.000689	-1.40497

Precision study performed under the same operating condition for intraday (Table 4.7)and interday (Table 4.8); results revealed for a short time interval and longtime respectively.In precision study, % RSD values obtained were less than 2.0% suggest that these methods have good precision and reproducibility. There is no intraday and interday variability by following of method. LOD:0.0392µg/ml and LOQ:0.1188 µg/ml.

4.9.4 Solution Stability of an Analytical Method

The results indicated that there was no considerable change in absorbance values of Modafinil estimated after 24 hrs. This clearly showed that the drug is stable in ACN: Water (35:65) for a period of at least 24 hrs and method can be used for further analysis.

Table 4.9 Stability Analysis of UV Method for Modafinil

Concentration (µg/ml)	Absorbance ± S.D.* (0 hr)	Absorbance ± S.D.* (24 hrs)
5.0	-0.01230 ± 0.000165	-0.01291 ± 0.000381
7.5	-0.01902 ± 0.000213	-0.01939 ± 0.000477
10.0	-0.02475 ± 0.000457	-0.02492 ± 0.000418
12.5	-0.03078 ± 0.000444	-0.03104 ± 0.000249
15.0	-0.03658 ± 0.000499	-0.03679 ± 0.000174
17.5	-0.04242 ± 0.000544	-0.04278 ± 0.000234
20.0	-0.04940 ± 0.000503	-0.04980 ± 0.000388

4.9.5 Interference Study

Concentration of excipients were taken at approximate level at which they are present in final formulation. The absorbance of Tween-80, Clove Oil and Capmul MCM C8 excipients at 232 nm was very high indicating that these excipients may interfere in the estimation of Modafinil. As per Fig. 4.4 the mixture of those excipients shown interference in estimation of Modafinil and therefore UV spectroscopy method cannot be used for the estimation of Modafinil in this formulation nor release study.

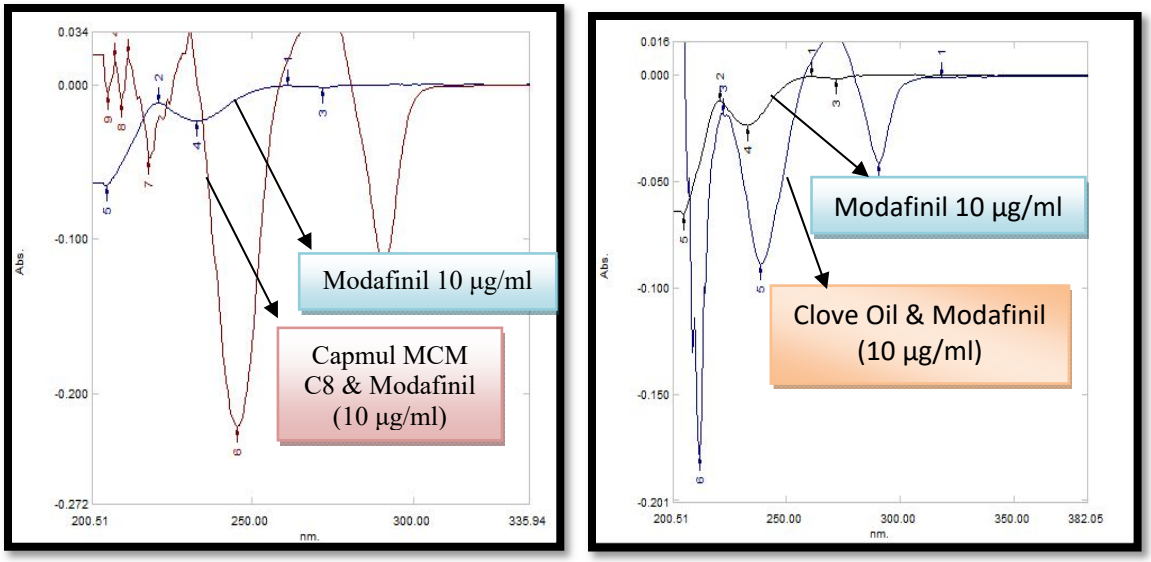

Fig. 4.4 Excipients Interference Chart for Modafinil by UV-visible Spectrophotometer

4.9.6 Calibration of Modafinil by HPLC Method:

In the Fig.4.5 first peak is of solvent peak and second peak is of Modafinil, recorded in the concentration range of 2.5-20 µg/ml.

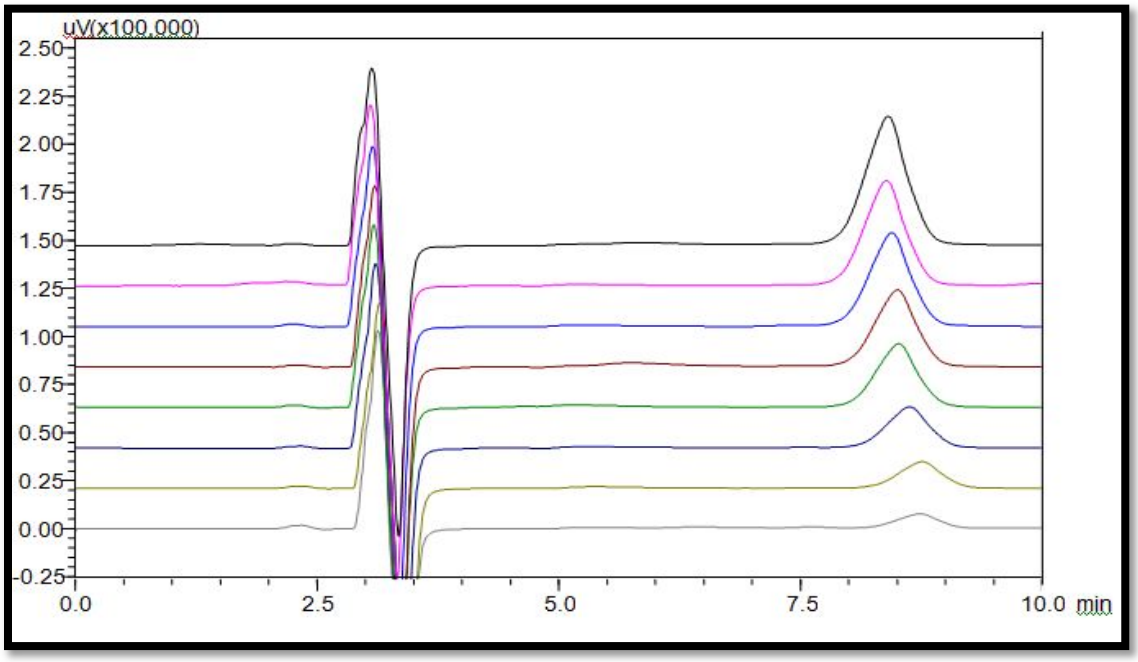

Fig. 4.5 Graphical Spectrum of Modafinil Calibration Plot by HPLC Method

Table 4.10 Area for the Calibration Plot of Modafinil by HPLC

Sr. No.	Concentration (µg/ml)	Mean Area (mV) ± S.D. (n=3)
1	2.5	237844.3±2289.8
2	5.0	457485.6±1910.7
3	7.5	652841.3±6744.7
4	10.0	1009339.3±1200.3
5	12.5	1206018.0 ±2489.1
6	15.0	1412801.3±4790.1
7	17.5	1683399.6±5510.6
8	20.0	2046301.3±4583.6

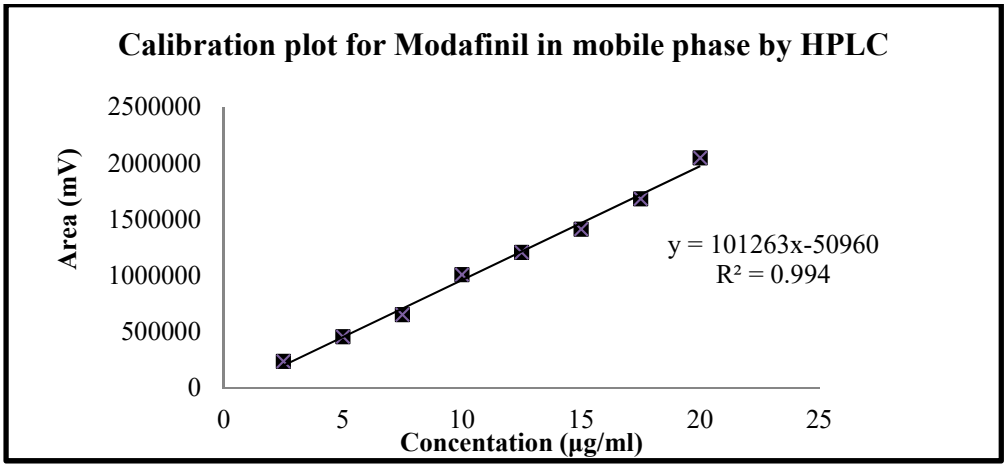

Fig. 4.6 Calibration Plot for Modafinil in Methanol: Water Mobile Phase by HPLC

As shown in Table 4.10 and Fig. 4.6, a linear increase in the peak area and is proportional with the increase in the concentration. Different parameters for analysis were discussed in the Table 4.11. Value of correlation coefficient 0.992 was obtained indicate that area and concentration of the drug were linearly related and Beer's law was found to be obeyed between 2.5-20 µg/ ml in HPLC. The method is highly sensitive and excipients have not interfering in estimation of Modafinil, therefore HPLC method used for the analysis of Modafinil in SMEDDS (Clove oil) formulation throughout study.

Table 4.11 Linearity of the Calibration Plot for Modafinil by HPLC

Parameters	Concentration range	Retention time (min)	Regression equation	Regression coefficient
Result	2.5-20 µg/ml	8.56±0.08	y = 101263x-50960	R^2=0.994

4.9.7 Validation of Modafinil by HPLC Method

Accuracy

The mean % recoveries for lower, intermediate and higher concentrations were found to be 101.92%, 100.98% and 100.52% respectively. These results show that any minor change in the drug concentration in the solutions can be accurately determined by the proposed analytical method. The results of this study recorded in Table 4.12.

Table 4.12 Accuracy of the HPLC Method for Modafinil

Sr. No.	% Drug Quantity added	Expected Concentration (µg/ml)	Recovered Concentration (µg/ml)	% Recovery
1. (Lower)	80%	5	5.096	101.92 %
2. (Intermediate)	100%	15	15.147	100.98%
3. (Higher)	120%	20	20.103	100.52%

In precision study, % RSD values obtained were less than 2.0% indicating that these methods have good precision and reproducibility. Results of intraday precision are represents in Table 4.13, show that there was no drastic change in the value of area of intraday validation. Also, % RSD < 2 indicate that method is precise and there is no intraday variability in following this method. LOD: 0.043839 µg/ml and LOQ: 0.132847µg/ml with linearity range 2.5-20 µg/ml.

Precision

Intraday Precision

Table 4.13 Intraday Precision Analysis of HPLC Method for Modafinil

Concentration (µg/ml)	Area at different time slot(mV)			Mean Area (mV)	SD	%RSD
	Slot 1	Slot 2	Slot 3			
2.5	238955	239367	235211	237844.333	2289.819	0.963
5.0	456381	456384	459692	457485.666	1910.741	0.418
7.5	658637	654449	645438	652841.333	6744.764	1.331
10.0	1009785	1007980	1010253	1009339.333	1200.248	0.119
12.5	1204090	1208476	1206079	1206018.000	2489.061	0.206
15.0	1412322	1417813	1408269	1412801.333	4790.021	0.339
17.5	1683169	1678008	1689022	1683399.667	5510.622	0.327
20.0	2042919	2049117	2051868	2047968.000	4583.809	0.224

Interday Precision

Table 4.14 Interday Precision Analysis of HPLC Method for Modafinil

Concentration (µg/ml)	Area at different days (mV)			Mean Area (mV)	SD	%RSD
	Day 1	Day 2	Day 3			
2.5	191814	187856	191838	190502.7	2292.112	1.203192
5	456381	445384	459692	453819.0	7490.167	1.650474
7.5	644586	664449	645438	651491.0	11230.040	1.723744
10	1003136	1005080	1026253	1011490	12822.320	1.267667
12.5	1203499	1184769	1209749	1199339	12999.200	1.083864
15	1482322	1517813	1511269	1503801	18887.230	1.255966
17.5	1713169	1678008	1699022	1696733	17691.910	1.042704
20	2002919	2049117	2016868	2022968	23695.390	1.171318

4.9.8 Solution Stability of a HPLC Analytical Method

Table 4.15 Solution Stability Analysis of HPLC Method for Modafinil

Conc. (µg/ml)	Mean Area (mV) ± S.D.(at 0 hr)	% RSD	Mean Area (mV) ± S.D.(after 24 hrs)	% RSD
2.5	237844.3±2289.8	0.962731	238044.3 ± 1847.2	0.77599
5	457485.6±1910.7	0.417652	457527.0 ± 2105.4	0.46017
7.5	652841.3±6744.7	1.03313	653351.6 ± 3840.2	0.587769
10	1009339.3±1200.3	0.118919	1009339.6 ± 1809.3	0.179256
12.5	1206018.0 ±2489.1	0.20639	1207145.0 ± 3208.4	0.265784
15	1412801.3±4790.1	0.33905	1414439.3 ± 4790.1	0.338657
17.5	1683399.6±5510.6	0.327349	1687547.0 ± 2598.6	0.153987
20	2046301.3±4583.6	0.223994	2049145.6 ± 3741.1	0.182569

The results of solution stability (Table 4.15) showed that prepared samples were stable in Methanol at least for a period of 24 hrs since % RSD was less than 2 % therefore drug can be analyzed after storing up to 24hrs.

4.9.9 Interference Study of Modafinil with Excipients in Formulation

Concentration of excipients were taken at approximate level at which they are present in final formulation. Chromatographic run of Tween-80, Clove Oil and Capmul MCM C8 excipients at 220 nm was performed and results were evaluated by comparing it with drug peak. Fig. 4.7 representsthe chromatogram of Modafinil along with excipients.Fig. 4.7 (A)

shows the noninterfering peak of clove oil (Black line) with absolute detection of the drug concentration but it show peak at 5 min and 12.47 min devoid of peak of Modafinil found at 8.47 min at the same retention time of the plain drug solution (Red line) of the same concentration. Similarly fig. 4.7 (B) shows no interference of the Capmul MCM C8 (brown line), Tween-80 (pink line) and PEG-400 (blue line) with the standard drug peak.

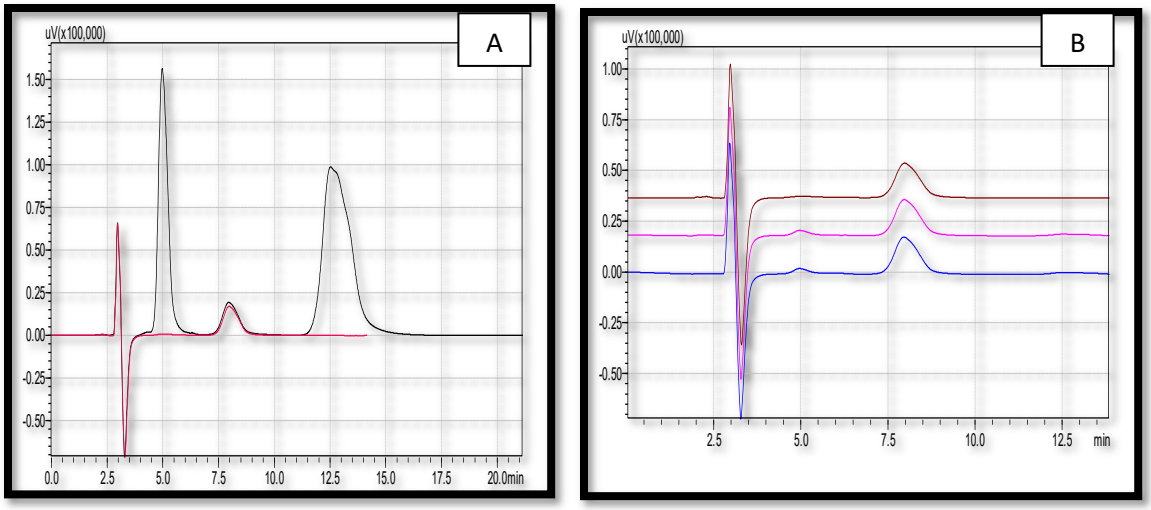

Fig. 4.7 Excipients Interference Chart of Modafinil by HPLC (A) Drug Peak (Red line) along with Clove Oil (Black line) (B), Drug Peak along with Capmul MCM C8 (brown line), Tween 80 (pink line) and PEG 400 (blue line)

4.9.10 Calibration Plot of Modafinil in Plasma using HPLC

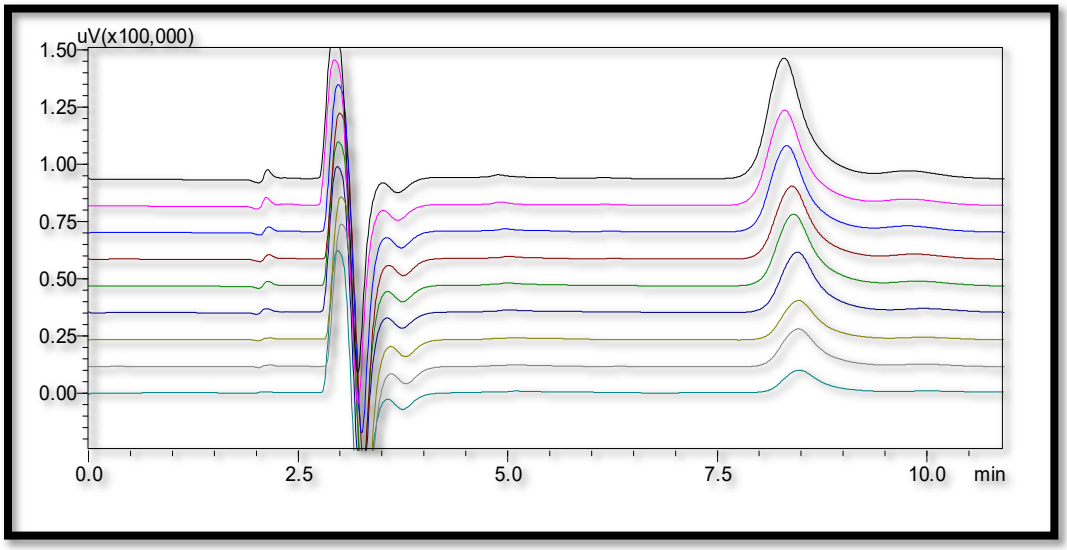

Fig. 4.8 HPLC Chromatogram of Modafinil in Plasma

The retention time for Modafinil was found to be at 8.62 min. The chromatographic conditions were same as discussed in Table 4.6. The HPLC chromatogram of Modafinil in plasma shown in Fig 4.8. The standard plot for Modafinil is shown in Fig. 4.9 and Table 4.16. The data for calibration plot of Modafinil in plasma by HPLC was fitted into a linear equation y=101263x - 50960 with correlation coefficient of R^2=0.994, which indicated the linearity of the plot.

Table 4.16 Area for the Calibration Plot of Modafinil by HPLC in Plasma

Sr. No.	Concentration (µg/ml)	Mean Area (mV) ± S.D.
1	1.0	119842.0 ± 1926.00
2	2.5	187698.3 ± 2041.00
3	5.0	450999.0 ± 1986.00
4	7.5	634514.7 ± 1298.00
5	10.0	1001499.0 ± 1368.21
6	12.5	1226339.0 ± 1149.00
7	15.0	1446135.0 ± 1142.00
8	17.5	1696733.0 ± 1286.84
9	20.0	2022968.0 ± 1386.00

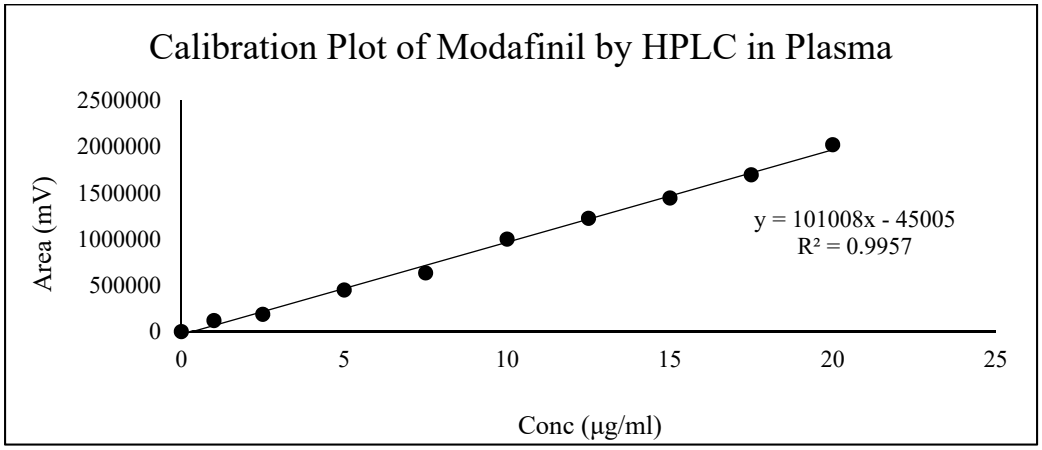

Fig. 4.9 Calibration Plot of Modafinil by HPLC in Plasma

Table 4.17 Linearity of the Calibration Plot for Modafinil in Plasma by HPLC

Parameters	Concentration range	Retention time (min)	Regression equation	Regression coefficient
Result	1-20 µg/ml	8.62 ± 0.08	y = 101008x - 45005	R^2=0.995

As shown in Table 4.16 and Fig. 4.8, there is a linear increase in the peak area which is proportional with the increase in the concentration. Table 4.17 shows different parameters of Modafinil analysis in plasma. From the value of correlation coefficient, it can be concluded that area and concentration of the drug were linearly correlated and Beer's law was found to be obeyed between 1-20 µg/ ml in HPLC. The method is highly sensitive and not interfering the analysis of Modafinil in plasma.

4.10 Analytical Method Development: Vinpocetine

List of Material and Instruments

Table 4.18 List of Material

Material	Manufacturer/Supplier
Vinpocetine	Gift Sample: Micro lab ltd., Bangalore
Methanol (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
Acetonitrile (A.R. & HPLC Grade)	Spectrochem Pvt. Ltd., Mumbai
KH_2PO_4	S.D. Fine Chemicals, Mumbai.
Polyethylene glycol- 400	Loba Chemicals, Mumbai.
Capmul MCM C8	Abitech Corporation Limited, Columbus, Ohio
Tween-80 (Analytical Grade)	S.D. Fine Chemicals, Mumbai.
Diethyl amine (Analytical Grade)	Loba Chemicals, Mumbai.
Sodium acetate (Analytical Grade)	S.D. Fine Chemicals, Mumbai.
Double Distilled Water	Prepared in the laboratory

Table 4.19 List of Instruments

Equipment /Instrument	Manufacturer
Bath sonicator	Insref, India

All other equipments are same as discussed in Table 4.2 section 4

4.11 Estimation of Vinpocetine by UV Spectroscopy

Preparation of Calibration Plot of Vinpocetine in UV Visible Spectroscopy

4.11.1 Preparation of Stock Solution

Accurately weighed (25 mg) Vinpocetine was transferred to 25 ml volumetric flask. Small quantity of Methanol was added to ensure complete solution of Vinpocetine and finally

volume was made up to the mark with Methanol (1 mg/ml). From the prepared solution (1mg/ml) of Vinpocetine, aliquot of 1 ml was transferred to 10 ml volumetric flask. Finally volume of stock solution was made up to the mark with Methanol (100 µg/ml).

4.11.2 Determination of λ_{max}

1 ml of the stock solution was transferred to a 10 ml volumetric flask and diluted with Methanol to make up the volume. The solution thus prepared (10 µg/ml) of Vinpocetine was scanned in the range of 200 nm-400 nm using methanol as blank.

4.11.3 Preparation of Calibration Plot by UV Spectroscopy

From the stock solution, aliquots of 0.4, 0.8, 1.2, 1.6, 2.0 ml were accurately withdrawn with the help of pipette and transferred to separate 10ml volumetric flasks and the volume was made up to the mark with Methanol to give final concentration of 4.0, 8.0, 12.0, 16.0, 20.0 µg/ml. The absorbance of all the prepared solutions was measured at the absorption maxima, using methanol as a blank. The readings were recorded in triplicate. Results are represented in Table 4.21 and Fig. 4.10.

4.12 Analytical Method Validation for the Estimation of Vinpocetine using UV Spectrophotometer (4)

For method validation Accuracy, Intraday & Interday Precision and Stability studies were performed same as discuss for Modafinil in section 4.4

4.13 Interference Study

In order to ascertain the non-interference of the excipients in estimation of Vinpocetine, solutions containing known concentration of each excipient were prepared in Methanol. The prepared solutions were scanned in the UV region between 200-400 nm using the respective blank. Also, to study the effect in presence of excipients, Vinpocetine solution (10 µg/ml) was spiked with known concentrations of each excipient (Capmul MCM, Tween 80, PEG-400) and scanned in the UV region between 200 nm-400 nm.

4.14 Estimation of Vinpocetine in formulation by UV spectroscopy

A definite volume of formulation Vinpocetine microemulsion or Vinpocetine mucoadhesive microemulsionwas taken in a 10 ml volumetric flask and diluted upto the

mark with methanol. The resultant solution was then sonicated for 3 min at ambient temperature and the absorbance measured at a wavelength of 314 nm against methanol as blank and recorded.

4.15 Estimation of Vinpocetine in Diffusion Media

Prepare stock solution as discussed in section 4.11. Appropriate aliquots (0.4 - 2 ml) of the stock solution were transferred to 10ml volumetric flasks and were diluted up to the mark with diffusion media (10% methanolic phosphate buffer saline pH 6.4) to get the solution having concentration 4 to 20µg/ml. The absorbance of the resulting solutions was measured using UV-Visible spectrophotometer having tungsten lamp as UV light source, at a wavelength of 314nm against diffusion media as a blank.

4.16 Estimation of Vinpocetine in Plasma by HPLC Method

Estimation of Vinpocetine in biological fluids/tissues have been reported by researchers.(11) With slight modification, the method mention was used for estimation of Vinpocetine in plasma. Estimation of Vinpocetine was carried out and validate in acetonitrilebefore estimation of Vinpocetine in plasma.

Table 4.20 Parameters for HPLC Method

Sr. No.	Parameters	Information
1	Mode	RP-HPLC
2	Detector	UV-visible spectroscopy
3	UV detection	λmax274 nm
4	Column	C18 (octadecylsilane-ODS), Supelco (250 mm × 4.6 mm i.d., 10µm particle size)
5	Mobile Phase	Acetonitrile: KH_2PO_4 (45:55) with 0.1% diethylamine, pH 3
6	Flow rate	1.5 ml/min
7	Injection size	20µl
8	Retention time	10.8 min
9	Stock solution conc.	1mg/ml
10	Serial conc. Range	1µg/ml - 20µg/ml, 200-1600 ng/ml

HPLC have an isocratic pump (Model LC-20 AT, Shimadzu, Japan), an ultra violet variable wavelength detector (Model SPD-20A, Shimadzu, Japan), a rheodyne injector (Model P/N 7725i, Made in USA), and the parameters used in the analysis are as follows.

Reversed phase high performance liquid chromatography (RP-HPLC) was conducted using a mobile phase of Acetonitrile: KH_2PO_4 (45:55) with 0.1% diethylamine and pH 3. The flow rate was 1.5mL/min, UV detection at 274 nm. The drug after extraction from plasma was chromatograph using a C18 reversed phase column.

4.16.1 Chemicals and Reagents

Acetonitrile was of HPLC grade and purchased from Merck chemicals, India. Triple distilled water used throughout the study. All other solvents and reagents used were analytical grade were filter through a 0.22 µm Ultipor® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

4.16.2 Mobile Phase Preparation

Mix Acetonitrile: KH_2PO_4 (45:55) with 0.1% diethylamine and adjust pH 3 in a reagent bottle, filter through 0.22 µm filter and sonicated the solution in bath sonicator for 3 minutes for 3 cycles to remove any air bubbles before use.

4.16.3 Preparation of Stock Solution

Accurately weighed (25 mg) Vinpocetine was transferred to 25 ml volumetric flask. Small quantity of acetonitrile (HPLC grade) was added to ensure complete solution of Vinpocetine and final volume was made up to the mark with acetonitrile (1 mg/ml).

4.16.4 Preparation of Calibration Plot of Vinpocetine by HPLC Method

From the stock solution, aliquots of 0.01, 0.02, 0.03, 0.04, 0.08, 0.12,0.16 and 0.2 ml were accurately withdrawn with the help of pipette and transferred to separate 10ml volumetric flasks and the volume was made up to the mark with acetonitrile to give final concentration of 1,2,3,4,8,12,16,20 µg/ml. The area of all the prepared solutions was then measured in triplicate in HPLC as per above analytical condition.The results were recorded in triplicate and are stated in Table 4.28.

4.17 Analytical Method Validation for the Estimation of Vinpocetine by HPLC

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

4.18 Estimation of Vinpocetine in Plasma

4.18.1 Calibration Plot of Vinpocetine in Plasma

Initially 10 mg of drug was dissolved in 10 ml of acetonitrile to make 1mg/ml solution of Vinpocetine. From this, 1ml of solution was diluted to 10 ml with acetonitrile to obtain final concentration of 100 µg/ml. From this stock solution, aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml, 1.2ml, 1.4ml and 1.6ml were taken and diluted upto 10ml with acetonitrile to make final concentrations in the range 2 µg/ml to 16 µg/ml. From this 0.1ml of drug sample was added to 0.1 ml of plasma and were diluted upto1ml with acetonitrile to precipitate plasma proteins. Final drug solution obtained was of 200, 400, 600, 800, 1000, 1200, 1400 and 1600 ng/ml respectively. The mixture was then centrifuged at 5000rpm for 10 minutes to separate precipitated proteins. The supernatant was collected, filtered with syringe, 20 µl of filtered supernatant of each of the prepared sample was injected into HPLC system using HPLC syringe through rheodyne auto injector and the calibration plot was prepared. Chromatographic condition was maintained as discussed in 4.16. Calibration plots were drawn by plotting peak area vs. drug concentration. The column was equilibrated by passing at least 150-200 ml of mobile phase.

4.18.2 Estimation of Vinpocetine in Brain Homogenate

Mobile phase was prepared in the same way as discussed in 4.16.2.

Preparation of Brain Homogenate

Rat was humanely sacrificed by exposing it to high amount of diethyl ether. Brain was isolated from the animal and kept in phosphate buffer pH 7.4. Prior to subjecting the brain to homogenization, it was separately taken in petri plate and minced properly as this ensures thorough homogenization of the brain. The brain was added to homogenizer tube containing phosphate buffer pH 7.4 and homogenized to give uniform dispersion. This

dispersion was further centrifuged for 10 minutes at 5000 rpm to precipitate the tissues and the supernatant collected was then stored at -70°C till further analysed.

Preparation of Drug Solutions

Initially 10 mg of drug was dissolved in 10 ml of acetonitrile to make 1mg/ml solution of Vinpocetine. From this 1ml of the solution was taken and diluted to 10ml with acetonitrile to obtain a final concentration of 100 µg/ml. From this, aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml, 1.2ml, 1.4ml and 1.6ml were taken and diluted upto 10 ml with acetonitrile to make final concentrations in the range 2 µg/ml to 16 µg/ml. 0.1ml of this drug sample was added to 0.1ml of brain homogenate and these samples were diluted upto 1ml with acetonitrile to precipitate plasma proteins and the final drug solutions obtained were of 200, 400, 600, 800, 1000, 1200, 1400 and 1600 ng/ml respectively. The mixture was then centrifuged at 5000rpm for 10 minutes to separate the precipitated proteins. The supernatant is then collected and filtered with syringe filter before injecting it in HPLC column. Then, 20 µl of filtered supernatant of each of the prepared sample was injected into HPLC system using HPLC syringe and the calibration plot was prepared.

4.19 Results and discussion

4.19.1 Determination of λmax and Calibration Plot for Vinpocetine

A simple UV spectroscopic method was developed from the literature. The arbitrary concentrated solution (100µg/ml) of Vinpocetine in methanol was screened. Two absorption maxima were obtained at 274nm and 314nm. Because of low sensitivity at 274nm for a given concentration, λmax of 314nm was used. The absorbance was found to be linear in the range of 4-20 µg/ml with R^2 value 0f 0.9997.

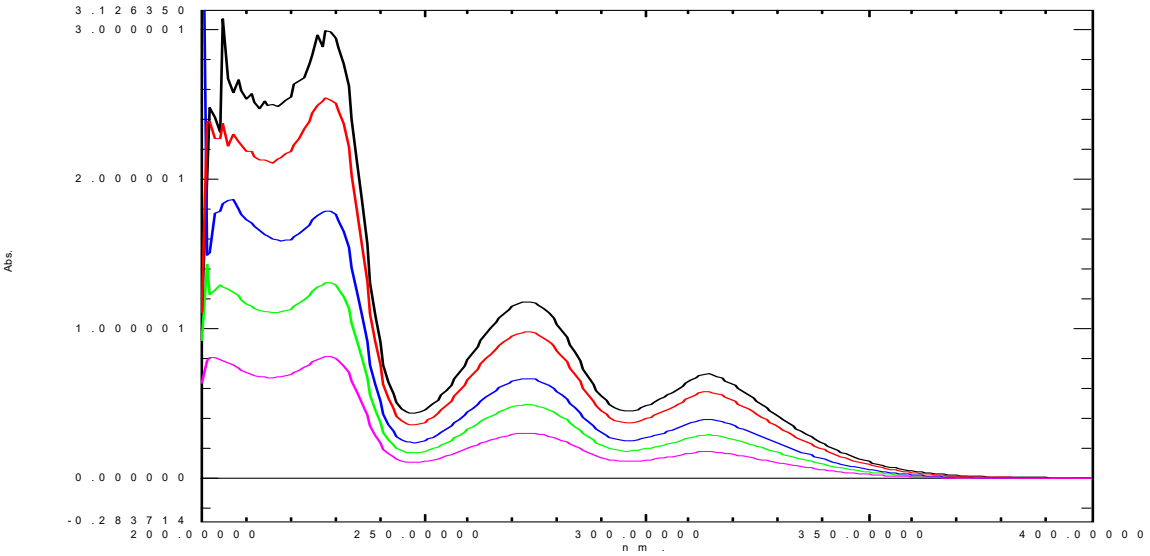

Fig. 4.10 Calibration Plot of Vinpocetine in Methanol by UV Spectroscopy

Table 4.21 Absorbance for the Calibration Plot of Vinpocetine at 314 nm in Methanol by UV Spectrometer

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D.*
1	4	0.099 ±0.00153
2	8	0.227 ± 0.00200
3	12	0.365 ± 0.00251
4	16	0.485 ± 0.00153
5	20	0.617 ± 0.00153

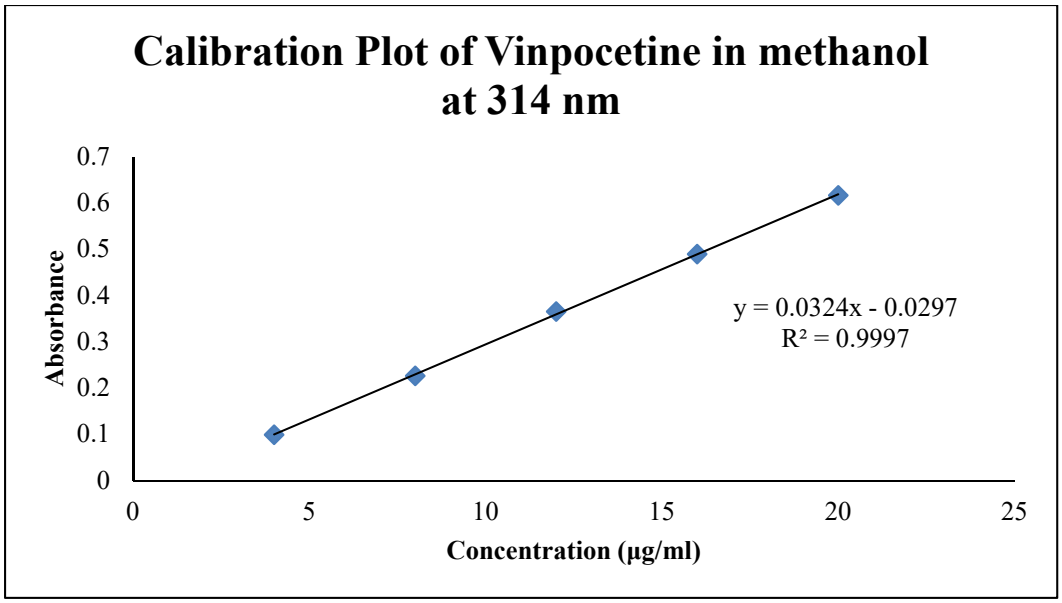

Fig. 4.11Calibration Plot of Vinpocetine in Methanol at 314nm

Table 4.22Linearity of Method of Analysis of Vinpocetine in Methanol by UV

Parameters	λmax	Concentration range	Regression equation	Regression coefficient
Result	314 nm	4-20 µg/ml	y = 0.0324x - 0.0297	R^2=0.9997

4.19.2 Validation of Analytical Method

Accuracy

The mean % recoveries for lower, intermediate and higher concentrations were found to be 100.33%, 100.5% and 100.14 % respectively. This result shows that any minor change in the drug concentration in the solutions can be accurately determined by the proposed analytical method. The results of this study are given in Table 4.23.

Table 4.23 Recovery Study for Accuracy of Vinpocetine Estimated by UV Method

Sr.No.	Quantity of Vinpocetine added%	Expected Concentration (µg/ml)	Recovered Concentration (µg/ml)	%Recovery
1.	80 (Lower)	9.0	09.03	100.33%
2.	100 (Intermediate)	12.0	12.06	100.50%
3.	120 (Higher)	14.0	14.02	100.14%

Precision

Intraday Precision

Table 4.24 Intraday and Interday Precision Analysis of Vinpocetine Estimated by UV method (1)

Sr. No.	Intraday Precision			Interday Precision		
Concentration (µg/ml)	Mean Absorbance	SD	% RSD	Mean Absorbance	SD	% RSD
4	0.099	0.001	1.0101	0.099	0.00152	1.5482
8	0.225	0.00321	1.42447	0.226	0.00200	0.885
12	0.366	0.00529	1.44577	0.365	0.00251	0.6889
16	0.490	0.001	0.20408	0.487	0.00264	0.5433
20	0.620	0.002	0.32258	0.616	0.00152	0.2478

Precision study performed under the same operating condition for intraday and interday (Table 4.24); results revealed for a short time interval and longtime respectively. In precision study, % RSD values obtained were less than 2.0% suggest that these methods have good precision and reproducibility. There is no intraday and interday variability by following of method. LOD: 0.1258 µg/ml and LOQ: 0.3813µg/ml

4.19.3 Stability of an Analytical Method

Table 4.25 Stability Analysis of UV Method for Vinpocetine

Concentration (µg/ml)	Absorbance ± S.D.* (0 hr)	Absorbance ± S.D.* (24 hrs)
8	0.225 ± 0.001	0.219 ± 0.007
12	0.366 ± 00153	0.345 ± 0.014
16	0.493 ± 0.00550	0.486 ± 0.006

*S.D. Standard deviation

The results indicated that there was no significant change in absorbance values of Vinpocetine estimated after 24 hrs. This clearly showed that the drug is stable in Methanol for a period of at least 24 hrs and and method can be used for further analysis.

4.19.4 Interference Study

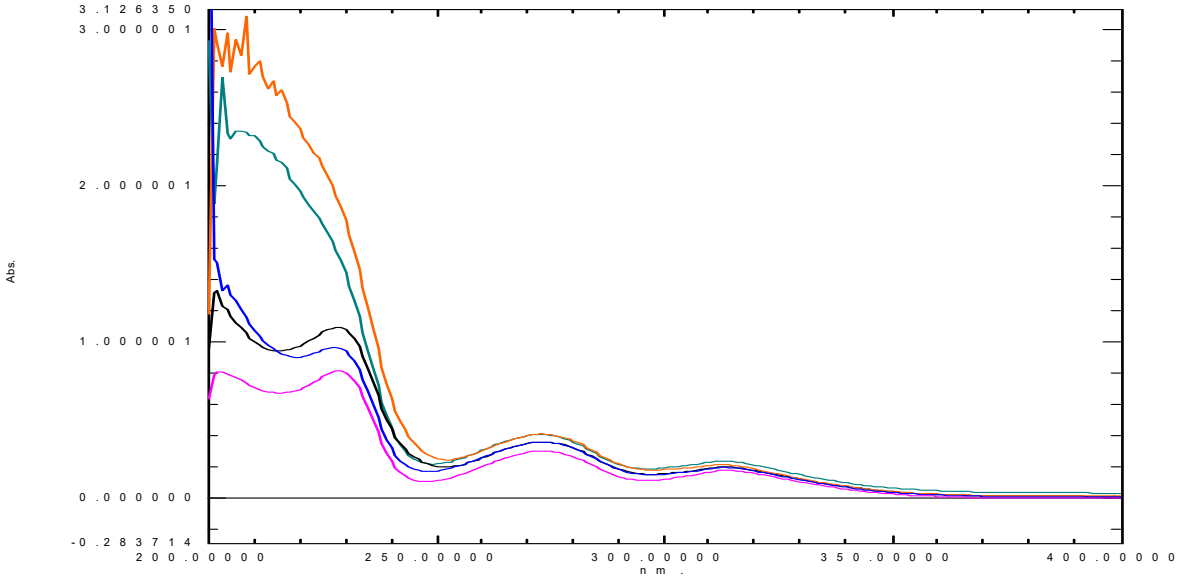

Fig. 4.12 Excipients Interference Chart of Vinpocetine by UV-visible Spectrophotometer (A) Drug peak (Pink line), (B) Drug peak along with Capmul MCM C8 (blue line), Tween-80 (Black line) and PEG-400 (blue line)

Concentration of excipients were taken at approximate level at which they are present in final formulation. The absorbance of Tween 80, PEG 400 and Capmul MCM C8 excipients at 314 nm was indicating that these excipients do not interfere in the estimation of Vinpocetine and hence UV Spectroscopy method can be used for estimation of Vinpocetine in formulation.

4.19.5 Estimation of Vinpocetine in Diffusion Media

Table 4.26 Absorbance of Vinpocetine at 314 nm in Diffusion Media

Sr. No.	Conc. (µg/ml)	Absorbance	SD	%RSD
1	4	0.096	0.0012	1.1945
2	8	0.206	0.0021	1.0072
3	12	0.357	0.0040	1.1204
4	16	0.490	0.0036	0.7358
5	20	0.615	0.0066	1.0662

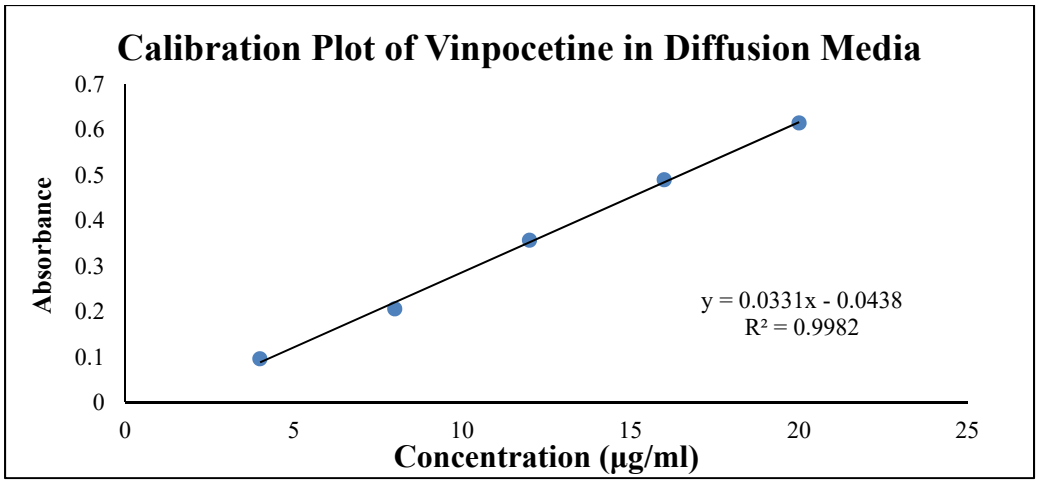

Fig. 4.13 Calibration plot of Vinpocetine in diffusion media at 314nm

Table 4.27 Linearity of Analytical Method of Vinpocetine in Diffusion Media

Parameters	λmax	Concentration Range	Regression Equation	Regression Coefficient
Result	314 nm	4-20 µg/ml	y = 0.0331x - 0.0438	R^2=0.9982

As shown in Table 4.26 and Fig. 4.13, a linear increase in the peak area and is proportional with the increase in the concentration. Different parameters for analysis were discussed in the Table 4.27. Value of correlation coefficient 0.9982 was obtained indicate that area and concentration of the drug were linearly related and Beer's law was found to be obeyed between 4-20 µg/ ml by UV Spectroscopy therefore it can be used for the analysis of Vinpocetine in diffusion study of Vinpocetine loaded Microemulsion.

4.19.6 Calibration Plot of Vinpocetine by HPLC Method

Table 4.28 Calibration Plot of Vinpocetine by HPLC

Sr. No.	Concentration (µg/ml)	Mean Area (mV) ± S.D. (n=3)
1	1	44761 ± 168.014
2	2	79245 ± 351.092
3	3	134059 ± 683.051
4	4	186144 ± 758.121
5	8	336373 ± 778.017
6	12	496473 ± 1083.21
7	16	670609 ± 998.756
8	20	812520 ± 1237.042

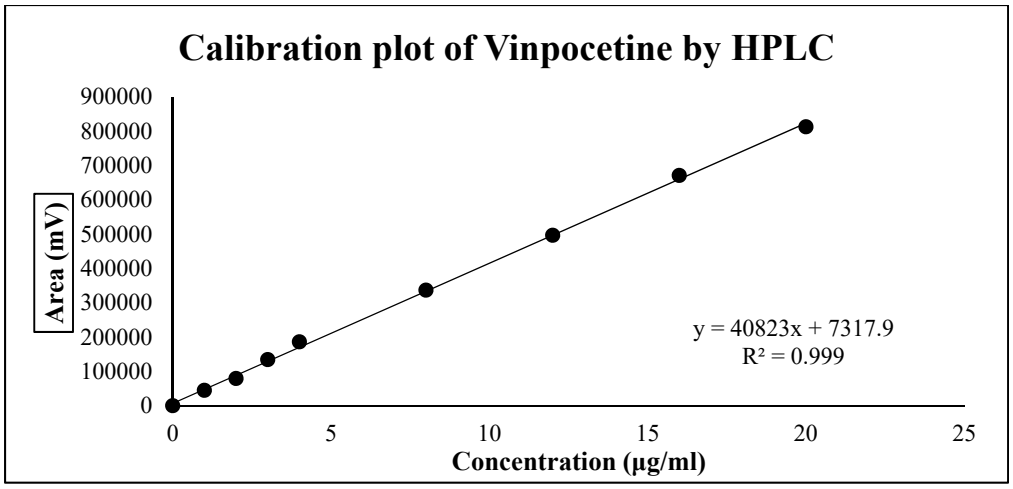

Fig.4.14 Calibration Plot for Vinpocetine by HPLC

Table 4.29 Linearity of Method of Analysis of Vinpocetine by HPLC

Parameters	Concentration range	Retention time (min)	Regression equation	Regression coefficient
Result	1-20 µg/ml	10.8 ± 0.07	y = 40823x + 7317.9	R^2=0.999

As shown in Table 4.28 and Fig. 4.14 there is a linear increase in the peak area and is proportional with the increase in the concentration. Different parameters for analysis were discussed in the Table 4.29. Value of correlation coefficient 0.999 was obtained indicate that area and concentration of the drug were linearly related and Beer's law was found to be obeyed between 1-20 µg/ml in HPLC. The HPLC method is highly sensitive and can be used for the analysis of Vinpocetine.

4.19.7 Validation of Vinpocetine by HPLC Method

Accuracy

The mean % recoveries for lower, intermediate and higher concentrations were found to be 99.9%, 101.47% and 99.22 % respectively. These results show that any minor change in the drug concentration in the solutions can be accurately determined by the proposed analytical method. The results of this study are given in Table 4.30.

Table 4.30 Accuracy of the HPLC Method for Vinpocetine

Sr. No.	% Drug Quantity added	Expected Concentration (µg/ml)	Recovered Concentration (µg/ml)	% Recovery
1. (Lower)	80%	4	3.996	99.9 %
2. (Intermediate)	100%	10	10.147	101.47 %
3. (Higher)	120%	15	14.883	99.22 %

Precision

Intraday Precision

Table 4.31 Intraday Precision Analysis of HPLC Method for Vinpocetine

Concentration (µg/ml)	Area at different time slot (mV)			Mean Area (mV)	SD	%RSD
	Slot 1	Slot 2	Slot 3			
4	187051	186598	187840	187163	628.5292	0.335819
8	330995	331956	332494	331815	759.382	0.228857
12	493451	492507	494416	493458	954.5193	0.193435
16	680113	681148	682063	681108	975.6152	0.143239

Interday Precision

Table 4.32 Interday Precision Analysis of HPLC Method for Vinpocetine

Concentration (µg/ml)	Area at different days (mV)			Mean Area (mV)	SD	%RSD
	Day 1	Day 2	Day 3			
4	187051	185017	188062	186710	1550.876	0.830634
8	330995	335042	337643	334560	3350.107	1.001347
12	493451	498823	489978	494084	4456.346	0.901941
16	680113	674854	688564	681177	6916.654	1.015397

The precision study operated under the same operating condition for intraday (Table 4.31) and interday (Table 4.32). In precision study, % RSD values obtained were less than 2.0% indicating that these methods have good precision and reproducibility. From the Table 4.31it can be concluded that there is no drastic change in the results during intraday validation. Here, mean of % RSD < 2 indicate that method is precise and there is no intraday variability in the following method. The LOD and LOQ of the HPLC method was found to be 0.042362 µg/ml and 0.12837 µg/ml with linearity range 1-20 µg/ml.

4.19.8 Solution Stability of HPLC Analytical Method

The results of solution stability (Table 4.33) showed that prepared samples were stable in acetonitrile atleast for a period of 24 hrs since % RSD was less than 2 % therefore drug can be analyzed after storing for 24 hrs.

Table 4.33 Solution Stability Analysis of HPLC Method for Vinpocetine

Concentration (µg/ml)	Mean Area (mV) ± S.D. (at 0 hr)	%RSD	Mean Area (mV) ± S.D. (after 24 hrs)	%RSD
1	44761 ± 0168.014	0.375358	43156 ± 0174.201	0.403654
2	79245 ± 0351.092	0.443046	78324 ± 0405.154	0.517280
3	134059 ± 0683.051	0.509515	133215 ± 0680.223	0.510620
4	186144 ± 0758.121	0.407277	187113 ± 0809.239	0.432487
8	336373 ± 0778.017	0.231296	335150 ± 0798.142	0.238145
12	496473 ± 1083.210	0.218181	495445 ± 1190.810	0.240352
16	670609 ± 0998.756	0.148933	671792 ± 1082.653	0.161159
20	812520 ± 1237.042	0.152248	813964 ± 1441.513	0.177098

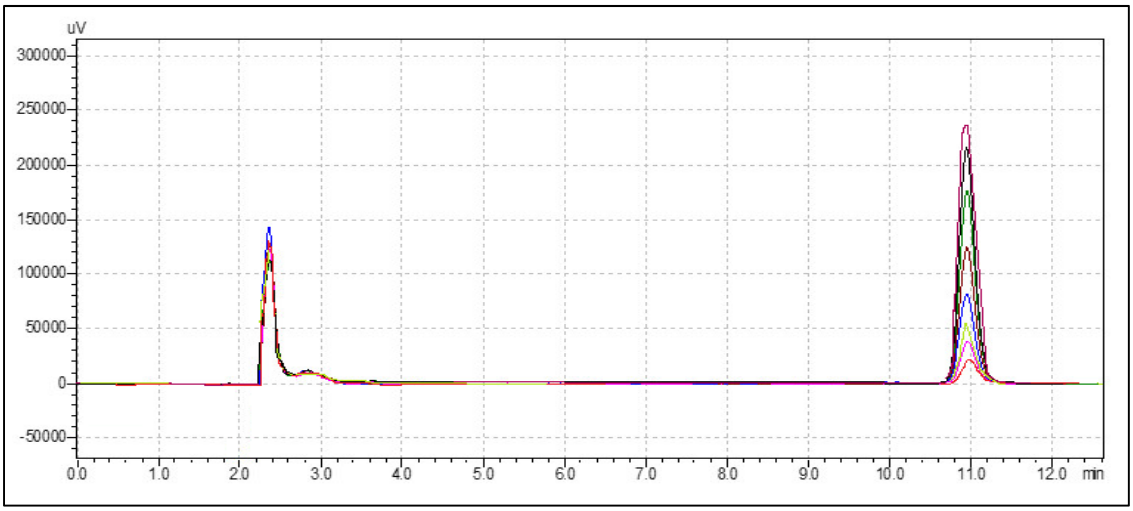

Fig.4.15 Graphical Spectrum of Vinpocetine Calibration Plot by HPLC Method

As shown in Table 4.34 and Fig. 4.16, a linear increase in the peak area and is proportional with the increase in the concentration. Different parameters for analysis were discussed in Table 4.35. Value of correlation coefficient 0.9977 was obtained indicate that area and concentration of the drug were linearly related and Beer'slaw was found to be obeyed between 200-1600 ng/ml in HPLC.

Table 4.34 Calibration Plot of Vinpocetine by HPLC

Sr. No.	Concentration (ng/ml)	Mean Area (mV) ± S.D. (n=3)
1	200	11159 ± 111.53
2	400	18216 ± 368.60
3	600	30546 ± 257.34
4	800	42637 ± 305.18
5	1000	52586 ± 584.91
6	1200	63828 ± 776.45
7	1400	73267 ± 542.72
8	1600	81322 ± 759.13

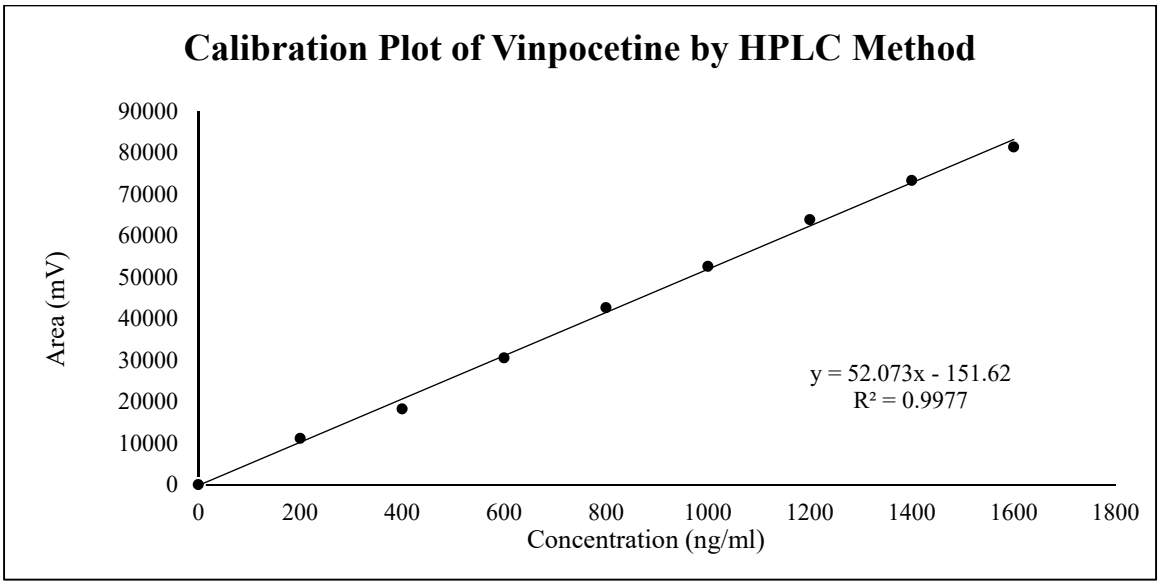

Fig. 4.16 Calibration Plot for Vinpocetine by HPLC Method

Table 4.35 Linearity of Method of Analysis of Vinpocetine by HPLC

Parameters	Concentration range	Retention time (min)	Regression equation	Regression coefficient
Result	200-1600 ng/ml	10.7 ± 0.07	y = 52.073x + 151.62	R^2=0.9977

Table 4.36 Calibration Plot of Vinpocetine in Plasma by HPLC

Sr. No.	Concentration (µg/ml)	Mean Area (mV) ± S.D. (n=3)
1	1	38746 ± 213.74
2	2	64417 ± 355.12
3	3	124955 ± 328.45
4	4	185251± 431.75
5	8	335318 ± 631.55
6	12	454651 ± 689.67
7	16	619041 ± 756.33
8	20	781230 ± 946.48

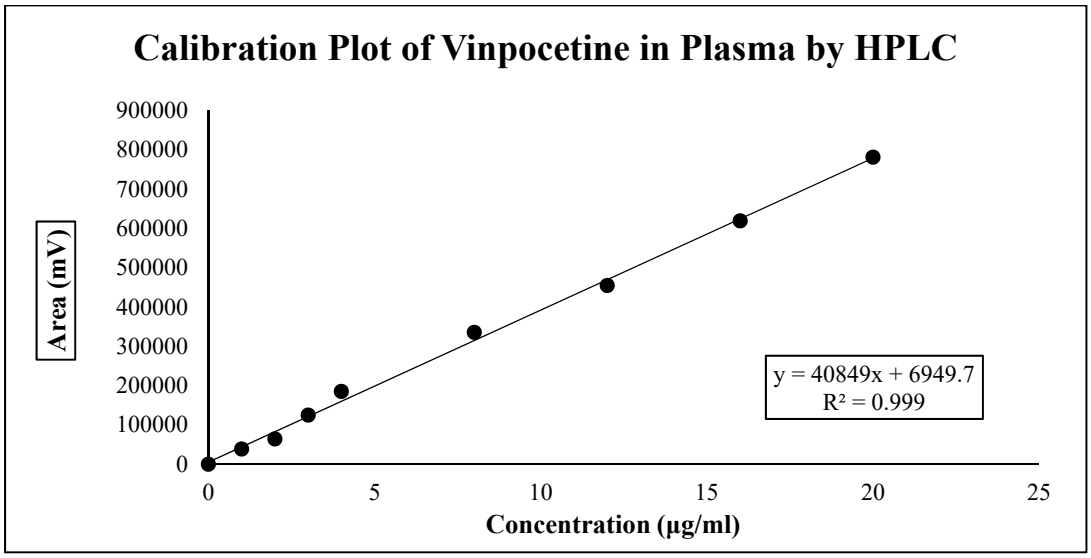

Fig. 4.17 Calibration Plot of Vinpocetine in Plasma by HPLC

Table 4.37 Linearity of Method of Analysis of Vinpocetine in Plasma by HPLC

Parameters	Concentration range	Retention time (min)	Regression Equation	Regression Coefficient
Result	1-20 µg/ml	10.3 ± 0.07	y = 40849x + 6949.7	R^2=0.999

As shown in Table 4.36 and Fig. 4.17, a linear increase in the peak area and is proportional with the increase in the concentration. Different parameters for analysis were discussed in Table 4.37. Value of correlation coefficient 0.999 was obtained indicate that area and concentration of the drug were linearly related and Beer's law was found to be obeyed between 1-20 µg/ml in HPLC.

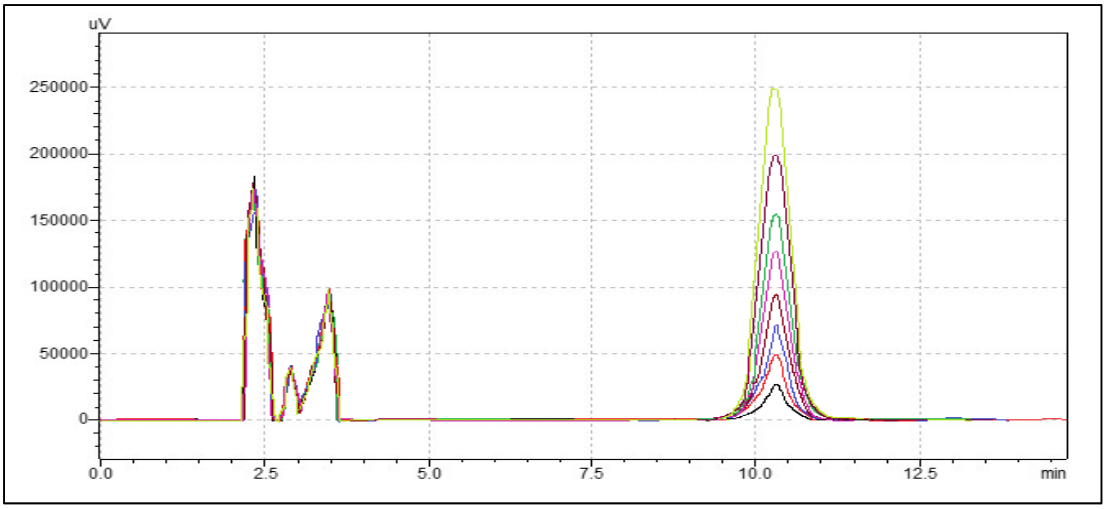

Fig.4.18 Graphical Spectrum of Vinpocetine Calibration Plot in Plasma by HPLC Method

Table 4.38 Area for the Calibration Plot of Vinpocetine in Plasma by HPLC

Sr. No.	Concentration (ng/ml)	Mean Area (mV) ± S.D.
1	200	10051 ± 241.63
2	400	18239 ± 348.61
3	600	29716 ± 108.56
4	800	40540 ± 427.84
5	1000	48614 ± 698.10
6	1200	61835 ± 764.18
7	1400	70257 ± 528.44
8	1600	77462 ± 814.72

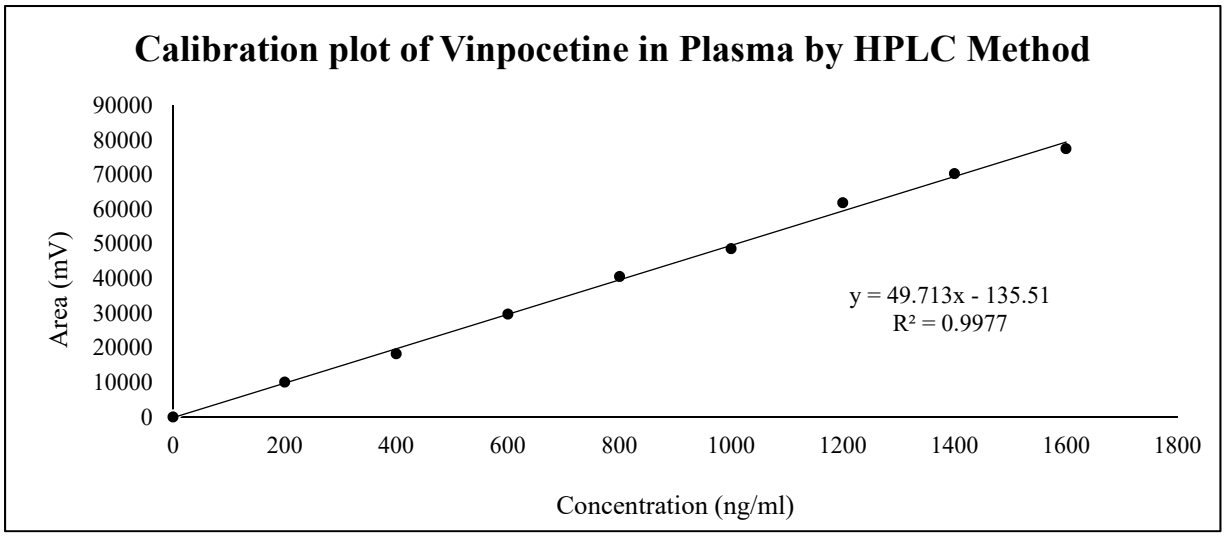

Fig. 4.19 Calibration Plot of Vinpocetine in Plasma by HPLC

As shown in Table 4.38 and Fig. 4.19, there is a linear increase in the peak area which is proportional with the increase in the concentration. Table 4.39 shows different parameters of Vinpocetine analysis in plasma. From the value of correlation coefficient, 0.9977; it can be concluded that area and concentration of the drug were linearly correlated and Beer's law was found to be obeyed between 200 - 1600 ng/ml in HPLC. The method is highly sensitive and not interfering the analysis of Vinpocetine in plasma.

Table 4.39 Linearity of Method of Analysis of Vinpocetine in Plasma by HPLC

Parameters	Concentration range	Retention time (min)	Regression Equation	Regression Coefficient
Result	200-1600 ng/ml	10.3 ± 0.07	y = 49.71x -135.51	R^2=0.9977

4.19.9 Estimation of Vinpocetine in Brain Homogenate

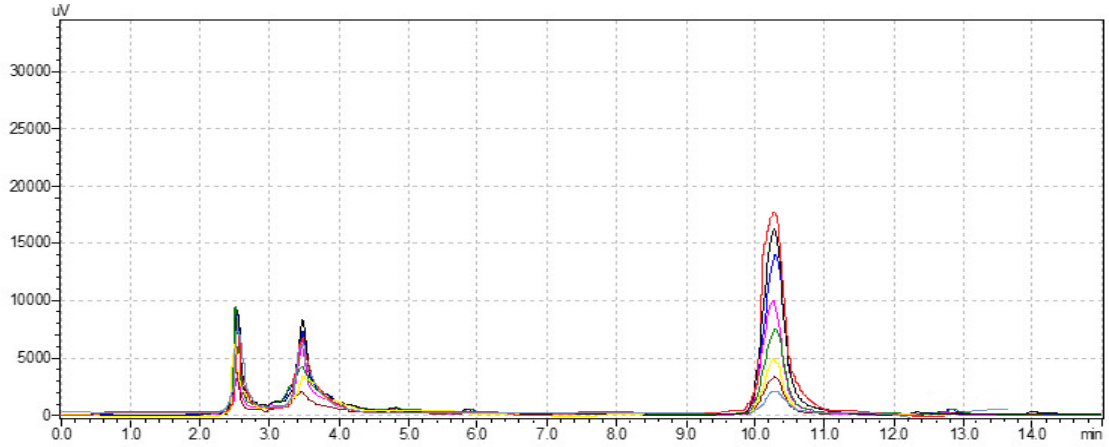

Fig. 4.20 Graphical Spectrum of Vinpocetine Calibration plot in Brain Homogenate by HPLC Method

Table 4.40 Area for the Calibration Plot of Vinpocetine in Brain Homogenate

Sr. No.	Concentration (ng/ml)	Mean Area (mV) ± S.D. (n=3)
1	200	9486 ± 87.02
2	400	16724 ± 116.27
3	600	27396 ± 104.36
4	800	38419 ± 385.42
5	1000	49765 ± 294.61
6	1200	60118 ± 486.74
7	1400	69631 ± 564.10
8	1600	78567 ± 498.79

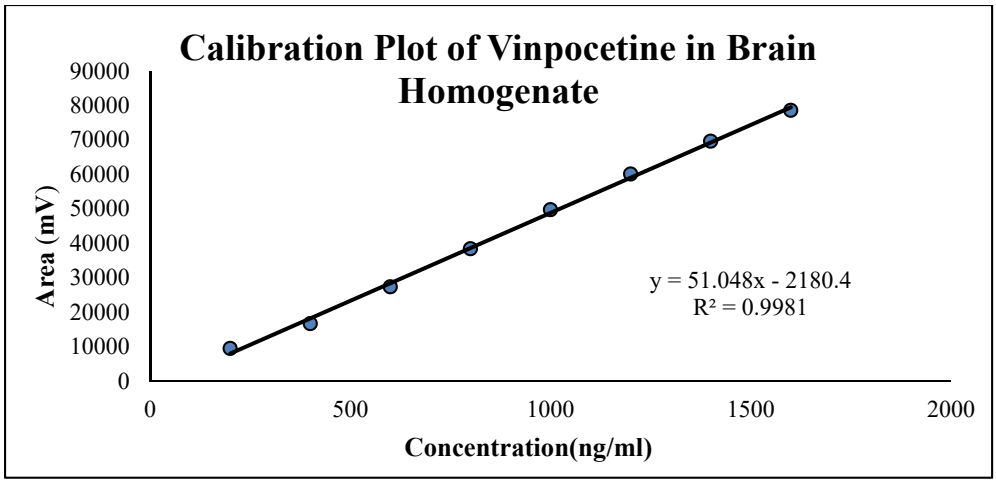

Fig. 4.21 Calibration Plot of Vinpocetine in Brain Homogenate by HPLC

Table 4.41 Linearity of Method of Analysis of Vinpocetine in Brain Homogenate by HPLC

Parameters	Concentration range	Retention time (min)	Regression Equation	Regression Coefficient
Result	200-1600 µg/ml	10.5 ± 0.06	y = 51.048x – 2180.4	R^2=0.9981

As shown in Table 4.40 and Fig. 4.21, there is a linear increase in the peak area which is proportional with the increase in the concentration. Table 4.41 shows different parameters of Vinpocetine analysis in brain homogenate. From the value of correlation coefficient, 0.9981; it can be concluded that area and concentration of the drug were linearly correlated and Beer's law was found to be obeyed between 200 - 1600 ng/ml in brain homogenate by HPLC. The method is highly sensitive and not interfering the analysis of Vinpocetine in brain homogenate.

4.20 References

1. Mothilal M, Kumar AH, Krishna MC, Manasa V, Manimaran V, Damodharan N. Formulation and evaluation of modafinil fast dissolving tablets by sublimation technique. J Chem Pharm Sci. 2013;6:147-54.
2. Pritam J, Amar C, Bhargav D, Shani P, Santsaran P, Hiren S. Development and validation of first order derivative UV-Spectrophotometric method for determination of

Sitagliptin in bulk and in formulation. International Journal of Drug Development and Research. 2011;3(4):194-9.

3. Nagulwar V, Bhusari K. Development of UV spectrophotometric first order derivative method for the simultaneous estimation of ritonavir and lopinavir in combined tablet dosage form. International Journal of Pharmaceutical Sciences and Research. 2012;3(7):2317.
4. Guideline IHT, editor Validation of analytical procedures: text and methodology Q2 (R1). International Conference on Harmonization, Geneva, Switzerland; 2005.
5. Huber L. Book: Validation and qualification in analytical laboratories, second edition: CRC Press; 2007.
6. Bhimanadhuni CN, Garikapati DR, Srinivas C. Development and validation of RP-HPLC method for determination of Duloxetine hydrochloride in bulk and dosage form. International Current Pharmaceutical Journal. 2012;1(5):98-102.
7. Khan AA, Panda SK, Sahoo SK, Dash AK. Stability indicating RP-HPLC method for determination of modafinil in bulk & its formulations. Int J Biol Pharma Res. 2011;2:39-44.
8. Schwertner HA, Kong SB. Determination of modafinil in plasma and urine by reversed phase high-performance liquid-chromatography. Journal of pharmaceutical and biomedical analysis. 2005;37(3):475-9.
9. Burnat P, Robles F, Do B. High-performance liquid chromatographic determination of modafinil and its two metabolites in human plasma using solid-phase extraction. Journal of Chromatography B: Biomedical Sciences and Applications. 1998;706(2):295-304.
10. Analytical method of Modafinil. Indian Pharmacopoeia 2007,volume I.242.
11. Abd Elbary A, Foda N, El-Gazayerly O, El Khatib M. Reversed phase liquid chromatographic determination of vinpocetine in human plasma and its pharmacokinetic application. Analytical letters. 2002;35(6):1041-54.