CONTENTS

3.1. INTRODUCTION

Analytical method development and validation are key elements of any pharmaceutical development program. Analytical method development is the process of selecting an accurate assay procedure to measure the desired component(s) of a formulation at various stages of product development. Validation is the process of proving that an analytical method is acceptable for use in laboratory to measure the concentration of subsequent samples. Validation helps the analyst in recognizing the behavior of developed method and in establishing its performance limits (1). The important parameters that may be evaluated during method validation are specificity, linearity, range, accuracy, precision, limits of detection (LOD) and limit of quantitation (LOQ). Changes encountered during formulation development also require

modifications to existing analytical methods which in turn, may require additional validation. The design and execution of these studies requires thorough knowledge of the product being tested as well as a good understanding of the analytical technique. Analytical instruments also play a major role in the process to achieve high quality and reliable data.

Present investigation required the analytical methods for the purpose of measuring vital formulation characteristics *viz.*, percent entrapment efficiency, *in vitro* drug release, ex vivo skin permeation/deposition, *in vivo* bioavailability and drug retention during stability testing. Several analytical methods for the determination of VPN as well as NPT in pharmaceutical dosage forms and in biological fluids were found in literatures (Table 3-1).

Table 3-1. Reported analytical methods for VPN and NPT

Analytical Methods	References
Vinpocetine (VPN)	
Ultraviolet-visible (UV) spectrophotometry	(2)
High performance liquid chromatography (HPLC)	(3, 4)
Ultrahigh performance liquid chromatography (UPLC)	(5)
Gas chromatography-mass spectroscopy (GC-MS)	(6)
Liquid chromatography-mass spectroscopy (LC-MS)	(7)
Sensitive enzyme immunoassay	(8)
Noopept (NPT)	
Ultraviolet-visible (UV) spectrophotometry	(9, 10)
High performance liquid chromatography (HPLC)	(10, 11)
Liquid chromatography-mass spectroscopy (LC-MS)	(12)

Depending on the theoretical amount of drugs expected to be present in analytical samples, a simple instrumental method like UV spectrophotometry to a more sensitive and sophisticated methods like high performance liquid chromatography (HPLC) were exploited. The analytical methods employed in present investigation are described subsequently.

3.2. MATERIALS

Vinpocetine was obtained as a gift sample from Covex S.A., Madrid, Spain. Noopept was purchased from Nootrico SA, New York, United States. PLGA (lactide/glycolide ratio 50:50) was a generous gift from Purac Biomaterials, Netherlands. Phospholipon® 90G and 90H

were kindly gifted by Lipoid GmBH, Germany. Poloxamer® 188 was purchased from BASF, India. Sodium deoxycholate was purchased from S.D. Fine Chemicals, Mumbai, India. Poly vinyl alcohol (MW, 6 kD) and Poly vinyl pyrollidone (MW, 3.5 kD) were purchased from Acros organics, Mumbai, India.

3.3. EQUIPMENTS

3.3.1. UV Spectrophotometer

A double beam UV-1800 spectrophotometer (Shimadzu, Japan) coupled with UV Probe v2.42 data handling software was used for spectrophotometric measurements. All the spectral measurements were made using matched quartz cuvettes of 1 ml capacity and 1 cm path length. The spectral bandwidth and wavelength scanning speed were 1 nm and 2800 nm/min, respectively.

3.3.2. High Performance Liquid Chromatographic (HPLC) system

The isocratic HPLC system comprising of LC-20AT Prominence solvent delivery module, a manual Rheodyne injector (20 µl fixed loop) and SPD-20A Prominence UV-Visible detector (Shimadzu, Japan) was used together with either LCsolution or Spinchrom Chromatographic Station® CFR v2.4.0.193 as data handling software. Supelco® C-18 column of 250 mm length, 4.6 mm internal diameter and 5 µm particle size (Sigma-Aldrich, India) pre-connected to a 2 cm guard column (Phenomenex, USA) was used to perform chromatographic separations.

3.4. REAGENTS & SOLUTIONS

3.4.1. Filtered double distilled water

Tap water was distilled using double distillation assembly (Pranava Lab Solutions, India) and passed through 0.22 µm membrane filter to prepare filtered double distilled water.

3.4.2. Ammonium acetate solution (1.54 %w/w)

6.16 g of ammonium acetate was accurately weighed and dissolved in 400 ml of filtered double distilled water.

3.4.3. Mobile phase for VPN estimation by HPLC

400 ml of ammonium acetate solution (1.54 %w/w) was mixed with 600 ml of HPLC grade acetonitrile. The mixture was filtered using 0.22 µm membrane filter and then sonicated for 10 min using ultrasonic bath. The resulting solvent mixture was used as mobile phase for HPLC based estimation of VPN.

3.4.4. Mobile phase for NPT estimation by HPLC

500 ml of HPLC grade acetonitrile, 500 ml of filtered double distilled water and 1 ml of glacial acetic acid were mixed together. The resulting solvent mixture was sonicated for 10 min using ultrasonic bath and used as mobile phase for HPLC based estimation of NPT.

3.5. METHODS

3.5.1. VPN estimation by UV Spectrophotometry

UV spectrophotometric methods in ACN (acetonitrile) and A2M8 (acetonitrile-methanol in 2:8 v/v ratio) were developed for estimation of VPN entrapped within polymeric nanoparticles and VPN entrapped within ultradeformable liposomes, respectively.

3.5.1.1. Preparation of Standard Stock Solutions of VPN

Standard stock solutions containing 100 µg/ml VPN were prepared in both the solvents mentioned above. Briefly, accurately weighed 10 mg of VPN were transferred to separate 10 ml calibrated volumetric flasks and dissolved in respective solvents. The volumes were made up to 10 ml with the same solvents to get 1000 µg/ml of standard VPN solutions. 1 ml of above solutions were further taken in separate 10 ml calibrated volumetric flasks and the volumes were made up to the mark to obtain standard stock solutions of 100 µg/ml.

3.5.1.2. Preparation of Standard VPN Solutions

Standard solutions of VPN in respective solvents were prepared using above 100 µg/ml standard stock solutions. Briefly, 0.5, 1.0, 1.5, 2.0 and 2.5 ml aliquots of VPN standard stock solutions were transferred to 10 ml calibrated volumetric flasks and diluted up to the mark with

respective solvents to obtain standard VPN solutions having concentrations ranging from 5 to 25 μg/ml.

3.5.1.3. Determination of Analytical Wavelengths

The spectrum of 10 μg/ml standard VPN solutions in both solvents were obtained by scanning the solutions over a wavelength (λ) range of 220-400 nm using UV Visible spectrophotometer against respective solvents as blank. The wavelengths with maximum absorbance (λ_{max}) were selected as analytical wavelengths for photometric measurements in respective solvents during preparation of calibration curves as well as during drug quantification in analytical samples.

3.5.1.4. Preparation of Calibration Curves

Absorbance of standard VPN solutions at respective analytical wavelengths were recorded using UV Visible spectrophotometer. The calibration curves between standard VPN concentrations (taken on X-axis) and their corresponding absorbance (taken on Y-axis) were generated.

3.5.2. Validation of UV Spectrophotometric methods for VPN

3.5.2.1. Linearity and range

The ability of any analytical method to obtain test results proportional to the concentration of analyte in the sample, directly or after well-defined transformation, is termed as linearity. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which linearity has been demonstrated with a suitable level of precision and accuracy (13). MS office excel software v2007 (Microsoft corporation) was used to apply linear regression on standard calibration data and the straight line equations as well as correlation coefficients (R^2) were generated to validate linearity.

3.5.2.2. Robustness

Robustness is typically assessed by the effect of small changes in analytical methods on system suitability parameters to ensure that the validity of the analytical procedure is maintained whenever used (14).

The robustness was validated by observing the change in the absorbance of VPN standard solutions measured at analytical wavelength immediately after preparation and after 24 h of storage at room temperature.

3.5.2.3. Sensitivity

To ascertain the smallest concentration of an analyte that can be reliably measured by an analytical method, limit of detection (LOD) and limit of quantification (LOQ) are frequently utilized. The LOD and LOQ of developed methods were determined from standard deviation of the response and the slope using **Eq. 3-1** and **Eq. 3-2** (15),

$$LOD = 3 \times \left(\frac{R}{S} \right) \qquad \textbf{Eq. 3-1}; \qquad LOQ = 10 \times \left(\frac{R}{S} \right) \qquad \textbf{Eq. 3-2}$$

Where, S= Slope of the linearity curve

R= Standard deviation of line (standard error of Y-predicted for each x in the regression)

3.5.2.4. Precision/ Repeatability

Precision represents the consistency and reproducibility of an analytical method and expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample (15). Multiple measurements for 10 μg/ml standard solutions were made on same day as well as on three consecutive days to determine intraday and interday precision, respectively. The precision of methods was reported in terms of percent relative standard deviation (% RSD).

3.5.2.5. Accuracy

The accuracy represents the closeness of agreement between the test results obtained by an analytical method to the accepted reference value (16). The standard addition method was used to determine accuracy where known amounts of standard drug (80, 100 and 120 %) were added to the pre-analyzed samples and the absorbance were measured. Accuracy was then assessed in terms of mean % recovery using **Eq. 3-3**.

$$\% \text{ recovery} = \left[\frac{(C_T - C_S)}{C_A}\right] \times 100 \qquad \text{Eq. 3-3}$$

Where, C_T = total drug concentration measured after standard addition;

C_S = drug concentration measured before standard addition;

C_A = theoretical increase in drug concentration by standard addition

3.5.2.6. Specificity

Specificity is the ability to measure the analyte for the presence of various components *viz.,* impurities, degradants, matrix, etc. which may be present (14). Here, the ability of the methods to accurately measure VPN in formulations was assessed via evaluation of interference by excipients (PLGA, Pol-188, P-90G, P-90H, SDC, PVA and PVP) using formulation prototype method (15). The formulation prototypes were prepared by spiking standard 10 µg/ml VPN solutions with other formulation components at their maximum fraction which were supposed to be present in analytical samples as given in Table 3-2.

Table 3-2. Formulation prototypes for determination of specificity of analytical methods

Ultradeformable liposomes	
Formulation components	**Concentration (µg/ml)**
P90G	500
P90H	250
SDC	40
PVA	4500
PVP	1500

Polymeric Nanoparticles	
Formulation components	**Concentration (µg/ml)**
PLGA	400
Pol-188	250
PVA	4500
PVP	1500

The absorption spectrum of these formulation prototypes were compared with that of standard VPN solutions in respective solvents and excipients' mix which were prepared using similar portions of all the excipients and omitting the drug.

3.5.3. VPN estimation by HPLC method

For estimation of VPN in drug release study samples and in rat plasma, an earlier reported RP-HPLC method (17) was used with slight modifications as described below.

3.5.3.1. Preparation of Standard Stock Solution of VPN

Standard stock solution containing 50 μg/mL VPN was prepared in acetonitrile. Briefly, accurately weighed 10 mg of VPN was transferred to 10 ml calibrated volumetric flasks and dissolved in acetonitrile. The volume was made up to 10 ml with the same solvent to get standard VPN solution of 1000 μg/mL. Further, 0.5 ml of this solution was diluted up to 10 ml in a separate calibrated volumetric flask to prepare standard VPN solution of 50 μg/mL.

3.5.3.2. Preparation of Standard VPN Solutions

Two different methods were developed to prepare standard solutions of VPN.

Method 1: 0.01 ml to 2 ml aliquots of VPN standard stock solutions were transferred to 10 ml calibrated volumetric flasks and diluted up to the mark with acetonitrile to achieve standard VPN solutions of 0.05, 0.10, 0.50, 1.0, 5.0 and 10 μg/mL concentrations. These standard VPN solutions were used to develop calibration curve for estimation of VPN in various drug release/permeation study samples.

Method 2 (Bioanalytical method for Standard VPN Solutions in Plasma): Aliquot ranging from 0.1 mL to 5.0 mL of standard stock solution of VPN was transferred to separate 100 mL volumetric flasks and diluted up to the mark with acetonitrile to produce working stock solutions having VPN concentrations of 0.05, 0.125, 0.25, 0.5, 1.0 and 2.5 μg/mL. From each working stock solutions of VPN, 0.1 mL aliquot was transferred in separate 5 ml screw capped tubes and 4.9 mL of human plasma was added in each tube to yield VPN concentrations of 0.001, 0.0025, 0.005, 0.01, 0.02 and 0.05 μg/mL. Protein precipitation method was utilized for VPN extraction from plasma. Briefly, 0.30 ml of each plasma sample was taken in separate ria vials. 2 ml of chilled Acetonitrile (at -20 °C) was added to all samples and vortexed for 5 min. The samples were centrifuged at 1700 g at 4 °C for 15 min. Supernatant layer was carefully transferred to ria vials and evaporated to dryness at 40 °C under the gentle stream of nitrogen. The dried residues were further reconstituted

with 30 µl of mobile phase and vortexed for 30 seconds to get the final VPN concentrations of 0.01, 0.025, 0.05, 0.10, 0.20 and 0.50 µg/ml. These standard VPN solutions were used to develop calibration curve for estimation of VPN in plasma samples collected during in vivo pharmacokinetic study.

3.5.3.3. Preparation of Calibration Curve

Isocratic LC-20AT/SPD-20A HPLC system (Shimadzu, Japan) with Supelco® C18 column (Sigma-Aldrich, India) was used for HPLC analysis of VPN. The resulting standard solutions of VPN were injected through Rheodyne® injectors with 20 µL sample loop. A mixture of ammonium acetate buffer (1.54%) and acetonitrile [ratio, 40:60 v/v] was used as mobile phase at a flow rate of 1 ml/minute at room temperature. The chromatograms were recorded at 280 nm detection wavelength for a run time of 25 minutes. The calibration curves between standard VPN concentrations (taken on X-axis) and their corresponding peak area (taken on Y-axis) were generated. MS office excel software v2007 (Microsoft corporation) was used to obtain the equation of best fit straight line and correlation coefficient.

3.5.4. Validation of HPLC method for VPN

The HPLC method for VPN was validated for linearity, robustness, sensitivity, precision and accuracy in a similar way as described in section 3.5.2.

3.5.5. NPT estimation by HPLC methods

For estimation of NPT in all analytical samples, an earlier reported RP-HPLC method (10) was used with slight modifications as described below

3.5.5.1. Preparation of Standard Stock Solutions of NPT

Standard stock solution containing 50 µg/mL NPT was prepared in acetonitrile. Briefly, 10 mg of NPT was accurately weighed, transferred to 10 ml calibrated volumetric flasks and dissolved in acetonitrile. The volume was made up to 10 ml with the same solvent to get standard NPT solution of 1000 µg/mL. Further, 0.5 ml of this solution was diluted up

to 10 ml in a separate calibrated volumetric flask to prepare standard NPT solution of 50 μg/mL.

3.5.5.2. Preparation of Standard NPT Solutions

Two different methods were developed to prepare standard solutions of NPT.

Method 1: 0.01 ml to 2 ml aliquots of NPT standard stock solutions were transferred to 10 ml calibrated volumetric flasks and diluted up to the mark with acetonitrile to achieve standard NPT solutions of 0.05, 0.10, 0.50, 1.0, 5.0 and 10 μg/mL concentrations. These standard NPT solutions were used to develop calibration curve for estimation of NPT in various *in vitro* as well as *ex vivo* samples.

Method 2 (Bioanalytical method for Standard NPT Solutions in Plasma): Aliquot ranging from of 0.25 mL to 20.0 mL of standard stock solution of NPT was transferred to separate 100 mL volumetric flasks and diluted up to the mark with Acetonitrile to produce working stock solutions having NPT concentrations of 0.125, 0.25, 0.50, 2.5, 5.0 and 10.0 μg/mL. Further, from each working stock solutions of NPT, 0.1 mL aliquot was transferred in separate 5 ml screw capped tubes and 4.9 mL of human plasma was added in each tube to yield NPT concentrations of 0.0025, 0.005, 0.01, 0.05, 0.10 and 0.20 μg/mL. Protein precipitation method was utilized for NPT extraction from plasma. Briefly, 0.30 ml of each plasma sample was taken in separate ria vials. 2 ml of chilled Acetonitrile (at -20°C) was added to all samples and vortexed for 5 min. The samples were centrifuged at 1700g at 4°C for 15 min. Supernatant layer was carefully transferred to ria vials and evaporated to dryness at 40°C under the gentle stream of nitrogen. The dried residues were further reconstituted with 30 μl of mobile phase and vortexed for 30 seconds to get the final NPT concentrations of 0.025, 0.05, 0.10, 0.50, 1.0 and 2.0 μg/ml. These standard NPT solutions were used to develop calibration curve for estimation of NPT in plasma samples collected during *in vivo* pharmacokinetic study.

3.5.5.3. Preparation of Calibration Curves

Isocratic LC-20AT/SPD-20A HPLC system (Shimadzu, Japan) with Supelco® C18 column (Sigma-Aldrich, India) was used for HPLC analysis of NPT. The resulting standard solutions of NPT were injected through Rheodyne® injectors with 20 µL sample loop. A mixture of acetonitrile, filtered double distilled water and glacial acetic acid in a ratio of 500:500:1 v/v was used as mobile phase at a flow rate of 0.5 ml/minute at room temperature. The chromatograms were recorded at 258 nm detection wavelength for a run time of 15 minutes. The calibration curves between standard NPT concentrations (taken on X-axis) and their corresponding peak area (taken on Y-axis) were generated. MS office excel software v2007 (Microsoft corporation) was used to obtain the equation of best fit straight line and correlation coefficient.

3.5.6. Validation of HPLC methods for NPT

The HPLC methods for NPT were validated for linearity, robustness, sensitivity, precision and accuracy in a similar way as described in section 3.5.2.

3.6. RESULTS & DISCUSSION

The observations and results obtained have been discussed below.

3.6.1. VPN estimation by UV spectrophotometry

The UV spectrum scans of 10 µg/ml standard solutions of VPN in A2M8 and ACN have been shown in **Fig. 3-1**. Three absorption maxima were observed at different wavelengths in the UV spectra of VPN in both solvents. The result reflected the inherent property of VPN where multiple electron transitions (from the ground state to the excited state of the molecule) with different energies, and therefore, different wavelengths are allowed. Fairly strong peaks with sufficient distance from measurement limits and with better peak shape were found at 273.8 and 274.6 wavelengths in A2M8 and ACN, respectively and thus selected as analytical wavelengths for respective solvents. The calibration data and calibration curves of VPN in selected solvents are given in **Table 3-3** and **Fig. 3-2**, respectively.

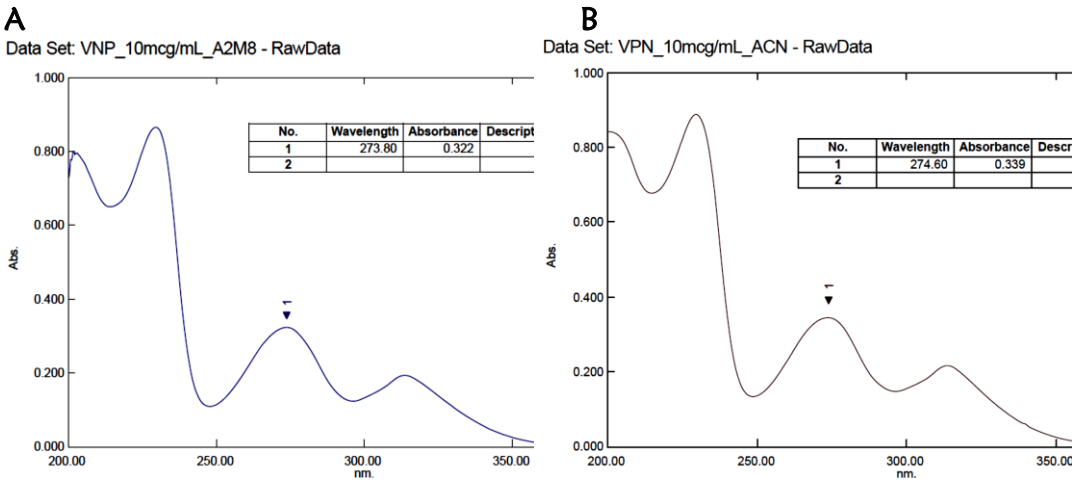


Fig. 3-1. UV Absorption Spectra of VPN in (A) A2M8 and (B) ACN

3.6.2. Validation of UV spectrophotometric methods for VPN

The regression analysis showed a positive correlation between concentration of VPN and absorbance values at respective λ_{max} with a good linearity ($R^2 \geq 0.999$, in both solvents). These results reflected that Beer's law was obeyed for selected VPN concentration range of 5 to 25 *µg*/ml in both solvents. Measurement of same standard solutions after storage for 24 hours at room temperature did not show any significant change in the absorbance values (**Table 3-3, Fig. 3-2**) indicating the stability of VPN in both solvents over the period of analysis.

Table 3-3. Absorbance data of VPN in A2M8 and ACN at 0 and 24 h for calibration and stability

	Concentration (µg/ml)	Absorbance[#]	
		In: A2M8 λ_{max}: 273.8 nm	In: ACN λ_{max}: 274.6 nm
At 0 h (initial)	0	0	0
	5	0.151 ± 0.004	0.170 ± 0.002
	10	0.322 ± 0.001	0.333 ± 0.005
	15	0.515 ± 0.006	0.508 ± 0.008
	20	0.675 ± 0.012	0.672 ± 0.005
	25	0.858 ± 0.006	0.844 ± 0.007
After 24 h	0	0	0
	5	0.150 ± 0.005	0.168 ± 0.005
	10	0.327 ± 0.009	0.329 ± 0.009
	15	0.519 ± 0.005	0.507 ± 0.012
	20	0.676 ± 0.004	0.668 ± 0.011
	25	0.863 ± 0.008	0.839 ± 0.009

[#] Mean ± SD (n=3)

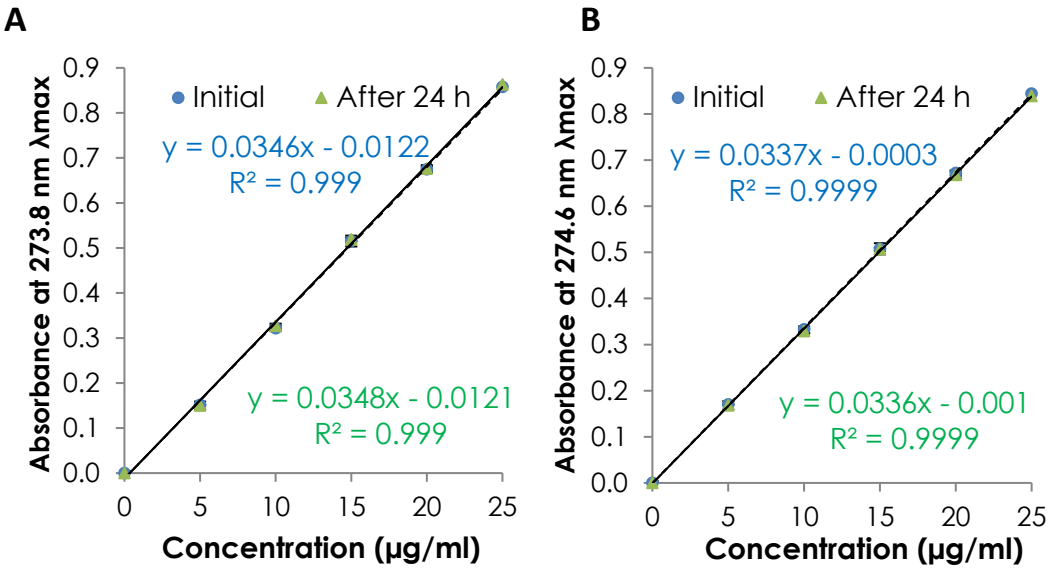


Fig. 3-2. Calibration curves of VPN at 0 h and after 24 h in (A) A2M8 and (B) ACN

Table 3-4 summarizes the limit of detection and the limit of quantification values of UV spectrophotometric methods for VPN in both solvents. The LOD and LOQ values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate quantification of drug present in their standard solutions.

Table 3-4. Sensitivity evaluation of UV methods of VPN in A2M8 and ACN

Solvents	Slope of line	SD of line	LOD (µg/ml)	LOQ (µg/ml)
A2M8	0.035	0.008	0.688	2.294
ACN	0.034	0.003	0.286	0.953

Intraday and interday precision data under the same operating conditions are summarized in **Table 3-5**. The results were found to be precise over the selected time interval as the % RSD values obtained for these UV spectrophotometric methods were within the acceptable range (< 2%) (15).

Table 3-5. Intraday and interday precision analysis of UV methods using 10 µg/ml standard VPN solutions

Solvents	Concentration prepared (µg/ml)	Concentration observed					
		Intraday precision			Interday precision		
		µg/ml	mean	% RSD	µg/ml	mean	% RSD
A2M8	10	9.96	9.98	1.27	9.87	9.97	1.40
		9.87			9.91		
		10.12			10.13		
ACN	10	9.91	9.93	0.25	9.96	10.02	0.49
		9.96			10.05		
		9.93			10.04		

The mean % recovery and % RSD values for lower, intermediate and higher concentration are summarized in **Table 3-6** for both solvents. The mean % recovery values, close to 100% with low relative standard deviation (% RSD ≤ 1.48%) represented high accuracy of the developed spectrophotometric methods (15).

Table 3-6. Accuracy evaluation of UV methods by standard addition technique using 10 µg/ml standard VPN solutions

Drug spiked (%)	Recovery							
	µg/ml	%	mean	% RSD	µg/ml	%	mean	% RSD
	A2M8				ACN			
80	17.87	99.28	100.39	1.01	18.06	100.33	100.46	0.12
	18.23	101.28			18.10	100.56		
	18.11	100.61			18.09	100.50		
100	20.11	100.55	101.22	0.90	19.94	99.70	100.20	0.44
	20.45	102.25			20.07	100.35		
	20.17	100.85			20.11	100.55		
120	22.41	101.86	100.24	1.48	21.89	99.50	100.11	0.59
	21.98	99.91			22.03	100.14		
	21.77	98.95			22.15	100.68		

The absorption spectra of the standard VPN solution in A2M8 and ACN were compared with that obtained for formulation prototype (Drug + Excipients) and excipient mixture (without drug) in same solvents.

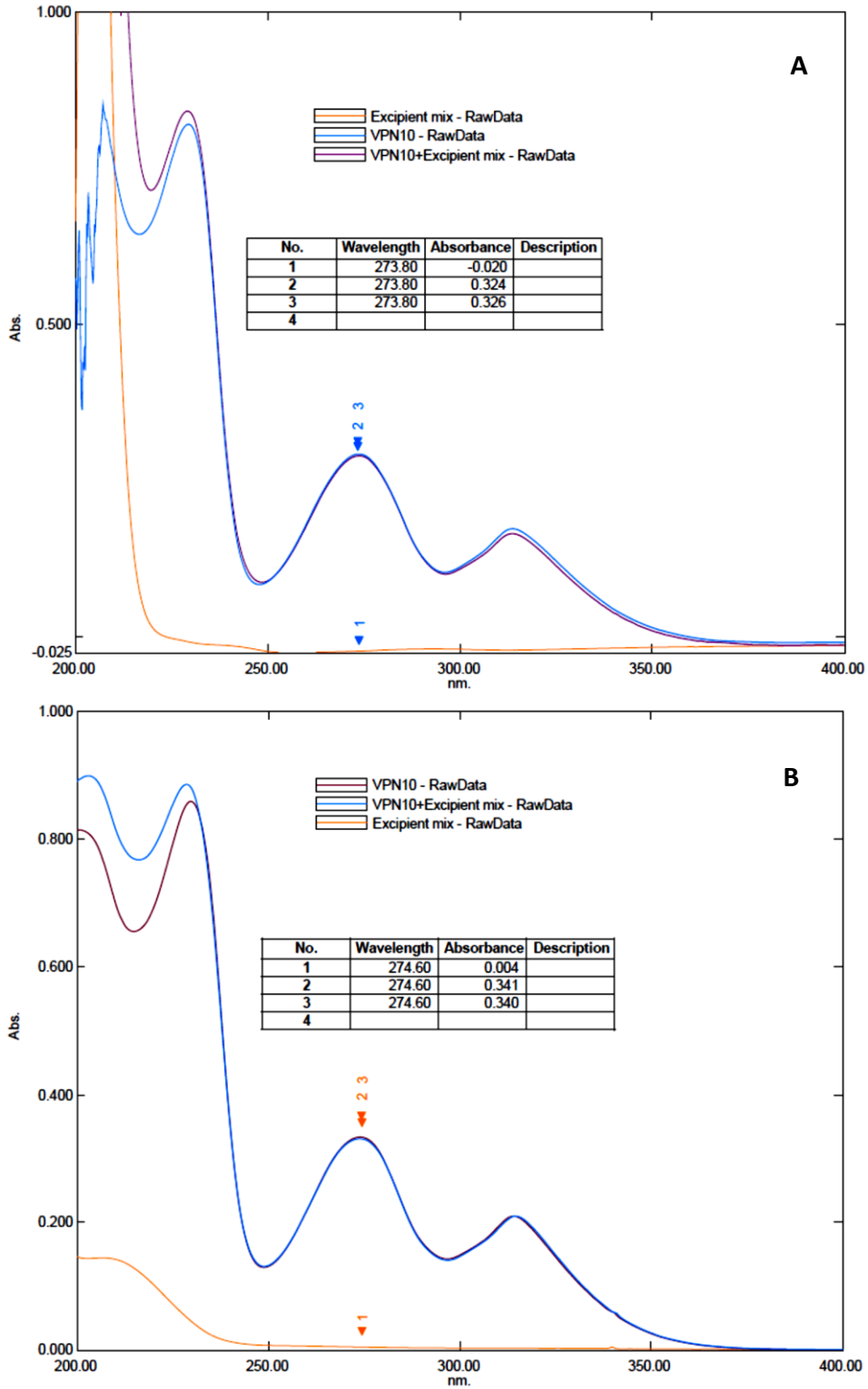


Fig. 3-3. Absorption spectra to demonstrate specificity of analytical methods for (A) ultradeformable liposomes and (B) polymeric nanoparticles formulations

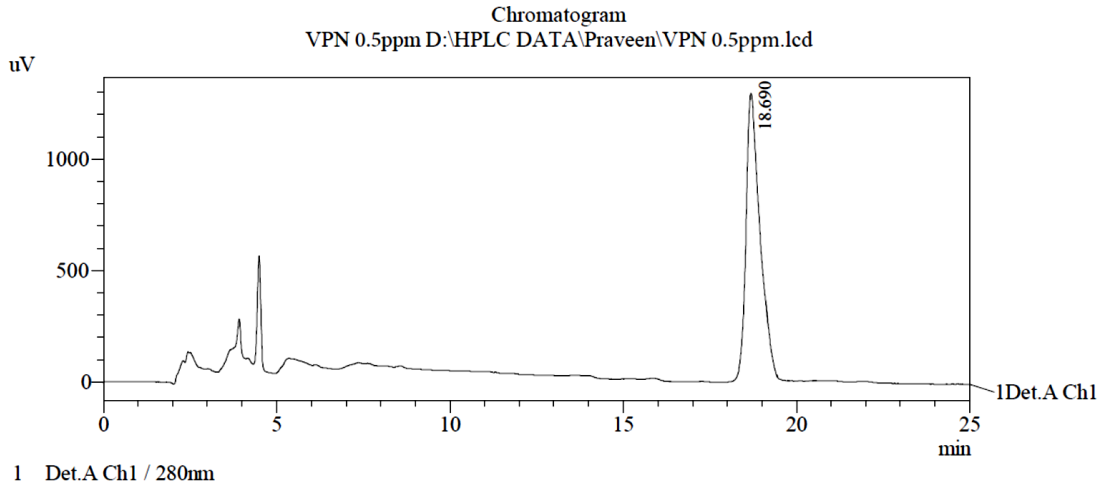
As shown in **Fig. 3-3**, no change in position or intensity of the drug's peak was observed in formulation prototypes of ultradeformable liposomes and polymeric nanoparticles as compared to standard VPN solutions indicating the absence of any interference by formulation

components at analytical wavelength. The absence of any overlapping or extraneous peaks in excipient mixtures at analytical wavelengths further suggested the specificity of the methods.

3.6.3. VPN estimation by HPLC method

Typical chromatograms obtained from RP-HPLC analysis using hydrophobic C18 column is given in **Fig. 3-4**. Sharp, symmetric peaks (average tailing factor < 0.95) were observed with average retention time of 18.5 min at 280 nm detection wavelength and 1 ml/min flow rate.

A



B

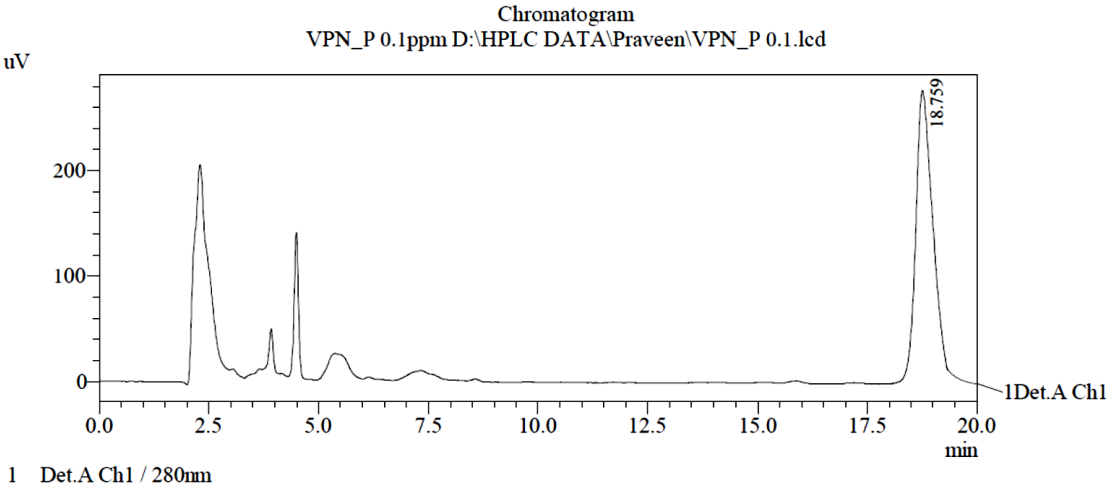


Fig. 3-4. Typical HPLC chromatograms of (A) 0.5 µg/mL VPN by method-1 and (B) 0.1 µg/mL VPN by method-2

The peak area values corresponding to selected concentration range of VPN for both the methods are given in **Table 3-7** and a calibration plots for the same are illustrated in **Fig. 3-5**.

3.6.4. Validation of HPLC method for VPN

The regression analysis of calibration data showed a positive correlation between concentration of VPN and peak area values with a good linearity (R^2 =1). The result reflected that Beer's law was obeyed for the selected VPN concentration range of 0.05 to 10 μg/mL by method-1 and 0.01 to 0.5 μg/mL by method-2.

Table 3-7. Peak area data of VPN at 0 and 24 h for calibration and stability

Concentration (μg/mL)	Peak area (μV*S)#			
	Method-1		Method-2	
	Initial	After 24 h	Initial	After 24 h
0.01	-	-	1244±32	1236±23
0.025	-	-	3107±28	3103±42
0.05	6221±50	6180±67	6159±46	6158±51
0.1	12429±61	12412±73	12415±45	12411±47
0.2	-	-	25014±29	25009±77
0.5	61594±79	61577±74	62846±82	62841±94
1.0	124148±91	124107±115	-	-
5.0	625342±129	625233±133	-	-
10.0	1256927±246	1256814±325	-	-

#Mean ± SD (n=3)

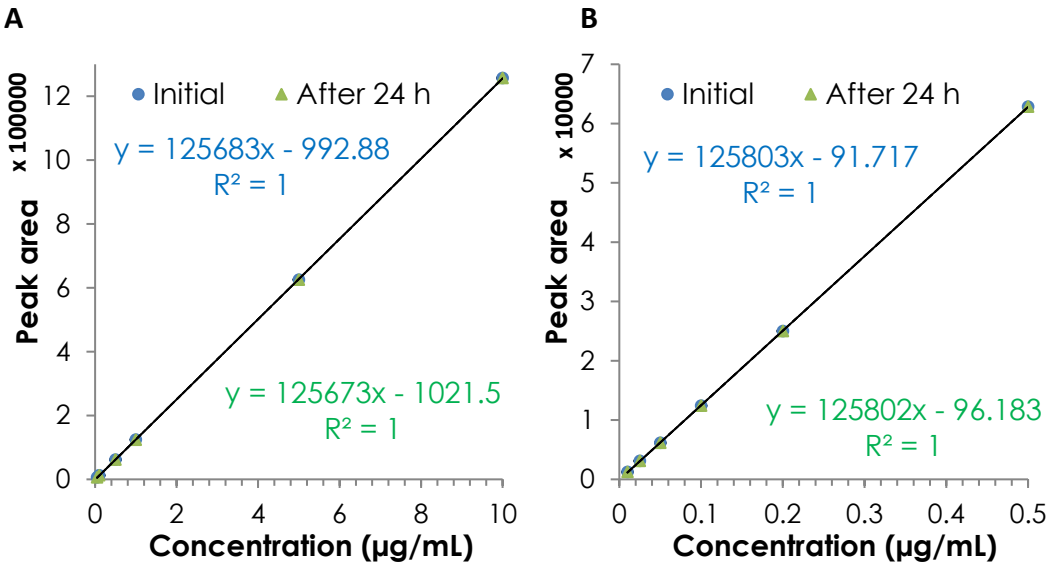


Fig. 3-5. Calibration curves of VPN at 0 h and after 24 h by HPLC (A) method-1 and (B) method-2

Measurement of same standard VPN solutions after storage for 24 hours at room temperature did not show any significant change in the peak area values (**Table 3-7**, **Fig. 3-5**) indicating the stability of standard VPN solutions over the period of analysis.

Table 3-8 summarizes the limit of detection and the limit of quantification values of HPLC methods for VPN estimation in analytical samples. The LOD values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate detection of VPN present in standard solutions.

Table 3-8. Sensitivity evaluation of HPLC method of VPN

Method	Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
1	125683	1366	0.033	0.109
2	125803	71.3	0.002	0.006

Intraday and interday precision data under the same operating conditions are summarized in **Table 3-9**. The results were found to be precise over the selected time interval as the % RSD values obtained for the HPLC methods were within the acceptable range (< 2%).

Table 3-9. Intraday and interday precision analysis of HPLC methods of VPN

	Concentration prepared (µg/mL)	Concentration observed					
		Intraday precision			Interday precision		
		µg/mL	mean	% RSD	µg/mL	mean	% RSD
Method-1	0.5	0.504	0.501	0.66	0.500	0.501	0.95
		0.498			0.501		
		0.500			0.496		
Method-2	0.1	0.100	0.101	1.30	0.101	0.100	1.44
		0.101			0.099		
		0.102			0.101		

The mean % recovery and % RSD values for lower, intermediate and higher concentrations are summarized in **Table 3-10**. The mean % recovery values, close to 100% with low relative standard deviation (% RSD < 2 %) represented high accuracy of the developed HPLC method.

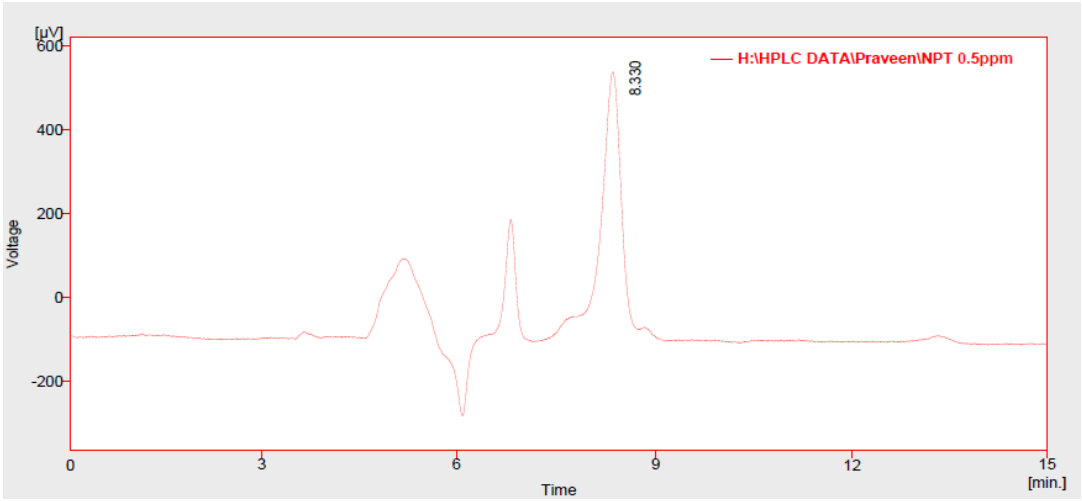
Table 3-10. Accuracy evaluation of HPLC methods of VPN by standard addition technique

Drug spiked (%)		Initial concentration (µg/mL)	Recovery			
			µg/mL	%	mean	% RSD
Method-1	80	0.5	0.897	99.67	99.48	0.20
			0.896	99.50		
			0.894	99.28		
	100	0.5	1.004	100.40	100.30	0.36
			1.006	100.60		
			0.999	99.90		
	120	0.5	1.107	100.64	100.00	0.58
			1.099	99.86		
			1.095	99.50		
Method-2	80	0.1	0.181	100.44	99.93	0.50
			0.179	99.44		
			0.180	99.89		
	100	0.1	0.201	100.60	100.40	0.91
			0.202	101.20		
			0.199	99.40		
	120	0.1	0.222	100.91	99.76	1.02
			0.219	99.36		
			0.218	99.00		

3.6.5. NPT estimation by HPLC methods

Typical chromatograms obtained from RP-HPLC analysis of NPT using hydrophobic C18 column is given in **Fig. 3-6**. Sharp, symmetric peaks (average tailing factor < 0.84) were observed with average retention time of 8.3 min at 258 nm detection wavelength and 0.5 ml/min flow rate.

A



B

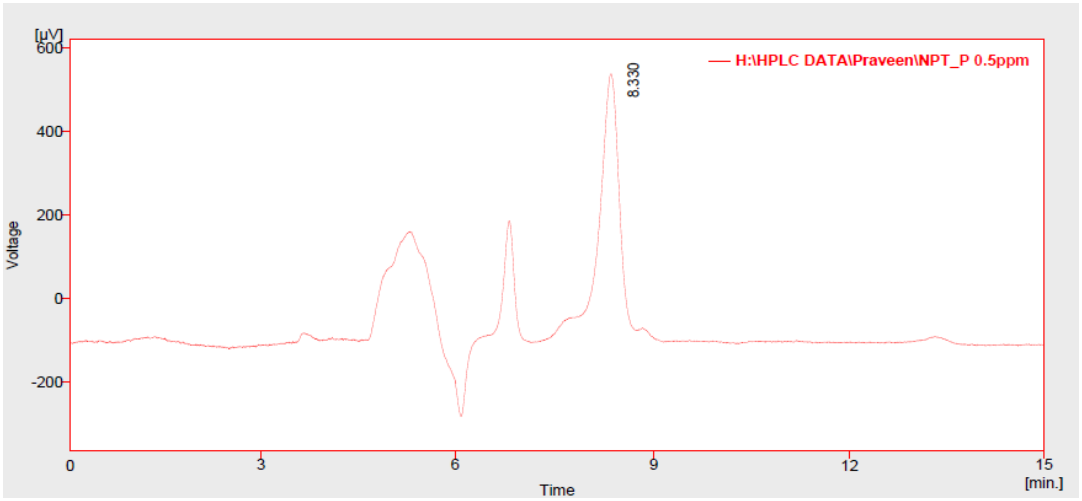


Fig. 3-6. Typical HPLC chromatograms of 0.5 µg/mL NPT by (A) method-1 and (B) method-2

The peak area values corresponding to selected concentration range of NPT for both the methods are given in **Table 3-11** and the calibration plots for the same are illustrated in **Fig. 3-7**.

3.6.6. Validation of HPLC methods for NPT

The regression analysis of calibration data showed a positive correlation between concentration of NPT and peak area values with a good linearity (R^2 =1). The result reflected that Beer's law was obeyed for the selected NPT concentration range of 0.05 to 10 μg/mL by method-1 and 0.025 to 2 μg/mL by method-2.

Table 3-11. Peak area data of NPT at 0 and 24 h for calibration and stability

Concentration (µg/mL)	Peak area (µV*S)#			
	Method-1		Method-2	
	Initial	After 24 h	Initial	After 24 h
0.025	-	-	911 ± 31	904 ± 24
0.05	1891 ± 50	1888 ± 67	1954 ± 47	1949 ± 53
0.1	4022 ± 61	4015 ± 73	4007 ± 30	3997 ± 49
0.5	20572 ± 79	20538 ± 74	20187 ± 61	20154 ± 72
1.0	41620± 91	41590 ± 115	40590 ± 86	40534 ± 95
2.0	-	-	81469 ± 74	81368 ± 88
5.0	208644 ± 129	208380 ± 133	-	-
10.0	418760 ± 246	418580 ± 325	-	-

#Mean ± SD (n=3)

Measurement of same standard NPT solutions after storage for 24 hours at room temperature did not show any significant change in the peak area values (**Table 3-11**, **Fig. 3-7**) indicating the stability of standard NPT solutions over the period of analysis.

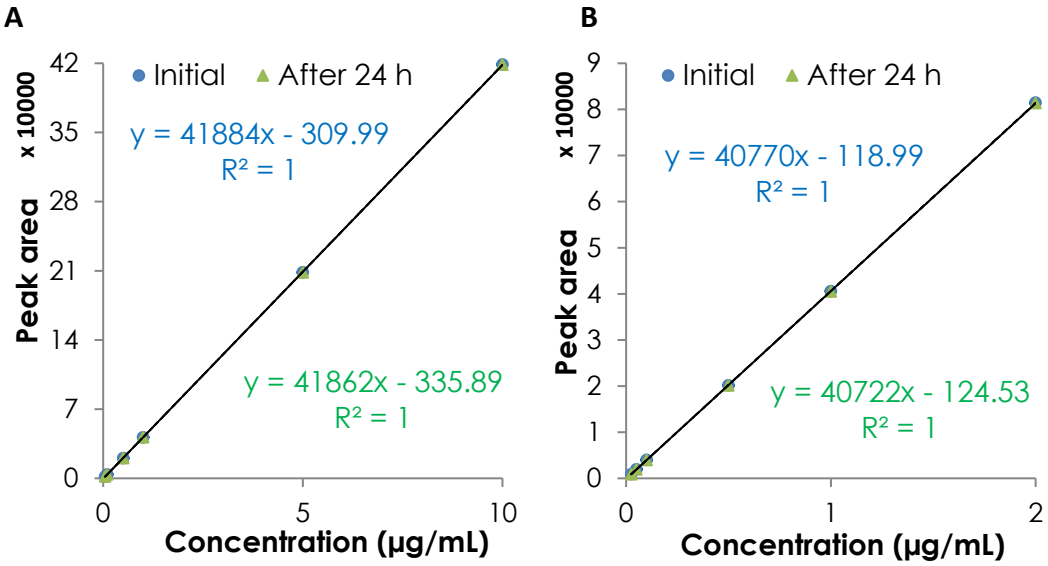


Fig. 3-7. Calibration curves of NPT at 0 h and after 24 h by HPLC (A) method-1 and (B) method-2

Table 3-12 summarizes the limit of detection and the limit of quantification values of HPLC methods for NPT estimation in analytical samples. The LOD values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate detection of NPT present in standard solutions.

Table 3-12. Sensitivity evaluation of HPLC methods of NPT

Method	Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
1	20942	138.7	0.02	0.066
2	40770	63.5	0.005	0.016

Intraday and interday precision data under the same operating conditions are summarized in **Table 3-13**. The results were found to be precise over the selected time interval as the % RSD values obtained for the HPLC methods were within the acceptable range (< 2%).

Table 3-13. Intraday and interday precision analysis of HPLC methods of NPT

	Concentration prepared (µg/mL)	Concentration observed					
		Intraday precision			Interday precision		
		µg/mL	mean	% RSD	µg/mL	mean	% RSD
Method-1	0.5	0.501	0.500	0.38	0.502	0.501	0.47
		0.502			0.498		
		0.498			0.503		
Method-2	0.5	0.506	0.499	1.10	0.498	0.501	0.66
		0.497			0.504		
		0.495			0.502		

The mean % recovery and % RSD values for lower, intermediate and higher concentrations are summarized in **Table 3-14**. The mean % recovery values, close to 100% with low relative standard deviations (% RSD < 2 %) represented high accuracy of the developed HPLC methods.

Table 3-14. Accuracy evaluation of HPLC methods of NPT by standard addition technique

Drug spiked (%)		Initial concentration (µg/mL)	Recovery			
			µg/mL	%	mean	% RSD
Method-1	80	0.5	0.904	100.39	100.00	0.54
			0.895	99.39		
			0.902	100.22		
	100	0.5	1.004	100.40	100.23	0.47
			1.006	100.60		
			0.997	99.70		
	120	0.5	1.099	99.91	100.03	0.47
			1.106	100.55		
			1.096	99.64		
Method-2	80	0.5	0.908	100.83	100.52	0.33
			0.905	100.56		
			0.902	100.17		
	100	0.5	1.006	100.60	100.47	0.19
			1.003	100.25		
			1.006	100.55		
	120	0.5	1.101	100.09	100.05	0.61
			1.094	99.41		
			1.107	100.64		

3.7. CONCLUSION

UV spectrophotometric as well as HPLC methods for quantification of VPN and NPT in various *in vitro*, *ex vivo* and *in vivo* experimental samples were successfully developed. Validation revealed that all the methods were linear, robust, sensitive, precise, accurate and specific.

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