4.

ANALYTICAL TECHNIQUES

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LIPID BASED DRUG DELIVERY SYSTEM

4.1 Materials

Iloperidone (ILO) and Vardenafil HCL Trihydrate (VDN) were received as gift sample from Alembic Pharmaceuticals Ltd. (Vadodara, India). Dopamine was purchased from Sigma-Aldrich, India.

Acetonitrile (LC/MS grade), Methanol (LC/MS grade) and Formic Acid (LC/MS grade) were purchased from Fluka™, Germany. Acetonitrile (HPLC grade), Methanol (HPLC grade) and Chloroform (HPLC grade) were purchased from Spectrochem Labs Ltd., India. Hydrochloric acid (AR grade) was purchased from SR Enterprise, India. Potassium Dihydrogen Phosphate (GR grade), Triethylamine (GR grade), Orthophosphoric acid (GR grade) and Potassium Hydroxide (AR grade) were purchased from S.D. Fine Chemicals, India. Dimethyl Formamide 9(GC headspace grade) was purchased from Fisher Chemicals, USA. 25 mm - 0.45 µ PTFE syringe filter and 25 mm - 0.45 µ Nylon syringe filter were purchased from Himedia, India. 47 mm - 0.45µ, Nylon membrane filter was purchased from Millipore, India. Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.45 µm pore size (Pall Life sciences, Mumbai, India). Oasis HLB® cartridge was received as gift sample from Waters (India). Human plasma was procured from Indu Blood Bank, Vadodara, India.

4.2 Analytical Methods

Different analytical techniques were used for quantification purpose.

A. For drugs → ILO and VDN

- UV-Visible Spectroscopy (UV)
- High Performance Liquid Chromatography (HPLC)
- Ultra-Performance Liquid Chromatography – Mass Spectrometer (UPLC-MS)

B. For residual solvent in formulation → methanol and chloroform

- Headspace - Gas Chromatography (HS-GC)

4.3 Analytical Techniques for Iloperidone

4.3.1 Estimation of ILO by UV-visible Spectroscopy [1]

A. Calibration curve in pH 6.8 phosphate buffer

- Standard stock solution preparation:
  Accurately weighed 10 mg ILO was dissolved in 1 mL of methanol and the volume was made up with pH 6.8 phosphate buffer to make final concentration of 100 µg/mL.
- Method for calibration curve:
  Aliquots from the ILO stock solution were taken in 10 mL volumetric flask and volume was made up with pH 6.8 phosphate buffer to get a range from 2 µg/mL to 30 µg/mL. The absorbance of the prepared solution was measured against blank (pH 6.8 phosphate buffer) at its absorbance maxima, 275 nm.
- Using least square mathematical regression analysis, line of best fit was obtained. Correlation coefficient value (R^2) was calculated to find the closeness of the data to the fitted line.

Table 4. 1 calibration data for ILO by UV in pH 6.8 phosphate buffer

Concentration (µg/mL)	Absorbance	%RSD
2	0.110 ± 0.004	0.375
4	0.242 ± 0.006	0.233
6	0.314 ± 0.014	0.436
8	0.443 ± 0.007	0.160
10	0.518 ± 0.005	0.100
15	0.760 ± 0.003	0.036
20	0.947 ± 0.049	0.517
25	1.227 ± 0.104	0.848
30	1.461 ± 0.086	0.585

Mean SD	Slope	LOD	LOQ
0.011	0.048	0.737	2.233

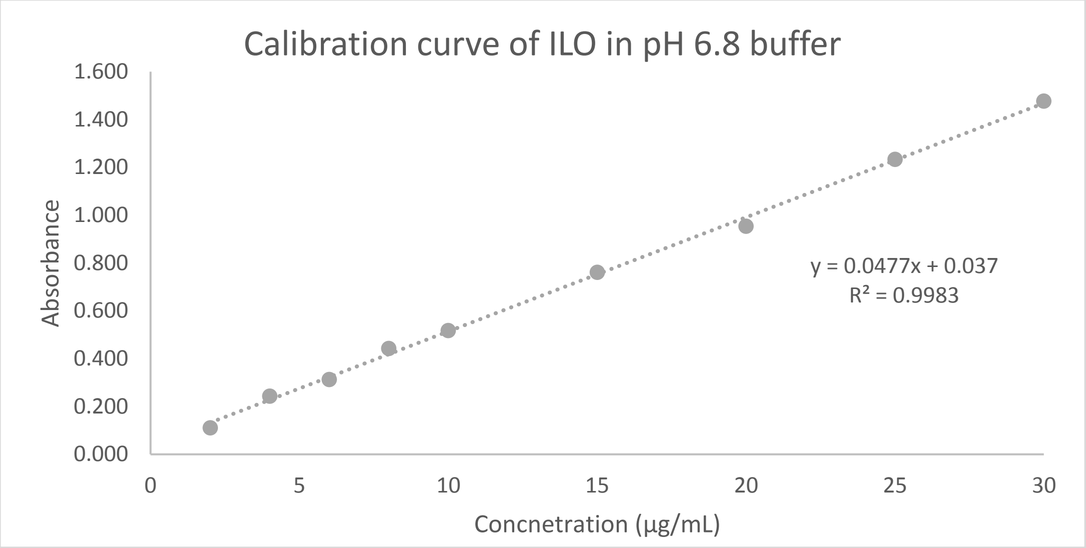

Figure 4.1 Calibration curve of ILO in pH 6.8 phosphate buffer

- ILO showed characteristic UV spectra when scanned from 200 to 400 nm. The λmax for ILO was found to be 275 nm. Hence, 275 nm was selected as analytical wavelength. Standard calibration curve was then drawn as shown in figure 4.1 using the range studied as shown in table 4.1. The 0.9983 value of R^2 obtained by linear regression analysis indicated linear relationship between absorbance and concentration of ILO in pH 6.8 phosphate buffer.
- LOD and LOQ were calculated using following formula:
  LOD = 3.3σ/S….. Equation 4.1
  LOQ = 10σ/S …..Equation 4.2
  Where, σ = std. deviation of line and S=slope of line
  LOD and LOQ was found to be 0.737 and 2.233 ppm respectively as shown in table 4.1. These indicated sensitivity of method for accurate quantification of drug in samples.

B. Calibration curve in Methanol [2,3]

- Standard stock solution preparation:
  Accurately weighed 10 mg ILO was dissolved in 10 mL of methanol with frequent shaking and the volume was make up with methanol to make final concentration of 100 µg/mL.

- Method for calibration curve:

  Aliquots from the ILO stock solution were taken in 10 mL volumetric flask and volume was make up with methanol to get a range from 2 µg/mL to 25 µg/mL. The absorbance of the prepared solution was measured against blank (methanol) at its absorbance maxima, 275 nm.

Table 4.2 Calibration data for ILO by UV in pH 6.8 phosphate buffer

Concentration (µg/mL)	Absorbance	%RSD
0	0.000 ± 0.000	0.000
2	0.087 ± 0.002	1.814
4	0.165 ± 0.003	1.889
6	0.225 ± 0.003	1.491
8	0.298 ± 0.004	1.479
10	0.422 ± 0.006	1.462
15	0.605 ± 0.010	1.662
20	0.855 ± 0.014	1.659
25	1.055 ± 0.019	1.803

Mean SD	Slope	LOD	LOQ
0.007	0.043	0.534	1.618

- In methanol also, the λmax for ILO was found to be 275 nm. Hence, 275 nm was selected as analytical wavelength. Standard calibration curve was then drawn as shown in figure 4.2 using the range studied as shown in table 4.2. The 0.9977 value of R^2 obtained by linear regression analysis indicated linear relationship between absorbance and concentration of ILO in pH 6.8 phosphate buffer.
- LOD and LOQ were found to be 0.543 and 1,618 ppm respectively as shown in table 4.2. These indicated sensitivity of method for accurate quantification of drug in samples.

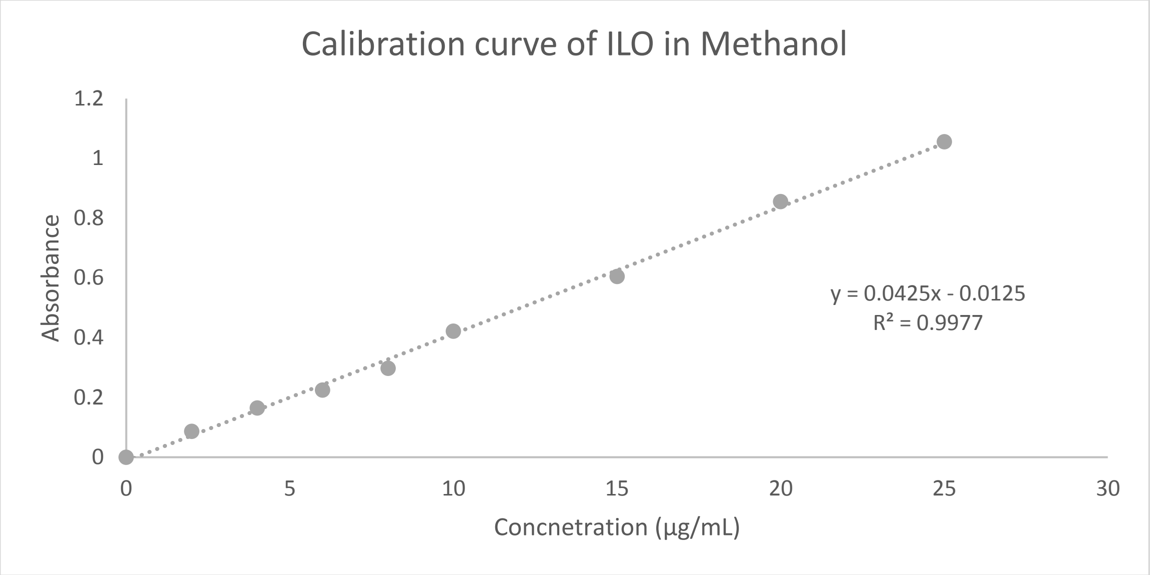

Figure 4.2 Calibration curve of ILO in methanol

4.3.2 Estimation of ILO by HPLC [4]

- Instrument: LC-20AD (Shimadzu, Japan)
- Chromatographic Condition: Column: ACE 5 C18 column, 250*4.6mm, 5 µm (Make: ACE, Part No: ACE-121-2546), Flow rate: 1.0 mL/minute, Injection volume: 20 µL, Run time: 10 minutes, Wavelength: 228 nm, Mode: Isocratic
- Mobile Phase:

  Mobile Phase consisted of aqueous and organic phase in ratio of 70:30.

  Aqueous phase - Buffer (pH 3.0) preparation:

  2.72 g of KH_2PO_4 was dissolved in sufficient quantity of water and diluted up to 1000 mL with water. To it, 1 mL of triethylamine was added. The pH was adjusted to 3.0 ± 0.05 with orthophosphoric acid. The buffer was mixed well and filtered through 0.45µ nylon membrane filter.

  Organic phase:

  Acetonitrile and methanol in ratio of 80:20 v/v was used.
- Standard stock solution preparation:

  Accurately weighed 10 mg ILO was dissolved in 100 mL of methanol to make final concentration of 100 µg/mL.

- Method for calibration curve:

  Aliquots from ILO stock solution were taken in 10 mL volumetric flask and volume was make up with mobile phase to get a range from 1 µg/mL to 50 µg/mL. The area for the range of standard solutions were measured.

Table 4.3 Calibration data for ILO by HPLC

Concentration (µg/mL)	Area (mV.s)	%RSD
0	0.000 ± 0.000	0.000
1	201.93 ± 3.65	1.81
2	232.03 ± 4.41	1.90
3	242.05 ± 2.68	1.11
4	256.32 ± 5.12	2.00
5	417.39 ± 5.96	1.43
10	621.85 ± 8.69	1.40
25	1283.26 ± 11.35	0.88
50	2580.63 ± 9.83	0.38

Mean SD	Slope	LOD	LOQ
5.09	48.72	0.34	1.04

- As shown in figure 4.4, the retention time of ILO was found to be 6.3 minutes. Linearity of HPLC method for estimation of ILO was established by regression analysis. The R^2 value was found to be 0.9932 which reflects linear correlation.
- The LOD and LOQ determination is based on signal/noise (S/N) ratio. It is based on analytical response of 10 and 3 times the background noise respectively. LOD and LOQ were found to be 0.34 and 1.04 ppm respectively. This indicated sensitivity of the method for accurate determination of drug in test sample.

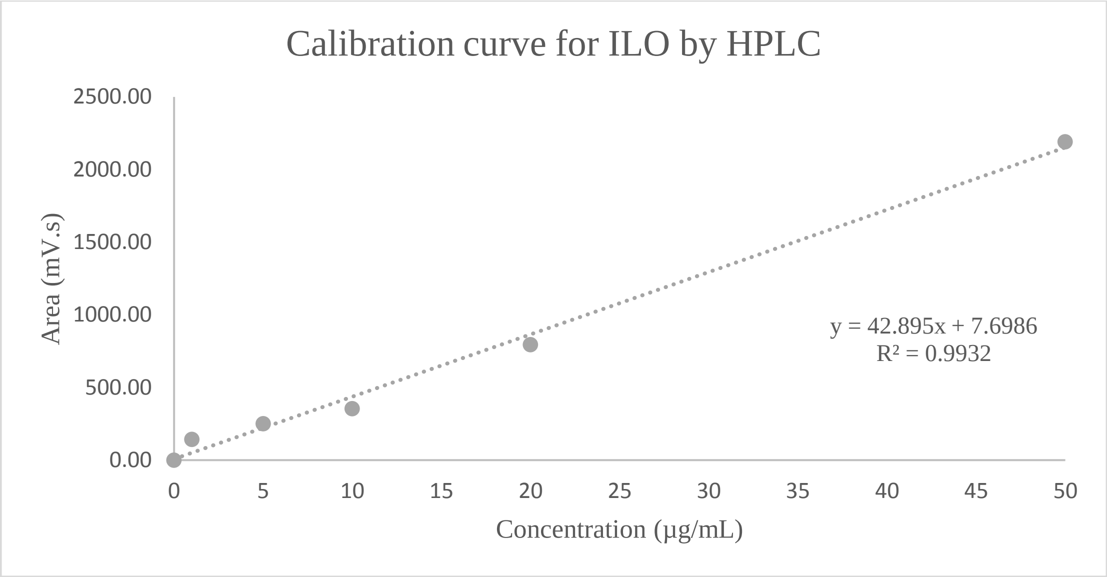

Figure 4.3 Calibration curve for ILO by HPLC

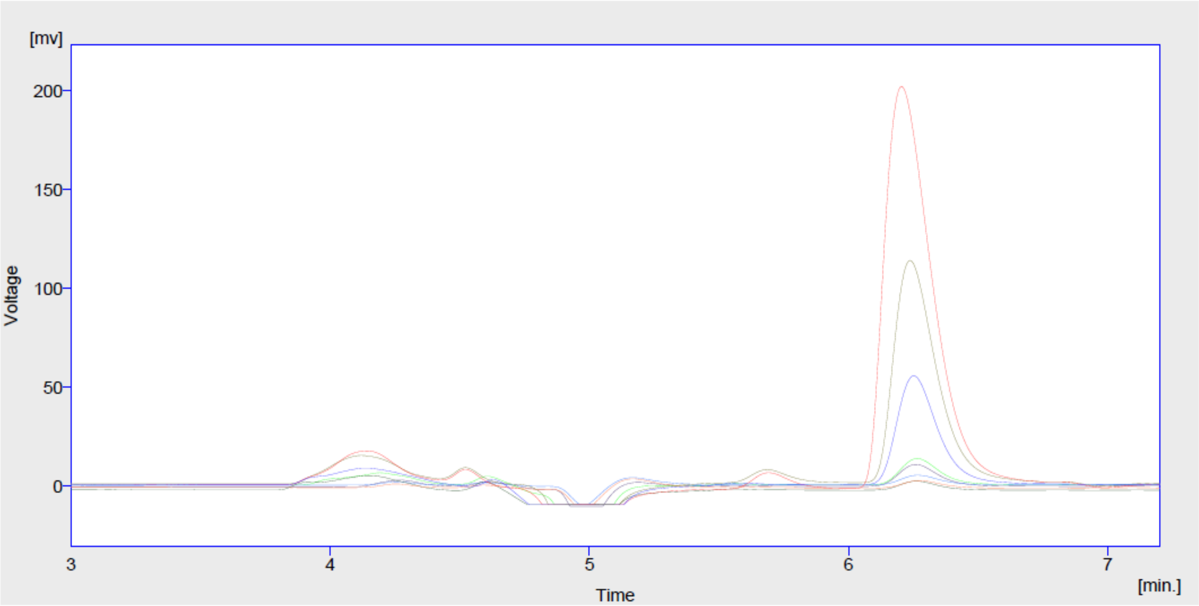

Figure 4.4 Chromatogram of ILO by HPLC

4.3.3 Estimation of ILO by UPLC-MS

UPLC-MS (ABSIEX QTRAP 4500 with ekspert^{TM} ultraLC 100-XL autosampler, make: SCIEX, DHR Holding Pvt. Ltd., India) with electrospray ionization (ESI) was used to quantify the amount of ILO in plasma for pharmacokinetic study.

Sample Preparation by Solid phase Extraction [5]

ILO was extracted from plasma by solid phase extraction (SPE) technique using Oasis HLB Cartridge (WATERS^{TM}). SPE assembly was Ezypress^{TM} HT48 (Orochem Laboratories) attached with the N_2 gas flow.

Step 1: Conditioning

The OASIS HLB™ cartridges (Waters, USA) were conditioned with 2 mL methanol. For this methanol was passed through the cartridges under N_2 environment.

Step 2: Sample pre-treatment:

The plasma (0.1 mL) separated from whole blood component at 3000 rpm at 4 °C was mixed with water (0.1 mL) and 8.3 mM phosphoric acid (0.2 mL). The sample was mixed properly by gentle shaking of the Eppendorf.

Step 3: Sample application:

The plasma sample was then slowly added to pre-conditioned cartridges.

Step 4: Rinsing:

To remove the plasma matrix components, rinsing was carried out using 8.3 mM phosphoric acid solution.

Step 5: Drying:

Under N_2 environment the cartridge was dried to remove any remnant of rinsing solvent. For this N_2 gas was passed through cartridge under pressure.

Step 6: Elution:

The sample (drug bonded in cartridge) was then eluted with methanol. Followed by N_2 passage at high pressure through the cartridge, any remaining amount of drug in methanol was collected in the vial.

Standard Range: For UPLC-MS, standard ILO concentration range was from 0.0005 ng/mL to 5 ng/mL in plasma.

LC condition: The ABSIEX UPLC-MS system contained micro-degasser with high-performance autosampler ekspert^{TM} ultraLC 100-XL. For LC separation, C18 column, 50*2.1 mm, 1.7 µm (Make: Aquity UPLC BEH) was used. Mobile Phase consisted of 35:65 v/v ratio of 1% V/V formic acid: methanol. Injection volume was 5 µL and the flow rate was 0.25 mL/min. Column and auto-sampler were operated at room temperature.

Tandem MS condition: Interface between UPLC and MS was ESI with positive ion mode polarity. The main working parameters were as follow: curtain gas, 25 psi; ion source gas 1, 50 psi; ion source gas 2, 50 psi; source temperature, 350 °C; Declustering potential (DP), 120 V; Collision energy (CE), 36.6 V; Collision cell exit potential (CXP), 18 V. Multiple reaction monitoring (MRM) transition was measured for Q1 → Q3 ion (427.2 m/z → 261.2 m/z). [6]

Table 4.4 Calibration data for ILO by UPLC-MS

Concentration (ng/mL)	Area (cps)	%RSD
5.00E-04	6.81E+02 ± 1.10E+01	1.62
5.00E-03	3.68E+03 ± 3.42E+01	0.93
5.00E-02	3.45E+04 ± 6.18E+01	0.18
5.00E-01	3.97E+05 ± 1.67E+02	0.04
5.00E+00	4.01E+06 ± 2.81E+02	0.01

Mean SD	Slope	LOD	LOQ
1.36E+02	8.02E+05	5.59E-04	1.70E-03

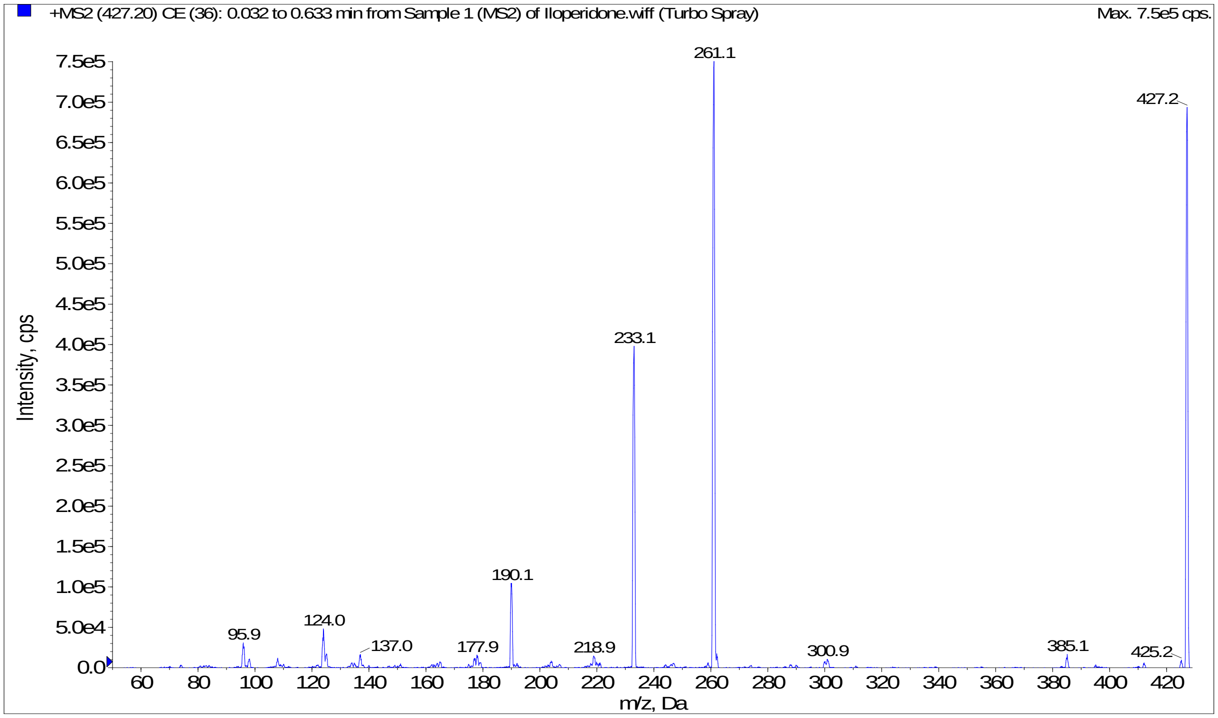

Figure 4.5 MRM transition of ILO for Mass detection

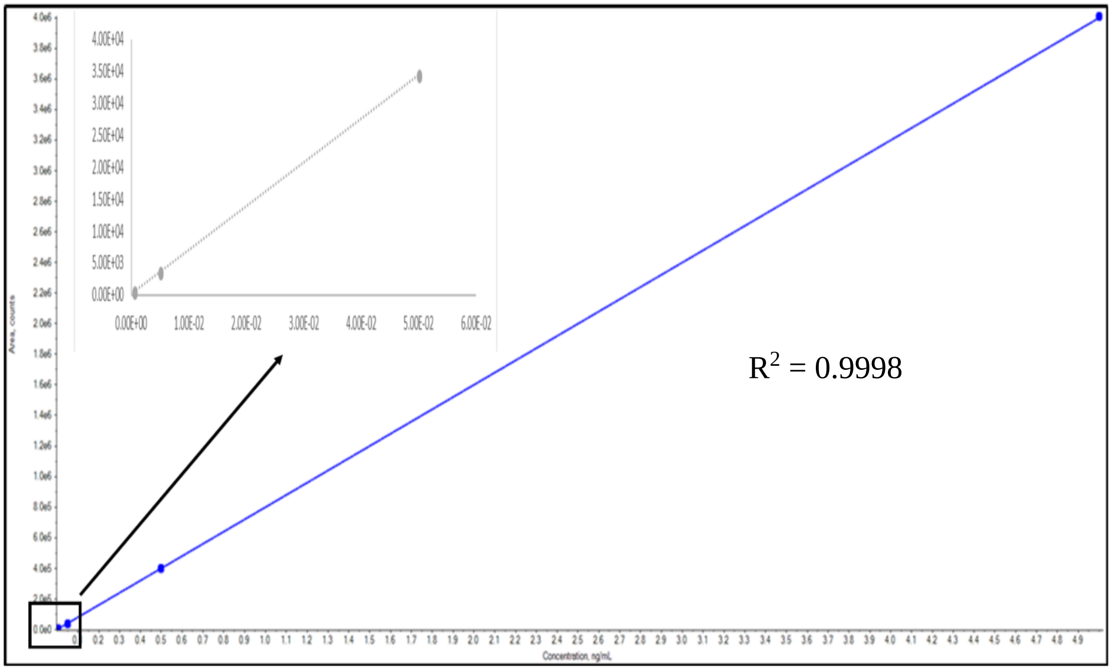

Figure 4.6 Calibration curve of ILO by UPLC-MS

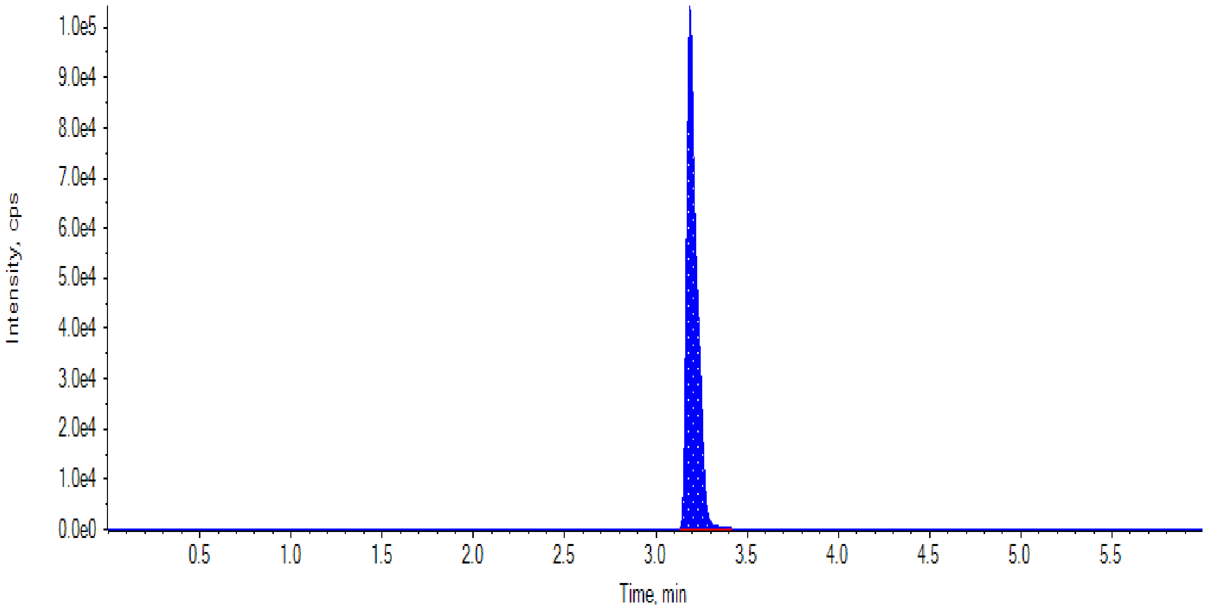

Figure 4.7 Retention time of ILO in UPLC-MS

- The LOQ for HPLC method developed was 1.04 ppm as shown in section 4.3.2 – table 4.3. This is very low determination of plasma samples obtained for pharmacokinetics study in animals. Hence, UPLC-MS method was developed to detect precisely minute concentration of drug in rat plasma.
- The R^2 value for regression analysis was found to be 0.9998. This indicated area and concentration of the drug was in significant linear correlation.
- The LOD and LOQ were found to be 5.59E-04 and 1.70E-03 ng/mL respectively. This indicated sensitivity of method in picogram concentration range. Hence, the UPLC-MS method can efficiently determine the plasma concentration of drug.

4.4 Analytical Techniques for Vardenafil HCl trihydrate

4.4.1 Estimation of VDN by UV [7]

A. Calibration curve in pH 6.8 phosphate buffer

- Standard stock solution preparation:

  Accurately weighed 10 mg VDN was dissolved in 1 mL of acetonitrile and the volume was made up with pH 6.8 phosphate buffer to make final concentration of 100 µg/mL.

- Method for calibration curve:

  Aliquots from VDN stock solution were taken in 10 mL volumetric flask and volume was made up with pH 6.8 phosphate buffer to get a range from 1 µg/mL to 25 µg/mL. The absorbance of the prepared solution was measured against blank (pH 6.8 phosphate buffer) at its absorbance maxima, 245 nm.

Table 4.5 Calibration data for VDN by UV in pH 6.8 phosphate buffer

Concentration (µg/mL)	Absorbance	%RSD
0	0.000 ± 0.00	0
1	0.159 ± 0.03	1.738
2	0.202 ± 0.04	1.916
3	0.236 ± 0.03	1.295
4	0.295 ± 0.04	1.216
5	0.412 ± 0.04	1.078
10	0.779 ± 0.10	1.230
15	0.989 ± 0.12	1.257
20	1.310 ± 0.15	1.119
25	1.519 ± 0.21	1.393

Mean SD	Slope	LOD	LOQ
0.010	0.060	0.539	1.633

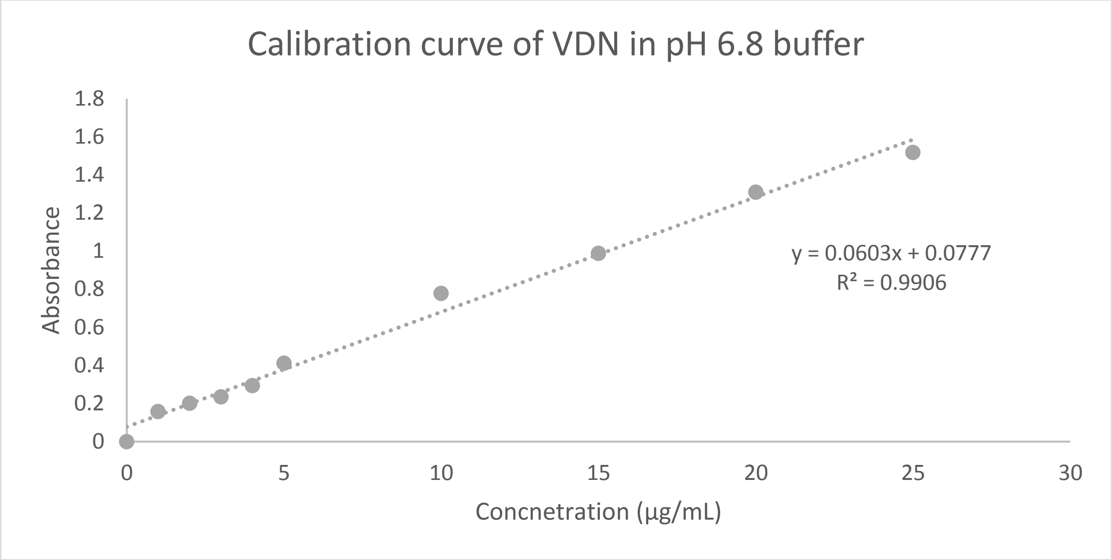

Figure 4.8 Calibration curve of VDN in pH 6.8 phosphate buffer

- VDN showed characteristic UV spectra when scanned from 200 to 400 nm. The λmax for VDN was found to be 245 nm. Hence, 245 nm was selected as analytical wavelength. Standard calibration curve was then drawn as shown in figure 4.9 using the range studied as shown in table 4.5. The 0.9906 value of R^2 obtained by linear regression analysis indicated linear relationship between absorbance and concentration of VDN in pH 6.8 phosphate buffer.
- LOD and LOQ was found to be 0.539 and 1.633 ppm respectively as shown in table 4.5. These indicated sensitivity of method for accurate quantification of drug in samples.

B. Calibration curve in Acetonitrile

- Standard stock solution preparation:

  Accurately weighed 10 mg VDN was dissolved in 10 mL of acetonitrile with frequent shaking and the volume was make up with acetonitrile to make final concentration of 100 µg/mL.
- Method for calibration curve:

  Aliquots from VDN stock solution were taken in 10 mL volumetric flask and volume was make up with methanol to get a range from 2 µg/mL to 20 µg/mL. The absorbance of the prepared solution was measured against blank (methanol) at its absorbance maxima, 245 nm.

Table 4.6 Calibration data for VDN by UV in Acetonitrile

Concentration (µg/mL)	Absorbance	%RSD
0	0.000 ± 0.000	0
2	0.097 ± 0.02	1.718
4	0.208 ± 0.02	1.002
6	0.270 ± 0.04	1.477
8	0.370 ± 0.05	1.275
10	0.430 ± 0.05	1.264
15	0.678 ± 0.13	1.947
20	0.907 ± 0.14	1.594

Mean SD	Slope	LOD	LOQ
0.006	0.045	0.420	1.272

- In acetonitrile also, the λmax for VDN was found to be 245 nm. Hence, 245 nm was selected as analytical wavelength. Standard calibration curve was then drawn as shown in figure 4.10 using the range studied as shown in table 4.6. The 0.9981 value of R^2 obtained by linear regression analysis indicated linear relationship between absorbance and concentration of VDN in acetonitrile.
- LOD and LOQ was found to be 0.420 and 1.272 ppm respectively as shown in table 4.6. These indicated sensitivity of method for accurate quantification of drug in samples.

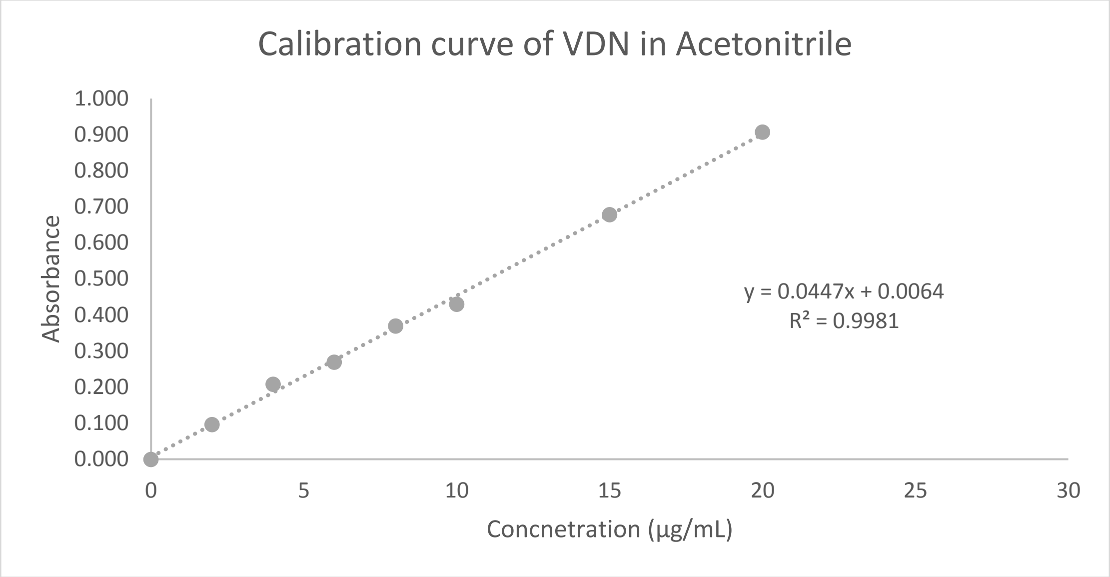

Figure 4.9 Calibration curve of VDN in acetonitrile

4.4.2 Estimation of VDN by HPLC [8]

- Instrument: LC-20AD (Shimadzu, Japan)
- Chromatographic Condition:

  Column: ACE 5 C18 column, 250*4.6mm, 5 µm (Make: ACE, Part No: ACE-121-2546), Flow rate: 1.0 mL/minute, Injection volume: 20 µL, Run time: 10 minutes, Wavelength: 220 nm, Mode: Isocratic
- Mobile Phase:

  Mobile Phase consisted of buffer and acetonitrile in ratio of 35:65.

  Buffer preparation:

  1.36 g of KH_2PO_4 was dissolved in sufficient quantity of water and dilute up to 1000 mL with water. The pH was adjusted to 6.5 ± 0.05 with KOH solution. The buffer was then filtered through 0.45µ nylon membrane filter.
- Standard stock solution preparation:

  Accurately weighed 10 mg VDN was dissolved in 100 mL of methanol to make final concentration of 100 µg/mL.

- Method for calibration curve:

Aliquots from VDN stock solution were taken in 10 mL volumetric flask and volume was make up with mobile phase to get a range from 2.5 µg/mL to 50 µg/mL. The area for the range of standard solution was measured.

Table 4.7 Calibration data for VDN by HPLC

Concentration (µg/mL)	Area (mV.s)	%RSD
2.5	18.07 ± 0.35	1.93
5	91.75 ± 1.60	1.74
10	198.15 ± 1.96	0.99
25	702.79 ± 2.75	0.39
50	1663.51 ± 11.83	0.71

Mean SD	Slope	LOD	LOQ
3.70	34.79	0.35	1.06

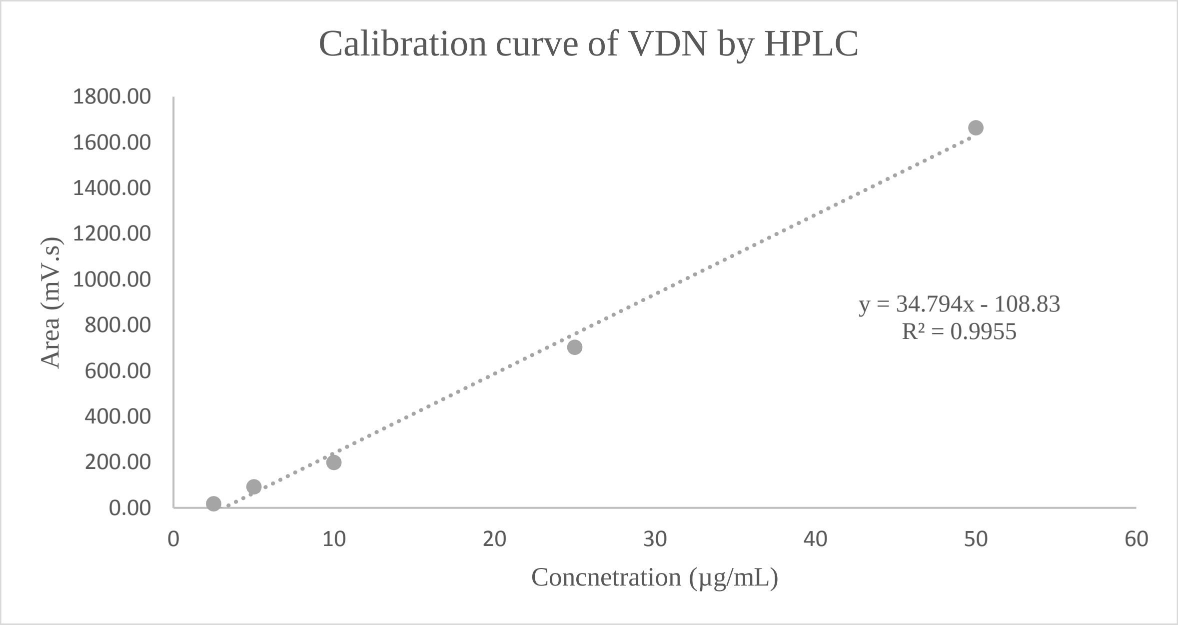

Figure 4.10 Calibration curve for VDN by HPLC

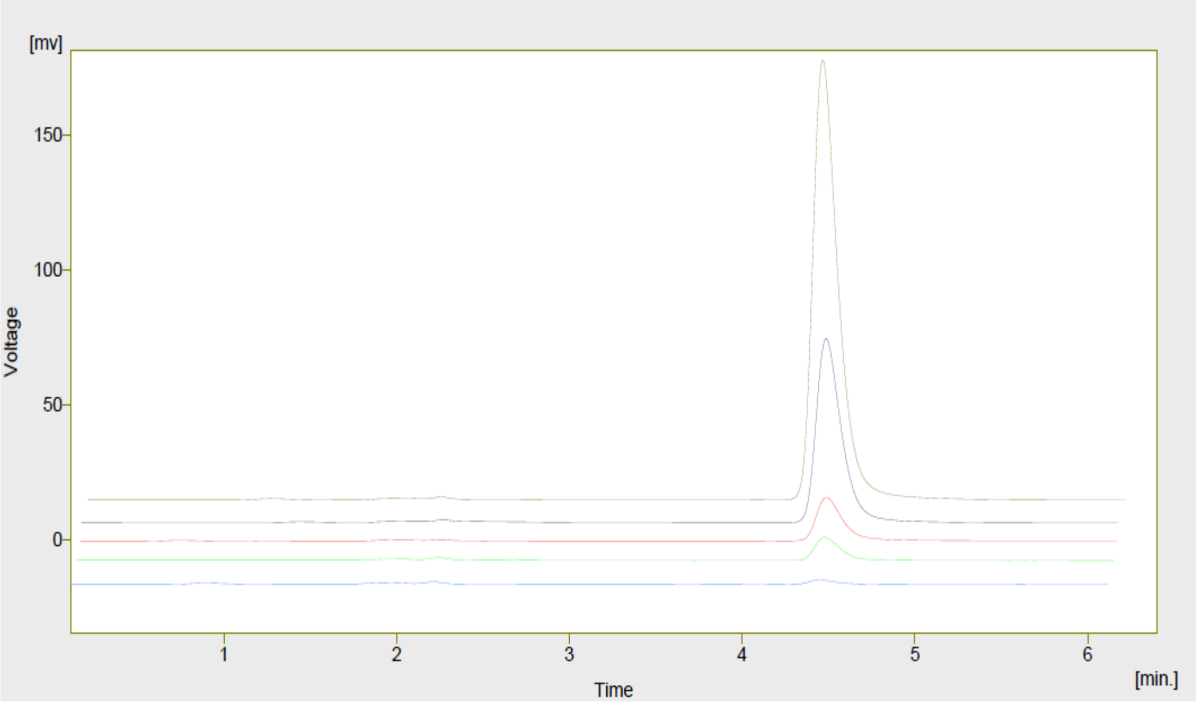

Figure 4.11 Chromatogram of VDN by HPLC

- As shown in figure 4.12, the retention time for VDN was found to be 4.5 min. Linearity of HPLC method for estimation of VDN was established by regression analysis. The R^2 value was found to be 0.9955 which reflects linear correlation.
- The LOD and LOQ were found to be 0.35 and 1.06 ppm respectively. This indicated sensitivity of the method for accurate determination of drug in test sample.

4.4.2 Estimation of VDN by UPLC-MS

UPLC-MS (ABSIEX QTRAP 4500 with ekspert^{TM} ultraLC 100-XL autosampler, make: SCIEX, DHR Holding Pvt. Ltd., India) with electrospray ionization (ESI) was used to quantify the amount of VDN in plasma for pharmacokinetic study.

Sample Preparation [9]:

VDN was extracted from plasma by solid phase extraction (SPE) technique using Oasis HLB Cartridge (WATERS^{TM}). SPE assembly was Ezypress^{TM} HT48 (Orochem Laboratories) attached with the N_2 gas flow.

Step 1: Conditioning

The OASIS HLB™ cartridges (Waters, USA) were conditioned with 2 mL methanol. For this methanol was passed through the cartridges under N_2 environment.

Step 2: Sample pre-treatment:

The plasma (0.1 mL) separated from whole blood component at 3000 rpm at 4 °C was mixed with water (0.1 mL) and 50 mM ammonium hydroxide solution, pH 9.0 (0.1 mL). The sample was mixed properly by gentle shaking of the Eppendorf.

Step 3: Sample application:

The plasma sample was then slowly added to pre-conditioned cartridges.

Step 4: Rinsing:

To remove the plasma matrix components, rinsing was carried out using ammonium hydroxide solution (pH 9.0).

Step 5: Drying:

Under N_2 environment the cartridge was dried to remove any remnant of rinsing solvent. For this N_2 gas was passed through cartridge under pressure.

Step 6: Elution:

The sample (drug bonded in cartridge) was then eluted with methanol. Followed by N_2 passage at high pressure through the cartridge, any remaining amount of drug in methanol was collected in the vial.

Standard Range: For UPLC-MS, standard VDN concentration range was from 0.01 ng/mL to 10 ng/mL in plasma.

LC condition: The ABSIEX UPLC-MS system contained micro-degasser with high-performance autosampler ekspert^{TM} ultraLC 100-XL. For LC separation, C18 column, 50*2.1 mm, 1.7 µm (Make: Aquity UPLC BEH) was used. Mobile Phase consisted of 35:65 v/v ratio of Formic acid (1%V/V): methanol. Injection volume was 5 µL and the flow rate was 0.25 mL/min. Column and auto-sampler were operated at room temperature.

Tandem MS condition: Interface between UPLC and MS was ESI with positive ion mode polarity. The main working parameters were as follow: curtain gas, 25 psi; ion source gas 1, 50 psi; ion source gas 2, 50 psi; source temperature, 350 °C; Declustering potential (DP), 186 V; Collision energy (CE), 75 V; Collision cell exit potential (CXP), 14 V. Multiple reaction monitoring (MRM) transition was measured for Q1 → Q3 ion (489.1 m/z → 151.1 m/z) [10].

Table 4.8 Calibration data for VDN by UPLC-MS

Concentration (ng/mL)	Area (cps)	%RSD
1.00E-02	3.74E+03 ± 1.76E+01	0.47
1.00E-01	4.39E+04 ± 3.30E+01	0.08
1.00E+00	4.27E+05 ± 1.82E+02	0.04
1.00E+01	3.79E+06 ± 1.90E+02	0.01

Mean SD	Slope	LOD	LOQ
3.78E+05	1.35E+02	1.18E-03	3.58E-03

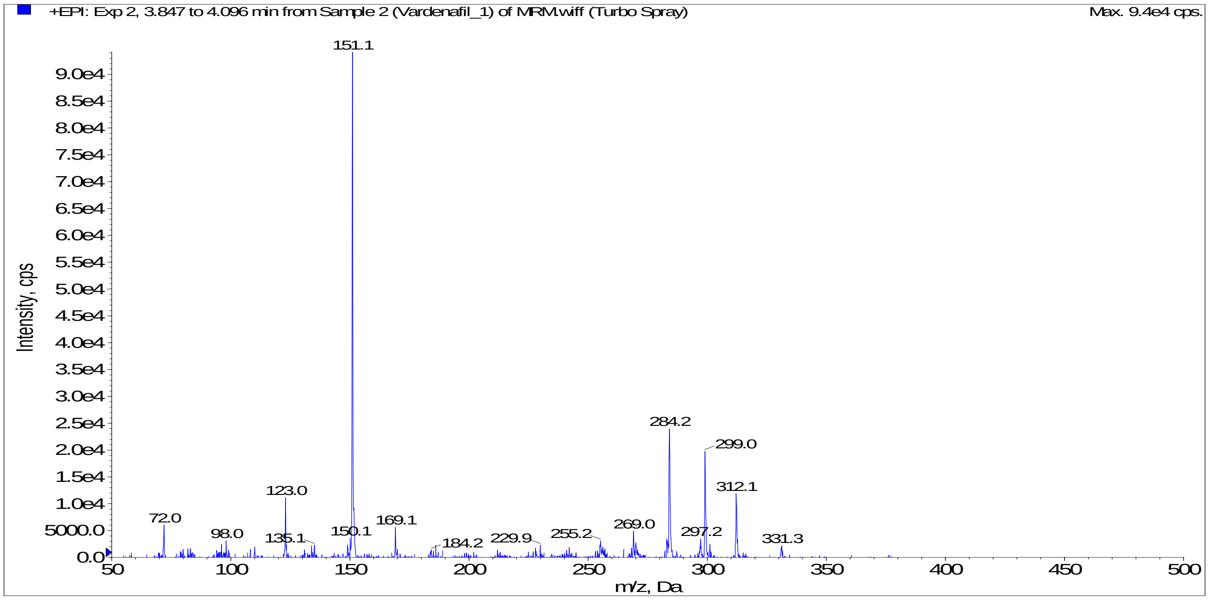

Figure 4.12 MRM transition of VDN for Mass detection

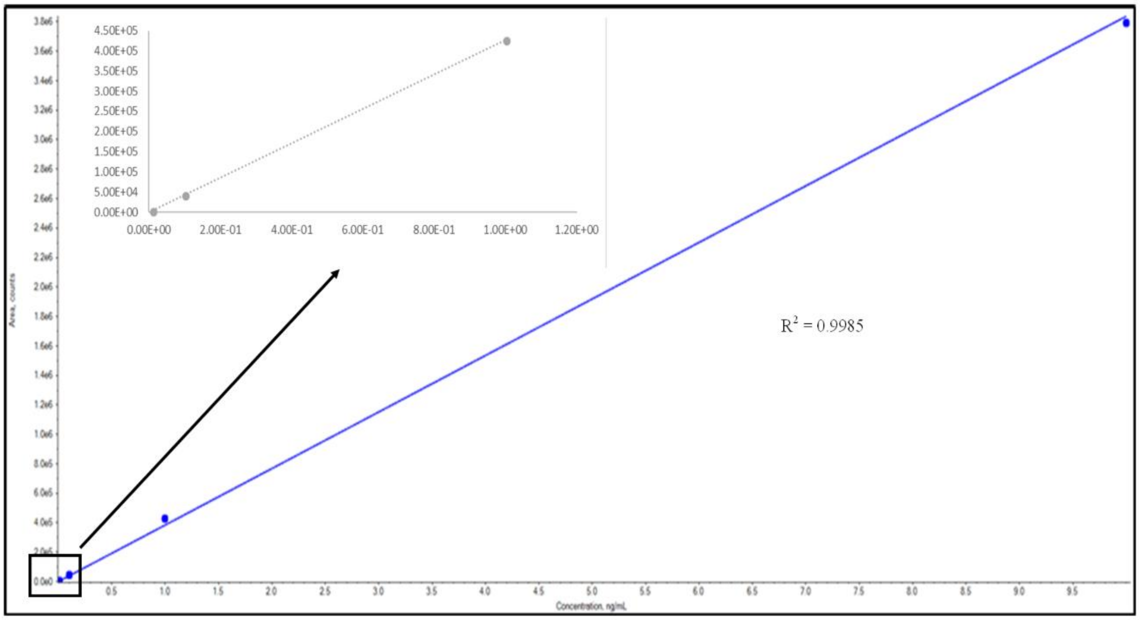

Figure 4.13 Calibration curve of VDN by UPLC-MS

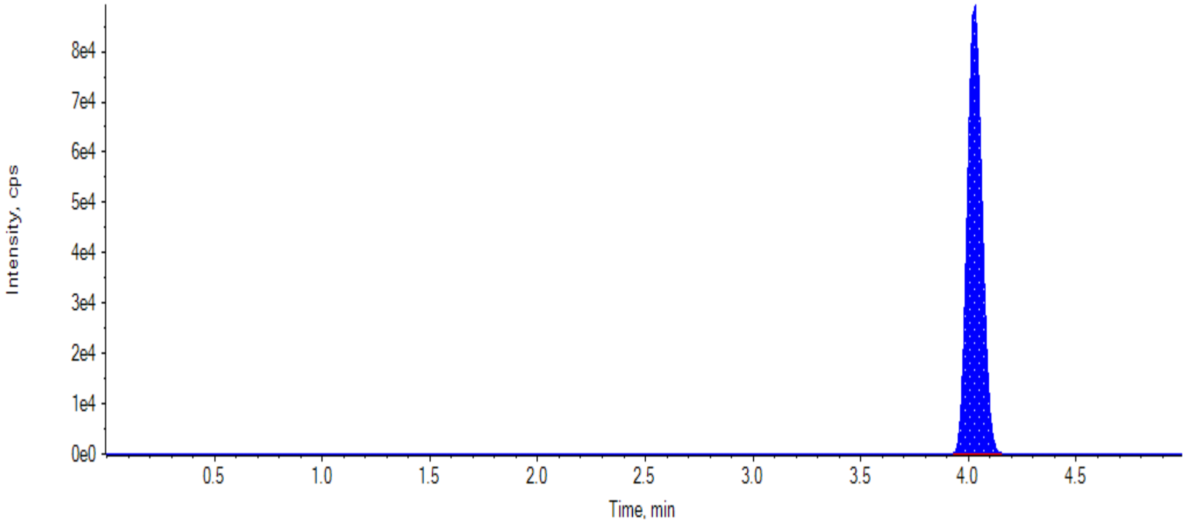

Figure 4.14 Retention time of VDN in UPLC-MS

- The LOQ for HPLC method developed was 1.06 ppm as shown in section 4.4.2 – table 4.7. This is very low determination of plasma samples obtained for pharmacokinetics study in animals. Hence, UPLC-MS method was developed to detect precisely minute concentration of drug in rat plasma.

- The R^2 value for regression analysis was found to be 0.9985. This indicated area and concentration of the drug was in significant linear correlation.
- The LOD and LOQ were found to be 1.18E-03 and 3.58E-03 ng/mL respectively. This indicated sensitivity of method in picogram concentration range. Hence, the UPLC-MS method can efficiently determine the plasma concentration of drug.

4.5 Estimation of residual solvents – Methanol and Chloroform by HS-GC [11]

GC – Clarus 500 (Perkin Elmer, India) with Headspace (HS) – turbomatrix 400 and a flame ionization detector (FID) was used to quantify the amount of residual solvent in formulation. Data were analyzed with total chrom navigator software.

The Clarus 500 GC system was attached with turbomatrix 40 – automatic HS sampler. The transfer channel from HS to GC colum was maintained at 100°C and the needle temperature was 115°C. The column oven temperature program was 40°C for 10 min, then ramped at 30°C/min to 240°C and held for 3.33 min. The injector and detector temperatures were 150°C and 250°C respectively. The carrier gas was helium. Samples were equilibrated at 60°C for 30 min prior to injection. The injection was performed in splitless mode onto a capillary column – DB-624 (30m x 0.53mm i.d., 3.0-µm film thickness, J&W scientific). The total run time was kept 20 min.

Standard preparation for methanol and chloroform:

Accurately weighed 2000 mg methanol and 40 mg chloroform were diluted up to 100 mL with DMF (GC grade) in a single 100 mL volumetric flask to obtain standard concentration of 40ppm chloroform and 2000ppm methanol.

Table 4.9 Area for Standard solution of chloroform – methanol mix obtained using HS-GC

	Area for Methanol (µV.s)	Area for Chloroform (µV.s)
Standard 1	511460	23702
Standard 2	525100	24344
Standard 3	520123	24404
Average ± SD	518894.3 ± 6902.508	24150 ±389.1375

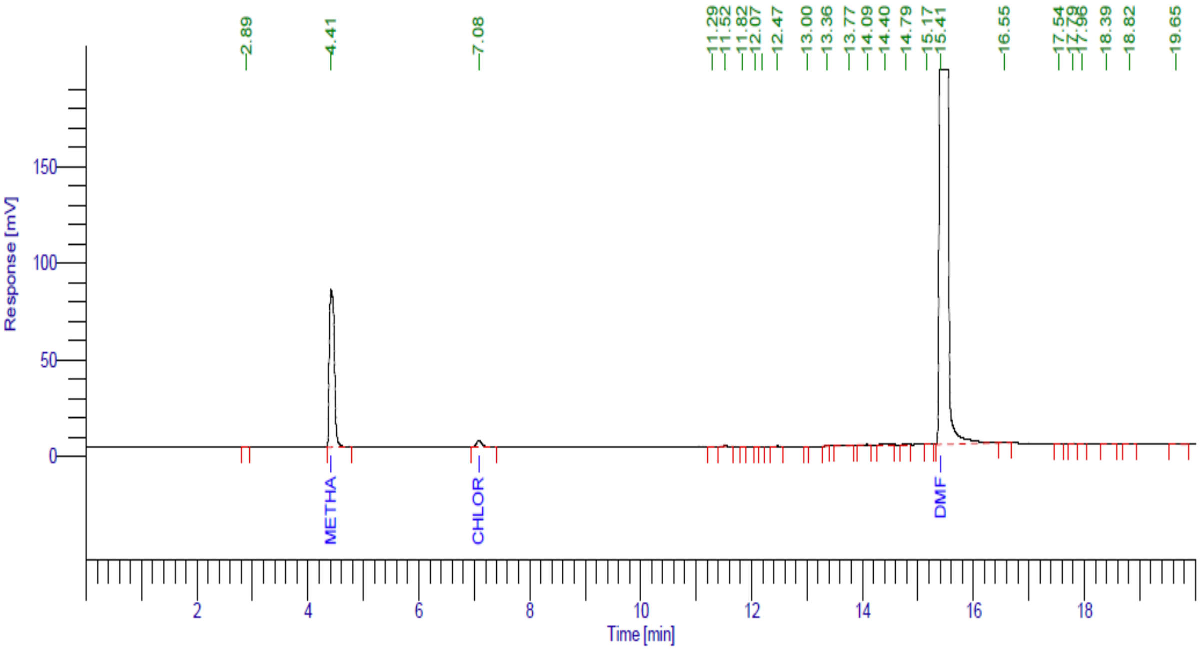

Figure 4.15 Chromatogram for standard solution of methanol – chloroform prepared in DMF

- Herein, as shown in table 4.10 and figure 4.20, for the standards area was found to be 518894.3 ± 6902.508 and 24150 ±389.1375 µV.s respectively for methanol and chloroform. Hence, any test sample which when tested indicates area greater than the reported for standards, will fail the limit for residual solvent. Therefore, drying time in rotary evaporation for Niosomes formulation development was optimized accordingly based on GC results.

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