Analytical Techniques

Chapter 3

3.1. INTRODUCTION

Analytical techniques are essential methods for quantification of different drugs at various phases of formulation development. They are used to determine the composition of formulation and are supportive in establishing crucial formulation characteristics of the prepared SMEDDS and NEs for parameters like drug content, % transmittance, Caco2 cell line permeability studies, stability studies, *in vitro* drug release behavior and *in vivo* bioavailability. The present analytical work comprises of simple, precise, rapid, sensitive and accurate method for the estimation of Dabigatran Etexilate (DE) and Nisoldipine (NISO) by UV spectrophotometry, one of the simplest instrumentation methods capable of drug estimation and HPLC per se sophisticated method used for the estimation of samples with very low quantity of the drug. Few analytical methods are reported for the determination of DE in pharmaceutical dosage forms and in biological fluids by UV [1], HPLC [2], LC/MS [3], LC/MS/MS [4] and UPLC-MS/MS [5]. Similarly, literature review reveals some analytical methods for the quantification of NISO in pharmaceutical dosage forms and in biological fluids by UV [6], HPLC [7] RP-HPLC-PDA [8] LC-MS/MS, [9] and HPTLC [10]. The analytical methods employed in present investigation are described below.

3.2. MATERIALS

DE and NISO were generously gifted by Alembic Research Centre (Vadodara, India). Water (HPLC grade) and Acetontrile (HPLC Grade) were purchased from Merck, USA. Ultipor® Nylon-66 membrane filter (0.22 µm) was purchased from Pall Life Sciences, USA. All the chemicals and reagents like Potassium phosphate, monobasic, Concentrated hydrochloric acid, Monosodium phosphate, Sodium lauryl sulphate, Sodium dihydrogen phosphate monohydrate, Ammonium dihydrogen phosphate, Trifluoroacetic acid and Sodium hydroxide were of AR Grade. Human plasma was obtained from Suraktam Blood Bank, Vadodara, India.

3.2.1. Instruments and Softwares

3.2.1.1. UV spectrophotometer

A double beam UV-Visible spectrophotometer (UV-1800, Shimadzu Corporation, Japan) with a fixed slit width of 1nm coupled with UVPROB V2.42 software was used for all spectrophotometric measurements. The wavelength scanning speed was kept fast with light mode change at 340nm wavelength. Quartz cuvettes (1 cm) were used for all the spectral measurements.

3.2.1.2. HPLC

HPLC was equipped with Shimadzu (Shimadzu Corporation, Kyoto, Japan) LC-20AT pump, and Shimadzu SPD-20A UV-Visible detector. Samples were injected manually through a Rheodyne injector valve with fixed loop at 20 µL. The separation was performed on Synergi^{TM} 4 µm Hydro-RP 80 Å, LC Column (150 x 4.6mm) (Phenomenex, Torrance, USA). The column was connected to a 2 cm guard column (Phenomenex, Torrance, USA) for plasma and cell line studies. Chromatographic data were recorded and processed using Spinchrome Chromatographic Station® CFR Version 2.4.0.193 (Spinchrome Pvt. Ltd., Chennai, India).

3.2.2. Reagents / solutions

3.2.2.1. Hydrochloric acid, 0.01N

0.85 mL of concentrated hydrochloric acid was carefully added to 500 mL of distilled water and finally the volume was made up to 1000 mL using distilled water.

3.2.2.2. Hydrochloric acid, 0.1N with 0.5% SLS

5.0 g SLS was dissolved in 1000 mL of 0.1N HCl Solution (8.5 mL of concentrated hydrochloric acid was carefully added to 500 mL of distilled water and finally the volume was made up to 1000 mL using distilled water).

3.2.2.3. Phosphate buffer, pH 6.8

Potassium phosphate, monobasic, 0.2 M:

27.22 g of potassium phosphate, monobasic was dissolved in distilled water and the volume was made up to 1000 mL.

Sodium hydroxide, 0.2 M:

22.4 g of NaOH was dissolved in distilled water and the volume was made up to 1000 mL.

Preparation of phosphate buffer (PB) pH 6.8:

50 mL of potassium phosphate, monobasic and 22.4 mL of 0.2M NaOH were taken in volumetric flask and diluted up to 200 mL with distilled water.

3.2.2.4. Phosphate buffer, pH 6.8 with 0.5% SLS

Monosodium phosphate, 0.2 M:

23.99 g of monosodium phosphate was dissolved in distilled water and the volume was made up to 1000 mL.

Sodium hydroxide, 0.2 M:

22.4 g of NaOH was dissolved in distilled water and the volume was made up to 1000 mL.

Preparation of phosphate buffer (PB) pH 6.8:

50 mL of 0.2M Monosodium phosphate and 22.4 mL of 0.2M NaOH were taken in volumetric flask and diluted up to 200 mL with distilled water.

Above procedure to prepare potassium phosphate, pH 6.8 with 0.5% SLS is similar to one reported in USP 30 but with slight modification [11]. Potassium phosphate, monobasic solution formed turbidity with SLS, therefore equivalent molar ratio of monosodium phosphate was used.

3.2.2.5. Phosphate buffer, pH 5.8

4.14 g Sodium dihydrogen phosphate monohydrate was dissolved in 1000 mL double distilled filtered water. pH was adjusted to 5.8 with 0.2M NaOH solution.

3.2.2.6. Phosphate buffer, pH 3.0

1.15 g Ammonium dihydrogen phosphate was dissolved in 1000 mL double distilled filtered water. pH was adjusted to 3.0 with Trifluoro Acetic acid (TFA) solution.

3.2.2.7. Phosphate buffer, pH 7.4

Preparation of phosphate buffer (PB) 7.4:

50 mL of 0.2M potassium phosphate, monobasic and 39.1 mL of 0.2M NaOH (section 3.2.2.3) were taken and diluted up to 200 mL with distilled water.

3.2.2.8. Phosphate buffer, pH 7.4 with 0.5% SLS

Preparation of phosphate buffer (PB) 7.4:
50 mL of 0.2M Monosodium phosphate and 39.1 mL of 0.2M NaOH (section 3.2.2.4) were taken and diluted up to 200 mL with distilled water.

Above procedure to prepare potassium phosphate, pH 7.4 with 0.5% SLS is similar to one reported in USP 30 but with slight modification [11]. Potassium phosphate, monobasic solution formed turbidity with SLS, therefore equivalent molar ratio of monosodium phosphate was used.

3.2.3. Mobile phase for HPLC of DE

Acetonitrile and Phosphate buffer, pH 5.8 were mixed in the ratio of 60:40 v/v. The solvent mix was filtered through 0.22 µm membrane filter (Ultipor® Nylon 66 membrane filter, Pall Life Sciences, USA), transferred to reagent bottle and degassed using bath sonication for 10 min.

3.2.4. HPLC Mobile phase for determination of NISO

Acetonitrile and Phosphate buffer, pH 3.0 were mixed in the ratio of 70:30 v/v. The solvent mix was filtered through 0.22 µm membrane filter (Ultipor® Nylon 66 membrane filter, Pall Life Sciences, USA), transferred to reagent bottle and degassed using bath sonication for 10 min.

3.3. METHODS

3.3.1. Estimation of DE by UV spectrophotometric methods

UV spectrophotometric methods for estimation of DE were developed in different media viz. 0.01N hydrochloric acid, phosphate buffer pH 6.8, phosphate buffer pH 7.4 and methanol.

3.3.1.1. Preparation of stock solutions

Stock solutions containing 100 µg/mL were prepared in all the above media. Accurately weighed quantities 10 mg of drug were transferred to 100 mL calibrated volumetric flasks and dissolved in different media. The volumes were made up to 100 mL with respective media. The resulting solutions (1000 µg/mL) were further diluted ten times with the same media to get stock solutions (100 µg/mL).

3.3.1.2. Preparation of standard solutions

Appropriate aliquots (0.25 to 3.0 mL) from the stock solutions of DE were transferred to 10 mL calibrated volumetric flasks and diluted up to the mark with respective media to obtain known final concentrations ranging from 2.5-30µg/mL for 0.01N HCl, pH 6.8 phosphate buffer, pH 7.4 phosphate buffer and methanol.

3.3.1.3. Determination of analytical wavelength

The spectrum scan of each standard solution was recorded using UV Visible spectrophotometer from 200 to 400 nm wavelength range against respective media as blank. The wavelengths with maximum absorbance (λ_{max}) were selected as analytical wavelengths for respective media.

3.3.1.4. Preparation of calibration curves

Absorbances of standard solutions were recorded at selected analytical wavelengths and the calibration curves were plotted between standard drug concentrations (X-axis) and observed absorbance (Y-axis). Regression equations of best fit straight line and correlation coefficients were generated in Microsoft office excel software to observe linearity. Experiment was performed in triplicate using freshly prepared stock solution every time.

3.3.2. Validation of UV spectrophotometric methods

3.3.2.1. Linearity and range

Linearity of an analytical method is the ability to bring forth the test results that are directly or by well-defined mathematical transformation, proportional to the concentration of the analyte in the samples within the given range [12]. Least square linear regression was performed using Microsoft office excel software to determine the

regression coefficient (R^2) and the equation for the best fitting line to demonstrate linearity.

3.3.2.2. Stability

The assessment of stability should show the reliability of an analysis with respect to intentional variations in method parameters to guarantee that the legitimacy of the analytical procedure is sustained whenever used [13]. Stability of DE analytical solutions in different media was ascertained as a measure of stability by observing the changes in the absorbance of the solutions at the analytical wavelength over a period of 24h at room temperature. The readings were recorded in triplicate.

3.3.2.3. Precision/ repeatability

Precision is a measure of the steadiness and reproducibility of a method. Precision of an analytical method is the degree of agreement among the individual test results giving very close values for repeated measurement of same sample when the procedure is applied repeatedly to multiple sampling of homogeneous sample [13]. Multiple measurements for same standard concentrations were made three times on same day as well as on three consecutive days to determine intraday and interday precision respectively [14]. All the solutions were prepared freshly each time. The % Relative Standard Deviation (% RSD) was calculated as a measure of precision.

3.3.2.4. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value [13]. To confirm the accuracy of proposed method, recovery study was performed by method of standard additions at different levels of drug concentrations [15]. Known amounts of standard drug (80%, 100% and 120%) were added to the pre-analyzed samples and the absorbance was measured. Basic concentration of sample chosen was 10 µg/mL of DE standard solution. The accuracy was calculated by using following formula:

$$\text{Accuracy} = \frac{\text{True value} - \text{Measured value}}{\text{True value}} \times 100 \quad \text{…Eqn. 3.3.1}$$

3.3.2.5. Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) are the quantification parameters. These are used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. Therefore, LOD and LOQ of developed methods were determined using following equations [14, 15],

$$LOD = 3.3 \times \left(\frac{R}{S}\right) \quad \text{.....Eqn. 3.3.2} \qquad LOQ = 10 \times \left(\frac{R}{S}\right) \quad \text{.....Eqn. 3.3.3}$$

S= Slope of the linearity curve

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

3.3.2.6. Specificity

Specificity is the capability to evaluate the analyte in the presence of components which may be expected to be present. Typically these comprise formulation additives [13]. The interference of formulation excipients (Capmul MCM C8, Cremophor EL, Transcutol HP) in the estimation of DE was determined in 0.01N HCl, pH 6.8 phosphate buffer, pH 7.4 phosphate buffer and methanol at 325nm, 316nm, 316nm and 315nm respectively using formulation prototype method. The formulation prototype was prepared by mixing one portion of drug with 3 portions of oil and 2 portion each of Cremophor EL and Transcutol HP. The absorbance of this formulation prototype and excipient mix without drug were compared with absorbance of pure drug solutions in respective media.

3.3.3. Estimation of DE by HPLC method

Quantitative estimation of DE was done by HPLC as described by Bernardi et.al. 2013 [2] with slight modifications.

3.3.3.1. Preparation of stock solution of drug

Stock solution containing 100 µg/mL drug was prepared by dissolving 10 mg drug in 10 mL acetonitrile and further diluting it to 10 times with acetonitrile.

3.3.3.2. Preparation of standard solutions of drug

Appropriate and accurate aliquots of the stock solution were transferred to 10 mL calibrated volumetric flasks and diluted up to the mark with acetonitrile to get known final concentrations ranging from 1 to 10 µg/mL.

3.3.3.3. Preparation of calibration curve

Shimadzu isocratic HPLC with a UV-visible detector was used for HPLC analysis. The mobile phase (ACN : Phosphate buffer, pH 5.8:: 60:40 % v/v)) was set to a flow rate of 1.0 mL/min at room temperature. 20 µL of standard drug solution was injected each time using syringe through rheodyne injector. The chromatograms were recorded at 230 nm detection wavelength for a run time of 10 min. The column was equilibrated by passing 100 mL of mobile phase. Calibration curve was drawn by plotting peak area of curve versus drug concentration.

3.3.4. Estimation of DE in plasma by HPLC method

Quantitative estimation of DE in plasma was done by HPLC in similar manner as explained in section 3.3.3 but with final concentrations ranging from 0.05 to 2 µg/mL.

3.3.4.1. Preparation of stock solution of drug

Stock solution containing 5 µg/mL drug was prepared by dissolving 10 mg drug in 10 mL acetonitrile and further 0.5mL of this solution was diluted 100 times with acetonitrile.

3.3.4.2. Preparation of standard solutions of drug

Appropriate and accurate aliquots of the stock solution were transferred to 2 mL eppendorff tubes, spiked with 0.2 mL of rat plasma and diluted up to 1 mL with acetonitrile to get known final concentrations ranging from 0.05 to 2 µg/mL. The separation of precipitate from organic phase was carried out by centrifugation at 4000 rpm for 10 min. The obtained organic phase (acetonitrile solution) was evaporated to dryness and used for analysis after reconstitution with 1 mL mobile phase consisting of mixture of ACN : Phosphate buffer, pH 5.8 in the ratio of 60:40 v/v.

3.3.4.3. Preparation of calibration curve

Shimadzu isocratic HPLC with a UV-visible detector was used for HPLC analysis. The mobile phase was set to a flow rate of 1.0 mL/minute at room temperature. 20 µL of standard drug solutions were injected each time using syringe through rheodyne injector. The chromatograms were recorded at 230 nm detection wavelength for a run time of 10 min. The column attached with guard column was equilibrated by passing 100 mL of mobile phase. Calibration curve was drawn by plotting peak area of curve versus drug concentration.

3.3.5. Validation of HPLC method

The parameters including linearity, accuracy and precision were evaluated using methods as described in section 3.3.2.

3.3.6. Estimation of NISO by UV spectrophotometric methods

UV spectrophotometric methods for estimation of NISO in different media including 0.1N HCl + 0.5% SLS, pH 6.8 phosphate buffer + 0.5%SLS, pH 7.4 phosphate buffer + 0.5%SLS and methanol were developed.

3.3.6.1. Preparation of stock solutions

Stock solutions containing 100 µg/mL were prepared in all the selected media. Accurately weighed quantities 10 mg of drug were transferred to 100 mL calibrated volumetric flasks and dissolved in different media. The volumes were made up to 100 mL with the same media. The resulting solutions (1000 µg/mL) were further diluted ten times with the same media to get stock solutions (100 µg/mL).

3.3.6.2. Preparation of standard solutions

Appropriate aliquots (0.2 to 1.0 mL) of the stock solutions of NISO were transferred to 10 mL calibrated volumetric flasks and diluted up to the mark with respective media to obtain known final concentrations ranging from 2 to 10 µg/mL.

3.3.6.3. Determination of analytical wavelength

The spectrum scan of each standard solution was recorded using UV Visible spectrophotometer from 200 to 400 nm wavelength range against respective medium as

blank. The wavelengths with maximum absorbance (λ_{max}) were selected as analytical wavelengths for respective media.

3.3.6.4. Preparation of calibration curves

Absorbances of standard solutions were recorded at selected analytical wavelengths and the calibration curves were plotted between standard drug concentrations (X-axis) and observed absorbance (Y-axis). Equations of best fit straight line and correlation coefficients were generated in MS office excel software to observe linearity.

3.3.7. Validation of UV spectrophotometric methods

The parameters including linearity and range, stability, precision, accuracy, sensitivity and specificity were evaluated using methods as described in section 3.3.2. For demonstration of specificity, the formulation prototype was prepared by mixing one portion of drug with 2 portions of Cremophor EL, 1 portion each of Peceol and Transcutol HP.

3.3.8. Estimation of NISO by HPLC method

Estimation of NISO by HPLC has been reported by Marinkovic et al., 2003 [7]. The same method was adopted here with a few modifications for estimation of NISO.

3.3.8.1. Preparation of NISO stock solution

Stock solution containing 100 µg/mL drug was prepared by dissolving 10 mg drug in 10 mL acetonitrile and further diluting it to 10 times with acetonitrile.

3.3.8.2. Preparation of NISO standard solutions

Appropriate and accurate aliquots of the stock solution were transferred to 10 mL calibrated volumetric flasks and diluted up to the mark with acetonitrile to get known final concentrations ranging from 1 to 10 µg/mL.

3.3.8.3. Preparation of calibration curve

Isocratic HPLC with a UV-visible detector was used for HPLC analysis. The mobile phase (ACN: Phosphate buffer, pH 3.0) was set to a flow rate of 1 mL/minute at room temperature. 20 µL of standard drug solutions were injected each time using syringe through rheodyne injector. The chromatograms were recorded at 238 nm detection

wavelength for a run time of 10 min. The column was equilibrated by passing 100 mL of mobile phase. Calibration curve was drawn by plotting peak area of curve versus drug concentration.

3.3.9. Estimation of NISO in plasma by HPLC method

Estimation of NISO by HPLC has been reported by Marinkovic et al., 2003 [7]. The same method was adapted here with a few modifications in mobile phase for estimation of NISO in plasma.

3.3.9.1. Preparation of NISO stock solution

Stock solution containing 5 µg/mL drug was prepared by dissolving 10 mg drug in 10 mL acetonitrile and further diluting 0.5 mL of this solution to 100 times with acetonitrile.

3.3.9.2. Preparation of NISO standard solutions

Appropriate and accurate aliquots of the stock solution were transferred to 2 mL eppendorff tubes, spiked with 0.2 mL of rat plasma and diluted up to 1 mL with acetonitrile to get known final concentrations ranging from 0.05 to 2 µg/mL. The separation of precipitate from organic phase was carried out by centrifugation at 4000 rpm for 10 min. The obtained organic phase (acetonitrile solution) was evaporated to dryness and used for analysis after reconstitution with 1 mL mobile phase consisting of mixture of ACN : Phosphate buffer, pH 3.0 in the ratio of 70:30 v/v.

3.3.9.3. Preparation of calibration curve

Isocratic HPLC with a UV-visible detector was used for HPLC analysis. The mobile phase was set to a flow rate of 1 mL/minute at room temperature. 20 µL of standard drug solutions were injected each time using syringe through rheodyne injector. The chromatograms were recorded at 233 nm detection wavelength for a run time of 10 minutes. The column was equilibrated by passing 100 mL of mobile phase. Calibration curve was drawn by plotting peak area of curve versus drug concentration.

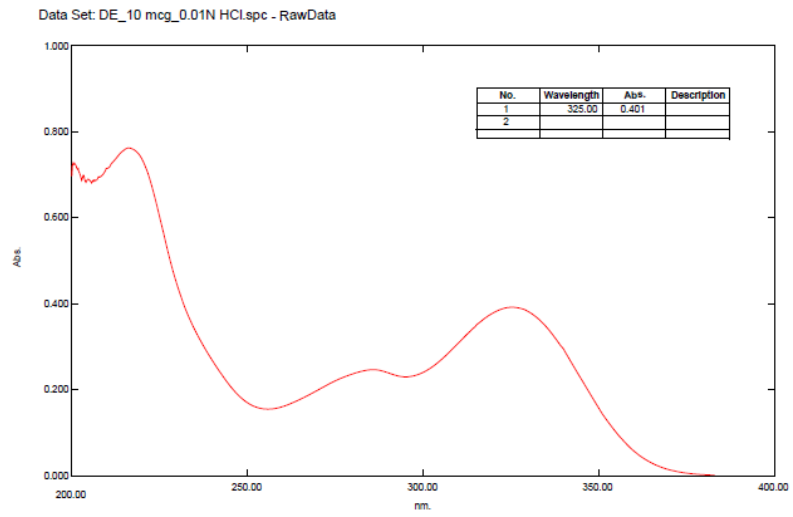
3.3.10. Validation of HPLC method

The parameters including linearity, accuracy, precision, stability and sensitivity were evaluated using methods as described in section 3.3.2.

3.4. RESULTS & DISCUSSION

3.4.1. Estimation of DE by UV spectrophotometry

DE showed characteristic UV spectra when scanned in the UV range between 200 nm and 400 nm. The UV spectrum showed absorption maxima at 325nm, 316nm, 316nm and 315nm for 0.01N HCl, pH 6.8 phosphate buffer, pH 7.4 phosphate buffer and methanol respectively (Figure 3.4.1). Hence, these were selected as analytical wavelengths for all respective media.



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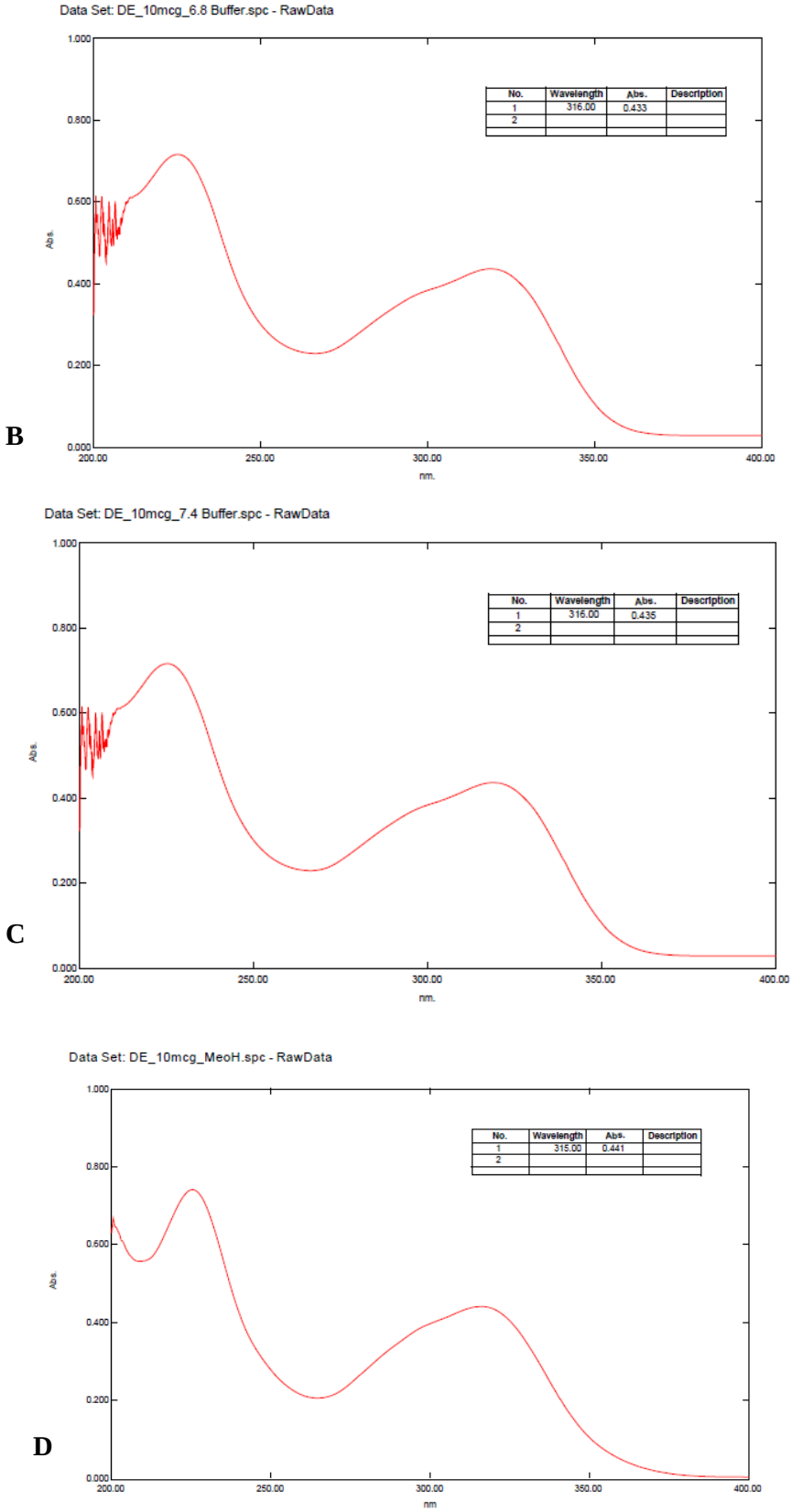


Figure 3.4.1: UV Absorption spectrum of DE in (A) 0.01N HCl, (B) phosphate buffer pH 6.8 (C) phosphate buffer pH 7.4 and (D) methanol

Calibration curves for all the media were then prepared using respective absorbance of standard drug solutions. The calibration data for DE in different media are summarized in Table 3.4.1. Standard calibration curves in various media with their regression equations and correlation coefficients (R^2) were then drawn as shown in Figure 3.4.2. The value of correlation coefficient indicated the linear relationship between absorbance and concentration of DE in each media.

Table 3.4.1: Calibration data for DE in various media

Concentration (µg/mL)	Absorbance[#]			
	0.01N HCl	Phosphate buffer pH 6.8	Phosphate buffer pH 7.4	Methanol
2.5	0.124± 0.004	0.119 ± 0.006	0.114 ± 0.003	0.122± 0.001
5	0.200± 0.006	0.226 ± 0.006	0.223 ± 0.006	0.234 ± 0.001
10	0.401± 0.009	0.433 ± 0.002	0.435 ± 0.005	0.441 ± 0.004
15	0.602± 0.028	0.612 ± 0.005	0.615 ± 0.003	0.667 ± 0.003
20	0.799± 0.017	0.805 ± 0.006	0.809 ± 0.003	0.865 ± 0.004
25	1.015± 0.019	0.992 ± 0.006	0.995 ± 0.005	1.092 ± 0.006
30	1.183± 0.036	1.172 ± 0.003	1.179 ± 0.003	1.241 ± 0.002

[#] Mean ± SD (n=3)

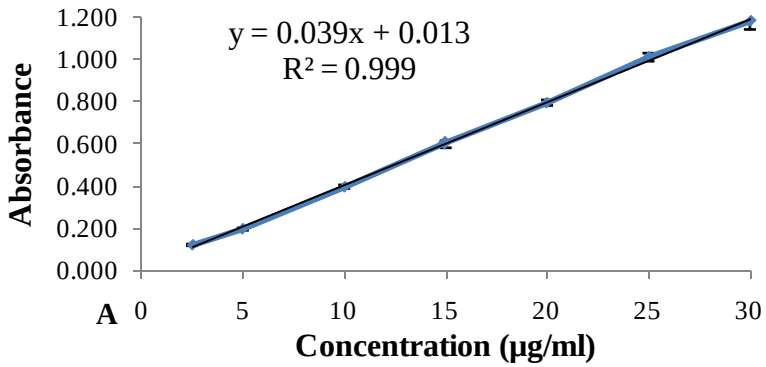
3.4.2. Validation of UV spectrophotometric methods

3.4.2.1. Linearity and Range

Regression analysis was performed on mean absorbance values of standard drug solutions in different media using MS office excel software 2007. As shown in Table 3.4.2, significantly high linear correlation ($R^2 \geq 0.999$) was evident among selected drug concentration range and their respective absorbance values for all the media. Range is defined as the interval between the upper and lower levels of analyte. These findings clearly indicated that Beer's law was obeyed in the drug concentration range of 2.5 to 30 µg/mL in all the respective media.

Table 3.4.2: Linear regression analysis of calibration data for DE in different media

Media	Calibration range (µg/mL)	Regression equation	Correlation coefficient (R^2)
0.01N hydrochloric acid	2.5-30	y = 0.039x - 0.013	0.999
Phosphate buffer pH 6.8	2.5-30	y = 0.038x - 0.036	0.999
Phosphate buffer pH 7.4	2.5-30	y = 0.038x - 0.032	0.999
Methanol	2.5-30	y = 0.041x - 0.025	0.999



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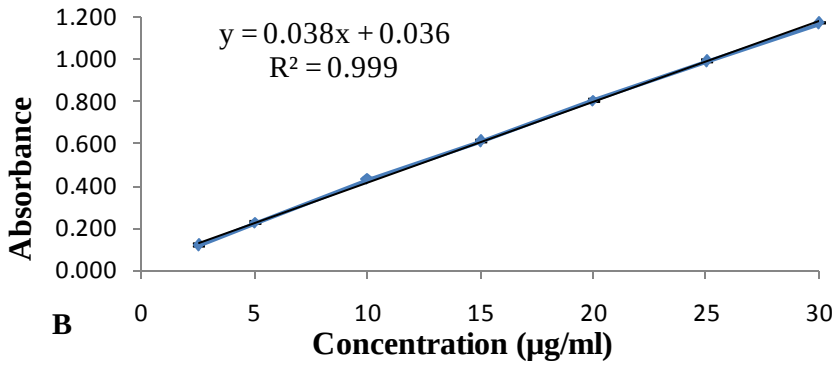

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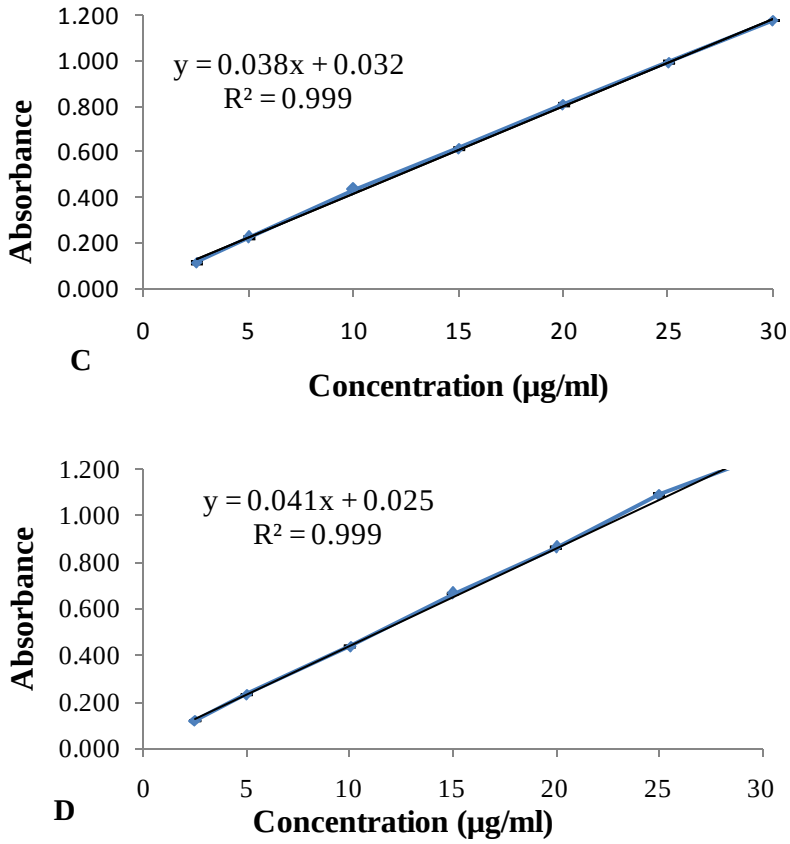


Figure 3.4.2: Calibration curves of DE in (A) 0.01N HCl, (B) phosphate buffer pH 6.8 (C) phosphate buffer pH 7.4 and (D) methanol

3.4.2.2. Stability

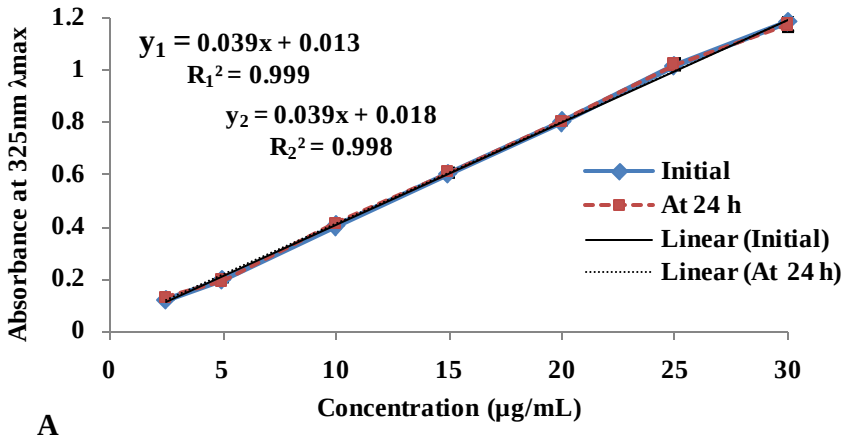
The stability of DE in all the four media was established over the period of 24h by measuring absorbance of the solution at 0 and 24 h. The results (Table 3.4.3) revealed insignificant difference between the initial and 24 h absorbance readings of same drug solutions. Regression analysis also showed insignificant change in the slope of straight line (Figure 3.4.3). Thus, it can be concluded that DE was stable in all four media over the period of analysis.

Table 3.4.3: Absorbance data of DE at 0 and 24 h for stability

Conc (µg/mL)	Absorbance[#]	
	Initial	**At 24 h**
0.01N hydrochloric acid		
2.5	0.124 ± 0.004	0.128 ± 0.006
5	0.200 ± 0.006	0.194 ± 0.009
10	0.401 ± 0.009	0.412 ± 0.011
15	0.602 ± 0.017	0.609 ± 0.013
20	0.799 ± 0.013	0.802 ± 0.012
25	1.015 ± 0.019	1.020 ± 0.021
30	1.183 ± 0.036	1.174 ± 0.031
Phosphate buffer pH 6.8		
2.5	0.119 ± 0.006	0.117 ± 0.005
5	0.226 ± 0.006	0.221± 0.004
10	0.433 ± 0.002	0.428 ± 0.003
15	0.612 ± 0.005	0.609 ± 0.002
20	0.805 ± 0.006	0.810 ± 0.007
25	0.992 ± 0.006	0.998 ± 0.005
30	1.172 ± 0.003	1.166 ± 0.004
Phosphate buffer pH 7.4		
2.5	0.114 ± 0.003	0.115 ± 0.003
5	0.223 ± 0.006	0.222± 0.005
10	0.435 ± 0.005	0.438 ± 0.004
15	0.615 ± 0.003	0.611 ± 0.002
20	0.809 ± 0.003	0.808 ± 0.005

25	0.995 ± 0.005	0.996 ± 0.004
30	1.179 ± 0.003	1.176 ± 0.003
Methanol		
2.5	0.122± 0.001	0.121 ± 0.002
5	0.234 ± 0.001	0.240 ± 0.003
10	0.441 ± 0.004	0.438 ± 0.007
15	0.667 ± 0.003	0.661 ± 0.009
20	0.865 ± 0.004	0.871 ± 0.008
25	1.092 ± 0.006	1.088 ± 0.003
30	1.241 ± 0.002	1.242 ± 0.005

[#] Mean ± SD (n = 3)



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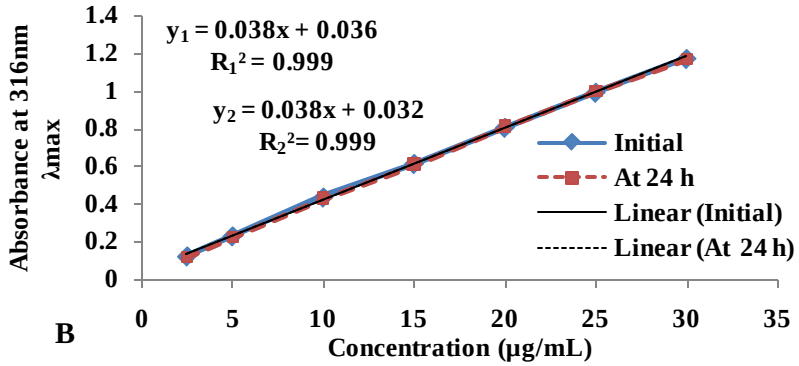

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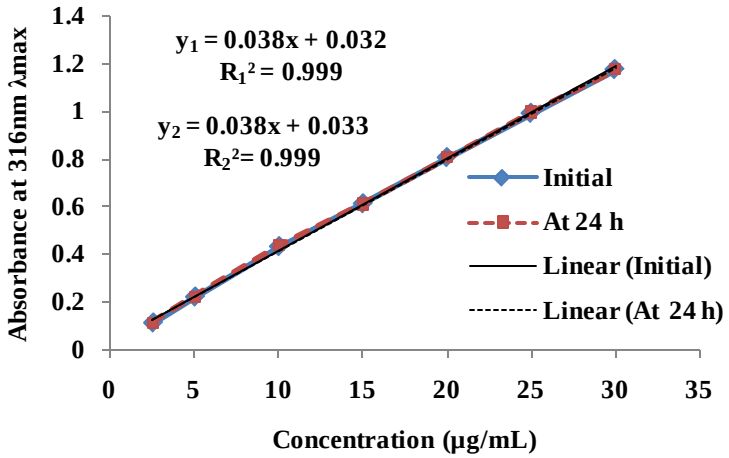

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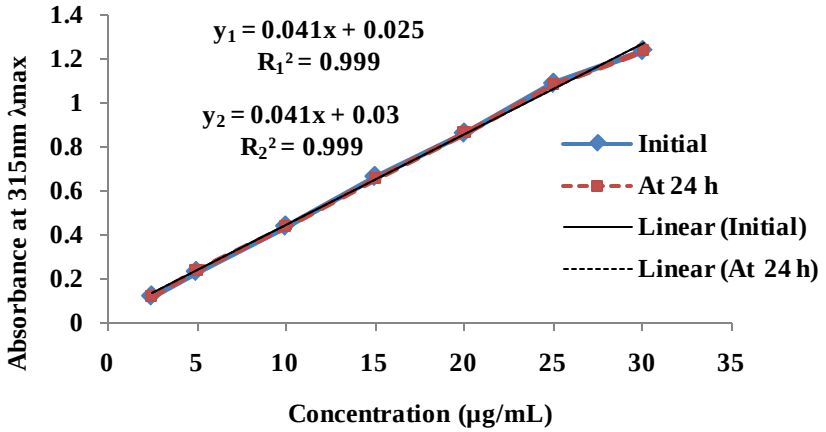

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Figure 3.4.3: Calibration curves of DE at 0 h and 24 h time points in (A) 0.01N HCl, (B) phosphate buffer pH 6.8 (C) phosphate buffer pH 6.8 and (D) methanol for stability [y_1, R_1= initial, y_2, R_2= at 24h]

3.4.2.3. Precision

It refers to the extent of variability of a group of measurements observed under similar conditions [16]. The data for intraday and interday precision are summarized in Table 3.4.4. The results were found to be precise under the same analytical working conditions over the interval of time. In addition, the RSD values obtained for the

analytical methods were within the acceptable range (< 5%) indicating that these methods are precise [14].

Table 3.4.4: Intraday and interday precision analysis of UV method for DE

Conc. (µg/mL)	Intraday Precision				Interday Precision			
	Absorbance			% RSD	Absorbance			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
0.01N hydrochloric acid								
2.5	0.123	0.129	0.121	**3.349**	0.125	0.129	0.131	**2.381**
5	0.205	0.201	0.193	**3.060**	0.192	0.191	0.201	**2.829**
10	0.391	0.408	0.403	**2.181**	0.400	0.398	0.402	**0.500**
15	0.621	0.596	0.589	**2.794**	0.584	0.581	0.584	**0.297**
20	0.814	0.789	0.795	**1.633**	0.772	0.787	0.775	**1.020**
25	1.012	0.998	1.036	**1.893**	0.978	1.026	0.989	**2.521**
30	1.222	1.152	1.174	**3.027**	1.130	1.163	1.154	**1.485**
Phosphate buffer pH 6.8								
2.5	0.121	0.119	0.116	**2.121**	0.113	0.114	0.111	**1.356**
5	0.227	0.219	0.231	**2.708**	0.225	0.215	0.226	**2.740**
10	0.433	0.439	0.428	**1.271**	0.431	0.441	0.432	**1.267**
15	0.614	0.611	0.61	**0.340**	0.615	0.609	0.614	**0.525**
20	0.804	0.800	0.81	**0.626**	0.806	0.805	0.809	**0.258**
25	0.999	0.99	0.987	**0.630**	0.997	0.991	0.992	**0.324**

30	1.178	1.171	1.167	**0.475**	1.175	1.177	1.179	**0.170**
Phosphate buffer pH 7.4								
2.5	0.114	0.111	0.116	**2.214**	0.116	0.121	0.117	**2.242**
5	0.223	0.224	0.229	**1.427**	0.224	0.219	0.221	**1.137**
10	0.435	0.431	0.429	**0.708**	0.435	0.441	0.432	**1.051**
15	0.614	0.619	0.613	**0.522**	0.615	0.609	0.614	**0.525**
20	0.809	0.805	0.811	**0.378**	0.809	0.811	0.804	**0.446**
25	0.995	0.991	0.988	**0.354**	0.992	0.996	0.989	**0.354**
30	1.174	1.169	1.173	**0.226**	1.179	1.171	1.174	**0.344**
Methanol								
2.5	0.122	0.121	0.123	**0.820**	0.121	0.123	0.125	**1.626**
5	0.234	0.233	0.235	**0.427**	0.239	0.237	0.238	**0.420**
10	0.440	0.438	0.445	**0.818**	0.445	0.441	0.449	**0.899**
15	0.667	0.67	0.665	**0.377**	0.661	0.668	0.664	**0.529**
20	0.865	0.869	0.861	**0.462**	0.859	0.861	0.855	**0.356**
25	1.099	1.088	1.089	**0.557**	1.087	1.089	1.081	**0.383**
30	1.261	1.258	1.259	**0.121**	1.258	1.252	1.261	**0.365**

3.4.2.4. Accuracy

Table 3.4.5 shows the data of recovery studies by standard addition method. The mean % recoveries for lower, intermediate and higher concentration are presented for all the media. The mean % recovery values, close to 100% with low standard deviation (SD < 0.4%) represent high accuracy of the analytical methods. These results revealed that

any small change in the drug concentration could accurately be determined by the proposed analytical methods.

Table 3.4.5: Standard addition data to measure accuracy of UV method in different media

Level (%)	Drug in solution (µg/mL)	conc. of drug added (µg/mL)	Total drug Recovered[#] (µg/mL)	% Analytical Recovery[#]
0.01N Hydrochloric acid				
80%	10	8	17.723 ± 0.018	98.46 ± 0.100
100%	10	10	19.932 ± 0.038	99.66 ± 0.190
120%	10	12	21.915± 0.026	99.61 ± 0.118
Phosphate buffer pH 6.8				
80%	10	8	17.856 ± 0.016	99.20 ± 0.089
100%	10	10	19.543 ± 0.027	97.72 ± 0.135
120%	10	12	21.842 ± 0.042	99.28 ± 0.191
Phosphate buffer pH 7.4				
80%	10	8	17.864 ± 0.012	99.24 ± 0.091
100%	10	10	19.621 ± 0.023	98.11 ± 0.132
120%	10	12	21.851 ± 0.042	99.32 ± 0.194
Methanol				
80%	10	8	17.736 ± 0.034	98.53 ± 0.189
100%	10	10	19.845 ± 0.055	99.23 ± 0.275
120%	10	12	21.768± 0.041	98.95 ± 0.186

[#]Mean ± SD (n = 3)

3.4.2.5. Sensitivity

LOD and LOQ were calculated using the formula as per equations 3.3.2 and 3.3.3.The values are presented in Table 3.4.6 for all the media. As indicated by the results, the concentration range selected for calibration was more than the LOD for all media. LOQ values also indicated the sensitivity of methods for accurate quantification of drug present in standard solutions [17].

Table 3.4.6: LOD and LOQ calculation from calibration data of DE in different media

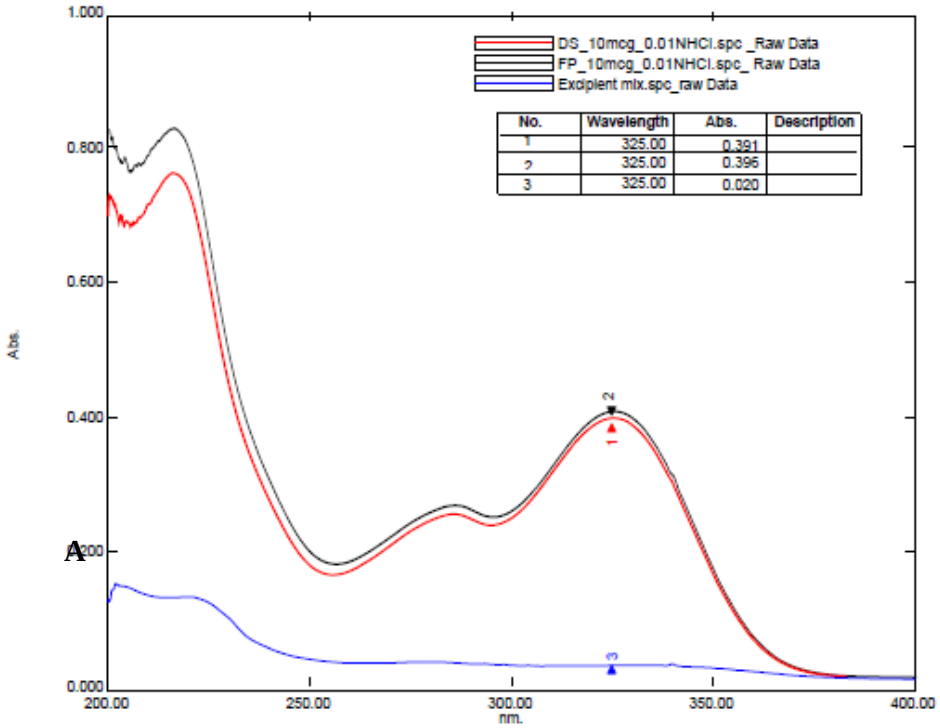
Media	Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
0.01N hydrochloric acid	0.041	0.012	1.048	3.176
Phosphate buffer pH 6.8	0.038	0.010	0.895	2.712
Phosphate buffer pH 7.4	0.038	0.011	0.996	3.018
Methanol	0.041	0.015	1.225	3.712

3.4.2.6. Interference and specificity study

The analytical specificity of these UV spectrophotometric methods were determined by comparing the absorbance values at 325nm, 316nm, 316nm and 315nm λ_{max} obtained for the drug solution, prototype formulation composition (Drug + Excipients) and excipient mixture (without drug) in 0.01N HCl, pH 6.8 phosphate buffer, pH 7.4 phosphate buffer and methanol respectively (Table 3.4.7). The representative graphs are shown in Figure 3.4.4. The absence of any overlapping or extraneous peaks in graph indicates the specificity of the UV method. Since DE peak could be clearly distinguished from the other peaks therefore, this method was considered to be specific for the analysis of DE.

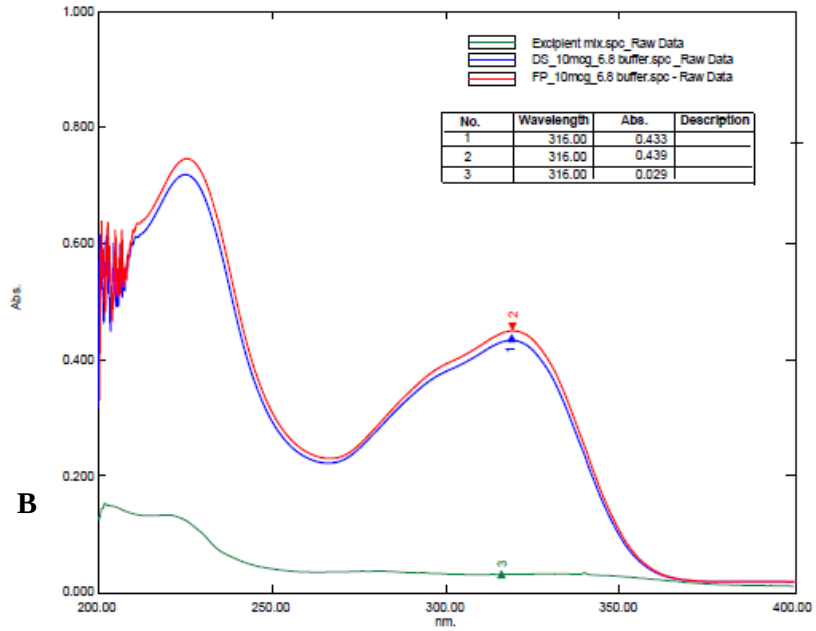
Table 3.4.7: Specificity and interference study of formulation components for DE

Media	Absorbance		
	Drug solution (10 µg/mL)	**Formulation prototype**	**Excipient mixture**
0.01N hydrochloric acid	0.391	0.396	0.020
Phosphate buffer pH 6.8	0.433	0.439	0.029
Phosphate buffer pH 7.4	0.435	0.440	0.028
Methanol	0.440	0.443	0.030

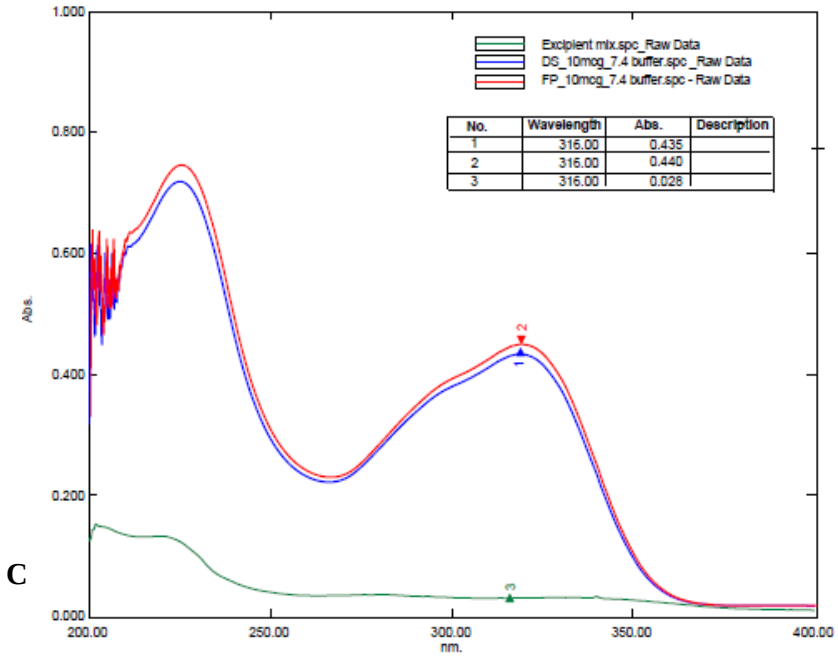


A

B


Excipient mix.spc_Raw Data
DS_10mcg_6.8 buffer.spc _Raw Data
FP_10mcg_6.8 buffer.spc - Raw Data
No. Wavelength Abs. Description
1 316.00 0.433
2 316.00 0.439
3 316.00 0.029
Abs.
nm.

C


Excipient mix.spc_Raw Data
DS_10mcg_7.4 buffer.spc _Raw Data
FP_10mcg_7.4 buffer.spc - Raw Data
No. Wavelength Abs. Description
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2 316.00 0.440
3 316.00 0.028
Abs.
nm.

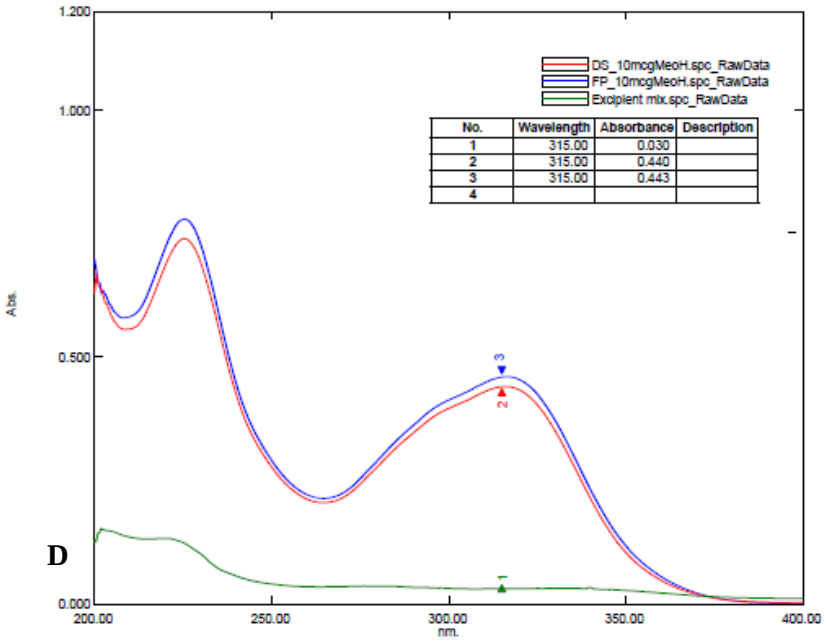


Figure 3.4.4: Specificity study graph between DE and excipients in (A) 0.01N HCl, (B) phosphate buffer pH 6.8, (C) phosphate buffer pH 7.4 (D) methanol

3.4.3. Estimation of DE by HPLC method

HPLC analytical method was developed and validated to estimate DE during permeability study in cell line studies for developed formulations [Self micro emulsifying Drug Delivery System-(SMEDDS) and Nanoemulsion (NE)]. Separation was attained using a mobile phase consisting of acetonitrile and phosphate buffer (pH 5.8) in the ratio of 60:40 (% v/v), pumped at a flow rate of 1 mL/ min. The retention time of DE was found to be 4.60 min at 230 nm for a run time of 10 min. Injections of 20 µl were made for each concentrations and peak area was noted (Table 3.4.8). Chromatogram was drawn at 10µg/mL at RT of 4.6 (Figure 3.4.5). All the readings were taken in triplicate. Calibration curve was plotted between concentration and peak area of DE (Figure 3.4.6). R^2 was found to be 1 in regression equation.

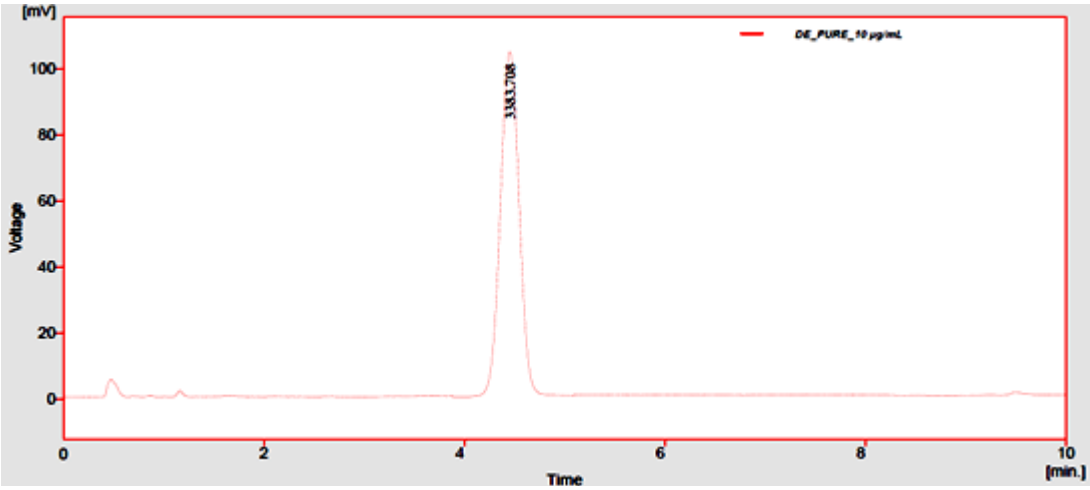


Figure 3.4.5. Chromatogram of DE solution by HPLC at 230nm

Table 3.4.8: Calibration data for estimation of DE by HPLC at 230 nm

Concentration (µg/mL)	RT	Peak area (µV*S)[#]
1.0	4.601	511.12 ± 10.22
2.5	4.613	996.41 ± 29.89
5.0	4.605	1787.44 ± 62.56
7.5	4.602	2592.44 ± 95.92
10.0	4.614	3393.70 ± 111.99

[#] Mean ± SD (n=3)

3.4.3.1. Validation of HPLC method

The HPLC method was validated for accuracy, precision, stability, sensitivity (LOD, LOQ) and linearity.

3.4.3.1.1. Linearity

Linearity of HPLC method for the estimation of DE was established by regression coefficient. Least square regression method was used to determine the regression coefficient, (R) and the equation for the best fitting line. Figure 3.4.6 shows linear regression equation with R^2 value equal to 1 which reflects linear correlation of the method used. Hence, Beer's law was found to be obeyed in the range of 1 to 10 µg/mL.

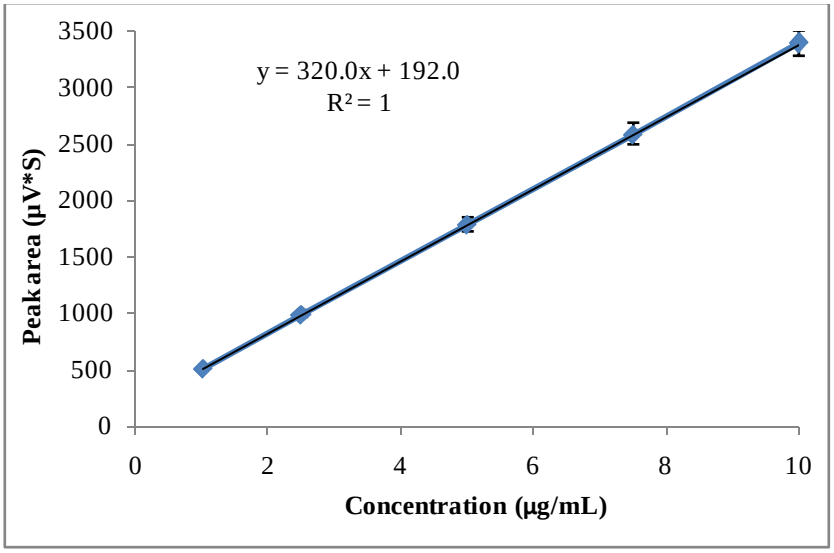


Figure 3.4.6. : Calibration curve of DE by HPLC at 230 nm

3.4.3.1.2. Accuracy

Accuracy of DE at three levels of standard addition (80%, 100%, and 120%) was done to confirm its recovery. The excellent recoveries of standard addition method with low %RSD justified the accuracy of the method (Table 3.4.9). These results revealed that any small change in the drug concentration could accurately be determined by the proposed analytical method.

Table 3.4.9: Accuracy of HPLC method for DE at 230nm

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found (µg/mL)	% Analytical Recovery
80%	1	0.8	1.78 ± 0.51	99.39 ± 0.47
100%	1	1.0	1.99 ± 0.97	99.80 ± 0.39
120%	1	1.2	2.19 ± 0.93	99.91 ± 0.45

3.4.3.1.3. Precision

The developed method was validated for intra-day and inter-day precision (3 consecutive days, n=3) at three different test concentrations. The average % RSD of intra-day and inter-day precision were found to be <5.0% which complies with the

standard acceptance limit of 5.0% and confirmed the precision of the method (Table 3.4.10).

Table 3.4.10: Intraday and interday precision analysis of HPLC method

Conc. (µg/mL)	Intraday Precision				Interday Precision			
	Peak area (µV*S)			% RSD	Peak area (µV*S)			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
1.0	519.25	512.25	513.84	**0.71**	504.25	502.74	506.35	**0.36**
2.5	1013.12	998.11	1011.65	**0.82**	997.32	992.51	999.6	**0.36**
5.0	1799.98	1788.65	1786.24	**0.41**	1800.56	1794.30	1794.68	**0.19**
7.5	2501.36	2515.65	2514.71	**0.31**	2518.87	2511.02	2512.25	**0.16**
10.0	3374.65	3371.54	3369.78	**0.07**	3371.24	3371.87	3376.36	**0.08**

3.4.3.1.4. Stability

The HPLC method was found to be robust when the stability of same DE solution was determined at 0 and 24h. The peak area was noted at different concentrations (Table 3.4.11) and calibration curve were plotted (Figure 3.4.6) and regression equation was determined. R^2 value of 1 suggests stability of drug solutions even after 24h. There is insignificant change in regression equation. Thus, it can be concluded that DE solution was stable over the period of analysis.

Table 3.4.11: Absorbance data of DE at 0 and 24 h for stability

Concentration (µg/mL)	Peak area (µV*S)[#]	
	Initial	At 24 h
1.0	511.12 ± 11.19	516.211 ± 10.32
2.5	996.41 ± 27.89	990.214 ± 29.70
5.0	1787.44 ± 65.56	1779.786 ± 62.29
7.5	2592.44 ± 90.92	2597.487 ± 96.10
10.0	3393.70 ± 109.99	3398.546 ± 115.15

[#]Mean ± SD (n = 3)

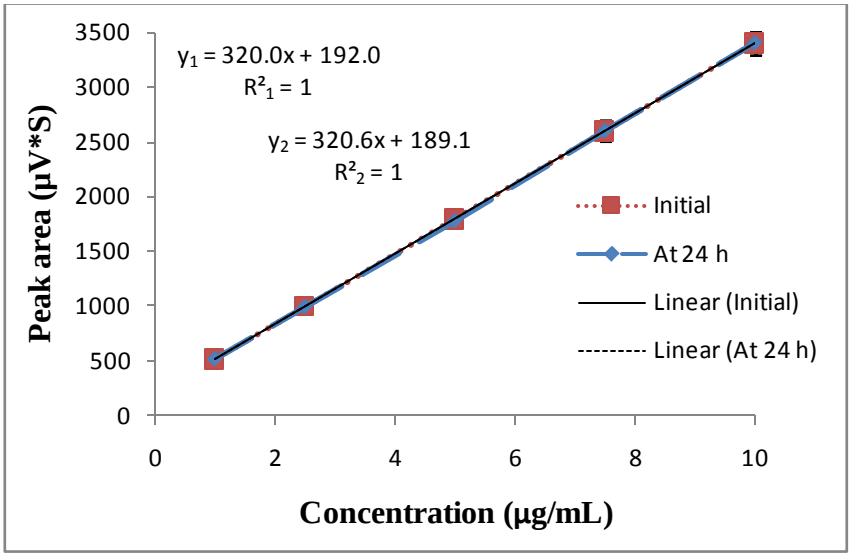


Figure 3.4.7.: Calibration curves of DE at 0 h and 24 h time points for stability by HPLC at 230 nm [y_1, R_1= initial, y_2, R_2= at 24h]

3.4.3.1.5. Sensitivity

The LOQ and LOD were determined based on a signal to noise (S/N) ratios and were based on analytical responses of 10 and 3 times the background noise respectively [18]. LOD and LOQ were calculated using the formulae described in section 3.3.2. Results indicate the sensitivity of methods for accurate quantification of drug present in standard solutions by HPLC (Table 3.4.12).

Table 3.4.12: LOD and LOQ calculation from calibration curve of DE by HPLC

Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
320.0	3.82	38.67	117.19

3.4.4. Estimation of DE in plasma by HPLC method

Quantitative estimation of DE in plasma was based on reverse phase chromatography using C18 column and 2cm guard column. Protein precipitation method was employed to separate the DE from plasma. The elution was done with the flow rate of 1.0 mL/min of mobile phase. The retention time of DE in plasma was also found to be 4.60 min at 230 nm of detection with a run time of 10 min. Chromatograph was drawn with 2.0 µg/mL concentration to elicit retention time (Figure 3.4.8). Peak area at different concentrations was noted in table 3.4.13. All the readings were taken in triplicate.

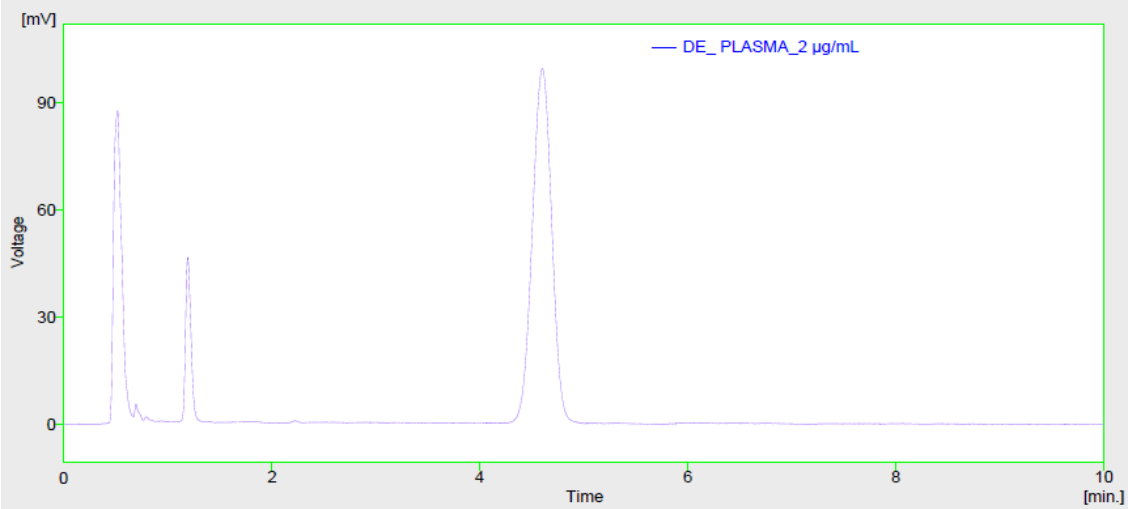


Figure 3.4.8: Chromatograph of DE in plasma by HPLC at 230nm

Table 3.4.13: Calibration data for estimation of DE in plasma by HPLC at 230 nm

Concentration (µg/mL)	RT	Peak area (µV*S)[#]
0.05	4.612	53.12 ± 4.84
0.10	4.602	105.23 ± 9.70
0.50	4.614	507.24 ± 24.36
1.00	4.603	1025.69 ± 28.11
2.000	4.605	2099.12 ± 35.23

[#] Mean ± SD (n=3)

3.4.4.1. Validation of HPLC method of DE in plasma

The HPLC method in plasma was validated for accuracy, precision, stability, sensitivity (LOD, LOQ) and linearity.

3.4.4.1.1. Linearity

The mean peak area values for DE in plasma by HPLC method are shown in Table 3.4.13. The value of correlation coefficient (R^2 = 0.999) indicated that area and concentration of the drug was in significant linear correlation (Figure 3.4.9). Beer's law was found to be obeyed in the range of 0.05 to 2 µg/mL. Results indicated the linearity of the method for selected calibration range**.**

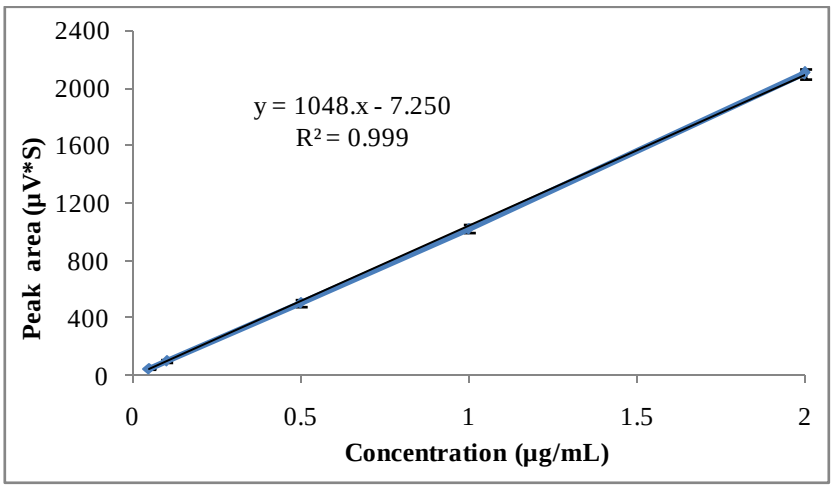


Figure 3.4.9. : Calibration curve of DE in plasma by HPLC at 230 nm

3.4.4.1.2. Stability

Peak area of same solutions at 0 and 24 h was measured using HPLC for stability study. Insignificant difference was observed between the initial and 24 h readings (Table 3.4.14). Regression analysis also showed insignificant change in the slope of straight line (Figure 3.4.10). Thus, it can be concluded that DE solution was robust throughout the period of analysis.

Table 3.4.14: Absorbance data of DE in plasma for stability using HPLC at 230nm

Concentration (µg/mL)	Peak area (µV*S)#	
	Initial	At 24 h
0.05	53.12 ± 4.84	2102.12 ± 8.26
0.1	105.23 ± 9.70	2102.12 ± 9.21
0.5	507.24 ± 24.36	2102.12 ± 26.47
1	1025.69 ± 28.11	2102.12 ± 29.71
2	2099.12 ± 35.23	2102.12 ± 37.54

#Mean ± SD (n = 3)

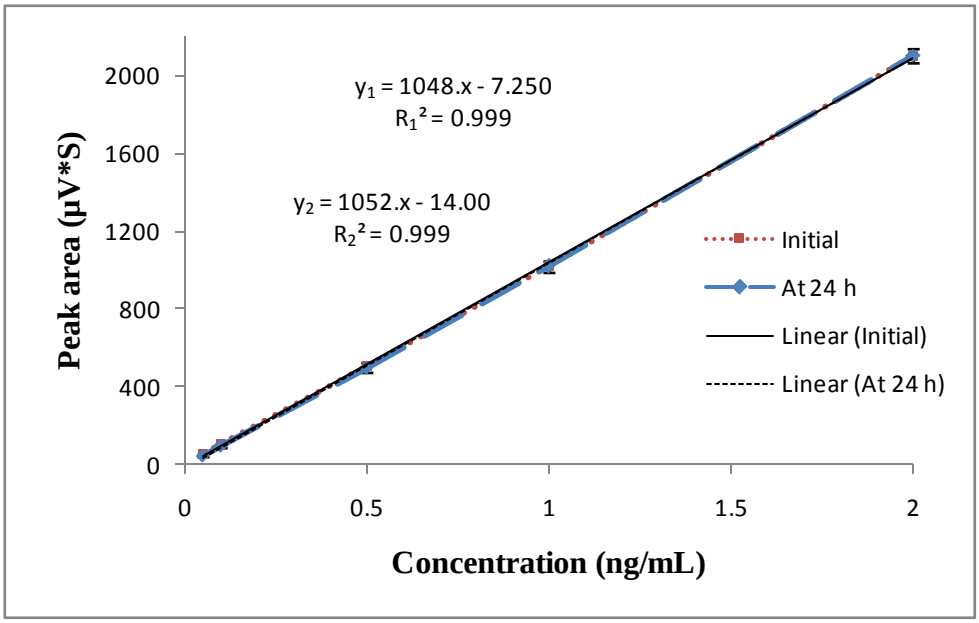


Figure 3.4.10: Calibration curves of DE at 0 h and 24 h time points for stability by HPLC in plasma at 230 nm [y_1, R_1= initial, y_2, R_2= at 24h]

3.4.4.1.3. Precision

The results for intraday and interday precision are summarized in Table 3.4.15. The data shows that analytical method adopted was precise under the same operating conditions over the interval of time. In addition, the RSD values were also within the acceptable range demonstrating that the methods followed were precise.

Table 3.4.15: Intraday and interday precision analysis of HPLC method in plasma

Conc. (µg/mL)	Intraday Precision				Interday Precision			
	Peak area (µV*S)			% RSD	Peak area (µV*S)			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
0.05	53.12	51.36	55.69	**4.07**	49.12	50.25	53.69	**4.67**
0.1	105.23	101.65	107.34	**2.74**	99.23	102.84	103.35	**2.21**
0.5	507.24	512.23	504.12	**0.80**	501.24	498.58	502.14	**0.37**
1	1025.69	1031.41	1028.98	**0.27**	1018.69	1014.36	1021.36	**0.35**
2	2099.12	2101.22	2098.64	**0.06**	2102.12	2004.39	2106.74	**2.79**

3.4.4.1.4. Accuracy

Recovery studies were carried out by standard addition or spiking method. Table 3.4.16 presents the mean % recoveries for lower, intermediate and higher concentration. The % recovery values, close to 100% represent high accuracy of the analytical method. These results revealed that any small change in the drug concentration possibly will accurately be determined by the proposed analytical method.

Table 3.4.16: Standard addition data to measure accuracy of HPLC method for DE in plasma

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found (µg/mL)	% Analytical Recovery
80%	0.05	0.04	0.0892 ± 0.01	99.11 ± 0.31
100%	0.05	0.05	0.0994 ± 0.03	99.40 ± 0.23
120%	0.05	0.06	0.1093 ± 0.02	99.36 ± 0.22

3.4.4.1.5. Sensitivity

LOD and LOQ were calculated using the formula described in section 3.3.2.5 and the values are presented in Table 3.4.17. LOD and LOQ values indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

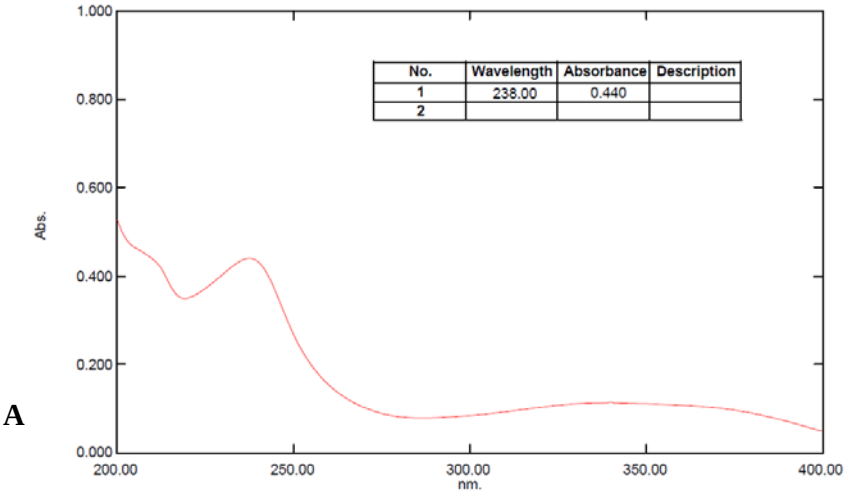
Table 3.4.17: LOD and LOQ calculation from calibration curve of DE in plasma by HPLC

Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
1048.0	13.49	0.042	0.128

3.4.5. Estimation of NISO by UV spectrophotometry

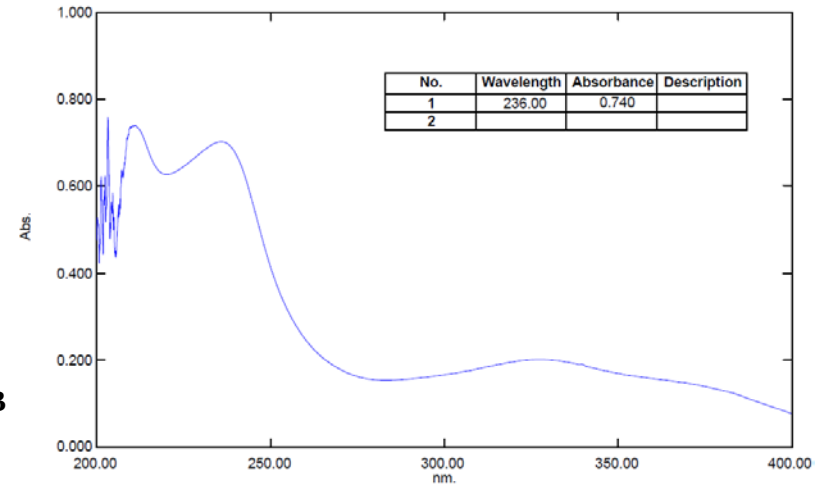
The UV spectrum obtained by scanning the standard solutions of NISO in 0.1N HCl with 0.5% SLS, pH 6.8 phosphate buffer with 0.5% SLS, pH 7.4 phosphate buffer + 0.5% SLS and methanol showed absorption maxima at 238nm, 236 nm, 236 nm and 236 nm respectively (Figure 3.4.11) and hence were selected as analytical wavelengths for respective media.

Data Set: Niso_10mcg_0.1NHCl+0.5%SLS.spc - RawData



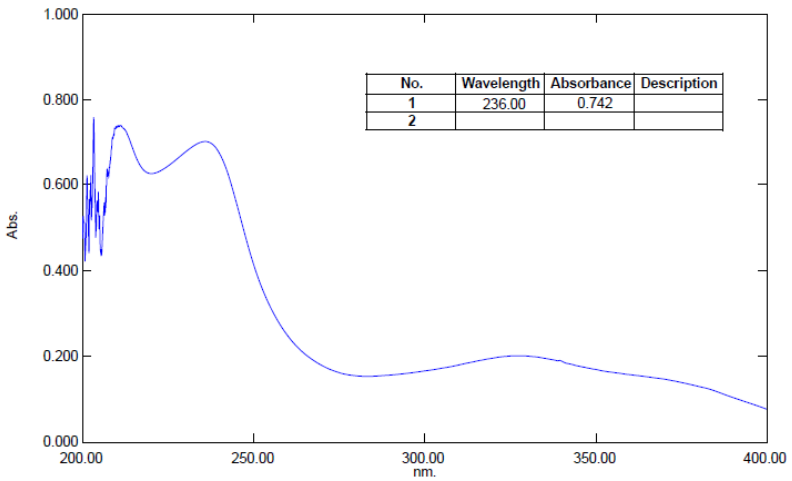
A

Data Set: Niso_10mcg_ 6.8Buffer+0.5%SLS.spc - RawData



B

Data Set: Niso_10mcg_ 7.4Buffer+0.5%SLS.spc - RawData



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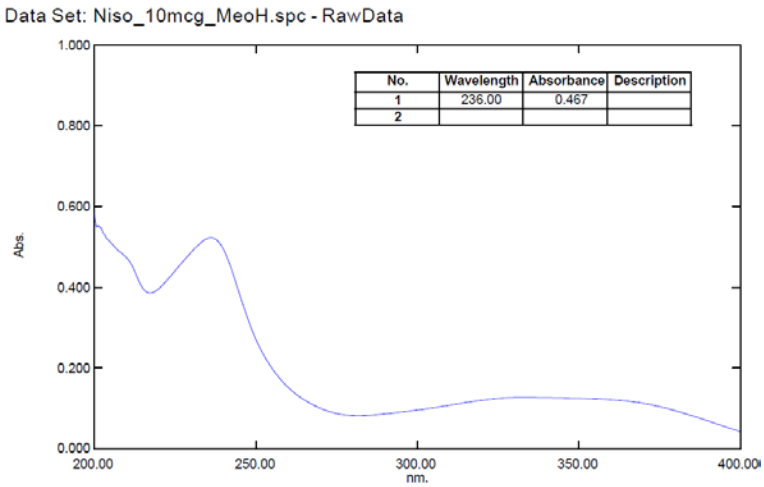


D

Figure 3.4.11: UV Absorption Spectrum of NISO in (A) 0.1N HCl with 0.5% SLS, (B) Phosphate buffer pH 6.8 with 0.5% SLS (C) Phosphate buffer pH 7.4 with 0.5% SLS and (D) Methanol

The absorbance of standard drug solutions was measured at their particular λ_{max} to prepare calibration curves in 0.1N HCl with 0.5% SLS, methanol (Table 3.4.18) and in pH 6.8 phosphate buffer, pH 7.4 phosphate buffer with 0.5% SLS (Table 3.4.19). Standard calibration curves in various media with their regression equations and correlation coefficients (R^2) (Figure 3.4.12) were drawn.

Table 3.4.18: UV spectrophotometric calibration data for NISO in 0.1N HCl + 0.5% SLS and Methanol

Concentration (µg/mL)	Absorbance[#]	
	0.1N HCl with 0.5% SLS	Methanol
2.5	0.120 ± 0.028	0.131 ± 0.024
5	0.217 ± 0.018	0.253 ± 0.019
10	0.448 ± 0.012	0.467 ± 0.010
15	0.646 ± 0.008	0.712 ± 0.009
20	0.895 ± 0.006	0.949 ± 0.002
25	1.100 ± 0.005	1.167 ± 0.002
30	1.318 ± 0.003	1.385 ± 0.008

[#] Mean ± SD (n=3)

Table 3.4.19: UV spectrophotometric calibration data for NISO in phosphate buffer pH 6.8 with 0.5% SLS and phosphate buffer pH 7.4 with 0.5% SLS

Concentration (µg/mL)	Absorbance[#]	
	Phosphate buffer pH 6.8 with 0.5% SLS	Phosphate buffer pH 7.4 with 0.5% SLS
2	0.125 ± 0.015	0.123 ± 0.014
4	0.280 ± 0.013	0.284 ± 0.011
6	0.436 ± 0.012	0.432 ± 0.013
8	0.617 ± 0.003	0.612 ± 0.014
10	0.740 ± 0.003	0.742 ± 0.001
12	0.906 ± 0.002	0.910 ± 0.011
14	1.078 ± 0.001	1.081 ± 0.002
16	1.209 ± 0.002	1.211 ± 0.004

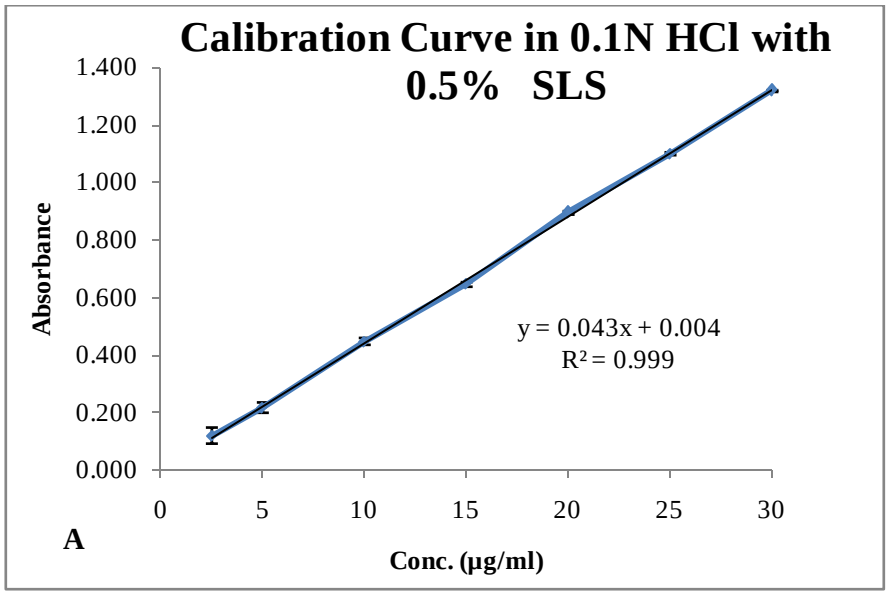
3.4.5.1. Validation of UV spectrophotometric methods

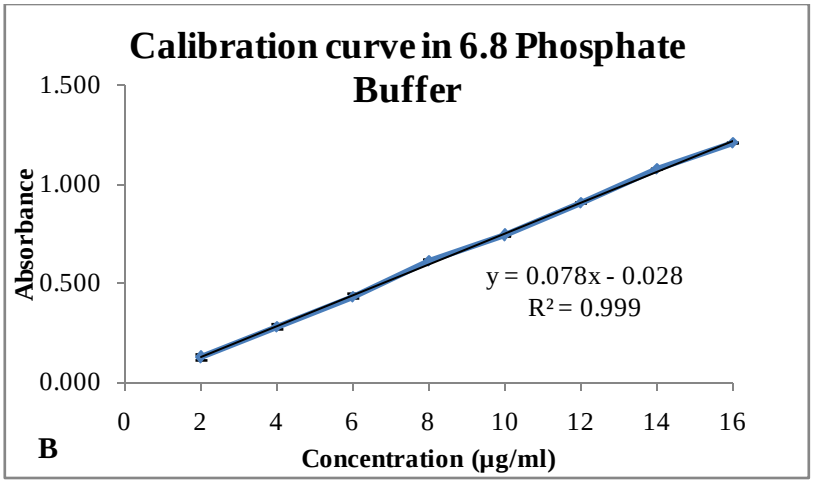
3.4.5.1.1. Linearity

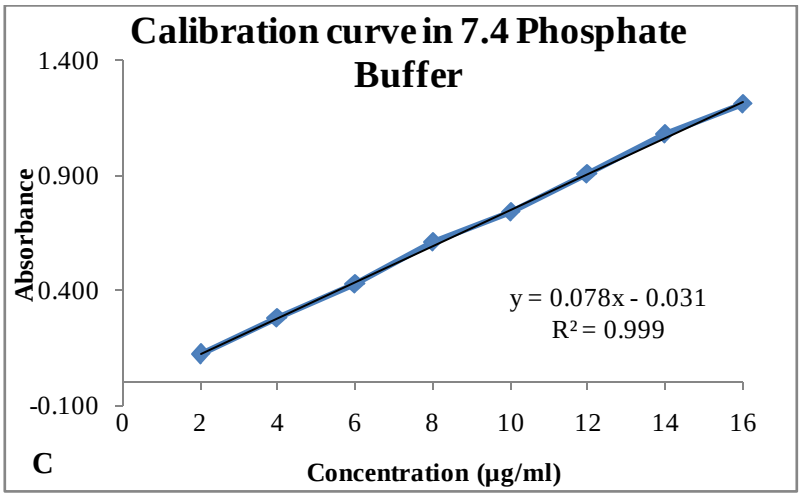
For all the four media's, significantly high linear correlation ($R^2 \geq 0.999$) was observed (Table 3.4.15) for the selected drug concentration range and their respective absorbance values. Regression analysis was performed using MS office excel software 2007. These findings clearly indicated that Beer's law was obeyed in the drug concentration range of 2.5 to 30 µg/mL in 0.1N hydrochloric acid with 0.5% SLS, methanol and 2-16 µg/mL in Phosphate buffer pH 6.8 with 0.5% SLS, Phosphate buffer pH 7.4 with 0.5% SLS .

Table 3.4.20: Linear regression analysis of calibration data for NISO in different media

Media	λ_{max} (nm)	Calibration range (µg/mL)	Regression equation	Correlation coefficient (R^2)
0.1N hydrochloric acid with 0.5% SLS	238	2.5 - 30	y = 0.043x + 0.004	0.999
Phosphate buffer pH 6.8 with 0.5% SLS	236	2 - 16	y = 0.078x - 0.028	0.999
Phosphate buffer pH 7.4 with 0.5% SLS	236	2 - 16	y = 0.078x - 0.031	0.999
Methanol	236	2.5 - 30	y = 0.045x + 0.020	0.999







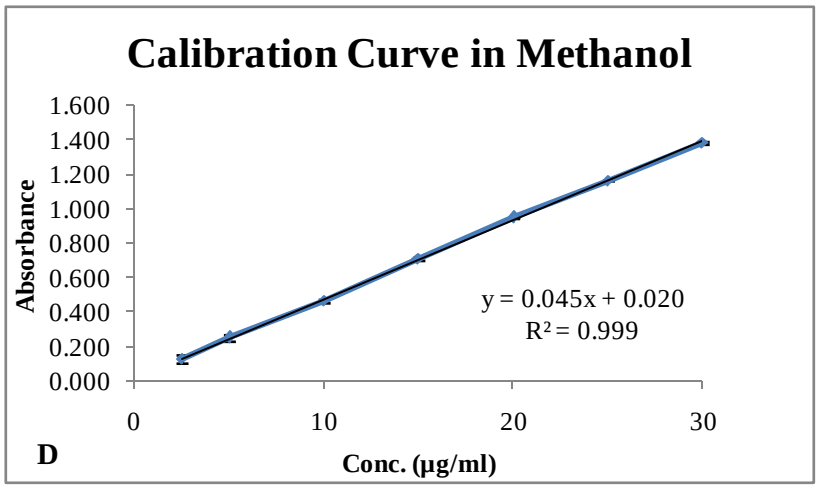


Figure 3.4.12: Calibration curves of NISO in (A) 0.1N HCl with 0.5% SLS (B) Phosphate buffer pH 6.8 with 0.5% SLS, (C) Phosphate buffer pH 6.8 with 0.5% SLS and (D) Methanol

3.4.5.1.2. Stability

The results of stability (Table 3.4.21) revealed insignificant difference between the initial and 24 h absorbance readings for the same drug solutions in different media. Insignificant change in the slope of straight line (Figure 3.4.13) confirmed stability of drug in different media up to 24 h. Thus, it can be concluded that NISO was stable in all four media over the period of analysis.

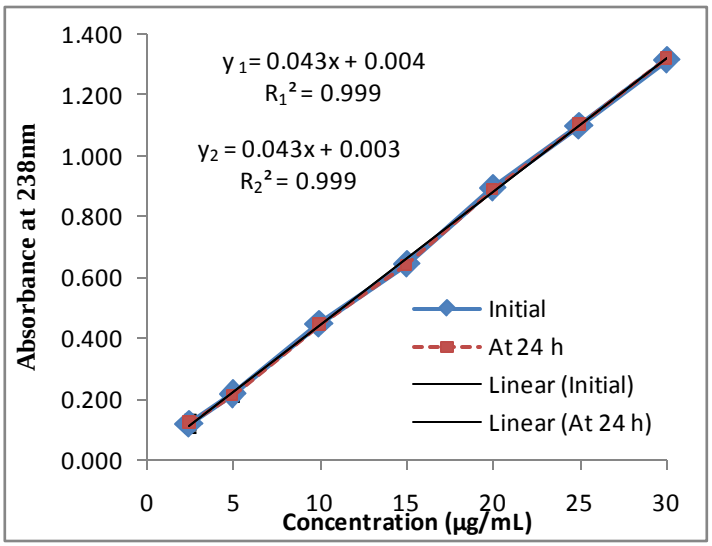
Table 3.4.21: Absorbance data of NISO at 0 and 24 h for Stability

Conc. (µg/mL)	Absorbance[#]	
	Initial	At 24 h
0.1N HCl with 0.5% SLS		
2.5	0.120 ± 0.028	0.126 ± 0.024
5	0.217 ± 0.018	0.213 ± 0.019
10	0.448 ± 0.012	0.447 ± 0.011
15	0.646 ± 0.008	0.641 ± 0.009
20	0.895 ± 0.006	0.891± 0.007
25	1.100 ± 0.005	1.103 ± 0.008
30	1.318 ± 0.003	1.320 ± 0.002
Phosphate buffer pH 6.8 with 0.5% SLS		
2	0.125 ± 0.015	0.127 ± 0.016
4	0.280 ± 0.013	0.276 ± 0.014
6	0.436 ± 0.012	0.431 ± 0.009
8	0.617 ± 0.003	0.621± 0.002
10	0.740 ± 0.003	0.742 ± 0.004
12	0.906 ± 0.002	0.911 ± 0.003

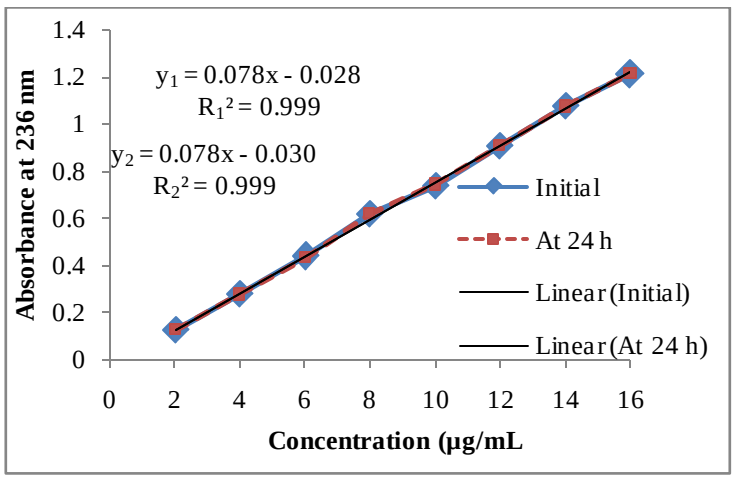
14	1.078 ± 0.001	1.074 ± 0.002
16	1.209 ± 0.002	1.213 ± 0.001
Phosphate buffer pH 7.4 with 0.5% SLS		
2	0.123 ± 0.014	0.124 ± 0.011
4	0.284 ± 0.011	0.281 ± 0.008
6	0.432 ± 0.013	0.434 ± 0.012
8	0.612 ± 0.014	0.618± 0.007
10	0.742 ± 0.001	0.746 ± 0.012
12	0.910 ± 0.011	0.907 ± 0.009
14	1.081 ± 0.002	1.084 ± 0.014
16	1.211 ± 0.004	1.215 ± 0.011
Methanol		
2.5	0.131 ± 0.024	0.133 ± 0.014
5	0.253 ± 0.019	0.251 ± 0.011
10	0.467 ± 0.010	0.472 ± 0.011
15	0.712 ± 0.009	0.718 ± 0.010
20	0.949 ± 0.002	0.946 ± 0.009
25	1.167 ± 0.002	1.171 ± 0.005
30	1.385 ± 0.008	1.381 ± 0.003

[#] Mean ± SD (n = 3)

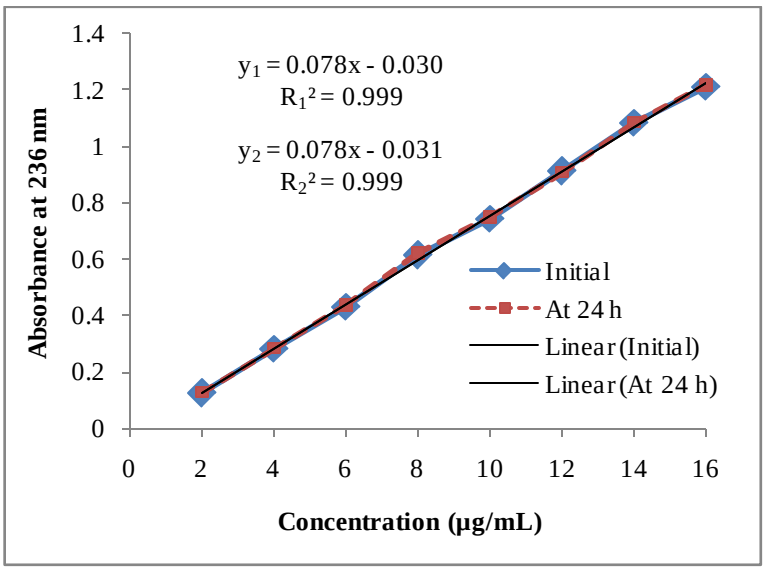
A


y 1= 0.043x + 0.004
R1² = 0.999
y2 = 0.043x + 0.003
R2² = 0.999
Absorbance at 238nm
Concentration (µg/mL)
Initial
At 24 h
Linear (Initial)
Linear (At 24 h)

B


y1 = 0.078x - 0.028
R1² = 0.999
y2 = 0.078x - 0.030
R2² = 0.999
Absorbance at 236 nm
Concentration (µg/mL
Initial
At 24 h
Linear (Initial)
Linear (At 24 h)

C


y1 = 0.078x - 0.030
R1² = 0.999
y2 = 0.078x - 0.031
R2² = 0.999
Absorbance at 236 nm
Concentration (µg/mL)
Initial
At 24 h
Linear (Initial)
Linear (At 24 h)

D

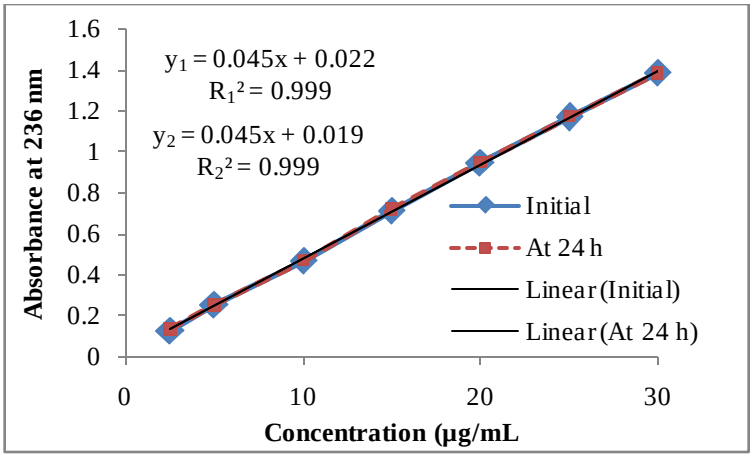


Figure 3.4.13: Calibration curves of NISO at 0 h and 24 h time points in (A) 0.1N HCl with 0.5% SLS, (B) phosphate buffer pH 6.8 with 0.5% SLS, (C) phosphate buffer pH 7.4 with 0.5% SLS and (D) methanol for stability study [y_1, R_1= initial, y_2, R_2= at 24h]

3.4.5.1.3. Precision

The results for intraday and interday precision were found to be precise under the same operating conditions over the interval of time (Table 3.4.22). The RSD values obtained for the analytical methods were within the acceptable range (< 2%) representing that the methods selected are precise [14].

Table 3.4.22: Intraday and Interday Precision Analysis of UV Method for NISO

Conc. (µg/mL)	Intraday Precision				Interday Precision			
	Absorbance			% RSD	Absorbance			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
0.1N hydrochloric acid with 5% SLS								
2.5	0.120	0.123	0.119	**1.725**	0.126	0.129	0.131	**1.956**
5	0.217	0.221	0.222	**1.203**	0.213	0.218	0.211	**1.685**
10	0.448	0.451	0.445	**0.670**	0.447	0.451	0.441	**1.128**
15	0.646	0.645	0.642	**0.323**	0.641	0.649	0.643	**0.646**

20	0.895	0.893	0.898	**0.281**	0.891	0.888	0.899	**0.637**
25	1.100	1.102	1.103	**0.139**	1.103	1.111	1.105	**0.376**
30	1.318	1.315	1.317	**0.116**	1.320	1.324	1.317	**0.266**
Phosphate buffer pH 6.8 with 0.5% SLS								
2	0.125	0.129	0.128	**1.635**	0.127	0.129	0.131	**1.550**
4	0.280	0.287	0.277	**1.824**	0.276	0.280	0.275	**0.955**
6	0.436	0.431	0.429	**0.835**	0.431	0.435	0.427	**0.928**
8	0.617	0.609	0.614	**0.659**	0.621	0.617	0.627	**0.810**
10	0.74	0.745	0.737	**0.546**	0.742	0.736	0.744	**0.562**
12	0.906	0.901	0.909	**0.446**	0.911	0.917	0.912	**0.352**
14	1.078	1.081	1.074	**0.326**	1.074	1.071	1.077	**0.279**
16	1.209	1.204	1.211	**0.298**	1.213	1.214	1.217	**0.171**
Phosphate buffer pH 7.4 with 0.5% SLS								
2	0.124	0.131	0.129	**0.128**	0.129	0.132	0.134	**0.132**
4	0.287	0.281	0.279	**0.282**	0.272	0.284	0.277	**0.278**
6	0.434	0.433	0.425	**0.431**	0.433	0.434	0.429	**0.432**
8	0.621	0.612	0.607	**0.613**	0.617	0.621	0.624	**0.621**
10	0.742	0.748	0.741	**0.744**	0.744	0.739	0.748	**0.744**
12	0.911	0.907	0.913	**0.910**	0.909	0.918	0.914	**0.914**

14	1.081	1.085	1.071	**1.079**	1.072	1.075	1.079	**1.075**
16	1.215	1.21	1.213	**1.213**	1.212	1.211	1.213	**1.212**
Methanol								
2.5	0.131	0.129	0.134	**1.916**	0.133	0.134	0.130	**1.573**
5	0.253	0.248	0.254	**1.277**	0.251	0.2487	0.254	**1.058**
10	0.467	0.462	0.469	**0.774**	0.472	0.477	0.469	**0.855**
15	0.712	0.718	0.71	**0.584**	0.718	0.714	0.711	**0.492**
20	0.949	0.941	0.947	**0.440**	0.946	0.941	0.949	**0.428**
25	1.167	1.161	1.163	**0.263**	1.171	1.174	1.165	**0.392**
30	1.385	1.388	1.381	**0.254**	1.381	1.377	1.384	**0.254**

3.4.5.1.4. Accuracy

Standard addition or spiking method was used for recovery study. The mean % recoveries for lower, intermediate and higher concentration were presented for all the four media (Table 3.4.23). High accuracy of the analytical method is reflecting from the mean % recovery values, which are close to 100% with less standard deviation (SD < 0.2%) values. Hence, these results revealed that any small change in the drug concentration could accurately be determined by the proposed analytical methods.

Table 3.4.23: Standard addition data to measure accuracy of UV Method for NISO

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found[#] (µg/mL)	% Analytical Recovery[#]
0.1N hydrochloric acid with 0.5% SLS				
80%	10	8	17.821 ± 0.019	99.01 ± 0.137
100%	10	10	19.922 ± 0.034	99.61 ± 0.119
120%	10	12	21.953 ± 0.022	99.79 ± 0.153
Phosphate buffer pH 6.8 with 0.5% SLS				
80%	10	8	17.861 ± 0.016	99.23 ± 0.128
100%	10	10	19.654 ± 0.023	98.27 ± 0.115
120%	10	12	21.953 ± 0.041	99.25 ± 0.116
Phosphate buffer pH 7.4 with 0.5% SLS				
80%	10	8	17.873 ± 0.012	99.29 ± 0.121
100%	10	10	19.802 ± 0.019	99.01 ± 0.109
120%	10	12	21.962 ± 0.039	99.83 ± 0.114
Methanol				
80%	10	8	17.812 ± 0.046	98.96 ± 0.613
100%	10	10	19.887 ± 0.054	99.44 ± 0.131
120%	10	12	21.799 ± 0.032	99.09 ± 0.112

[#]Mean ± SD (n = 3)

3.4.5.1.5. Sensitivity

Results of LOD indicated that the concentration range selected for calibration was well above its limit of detection for all four media. LOQ values represents the

sensitivity of methods for accurate quantification of drug present in standard solutions (Table 3.4.24).

Table 3.4.24: LOD and LOQ calculation from calibration data of NISO in different media

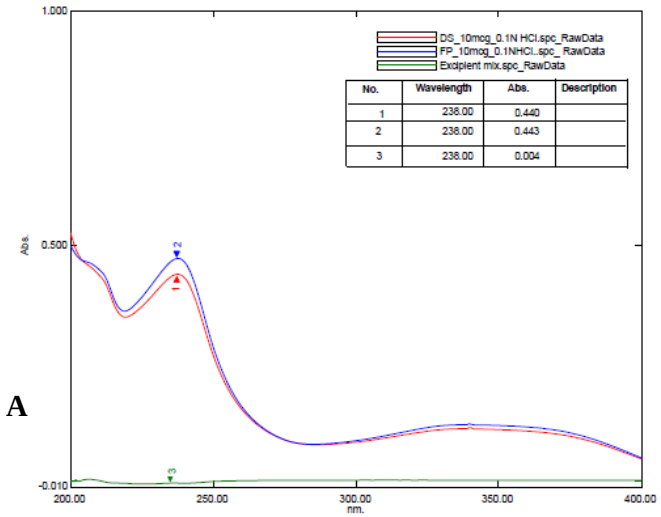
Media	Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
0.1N hydrochloric acid with 0.5% SLS	0.043	0.011	0.809	2.452
Phosphate buffer pH 6.8 with 0.5% SLS	0.078	0.013	0.531	1.610
Phosphate buffer pH 7.4 with 0.5% SLS	0.078	0.011	0.487	1.476
Methanol	0.045	0.009	0.679	2.056

3.4.5.1.6. Interference and specificity study

No interference was observed due to excipients and formulation prototype determined by comparing the absorbance values obtained for the drug solution in selected media determined using UV spectrophotometric methods (Table 3.4.25). The absence of any overlapping or extraneous peaks in graph indicated the specificity of the UV spectrophotometric method (Figure 3.4.14). Since NISO peak could be clearly distinguished from the other peaks therefore, this method was said to be specific for the analysis of NISO.

Table 3.4.25: Specificity and interference study of formulation components for NISO

Media	Absorbance		
	Drug solution (10 µg/mL)	**Formulation prototype**	**Excipient mixture**
0.1N hydrochloric acid with 0.5% SLS	0.440	0.443	0.004
Phosphate buffer pH 6.8 with 0.5% SLS	0.740	0.739	0.002
Phosphate buffer pH 7.4 with 0.5% SLS	0.742	0.741	0.003
Methanol	0.467	0.465	0.003



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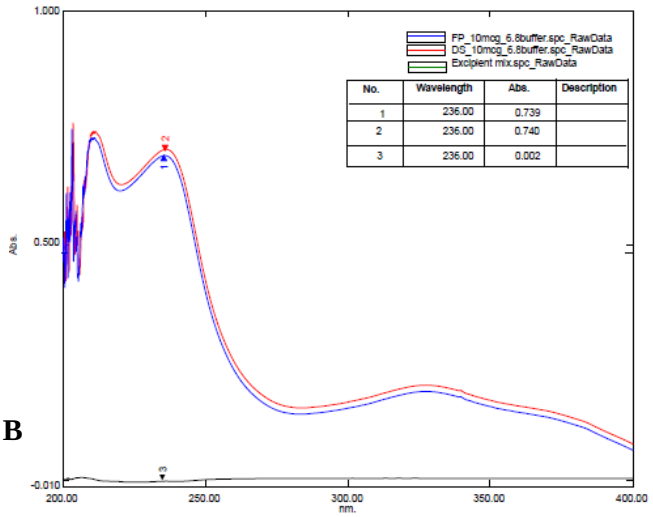

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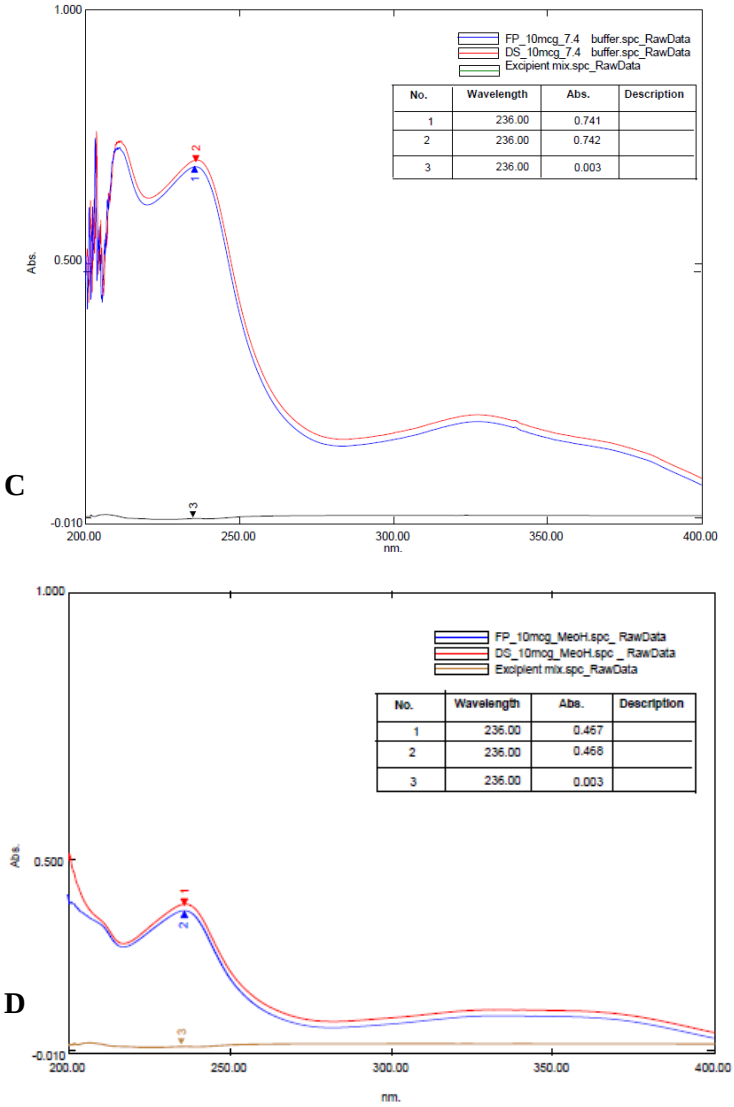

Figure 3.4.14: Specificity and interference study graph between NISO and excipients in (A) 0.1N HCl with 0.5% SLS, (B) phosphate buffer pH 6.8 with 0.5% SLS, (C) phosphate buffer pH 7.4 with 0.5% SLS and (D) methanol

3.4.6. Estimation of NISO by HPLC method

HPLC method was based on reverse phase chromatography on a C18 column. Method was developed and validated to estimate drug concentration during cell line studies. Peak area was recorded using UV detector at 235nm detection wavelength, with the flow rate of 1 mL/min of mobile phase for a run time of 8 min and retention time was found to be 4.0 min for NISO (Table 3.4.26). The chromatogram was drawn at 10 µg/mL (Figure 3.4.15). All the readings were taken in triplicate. Calibration curve was

plotted between concentration and peak area of NISO (Figure 3.4.16). R^2 value was found to be 1 in regression equation.

Table 3.4.26: Calibration data for NISO by HPLC

Concentration (µg/mL)	RT	Peak area (µV*S)#
1	4.073	636.70 ± 15.28
2.5	4.043	1050.49 ± 31.51
5	4.019	1732.63 ± 51.98
7.5	4.032	2410.53 ± 81.96
10	4.021	3101.15 ± 124.05

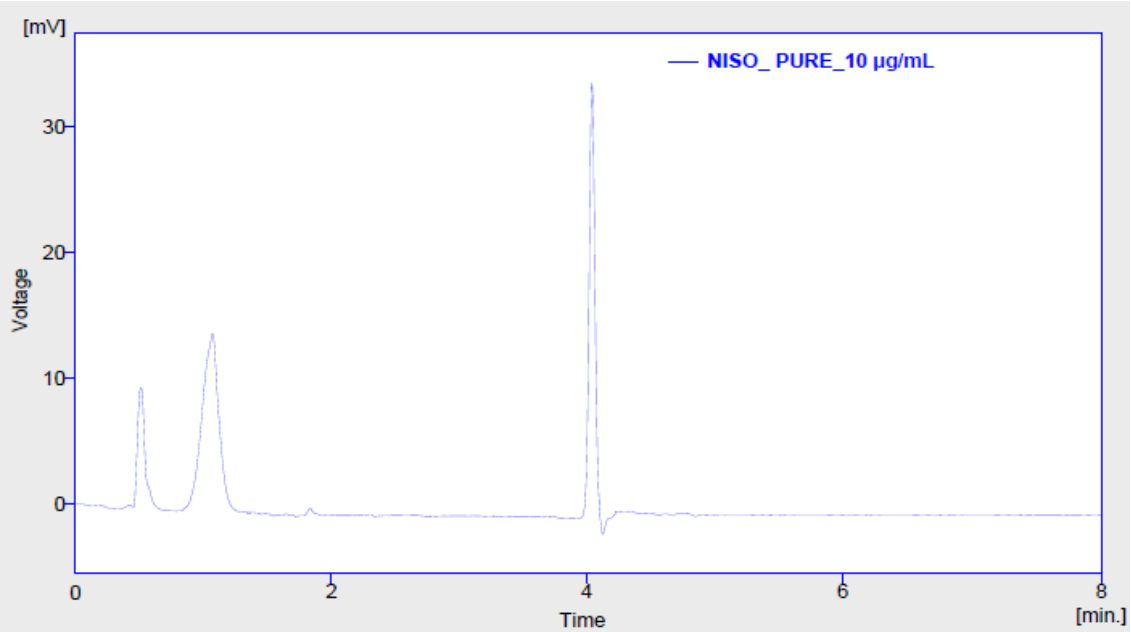

Figure 3.4.15: Chromatogram of NISO solution by HPLC at 235nm

3.4.6.1.Validation of HPLC method

3.4.6.1.1. Linearity

Significant linear correlation was observed between the mean peak area and different concentrations of the drug with less standard deviation using HPLC (Table 3.4.26). It is further confirmed by the value of correlation coefficient which is found to be 1 (Figure 3.4.16). Beer's law was found to be obeyed in the range of 1 to 10 µg/mL. Hence, linearity of the method is indicated for selected calibration range**.**

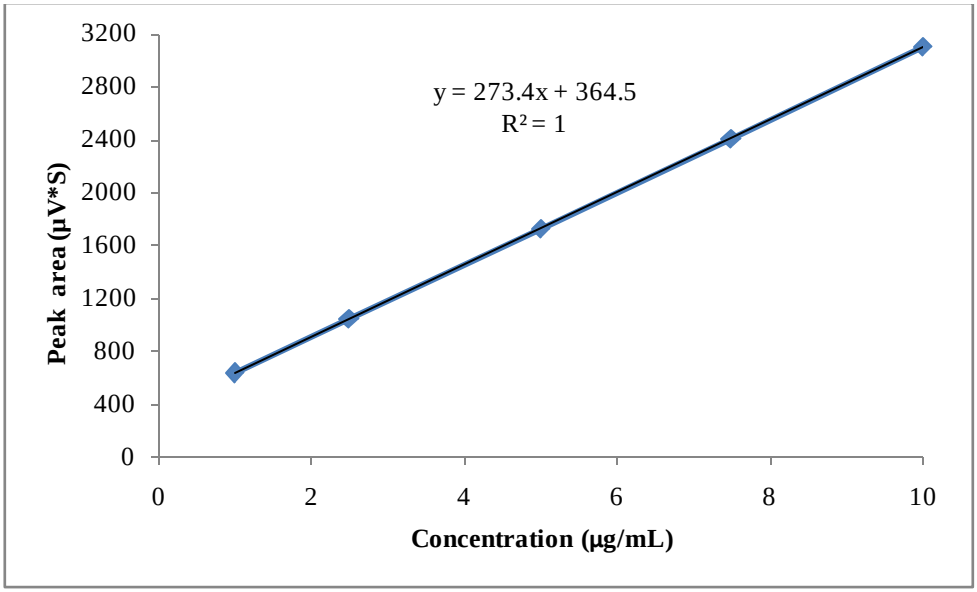

Figure 3.4.16: Calibration curve of NISO by HPLC

3.4.6.1.2. Accuracy

Accuracy of HPLC method was performed for NISO by standard addition or spiking method. The mean % recovery values for lower, intermediate and higher concentration were obtained close to 100% with less standard deviations that represents high accuracy of the analytical method chosen (Table 3.4.27). These results revealed that any small change in the drug concentration could be determined accurately by the proposed analytical method.

Table 3.4.27: Standard addition data to measure accuracy of HPLC method for NISO

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found (µg/mL)	% Analytical Recovery
80%	1	0.8	1.79 ± 7.54	99.44 ± 0.36
100%	1	1.0	1.99 ± 4.63	99.50 ± 0.25
120%	1	1.2	2.18 ± 3.89	99.09 ± 0.56

3.4.6.1.3. Precision

The results of intraday and interday precision were found to be precise under the same operating conditions over the interval of time (Table 3.4.28). In addition, the RSD values achieved for the analytical methods were within the acceptable range (< 2.0) indicating that the method is precise.

Table 3.4.28: Intraday and Interday Precision Analysis of HPLC Method for NISO

Conc. (µg/mL)	Intraday Precision				Interday Precision			
	Peak area			% RSD	Peak area			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
1	646.77	615.74	664.97	**0.039**	624.76	633.57	693.41	**0.057**
2.5	1050.49	1131.02	1094.35	**0.037**	1095.81	1190.35	1104.56	**0.048**
5	1732.63	1749.82	1638.89	**0.035**	1653.34	1714.28	1749.45	**0.028**
7.5	2410.53	2323.67	2423.46	**0.023**	2407.41	2401.12	2517.15	**0.027**
10	3101.15	3142.21	3197.51	**0.015**	3096.75	3119.41	3195.46	**0.016**

3.4.6.1.4. Stability

NISO in solution was kept over a period of 24 h and peak area of same solutions at 0 and 24 h was measured using HPLC. Insignificant difference was observed between the initial and 24 h readings (Table 3.4.29). Regression analysis also showed insignificant change in the slope of straight line (Figure 3.4.17). Thus, it can be concluded that NISO solution was stable throughout analysis.

Table 3.4.29: Absorbance data of NISO at 0 and 24 h for analytical stability

Concentration (µg/mL)	Peak area (µV*S)[#]	
	Initial	At 24 h
1	636.70 ± 1.552	664.76 ± 1.234
2.5	1050.49 ± 3.151	1065.81 ± 2.947
5	1732.63 ± 5.198	1744.12 ± 5.241
7.5	2410.53 ± 8.196	2425.53 ± 7.389
10	3101.15 ± 12.405	3096.75 ± 10.898

[#] Mean ± SD (n = 3)

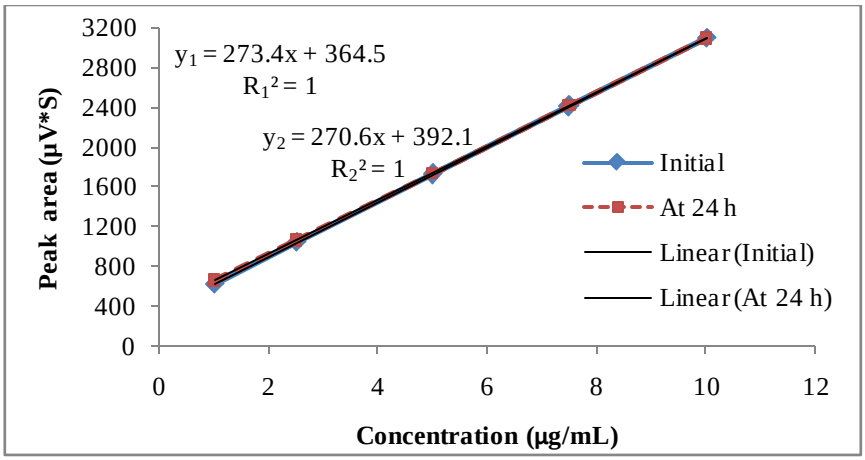

Figure 3.4.17: Calibration Curves of NISO at 0 h and 24 h time points for Stability by HPLC [y_1, R_1= initial, y_2, R_2= at 24h]

3.4.6.1.5. Sensitivity

LOD and LOQ were calculated using the formula described in section 3.3.2.5 and the values are presented in Table 3.4.30. LOD and LOQ values indicated the sensitivity of the method for accurate quantification of drug present in standard solutions.

Table 3.4.30: LOD and LOQ calculation from calibration curve of NISO by HPLC

Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
273.4	3.457	0.042	0.126

3.4.7. Estimation of NISO in plasma by HPLC method

Reverse phase chromatography technique was used to estimate NISO in plasma by HPLC method. Drug was separated from plasma using protein precipitation method. Same chromatographic conditions were used as that of pure drug (section 3.4.6). Chromatograph was drawn with 2.0 µg/mL concentration to show retention time (Figure 3.4.18). Peak area at different concentrations were noted (Table 3.4.31). All the readings were taken in triplicate.

Table 3.4.31: Calibration data for NISO in plasma by HPLC

Concentration (µg/mL)	RT	Peak area (µV*S)[#]
0.05	4.053	34.91 ± 1.217
0.1	4.067	66.42 ± 2.675
0.5	4.037	313.22 ± 4.666
1.0	4.030	602.45 ± 7.810
2.0	4.040	1238.64 ± 11.045

[#] Mean ± SD (n = 3)

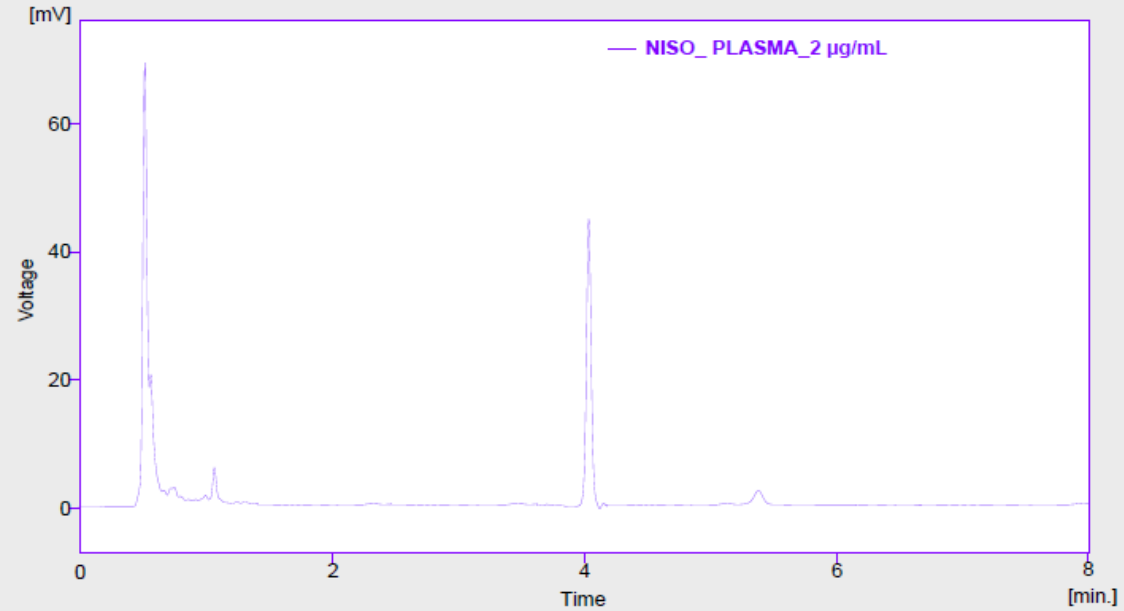

Figure 3.4.18: Chromatogram of NISO in plasma by HPLC at 235nm

3.4.7.1. Validation of HPLC method of NISO in plasma

3.4.7.1.1 Linearity

Mean peak area and different concentrations of the drug in plasma using HPLC are as shown in table 3.4.31. Calibration curve and value of correlation coefficient $R^2 = 0.999$ indicates linearity of the HPLC method in plasma (Figure 3.4.19).

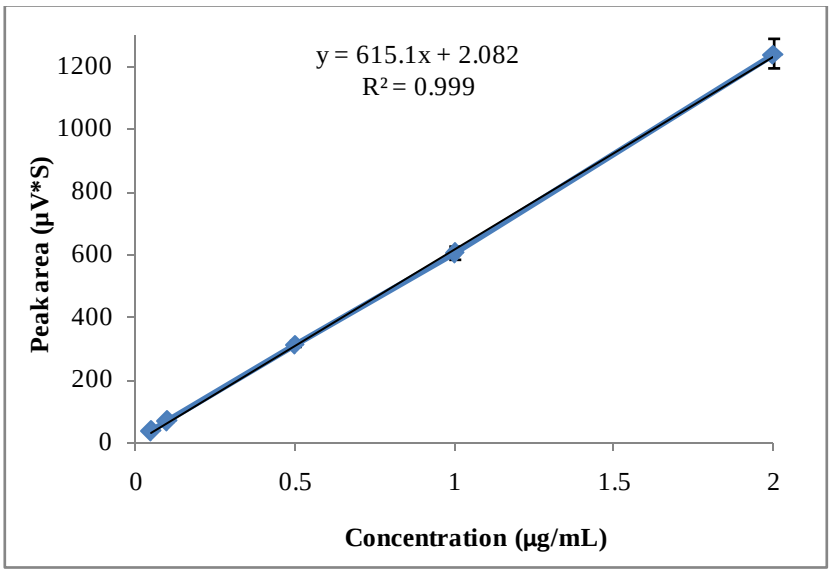

Figure 3.4.19: Calibration curve of NISO in plasma by HPLC

3.4.7.1.2. Accuracy

Accuracy of HPLC method in plasma was performed for NISO by standard addition or spiking method similar to method as used for pure drug (section 3.4.6.1.2). Analytical recovery was found to be > 99.0 % as shown in Table 3.4.32. Hence, any small change in the drug concentration could accurately be determined by the proposed analytical method in plasma.

Table 3.4.32: Standard addition data to measure accuracy of HPLC method for NISO in plasma

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found (µg/mL)	% Analytical Recovery
80%	0.05	0.04	0.0894 ± 0.02	99.33 ± 0.32
100%	0.05	0.05	0.0992 ± 0.04	99.20 ± 0.29
120%	0.05	0.06	0.1094 ± 0.03	99.45 ± 0.27

3.4.7.1.3. Precision

The results of intraday and interday precision were found to be precise under the same operating conditions over the interval of time (Table 3.4.33). In addition, the RSD values achieved for the analytical methods were within the acceptable range indicating that the method is precise.

Table 3.4.33: Intraday and interday precision analysis of HPLC method for NISO in plasma

Conc. (µg/mL)	Intraday Precision				Interday Precision			
	Peak area			% RSD	Peak area			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
0.05	34.91	44.57	33.49	**0.160**	39.87	42.57	31.52	**0.152**
0.1	66.42	75.18	80.32	**0.095**	76.35	81.11	69.32	**0.078**
0.5	313.22	324.54	329.88	**0.026**	315.36	328.43	331.12	**0.026**
1.0	602.45	632.28	618.34	**0.024**	643.23	604.56	621.26	**0.031**
2.0	1238.64	1299.23	1286.75	**0.025**	1241.23	1285.65	1264.48	**0.018**

3.4.7.1.4. Stability

NISO solution was kept over the period of 24 h and peak area of same solutions at 0 and 24 h was measured using HPLC. As shown in table 3.4.34, no significant difference was observed between the initial and 24 h readings. Slope of straight line also showed insignificant change in regression analysis as shown in figure 3.4.20. Thus, it can be concluded that NISO solution was stable throughout analysis.

Table 3.4.34: Absorbance data of NISO at 0 and 24 h for analytical stability in plasma

Concentration (µg/mL)	Peak area (µV*S)[#]	
	Initial	At 24 h
0.05	34.91	32.57
0.10	66.42	61.11
0.50	313.22	318.43
1.00	602.45	604.56
2.00	1238.64	1235.65

[#] Mean ± SD (n = 3)

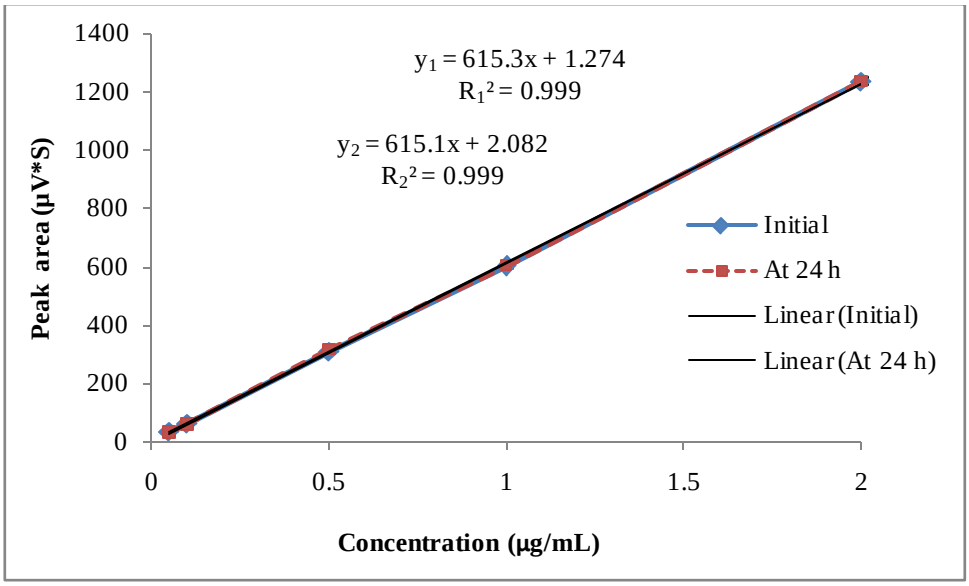

Figure 3.4.20: Calibration curves of NISO at 0 h and 24 h time points for stability in plasma by HPLC [y_1, R_1= initial, y_2, R_2= at 24h]

3.4.7.1.5. Sensitivity

LOD and LOQ values (Table 3.4.35) represents sensitivity of the HPLC method in plasma selected for accurate quantification of NISO present in standard solutions.

Table 3.4.35: LOD and LOQ calculation from calibration curve of NISO by HPLC in plasma

Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
615.1	9.706	0.052	0.158

3.5. Conclusions

Analytical methods for UV spectrophotometry and HPLC were successfully developed and validated for the quantitative estimation of DE and NISO. The spectrometric analysis for both the drugs were carried out between 200 to 400 nm using four different media viz., 0.01N HCl, phosphate buffer pH 6.8, phosphate buffer pH 7.4 and methanol for DE and in 0.1N HCl + 0.5% SLS, pH 6.8 phosphate buffer + 0.5% SLS, pH 7.4 phosphate buffer + 0.5% SLS and methanol for NISO. The UV absorption by DE was observed at 325 nm, 316 nm, 316 nm and 315 nm as λ_{max} while NISO showed absorption maxima at 238 nm, 236 nm, 236 nm and 236 nm in their respective media. Regression analysis was performed on the experimental data. Strong linear correlation with R^2 = 0.999 was observed between the concentration range of 2.5 to 30 µg/mL for all four media of DE. For the spectrophotometric analysis of NISO a strong linear correlation with R^2=0.999 in all four media was observed between the concentration of the drug and absorbance obtained for a concentration range of 2.5 to 30 µg/mL for 0.1N HCl + 0.5% SLS and methanol while concentration range of 2 to 16 µg/mL was observed for pH 6.8 and pH 7.4 phosphate buffer of NISO . The analytical methods for estimation of DE as well as NISO were found to be precise and represented accuracy in measuring the additional drug concentrations spiked in standard drug solutions. The developed UV methods for both the drugs were found to be specific and showed no interference with other formulation components.

HPLC method was developed and validated to estimate DE and NISO during permeability study in cell line studies for developed formulations (SMEDDS and NE).

HPLC methods for the quantitative estimation of DE and NISO in plasma were also successfully developed and validated. The chromatographic separation of DE and NISO was carried out on a Synergi^{TM} 4 µm Hydro-RP 80 Å, LC Column (150 x 4.6mm) (Phenomenex, Torrance, USA) column using a reported (slightly modified) mobile phase. The flow rate was maintained at 1.0 mL/min. The UV absorption was read at 230 nm for DE and 235 nm for NISO. Similarly, the chromatographic separation of NISO was carried out on a C18 column (Phenomenex) using a reported mobile phase. The developed HPLC methods were found to be specific for both DE and NISO. A strong linear correlation with an R^2 = 1 and 0.999 was observed between the concentration of the drugs and peak area obtained upon chromatographic extraction over a concentration range of 1 to 10 µg/mL and 0.05 to 2 µg/mL for routine and plasma methods respectively. The methods were found to measure the concentrations with significant precision and accuracy.

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