3.1 Introduction

Analytical methods are important tools for drug quantification at various stages of formulation development. They are helpful in establishing crucial formulation characteristics like entrapment efficiency, stability (in terms of percent drug retained), *in vitro* drug release behavior and *in vivo* bioavailability etc. Instrumental analysis is very sensitive and accurate measure of estimation. Hence, UV spectrophotometry, one of the simplest instrumentation methods capable of drug estimation, was used in the present study. HPLC is more sophisticated method used for the estimation of samples with very low quantity of the drug. Literature survey reveals several analytical methods for the determination of Metoprolol Succinate (MS) in pharmaceutical dosage forms and in biological fluids by LC [1], UV [2, 3], HPLC [4] and LC-MS/MS [5]. Similarly, for determination of Metformin hydrochloride (MH) in pharmaceutical dosage forms and in biological fluids, UV [1, 6] and HPLC [7] methods have been reported. The analytical methods employed in present investigation are described below.

3.2 Materials, Instruments and Reagents

3.2.1 Materials

MS and MH were provided by Alembic Research Centre (Vadodara, Gujarat, India) as gift samples. Methanol (HPLC grade), 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 other materials namely, concentrated hydrochloric acid, sodium dihydrogen phosphate monohydrate, ortho phosphoric acid, potassium phosphate monobasic, Sodium Hydroxide and triethylamine were of AR Grade.

3.2.2 Instruments and softwares

3.2.2.1 UV spectrophotometer

Spectrophotometric measurements were carried out on a double beam UV-Visible spectrophotometer (UV-1800, Shimadzu Corporation, Japan) with a fixed slit width of 1nm coupled with UVPROB V2.42 software. The spectral bandwidth was 1 nm and the wavelength scanning speed was 2800 nm/min. Matched quartz cuvettes (1 cm) were used for all the spectral measurements.

3.2.2.2 HPLC

The chromatographic system was LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20µl fixed loop and SPD-20A Prominence UV-Visible detector (Shimadzu, Kyoto, Japan). The separation was performed on a C-18 column (250 mm [L] x 4.6 mm [ID] x 5 µm [particle size]; Phenomenex, Torrance, USA) connected to a 2 cm guard column. Chromatographic data were recorded and processed using Spinchrome Chromatographic Station® CFR Version 2.4.0.193 (Spinchrome Pvt. Ltd., Chennai, India).

3.2.3 Reagents / solutions

3.2.3.1 Hydrochloric acid, 0.1N

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.

3.2.3.2 Phosphate buffer, pH 3

6.9 g of sodium dihydrogen phosphate monohydrate was dissolved in 500 mL of distilled water and the volume was made up to 1000 mL using distilled water. The pH was finally adjusted to 3.0 using ortho phosphoric acid.

3.2.3.3 Phosphate buffer, pH 6.8

Potassium phosphate, monobasic, 0.2 M: 27.22 gm 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 gm of NaOH was dissolved in distilled water and the volume was made up to 1000 mL.

Preparation of phosphate buffer (PB) 6.8: 50 mL of potassium phosphate, monobasic and 22.4 mL of 0.2M NaOH were taken and diluted up to 200 mL with distilled water.

3.2.3.4 Mobile phase for HPLC of MS

Phosphate buffer (pH 3, containing 0.5% triethylamine), methanol and acetonitrile were mixed in a ratio of 90:1:9, respectively to serve as mobile phase. 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 minutes.

3.2.3.5 Mobile phase for HPLC of MH

Methanol and 0.05 mol/L of ammonium dihydrogen phosphate were mixed in a ratio of 35:65 v/v, respectively to serve as mobile phase. The solvent mix was filtered and degassed in a similar manner as described in previous section 3.2.3.4.

3.3 Methods

3.3.1 Estimation of MS by UV spectrophotometric methods

UV spectrophotometric methods for estimation of MS in different solvents including 0.1N hydrochloric acid, phosphate buffer pH 3, phosphate buffer pH 6.8 and distilled water were developed.

3.3.1.1 Preparation of stock solutions

Stock solutions containing 0.1 mg/mL (100 µg/mL) were prepared in all the selected solvents. Accurately weighed quantities (100 mg) of drug were transferred to 100 mL calibrated volumetric flasks and dissolved in different solvents. The volumes were made up to 100 mL with the same solvents. The resulting solutions (1000 µg/mL) were further diluted ten times with the same solvents to get stock solutions (100 µg/mL).

3.3.1.2 Preparation of standard solutions

Appropriate aliquots (0.4 to 2.0 mL) of the stock solutions of MS were transferred to 10 mL calibrated volumetric flasks and diluted up to the mark with respective solvents to obtain known final concentrations ranging from 4 to 20 µg/mL.

3.3.1.3 Determination of analytical wavelength

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

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). Equations of best fit straight line and correlation coefficients were generated on MS office excel software to observe linearity.

3.3.2 Validation of UV spectrophotometric methods

3.3.2.1 Linearity and range

Linearity of an analytical method is the ability to elicit the test results that are directly or by well-defined transformation proportional to the concentration of the analyte in the samples within the given range [8]. Linear regression was performed using MS office excel software and the correlation coefficient (R^2) was generated to demonstrate linearity.

3.3.2.2 Robustness

The evaluation of robustness should show the reliability of an analysis with respect to deliberate variations in method parameters to ensure that the validity of the analytical procedure is maintained whenever used [9]. Stability of MS analytical solutions in different solvents was ascertained as a measure of robustness 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 consistency and reproducibility of a method. The precise analytical method is the degree of agreement among the individual test results gives very close values for repeated measurement of same sample when the procedure is applied repeatedly to multiple sampling of homogeneous sample [10]. Multiple measurements for same standard concentrations were made on same day as well as on three consecutive days to determine intraday and interday precision, respectively. 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 [11]. The accuracy of the method was determined by calculating the recoveries of the analyte by the method of standard additions different levels of drug concentrations. Known amounts of standard drug (80%, 100% and 120%) were added to the pre-analyzed samples and the absorbance was measured. Accuracy is assessed as the mean % recovery of the added pure drug which was calculated using following equation

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

C_T= total drug conc. measured after standard addition;
C_S= drug concentration in solution sample;
C_A= drug concentration added to solution

3.3.2.5 Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ are used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. So, LOD and LOQ of developed methods were determined using following equations [10],

$$LOD = 3 \times \left(\frac{R}{S}\right) \qquad LOQ = 10 \times \left(\frac{R}{S}\right)$$

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 ability to assess unambiguously the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. [9]. The interference of formulation excipients

(Celphere, Ethyl Cellulose, Hydroxy Propyl Cellulose and Eudragit E) in the estimation of the MS was determined in all the four solvents at 274 nm using formulation prototype method [10]. The formulation prototype was prepared by mixing one portion of drug with 5 portions of Celphere and 1 portion each of Ethyl Cellulose, Hydroxy Propyl Cellulose and Eudragit E. The absorbance of this formulation prototype was compared with absorbance of pure drug solutions in respective solvents and excipients' mix which was prepared using similar portions of all the excipients and omitting the drug.

3.3.3 Estimation of MS in plasma by HPLC method

Estimation of MS by HPLC has been reported in USP30-NF 25 [1]. The same method was adapted here with a few modifications for estimation of MS in plasma.

3.3.3.1 Preparation of stock solution of drug

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

3.3.3.2 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 50 to 2000 ng/mL. The plasma proteins thus precipitated were separated 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.

3.3.3.3 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 1.4 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 280 nm detection wavelength for a run time of 10 minutes. The column was equilibrated by passing at least 100-150 mL of mobile phase. Calibration curve was drawn by plotting peak area of curve versus drug concentration.

3.3.4 Validation of HPLC method

The parameters including linearity and range, stability, Precision, Accuracy and Sensitivity were evaluated using methods as described in section 3.3.2.

3.3.5 Estimation of MH by UV spectrophotometric methods

UV spectrophotometric methods for estimation of MH in different solvents including 0.1N hydrochloric acid, phosphate buffer pH 6.8 and distilled water were developed.

3.3.5.1 Preparation of stock solutions

Stock solutions containing 0.1 mg/mL (100 µg/mL) were prepared in all the selected solvents. Accurately weighed quantities (100 mg) of drug were transferred to 100 mL calibrated volumetric flasks and dissolved in different solvents. The volumes were made up to 100 mL with the same solvents. The resulting solutions (1000 µg/mL) were further diluted ten times with the same solvents to get stock solutions (100 µg/mL).

3.3.5.2 Preparation of standard solutions

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

3.3.5.3 Determination of analytical wavelength

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

3.3.5.4 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 on MS office excel software to observe linearity.

3.3.6 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 3 portions of Celphere, 0.5 portion each of Eudragit® RS and Eudragit E, 0.15 portion of Talc UM and 0.1 portion each of PVP K30, Eudragit® RL and Tri Ethyl Citrate.

3.3.7 Estimation of MH in plasma by HPLC method

Estimation of MH by HPLC has been reported by Hu et al., 2006 [7]. The same method was adapted here with a few modifications for estimation of MH in plasma.

3.3.7.1 Preparation of stock solution of drug

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

3.3.7.2 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 50 to 2000 ng/mL. The plasma proteins thus precipitated were separated 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.

3.3.7.3 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 at least 100-150 mL of mobile phase. Calibration curve was drawn by plotting peak area of curve versus drug concentration.

3.3.8 Validation of HPLC method

The parameters including linearity and range, stability, Precision, Accuracy and Sensitivity were evaluated using methods as described in section 3.3.2.

3.4 Results & Discussion

3.4.1 Estimation of MS by UV spectrophotometry

The UV-Visible spectrum obtained by scanning the standard solutions of MS in different solvents showed absorption maxima at 274 nm (Figure 3.4.1) and hence, 274 nm was selected as analytical wavelength for all the four solvents.

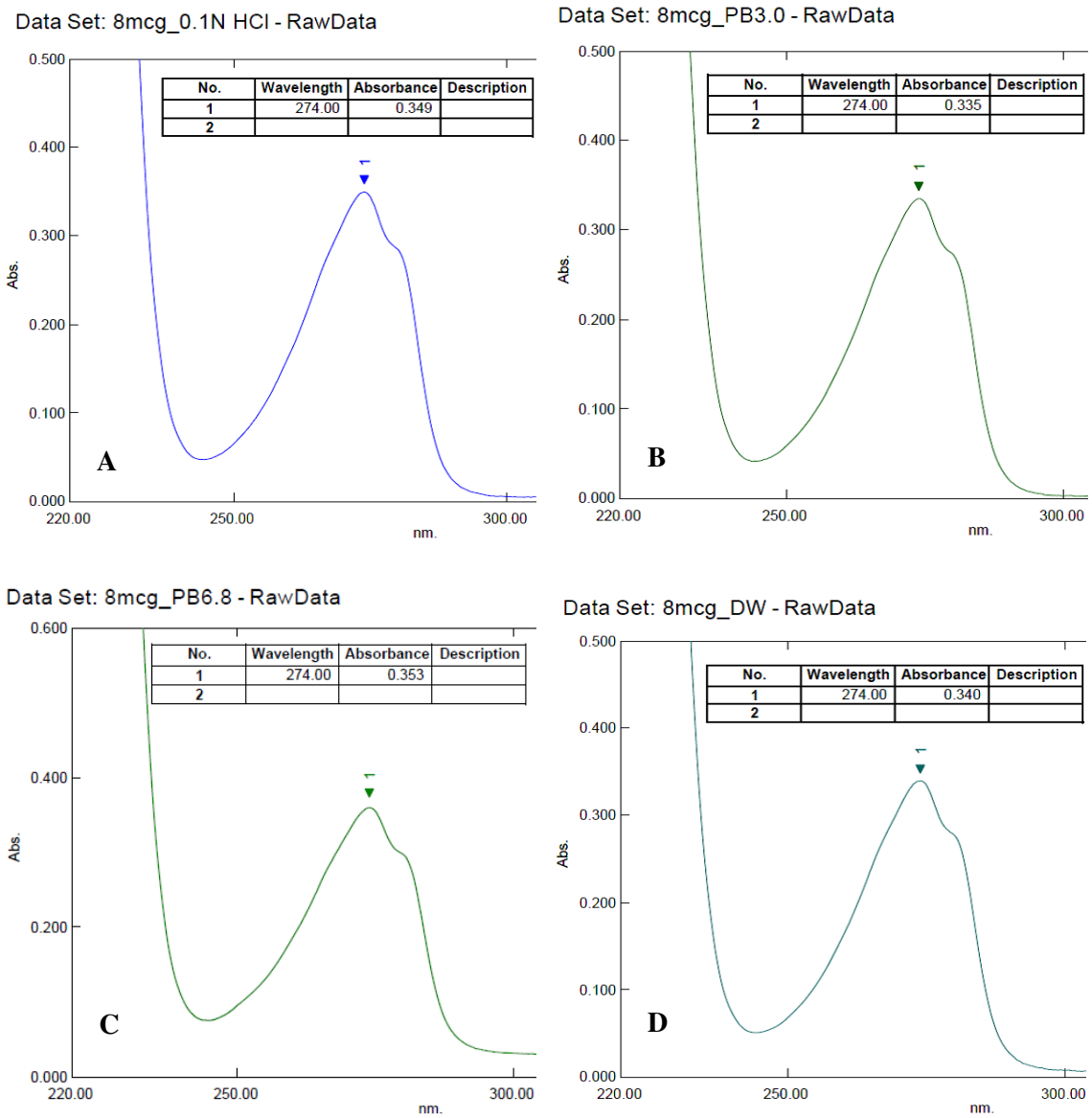

Figure 3.4.1: UV Absorption spectrum of MS in (A) 0.1N HCl, (B) Phosphate buffer pH 3, (C) Phosphate buffer pH 6.8 and (D) Distilled water

The absorbance of standard drug solutions was measured at 274 nm to prepare calibration curves. The calibration data for MS in different solvents are summarized in Table 3.4.1. Figure 3.4.2 shows the standard calibration curves in various solvents with their regression equations and correlation coefficients (R^2).

Table 3.4.1: Calibration data for MS at 274 nm in various solvents

Concentration (µg/mL)	Absorbance[#]			
	In 0.1N HCl	In phosphate buffer pH 3.0	In phosphate buffer pH 6.8	In distilled water
4	0.176 ± 0.003	0.171 ± 0.003	0.185 ± 0.003	0.170 ± 0.001
8	0.348 ± 0.006	0.337 ± 0.002	0.346 ± 0.006	0.338 ± 0.003
12	0.509 ± 0.009	0.498 ± 0.003	0.519 ± 0.010	0.488 ± 0.007
16	0.681 ± 0.014	0.669 ± 0.010	0.722 ± 0.014	0.671 ± 0.013
20	0.913 ± 0.016	0.850 ± 0.015	0.901 ± 0.010	0.858 ± 0.013

[#] Mean ± SD (n=3)

3.4.2 Validation of UV spectrophotometric methods

3.4.2.1 Linearity

Regression analysis was performed on mean absorbance values of standard drug solutions in different solvents using MS office excel software v2007. As shown in Table 3.4.2, significantly high linear correlation ($R^2 \geq 0.994$) was evident among selected drug concentration range and their respective absorbance values for all the four solvents. These findings clearly indicated that Beer's law was obeyed in the drug concentration range of 4 to 20 µg/mL in all the solvents.

Table 3.4.2: Linear regression analysis of calibration data for MS in different solvents

Solvents	Calibration range (µg/mL)	Regression equation	Correlation coefficient (R^2)
0.1N hydrochloric acid	4 - 20	y = 0.045x - 0.016	0.994
Phosphate buffer pH 3.0	4 - 20	y = 0.042x - 0.002	0.999
Phosphate buffer pH 6.8	4 - 20	y = 0.045x - 0.008	0.998
Distilled water	4 - 20	y = 0.042x - 0.007	0.998

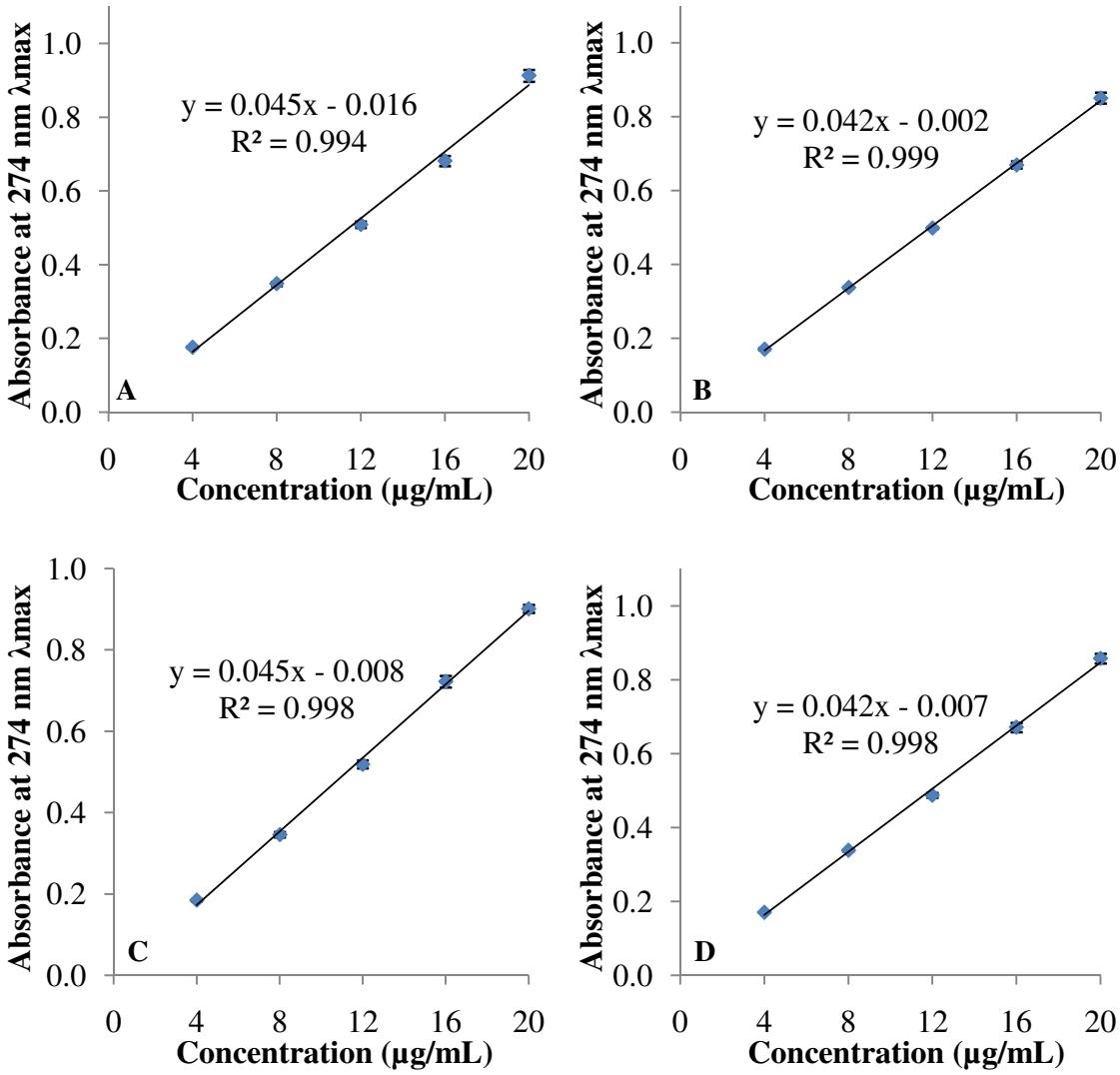

Figure 3.4.2: Calibration curves of MS in (A) 0.1N HCl, (B) Phosphate buffer pH 3, (C) Phosphate buffer pH 6.8 and (D) Distilled water

3.4.2.2 Stability

The stability of the drug in all the four solvents was ascertained 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 MS was stable in all four solvents over the period of analysis.

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

Conc (µg/mL)	Absorbance[#]	
	Initial	**At 24 h**
0.1N hydrochloric acid		
4	0.176 ± 0.003	0.172 ± 0.014
8	0.348 ± 0.006	0.344 ± 0.009
12	0.509 ± 0.009	0.497 ± 0.021
16	0.681 ± 0.014	0.679 ± 0.033
20	0.913 ± 0.016	0.902 ± 0.052
Phosphate buffer pH 3.0		
4	0.171 ± 0.003	0.167 ± 0.011
8	0.337 ± 0.002	0.328 ± 0.013
12	0.498 ± 0.003	0.489 ± 0.030
16	0.669 ± 0.010	0.664 ± 0.016
20	0.850 ± 0.015	0.848 ± 0.031
Phosphate buffer pH 6.8		
4	0.185 ± 0.003	0.181 ± 0.006
8	0.346 ± 0.006	0.346 ± 0.043
12	0.519 ± 0.010	0.510 ± 0.031
16	0.722 ± 0.014	0.710 ± 0.029
20	0.901 ± 0.010	0.893 ± 0.038
Distilled water		
4	0.170 ± 0.001	0.164 ± 0.004
8	0.338 ± 0.002	0.336 ± 0.005
12	0.488 ± 0.007	0.481 ± 0.011
16	0.671 ± 0.013	0.669 ± 0.034
20	0.858 ± 0.013	0.843 ± 0.022

[#] Mean ± SD (n = 3)

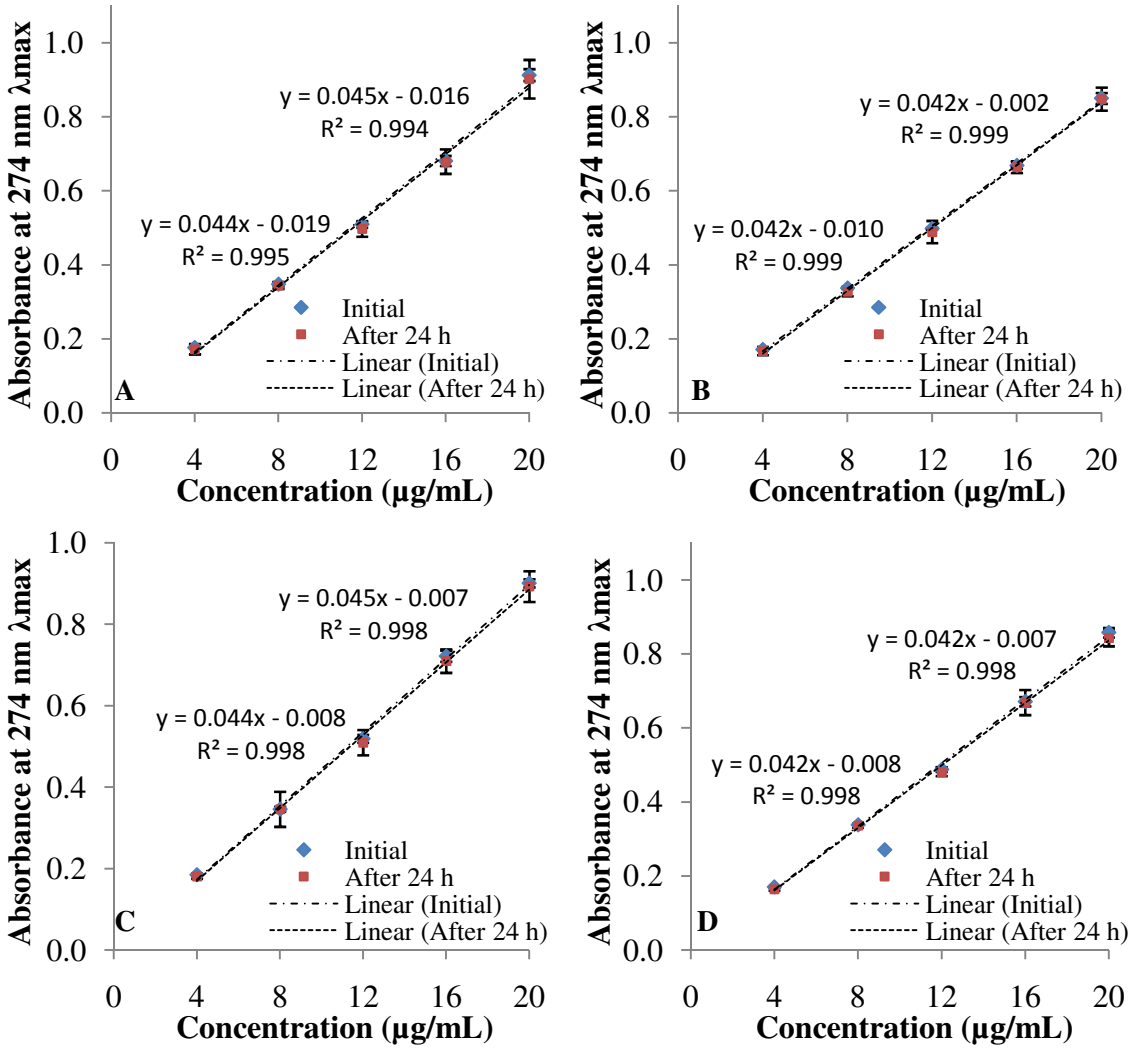

Figure 3.4.3: Calibration Curves of MS at 0 h and 24 h time points in (A) 0.1N HCl, (B) Phosphate buffer pH 3, (C) Phosphate buffer pH 6.8 and (D) Distilled water for stability study

3.4.2.3 Precision

The data for intraday and interday precision are summarized in Table 3.4.4. The results were found to be precise under the same operating conditions over the interval of time. In addition, the RSD values obtained for the analytical methods were within the acceptable range (< 2%) indicating that these methods are precise [10].

Table 3.4.4: Intraday and Interday Precision Analysis of UV Method for MS

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								
4	0.178	0.176	0.173	**1.43**	0.175	0.170	0.169	**1.88**
8	0.353	0.349	0.342	**1.60**	0.349	0.347	0.355	**1.19**
12	0.518	0.501	0.507	**1.69**	0.501	0.494	0.482	**1.95**
16	0.697	0.674	0.673	**1.99**	0.674	0.675	0.680	**0.48**
20	0.931	0.901	0.906	**1.76**	0.901	0.867	0.881	**1.94**
Phosphate buffer pH 3.0								
4	0.174	0.170	0.168	**1.79**	0.170	0.166	0.168	**1.19**
8	0.339	0.337	0.335	**0.59**	0.337	0.332	0.341	**1.34**
12	0.495	0.500	0.500	**0.58**	0.500	0.487	0.481	**1.98**
16	0.671	0.678	0.659	**1.44**	0.678	0.682	0.681	**0.31**
20	0.845	0.839	0.867	**1.73**	0.839	0.818	0.829	**1.27**
Phosphate buffer pH 6.8								
4	0.182	0.186	0.186	**1.39**	0.186	0.179	0.183	**1.77**
8	0.341	0.353	0.344	**1.83**	0.344	0.341	0.352	**1.67**
12	0.508	0.524	0.526	**1.88**	0.526	0.520	0.526	**0.67**
16	0.710	0.719	0.738	**1.93**	0.738	0.742	0.731	**0.76**
20	0.890	0.906	0.908	**1.06**	0.908	0.894	0.905	**0.84**
Distilled water								
4	0.170	0.171	0.170	**0.34**	0.170	0.168	0.174	**1.79**
8	0.336	0.341	0.338	**0.74**	0.338	0.347	0.342	**1.32**
12	0.482	0.495	0.486	**1.37**	0.486	0.495	0.486	**1.06**
16	0.684	0.659	0.671	**1.86**	0.671	0.658	0.649	**1.68**
20	0.855	0.872	0.846	**1.54**	0.846	0.816	0.819	**2.00**

3.4.2.4 Accuracy

Table 3.4.5 shows the data of recovery studies by standard addition or spiking method. The mean % recoveries for lower, intermediate and higher concentration are presented for all the four solvents. The mean % recovery values, close to 100% with low standard deviation (SD < 0.4%) represent high accuracy of the analytical methods [10]. 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 solvents

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found[#] (µg/mL)	% Analytical Recovery[#]
0.1N Hydrochloric acid				
80%	10	8	17.834 ± 0.019	99.08 ± 0.10
100%	10	10	19.821 ± 0.038	99.11 ± 0.19
120%	10	12	21.905± 0.026	99.57 ± 0.12
Phosphate buffer pH 3.0				
80%	10	8	17.756 ± 0.026	98.64 ± 0.15
100%	10	10	19.473 ± 0.017	97.37 ± 0.09
120%	10	12	21.832 ± 0.031	99.24 ± 0.14
Phosphate buffer pH 6.8				
80%	10	8	17.815 ± 0.024	98.97 ± 0.13
100%	10	10	19.773 ± 0.045	98.87 ± 0.23
120%	10	12	21.758± 0.030	98.90 ± 0.14
Distilled water				
80%	10	8	17.744 ± 0.060	98.58 ± 0.33
100%	10	10	19.861 ± 0.037	99.31 ± 0.19
120%	10	12	21.782 ± 0.051	99.01 ± 0.23

[#]Mean ± SD (n = 3)

3.4.2.5 Sensitivity

LOD and LOQ were calculated using the formula described above and the values are presented in Table 3.4.6 for all the four solvents. As indicated by the results, the concentration range selected for calibration was well above the LOD for all four solvents. LOQ values also indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

Table 3.4.6: LOD and LOQ calculation from calibration data of MS in different solvents

Solvents	Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
0.1N hydrochloric acid	0.045	0.024	1.578	5.283
Phosphate buffer pH 3.0	0.042	0.007	0.474	1.581
Phosphate buffer pH 6.8	0.045	0.013	0.859	2.864
Distilled water	0.042	0.013	0.914	3.048

3.4.2.6 Interference and specificity study

The analytical specificity of these UV spectrophotometric methods were determined by comparing the absorbance values at 274 nm λ_{max} obtained for the drug solution in selected solvent with that obtained for formulation prototype (Drug + Excipients) and excipient mixture (without drug) in same solvent (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 MS peak could be clearly distinguished from the other peaks therefore, this method was said to be specific for the analysis of MS.

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

Solvents	Absorbance at 274 nm λ_{max}		
	Drug solution (8 µg/mL)	**Formulation prototype**	**Excipient mixture**
0.1N hydrochloric acid	0.349	0.351	0.003
Phosphate buffer pH 3.0	0.335	0.343	0.008
Phosphate buffer pH 6.8	0.353	0.356	0.004
Distilled water	0.340	0.345	0.006

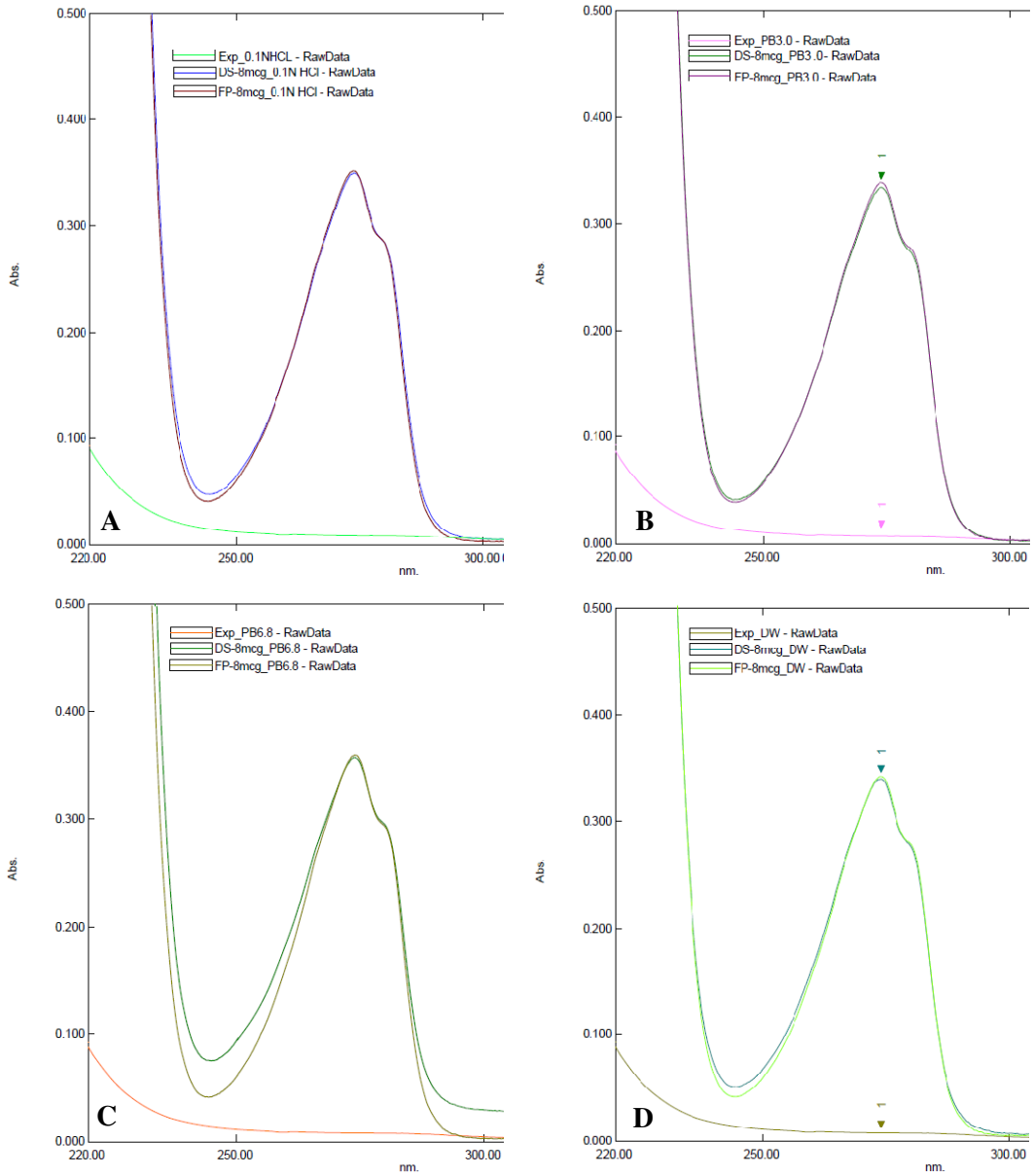

Figure 3.4.4: Specificity and Interference Study Graph between MS and Excipients in (A) 0.1N HCl, (B) Phosphate buffer pH 3, (C) Phosphate buffer pH 6.8 and (D) Distilled water

3.4.3 Estimation of MS in plasma by HPLC method

HPLC method was based on reverse phase chromatography on a C18 column [4]. The elution was done with the flow rate of 1.4 mL/min of mobile phase and the retention time of MS was found to be 4.9 min at 280 nm of detection (Figure 3.4.5).

Table 3.4.8: Calibration data for MS by HPLC

Concentration (ng/mL)	Peak area (µV*S)[#]
50	2576 ± 16
100	3052 ± 30
500	6896 ± 122
1000	11698 ± 122
2000	21278 ± 263

[#] Mean ± SD (n=3)

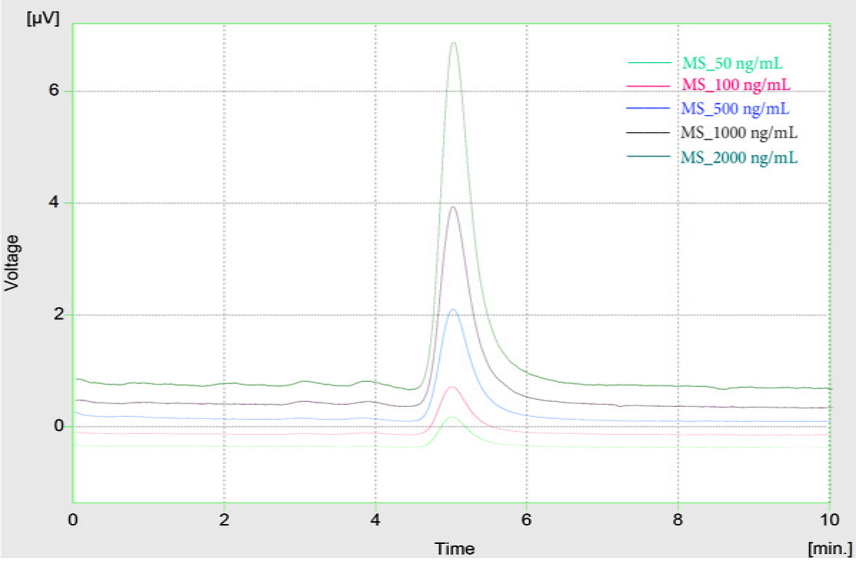

Figure 3.4.5: Typical overlay plot of MS by HPLC

3.4.4 Validation of HPLC method

3.4.4.1 Linearity

The mean peak area values along with the standard deviation for MS by HPLC method are shown in Table 3.4.8 and Figure 3.4.6. The value of correlation coefficient (R^2 = 0.999) indicated that area and concentration of the drug was in significant linear correlation. Beer's law was found to be obeyed in the range of 50 to 2000 ng/mL. Results indicated the linearity of the method for selected calibration range**.**

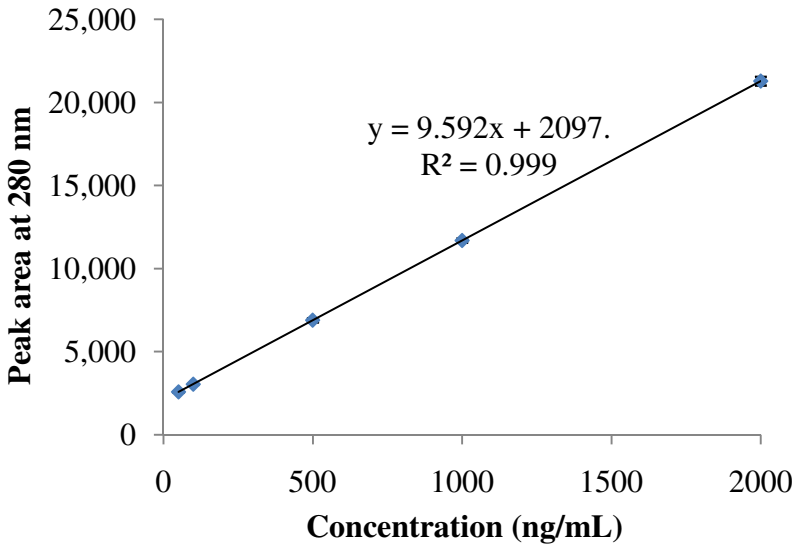

Figure 3.4.6: Calibration curve of MS by HPLC

3.4.4.2 Stability

The stability of the drug in solution was ascertained over the period of 24 h by measuring peak area of same solutions at 0 and 24 h using HPLC. The results (Table 3.4.9) revealed insignificant difference between the initial and 24 h readings. Regression analysis also showed insignificant change in the slope of straight line (Figure 3.4.7). Thus, it can be concluded that MS solution was stable over the period of analysis.

Table 3.4.9: Absorbance data of MS at 0 and 24 h for analytical stability

Concentration (ng/mL)	Peak area (µV*S)[#]	
	Initial	At 24 h
50	2576 ± 16	2515 ± 36
100	3052 ± 30	2984 ± 67
500	6896 ± 122	6831 ± 146
1000	11698 ± 122	11573 ± 201
2000	21278 ± 263	21176 ± 342

[#]Mean ± SD (n = 3)

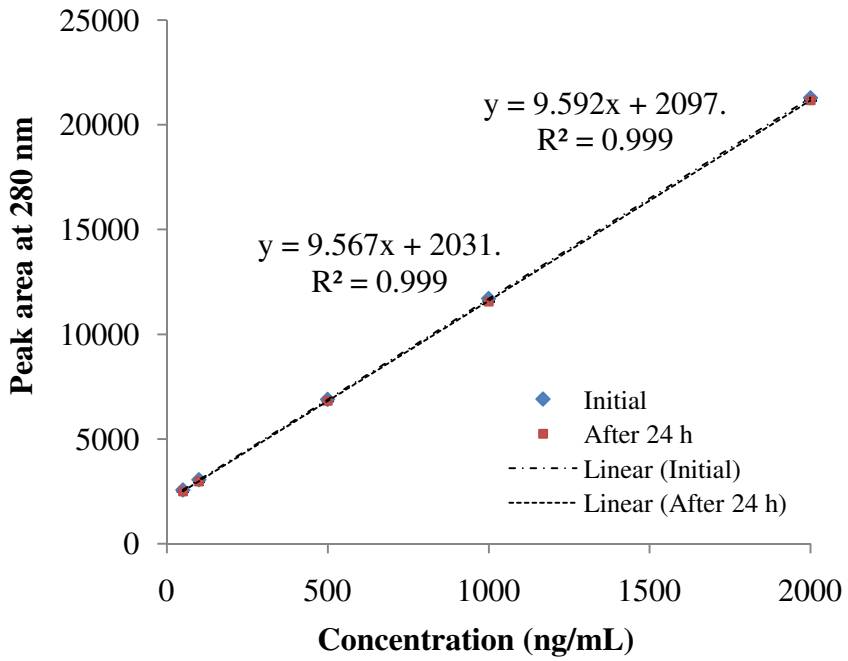

Figure 3.4.7: Calibration Curves of MS at 0 h and 24 h time points for stability study by HPLC

3.4.4.3 Precision

The data for intraday and interday precision are summarized in Table 3.4.10. The results were found to be precise under the same operating conditions over the interval of time. In addition, the RSD values obtained for the analytical methods were within the acceptable range [10] indicating that these methods were precise.

Table 3.4.10: Intraday and Interday Precision Analysis of HPLC Method

Conc. (ng/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	
50	2559	2590	2580	**0.61**	2580	2517	2604	**1.75**
100	3067	3071	3018	**0.97**	3018	3107	3024	**1.63**
500	6774	7018	6897	**1.77**	6897	6851	6864	**0.35**
1000	11676	11830	11588	**1.05**	11588	11772	11714	**0.80**
2000	21089	21167	21578	**1.23**	21578	21385	21197	**0.89**

3.4.4.4 Accuracy

Table 3.4.11 shows the data of recovery studies by standard addition or spiking method. The mean % recoveries for lower, intermediate and higher concentration were presented. The excellent mean % recovery values, close to 100% with less standard deviations (SD < 0.7%) represent high accuracy of the analytical method [10]. These results revealed

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

Table 3.4.11: Standard addition data to measure accuracy of HPLC method for MS

Spiking	Drug in solution (ng/mL)	Spiked drug (ng/mL)	Total drug found (ng/mL)	% Analytical Recovery
80%	500	400	889 ± 5.5	98.78 ± 0.61
100%	500	500	986 ± 6.8	98.60 ± 0.68
120%	500	600	1099 ± 4.1	99.91 ± 0.37

3.4.4.5 Sensitivity

LOD and LOQ were calculated using the formula described above and the values are presented in Table 3.4.12. LOD and LOQ values indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

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

Slope of line	SD of line	LOD (ng/mL)	LOQ (ng/mL)
9.592	6.140	1.920	6.401

3.4.5 Estimation of MH by UV spectrophotometry

The UV-Visible spectrum obtained by scanning the standard solutions of MH in 0.1 N HCl, phosphate buffer (pH 6.8) and distilled water showed absorption maxima at 230, 233 and 232 nm, respectively (Figure 3.4.8) and hence were selected as analytical wavelengths for respective solvents.

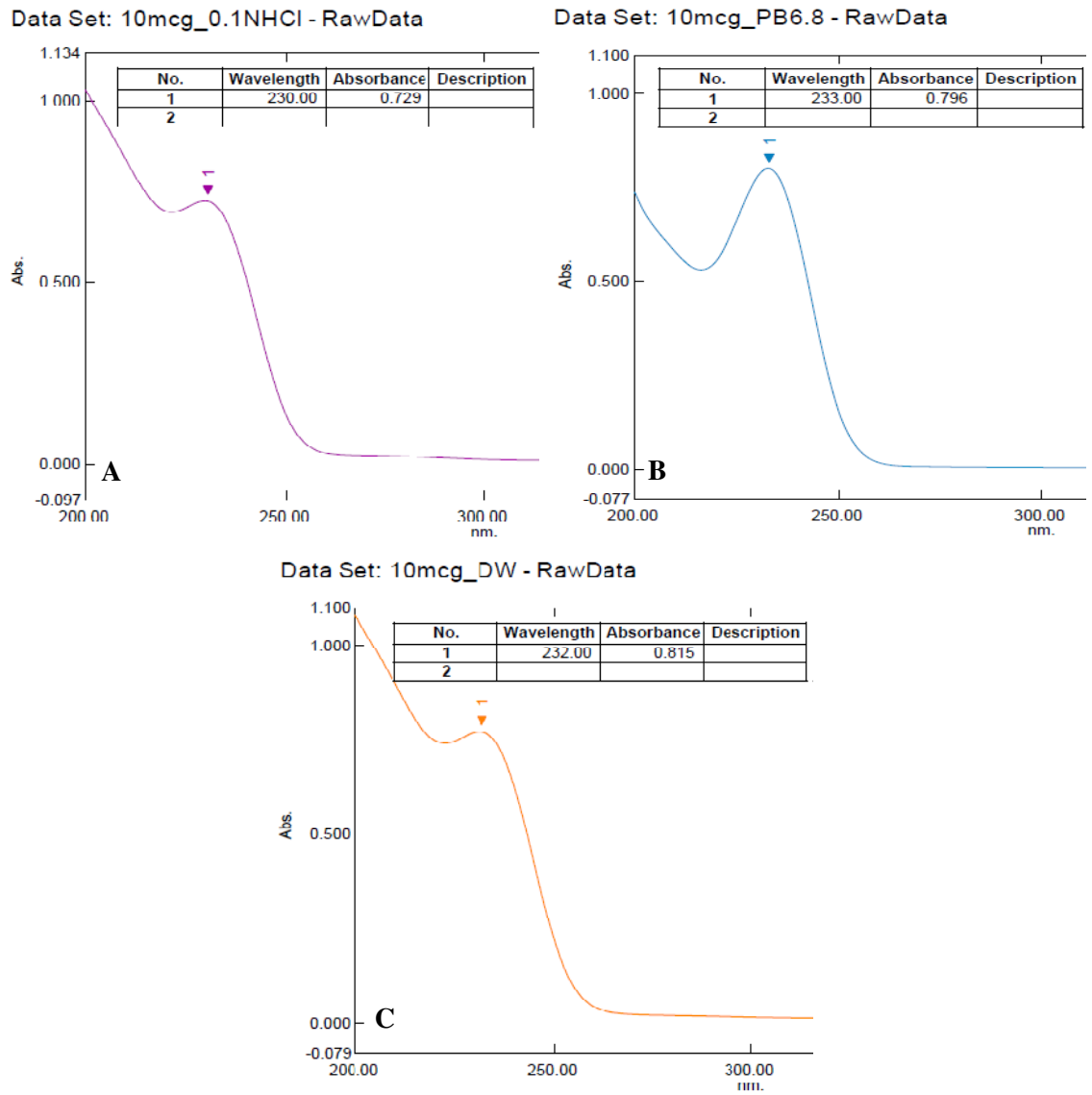

Figure 3.4.8: UV Absorption Spectrum of MH in (A) 0.1N HCl, (B) Phosphate buffer pH 6.8 and (C) Distilled water

The absorbance of standard drug solutions was measured at respective λ_{max} to prepare calibration curves as summarized in Table 3.4.13. Figure 3.4.9 shows the standard calibration curves in various solvents with their regression equations and correlation coefficients (R^2).

Table 3.4.13: UV spectophotometric calibration data for MH

Concentration (µg/mL)	Absorbance[#]		
	0.1N HCl (λ_{max}, 230 nm)	**Phosphate buffer pH 6.8** (λ_{max}, 233 nm)	**Distilled water** (λ_{max}, 232 nm)
2	0.159 ± 0.003	0.169 ± 0.003	0.164 ± 0.003
4	0.279 ± 0.004	0.327 ± 0.004	0.325 ± 0.005
6	0.421 ± 0.006	0.474 ± 0.006	0.484 ± 0.010
8	0.545 ± 0.003	0.660 ± 0.011	0.653 ± 0.010
10	0.723 ± 0.007	0.800 ± 0.011	0.810 ± 0.014

[#] Mean ± SD (n=3)

3.4.6 Validation of UV spectrophotometric methods

3.4.6.1 Linearity

Regression analysis was performed on mean absorbance values of standard drug solutions in different solvents using MS office excel software v2007. As shown in Table 3.4.14, significantly high linear correlation ($R^2 \geq 0.995$) was evident among selected drug concentration range and their respective absorbance values for all the three solvents. These findings clearly indicated that Beer's law was obeyed in the drug concentration range of 2 to 10 µg/mL in all the solvents.

Table 3.4.14: Linear regression analysis of calibration data for MH in different solvents

Solvents	λ_{max} (nm)	Calibration range (µg/mL)	Regression equation	Correlation coefficient (R^2)
0.1N hydrochloric acid	230	2 - 10	y = 0.069x - 0.007	0.995
Phosphate buffer pH 6.8	233	2 - 10	y = 0.079x - 0.007	0.998
Distilled water	232	2 - 10	y = 0.081x - 0.000	0.999

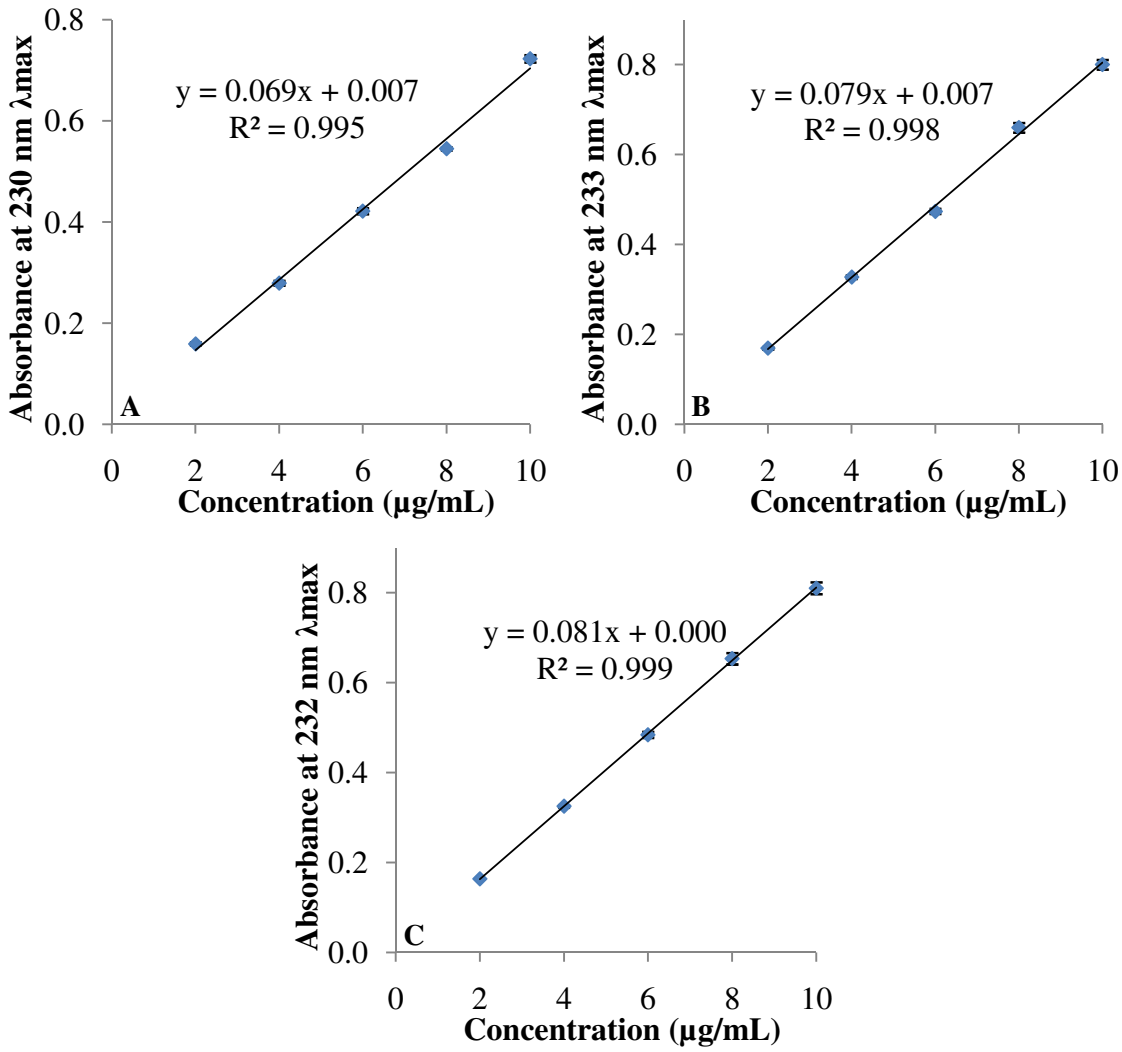

Figure 3.4.9: Calibration curves of MH in (A) 0.1N HCl, (B) Phosphate buffer pH 6.8 and (C) Distilled water

3.4.6.2 Stability

The stability of the drug in all the three solvents was ascertained over the period of 24h by measuring absorbance of the solution at 0 and 24 h. The results (Table 3.4.15) 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 3.4.10). Thus, it can be concluded that MH was stable in all three solvents over the period of analysis.

Table 3.4.15: Absorbance data of MH at 0 and 24 h for analytical stability

Conc (µg/mL)	Absorbance[#]	
	Initial	**At 24 h**
0.1N hydrochloric acid		
2	0.159 ± 0.003	0.156 ± 0.005
4	0.279 ± 0.004	0.278 ± 0.007
6	0.421 ± 0.006	0.417 ± 0.011
8	0.545 ± 0.003	0.543 ± 0.013
10	0.723 ± 0.007	0.718 ± 0.012
Phosphate buffer pH 6.8		
2	0.169 ± 0.003	0.167 ± 0.004
4	0.327 ± 0.004	0.323 ± 0.005
6	0.474 ± 0.006	0.471 ± 0.009
8	0.660 ± 0.011	0.658 ± 0.012
10	0.800 ± 0.011	0.796 ± 0.015
Distilled water		
2	0.164 ± 0.003	0.163 ± 0.004
4	0.325 ± 0.005	0.321 ± 0.009
6	0.484 ± 0.010	0.482 ± 0.011
8	0.653 ± 0.010	0.649 ± 0.014
10	0.810 ± 0.014	0.806 ± 0.013

[#] Mean ± SD (n = 3)

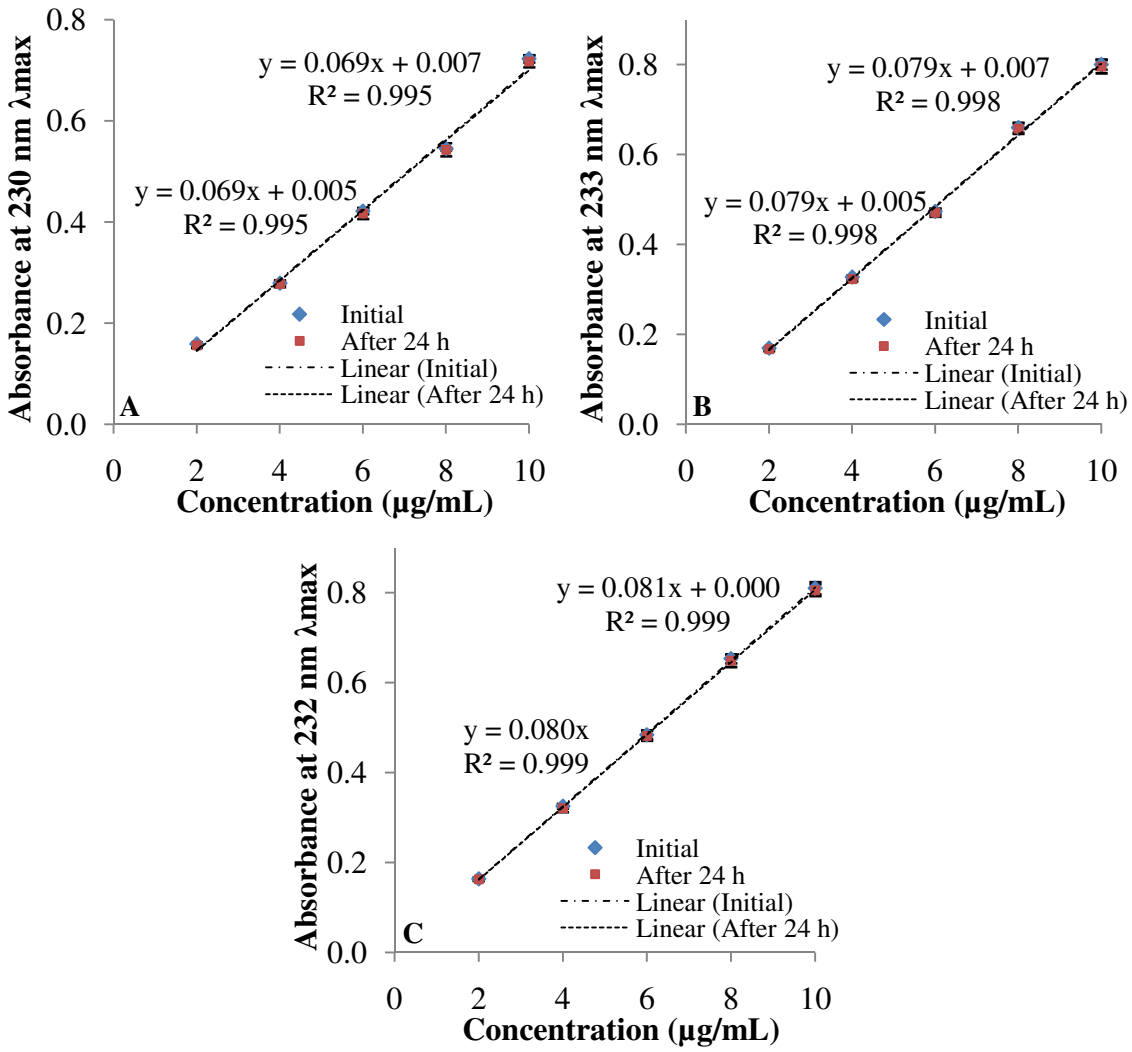

Figure 3.4.10: Calibration Curves of MH at 0 h and 24 h time points in (A) 0.1N HCl, (B) Phosphate buffer pH 6.8 and (C) Distilled water for stability study

3.4.6.3 Precision

The data for intraday and interday precision are summarized in Table 3.4.16. The results were found to be precise under the same operating conditions over the interval of time. In addition, the RSD values obtained for the analytical methods were within the acceptable range (< 2%) indicating that these methods are precise [10].

Table 3.4.16: Intraday and Interday Precision Analysis of UV Method for MH

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								
2	0.159	0.156	0.162	1.89	0.159	0.158	0.154	1.69
4	0.281	0.282	0.274	1.56	0.281	0.273	0.276	1.46
6	0.417	0.428	0.419	1.39	0.417	0.424	0.422	0.86
8	0.541	0.546	0.547	0.59	0.541	0.539	0.550	1.08
10	0.729	0.715	0.724	0.98	0.729	0.728	0.720	0.68
Phosphate buffer pH 6.8								
2	0.167	0.172	0.168	1.57	0.168	0.174	0.171	1.75
4	0.330	0.323	0.329	1.16	0.329	0.332	0.327	0.76
6	0.474	0.468	0.479	1.16	0.479	0.477	0.467	1.36
8	0.655	0.652	0.672	1.64	0.672	0.661	0.664	0.85
10	0.792	0.796	0.812	1.32	0.812	0.795	0.806	1.07
Distilled water								
2	0.162	0.167	0.162	1.76	0.162	0.168	0.167	1.94
4	0.323	0.331	0.321	1.63	0.323	0.324	0.330	1.16
6	0.486	0.493	0.474	1.98	0.486	0.482	0.489	0.72
8	0.642	0.660	0.658	1.51	0.642	0.651	0.664	1.70
10	0.795	0.821	0.815	1.68	0.795	0.803	0.811	1.00

3.4.6.4 Accuracy

Table 3.4.17 shows the data of recovery studies by standard addition or spiking method. The mean % recoveries for lower, intermediate and higher concentration were presented for all the three solvents. The mean % recovery values, close to 100% with less standard deviation (SD < 0.4%) represent high accuracy of the analytical methods [10]. These results revealed that any small change in the drug concentration could accurately be determined by the proposed analytical methods.

Table 3.4.17: Standard addition data to measure accuracy of UV Method for MH in different solvents

Spiking	Drug in solution (µg/mL)	Spiked drug (µg/mL)	Total drug found[#] (µg/mL)	% Analytical Recovery[#]
0.1N hydrochloric acid				
80%	5	4	9.083 ± 0.048	100.92 ± 0.53
100%	5	5	9.964 ± 0.051	99.64 ± 0.51
120%	5	6	10.937 ± 0.039	99.42 ± 0.35
Phosphate buffer pH 6.8				
80%	5	4	8.926 ± 0.038	99.18 ± 0.42
100%	5	5	9.894 ± 0.031	98.94 ± 0.31
120%	5	6	11.085 ± 0.056	100.77 ± 0.51
Distilled water				
80%	5	4	8.973 ± 0.057	99.70 ± 0.63
100%	5	5	9.918 ± 0.063	99.18 ± 0.63
120%	5	6	11.028 ± 0.045	100.25 ± 0.41

[#]Mean ± SD (n = 3)

3.4.6.5 Sensitivity

LOD and LOQ were calculated using the formula described above and the values are presented in Table 3.4.18 for all the three solvents. As indicated by the results, the concentration range selected for calibration was well above the LOD for all three solvents. LOQ values also indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

TABLE 3.4.18: LOD and LOQ calculation from calibration data of MH in different solvents

Solvents	Slope of line	SD of line	LOD (µg/mL)	LOQ (µg/mL)
0.1N hydrochloric acid	0.070	0.018	0.773	2.576
Phosphate buffer pH 6.8	0.080	0.011	0.425	1.416
Distilled water	0.081	0.003	0.109	0.363

3.4.6.6 Interference and specificity study

The analytical specificity of these UV spectrophotometric methods were determined by comparing the absorbance values obtained for the drug solution in selected solvent with that obtained for formulation prototype (Drug + Excipients) and excipient mixture

(without drug) in same solvent (Table 3.4.19). The representative graphs are shown in Figure 3.4.11. The absence of any overlapping or extraneous peaks in graph indicates the specificity of the UV method. Since MH peak could be clearly distinguished from the other peaks therefore, this method was said to be specific for the analysis of MH.

Table 3.4.19: Specificity and Interference Study of formulation components for MH

Solvents	Absorbance at 274 nm λ_{max}		
	Drug solution (10 µg/mL)	Formulation prototype	Excipient mixture
0.1N hydrochloric acid	0.729	0.733	0.003
Phosphate buffer pH 6.8	0.796	0.802	0.006
Distilled water	0.815	0.819	0.004

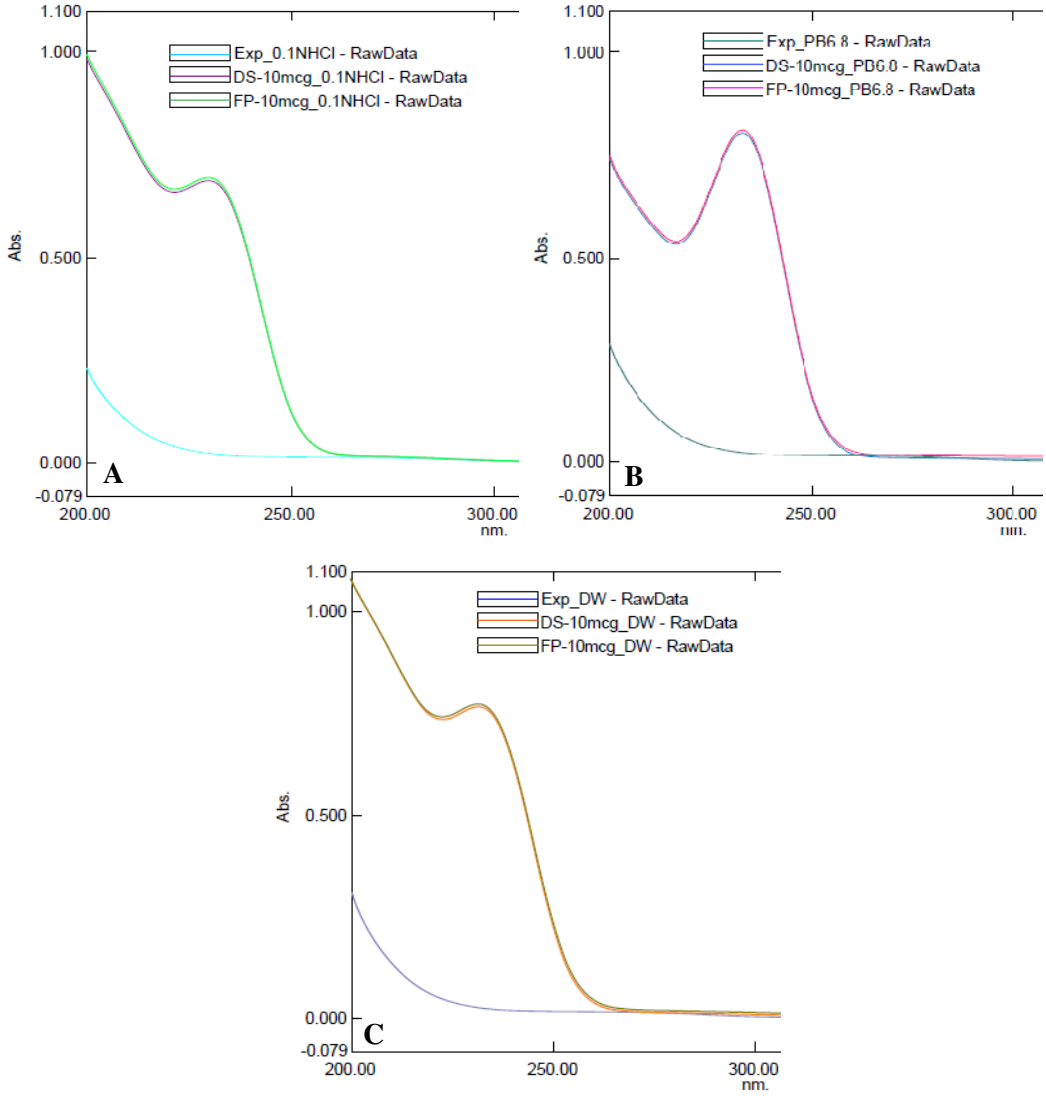

Figure 3.4.11: Specificity and Interference Study Graph between MH and Excipients in (A) 0.1N HCl, (B) Phosphate buffer pH 6.8, and (C) Distilled water

3.4.7 Estimation of MH in plasma by HPLC method

HPLC method was based on reverse phase chromatography on a C18 column [7]. The elution was done with the flow rate of 1 mL/min of mobile phase and the retention time of MH was found to be 5.1 min at 233 nm of detection (Figure 3.4.12).

Table 3.4.20: Calibration data for MH by HPLC

Concentration (ng/mL)	Peak area (µV*S)[#]
50	4734 ± 71
100	6418 ± 43
500	19983 ± 149
1000	36954 ± 249
2000	70789 ± 742

[#] Mean ± SD (n = 3)

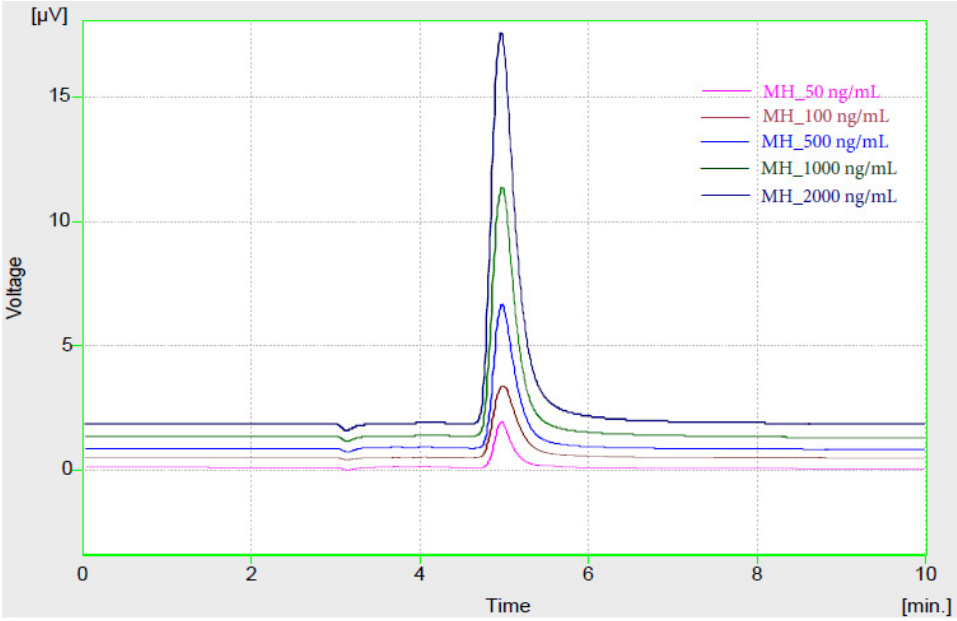

Figure 3.4.12: Typical overlay plot of MH by HPLC

3.4.8 Validation of HPLC method

3.4.8.1 Linearity

The mean peak area values along with the standard deviation for MH by HPLC method are shown in Table 3.4.20 and Figure 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. Beer's law was found to be obeyed in the range of 50 to 2000 ng/mL. Results indicated the linearity of the method for selected calibration range**.**

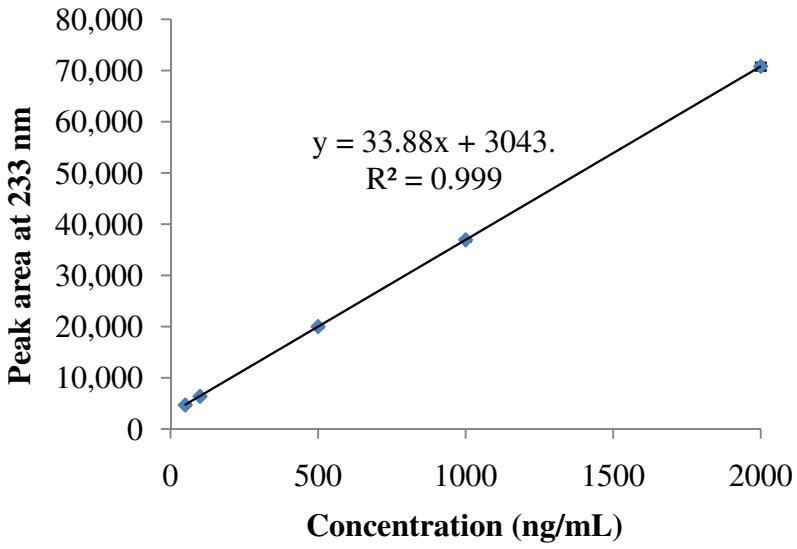

Figure 3.4.13: Calibration curve of MH by HPLC

3.4.8.2 Stability

The stability of the drug in solution was ascertained over the period of 24 h by measuring peak area of same solutions at 0 and 24 h using HPLC. The results (Table 3.4.21) revealed insignificant difference between the initial and 24 h readings. Regression analysis also showed insignificant change in the slope of straight line (Figure 3.4.14). Thus, it can be concluded that MH solution was stable over the period of analysis.

Table 3.4.21: Absorbance data of MH at 0 and 24 h for analytical stability

Concentration (ng/mL)	Peak area (µV*S)[#]	
	Initial	At 24 h
50	04734 ± 071	04698 ± 081
100	06418 ± 043	06384 ± 057
500	19983 ± 149	19935 ± 165
1000	36954 ± 249	36873 ± 251
2000	70789 ± 742	70756 ± 802

[#] Mean ± SD (n = 3)

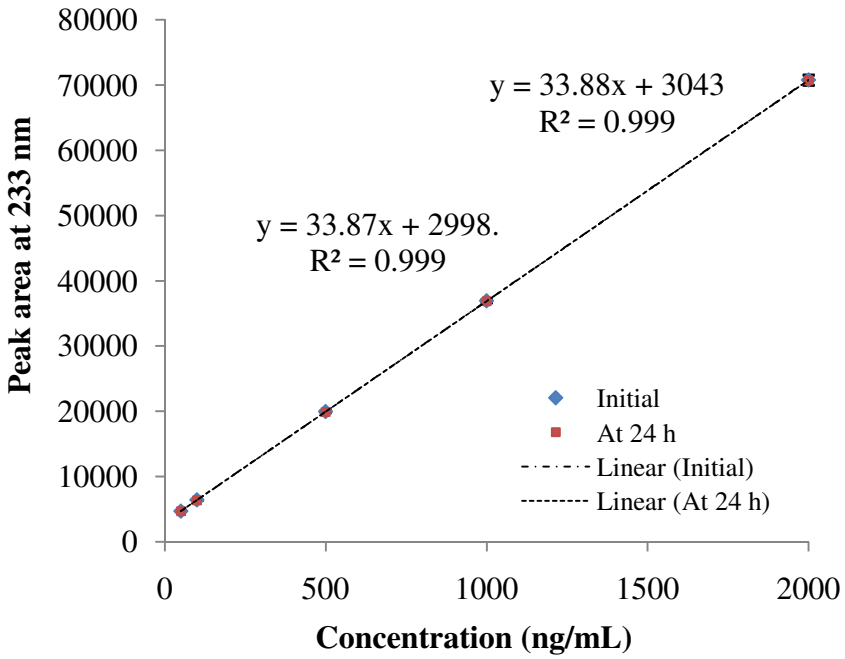

Figure 3.4.14: Calibration Curves of MH at 0 h and 24 h time points for stability study by HPLC

3.4.8.3 Precision

The data for intraday and interday precision are summarized in Table 3.4.22. The results were found to be precise under the same operating conditions over the interval of time. In addition, the RSD values obtained for the analytical methods were within the acceptable range [10] indicating that the method is precise.

Table 3.4.22: Intraday and Interday Precision Analysis of HPLC Method for MH

Conc. (ng/mL)	Intraday Precision				Interday Precision			
	Peak area			% RSD	Peak area			% RSD
	Set 1	Set 2	Set 3		Day 1	Day 2	Day 3	
50	4754	4655	4793	**1.50**	4655	4715	4771	**1.23**
100	6417	6376	6462	**0.67**	6376	6324	6493	**1.35**
500	19925	19872	20153	**0.75**	19872	20132	20076	**0.68**
1000	36848	36776	37238	**0.67**	36776	36638	37347	**1.02**
2000	71641	70442	70283	**1.05**	70442	70623	71429	**0.74**

3.4.8.4 Accuracy

Table 3.4.23 shows the data of recovery studies by standard addition or spiking method. The mean % recoveries for lower, intermediate and higher concentration were presented. The mean % recovery values, close to 100% with less standard deviations (SD ≤ 0.71%)

represent high accuracy of the analytical method [10]. These results revealed that any small change in the drug concentration could accurately be determined by the proposed analytical method.

Table 3.4.23: Standard addition data to measure accuracy of HPLC method for MH

Spiking	Drug in solution (ng/mL)	Spiked drug (ng/mL)	Total drug found (ng/mL)	% Analytical Recovery
80%	500	400	902 ± 7.3	100.22 ± 0.81
100%	500	500	995 ± 6.6	99.50 ± 0.66
120%	500	600	1084 ± 7.8	98.55 ± 0.71

3.4.8.5 Sensitivity

LOD and LOQ were calculated using the formula described above and the values are presented in Table 3.4.24. LOD and LOQ values indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

Table 3.4.24: LOD and LOQ calculation from calibration curve of MH by HPLC

Slope of line	SD of line	LOD (ng/mL)	LOQ (ng/mL)
33.880	21.091	1.868	6.225

3.5 Conclusions

Rapid analytical methods for the quantitative estimation of MS and MH using UV spectrophotometry were successfully developed and validated. The spectrometric analysis of MS were carried out between 200 - 400 nm using four different solvents viz., 0.1N HCl, phosphate buffer pH 3.0, phosphate buffer pH 6.8 and distilled water. The UV absorption by the drug was observed at 274 nm λ_{max} for all four solvents. A strong linear correlation with $R^2 \geq 0.994$ in all four solvents was observed between the concentration of the drug and absorbance obtained for a concentration range of 4 to 20 µg/mL. The spectrometric analysis of MH were carried out between 200 - 400 nm using three different solvents viz., 0.1 N HCl, phosphate buffer (pH 6.8) and distilled water and the absorption maxima were found to be 230, 233 and 232 nm, respectively. A strong linear correlation with $R^2 \geq 0.995$ in all three solvents was observed between the concentration of the drug and absorbance obtained for a concentration range of 2 to 10 µg/mL. The analytical methods for estimation of MS as well as MH were found to be precise and also depicted accuracy in measuring the additional drug concentrations spiked in standard

drug solutions. The developed UV methods were found to be specific and showed no interference with other formulation components.

HPLC methods for the quantitative estimation of MS and MH in plasma were also successfully developed and validated. The chromatographic separation of MS was carried out on a C18 column (Phenomenex) using a reported mobile phase. The flow rate was maintained at 1.4 mL/min. The UV absorption by the effluents was read at 280nm. Similarly, the chromatographic separation of MH was carried out on a C18 column (Phenomenex) using a reported mobile phase. The flow rate was maintained at 1 mL/min. The UV absorption by the effluents was read at 233 nm. The developed HPLC methods were found to be specific for both MS and MH. A strong linear correlation with an R^2 = 0.999 was observed between the concentration of the drugs and peak area obtained upon chromatographic extraction over a concentration range of 50 to 2000 ng/mL. The methods were found to measure the concentrations with significant precision and accuracy.

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