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REVERSE PHASE HPLC METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF MELOXICAM IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

Meloxicam (MEL) is an oxicam derivative and a member of the enolic acid group of non-steroidal anti-inflammatory drugs (NSAIDs). Most of the reported methods for HPLC analysis of Meloxicam are cumbersome, time-consuming and expensive. Reverse phase chromatographic analysis was performed on a C18 Hi Q Sil column with methanol–water-orthophosphoric acid (80:19.9:0.1 % v/v) at a flow rate of 1ml/min and detection wavelength of 360 nm. System suitability tests essential for the assurance of quality performance of the method were performed. The method was validated for accuracy, precision, reproducibility, specificity and robustness, limit of detection (LOD) and limit of quantification (LOQ). A single sharp peak was obtained for MEL at Rt of 4.38 ± 0.02 min. The polynomial regression data for the calibration plots exhibited linear relationship (r = 0.999) over a concentration range of 4–20µg/ml and the linear regression equation was *y = 43754x - 2094*. Accuracy ranged from 99.33 to 100.45% and the % coefficient of variation (CV) for both intra-day and inter-day precision was less than 2%. The LOD and LOQ values were 480 ng/ml and 680 ng/ml, respectively. The proposed method gave good resolution for MEL. System suitability tests and statistical analysis performed prove that the method is precise, accurate and reproducible, hence can be employed for routine analysis of MEL in bulk and commercial formulations.

INTRODUCTION: Meloxicam, 4- hydroxy-2- methyl-*N*- (5- methyl- 2- thiazolyl)- 2H- 1, 2- benzothiazine- 3- carboxamide- 1, 1-dioxide (**Fig. 1**) is a potent non-steroidal anti-inflammatory drug (NSAIDs) of the enolic acid class of oxicam derivatives which shows preferential inhibition of cyclo-oxygenase-2 (COX-2) and inhibits prostaglandin synthesis. Therapeutically Meloxicam (MEL) exhibits anti-inflammatory, analgesic and anti-pyretic activities; it is very efficient for the treatment of rheumatoid arthritis, osteoarthritis, and other joint diseases. Its therapeutic benefits combined with a good gastrointestinal tolerability are well-documented in comparison with other NSAIDs [1-3].

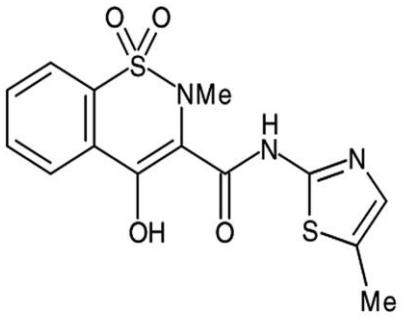


FIG. 1: STRUCTURE OF MELOXICAM

Various analytical techniques are reported for the analysis of MEL in pharmaceuticals like, UV spectrophotometry [4-5], fluorimetry [4], capillary electrophoresis [6], pulse polarography [7], electrochemical oxidation [8], electrochemical reduction [9] and voltametry [10]. HPLC is the most commonly used method for analysis of MEL. Few HPLC methods for estimation of MEL in pharmaceutical dosage forms as well as biological fluids; some of them make use of buffer in the mobile phase and they are cumbersome, time-consuming and expensive [11-14]. Method validation is an essential step in drug analysis and it confirms that the analytical procedure employed for the analysis is suitable for its intended use and shows reliability of the results produced by any method. The primary objective of the present work was thus to develop and validate HPLC method for MEL, which could also be employed for the routine analysis of the drug in pharmaceutical dosage forms. In the proposed method, the mobile phase was used directly for the dilution of the formulation after filtration, and then further used for analysis. Direct use of the mobile phase as diluent for formulations in quantitative analysis minimizes errors that occur during tedious extraction procedures.

MATERIAL AND METHODS:

Materials: MEL was obtained as a gift sample from Zest Pharma, Indore, India. Acetonitrile, Methanol, Ortho phosphoric acid, Acetic acid (HPLC– grade) was purchased from Merck, India. Millipore purification system was used for high purity water. All other chemicals and reagents employed were of analytical grade and were purchased from S.D. Fine Chemicals, India.

Chromatography method: The chromatograph system comprised of a Jasco PU-980 pump equipped with a Jasco UV-975 detector and a Rheodyne injector with a 20-microlitre loop. Data integration was done using a Borwin software package V1.21. Samples were injected into a Hi-Q-Sil C-18 column (4.6 x 250mm, 5µ particle size). Mobile phase flow rate was 1ml/min. The drug was analyzed at a wavelength of 360nm.

Method development: Initial trial experiments were conducted, with a view to select a suitable solvent system for the accurate estimation of the drug and to achieve good resolution between the drug and the degradation products. The suitability of the mobile phase was decided on the basis of the sensitivity of the assay, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents. These included methanol-water (50:50 % v/v), methanol-water (80: 20 % v/v), methanol-water-glacial acetic acid (55: 45:5 % v/v), methanol-water- orthophosphoric acid

(80: 19.9: 0.1 % v/v), methanol- water, (70: 30 % v/v) and methanol-water- orthophosphoric acid (70: 29: 1 %v/v). A mobile phase system comprising of methanol- water- orthophosphoric acid (80: 19.9: 0.1 % v/v) was found to be optimum. The same solvent mixture was used for the extraction of the drug from the formulation containing excipients. The solvents were mixed, filtered through a membrane filter of 0.45 micron pore and degassed before use.

Method Validation:

Linearity: A series of standard curves were prepared over a concentration range of 4-20 µg/ml from a stock solution of MEL (1mg/ml) in Methanol. Dilutions were prepared in the mobile phase: methanol–water- orthophosphoric acid (80:19.9:0.1 % v/v). The procedure for analysis follows that described earlier under the subsection, 'Chromatography method'. The data from peak area versus drug concentration plots were treated by linear least square regression analysis. The standard curves were evaluated for intra-day and inter-day reproducibility. Each experiment was repeated in triplicate.

Precision: Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. The three components of precision, i.e., repeatability, intermediate precision and reproducibility, in accordance with ICH recommendations, were determined as follows:

Repeatability: Injection repeatability: Five injections of 12 µg/mL solution of MEL were analyzed and %RSD calculated for injection repeatability. Intra-day variation: Measurement of intra-day variation of MEL solutions at three different concentrations (8, 12 and 16 µg/mL) was carried out by injecting the samples on the same day at different time intervals.

Analysis repeatability: It was obtained by determining the relative standard deviation (RSD) of replicate samples (n=3) of the accuracy study.

Intermediate Precision (Inter-day Variation): Measurement of inter-day variation of MEL solutions at three different concentrations (8, 12 and 16 µg/mL) in triplicate on three consecutive days were determined.

Reproducibility: The reproducibility of the method was checked by determining precision on the same instrument, but by a different analyst. For both intra-day and inter-day variation, solutions of MEL at three different concentrations (8, 12, and 16 µg/mL) were analyzed in triplicate.

Accuracy: Accuracy is the measure of how close the experimental value is to the true value. Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method. Previously analyzed samples of MEL (12 µg/ml) were spiked with 50, 100, and 150% extra MEL standard and the mixtures were analyzed by the proposed method. The experiment was performed in triplicate. Recovery (%), RSD (%) and standard error of mean (SEM) were calculated for each concentration.

LOD and LOQ: In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ) values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level while ten times the noise value gave the LOQ.

Robustness: The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions on the determination of MEL. Robustness was determined by using reagents from two different lots and two different manufacturers.

Sample Solution Stability: The stability of the drug in solution during analysis was determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of the drug solution for 72 h under laboratory bench conditions (25±1°C) and under refrigeration (8±0.5°C). An accurately weighed quantity of the pure drug was dissolved in methanol and suitably diluted with mobile phase to get a final concentration of 12µg/ml. The solution was subjected to HPLC analysis immediately and after a period of 24, 48 and 72 h.

Specificity/Selectivity: To assess the method selectivity marketed tablets were analyzed. Ten tablets (strength: 15 mg/tablet) were crushed and triturated well in a mortar. A powder sample, equivalent to 15mg of MEL, was accurately weighed and transferred to a 25ml volumetric flask. The drug was extracted into methanol and mixed thoroughly for 30 min using a sonicator. The solution was filtered through 0.45 micron pore filter after making up the volume, adequately diluted with mobile phase and analyzed by the proposed HPLC method. The possibility of interference of excipients with the analysis was studied.

System Suitability Tests: The chromatographic systems used for analyses must pass the system suitability limits before sample analysis can commence. The capacity factor (K), injection repeatability (as described earlier in the subsection, 'Precision'), tailing factor (T), theoretical plate number (N) and resolution (Rs) for the principal peak and its degradation product were the parameters tested on a 12 µg/mL sample of MEL to assist the accuracy and precision of the developed HPLC system.

Data Analysis: The r value for the calibration plot, SD, RSD, and SEM were determined using Microsoft Excel 2007 application.

RESULTS:

Method development: Methanol- water- ortho phosphoric acid (80: 19.9: 0.1 % v/v) was selected as the optimum mobile phase. Under these conditions the retention time and tailing factor were 4.38±0.02 min and 1.00 respectively. A typical chromatogram is represented in **Fig. 3** (**d**).

Method validation:

Linearity: Peak area versus drug concentration was plotted to construct a standard curve for MEL. The polynomial regression for the calibration plots showed good linear relationship with coefficient of correlation, r= 0.999±0.0091; slope= *43754*±126.65 and intercept= *2094*±59.33 (n= 6) over the concentration range studied. The range of reliable quantification was set at 4-20µg/ml as no significant difference was observed in the slopes of the standard curves in this range. The linear regression data for the calibration plot is indicative of a good linear relationship between peak area and concentration over a wide range (**Fig. 2**). The correlation coefficient was indicative of high significance. The low values of the standard deviation, the standard error of slope, and the intercept of the ordinate showed the calibration plot did not deviate from linearity.

Precision: Precision was measured in accordance with ICH recommendations. Five consecutive injections of 12 µg/mL solution of MEL by the proposed method showed excellent injection repeatability with RSD of only 0.55%. Repeatability of sample injection was determined as intra-day variation while intermediate precision was determined by measuring inter-day variation for triplicate determination of MEL at three different concentrations. The results of the determination of repeatability, intermediate precision and reproducibility are listed in **Table 1**. Reproducibility was checked by measuring the precision of the proposed method with analysis being performed by

another person. The low RSD values indicate the repeatability and reproducibility of the method.

Recovery: The recovery of the method, determined by spiking a previously analyzed test solution with additional drug standard solution, was found to be in the range of 99.33-100.45%. The values of recovery (%), RSD (%) and SEM listed in **Table 2** indicate the method is accurate.

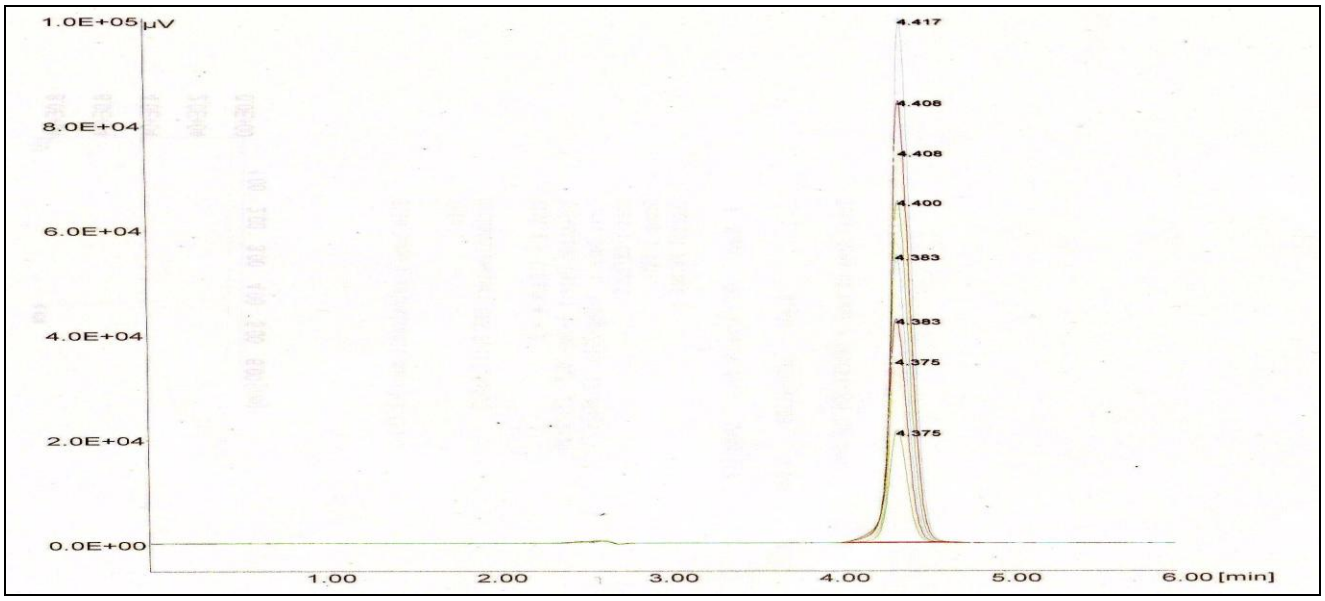


FIG. 2: LINEARITY BETWEEN PEAK AREA AND CONCENTRATION OVER A WIDE RANGE

TABLE 1: PRECISION OF METHOD

Intra- day and inter-day precision						
Conc. (µg/mL)	Repeatability (Intra- day precision)			Intermediate precision (Inter- day)		
	Mean area ± SD*	SEM	RSD (%)	Mean area ± SD*	SEM	RSD (%)
8	331946 ±3116	1799.4	0.930	339979 ±4130	2384.5	1.260
12	533545 ±5748	3318.8	1.077	530291 ±1737	1002.9	0.327
16	700544 ±2896	1672.1	0.413	698364 ±2315	1336.5	0.331
Reproducibility						
Conc. (µg/mL)	Repeatability (Intra- day precision)			Intermediate precision (Inter- day)		
	Mean area ± SD*	SEM	RSD (%)	Mean area ± SD*	SEM	RSD (%)
8	333361 ±1325	765.2 0.397	0.397	341839±6390	3689.5	1.860
12	524268 ±854	493.04 0.162	0.162	528732 ±7031	4059.8	1.320
16	696983 ±2722	1571.4 0.390	0.390	699009 ±6470	3735.5	0.925

TABLE 2: ACCURACY OF METHOD

Amount (%) of drug added to analyte SEM	Theoretical content (µg/ml)	Conc. Found (µg/ml) ± SD*	Recovery (%)	RSD (%)	SEM
0	12	11.92±0.170	98.33	1.426	0.098
50	18	17.98±0.246	99.88	1.368	0.142
100	24	24.11±0.104	100.45	0.431	0.060
150	30	29.92±0.158	99.73	0.528	0.158

*n = 3, SEM = standard error of mean

Detection and Quantification limits: The limit of detection was found to be 480ng/ml where the drug could be detected without any noise. The limit of quantification was 680ng/ml. This indicated the method can be used for detection and quantification of MEL over a very wide range of concentrations.

Robustness: There was no significant change in the retention time of MEL when reagents (acetonitrile and glacial acetic acid) from different lots and different manufacturers were used. The concentration of the solution analyzed was 12 µg/mL and the % RSD ranged from 0.132 to 1.366 %. The low values of the RSD indicated the robustness of the method.

Stability: There was no significant change in analyte composition (sample concentration = 12 µg/mL) over a period of 72 h. The mean RSD between peak areas, for the samples stored under refrigeration (8 ± 1°C) and at laboratory temperature (25 ± 1°C) was found to be 1.126% and 0.983% respectively, suggesting that the drug solution can be stored without any degradation over the time interval studied.

Specificity (Analysis of MEL from marketed tablets): A single peak was observed at the retention time of MEL when a suitably diluted solution of the tablet formulation was chromatographed. No interaction was observed between MEL and excipients present in the tablets. The MEL content was found to be 99.58% and the RSD was 0.97%. The low RSD indicated the suitability of this method for routine analysis of MEL in pharmaceutical dosage forms.

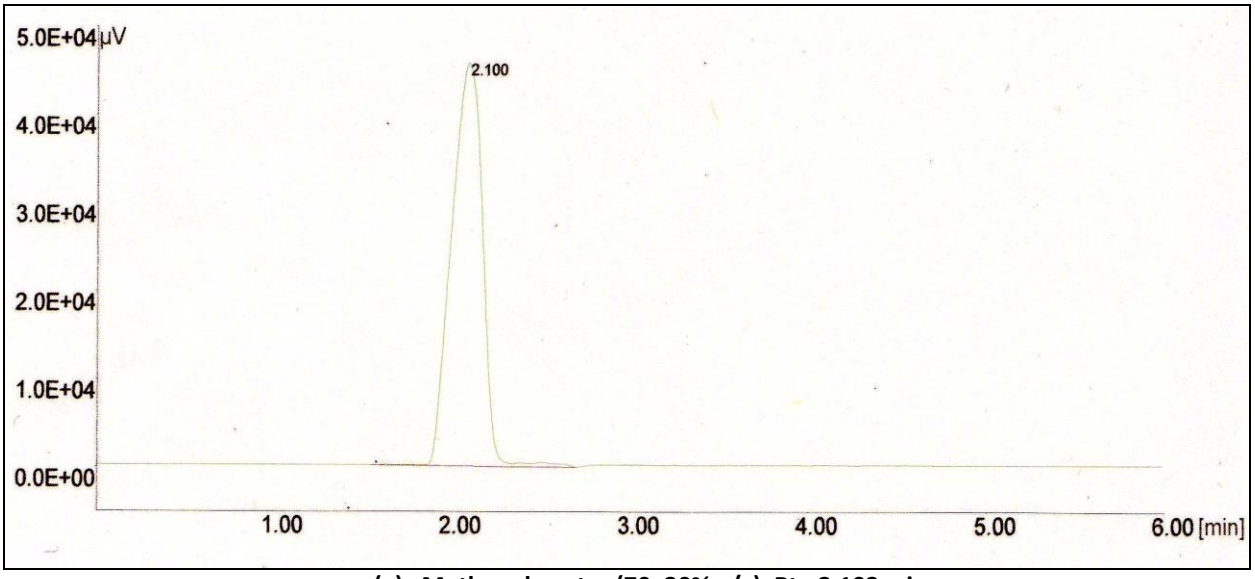
System Suitability Tests: The results of the system suitability tests assure the adequacy of the proposed HPLC method for routine analysis of MEL. The capacity factor (k) was found to be 1.88, indicating that the MEL peak is well resolved with respect to the void volume. The RSD of five consecutive injections performed under the precision test was found to be 0.58% and thus shows good injection repeatability. The tailing factor (T) for MEL peak was found to be 1.00, reflecting good peak symmetry. The theoretical plate number (N) was found to be 7444, thus demonstrating good column efficiency.

DISCUSSION: The final decision on mobile phase composition and flow rate was made on the basis of peak shape, peak area, tailing factor, baseline drift and time required for analysis. The solvent system selected [methanol- water-orthophosphoric acid (80: 19.9: 0.1 % v/v)] gave good resolution of drug peak (Fig. 3d). No internal standard was used because no extraction or separation step was involved. Methanol-water (70:30 %v/v) did not furnish a sharp, well-defined peak (**Fig. 3a**). Other mobile phases tried resulted either in much lower sensitivity, delayed retention time or poor peak shapes, and so were not considered (**Fig. 3**).

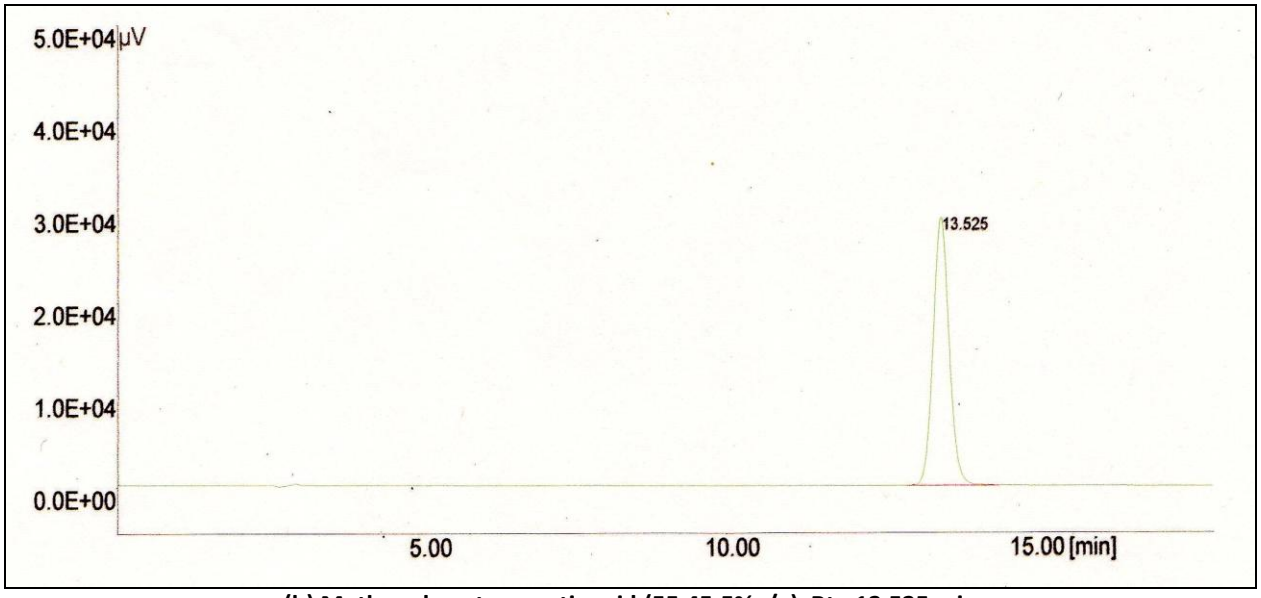
The proposed HPLC method of analysis was also found to be precise and accurate, as depicted by the statistical data of analysis. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration plots and obedience to Beer's laws. The RSD values and the slopes and intercepts of the calibration graphs indicate the high reproducibility of the proposed method. The method was also found to be robust as there was no significant change in the peak area, peak shape and retention time of MEL.

Furthermore, the low values of LOD and LOQ indicate that the method can be employed over a wide concentration range for linearity. This method is also highly sensitive and could effectively separate the drug from its degraded

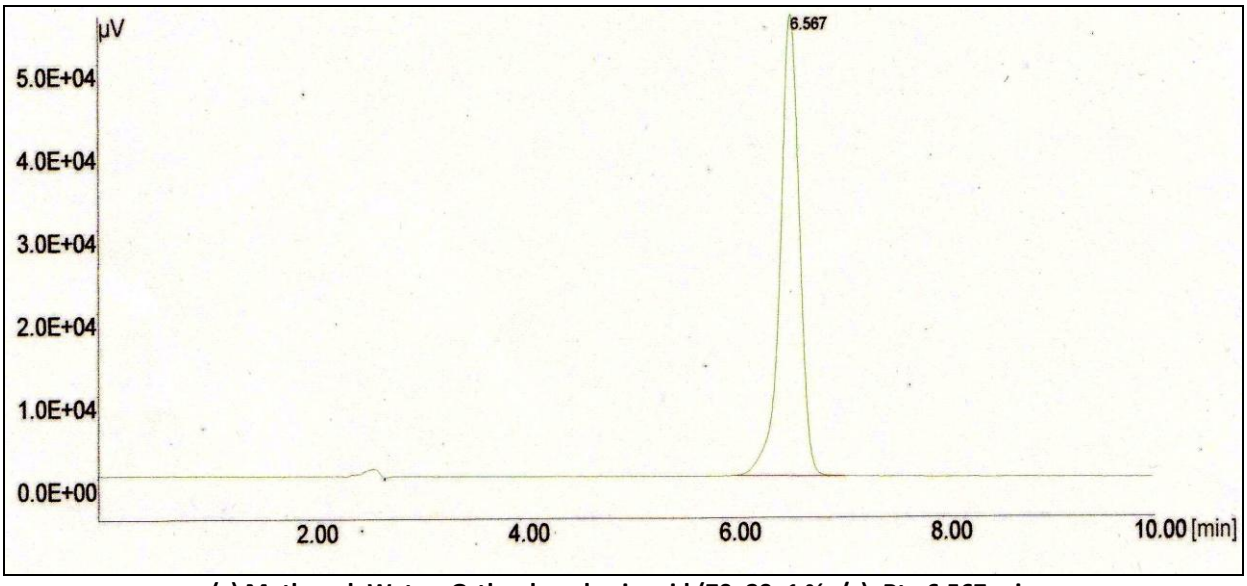
product. MEL is a thiazolyl substituted benzothiazine carboxamide. Solution of MEL is stable at room temperature. The system suitability tests performed verified the resolution, column efficiency and repeatability of the chromatographic system and ensured that the equipment, electronics, and analytical operations for the samples analyzed could be constituted as an integral system that can be evaluated as a whole.



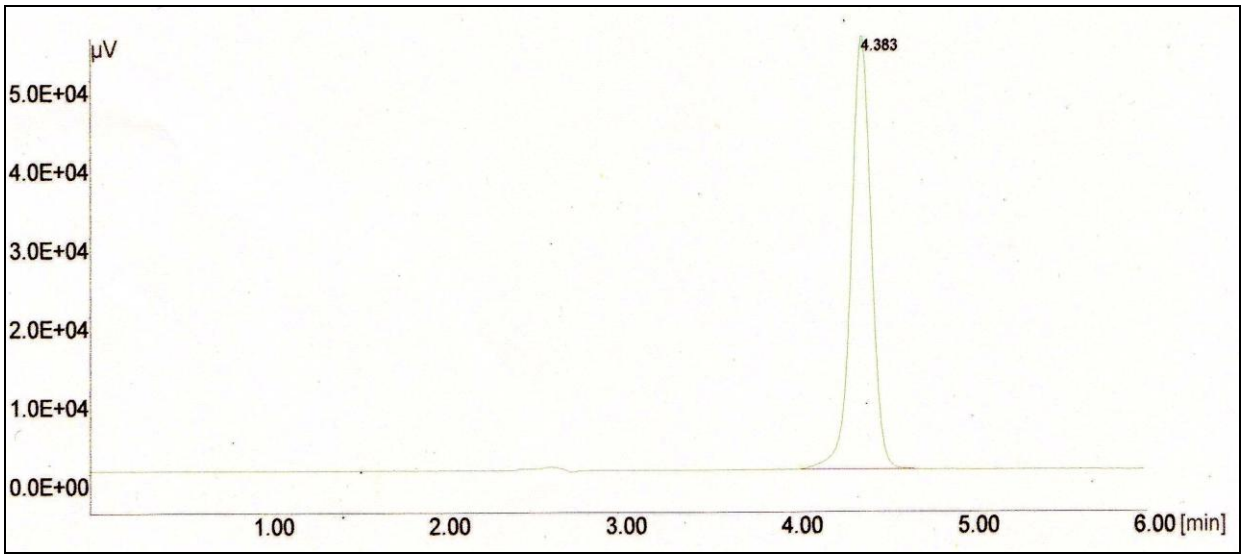
(a) Methanol: water (70: 30% v/v), Rt= 2.103 min



(b) Methanol: water: acetic acid (55:45:5%v/v), Rt= 13.525 min



(c) Methanol: Water: Orthophosphoric acid (70: 29: 1 %v/v), Rt= 6.567 min



(d) Methanol-water-orthophosphoric acid (80:19.9:0.1 %v/v) Rt= 4.383 min

FIG. 3 (a-d): PEAKS OBTAINED FOR MEL USING DIFFERENT MOBILE PHASES

CONCLUSION: The HPLC method developed is accurate, precise, reproducible and specific. The method is linear over a wide range, economical and utilizes a mobile phase which can be easily prepared. All these factors make this method suitable for quantification of MEL in bulk drugs and in pharmaceutical dosage forms. It can therefore be concluded that use of this method can save much time and money and it can be used even in small laboratories with very high accuracy and precision. The method can also be used for the routine analysis of MEL in bulk preparations of the drug and in pharmaceutical dosage forms without any interference.

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