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An Efficient RP-HPLC Method for the Estimation of Melphalan in Pharmaceutical Formulations

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ABSTRACT

A simple, convenient and efficient Reverse Phase High performance liquid chromatographic (HPLC) method for the estimation of Melphalan (MF: $C_{13}H_{18}Cl_2N_2O_2$; MW: 305.2) in pharmaceutical formulations was developed and validated. The method was developed using the mobile phase 0.01M KH_2PO_4, Acetonitrile and Methanol in the ratio 30:45:25(v/v/v) at a pH 3.8 with a flow rate of 1mL/min, at a wavelength of 210nm on an isocratic HPLC (PEAK-7000) system. The chromatogram obtained is a high resolution chromatogram with good tailing factor (<2). The operating pressure is 24-30 Mpa at room temperature and the total run time is 8min. The Limit of Detection (LOD) and Limit of Quantification (LOQ) values obtained are 0.05 and 0.165ppm respectively. The applicability of the method was also tested with commercial sample of alkeran-5mg formulation.

Keywords: Melphalan, HPLC estimation, Alkeran, Validation.

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INTRODUCTION

Chemically Melphalan [1] (L-PAM, MF: $C_{13}H_{18}Cl_2N_2O2$; MW: 305.2) (Fig.1) is 4-[bis(Chloroethyl)amino]-phenyl alanine and is soluble in 95% ethanol and 1 drop of 6N HCl. It is used to treat multiple myeloma [2, 3], ovarian cancer and occasionally malignant melanoma. A survey of literature revealed that there are some HPLC methods [4-7] and LC-MS [8] methods for the estimation of Melphalan. In the present investigation an attempt was made to develop a prominant RP-HPLC method for the estimation of L-PAM in pharmaceutical dosage forms.

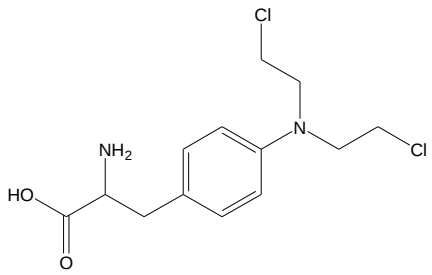

Fig.1. The structure of Melphalan [IUPAC Name: 4-[Bis(chloroethyl)amino]-phenyl alanine.

MATERIALS AND METHODS

Equipment

Analysis was carried out using PEAK 7000 isocratic HPLC with rheodyne manual sample injector with switch (77251) and the column used was Analytical column kromosil 100-5 C-18 (250x4.6mm). Electronic balance used was ELB300, DIGISUN pH meter was used for all pH measurements.

Chemicals and reagents

Melphalan reference standard was a kind gift of V.V.MED Laboratories, Hyderabad and the tablet formulation (Alkeran-5mg) used for testing the method was purchased from local market. The solvents used were Methanol and Acetonitrile of HPLC grade and KH2PO4 (GR) of Merck manufactures.

Optimized Chromatographic Conditions

Chromatographic analysis of the Melphalan was done using a Kromasil C18, (250x4.6mm, 5µm). The mobile phase composition used was Acetonitrile 45%, 0.01M KH2PO4 30% and Methanol 25% was filtered through 0.5µ nylon membrane filter before use and the pH was at 3.8. The analysis was carried out in isocratic mode at a flow rate of 1ml/min. The detector wavelength is 210nm and the operating pressure is 24-30 Mpa at room temperature. The injecting volume is 20µL and the total run time is 8min.

Preparation of standard solutions

Pure standards of Melphalan were used as external standards in the analysis. Different concentrations of the standards were used based on the range required to plot a suitable calibration curve. About 10mg of melphalan drug transferred into a 10ml volumetric flask and made up to the mark by using methanol. The flask containing standard stock solution was sonicated for 10 minutes to degas it. The standard solution was then filtered with 0.45µm membrane filter paper. A series of different dilutions (2-10ppm) were prepared using above stock solution with mobile phase(0.01M KH_2PO_4, Acetonitrile and Methanol in the ratio 30:45:25(v/v/v)).

Sample preparation

8ppm of sample solution was prepared by accurately weighing the required amount of the drug and transferring it into a 100ml volumetric flask and added mobile phase. The sample solution was then filtered with 0.45µm membrane sample filter.

Procedure for analysis

With the optimized chromatographic conditions set for Melphalan a study base line was recorded and stabilized for abut 30min. After the stabilization of base line successive aliquots of the sample solution were recorded, until the reproducibility of the peak areas was adequate. The sample was injected into the column at flow rate of 1ml/min.

RESULTS AND DISCUSSION

In addition to the earlier methods [4-8], recently an RP-HPLC method [9] was reported for the estimation of L-PAM in tablet dosage forms using Acetonitrile, water and 1% ortho phosphoric acid in the ratio 70:27:3(v/v/v) as mobile pahse. However, in the present investigation, an efficient alternative RP-HPLC method to the above method was developed for the estimation of L-PAM in pharmaceutical formulations. The method was in good agreement in respect of linearity(0.999), accuracy(99.40%), run time(8min) when compared with above mentioned HPLC methods [4-9]. The method was developed according to ICH guide lines [10,11]. The standard chromatograms of the L-PAM was given in Fig 2.

Method Validation

After the completion of HPLC method development the method was validated in terms of different parameters like linearity, specificity, precision, accuracy, LOD and LOQ.

Evaluation of linearity

The Linearity of the method was evaluated by analyzing different concentrations of the standard solutions. Melphalan solutions of 2ppm, 4ppm, 6ppm, 8ppm, 10ppm with standard

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100% pure Melphalan in the mobile phase were prepared and analyzed. After analysis the area of peaks were recorded and were reported in Table-1. It was found that there were no notable changes in the chromatograms for flow rate variation, column temperature variation and mobile phase variation for a wide range of drug concentration of 2-10ppm.

A plot was drawn by taking concentration on x-axis and area of the peaks on y-axis. It was found that a straight line satisfying linearity condition i.e. the correlation coefficient (r^2=0.999, Fig. 3); of regression was found almost equal to 1 and is also more efficient than the earlier method[9] (r^2=0.998).

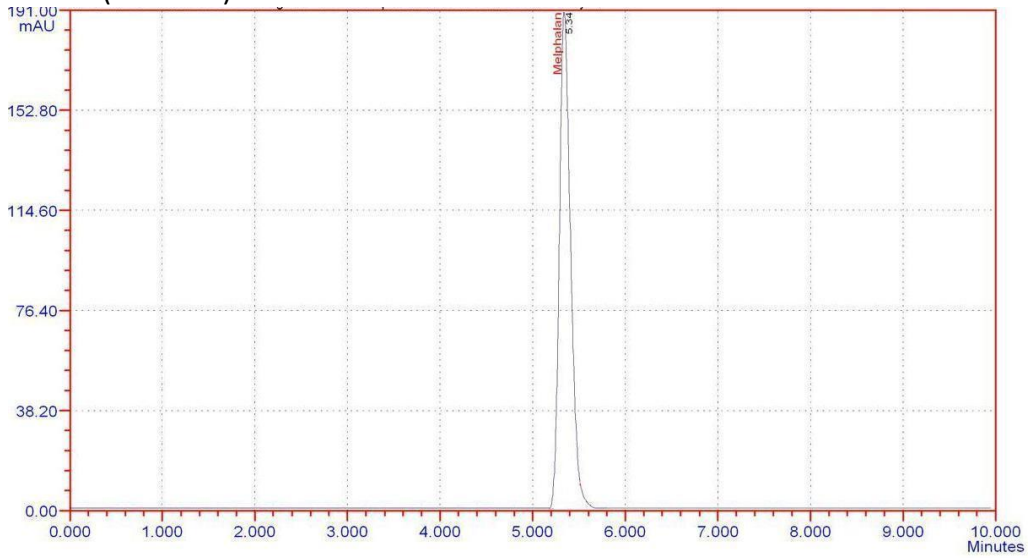

Figure 2: Standard chromatogram of Melphalan

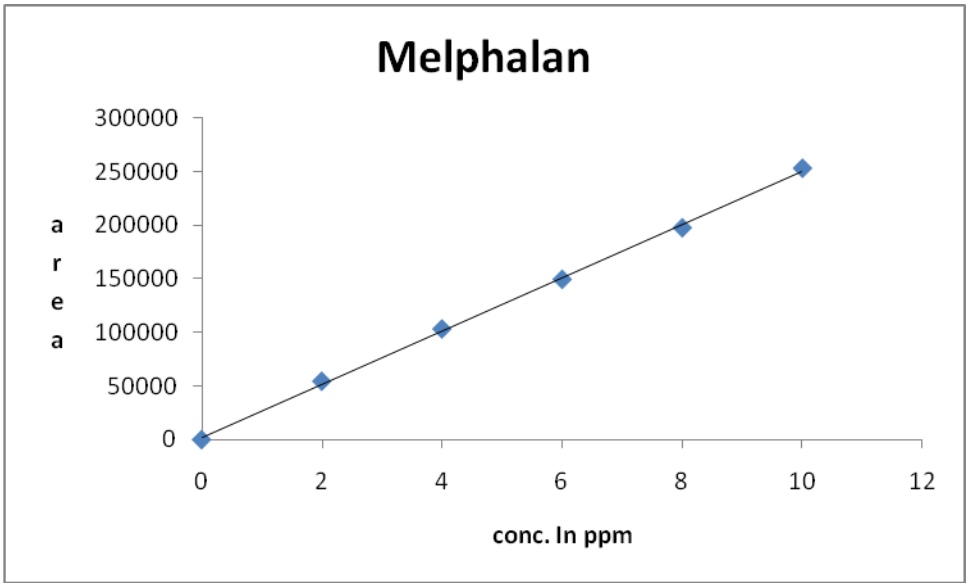

Figure 3: Linearity of Melphalan

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Table 1. Linearity data of Melphalan for the developed method.

S.NO	CONC ppm	AREA
1	2	54330.3
2	4	102982.7
3	6	149167.6
4	8	197145.7
5	10	252559.3

INTERCEPT = 4050.82
SLOPE = 24531.05
r^2 = 0.999

Accuracy (% Recovery)

To study of the reliability, validity, suitability and accuracy of the method, recovery experiments were carried out for Melphalan in 3 stages and the recovery of the drug was calculated. For this, the recovery studies were performed using standard addition method i.e. a known quantity of pure drug was added to the pre-analysed sample formulation. The results of these experiments from the linearity curve were given in Table 2. The % recovery of the drug in each stage is calculated by using the formula given below. The average recovery of the Melphalan from the linearity curves is 99.26%.

$$\% \text{ recovery} = [(b-a)/c] \text{ X}100$$

Where a- The amount of drug found before the addition of standard drug.
b- The amount of drug found after the addition of standard drug.
c- The amount of standard drug added.

The average % of recovery of melphalan was also calculated by standard chromatograms obtained at 6ppm concentration of the pure and sample drugs. The data obtained were given in Table 3 and from these chromatograms the average recovery was found to be 99.40%. The results obtained were more reliable and in good agreement in terms reliability, suitability and accuracy when compared to earlier methods [4-9]. The percentage of Melphalan found in alkeran(5mg) formulation is 1%.

Table 2. Recovery of the drug at stage 1 from linearity curve.

Labeled amount	Amount added	Total amount	% of recovery	Mean
4	2	6	98.88	99.26%
4	4	8	99.80	
4	6	10	99.12	

Table 3. Accuracy data of the developed method from chromatograms.

Conc. 6ppm	AREA	TH.PLATES	99.40%
STANDARD	149167.6	8899	
SAMPLE	148281.3	8960	

ISSN: 0975-8585

Intermediate Precision

A standard solution (8ppm) of drug substance was injected six times and corresponding peak areas were recorded. The % RSD found were less than 1%. The value of the %RSD obtained in intraday precision is 0.895(Table 4) and the inter day precision is 0.529(Table 5). The values of %RSD within a day, day to day variation (<1%) proves that the method is precise.

Table 4. Intraday precision

TEST.1	CONC 8ppm			PRECISSION
	INJECTION	AREA	TH.P	
	1	199656.4	8408.96	
	2	195644.9	8654.66	
Intraday	3	194845.6	8698.40	%R.S.D = 0.895
	4	196355.0	8609.95	
	5	195086.0	8682.02	
	6	195889.8	8640.89	

Table 5. Inter-day precision

TEST.1	CONC 8ppm			PRECISSION
	INJECTION	AREA	TH.P	
	1	201019.7	8336.27	
	2	203174.0	8216.75	
Inter-day	3	201993.6	8275.05	%R.S.D = 0.529
	4	201384.7	8319.03	
	5	200928.6	8334.94	
	6	200045.9	8385.89	

Specificity of the method

The specificity of the method was determined by observing any interference encountered from the ingredients present in the sample formulation. The test results obtained were compared with that of test results those obtained for standard drug. In the present study, it was shown that those ingredients are not interfering with the proposed method.

Ruggedness

Inter-day variations were performed by using six replicate injections of standard and sample solutions of concentrations which were prepared and analyzed by different analyst on two different days over a period of one week. Ruggedness also expressed in terms of percentage relative standard deviation, which is in good agreement for the developed method.

Robustness and system suitability

Robustness of the method was carried out by varying two parameters slightly from the Optimized chromatographic conditions, such as flow rate, column temperature and mobile

phase. It was found that there were no notable changes in the chromatograms for flow rate variation, column temperature variation and mobile phase variation. The robustness limit for the above parameter variations was well within the acceptable limit and is less than 2%. This shows that the method is having good system suitability under the given set of conditions.

Limit of Detection and limit of Quantification (LOD and LOQ)

The limit of Detection (LOD) and limit of Quantification (LOQ) (Table 6) of the developed method were determined by injecting increasingly low concentrations of the standard solutions by following the developed HPLC method. The LOD is the smallest concentration of the analyte which gives a measurable response. The LOD for Melphalan was found to be 0.165ppm. The LOQ is the smallest concentration of the analyte, which gives response that can be absolutely quantified. The LOQ for Melphalan was found to be 0.05ppm. The results of LOD and LOQ supported the sensitivity of the developed method.

Table 6 LOD and LOQ

Limit of Detection (L. O. D.)	0.165ppm
Limit of Quantification (L. O. Q)	0.05ppm

CONCLUSION

A simple, efficient and convenient RP-HPLC method was developed and validated interms of linearity, accuracy, precision, etc. The proposed method is also applicable to the analysis of Melphalan in pharmaceutical formulations.

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