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Simaltaneous Determination of Melphalan and Its Process-Related Impurities Using a Stability-Indicating and Validated Reverse Phase Hplc Method in a Short Run Time.

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Abstract: *Degradation pathway of melphalan was validated by stability indicating reverse phase liquid chromatographic method. melphalan was subjected to stress condition using acid, base or by oxidation, and photolysis. Significant degradation was observed in acid and base stress conditions. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 98.2 %. Efficient chromatographic separation was achieved on a Luna C18 (4.6 x 150 mm) 3 µm stationary phase with simple mobile phase combination. In the developed LC method, the resolution of melphalan and potential impurities such as Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate was found to be more than 2.0. Regression analysis showed correlation coefficient (r) of greater than 0.999 for melphalan and eight potential impurities. This method was capable of detecting the impurities of melphalan at a level of 0.02 % with respect to test concentration of 0.5 mg/mL. The developed rapid LC method was showed specificity, linearity, accuracy, precision and robustness for impurities.*
Key words: *Melphalan, method development, validation, precision and robustness.*

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I. Introduction

Method development is a multi step process involves adoption of existing method, making minor changes suitable for the novel application and developing a method for the estimation of drug using a HPLC. Method validation is a process normally followed to acceptability of a analytical techniques used to determine drugs in pharmaceutical dosages.

Melphalan is chemically known as 4-[bis (2-chloroethyl) amino]-L-phenylalanine [1-3]. It is L-phenylalanine derivative of nitrogen mustard (L-PAM). It is a bifunctional alkylating agent, active against of multiple myeloma, lymphomas, malignant melanoma and ovarian carcinoma [4-5]. The molecular formula is $C_{13}H_{18}C_{l2}N_2O_2$ and the molecular weight is 305.20. The structural formula of melphalan was depicted in Figure 1 [6]. Active melphalan is a L-isomer and was first synthesized in 1953 by Bergel and Stock [7]. The D-isomer is less active and requires high dosage to produce effects on chromosomes. The racemic (DL-) form is known as sarcolysin [8]. Melphalan is normally insoluble but melphaln hydrochloride is soluble in water and has a pKa1 of ~2.5. The injection form of melphalan hydrochloride was supplied as a sterile, non pyrogenic, freeze-dried powder. Each single-use vial contains melphalan hydrochloride equivalent to 50 mg melphalan and 20 mg povidone. It is reconstituted using the sterile diluents. Sodium citrate 0.2 g, propylene glycol 6.0 mL, ethanol (96%) 0.52 mL, and water in a total of 10 mL [9]. Melphalan hydrochloride injection is administered intravenously. Some of the chromatographic methods and Moss spectroscopy methods were reported for the estimation of melphalan in tablets dosage forms. Melphalan can be determined by different spectrophotometric methods [10-19]

The present study describes a simple, convenient efficient and robust HPLC method for the simultaneous estimation of melphalan content and related impurities in melphalan drugs.

II. Experimental

2.1 Chemicals:

The melphalan and its related impurities such as Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate used in the present study were of > 99% purity and provided by PS3 LABORATORIES LIMITED LLP, Hyderabad. Acetonitrile, trifloro acetic and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany).All other chemicals used in the present were analytical grade were obtained from commercial source.

2.2 Equipment's:

The Liquid chromatography system was used for the method development and method validation. The system consists of binary separation module equipped with an auto sampler and a photo diode array detector (Waters, USA). The output signal was monitored and processed using Empower software (Empower 3) view sonic computer. Photo stability and thermal stability studies were carried out using photostability chamber (Newtronic life care, India). Dry hot air oven (Newtronic life care, India), respectively.

2.3 Chromatographic conditions:

The chromatographic column, Luna C18 (4.6 x 150 mm, 3 µm) was used with a mobile phase-A containing 750 mL of water, 250 mL of acetonitrile and 1 mL of trifloro acetic and mobile phase-B containing 250 mL of water, 750 mL of acetonitrile and 1 mL of trifloro acetic. The flow rate was fixed at 1.0 mL/min, as autosampler, column temperatures were maintained at 25 and 5°C respectively and detection wavelength was set at 265 nm. 10µL of sample methanol was injected into system. Total system run was carried out for 40 min. The gradient program was fixed as shown in the table 1.

2.4 Preparation of standard solutions and sample solutions:

The system suitability test solution (0.5 mg/ml) was prepared by suspending melphalan standard in methanol using ultrasonic bath. The quantification test solution was prepared by diluting system suitability test solution with methanol (1:100). The limit of quantification test solution (0.05%) was prepared by diluting quantification test solution to with methanol (1:20). Unless otherwise stated freshly prepared solution was used. The commercially obtained melphalan sample was reconstituted with 10 mL water and diluted with methanol (5 mg/mL). The sample solution was transferred to 10 mL sample solvent and filtered through a 0.45µm nylon disk membrane filter.

III. Method Development

3.1 Method development and optimization

Method development was initiated by multiple experiments. In experimental setup-1 mobile phase- A consisting of 950 mL of water, 50 mL of acetonitrile and 1 mL of trifloroacetic acid and mobile phase-B consisting of 50 mL of water, 950 mL of acetonitrile and 1 mL of trifloroacetic acid, stationary phase (Waters C18 150 x 4.6 mm, 5µm) detection wavelength 262 nm and column oven temperature 25°C were used. The Injection volume 10 µL of impurity mixture, was analyzed. In experimental setup-2. Luna C18 (4.6 x 150 mm, 3 µm) column was used with a mobile phase-A containing of 950 mL of water, 50 mL of acetonitrile and 1 mL of trifloroacetic acid and mobile phase -B consisting of 50 mL of water, 950 mL of acetonitrile and 1 mL of trifloroacetic acid were used with the flow rate of 1.0 mL/min, column temperature of 30°C, auto sampler temperature of 5°C and detection wavelength of 262 nm. In experimental setup- 3, same mobile phase A and B, stationary phase , flow rate of 1.0 mL/min, detection wavelength of 260 nm and column oven temperature of 25°C were used for separation of melphalan related impurities.

3.2 Method validation parameters:

3.2.1 Stress studies / Specificity:

The stress studies/specificity was used to evaluate the ability of the method to resolve possible substance, degradation products of melphalan and its other impurities. For validation of method impurity samples and degradation products (5 PPM) were spiked in the melphalan sample. Forced degradation studies were performed on melphalan to infer stability-indicating property and specificity of validating method. The stress conditions used the degradation study were light (ICH Q1B), heat (100°C), acid (1N Hydrochloric acid), base (0.01 M sodium hydroxide) and oxidation (30% Hydrogen peroxide). The samples were exposed for 24 h, to heat and light studies where as samples were treated for 8 h with acid and base hydrolysis and also for oxidation studies. The peak purity of the melphalan stressed samples were validated using photo diode array detector (water's).The purity angle was set within the purity threshold limit for all of the stressed samples and contents of impurities were calculated for the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) was calculated for all of the samples.

3.2.2 Method validation:

The developed method was validated as per ICH recommendations. The system precision was investigated by injecting six individual preparations (5µg/ml) of melphalan spiked with specification level of each of Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate. The %RSD of the areas of above mentioned melphalan impurities was calculated. The intermediate precision of method was confirmed by different analysts and different instruments. The precision

of the method was further evaluated by the analysis of six independent analysis of a test samples of melphalan against a qualified reference standard. The %RSD of six independent test values was calculated.

3.2.3 Limit of detection (LOD) and limit of quantification (LOQ):

The LOD and LOQ of Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate and melphalan were evaluated at 3:1 and 10:1 signal-to noise ratio by injecting a series of dilute solutions with known concentrations. The precision study was also performed at the LOQ level by injecting six individual samples and calculated the %RSD of the areas.

3.2.4 Linearity:

Linearity test solutions were prepared by diluting stock solution from 50 to 150% of the analyte for the related substance method. The peak area versus concentration details were analyzed with least-squares linear regression. The linearity test solutions were prepared by diluting the impurity stock solution to the required concentrations for the related substance method. The solutions were diluted at six different concentration levels from the LOQ to 150%.The slope and y-intercept of the calibration curve was observed. The peak area versus concentration data was analyzed using least squares linear regression. The linearity test solutions were prepared by diluting the impurity stock solution to the required concentrations for the related substance method. The slope and y-intercept of the calibration curve were reported.

3.2.5 Accuracy:

The accuracy of the related substance method was evaluated in triplicate at three concentration levels, 50, 100 and 150% and the percent recovery was calculated. The impurities, Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate were spiked into melphalan and recovery experiments were performed to determine the accuracy of the related substance method for quantification of impurities. The study was carried out in triplicate at 0.5, 1.0 and 1.5 % of the analyte concentration (0.5 mg/ml). The percent recovery of above mentioned impurities was calculated.

3.2.6 Robustness:

The robustness of developed method of evaluated at different experimental conditions such as flow rate and column temperature. The flow rate of the mobile phase was maintained 1.0 ml/min. To study the effect of the flow rate on the resolution, the flow rate was changed every time by 0.1 units from 0.9 and 1.1 ml/min. The effect of column temperature on the resolution was studied from 20°C -30°C. The detector wavelength was maintained 260 nm. To study the effect of the wavelengths on the resolution, the wavelength was changed to 258 and 262 nm. To study the effect of the organic solvent ratio on the resolution, the organic solvent ratio was changed ± 2.0%. In all these varied conditions, the components of the mobile phase was remain same.

3.2.7 Solution stability and mobile phase stability:

The stability of melphalan solution in the proposed method was tested out by placing the sample and reference standard solutions in a tightly capped volumetric flasks at room temperature for 8 h. The sample solution was assayed at 8 h interval during the study period. The mobile phase stability was evaluated by assaying the freshly prepared sample solution against freshly prepared reference standard solution at 6 h interval. The prepared mobile phase maintained same during the study period. The %RSD of the melphalan impurities was calculated for the mobile phase as well as solution stability experiments. The amount of Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate was determined at 6 h intervals up to the study period. The stability of mobile phase was evaluated for 72 h by injecting the freshly prepared sample solution at every 8 h interval. The content of melphalan and its impurities Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate were determined in the test solution. The prepared mobile phase was maintained remain same during the study period.

IV. Results And Discussion

4.1 Method development and optimization

The information of active pharmaceutical ingredient melphalan and official product methods were not available in USP, BP and EP pharmacopeia. According to API route of synthesis, there were seven specified impurities in melphalan drug such as Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate. The current study was aimed to separate melphalan from impurities and degradants in a shorter run time using a RP-HPLC stability indicative method. The injection sequence of related substance and assay method of melphalan was depicted in the table 1. The results of experimental setup 1 of method development study reveals that the impurity peaks not separated and peak

shapes were not satisfactory. The results of experimental setup-2 was also not satisfactory for impurity peak separation and peak shapes. However, in experimental setup-3 separation of impurity peaks with sharp peak shapes was achived. These results indicate that Luna (C18 4.6 x 150 mm, 3 µm) sample cooler temperature 5°C and column oven temperature was 25°C found to be ideal for separation and sharp peak shapes.

Optimization of mobile phase was performed based on resolution of the drug, asymmetric factor and theoretical plates obtained for melphalan. The results indicate that mobile phase A consisting of 950 mL of water, 50 mL of acetonitrile and 1 mL of trifloroacetic acid and mobile phase B consisting of 50 mL of water, 950 mL of acetonitrile and 1 mL of trifloroacetic acid, a mobile phase flow rate of 1.0 ml/min was found to be satisfactory, achieved good separation and showed symmetric peak for melphalan and related impurities (Table 2). The results reveals that column oven temperature of 25°C, sample cooler temperature of 5°C. Retention time of 15 min. and detection wavelength at 260 nm were found to be ideal parameter for complete resolution of the peaks with clear baseline separation in the estimation of melphalan using reverse phase HPLC.

Table 1. HPLC conditions for gradient separation melphalan impurities.

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	1.0	95	5
15	1.0	65	35
28	1.0	30	70
32	1.0	0	100
36	1.0	0	100
36.1	1.0	95	5
40	1.0	95	5

4.0.1 Stress studies / Specificity:

The stressed samples of melphalan were subjected to forced degradation studies using light and heat. Significant degradation of melphalan and its products was detected with thermal, acid and oxidation, which may lead to formation of unknown degradation products with RRT of 1.19 (Table 3).

Table 3. Retention time of melphalan and its impurities and degradants

S.No.	Name of the impurity	Retention time in (min)	RRT
1	Monohydroxy	7.1	0.43
2	Dimer	17.7	1.09
3	Melphalan	16.2	1.00

The peak purity test using PDA detector confirmed that the melphalan peak and the degraded products peaks were homogeneously pure in all the analyzed stress samples. The degradation studies against a qualified reference standard of melphalan reveals that the mass balance of the stressed samples was close to 99.5%. These results further indicate that the developed method for estimation of melphalan was not affected by the presence of melphalan and monohydroxy, Dimer and its degradation products, confirming the stability-indicating power (Table 4).

Table 4. Effect of different stress conditions on a resolution melphalan and its impurities by newly developed HPLC method

S.No.	Conc. (PPM)	Stress condition	ML (%w/w)	MM (%w/w)	MD (%w/w)	MP (%w/w)	MEE (%w/w)	ME (%w/w)	MM (%w/w)	MDY (%w/w)	Unknown impurity (%w/w)	Total impurities (%w/w)
1	500	Acidic degradation, Hydrochloric acid 1 M, 1 Hour	98.74	1.00	0.08	0.00	0.00	0.00	0.00	0.12	0.0	1.26
2	500	Basic degradation, Sodium hydroxide 0.01 M,50 minutes	90.87	1.22	0.15	0.00	0.00	4.42	0.00	2.64	0.19	9.27
3	500	Oxidative stress, Hydrogen peroxide 30%, 90 minutes	91.86	5.87	0.30	0.00	0.00	1.96	0.00	0.00	0.00	8.13
4	500	Heat degradation,100°C, 24 hours	99.63	0.05	0.12	0.00	0.00	0.20	0.00	0.00	0.00	0.37
5	500	Light degradation	99.78	0.07	0.07	0.00	0.00	0.08	0.00	0.00	0.00	0.22

ML: Melphalan, **MM:** Monohydroxy **MD**: Dimer, **MP**: Phthalimido, **MEE**: Ethyl ester
ME: Elongated, **MM:** Monolakylate, **MDY**: Dihydroxy

4.0.2 System suitability and System precision:
The melphalan limit of quantification solution and system suitability solution were injected into the respective chromatographic condition and recorded the melphalan peak areas. The observed signal to noise was found to be 20.6 and % RSD for six replicate injections was observed as 3.0% (Table 5).

4.0.3 Method precision:
The results of method precision study of six individual sample solutions within 10.0 % RSD show that % RSD of unknown impurity and total impurities were 9.8 and 7.0, respectively (Table 6).

Table 6. Method precision for separation of melphalan and its impurities by developed HPLC method

S.No.	MM	MD (Dimer)	MP	MEE	ME	MM	MDY	Unknown Impurity	Total Impurities
1	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.04	0.08
2	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.04	0.07
3	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.05	0.08
4	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.04	0.07
5	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.04	0.07
6	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.04	0.07
Average								**0.04**	**0.07**
STDEV								**0.00**	**0.01**
%RSD								**9.8**	**7.0**

4.0.4 Intermediate precision:
The method precision study of six individual sample solutions within 10.0 % RSD show that % RSD of unknown impurity and total impurities were 8.4 and 7.0, respectively (Table 7).

Table 7. Intermediate precision for separation of melphalan and its impurities

S.No.	MM	MD (Dimer)	MP	MEE	ME	MM	MDY	Unknown Impurity	Total Impurities
1	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.05	0.07
2	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.05	0.08
3	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.05	0.07
4	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.05	0.08
5	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.04	0.07
6	BQL	BQL	BDL	BDL	BDL	BDL	BDL	0.05	0.07
Average								**0.05**	**0.07**
STDEV								**0.00**	**0.01**
%RSD								**8.4**	**7.0**

ML: Melphalan, **MM:** Monohydroxy **MD**: Dimer, **MP**: Phthalimido, **MEE**: Ethyl ester **ME**: Elongated, **MM:** Monolakylate, **MDY**: Dihydroxy

4.0.5 Accuracy:
The percent recovery of melphalan was ranged from 0.03 to 0.15 at the 100% level of unknown impurities in the melphalan drug Figure 3. The results on the accuracy of method was depicted in the (Table 8).the results indicate that the recovery at LOQ, 50,100 and 150% was 110,109,95.6 and 95.6 respectively indicating accuracy of developed method.

Table 8. Data derived from of melphalan by proposed HPLC method

S.No.	Level	Melphalan
1	LOQ	0.03
2		0.04
3		0.03
1	50%	0.05
2		0.06
3		0.05

1	100%	0.09
2		0.10
3		0.09
1	150%	0.14
2		0.14
3		0.15
Average	LOQ	**0.03**
STDEV		**0.0026**
%RSD		**8.0**
Average	50%	**0.05**
STDEV		**0.0012**
%RSD		**2.1**
Average	100%	**0.10**
STDEV		**0.0065**
%RSD		**6.8**
Average	150%	**0.14**
STDEV		**0.0058**
%RSD		**4.0**
Recovery at LOQ %		**110.0**
Recovery at 50 %		**109.0**
Recovery at 100 %		**95.6**
Recovery at 150 %		**95.6**

4.0.6 Linearity:

The linear calibration plot of the method was obtained in the tested calibration range of 50-150% level and the correlation coefficient obtained was > 0.999. These results indicate significant correlation between the peak areas and analyte concentration. The linear calibration plot for the related substance was determined in the calibration range for melphalan was 150% with respective to LOQ, a correlation coefficient of greater than 0.999 was obtained. In this range, the linearity was checked for the related substance at same concentration range for three consecutive days. The % RSD values of the slope and y-intercept of the calibration curves were achieved within 10%. These results showed significant correlation between the peak areas and concentrations of melphalan. The residuals were within ± 10% scattered with respect to 100% concentration response. The sensitivities were scattered within ± 10% with respect to 100% concentration sensitivity (Table 9).

Table 9. Regression and precision data analysis of melphalan by developed HPLC method

Name of the Impurity	Melphalan
LOD %	0.004%
LOQ %	0.012
Slope (m)	10845
Intercept (C)	16.73
Correlation coefficient	0.9999
Precision (%RSD)	5.2

4.0.7 Limit of quantification (LOQ) precision:

The LOQ precision study was carried out within 10.0 % RSD. The results show that % RSD of melphalan were 2.01 (Table 10).

4.0.8 Robustness:

At deliberately, modified chromatographic conditions such as flow rate, pH, solvent ratio and column temperature etc., the resolution between the closely eluting impurities, Dimer impurity, monohydroxy impurity, phthalimide, ethyl ester, elongated melphalan, dihydroxy and monolakylate resolution was greater than 2.0. The variability of both melphalan and the impurities area response was within ±5% emphasizing the robustness of the developed HPLC method.

4.0.9 Solution stability and Mobile phase stability:

The results of solution stability and mobile phase stability indicate the % RSD of the related substances method of melphalan was less than 1%. These results indicate that no significant changes in the content of melphalan during the solution and mobile phase stability experiments The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the related substance determinations were stable up to 96 h. The mobile phase was proven to be proved stable upto one-week days.

V. Conclusion

The degradation pathway of melphalan was established as per ICH recommendations. The gradient LC method was developed and used for stress studies and also for quantification impurities of melphalan drug. The behavior of melphalan under various stress conditions was studied. The thermal and all the degradation products and processed impurities were well separated from the melphalan and related impurities demonstrates the stability-indicating power of the method. This method is sensitive and can detected up to 0.02% impurities. This method was also precise, accurate and stability indicative. Within the recommendations of ICH. The developed method can be used to determine the impurities in melphalan injection in the routine and stability sample analysis.

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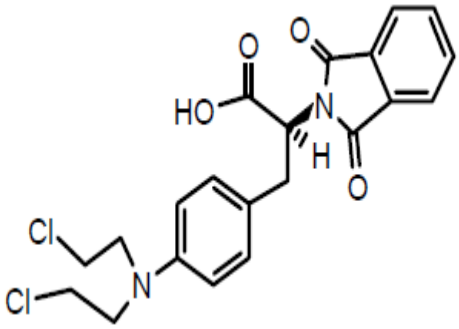
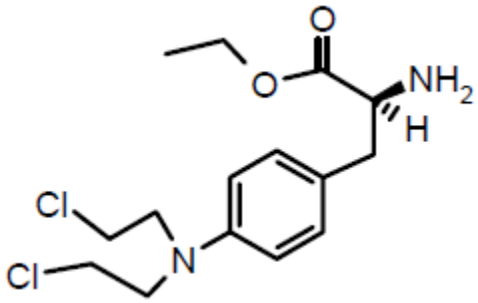
Figure. 1: Chemical structure of Melphalan **and its impurities.**

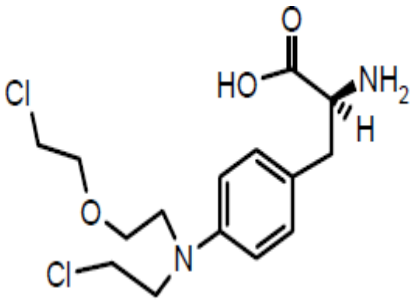
a) Phthalimido



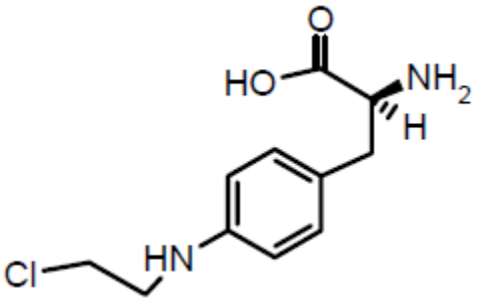
b) Ethyl ester



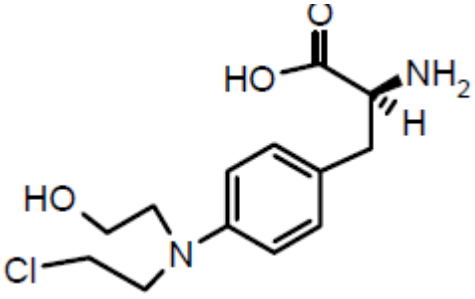
c) Elongated



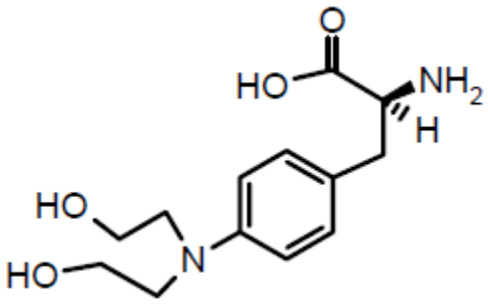
d) Monolakylate



e) Monohydroxy



f) Dihydroxy



g) Dimer **h) Melphalan**

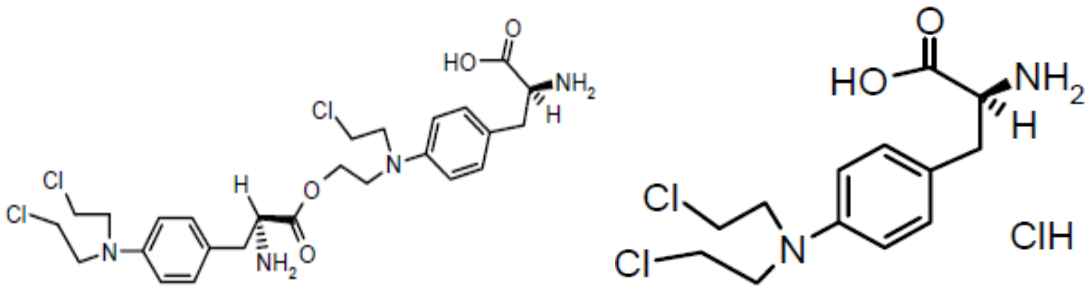
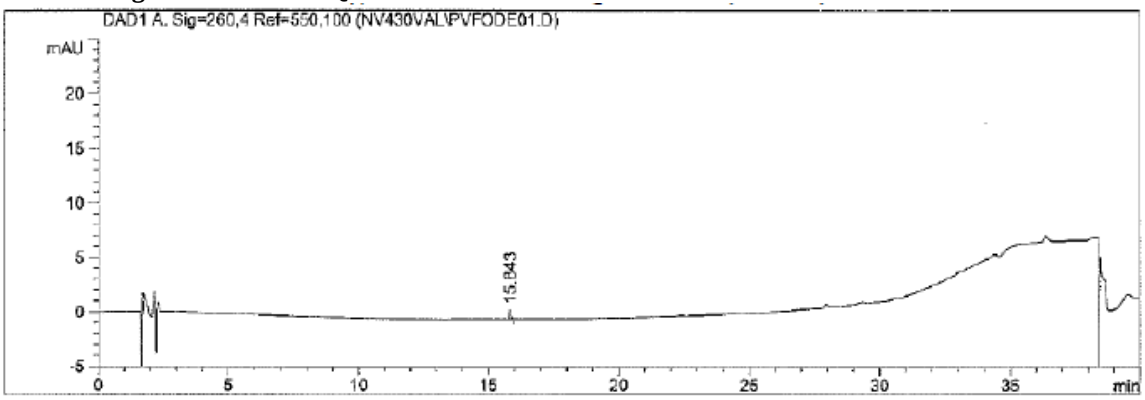
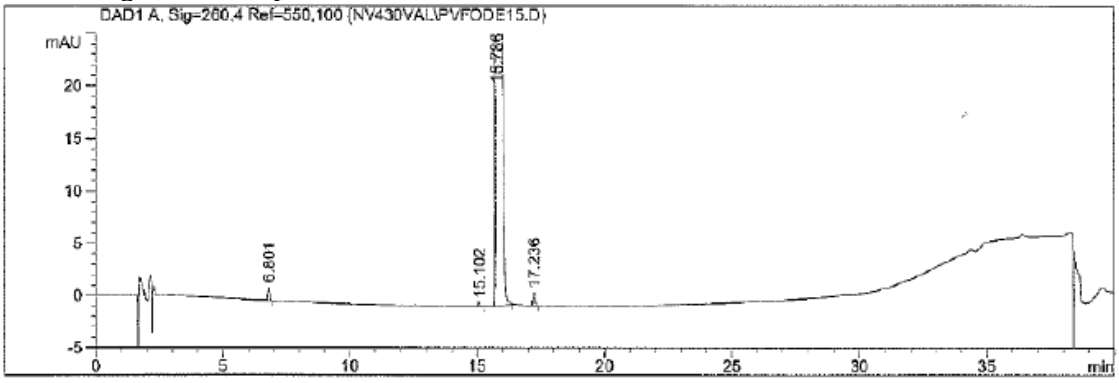


Figure. 2: Typical HPLC chromatograms of melphalan and impurities under different stress conditions.

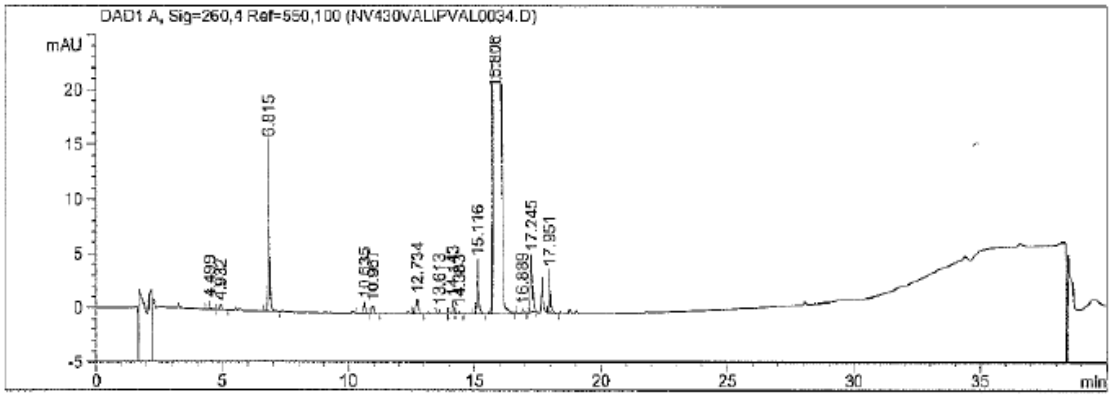
a) Chromatogram of Limit of Quantification solution



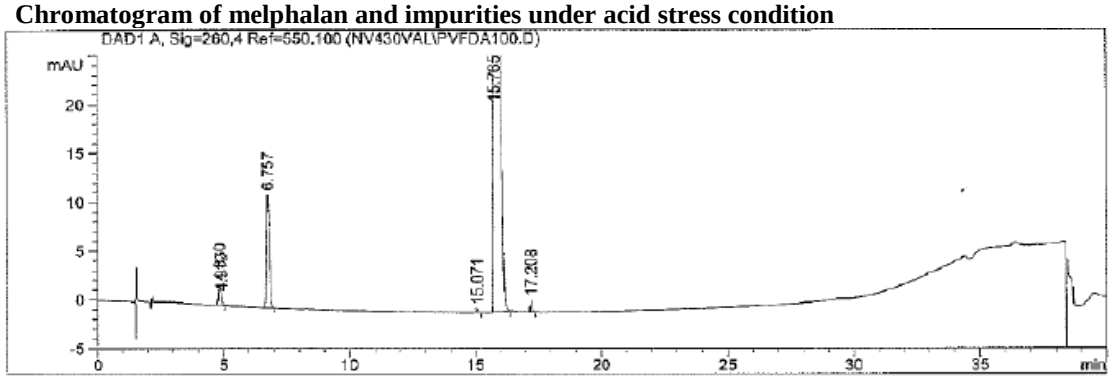
b) Chromatograms of sample solution



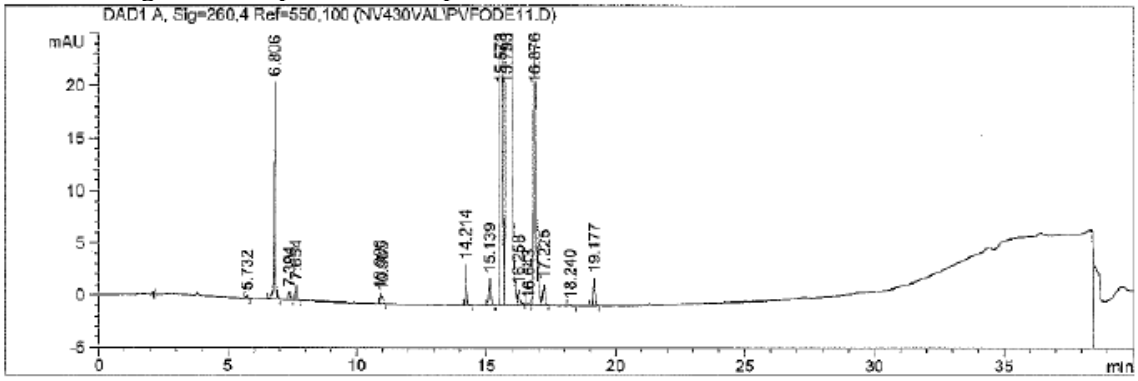
c) Chromatogram of melphalan and impurities mixture



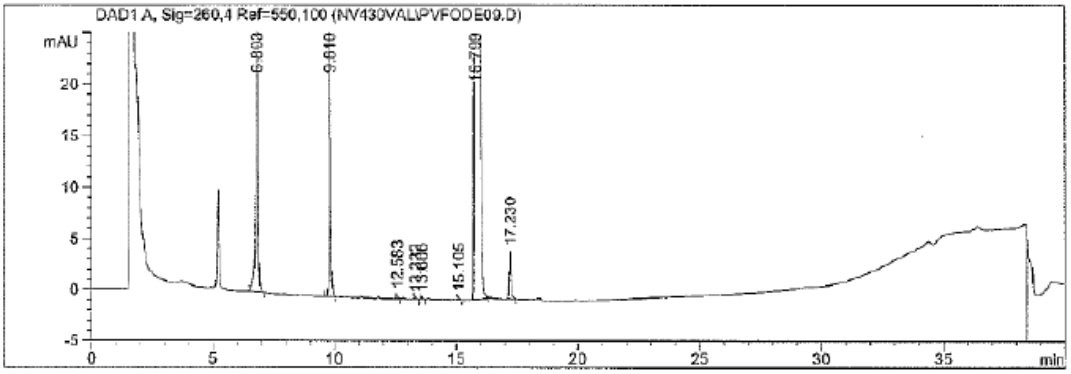
d) Chromatogram of melphalan and impurities under acid stress condition



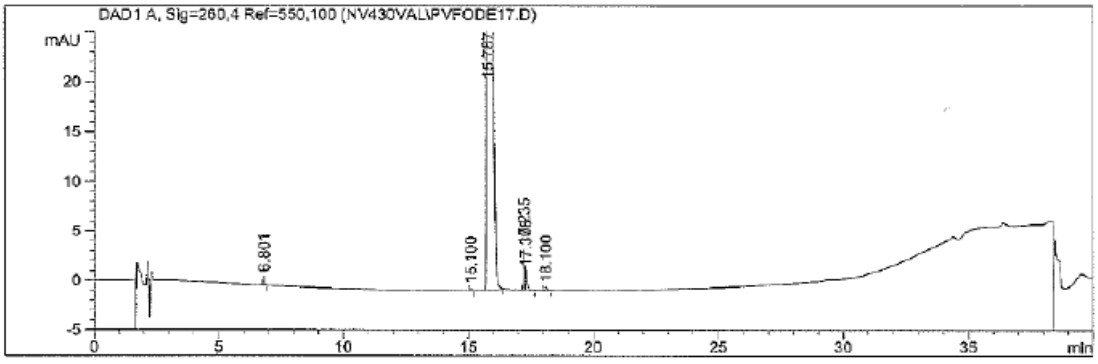
e) Chromatogram of melphalan and impurities under base stress condition



f) Chromatogram of melphalan and impurities under oxidative stress condition



g) Chromatogram of melphalan and impurities under heat stress condition



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