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Cleaning Method Validation for Determination of Residues in Anti-Cancer Drug product for Injection by Using RP-HPLC Technique

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Abstract

Cleaning method validation is a part of good practices in pharmaceutical sector and need to seek the contamination present in production area. OBJECTIVE: An RP-HPLC method for the cleaning samples analysis for Anti-Cancer Drug product Melphalan Hydrochloride for Injection was develop and validated. The method was developed and validated for quantitation of Cleaning of residue left in the vessels of the production equipment's. The analytical method used to determine the complete cleaning for Melphalan Hydrochloride for Injection by HPLC method. METHOD: The Cleaning validation was performed on Inertsil ODS,C18,(150mm×4.6mm,5µm) chromatographic column temperature at 40°C, and the detection wavelength was set at 260nm. Mobile Phase-A:10mL ammonium acetate, 88mL Purified water, 10mL acetic acid, 2mL Triethylamine, and Mobile Phase-B: 1mL Buffer, 10mL acetonitrile,190mL Purified water, and at a flow rate of 1.5 mL/min, and the injection volume was 10µL. RESULTS: The validation results of Melphalan Hydrochloride were linear over the concentration from 0.10 µg/mL to 0.75µg/mL (r = 1.00) and the recoveries from LOQ to 150% and precision ranged from 50% to 76% (RSD < 30%, n =9). CONCLUSION: This method has been validated successfully and all the results met the acceptance criteria. Hence method of Cleaning Validation is suitable for testing of residue sample of Melphalan Hydrochloride for Injection.

Keywords: *Anti-Cancer; Cleaning; Residue; Injection; HPLC; validation; Swabs and Column.*

1. Introduction

In pharmaceutical manufacturing, particularly for highly potent compounds like anti-cancer drugs, maintaining stringent cleanliness standards is critical to ensure patient safety and product efficacy [1]. Cleaning method validation is a crucial process that ensures that equipment used in the production of anti-cancer drug product injections is thoroughly cleaned and free of contaminants, including residual drug substances, cleaning agents, and microbial contaminants [2]. Anti-cancer drugs, being cytotoxic and often hazardous, present unique challenges in cleaning due to their potency and potential health risks, even at trace levels. Hence, a robust and validated cleaning procedure must be in place to ensure that no carryover from previous batches contaminates subsequent products. This is vital not only to meet regulatory standards but also to safeguard against cross-contamination and protect patients from unintended exposure to active pharmaceutical ingredients (APIs) or impurities [3]. As per FDA guidelines, two primary sampling methods are widely accepted for cleaning validation: direct surface sampling with swabs and the use of rinse solutions [4]. A key challenge in cleaning validation lies in establishing a reliable sampling process and developing highly sensitive analytical techniques to detect any residual traces of active pharmaceutical ingredients (APIs) that may remain on the equipment surfaces post-cleaning [5]. HPLC is frequently utilized to evaluate cleaning efficiency because of its exceptional sensitivity. The purpose of this study was to validate a straightforward reverse-

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phase HPLC (RP-HPLC) method for accurately measuring residual Melphalan hydrochloride on production equipment surfaces and to verify the effectiveness of the cleaning process. Several liquid chromatography (LC) methods have been proposed for the determination of Melphalan in pharmaceutical formulations [7-12]. However, these methods lack the sensitivity required to detect trace-level residues of Melphalan hydrochloride [9]. A comprehensive literature review indicates that no validated cleaning procedure specifically for Melphalan hydrochloride is currently available [10-12]. To address this gap, we developed a reverse-phase high-performance liquid chromatography (RP-HPLC) method to quantify trace-level residues of Melphalan hydrochloride. This method is applied to swab and rinse samples collected from manufacturing equipment and surfaces after cleaning. The analytical method underwent validation for specificity, linearity, precision, accuracy, limits of detection (LOD), and quantification (LOQ) in line with ICH guidelines [6&13].

2. Material and Methods

2.1.Chemical and Reagents

Melphalan, working standard was from kindos pharma, (purity ≥ 99.97%); Acetonitrile was purchased from Fischer Chemical. (purity ≥ 99.9%); Methanol was purchased from Fischer Chemical, US, (purity ≥ 99.97%); Triethylamine was purchased from Kermel, (purity ≥ 99.99%); Glacial acetic acid, was purchased from Knowles, (purity ≥ 99.99%); Ammonium acetate, was purchased from Kelong, China,(purity ≥ 99.99%); All reagents were analytical grade and the Glass Plate-Surface area 100cm^2 in this study were obtained from Kindos pharma.

2.2.HPLC Apparatus and Conditions

HPLC was achieved on an Thermo U3000 and Agilent 1260.The separation was done on a Inertsil ODS,C18,(150mm×4.6mm,5µm). The flow rate was set at 1.5 mL/min with column temperature 40°C and Sample tray controlled temperature at 5 °C. Detector was set at the wavelength of 260nm, runtime was set 20min, and injection volume was 10 µL for every samples and standard.

2.3.Standard and Samples Preparations

Buffer Preparation: Weigh accurately about 10g of ammonium acetate and transfer into 100-mL volumetric flask, add 88mL of purified water mix well to dissolve and add 10 mL glacial acetic acid and 2mL Triethylamine and mix well in the ratio (10/88/10/2: w/v/v/v).

Mobile phase-A preparation: Mix Buffer solution 10mL and Acetonitrile 100mL and 1900mL of purified water mix well and filter through a 0.45µm filter and degas (10/100/1900: v/v/v).

Mobile Phase-B preparation: Mix Buffer solution 10mL and Acetonitrile 800mL and 1200mL of purified water mix well and filter through a 0.45µm filter and degas (10/800/1200: v/v/v).

Diluent Preparation: Methanol.

Standard Stock Solution: Weigh accurately about 25 mg Melphalan Hydrochloride standard into a 200-mL volumetric flask, add diluent and sonicate to dissolve and dilute to volume with diluent, mix well. Transfer 1.0mL of Standard Stock Solution into a 50-mL volumetric flask, and make up to volume with diluent, mix well.

Standard solution: Transfer 2.0mL above solution into a 10-mL volumetric flask, and make up to volume with diluent, and mix well.

2.4.Swab Blank solution for Glass plate

1. **Preparation of swab Blank solution for Glass plate:**

For glass plate: Pipette 1.0mL of diluent and soil the surface with small droplets using the pipette tip within the intended sampling boundary (10cm×10 cm). Dry the glass plate by suitable way.

Swab: Soak the swab (flat head, 25mm long and 12.7mm wide) in 30 mL diluent in a 50mL beaker and eliminate excess solvent by pressing the swab gently against the beaker diluent. Swab the area (10cm×10cm) at least 2times vertically and 2times horizontally, holding the swab parallel to the surface, pressing the head of the swab against it with uniform pressure, and covering the surface entirely with the swabbed lanes. Swab the horizontal lanes with one side of the head of the swab and the vertical lanes with the other side. Then swab the plate in the same manner with another one dry swabs to remove residual liquid.

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Extraction: Soak the swabs in a test tube (2 swabs, one wet and one dried swab) containing 5.0mL of diluent, accurately transferred, and vortex about 1 minutes.

2. **Spike Sample Solution**

Spike: Pipette 1.0mL of Standard Stock Solution and soil the surface with small droplets using the pipette tip within the intended sampling boundary (10 cm×10 cm). Dry the glass plate by any suitable way. Soak the swabs in a test tube (2 swabs, one wet and one dried swab) containing 5.0mL of diluent, accurately transferred, and vortex about 1 minutes.

2.5.Method Validation

Validation of the method was done according to the International Conference on Harmonization guideline. The method was validated for System suitability, specificity, accuracy, precision, linearity& range, limit of detection (LOD),limit of quantification (LOQ) and solution stability[6].

- **System suitability:** The method should be suitable for its intended use and should be evaluated during routine use to ensure continued performance. System suitability was demonstrated prior to each experiment. The standard solution was used to evaluate the system suitability. The %RSD of peak areas of Melphalan Hydrochloride from 6standard injections were determined. The system suitability requirement was met for all experiments. The test results were summarized in Table 1.

Table 1 System Suitability Results –Area RSD of Standard Solution

S.No	Peak Area
1	12085
2	12241
3	11633
4	11843
5	11886
6	11825
Average	11919
SD	1060.1
%RSD	2

Specificity: The method should be specific for the analyte of interest and should not respond to other substances in the sample matrix. To evaluate the specificity, Diluent Swab Blank solution, standard solution and sample solution were injected into the HPLC system, and chromatograms were recorded. There was no any interference observed from diluent at the retention time of Melphalan Hydrochloride. No Melphalan Hydrochloride peak was detected in the sample solution. The retention time of Melphalan Hydrochloride peak in the swab sample solution correspond to that in the standard solution. The peak match of Melphalan Hydrochloride peak was determined in standard solution and swab sample solution. The specificity requirement was met. The test results are summarized in Table 2.

Table 2 Specificity Results

Blank, Placebo solution and Standard and Spiked sample were not give any interfering peak at the retention times of Melphalan Hydrochloride peak.	
Solution	**Retention time**
Swab sample solution	8.848
Standard solution	8.858

Diluent solution and Swab Blank solution has not given any interfering peak at the retention time of Melphalan Hydrochloride peak. The retention time of Melphalan Hydrochloride in the Swab sample solution correspond to that in the standard solution. Hence the method is found to be specific.

Limit of Quantification (LOQ): The method should be able to quantify the analyte of interest with acceptable precision and accuracy at the lowest concentration of interest. The limit of quantitation was determined by injecting the LOQ solution in six times and calculated signal to noise ratio. The Limit of Quantitation was determined by injecting the LOQ solution six times and calculated signal to noise ratio (S/N) and %RSD of response for Melphalan Hydrochloride Peak Area. The %RSD of peak area of Melphalan Hydrochloride in six replicate injections of LOQ solution was not more than 20%. The S/N ratio for the peak of Melphalan Hydrochloride in six replicate injections of LOQ solution was NLT 10. LOQ is 0.1035µg/mL and all the test results are summarized in Table 3.

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Table 3 The Results of LOQ

S.No	Peak Area	S/N
1	2325	37
2	2264	41
3	2302	34
4	2289	47
5	2276	32
6	2260	35
%RSD	1%	N/A

Limit of Detection (LOD): The method should be able to detect the lowest concentration of the analyte of interest with acceptable sensitivity. The Limit of Detection was determined by injecting the LOD solution two times and calculated signal to noise ratio (S/N) of response for Melphalan Hydrochloride. The S/N ratio for the peak of Melphalan Hydrochloride in two replicate injections of LOD solution was NLT 3. LOD is 0.03g/mL and all the test results are summarized in Table 4.

Table 4 The Results of LOD

LOD				
Injection		1	2	Concentration (µg/mL)
Melphalan Hydrochloride	S/N	5	5	0.03

The Signal-to-Noise ratio of Melphalan Hydrochloride in LOD Solution was not less than 3.

Linearity: The method should be linear over the range of concentrations expected in the sample. Linearity can be assessed by analyzing samples with varying concentrations and evaluating the linearity of the calibration curve. The Linearity and Range were verified by determining the response of Melphalan Hydrochloride Peak at five concentration levels in triplicate, from LOQ to 150% of sample concentration. Determined linearity plots with concentration levels of Melphalan Hydrochloride as x-values and detector response as y-values respectively. The statistical analysis includes correlation coefficient R, % y-axis intercept, slope of the line of regression and residual sum of square. All the test results met the acceptance criteria and are summarized in Table 5.

Table 5 The Results of Linearity and Range

Level	concentration (µg/mL)	Peak Area		
LOQ	0.10µg/mL	0.0379	0.0393	0.0382
60 %	0.30µg/mL	0.1130	0.1111	0.1102
100 %	0.50µg/mL	0.1820	0.1848	0.1782
120 %	0.60µg/mL	0.2193	0.2188	0.2163
150 %	0.75µg/mL	0.2682	0.2713	0.2715
Linearity Equation	Y=357.49x+0.0035			
correlation coefficient (R≥0.99)	1.00			
% y-intercept （NMT25 %）	2%			
Slope	357.49			
Residual sum (Q)	4.58×10^{-5}			

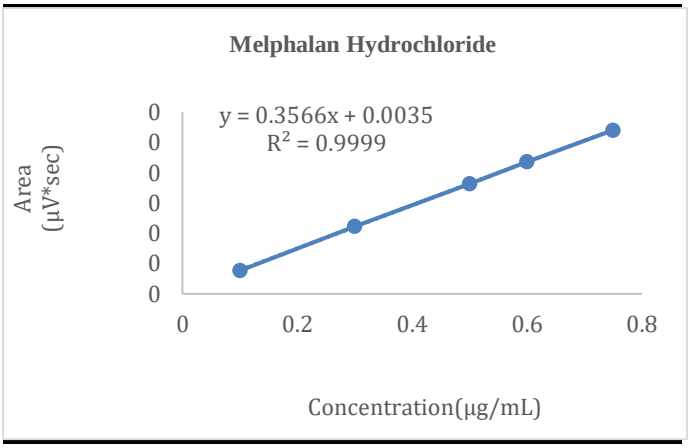

Figure 1 Linear Diagram of Melphalan Hydrochloride

The correlation coefficient (R) was not less than 0.99. %y intercept was not more than ±25% of standard response. All the slope of the regression line and residual sum of square.

Range: The range of the method should be appropriate for the intended use of the method. The range was evaluated by determining at 5

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concentration levels in triplicate from LOQ to 150%level with respect to standard solution. The range results were listed in Table 6.

Table 6 The Results of Range

Name of compound	LOQ~150%
Melphalan Hydrochloride	0.10µg/mL~0.75µg/mL

Accuracy: The method should be able to measure the analyte of interest with acceptable accuracy. The accuracy was evaluated by determining %Recovery of Melphalan Hydrochloride spike sample solution at four concentration levels in triplicate from LOQ to 150% of limit concentration. The %RSD of recoveries was NMT 15%. Reported recoveries mean, standard deviation & confidence interval of content for Melphalan Hydrochloride. All the test results are summarized in Table 7.

Table 7 The Results of Accuracy

Accuracy			
Level	Added Concentration (µg/mL)	Tested Concentration (µg/mL)	%Recovery
LOQ-1	0.10µg/mL	0.10µg/mL	100%
LOQ-2	0.10µg/mL	0.11µg/mL	110%
LOQ-3	0.10µg/mL	0.11µg/mL	110%
100%-1	0.50µg/mL	0.50µg/mL	100%
100%-2	0.50µg/mL	0.51µg/mL	102%
100%-3	0.50µg/mL	0.49µg/mL	98%
150%-1	0.75µg/mL	0.74µg/mL	99%
150%-2	0.75µg/mL	0.75µg/mL	100%
150%-3	0.75µg/mL	0.75µg/mL	100%
Mean of Recovery			102%
SD of Recovery			5
%RSD			4%
Confidence interval of Recovery			[99%,106%]

The %recovery of Melphalan Hydrochloride was between 80%~120% and for LOQ level the recovery is between 70%~130%. The %RSD was not more than 15%. The mean, standard deviation and confidence interval of recovery for Melphalan Hydrochloride. Hence the method is Accurate

Precision: The method should be reproducible and precise. Precision can be evaluated by analyzing multiple samples and determining the variation in the results obtained. The repeatability was evaluated by determining the recovery of Melphalan Hydrochloride residue from the Glass plate with 60%, 100% & 150% limit concentration level (in triplicate). All the test results met the acceptance criteria and are summarized in Table 8.

Table 8 The Results of Repeatability

Solution	Area	Test (µg)	Add (µg)	%Recovery
60%	5345	1.1809	1.5520	76%
60%	4882	1.0785	1.5520	69%
60%	5193	1.1472	1.5520	74%
100%	8038	1.7758	2.5866	69%
100%	8473	1.8719	2.5866	72%
100%	7664	1.6931	2.5866	65%
150%	12336	2.7252	3.8799	70%
150%	11361	2.5100	3.8799	65%
150%	8712	1.9247	3.8799	50%
Average(n=9)				68%
Standard deviation (n=9)				8%
%RSD (n=9)				11%
Confidence interval				[62%,74%]

The %RSD of the Melphalan Hydrochloride for 9 Injections is NMT 30% (n=9), Hence the method is Precise.

Solution Stability: The stability of standard solution and sample spike solution was evaluated by storing the solutions at 5°C temperature and injecting at suitable time interval. The Spike Sample solution and standard solution of Melphalan Hydrochloride spike

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sample solution are stored at 5°C. Injected the solutions at suitable time interval for not less than 24 hours.The standard solution was stable up to 43 hours and Spike sample solution was stable up to 40 hours at the 5°C. The test results are summarized in Table 9 to Table 10.

Table 9 The Results of Standard Solution Stability

Standard Solution Stability			
Time		0h	43h
Melphalan Hydrochloride	Area	0.1821	0.1817
	%RSD	N/A	1%

The %RSD of peak areas of Melphalan Hydrochloride for each time point should be NMT 5.0% in standard solution and the standard solution is stable up to 43 hours at the 5°C.

Table 10 The Results of Sample Solution Stability

Spike Sample Solution Stability					
Time		0h	16h	24h	40h
Melphalan Hydrochloride	Area	0.1464	0.1436	0.1495	0.1464
	%RSD	N/A	2%	2%	0%

The Spike Sample solution is stable up to 40 hours at the 5°C.

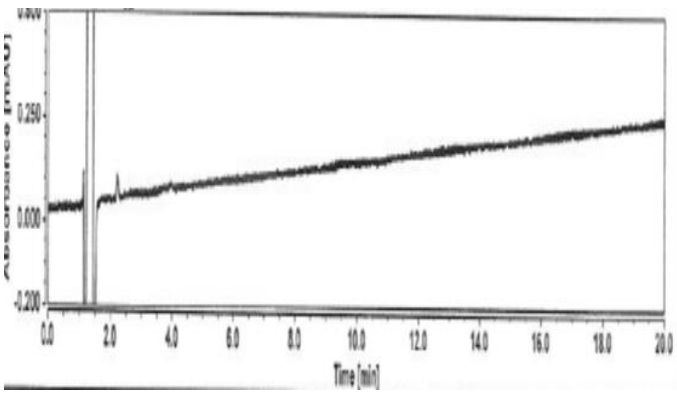

Figure 2 Chromatogram of Swab Blank Solution

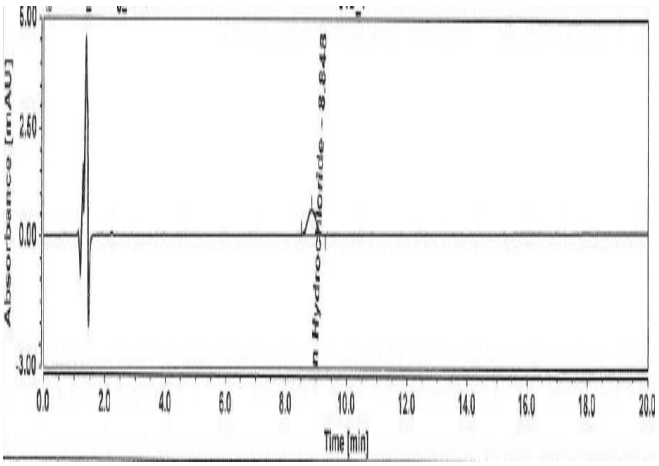

Figure 3 Chromatogram of Standard Solution

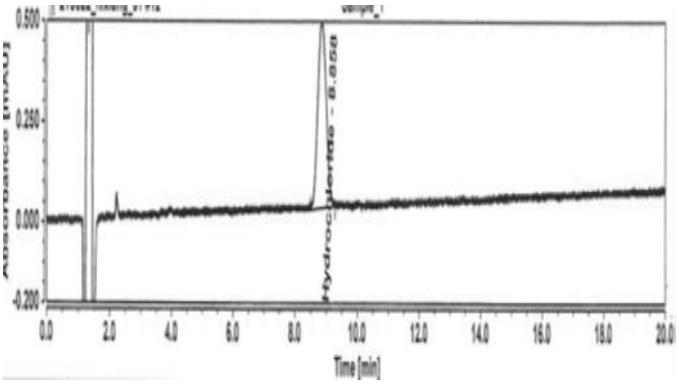

Figure 4 Chromatogram of Sample Solution

3. Results and Discussion

The Cleaning validation results indicate that the RP-HPLC method developed for the determination of Melphalan Hydrochloride Residue content in Melphalan Hydrochloride for Injection is suitable for its intended use (Refer Figures 1–4). System suitability was demonstrated prior to each experiment, and the system suitability requirement was met for all experiments. Specificity was evaluated, and no interference was observed from diluent, swab blank or other substances in the sample matrix. Accuracy and precision were also evaluated, and the results show that the method is accurate and precise for the determination of Melphalan Hydrochloride in residue content. The limit of quantitation and limit of detection were determined, and the method was able to quantify and detect the analyte of interest at the lowest concentration of interest with acceptable precision and accuracy. The linearity and range were also evaluated, and the

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method demonstrated linearity and range from LOQ to 150% level with respect to the standard solution. The solution stability of the standard solution was stable for 43 hours at 5°C, and the stability of the Spike sample solution was stable for 40 hours at 5°C. The method has been validated successfully, and all results met the respective acceptance criteria.

Conclusion

The RP-HPLC method for cleaning validation for the determination of Melphalan Hydrochloride residue in Melphalan Hydrochloride for Injection is suitable for routine testing. The method demonstrated specificity, accuracy, precision, LOD, LOQ, linearity, range, and stability. The samples of the drug product were also acceptable. The Cleaning method has been validated successfully and all results met the respective acceptance criteria. Hence this method is suitable for testing of Melphalan Hydrochloride residue content.

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