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Rapid and Simple Flow Injection Analysis-Tandem Mass Spectrometric (FIA-MS/MS) Method for the Quantification of Melphalan in Lipid-Based Drug Delivery System.

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Abstract

The use of the anticancer drug melphalan is limited due to its poor water solubility. To address such limitation, it is incorporated within a novel delivery system using β -cyclodextrin-gemini surfactants (18:1 β CDg). Herein, two fast and simple FIA-MS/MS methods are developed for the quantification of melphalan (Mel) within the drug delivery system so that the solubilization efficiency of the system can be assessed. FIA-MS/MS methods are developed using a triple quadrupole-linear ion trap mass spectrometer, equipped with electrospray ionization (ESI) in the positive ion mode. A deuterated form of melphalan (melphalan-d8) was used as an internal standard (IS). The methods were validated according to the FDA guidance. A linearity in the range of 2–100 ng/mL and accuracy and precision below 15% were observed for all standard points and quality control samples. The intra- and inter-day variations, freeze-thaw stability were within the acceptable range according to the criteria set by regulatory guidelines. On the other hand, other stability measures, such as room temperature stability and long term stability did not meet the required guidelines in some cases, indicating the need for quick sample analysis upon preparation. Such a fact could have been overlooked if full method validation was not performed. The developed methods were applied to determine the encapsulation/solubilisation of [18:1 β CDg\Mel] delivery system. 18:1 β CDg enhances the aqueous solubility of melphalan without the need for co-solvent. The highest melphalan solubility was observed at the 18:1 β CDg\Mel complex molar ratio of 2:1. This study demonstrated that a fast analysis for the purpose of quantifying a chemically unstable drug, such as melphalan is feasible and important for the development of commercial dosage forms.

Keywords: Flow Injection Analysis, Mass Spectrometry MSMS, Melphalan, β -cyclodextrin Drug Delivery

1. Introduction

Several effective chemotherapeutic agents, such as melphalan, show unfavourable solubility or stability due to their physicochemical properties that affect their therapeutic use.¹⁻³ Melphalan (L-phenylalanine mustard) (Figure 1-A) is an alkylating agent primarily used in the management of multiple myeloma and as an adjunctive therapy in regionally in-transit metastatic melanoma.⁴ It is a lipophilic drug with low aqueous solubility, requiring the use of a co-solvent (namely, propylene glycol) for parenteral administration. Propylene glycol can cause adverse effects when used in high concentrations, including, nephrotoxicity, cardiac arrhythmia, and metabolic acidosis.⁵ In addition to poor solubility, melphalan shows minimal chemical stability in aqueous medium as it hydrolyzes into hydroxymelphalan.⁶ Therefore, attempts to improve melphalan's solubility and stability were conducted by either chemical modifications or by engineering novel drug delivery systems, such as nano-systems.⁷⁻¹⁰

Nanotechnology is being applied for developing novel therapies that can improve the solubility of poorly soluble drugs.¹¹ For example, cyclodextrin-based drug delivery systems have been widely investigated to improve the delivery of melphalan as well as other chemotherapeutics.¹²⁻¹⁵ β -Cyclodextrin (β -CD) is a natural product with low toxicity.¹⁶ It has the ability to enhance the delivery of hydrophobic drugs by forming guest-host complexes.¹⁶ β -CD has cyclic ring structure containing seven D-(+) glucopyranose units attached by α -(1,4) glucosidic bonds which create a lipophilic inner cavity and hydrophilic outer surface. Such architecture creates a moiety capable of interacting with a large variety of guest molecules to form non-covalent inclusion complexes. To enhance the drug delivery capacity of the β -CD, we conjugated the molecule to gemini surfactants moieties. Gemini surfactants are composed of two hydrophobic portions, commonly quaternary amines, and two hydrophilic portions with the two polar groups connected via a spacer region. They have the ability to interact electrostatically with the negatively charged cell surface, enhancing cellular uptake of the delivery system¹⁷.

The resulting conjugate, 18:1-7N β CD-18:1 (18:1 β CDg) (Figure 1-C) combines the encapsulation properties of β -CD and the cell-penetrating ability of cationic gemini surfactants.¹⁸⁻²⁰ This novel drug

delivery system can incorporate lipophilic drugs, such as melphalan, into the β -CD moiety and form nanoplatform.²¹ Recently, we showed the efficiency of β -CD-modified gemini surfactant (12 β CDg) in improving the delivery of melphalan in melanoma cell line.²² The IC₅₀ of melphalan improved significantly and demonstrated efficiency in melphalan-resistant melanoma. To optimize the stability of the complex and to maximize the solubilisation efficiency, various analytical methods were developed. Higuchi and Connors described an analytical approach to study the CD/drug solubility relationship known as the phase-solubility method.²³ This method examines the effect of the solubilizer (CD or a ligand) on the drug being solubilized (the substrate). Therefore, the development of analytical methods to determine the encapsulation/solubilisation efficiency of the β -CD moiety is critical in the development of efficient β -CD modified drug delivery system.

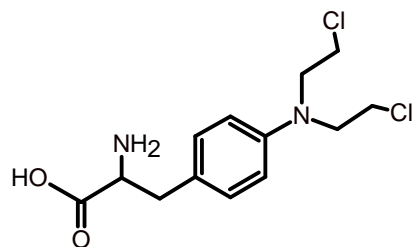
Numerous high-performance liquid chromatography (HPLC) analytical methods have been developed to quantify melphalan in pharmaceutical and biological systems for pharmacokinetic and quality control studies.²⁴⁻³⁰ For instance, Cummings and co-workers described an HPLC method for the determination of nitrogen mustard compounds, including melphalan, in plasma samples after chemical derivatization process with diethyldithiocarbamic acid (DDTC).²⁹ C₁₈ column was used with a linearity range of 0.1 – 100 g/mL. However, this method lacked sensitivity and required long sample preparation time (30 min) with a run time of 14.6 minutes. Pinguet *et al* developed a simplified HPLC method, requiring 3.3 min run time.³⁰ Despite improvements, the lower limit of quantification (LLOQ) at 20 ng/mL may not be sufficient for many pharmaceutical applications. To improve melphalan quantification, mass spectrometry (MS) have also been used.³¹⁻³⁵ A rapid HPLC-MS method for the quantification of melphalan in serum and plasma demonstrated a retention time of \approx 2 min with LLOQ of 2 ng/mL.³² However, the method requires a relatively complicated extraction procedure with specialized instrumentation (96 well solid phase extraction (SPE) blocks). The use of HPLC-tandem mass spectrometry (MS/MS) was reported by Mirkou and colleagues using two different HPLC conditions/columns and two extraction methods.³³ In the first method, a cold methanol protein

precipitation is applied prior to injection onto a C₁₈ column while the second one was based on solid phase extraction followed by hydrophilic interaction chromatography (HILIC)-MS/MS. The linearity of the methods were 1-500 ng/mL and 25-200 ng/mL, respectively with ≈ 7 min retention time in both cases.

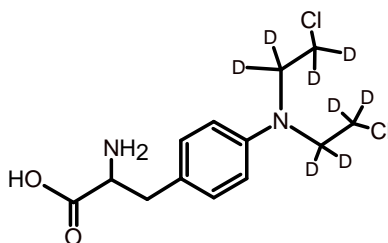
Despite the wide use of HPLC-MS, a simpler MS-based approach for quantification is flow injection analysis-tandem mass spectrometry (FIA)-MS/MS in which the analytical column is removed and quantification is reliant on MS response in multiple reaction monitoring mode (MRM). (FIA)-MS/MS has been widely utilized as a simple and high throughput methods in comparison to LC-hyphenated techniques. It provides cost and time effective strategy that have been explored for the analysis of pharmaceuticals in different matrices.³⁶⁻³⁹ For example, a fully validated simple FIA-MS/MS method was recently developed to quantify metformin in dog serum.³⁶ The method was simple and fast (2 min run time), achieving a sensitivity comparable to the relatively complicated conventional HPLC-MS/MS methods.^{40,41} The main shortcoming of FIA, however, is seen in complex matrices, particularly with multi-analyte analysis in which the linearity range of the method can be affected. In addition, the removal of the LC column can cause saturation of the MS detector with either the analyt(s) or matrix components, which can negatively affect method sensitivity.⁴² However, the advances in sample extraction techniques and in ionization efficiency (especially in ESI and nano-ESI) can overcome such hurdles, which is reflected in the large number of methods employing FIA.⁴³

In this paper, we developed and validated a rapid and simple FIA-MS/MS methods that were applied for the quantification of melphalan in 18:1 β CDg surfactant formulations. The methods were used to assess the solubilisation efficiency of 18:1 β CDg as a potential drug delivery system for melphalan.

(A)



(B)



(C)

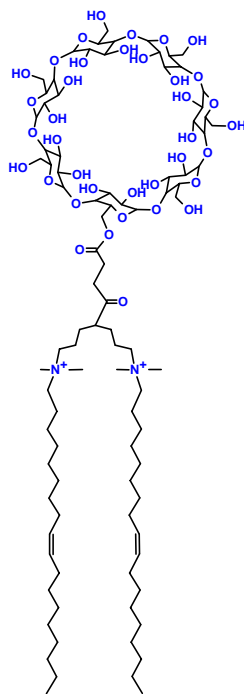


Figure 1: chemical structure of (A) melphalan, (B) melphalan-d₈ and (C) 18:1-7NβCD-18:1 (18:1βCDg) gemini surfactant

2. Material & Methods

2.1. Materials and apparatus

Melphalan (Mel, 99 % Purity) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Melphalan-d8 dihydrochloride (Mel-d8), used as internal standard (IS), was purchased from Toronto Research Chemicals Inc (Toronto, ON, Canada). Synthesis and characterization of 18:1-7N β CD-18:1 gemini surfactant was previously described.^{20,21} Formic acid was purchased from EMD (Gibbstown, NJ, USA). Water (LC-MS grade), acetonitrile (LC-MS grade) and Dimethyl Sulfoxide DMSO (Certified ACS) were purchased from Fisher (Ottawa, ON, CA). Chemical structure of the drug, IS and 18:1 β CDg are shown in Figure 1.

2.2. Standard Preparation

2.2.1. Method #1:

Stock solutions of melphalan (1 mg/mL) was prepared in 10% acidified ethanol (0.1% HCl), to achieve complete aqueous solubility, and stored at -80 °C. Fresh standard solutions were prepared daily by serial dilutions in water (LC/MS grade) to eight standard points in a range from 3 ng/mL to 100 ng/mL. The stocks of standard solutions were stored at -80 °C for method validation. A stock solution of Melphalan-d8 (1 mg/mL) in DMSO was prepared and stored at -20 °C. Working stock of melphalan-d8 (4 μ g/mL in DMSO) was prepared daily and 10 μ L was added to each standard and sample to a final concentration of 40 ng/mL. The purpose of this method is to compare the solubility of melphalan between acidified aqueous solution and the 18:1 β CDg gemini surfactant formulations (i.e. control).

2.2.2. Method #2:

Method#2 was developed to quantify the analyte in lipid-based drug delivery systems. Stock solution of melphalan (1 mg/mL) was prepared in acidified ethanol (0.1% HCl). An appropriate volume of melphalan solution was mixed with 18:1 β CDg gemini surfactant aqueous solution to yield a final molar ratio of 2:1 [18:1 β CDg-to-Mel]. 18:1 β CDg\Mel formulation were frozen at -80 °C for 2 h. After freezing, the

formulation was transferred to a Labconco® Freezone Plus 6 L cascade freeze dryer (Labconco, Kansas City, MO, USA) at -80 °C and 0.03 mBar vacuum and lyophilized for 24 h. Lyophilized formulations were rehydrated to a final concentration of 1 mg/mL melphalan. Eight standard points in a range from 3 ng/mL to 100 ng/mL (melphalan) were prepared. Melphalan-d8 (IS) were spiked in all standards at final concentration of 40 ng/mL. The stocks of standard solutions were stored at -80 °C for method validation.

2.3. Mass Spectrometry conditions

FIA–MS/MS methods were developed and validated using a 1200 Agilent High Performance Liquid Chromatography (HPLC) system (Mississauga, ON, CA) interfaced by connector to an ABSciex QTRAP 4000, a hybrid quadrupole linear ion trap mass spectrometer (Q-LIT), equipped with a Turbo-V IonSpray® electrospray ionization (ESI) source (Concord, ON, CA). ABSciex Analyst software (Version 1.6.0) was used for system control and quantification.

Sample injection was performed using 1200 Agilent auto-injector set to 4 °C and 2 µL of each sample was delivered with isocratic mobile phase consisting of water/acetonitrile (50:50, v/v) with 0.1% formic acid at a flow rate of 0.2 mL/min for a run time of 2 minutes. Multiple reaction monitoring (MRM) was achieved using (ESI) source in the positive ion mode. Source temperature was set to 600 C, ion spray voltage (ISV) 5500 V, curtain gas (CUR) 40, nebulizer gas (GS1) 40, heater gas (GS2) 40, collision gas (CAD) 6 and using an exit potential of 16 V for all MRM transitions. Dwell time for all transitions was 150 ms at unit resolution. Nitrogen was used as the gas for all cases. Table 1 summarizes the monitored ion transitions, for both the analyte and the internal standard, and the corresponding MS conditions.

Acceptance criteria for the ratio between the quantifier/qualifier ions was set at $\pm 20\%$ as recommended by the Commission of the European communities⁴⁴. In fact all our acquired data showed variations less than 7% except for two points showing 13% and 18%.

	Melphalan		Melphalan-d8 (IS)	
	305 → 168 (Quantification)	305 → 246 (Conformation)	314 → 172 (Quantification)	314 → 254 (Conformation)
De-clustering potential (DP)	55	50	56	52
Collision energy (CE)	45	33	38	35
Collision exit potential (CXP)	16	16	16	16

Table 1: MRM transitions for melphalan and melphalan-d8 (IS) and optimized MS conditions

2.4. Method Validation

FDA Guidance for Bioanalytical Method Validation was used as a guideline to validate both FIA-MS/MS methods,⁴⁵ to ensure the a validity of the quantitative data. The following parameters were evaluated for method validation: selectivity, accuracy, precision, sensitivity, recovery, reproducibility, matrix effect, dilution integrity and stability.

Selectivity was tested at the lower limit of quantification (3 ng/mL) for the ability to distinguish the analyte in the presence of other components in the formulation. Five sets of the formulation were used.

A standard (calibration) curve of eight concentration points, ranging from 3 ng/mL to 100 ng/mL , were constructed for each method by determining the best fit of peak area ratios (peak area ratio of the analyte to internal standard) versus the analyte concentration. A linear regression analysis weighing the standard curve with 1/X was applied for best fit.

To evaluate the accuracy and precision of the standard curve, five replicate of each quality control (QC) sample (LLOQ = 3 ng/mL, low QC (LQC) = 9 ng/mL, middle QC (MQC) = 35 ng/mL and high QC (HQC) = 80 ng/mL) were used. Accuracy of QCs passed only if the mean values of the QC were within 15% of the actual concentration except for (LLOQ) where it should be $\pm 20\%$ of the nominal value. For precision, each value of QC replicates must not exceed 15% of the coefficient of variation (CV) except for (LLOQ) where is should be $\pm 20\%$ of the CV. Inter- and intra-day accuracy and precision were also evaluated in a similar manner.

Dilution integrity was evaluated to assess the effect of dilution on the accuracy and precision of undiluted sample. Formulations were prepared without the drug as described above and spiked with 1 µg/mL of the drug. Spiked samples were then diluted with acidified water to 100 ng/mL (10x) and 10 ng/mL (100x).

The stability of melphalan in formulation matrix was tested using freshly prepared calibration curve and QC samples for a) short-term stability (at bench top and auto-sampler); b) long-term stability at -80 °C; and c) freeze / thaw stability. A set of five replicates of each QC were prepared for each stability evaluation. For bench top short-term stability, samples stored at room temperature for 12 and 24 hr and re-analyzed. Another set of QC samples were prepared and analyzed then stored in the auto-sampler for 24 h at 4 °C and reinjected. QC samples for the freeze / thaw stability were stored in the -80 °C freezer and were subjected to three freeze–thaw cycles. The samples were allowed to thaw completely and remained at room temperature for at least 1 h. Then samples were re-analyzed and returned to the -80 °C freezer for the next freeze–thaw cycle. The long-term stability was conducted on QC samples stored at -80 °C for 30 days.

2.5. Method Application, Solubility Evaluation

The developed methods were used to determine the solubilizing efficiency of cyclodextrin-modified gemini surfactant. 18:1βCDg\Mel formulations were prepared in different carrier-to-drug molar ratios (0:1, 1:1, 2:1, 3:1 and 5:1) as shown in Table 2. First, gemini surfactant stock solutions were prepared. Then, 1 mg of melphalan powder was added to each vial and placed in orbital shaker for 1 hour. Samples were subsequently lyophilized as described above. Upon freeze drying, samples were re-hydrated with 1 mL water and filtered through 0.45 µM syringe filter to remove any insoluble melphalan. Samples were diluted 100X in acidified water and IS spiked in each sample to a final concentration of 40 ng/mL. For samples containing free drug, Method 1 was used to quantify the analyte and for samples containing melphalan:18:1βCDg complex, Method 2 was used.

Formulation ID	Molar Ratio 18:1 β CDg/Mel	Amount of 18:1 β CDg mg (Molarity)	Amount of melphalan mg (Molarity)
1	0:1	0	1 (3.28 mM)
2	1:1	6.49 (3.28 mM)	1 (3.28 mM)
3	2:1	12.98 (6.56 mM)	1 (3.28 mM)
4	3:1	19.47 (9.84 mM)	1 (3.28 mM)
5	5:1	32.41 (16.4 mM)	1 (3.28 mM)

Table 2: molar ratios of 18:1 β CDg\Melphalan formulations evaluated in this work

3. Results and Discussion

3.1. Method development and selectivity

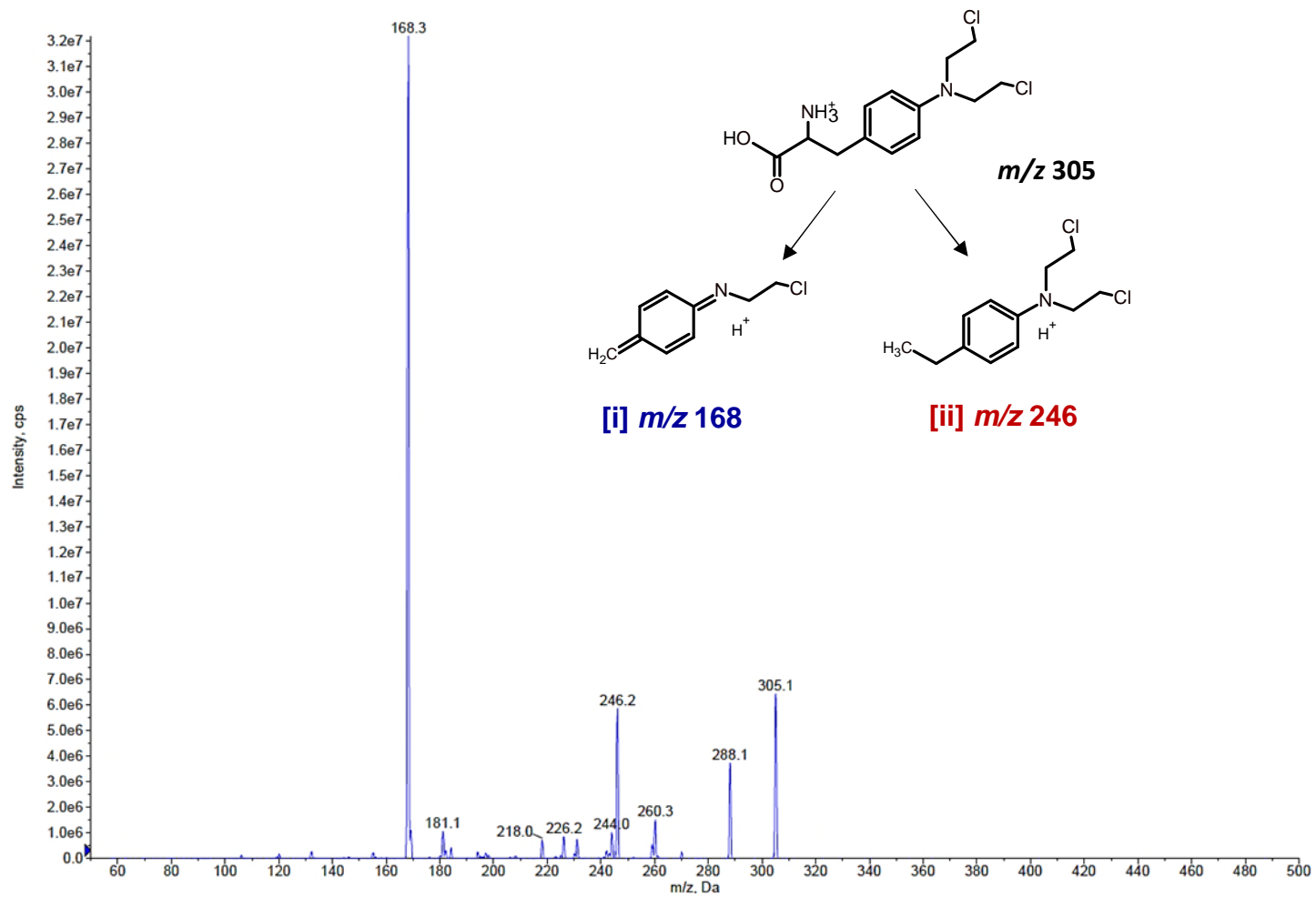
The main purpose of this work is to develop fast and simple FIA-MS/MS quantification methods for melphalan by removing the need for chromatographic separation while solely utilizing the MRM capability of the MS instrument, ensuring specificity and selectivity. In addition, no extraction process was needed for the drug from the formulation, indicating the simplicity of the analytical procedure. The methods were developed to assess the novel β -CD modified drug delivery system 18:1 β CDg and for quality control purposes.

To ensure the robustness of the methods that are reliant only on the MS, the following strategies were adopted. First, two MRM transitions were selected and the monitored product ions for melphalan were diagnostic with relatively high abundance [Figure 2]. In addition, the MS conditions (i.e. DP, CE and CXP) were optimized for each MRM transition to ensure the ions stability and abundance [Table 1].

Second, a deuterated melphalan analogue that bears 8 deuterium atoms (melphalan-d8) was used as an internal standard to account for any possible effects of formulation excipients/drug delivery system on the ionization and quantification of the drug. The internal standard (melphalan-d8) was spiked into standard curve samples, the QCs, and experimental samples to give a final concentration of 40 ng/mL prior to sample analysis. The similarity in the physicochemical properties of the deuterated internal standard (melphalan-d8) to the analyte (melphalan) confirmed the validity of the data and prevented any crosstalk between the two molecules. Figure 2 shows the MS/MS spectra of melphalan (Figure 1A) and the internal

standard melphalan-d8 (Figure 1B) and the proposed structures of the selected product ions. The ion observed at m/z 168 for melphalan and at m/z 172 for melphalan-d8 are the most abundant ions in the spectra and they were selected as the MRM transition for quantification. The second MRM transition, 305 \rightarrow 246 for melphalan and 314 \rightarrow 254 for melphalan-d8, were used as a confirmation transitions.

Finally, two FIA-MS/MS methods were developed and validated in this work: method#1 to quantify melphalan in aqueous solution with the use of a co-solvent and method#2 to quantify the drug from 18:1 β CDg delivery system to eliminate any possible interferences caused by formulation excipients. A typical FIA-MS/MS chromatogram for a double blank of 18:1 β CDg drug delivery system (control: not spiked with melphalan nor internal standard) is presented in Figure 3A. It shows no interference from the drug delivery system components at the analyte/internal standard retention time (0.17 min) or during the full run time (2 min). Similar observations were reported during the construction of standard curve at different melphalan concentrations.



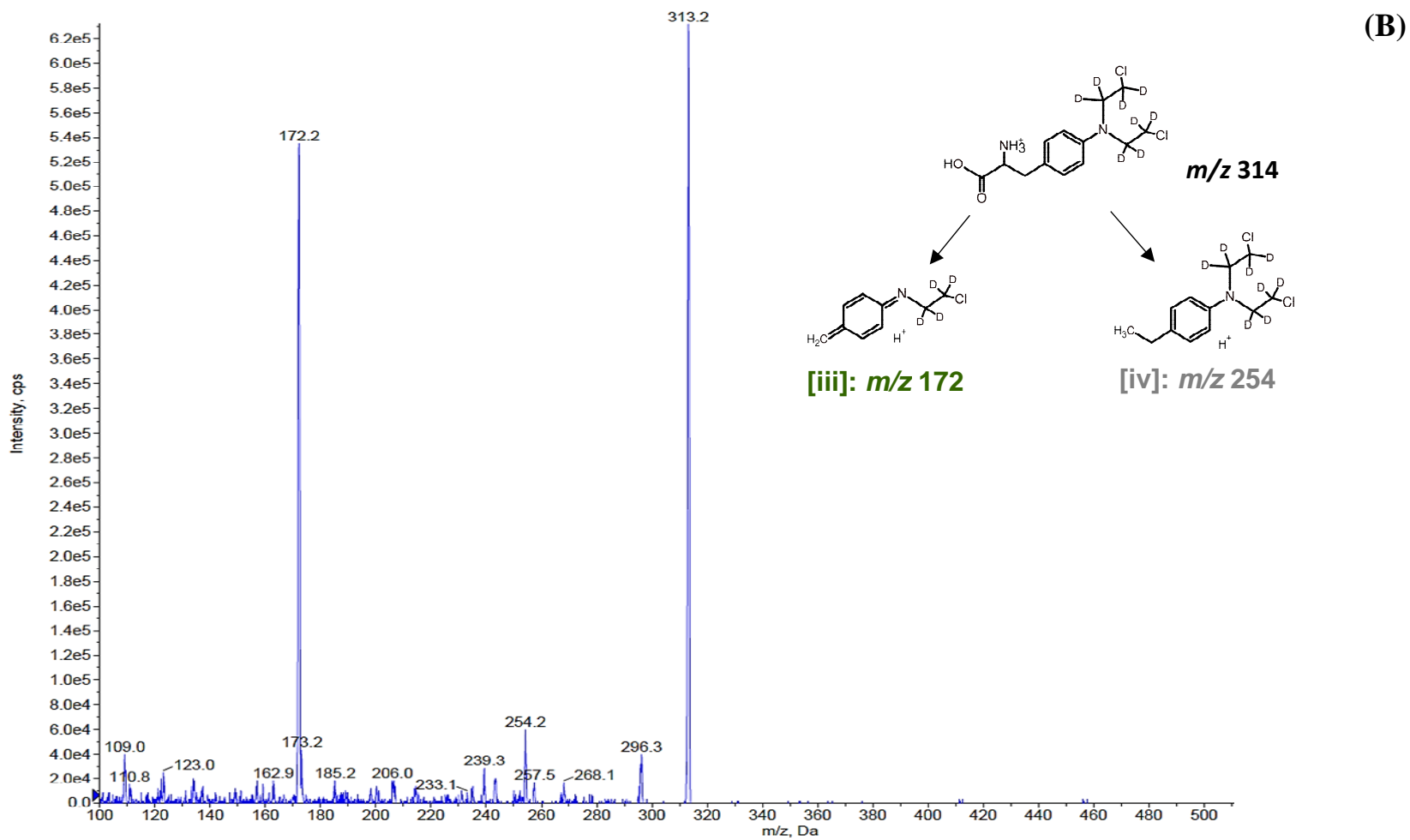
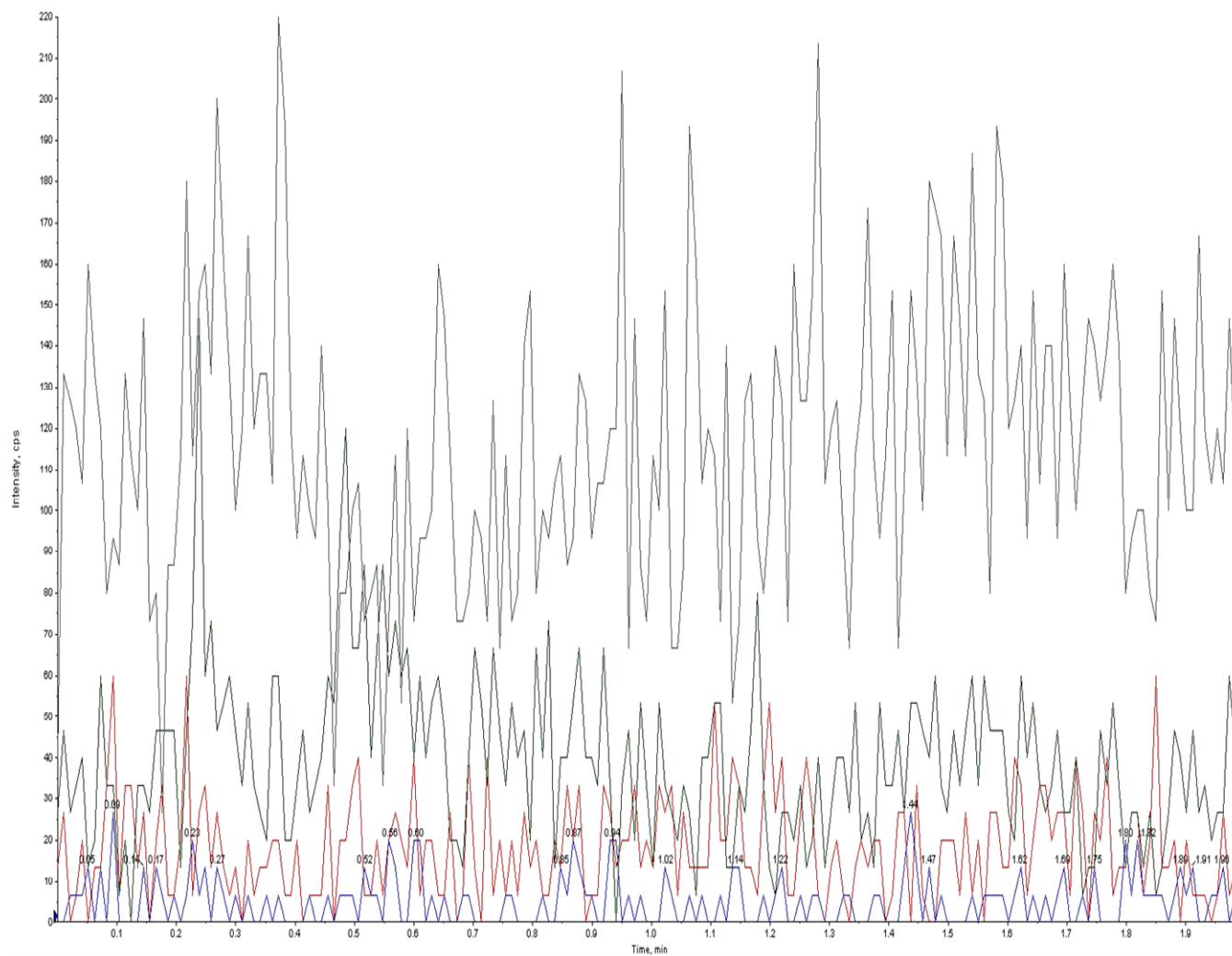


Figure 2: MSMS spectra of $[M+H]^+$ of melphalan (A) and the internal standard melphalan-d8 (B), the product ions used in MRM are shown

(A)



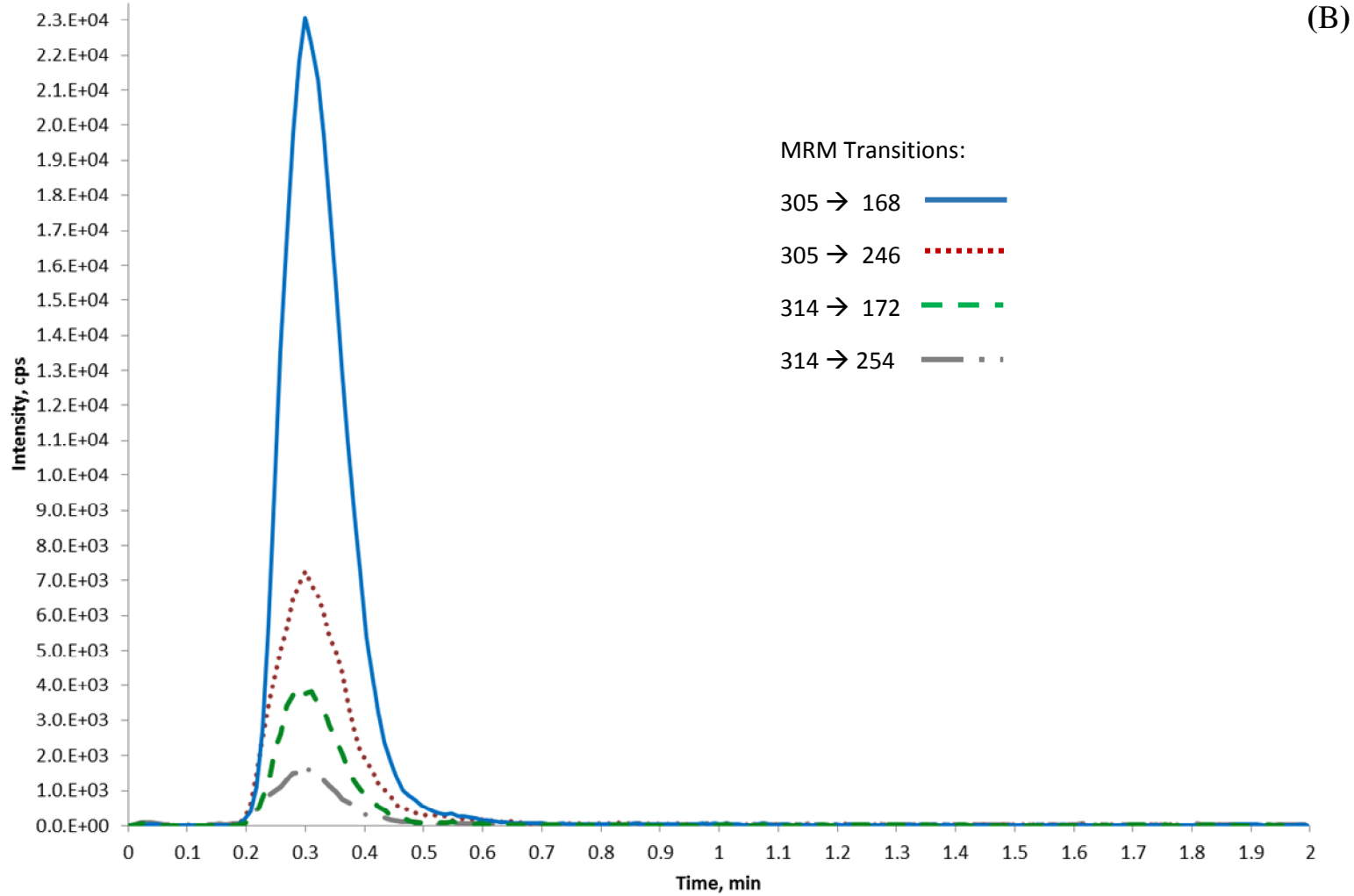


Figure 3: FIA-MS/MS chromatograms for method#2 (A) double blank 18:1 β CDg drug delivery system (B) 18:1 β CDg drug delivery system spiked with melphalan (10 ng/mL) and melphalan-d8 (40 ng/mL)

3.2. Linearity and sensitivity

An 8-point standard curve was constructed (Figure 4, Method#1) by calculating the peak area ratio of melphalan to melphalan-d8 versus melphalan concentration and establishing a linear regression analysis weighting the standard curve with 1/X for best fit. The average correlation coefficient (n = 3) was found to be 0.9944 for method 1 and 0.9991 for method 2. The LLOQ was 3 ng/mL and ULOQ was 100 ng/mL. Such values are ideal for the intended application and provide the needed quantitative power to assess the solubilization potential of the novel formulations without the need for tedious chromatographic separation.

3.3. Accuracy and precision

The precision and accuracy were evaluated according to the FDA guidelines and are shown in Tables 3 and 4. The LQC (9 ng/mL) was 3 fold higher than the LLOQ and the HQC was 80% of the ULOQ, as stipulated by the regulatory guidelines.

The intra-day precision and accuracy (Table 3) of the methods were evaluated by running three sets of QCs in three different days with five replicates of each QC in each run. The accuracy was reported as a percentage of the theoretical concentration ranging from 89.89 to 103.73% for method 1 and from 89.07 to 107.87% for method 2. The precision was also within the acceptable range as the coefficient of variation (CV%) was in the range of 1.69 – 9.35% and 0.9 – 10.39 % for methods 1 and 2, respectively.

Similarly, the inter-day accuracy and precision data for the 15 replicates from the three-day runs yielded a precision in range of 1.08 and 5.56 % and accuracy between 93.62% and 101.56% for method # 1. The inter-day precision and accuracy range for method # 2 were 1.49 to 6.43 and 93.9 to 100.47% respectively (Table 4). All these values were within the acceptable range according to the FDA guidelines.

3.4. Stability

Determining the chemical stability of QC samples in different storage conditions is essential for ensuring the accuracy of the method. The suitability of the experimental and storage conditions are assessed. Stability of the QC samples at bench top (stability at room temperature) for 24 hrs results showed over 50% reduction in the initial concentration of all QC samples for both methods. This is expected as hydrolysis of melphalan is fast in an aqueous solution at room temperature.^{6,33} Stability of QCs in the auto-sampler (at 4 °C) for 24 hrs showed 30% reduction of the initial concentrations for all QCs for method#1. For method # 2, at auto-sampler condition (4 °C) for 24 hrs, the precision and accuracy for the 5 replicates were 93.2 % to 98.52 and 2.72 to 6.72%, respectively. Such data suggests the need for quick analysis of melphalan samples to ensure no loss of the analyte during the analysis; hence the value of developing fast FIA-MS/MS method.

For the freeze-thaw stability, each batch of the QC samples was subjected to three freeze-thaw cycles. Upon the third cycle, the precision and accuracy were in the range of 93.55-102.8% and 5.01-6.6%, respectively for method#1 in comparison to 92.67-104.22% and 3.32-6.38% for method#2. In both cases, the analyte was stable after three cycles of freezing and thawing, meeting the required regulatory criteria of $\pm 15\%$.

For the long term stability (35 days at -80 °C), the precision and accuracy failed to meet the FDA guidelines as more than 2 QCs in each level were out of the range of 15-20% for method#1. On the contrary, the precision and accuracy of QC replicates were between 91.7 to 100.6% and 3.34 to 8.76 for method#2. These results indicate that samples for method 2 can be stored up to one month at -80 without affecting the method's validity. Such discrepancy between the two methods is most likely due to ability of β -CD to preserve the physiochemical stability of melphalan during freezing and storage.

In summary, Method#2, with the 18:1 β CDg/melphalan complexes, showed acceptable stability in all conditions, except for room temperature. However, significant instability was observed for method 1. As

a result, all experimental samples were freshly prepared and analyzed including the standard curve and QC samples to ensure the accuracy and the validity of the results.

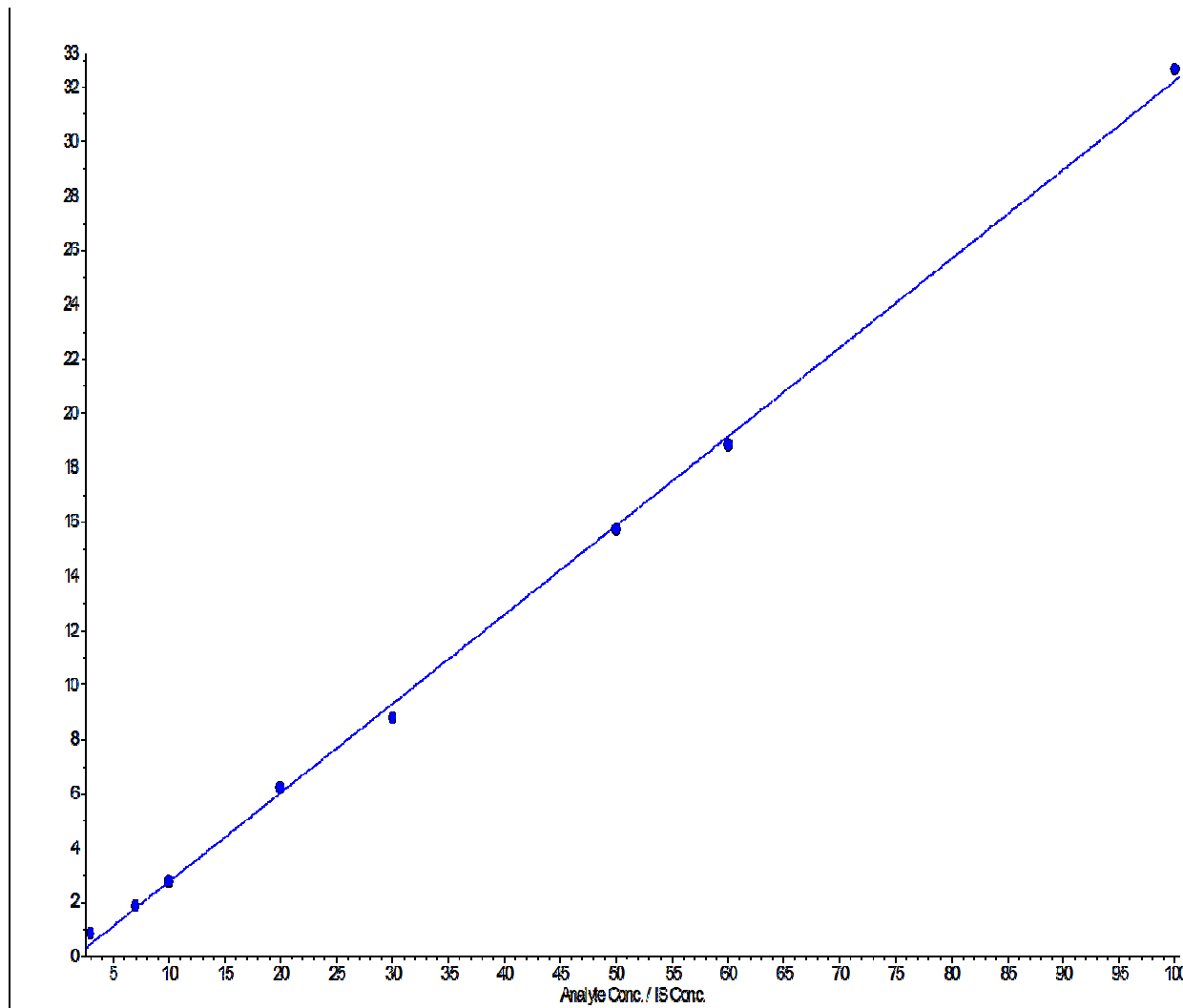


Figure 4: A typical 8 points standard curve ranging from 3 ng/mL to 100 ng/mL with a regression coefficient of 0.9995 (Method # 1)

QUALITY CONTROL	Replicates	Analysis day	METHOD # 1			METHOD # 2		
			Observed concentration (mean ± SD; ng/mL)	Precision (RSD%)	Accuracy (%)	Observed concentration (mean ± SD; ng/mL)	Precision (RSD%)	Accuracy (%)
LLOQ (3 NG/ML)	5	1	2.93 ± 0.13	4.59	97.67	3.24 ± 0.089	2.73	107.87
	5	2	3.11 ± 0	4.07	103.73	2.93 ± 0.3	10.39	97.60
	5	3	3.10 ± 0.08	2.67	103.27	2.88 ± 0.18	6.09	95.93
LQC (9 NG/ML)	5	1	9.04 ± 0.16	1.81	100.44	8.88 ± 0.08	0.9	98.62
	5	2	8.85 ± 0.15	1.69	98.311	8.02 ± 0.39	4.87	89.07
	5	3	8.92 ± 0.41	4.57	99.13	8.93 ± 0.35	3.92	99.27
MQC (35 NG/ML)	5	1	33.14 ± 1.39	4.21	94.69	32.48 ± 1.79	5.5	92.8
	5	2	31.46 ± 1.31	4.18	89.89	33.42 ± 1.94	5.8	95.48
	5	3	33.7 ± 3.15	9.35	96.29	32.7 ± 1.50	4.59	93.43
HQC (80 NG/ML)	5	1	77.64 ± 5.17	6.62	97.05	78.8 ± 4.88	6.19	98.5
	5	2	73.2 ± 4.87	1.88	91.5	78.26 ± 3.28	4.2	97.83
	5	3	81.82 ± 2.48	3.12	102.27	80.82 ± 2.66	3.29	101.03

[Table 3]: Intra-day accuracy and precision

Quality control	Concentration (ng/mL)	Replicates	Method # 1			Method # 2		
			Observed concentration (mean \pm SD; ng/mL)	Precision (RSD%)	Accuracy (%)	Observed concentration (mean \pm SD; ng/mL)	Precision (RSD%)	Accuracy (%)
LLOQ	3	15	3.05 \pm 0.10	3.32	101.56	3.01	6.43	100.47
LQC	9	15	8.94 \pm 0.09	1.08	99.3	8.61	5.97	95.65
MQC	35	15	32.77 \pm 1.17	3.56	93.62	32.87	1.49	93.9
HQC	80	15	77.55 \pm 4.31	5.56	96.94	79.29	1.7	99.12

[Table 4]: Inter-day accuracy and precision

3.5. Application

Both methods described above were utilized to characterize and evaluate the 18:1 β CDg delivery system as a potential carrier for melphalan. β -CD has the ability to form an inclusion complex with hydrophobic drugs.¹⁶ This property of β -CD has been employed widely in the pharmaceutical industry to increase the aqueous solubility of hydrophobic moieties with several β -CD-based formulations available in the market, such as VFEND[®] (voriconazole, an azole antifungal agent) and NEXTERONE[®] (amiodarone, an antiarrhythmic medication) among others, and hundred in clinical trials.¹⁶ Previously, our group reported that the β CD modified gemini surfactant (12 β CDgemini) are capable of forming guest/host complexes with a hydrophobic anticancer molecule.¹⁸

To investigate the efficiency of the novel 18:1 β CDg drug delivery system in increasing the solubility of melphalan, the validated methods were applied (Figure 5). 18:1 β CDg\Mel complexes were prepared in different β -CD gemini (host) to melphalan (guest) molar ratios. At [0:1] ratio (i.e., without the use of 18:1 β CDg) the aqueous solubility of melphalan was 203 ± 16.60 $\mu\text{g/mL}$ as determined by method #1. Increasing the molar ratio to [1:1] resulted in an almost 85% increase in melphalan's solubility (377 ± 16.4 $\mu\text{g/mL}$). The highest level of solubilization was achieved when the 18:1 β CDg to Mel molar ratios was at [2:1], with melphalan solubility reaching 605 ± 5 $\mu\text{g/mL}$ which is more than two-fold increase compared to melphalan aqueous solution. Upon increasing the molar ratio above [2:1], a substantial reduction in melphalan's solubility was observed at 450 ± 6.5 $\mu\text{g/mL}$ and 507 ± 25.2 $\mu\text{g/mL}$ for [4:1] and [5:1] molar ratios, respectively. We propose that at [2:1] 18:1 β CDg\Mel molar ratio, two 18:1 β CDg molecules encapsulate one melphalan molecule forming stable inclusion complexes leading to the higher solubilizing effect. This result is in support of the previous model proposed by our group.¹⁸

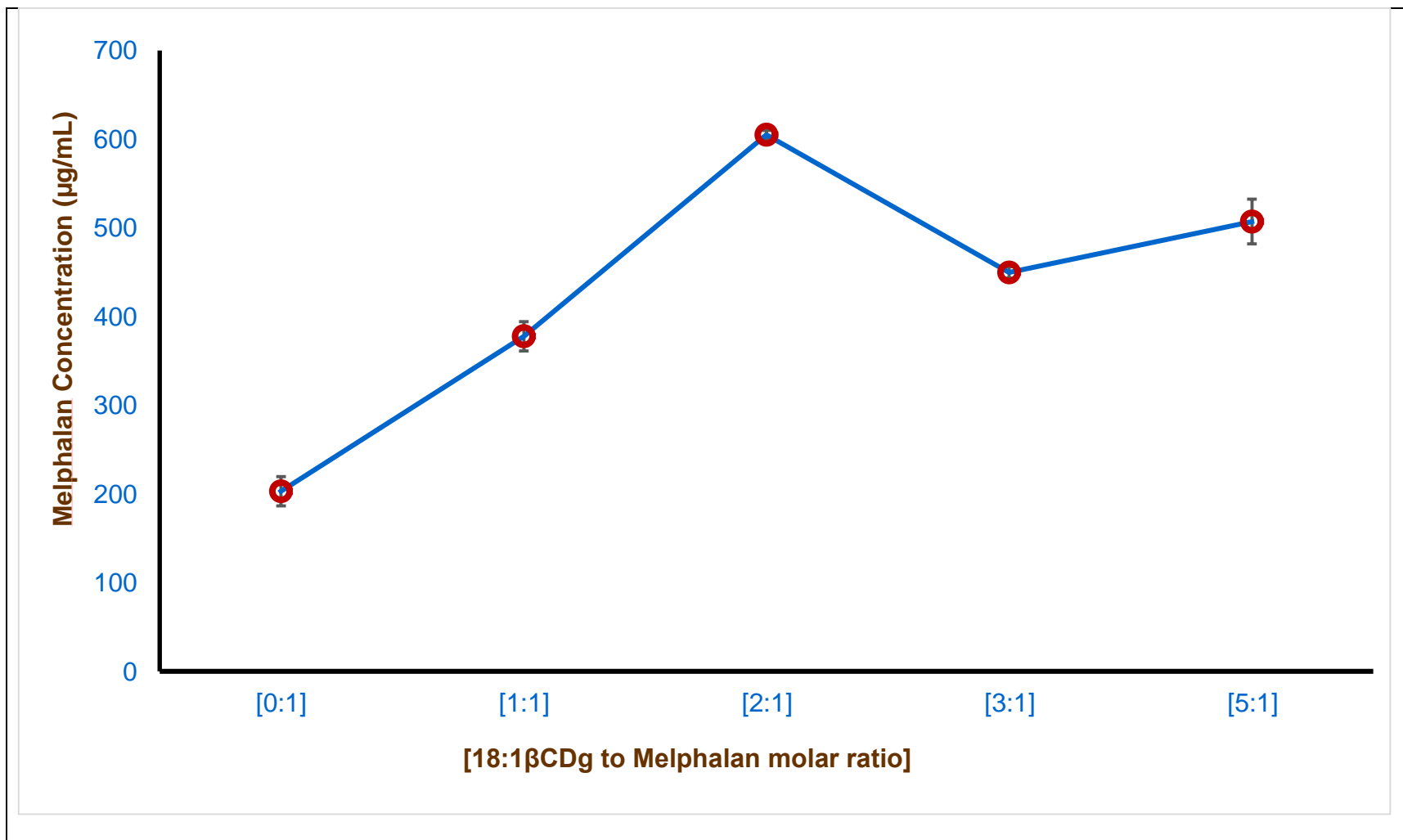


Figure 5: Solubilization Efficiency of β -CD-gemini surfactant: determined by FIA-MS/MS analysis (n=3, \pm SD)

4. Conclusion

In this study, two FIA-MS/MS methods for melphalan were successfully developed and validated. The methods were applied for the determination of melphalan in novel 18:1 β CDg drug delivery system. The simplicity and speed of the methods were appropriate with the requirement of efficient analytical approaches to quantify highly unstable drugs such as melphalan. In fact, to process a batch of 96 samples will only require 5.6 hours and we demonstrated that the samples are stable in such timeframe within an autosampler of an HPLC system. The removal of the HPLC column did not affect the sensitivity, accuracy, and precision of the methods as they were validated according to FDA guidelines. An important finding that could have been overlooked if a full validation was not conducted is the stability of the samples. It was shown that fresh samples should be prepared and analyzed as various storage conditions resulted in degradation beyond the acceptable limit of 15%.

The sensitivity and specificity of the methods allowed for the characterization of the solubility efficiency of a novel β -CD conjugated drug delivery system and it was shown that the solubility was significantly enhanced by 18:1 β CDg delivery system. Such findings are critical as increasing the solubility of melphalan will enhance the bioavailability of the drug in a biological system and improve its chemotherapeutic effects. In addition, the results provide essential data in understanding the basic behavior governing the use of the novel drug delivery system, 18:1 β CDg surfactants. Currently, the pharmacokinetics of the formulation is being assessed in animal model using FIA-MS/MS and it will be reported upon completion.

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