

## RP-HPLC method combined with ultrafiltration for simultaneous analysis of Melphalan and Topotecan in human plasma samples

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(Received November 10, 2023; Revised February 09, 2024; Accepted February 15, 2024)

**Abstract:** Pharmaceutical analysis still attracts the attention of researchers, and pharmaceutical analysis methods specific to the purpose of the application are needed in routine applications. In this study, the RP-HPLC method was developed for the simultaneous analysis of two different cancer drugs from human blood plasma due to simultaneous use. Melphalan and topotecan are licensed drugs that have been used for a long time. In routine practice, the simultaneous use of these two drugs is limited. However, studies have found that two active substances were used together in high-dose chemotherapy applications. This situation encourages the development of methods for the simultaneous analysis of both active substances in spiked human blood plasma samples. In this study, melphalan and topotecan were analyzed by RP-HPLC in 17 minutes, including post-run, with a gradient elution program using a Supercos 5  $\mu$ m C18 100 Å LC Column (100 x 4.6 mm) when the flow rate was 1.0 mL min<sup>-1</sup>. The method was linear in the 1.0- 20.0  $\mu$ g/mL range for both active substances. The detection wavelength was 261 nm. The accuracy and precision of the validated analytical method were demonstrated through intraday and interday studies. The analyte was freed from the remaining plasma components due to filtering the supernatant (after precipitation of the plasma proteins with methanol) with an ultrafilter (having 10 kDa pores). The simple methodology used in this study can be easily adapted to any pre-clinical or clinical application where analysis of melphalan and topotecan in plasma is required.

**Keywords:** Melphalan; Topotecan; RP-HPLC; pharmaceutical analysis; ultrafiltration; validation. © 2024 ACG Publications. All rights reserved.

### 1. Introduction

Cancer is a multifaceted disease characterized by the unchecked proliferation and division of cells. A combination of genetic and environmental factors influences its development. It may impact a singular organ or spread through metastasis. External contributors such as tobacco, chemicals, radiation, infectious agents, and internal factors like inherited mutations, hormonal imbalances, immune conditions, and

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sporadic genetic alterations collectively initiate or promote carcinogenesis. Treatment opportunities, including radiotherapy, chemotherapy, surgery, immunotherapy, hormone therapy, targeted therapies, and gene therapy, can be applied independently or in combination to address cancer [1, 2]. Chemotherapy is one of the most employed treatment method for cancer [3]. Antineoplastic drugs affect cancer cells to stop their proliferation [4].

Melphalan is an antineoplastic agent belonging to the class of nitrogen mustard alkylating agents, chemically referred to as 4-(Bis(2-chloroethyl)amino) phenylalanine [1]. Melphalan is commonly used to treat various malignancies, including breast and ovarian cancers and retinoblastoma [3].

Topotecan is a semi-synthetic alkaloid derivative with similar anticancer activity to camptothecin and it is safer when compared [5]. It is used in the treatment of ovarian cancer, small cell lung, and uterine cancer. It disrupts the normal function of type-1 DNA topoisomerase and inhibits the replication of rapidly dividing cells [6].

Melphalan has been reported to be analyzed using various analytical techniques. Qualitative analysis can be performed using colorimetry. Quantitative analyses are reported to be carried out using various chromatographic techniques, including HILIC and RP-HPLC. The reported techniques are overviewed in Table 1 [7-12].

**Table 1.** The analytical techniques reported for the determination of melphalan in human plasma.

Matrix	Technique	Mobile phase/ Solvents/ Dissolution medium used	Column/Spectrophotometer/ Electrode	$\lambda_{max}$ (nm)/ion transition	Flow rate (mL/min)	LOD-LOQ ( $\mu\text{g/mL}$ )	Ref
Plasma	UPLC-UV	25 mM NH <sub>4</sub> AC 0.1% HOAC : Acetonitrile (gradient elution)	C 18 (50 x 2.1 mm, 1.7 $\mu\text{m}$ )	261 nm	0.6	N.A-0.1	[7]
Plasma	TFLC-MS	Water:Methanol:Ammonium formate buffer (gradient elution)	C 18 (3,0 x 50 mm, 5 $\mu\text{m}$ )	Primary transition 305.1 >246.0 Secondary transition 305.1 >288.0 <i>m/z</i>	0.7	N.A-0.01	[8]
Plasma	HPLC	Sodium phosphate citrate buffer (0.016 M, pH 3.75): acetonitrile (87:13)	C18 (5 cm $\times$ 4.6 mm, 5- $\mu\text{m}$ )	254 nm	1	0.1-0.5	[9]
Plasma	(FIA)-MS/MS	water/acetonitrile (50:50, v/v) with 0.1% formic acid	-	Primary transition 305>168 Secondary transition 305>246.0 <i>m/z</i>	0.2	N.A-0.003	[10]
Plasma	LC-MS/MS	Method 1: water with 0.1% formic acid:acetonitrile with 0.1% formic acid (gradient elution) Method 2: Water:acetonitrile (gradient elution)	Method 1: C18 (5 $\mu\text{m}$ , 250 mm $\times$ 2.1 mm) Method 2: ;HILIC (3 m, 150 mm $\times$ 2.1 mm)		0.2		[11]
Plasma	HPLC	methanol, purified water and acetic acid (49.5:49.5:1, v/v)	Ultrasphere ODS (5 $\mu\text{m}$ )	261 nm	2	0.08-0.2	[12]

High-performance liquid chromatographic methods have been reported to determine topotecan concentrations in blood and urine samples. These methods are briefly given in Table 2 [13-15].

The physicochemical properties of melphalan and topotecan are given in the Supporting Information section, and their chemical structures are given in Figure 1. Up to now, various studies have been reported to determine melphalan and topotecan in human plasma samples. These are given in Tables 1 and 2, respectively.

**Table 2.** The analytical techniques reported for the determination of topotecan in human plasma

Matrix	Technique	Mobile phase/Solvents/Dissolution medium used	Column/Spectrophotometer/Electrode	$\lambda_{\max}$ (nm)/ion transition	Flow rate (mL/min)	LOD-LOQ ( $\mu\text{g/mL}$ )	Ref.
Plasma	HPLC	ammonium acetate buffer, acetonitrile and triethylamine (84:16:1.5, v/v) containing tetrabutyl ammonium hydrogen sulfate (TBAHS) (2 mM) with a pH of 5	Novapack C18 (4 $\mu\text{m}$ , 250 mm x 4.60 mm)	Detected fluorimetrically with an excitation wavelength of 380 nm and an emission wavelength of 527 nm.	1	0,015 ng/mL-0.05 ng/mL	[13]
Plasma	HPLC	methanol-0.1 M hexane-1-sulfonic acid in methanol-0.01 M N,N,N',N'-tetramethylethylenediamine (TEMED) in distilled water pH 6.0 (25:10:65, v/v)	Zorbax SB-C18 Stable Bond	Fluorescence Detector (with an excitation wavelength of 361 nm and an emission wavelength of 527 nm with an 40 nm bandwidth)	1	NA-0.05 ng/mL	[14]
Serum	HPLC	acetonitrile-water (70:30, 0.1% trifluoroacetic acid)	Phenomenex Luna C-18 (4.6 x 250 mm, dp = 5 $\mu\text{m}$ )	227 nm	1.2	0.023-0.07	[15]

Combined intravitreal melphalan and topotecan for refractory or recurrent vitreous seeding from retinoblastoma is a well-known application in routine therapy [16-18]. High-dose chemotherapy followed by autologous stem cell transplantation is standard application for multiple myeloma [19, 20]. In such cases, combined usage of these chemotherapeutics is vital [19, 21-23]. Monetizing the levels of these active pharmaceuticals in plasma after applications can be essential to understanding the behaviors of these two agents and arranging the therapeutical dosage. Therefore, in this study, a RP-HPLC-UV method combined with an ultrafiltration (UF) procedure to extract the sample was developed and validated to analyze melphalan and topotecan in plasma samples.

## 2. Experimental

### 2.1. Chemicals and Reagents

Melphalan and topotecan solutions were prepared using commercial hydrochloride salts of these active pharmaceutical ingredients (lyophilized powder for infusion solutions having  $\geq 97\%$  purity). The final concentrations of melphalan and topotecan solutions were 271  $\mu\text{g/mL}$  and 187  $\mu\text{g/mL}$ , respectively. An appropriate portion of these solutions were aliquoted (500  $\mu\text{L}$ ) and kept at  $-80\text{ }^\circ\text{C}$  till the experiments. Methanol (MeOH) was HPLC grade and purchased from Merck (Darmstadt, Germany). MilliQ water was

obtained from Barnstead Millipore Laboratory Water System. Commercially available lyophilized human citrated plasma samples (Sigma Aldrich, USA) were supplied to spike the melphalan and topotecan.

## 2.2. Apparatus and Chromatographic Conditions

The HPLC analyses were performed using the Shimadzu HPLC system including an SDP-20A UV-VIS detector, LC-20AD Puma, SIL-20AC HT autosampler, and a CTO-10AS VP column oven. Separation was performed while using a Supelco 5  $\mu\text{m}$  C18 100  $\text{\AA}$  LC Column (100 x 4.6 mm) at 30°C. The flow rate was 1.0 mL min<sup>-1</sup> in a gradient elution mode for MeOH:Water mixture. The gradient elution program started using 20% MeOH and reached 65% at the 7<sup>th</sup> minute and 80% at the 10<sup>th</sup> minute. The column was turned back into initial conditions and washed for seven minutes in post-run period. An injection volume of 20  $\mu\text{L}$  was utilized, and UV detection was set at 261 nm.

## 2.3. Preparation of the Calibration Curves

Melphalan (271  $\mu\text{g/mL}$ ) and Topotecan (187  $\mu\text{L/mL}$ ) stock solutions were stored at -80 °C to prepare working standard solutions. Working standard solutions ranging from 1.0 to 20.0  $\mu\text{g/mL}$  were freshly prepared by diluting the stock solutions with a MeOH:Water mixture (50:50 v/v) immediately before the injections to generate the calibration curve.

## 2.4. Preparation of the Sample Solutions

Standard solutions (2.0, 5.0, 10.0  $\mu\text{g/mL}$ ) were spiked into commercial plasma samples. In an Eppendorf tube, 500  $\mu\text{L}$  of plasma sample was treated with 500  $\mu\text{L}$  of methanol to precipitate the proteins. After vortexing for 1 minute and subsequent centrifugation, the resulting supernatant was transferred to a Merck Millipore-0.5 Centrifugal Filter with 10 kDa pores. Centrifugation at 12,000 rpm for 10 minutes was performed, and the supernatants were then transferred to vials for HPLC analysis. The results were evaluated, accounting for the dilution factor (2x).

# 3. Results and discussion

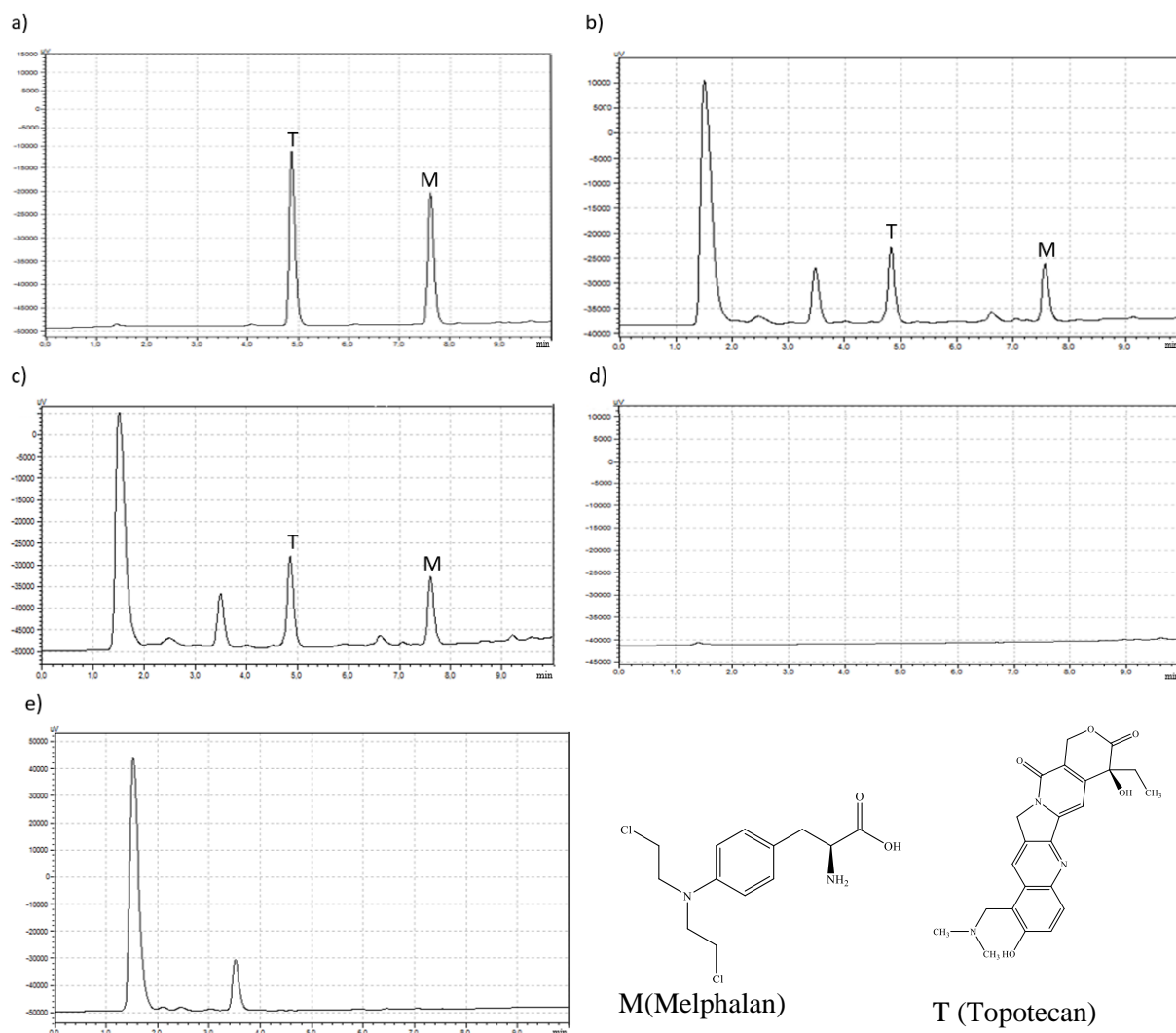
## 3.1. Chromatographic Separation

Our initial experiments failed to analyze melphalan and topotecan in isocratic elution mode. Therefore, we applied a gradient elution program, and both substances were separated with sufficient resolution in 8 minutes. The analysis time, including post-run, was 17 minutes in total. Under the optimum conditions, the plasma solutions including melphalan and topotecan were passed through the ultrafilter, and the chromatograms of the mobile phase, standard, spiked plasma samples, filtrated plasma samples, and blank plasma samples are given in Figure 1. Using an ultrafilter allowed us not to dilute samples one more time before the injections. Otherwise, the precipitated proteins might have contaminated our experiments without using an ultrafilter.

## 3.2. Validation

The 5.0  $\mu\text{g/mL}$  standard solution of melphalan and topotecan was used for system suitability testing in HPLC. Six consecutive injections were performed, and the number of plates, tailing factor, resolution, and reproducibility (retention time and peak height) were calculated. Results and reference values are given in Table 3. As it is seen, the results were within the reference values.

Standard, spiked, and blank chromatograms were compared to assess the analytical method's specificity. The chromatograms are given in Figure 1. The examination confirmed that no peaks from matrix components interfered with the analyte peaks. These results confirmed the specificity of the method.



**Figure 1.** Chromatograms taken under the optimum conditions (column: Supercio 5  $\mu\text{m}$  C18 100  $\text{\AA}$  LC Column (100 x 4.6 mm), flow rate: 1.0 mL min<sup>-1</sup>, injection volume: 20  $\mu\text{L}$ , UV detection: 261 nm) a) the standard M (melphalan) and T (topotecan) solution 5.0  $\mu\text{g/mL}$ , b) M and T spiked into plasma 5.0  $\mu\text{g/mL}$  (proteins were precipitated and filtrated), c) M and T spiked into plasma as 5.0  $\mu\text{g/mL}$  (proteins were precipitated but not filtrated), d) mobile phase, and e) blank plasma.

The melphalan and topotecan standards calibration curve was generated by plotting compound concentration against peak area ratio. The resulting graph exhibited linearity up to 20  $\mu\text{g/mL}$ . Subsequently, nine standard solutions, ranging from 1.0 to 20.0  $\mu\text{g/mL}$  for melphalan and topotecan, falling within this linear range, were prepared, and introduced into the HPLC system. Linearity was assessed using linear regression analysis. From the calibration graphs, regression equations were derived, and the standard deviations of the slope ( $S_b$ ) and intercept ( $S_a$ ) were calculated, as summarized in Table 4.

**Table 3.** System suitability tests results and reference values.

Chromatographic Parameters	Mean	Standard deviation	% RSD	Reference Values
R.Time'T	4.9	0.002	0.05	RSD<% 1
R.Time'M	7.6	0.002	0.03	
Peak Height'T	38079	49.2	0.13	RSD<% 1
Peak Height'M	31075.83	139.4	0.45	
k'T	2.48	0.002	0.08	k' > 2
k'M	4.44	0.002	0.04	
tf'T	1.35	0.001	0.10	Tf < 2
tf'M	1.32	0.005	0.39	
Rs'T	3.66	0.02	0.65	Rs > 2,0
Rs'M	6.27	0.51	8.18	
N'T	8091	8.33	0.10	N > 2000
N'M	18065	57.15	0.32	
A (Separation factor)	<b>1.79</b>			$\alpha > 1$

The regression equation is  $y=47268x+1489$  for melphalan and  $y=60170x+864$  for topotecan. The regression coefficient is 0.9998 for both graphs. The standard deviation of the slope of the calibration equation created for Melphalan is 4442, and the standard deviation of the intercept is 1150. The standard deviation of the slope of the calibration equation created for Topotecan is 3022, and the standard deviation of the intercept is 1787. The calibration curve y values refer to the concentration as  $\mu\text{g/mL}$ , and the x values are peak areas.

The limit of detection (LOD) and quantification (LOQ) were determined based on the signal-to-noise ratio. The LOD represents the lowest concentration level at which the peak height is three times the baseline noise. The LOQ is the lowest concentration level with a peak height possessing a signal-to-noise ratio greater than 10, with precision (RSD%) and accuracy (Bias%) within  $\pm 10\%$ . For the HPLC method, the limit of detection (LOD) and limit of quantification (LOQ) values were determined to be 0.3 and 0.9  $\mu\text{g/mL}$  for Melphalan and 0.2 and 0.5  $\mu\text{g/mL}$  for Topotecan, respectively.

**Table 4.** Linearity of the developed method (n=6).

Paramaters	M	T
Regression equation*	$y=47268x+1489$	$y=60170x+864$
Standard error of intercept	1150	1787
Standard error of slope	4442	3022
Regression coefficient ( $R^2$ )	0.9998	0.9998
Number of data points	7	9
LOD ( $\mu\text{g/mL}$ )	0.3	0.2
LOQ ( $\mu\text{g/mL}$ )	0.9	0.5

\* x: concentration as  $\mu\text{g/mL}$ , y: peak area

Three different concentrations of standard Melphalan and Topotecan solutions within the linear range were analyzed over six consecutive days (inter-day precision) and six times within the same day (intra-day precision). The values obtained for relative standard deviation (RSD) and bias in both intra and inter-day studies indicated satisfactory precision and accuracy of the method. The summarized results are given in Table 5.

**Table 5.** Accuracy and precision of the developed method (n=3).

		<b>Intra-day</b>				
		Added ( $\mu\text{g/mL}$ )	Found <sup>a</sup> ( $\mu\text{g/mL}$ )	Precision RSD %	Accuracy <sup>b</sup> Bias %	
<b>M</b>		2.0	2.05 $\pm$ 0.04	3.50	-2.43	
		5.0	5.08 $\pm$ 0.04	1.66	-1.57	
		10.0	10.00 $\pm$ 0.02	0.34	0.01	
			<b>Inter-day</b>			
			Added ( $\mu\text{g/mL}$ )	Found ( $\mu\text{g/mL}$ )	Precision RSD %	Accuracy Bias %
			2.0	2.38 $\pm$ 0.07	5.11	-19.05
		5.0	5.73 $\pm$ 0.06	1.93	-14.63	
		10.0	10.53 $\pm$ 0.03	0.52	-5.33	
		<b>Intra-day</b>				
		Added ( $\mu\text{g/mL}$ )	Found <sup>a</sup> ( $\mu\text{g/mL}$ )	Precision RSD %	Accuracy <sup>b</sup> Bias %	
<b>T</b>		2.0	1.96 $\pm$ 0.02	2.24	1.80	
		5.0	5.00 $\pm$ 0.01	0.40	-0.07	
		10.0	10.13 $\pm$ 0.03	0.50	-1.25	
			<b>Inter-day</b>			
			Added ( $\mu\text{g/mL}$ )	Found ( $\mu\text{g/mL}$ )	Precision RSD %	Accuracy Bias %
			2.0	1.92 $\pm$ 0.004	0.43	3.84
		5.0	5.01 $\pm$ 0.002	0.07	-0.14	
		10.0	9.67 $\pm$ 0.02	0.36	3.29	

<sup>a</sup>Found : mean  $\pm$  standard error (n=3)<sup>b</sup>Bias : [(Found - Added) / Added] x 100

The ruggedness study assessed the impact of different analysts. To check the ruggedness of the method, 1.0 mL of a 5.0  $\mu\text{g/mL}$  melphalan and topotecan standard solutions, prepared by a different analyst from the stock solution, were repetitively injected into the HPLC (n=6). The calculated concentration values were compared statistically within each other under optimal conditions using Wilcoxon signed-rank test. Since  $t_{\text{calculated}} > t_{\text{theoretical}}$ , it indicated no significant difference between the two analysts and the method was rugged ( $p > 0.05$ ).

Spiked plasma samples were created by adding standard solutions to commercial plasma samples, representing substances at three concentration levels (2.0, 5.0, 10.0  $\mu\text{g/mL}$ ) to mimic real samples. The recovery calculation, based on the injection values, yielded satisfactory results. The analysis outcomes are detailed in Table 6.

**Table 6.** Analysis of the spiked plasma samples (n=6)

Added ( $\mu\text{g/mL}$ )	Recovery %	
	M	T
2.0	104.43 $\pm$ 2.49	103.45 $\pm$ 4.13
5.0	97.21 $\pm$ 0.31	99.88 $\pm$ 1.96
10.0	101.75 $\pm$ 0.61	98.57 $\pm$ 2.15

### 3.3. Limitations and Advantages of the Study

Although the study was designed to enable the analysis of melphalan and topotecan used in combination from plasma, the situations in which these two active substances are used together are very limited, as mentioned in the introduction section. It has been reported that plasma concentrations of melphalan and topotecan remain very low due to *intra-vitreous* use in retinoblastoma treatment, as seen in previous studies [24, 25]. In this case, using the developed method in monitoring plasma concentration is hard for *intra-vitreous* applications. In a study published by Taich et al. [26], they used an HPLC equipped with a fluorescence detector and reported that the LOQ values of melphalan and topotecan were 50 and 5 ng/mL, respectively. Therefore, a fluorescence or mass-spectrometry detector could help monitor the level of these compounds in plasma when an *intra-vitreous* application is performed.

However, using both active substances in high-dose chemotherapeutic applications will also need methods that allow plasma monitoring of these active substances in routine treatments and alternative treatment interventions. Although fluorescence or mass spectrometry (MS) detectors provide better sensitivity in general cases, UV detector is a gold standard for HPLC analysis. Therefore, we developed the RP-HPLC-UV method using an easy-to-find C18 column (C18 100 Å LC Column (100 x 4.6 mm), a UV-detector, and a really basic mobile phase to prepare (MeOH:Water mixture in a gradient elution mode). This common equipment will allow researchers to apply the developed methodology in their laboratory for further application where Melphalan and Topotecan need to be analyzed in human plasma samples.

Despite the method's limitations regarding sensitivity, the prominent aspect is the contribution of the ultrafiltration technique used to remove the matrix components in plasma samples, where proteins are precipitated using organic solvent-methanol at a ratio of 1:1. In routine applications using protein precipitations, this ratio is up to 1:3 for plasma samples. The improvement of ultrafiltration on cleaning the sample was observed, especially in a post-run period when some residual peaks from the supernatant were not observed. Ultrafiltration prevented further dilution of the solution and increased the column life by cleaning the sample. In addition, the stability issues of Melphalan, as we see in our initial results and reported in the literature [27], forced us to develop a rapid sample preparation step. Ultrafiltration provided an effective and faster methodology than solid or liquid phase extraction.

### 3.4. Uncertainty Budget Estimation

Uncertainty is defined as a parameter linked to the outcome of a measurement, describing the spread of values that could reasonably be assigned to the measurement. In chemical analysis, the term measurand often pertains to the concentration of an analyte [28]. Uncertainty reflects variations in the analytical result.  $u_{\text{linearity}}$  values were measured based on equation 1 and found to be 9.39 (melphalan) and 5.02 (topotecan).

$$u_{\text{Linearity}} = \frac{(\text{Standard Error of Slope} * 100)}{\text{Slope}} \quad \text{Equation 1}$$

$u_{\text{standard}}$  values for both melphalan and topotecan were 1.73 since these are the pharmaceutical-grade bulks of the approved commercial drugs, and their purities were  $\geq 97\%$ .

$$u_{\text{Standard}} = \frac{100 - \% \text{Purity}}{\sqrt{3}} \quad \text{Equation 2}$$

$u_{\text{repeatability}}$  is a value associated with RSD% of the consecutive injections or the results for the samples prepared intra-day or interday. To calculate the repeatability, the repeatability of the injections (including the injection volume of the instrument) and the sample preparation (including pipetting, measuring volume, protein precipitation yield, and ultrafiltration) were calculated for both compounds and found to be a maximum value 5.11 for melphalan and 2.24 for topotecan, respectively. Our results confirmed that the variation while pipetting, weighing, and losing some of the analytes while ultrafiltration and precipitating the proteins affects our results and causes variations.  $u_{\text{combined}}$  was calculated as 10.82 for melphalan and 6.34 for topotecan.



## 4. Conclusions

In this study, a method was developed that enables the analysis of melphalan and topotecan simultaneously in plasma by RP-HPLC. The developed method was validated in terms of selectivity, linearity, accuracy, precision (interday and intraday), sensitivity, and ruggedness according to ICH guidelines. Based on our results, the method was selective. The intraday and interday precision and accuracy study showed that the method was precise and accurate. The recovery of melphalan in plasma was found to be 104.43, 97.21 and 101.75% for 2.00, 5.00 and 10.00 µg/mL, respectively. The recovery of topotecan in plasma was found to be 103.45, 99.88 and 98.57% for 2.00, 5.00 and 10.00 µg/mL, respectively. Although the sensitivity of the developed method is not able to determine lower concentrations, this method could be applied for any pharmacokinetic application where the plasma concentration covers our range in high-dose applications.

## Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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