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Assays for the quantification of melphalan and its hydrolysis products in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Two high-performance liquid chromatography tandem mass spectrometry (LC–MS/MS) assays are described for the quantification of melphalan in human plasma. N-phenyldiethanolamine was tested as internal standard. The first assay consisted of a protein precipitation by cold methanol and a reversed-phase HPLC whereas the second one was based on a solid phase extraction and a hydrophilic interaction chromatography. Both provided a very satisfactory mean extraction yield with a small volume of sample. The first method was simple, rapid and used as a routine assay. The second one was developed in order to determine melphalan hydrolysis products and to avoid scarce cases when interferences from biological matrix alter the quantification of melphalan using the first method. The two assays were linear and sensitive in the range of 1–500 ng/mL for the first one and in a range of 25–2000 ng/mL for the second one. Concentrations out of the range fixed with the first method were also validated. The procedure was reliable with precision and accuracy below 10%. All compounds were detected after positive mode electrospray ionization in selected reaction monitoring mode. These new analytical procedures were developed for melphalan pharmacokinetic studies or therapeutic drug monitoring.

1. Introduction

Melphalan (4-(bis(2-chloroethyl)amino)-L-phenylalanine) is a cytotoxic drug that acts as a bifunctional alkylating agent on DNA. This derivative of nitrogen mustard is mainly indicated in the treatment of multiple myeloma and secondary in the treatment of breast and ovary carcinoma, neuroblastoma, Hodgkin's disease, polycythaemia vera and in amyloidosis [1,2]. It can be included in the protocol of haematopoietic stem cell transplantation. It is also used in the treatment of malignant melanoma and soft-tissue sarcomas by heated intra-arterial regional limb perfusion (ILP) [3].

Melphalan has been available for more than forty years and several analytical methods have been developed for its quantification in biological matrix [4,5]. Most common problems encountered were its spontaneous hydrolysis and sample pre-treatment. Melphalan is unstable in plasma at ambient temperature. It is hydrolyzed to an unstable compound, the monohydroxymelphalan (MHM), which in its turn, is hydrolyzed to a stable compound, the dihydroxymelphalan (DHM). The degradation kinetic and a method to obtain these products have been reported. Both were also detected after intravenous injection in patient's plasma but they did not present any cytotoxic activity [6].

Assays for the determination of melphalan were mainly based on HPLC coupled with UV, fluorescence or electrochemical detection [7–18]. Melphalan was usually extracted from the matrix by protein precipitation. Nevertheless, these assays could not reach satisfactory limits of quantification or were not applied to human blood samples. Sensitive detection of melphalan by LC–MS/MS was previously described in two studies. Bauer et al. proposed a method based on LC–APCI/MS/MS for the quantification of melphalan in rat tissue [19]. Davies et al. described an assay (LC–ESI/MS/MS) from human plasma with sample pre-treatment based on a solid phase extraction with a robotic sample processor and [$^{13}C_4{}^2H_4$]-melphalan was used as internal standard [20]. However, in both cases no determination of melphalan hydrolysis products was provided.

In our analytical report, two methods for the quantification of melphalan by LC–MS/MS were validated. The first method (method 1) consisted of a protein precipitation by cold methanol and a reversed-phase high-performance liquid chromatography. The second method (method 2) was based on a solid phase extraction and

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a hydrophilic interaction chromatography (HILIC). The aim of this study was to set up a rapid and sensitive quantification of melphalan in pharmacokinetic investigations using method 1. The second method allowed also the determination of its hydrolysis products that constitutes relevant parameters to assess the degradation of melphalan in various conditions. The method 2 may also be used as alternative assay for the scarce cases when interferences from biological matrix alter the quantification of melphalan using the method 1.

2. Experimental

2.1. Reagents and chemicals

Melphalan and N-phenyldiethanolamine (N-PEA) which was used as internal standard (IS) were purchased from Sigma (Steinhein, Germany). The powder of melphalan was stored at ambient temperature in the dark as well as N-PEA which required a slight nitrogen stream for its conservation. The dihydroxymelphalan (DHM) was prepared from an aqueous solution of melphalan completely hydrolyzed at 60 °C for 4 h according to the literature [4,15]. It was stored at −80 °C. Formulas of all compounds are reported in Table 1. Milli-Q deionized water was provided by Millipore (Eschborn, Germany) and HPLC-grade acetonitrile by Merck (Darmstadt, Germany). Acetic acid, formic acid and ammonium hydroxide solution for LC/MS were purchased from Fluka (Steinhein, Germany). Methanol was supplied by Carlo Erba (Milano, Italy). Drug-free normal human plasma was obtained from the regional blood bank (EFS Rhône-Alpes, France).

2.2. Standard solutions and calibration

Stock solutions (1 mg/mL) were prepared in methanol acidified at 0.1% by formic acid for melphalan and in methanol only for N-PEA. They were stored at −80 °C. Standard solutions were obtained daily by successive dilutions in methanol.

2.2.1. Method 1

Drug-free normal human plasma (475 μL) was spiked with 25 μL of the appropriate diluted solutions to give final concentrations of calibration standards at 1, 2.5, 10, 50, 200 and 500 ng/mL. In addition, quality control (QC) samples were made at 1.5, 35 and 350 ng/mL from these standard solutions. Assessment of accuracy for extended concentration range was carried out with a quality control at 1000 ng/mL in order to confirm the possibility of using the method at higher concentrations. Finally, solution of melphalan at 2000 and 8000 ng/mL in plasma was respectively diluted by 1/5 and 1/20 to obtain a final concentration of 400 ng/mL that was extracted in triplicate on three separate days. This was done to validate the dilution procedure performed from patient samples when the concentration was over 1000 ng/mL.

2.2.2. Method 2

Drug-free normal human plasma (450 μL) was spiked with 50 μL of the appropriate diluted solutions to give final concentrations of calibration standards at 25, 75, 250, 750 and 2000 ng/mL. In addition, quality control (QC) samples were made at 50, 150 and 1000 ng/mL from these standard solutions.

The dihydroxymelphalan (DHM) was obtained as described above. Standard solutions of DHM were then obtained daily by successive dilutions in water. Calibration standards at 12.5, 37.5, 125, 375 and 1000 ng/mL of DHM using method 2 were prepared with QC samples at 25, 75 and 500 ng/mL.

2.3. Sample preparation

All samples were kept in ice during the sample preparation procedure.

2.3.1. Method 1

A volume of 40 μL of a solution containing 50 ng/mL of N-PEA was added to 100 μL of plasma samples. After protein precipitation by 500 μL of cold methanol, sample was mixed and centrifuged for 10 min at 13,000 × *g* at 6 °C. Then, the clear supernatant was transferred to a glass vial.

2.3.2. Method 2

A volume of 30 μL of a solution containing 100 ng/mL of N-PEA was added to 200 μL of plasma samples. This volume was passed through an Oasis MAX column (Waters, Milford, USA) conditioned with Milli-Q deionized water and methanol. After washing by ammonium hydroxide solution (5%), the compounds were eluted by methanol/Milli-Q deionized water (20/80, v/v) and by 1.5% formic acid in methanol. The eluent was evaporated under a slight nitrogen stream and reconstituted in 1 mL of mobile phase. It was then transferred in a glass vial.

Finally, in both cases, the glass vial was kept at +5 °C in the autosampler, and 10 μL was injected into the chromatographic device.

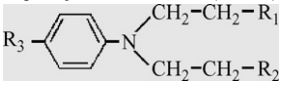
2.4. HPLC conditions

The high-performance liquid chromatography system included a ThermoElectron Surveyor MS pump and a Surveyor autosampler injector (ThermoElectron, San Jose, USA). The column oven temperature was maintained at +20 °C and the mobile phase flow rate was 200 μL/min.

2.4.1. Method 1

Compounds were separated on a X-Terra RP C18 column (Waters, Milford, USA; 5 μm, 250 mm × 2.1 mm i.d.) with a X-Terra guard cartridge (10 mm × 2.1 mm i.d.). Gradient elution with water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid was applied. The total run was set at 15 min (Table 2).

Table 1
Structure of melphalan, monohydroxymelphalan (MHM), dihydroxymelphalan (DHM) and the internal standard N-phenyldiethanolamine (N-PEA).



	R 1	R 2	R 3
Melphalan	Cl	Cl	NH_2 | $CH-CH_2$ | $COOH$
MHM	OH	Cl	
DHM	OH	OH	
N-PEA	OH	OH	H

Table 2
Method 1: mobile phase composition and gradient evolution.

Time (min)	Formic acid, 0.1%	Acetonitrile + formic acid, 0.1%
0	80	20
1	80	20
2.5	10	90
8	10	90
8.1	80	20
15	80	20

Table 3
Method 2: mobile phase composition and gradient evolution.

Time (min)	Acetic acid (100 mM)	Water	Acetonitrile
0	14	10	76
1	14	10	76
4	14	46	40
10	14	46	40
10.1	14	10	76
20	14	10	76

2.4.2. Method 2

Compounds were separated on an Atlantis HILIC Silice column (Waters, Milford, USA; 3 μm, 150 mm × 2.1 mm i.d.). Gradient elution with water and acetonitrile was used. During all the run, the percentage of acetic acid 100 mM in the mobile phase was set at 14%. The total run was fixed at 20 min (Table 3).

2.5. Mass spectrometry conditions

Analytes mass spectra were determined by a Quantum-Ultra triple-quadrupole mass spectrometer (ThermoElectron, San Jose, USA) equipped with an Ion Max atmospheric pressure ionization (API) source. After vaporization and positive ionization by an electrospray ionization (ESI) probe, the sample ions $[M+H]^+$ entered in the mass spectrometer. The best position (x, y, z) of the ESI probe was performed using a solution of melphalan. The collision gas used in the second quadrupole was argon at a pressure of 1.5 mTorr. Spray voltage and capillary temperature were maintained at 3.5 kV and 300 °C, respectively. The pressures of the nitrogen sheath gas and auxiliary gas were set at 40 and 10 units (arbitrary units), respectively. These ions were detected by selected reaction monitoring (SRM) with specific transitions and collision energy summarized in Table 4 and with a scan time of 500 ms.

2.6. Calibration curves and validation procedure

Calibration curves were constructed through a linear least-squared regression model. The ion abundance peak area ratios

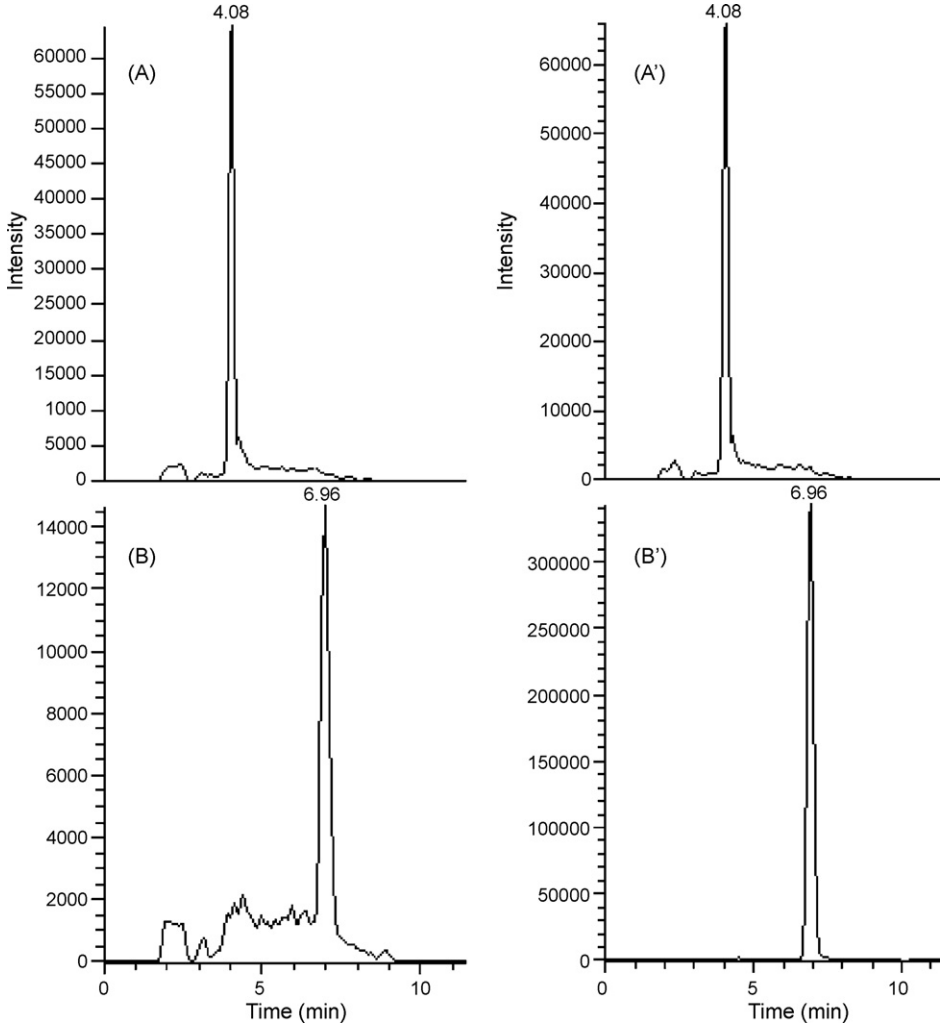


Fig. 1. Chromatograms obtained with the method 1 (see text for details). Left: SRM chromatograms from human blank plasma spiked with I.S. and melphalan. Right: SRM chromatograms from patient treated with melphalan (blood sample was collected 90 min after the end of the administration). A and A′ represent the specific transitions of the I.S., B and B′ represent the specific transitions of melphalan at 1 ng/mL corresponding to the first calibration curve, and at 200 ng/mL, respectively.

Table 4
SRM parameters of melphalan, MHM, DHM and N-PEA.

Compounds	SRM transition (m/z)	Collision energy (eV)
Melphalan	305.0 > 246.0	25
MHM	287.0 > 270.0, 228.0	20
DHM	269.1 > 252.1	20
N-PEA	182.1 > 120.1, 106.1	25

melphalan/IS and DHM/IS were function of the concentration (C) of the calibration standards with a weight-factor of $1/C$. Control samples and plasma samples were then calculated from the equation of the regression. An analysis of variance was used to test the linearity of the response. Fisher–Snedecor's F-test ($p < 0.05$) was used to determine the significance of the slope and the validity of the linear calibration curves, and Cochran's test ($p < 0.05$) assessed homocedasticity.

Validation procedure consisted in analysis of seventeen calibration curves prepared as a single replicate on seventeen different days for the method 1 and five calibration curves on five different days for the method 2. Analysis of each QC samples in six replicates was added to four and three of this run calibration curves for methods 1 and 2, respectively. Lower and upper limit of quantification were defined, respectively as the concentration of the lowest and the upper calibration standard.

The accuracy and precision of the assay were assessed by the mean relative percentage deviation from the nominal concentrations and the within-run precision (WRP) and between-run precision (BRP), respectively. The within-run precision was determined as $\mathrm{WRP} = 100 \times (\sqrt{\mathrm{MS_{wit}}}/\mathrm{GM})$. The between-run precision was estimated as $\mathrm{BRP} = 100 \times (\sqrt{((\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n)}/\mathrm{GM})$. $\mathrm{MS_{wit}}$, $\mathrm{MS_{bet}}$, n and GM represent the within-groups mean square, the between-groups mean square, the number of replicate observations within each run and the grand mean, respectively. These parameters were calculated using the software Statview for windows version 5.0 (SAS Institute, Cary, USA).

The extraction efficiency was measured by comparison of extracted plasma samples and methanol samples at the same concentrations than the standard curve.

2.7. Stability of melphalan

Duplicate QC samples (1.5, 35 and 350 ng/mL) of melphalan in plasma were used to investigate the short-term molecule stability after three freeze/thaw cycles (seven days intervals) and long-term stability (two months) at −80 °C.

Melphalan stability in the autosampler maintained at +5 °C was tested by re-analyzing six replicates of QC samples at 35 ng/mL 7 h after the first injection.

A blank plasma sample spiked with 1 μg/mL of melphalan was maintained at ambient temperature for 24 h, and analyzed every hour during 8 h and then at 24 h. This study was made in triplicate and was also performed at +4 °C as a single replicate.

2.8. Clinical samples

Quantification of melphalan was carried out on clinical samples obtained from a sixty-six-year-old man who received a heated intraperitoneal intraoperative melphalan perfusion. Melphalan was determined in plasma before the beginning of the perfusion and then at 30, 60, 90 and 120 min. Determination of melphalan was also performed from two patients treated by I.V. perfusion of melphalan (15 min). In both cases, blood samples were taken at 0 (before the beginning of the perfusion), and at 10, 30, 90 and 120 min after the end of the perfusion (patient 1, two kinetics with different doses) and at 25, 50, 140 and 260 min after the end of the perfusion (patient 2). Blood samples were collected in 5 mL glass tubes containing lithium heparin and kept in ice. After centrifugation at 3500 × g for 10 min at +4 °C, plasma was frozen at −80 °C until analysis.

3. Results and discussion

3.1. Liquid chromatography

Method 2 was developed in order to assess the potential degradation of melphalan in samples. This may be interesting to explain, for example, melphalan concentration in blood lower than those

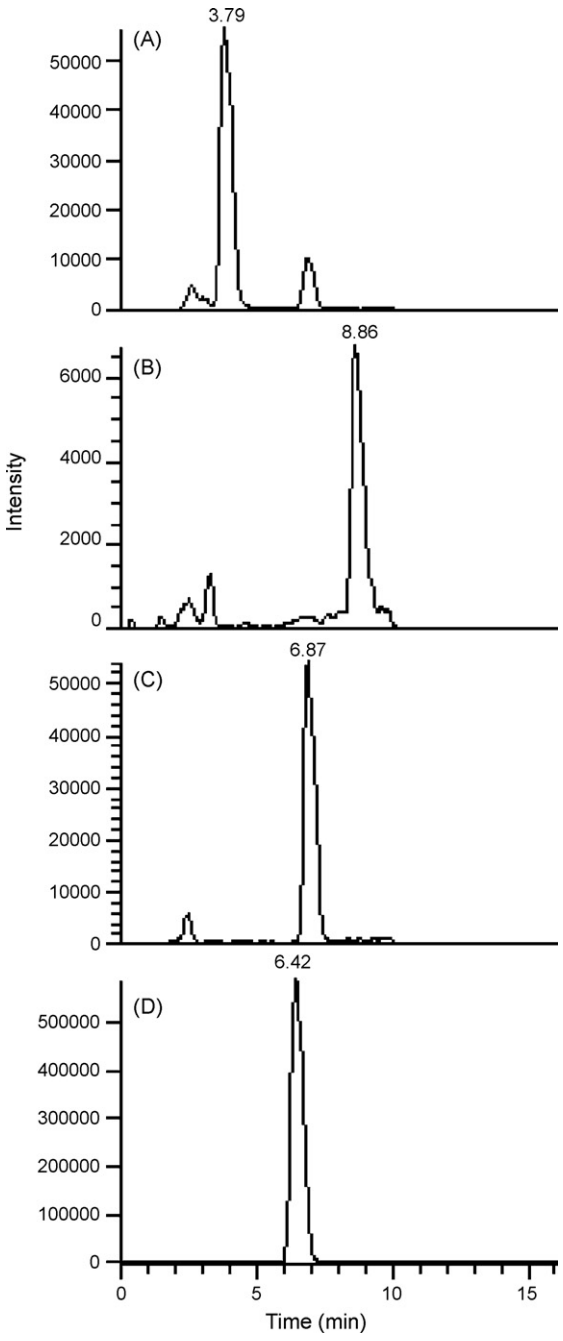


Fig. 2. SRM chromatograms obtained with the method 2 (see text for details) from patient treated with melphalan (blood sample was collected 90 min after the end of the administration). A–D represent the specific transitions of the N-phenyldiethanolamine (I.S.), the dihydroxymelphalan, the monohydroxymelphalan and the melphalan (220 ng/mL), respectively.

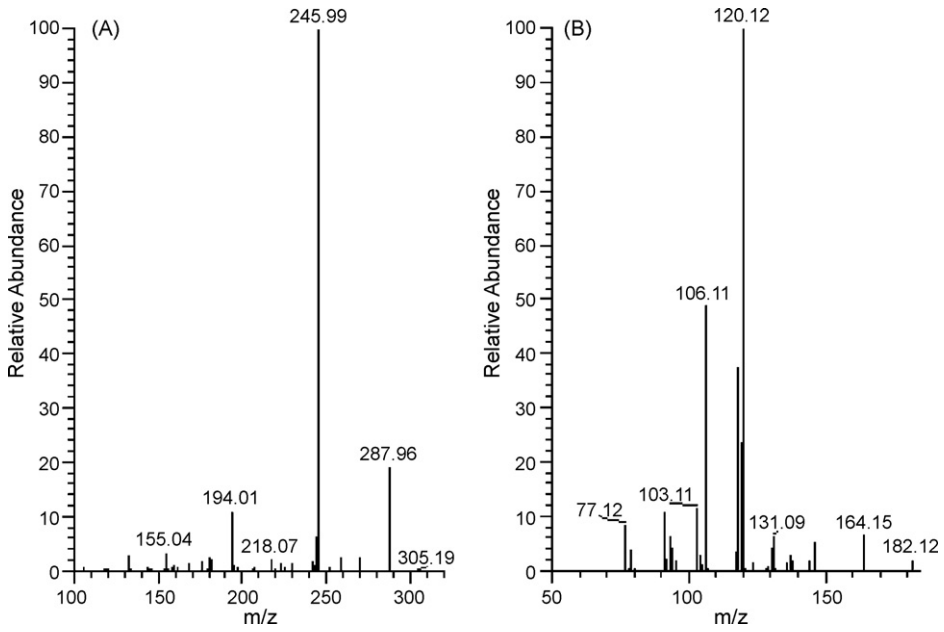


Fig. 3. Product ion mass spectrum of melphalan (A) and N-phenyldiethanolamine (internal standard) (B).

expected. Initially, determination of MHM and DHM was tested with method 1. Unfortunately, ion suppression was observed for both products while no matrix effect was observed for melphalan (data not shown). The solid phase extraction (SPE) associated to HILIC gave a good signal for melphalan hydrolysis products by the elimination of the endogenous compounds from matrix. An estimation of MHM was given since the pure compound was not available and it was impossible to stop the hydrolysis of MHM to DHM in order to obtain a pure solution of MHM. Finally, the method 2 allowed the quantification of melphalan in scarce cases where interference might be observed using protein precipitation sample procedure.

Fig. 1 reports the chromatograms obtained with the method 1 for N-PEA and melphalan. Fig. 2 shows the chromatograms of N-PEA, melphalan and its hydrolysis products that are eluted according to their increasing polarities. Good peak shapes and sensitivity were obtained for all compounds with both assays.

3.2. Mass spectrometry

Formic and acetic acid used in the mobile phase enhanced the formation of protonated $[M+H]^+$ ions. For the melphalan, the specific $[M+H]^+$ ion transition $305 \rightarrow 246$ was chosen because it was the major ion product and a better signal was obtained contrary to the transition $305 \rightarrow 288$ previously used [19] (Fig. 3). The $305 \rightarrow 246$ m/z transition involved a loss in the alanine side chain while $305 \rightarrow 288$ m/z transition revealed the loss of the amine group and the formation of a double bound. The same fragmentation pathway was observed for melphalan hydrolysis products. The major ion product was 270 and 252.1 for MHM and DHM, respectively. Signal intensities for the two SRM transitions of MHM (ions 270 and 228) and N-PEA (ions 120.1 and 106.1) were summed in order to increase the signal-to-noise ratio.

3.3. Calibration curves

Calibration curves of melphalan (peak area ratios melphalan/IS versus melphalan concentration) were defined on seventeen different days for the method 1 and five different days for the method 2. Data were fitted by the weighting factor $1/C$ through a linear least-squares regression curve. Thus, linear detection response was defined from 1 to 500 ng/mL and from 25 to 2000 ng/mL for methods 1 and 2, respectively with a coefficient of determination (r^2) was at least equal to 0.997. Inter-day precision of concentration calibration standard never exceeded by 8% and the accuracy was always below 6% for method 1 and equal to 2 and 5% for method 2, respectively (Table 5).

The quantification of DHM was based on the complete degradation of melphalan, according to the appropriate conditions, to DHM in a unimolecular reaction [21]. After the incubation (4 h at +60 °C), it was verified that no trace of melphalan and MHM was present in the incubation mixture. Moreover, the peak area for DHM remained stable overtime. Calibration curves of DHM (peak areas ratios DHM/IS versus DHM concentration) were made as a single replicate on five different days using method 2. Data were fitted by the weighting factor $1/C$ through a linear least-squares regression curve. Linear detection response was found from 12.5 to 1000 ng/mL with a coefficient (r^2) of the linear regression was at least equal to 0.998. Precision and accuracy of concentration calibration standard were always below 10% (Table 6).

Table 5
Inter-day validation of the determination of melphalan with methods 1 and 2. Data from n calibration curves prepared as a single replicate and analyzed on n different days.

Melphalan (ng/mL)			
Spiked	Found (mean ± S.D.)	Precision (%) (between-run)	Accuracy (%)
Method 1, $n = 17$			
1	0.95 ± 0.07	7.2	94.9
2.5	2.50 ± 0.12	4.8	100.1
10	10.4 ± 0.3	3.3	103.9
50	50.7 ± 1.7	3.3	101.5
200	201 ± 4	2.1	100.5
500	498 ± 5	0.9	99.7
Method 2, $n = 5$			
25	26.1 ± 0.2	0.8	104.6
75	75.9 ± 1.1	1.5	101.2
250	233 ± 2	0.8	93.3
750	752 ± 13	1.7	100.2
2000	2013 ± 11	0.5	100.6

Table 6
Inter-day validation of the determination of dihydroxymelphalan (DHM) with method 2. $n = 5$ calibration curves prepared as a single replicate, five different days.

DHM (ng/mL)			
Spiked	Found (mean ± S.D.)	Precision (%) (between-run)	Accuracy (%)
12.5	13.2 ± 0.8	5.8	105.9
37.5	37.2 ± 1.8	4.9	99.1
125	117 ± 3	2.6	94.3
375	375 ± 19	5.2	100.0
1000	1007 ± 19	1.9	100.3

Table 7
Assessment of accuracy and precision obtained with methods 1 and 2. Data from six replicates for each quality control and extracted and analyzed on n different days.

Melphalan (ng/mL)		Precision (%)		Accuracy (%)
Spiked	Found (mean ± S.D.)	Within-run	Between-run	
Method 1, $n = 4$				
1.5	1.53 ± 0.14	7.8	5.2	101.9
35	36.9 ± 1.2	2.6	2.5	105.5
350	348 ± 9	2.1	1.4	99.5
Method 2, $n = 3$				
50	53.8 ± 4.0	6.3	4.8	107.5
150	145 ± 11	4.5	6.7	96.6
1000	1013 ± 64	2.6	6.9	101.3

Table 8
Assessment of accuracy and precision performed for dihydroxymelphalan (DHM) with method 2. Data from six replicates for each quality control and extracted and analyzed on three different days.

DHM (ng/mL)		Precision (%)		Accuracy (%)
Spiked	Found (mean ± S.D.)	Within-run	Between-run	
25	25.7 ± 2.4	7.2	6.5	102.9
75	74 ± 5	3.7	6.9	99.1
500	494 ± 31	5.5	3.3	98.8

3.4. Precision, accuracy and recovery

Precision within-run and between-run and accuracies obtained from each quality control of melphalan and DHM concentration were below 10% for the two methods (Tables 7 and 8).

Validation of concentrations over the range and dilution procedure was done using method 1 since the method is performed in first intention in our routine laboratory. Human plasma spiked with 1 μg/mL of melphalan and quantified without dilution, showed a very good precision and accuracy. Results of analysis from plasma sample spiked at 2000 and 8000 ng/mL and diluted by 1/5 and 1/20, respectively revealed also a very satisfactory precision and accuracy. All results are summarized in Table 9.

Extraction yield was obtained from the peak area ratio of melphalan or DHM or IS extracted/unextracted. A very satisfactory

Table 9
Assessment for extended concentration range and validation of dilution procedure using method 1 ($n = 3$ for each day, three days).

Melphalan (ng/mL)		Precision (%)		Accuracy (%)
Spiked	Found (mean ± S.D.)	Within-run	Between-run	
1000[a]	1036 ± 40	3.1	2.6	103.7
2000[b]	2034 ± 154	5.8	5.6	101.7
8000[b]	8028 ± 202	2.2	1.7	100.4

[a] Samples not diluted and quantified with calibration curve from 1 to 500 ng/mL.
[b] Samples diluted prior to extraction in a blank plasma at 1/5 and 1/20, respectively.

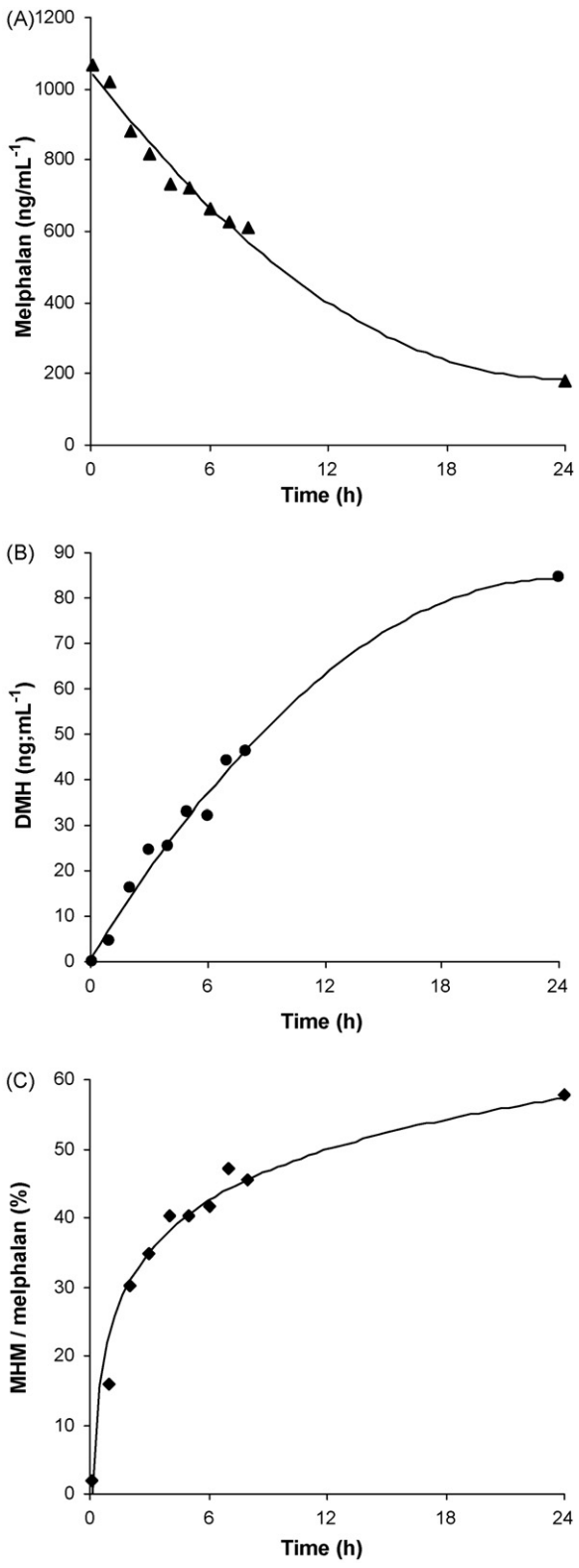


Fig. 4. Evolution of melphalan and its hydrolysis products according to time at ambient temperature (human plasma spiked with 1 μg/mL of melphalan). (A) Concentration of melphalan, (B) concentration of dihydroxymelphalan (DHM), (C) peak area ratio between monohydroxymelphalan (MHM) and melphalan.

mean extraction yield reaching 99% was found for N-PEA with the two methods. The mean recovery calculated from all calibration standards for melphalan was equal to 87% and 95% with methods 1 and 2, respectively. Concerning the DHM, the extraction efficiency was higher than 90%.

3.5. Assessment of stability of melphalan

According to the literature, neither MHM nor DHM were detected in calibration standard solutions of melphalan since preparation in ice prevented melphalan from hydrolysis [6]. Analyzing in duplicate each quality control samples after three freeze/thaw cycles at seven days interval tested stability of melphalan. The stability (decrease inferior to 10%) was only observed after two cycles. No significative decrease was observed when samples were stored at −80 °C for two months. Assessment of the stability at room temperature using human plasma spiked with 1 μg/mL of melphalan, revealed a linear decrease for the first 8 h. At 24 h, its concentration fell by 80% and data were fitted through an exponential model (Fig. 4). MHM constitutes the main hydrolysis product while DMH level production was low. A same solution kept at +4 °C during 24 h remained stable. The study of quality control sample at 35 ng/mL re-analyzed after 7 h in the autosampler (+5 °C) showed no significant degradation of melphalan (data not shown).

3.6. Clinical samples

Quantification of melphalan was performed in plasma samples of a sixty six-year-old man who received a heated intraperitoneal intraoperative melphalan. The plasma determination for melphalan, performed at two different days, was 374, 276, 200 and 151 ng/mL with method 1 and 378, 291, 220 and 161 ng/mL with method 2 for T30, T60, T90 and T120 min, respectively, showing a good correlation. The ratio MHM/melphalan in plasma increases according to time as: 9.6, 10.4, 13.0 and 15.6%, respectively for T30, T60, T90 and T120 min. For the same times traces of DHM were detected at 10.7, 19.8, 17.8 and 20.2 ng/mL. Thus only drastic conditions (temperature and/or time) as described above lead to their yield. The ratio MHM/melphalan in intraperitoneal liquid perfusion was equal to 8.75, 10.9 and 9.3% for T30, T60 and T90 min while the ratio DHM/melphalan was equal to 0.6, 1.1 and 1.0%. The gradual apparition of hydrolysis products in plasma may be due to the exposition of melphalan at temperature about +40 °C during the intraperitoneal perfusion. However, degradation after blood collection may also explain the detection of hydrolysis products if sample was not immediately stored in ice.

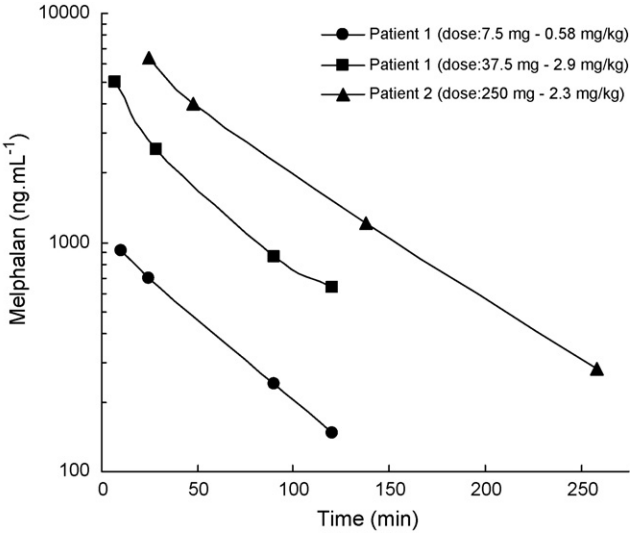


Fig. 5. Kinetics from two patients treated by melphalan perfusion for 15 min.

Three kinetics were also performed in plasma after 15 min perfusion of melphalan as shown in Fig. 5. Patient 1 received a test dose (D1: 7.5 mg) and one week later a plain dose (D2: 37.5 mg). Patient 2 received a dose equal to 250 mg. The concentrations were from 146 to 6350 ng/mL, thus according to dilution procedure our method allows the quantification of melphalan in a large range. For the patient 1 it was observed that the plasma concentrations increase in the same ratio than the doses. For both patients, the ratio MHM/melphalan in plasma increases according to time from 3.9 to 12.4%. Based on our stability data (see Fig. 4) these values indicate a moderate degradation of melphalan, and suggest that samples were maintained less than 30 min at room temperature between the collection and the freezing storage.

4. Conclusion

Several pharmacokinetic studies of melphalan are reported in the literature in order to optimize its use for antineoplastic chemotherapy because of its leukaemogenic potential. The pharmacokinetic of this drug is often investigated in protocol of heated intra-arterial regional limb perfusion (ILP) [22]. We developed two methods for the human plasma quantification of melphalan by LC–MS/MS. The first method allows an accurate determination in a wide range of concentrations with a simple and rapid sample preparation procedure adequate for routine use. The second method using a SPE and HILIC approach allows the quantification of melphalan and the DHM and the assessment of the MHM production. Although MHM and DHM are pharmacologically inactive, they are present in vivo due to a metabolic conversion from the parent drug melphalan. Their determination may be useful to understand the pharmacokinetic of the parent drug in specific conditions such as ILP. Furthermore MHM determination and at least extent DHM, are high relevant parameters to assess a failure during the storage of samples. This could explain an unexpected low concentration of melphalan for pharmacokinetic study or therapeutic drug monitoring. These assays with a small volume of plasma sample were selective, sensitive, reproducible and accurate, according to the device performance.

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