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High-performance liquid chromatographic assay for melphalan in human plasma
Application to pharmacokinetic studies

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Abstract

This paper describes a simple, rapid and reproducible high-performance liquid chromatographic method (HPLC) with ultraviolet absorbance detection for the analysis of melphalan in plasma. The HPLC column was an Ultrasphere ODS (5 μm) and the eluent was composed of methanol, purified water and acetic acid (49.5:49.5:1, v/v). The detection was performed at 261 nm. The method involved a simple treatment of the samples with methanol. The propylparaben was used as internal standard. Linear detection response was obtained for concentrations ranging from 50 to 2500 ng/ml. Recovery from plasma proved to be more than 90%. Precision, expressed as C.V., was in the 0.5 to 9% range. Accuracy ranged from 95 to 102%. This method was used to determine the pharmacokinetic parameters of melphalan following high-dose (140 mg/m^2) intravenous administration in patients with advanced malignancies undergoing peripheral blood hematopoietic progenitor–cell transplantation.

Keywords: Melphalan; Pharmacokinetics

1. Introduction

Melphalan(*p*-[*bis*(chloro-2-ethyl)amino]-L-phenylalanine), an alkylating agent of the bischloroethylamine type, was introduced into clinical use in the late 1950's and has since established itself as an agent with a wide spectrum of antitumor activity [1,2]. It exerts a cytotoxic effect through the formation of interstrand or intrastrand DNA cross-links or DNA-protein cross-links via the two chloroethyl groups of the molecule [1]. It is extensively used in the treatment of multiple myeloma, ovarian cancer, breast cancer, neuroblastoma, regionally advanced malignant melanoma and localized soft tissue sarcoma [3]. High-dose intravenous melphalan with or without autologous bone marrow is under evaluation for the treatment of a variety of refractory solid and hematologic malignancies [1,4].

Some analytical methods have been developed to quantify melphalan in biological samples after administration of therapeutic doses. Mass spectrometry [5] and gas chromatography–chemical ionisation–mass spectrometry [6] provide good results, but they involve the use of expensive equipment and time-consuming sample pretreatment and are not easily available for routine drug monitoring. High-performance liquid chromatographic (HPLC) methods with

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ultraviolet, fluorimetric or electrochemical detection have been also developed [7–14].

This paper describes a rapid, specific, reliable and sensitive analytical method based on reversed-phase liquid chromatography for the quantitation of melphalan in plasma. The procedure involves the addition of an internal standard followed by a treatment of the samples with methanol. This method was validated according to validation procedures, parameters and acceptance criteria based on USP XXIII guidelines [15] and recommendations of Shah et al. [16]; moreover, stability tests under various conditions have been performed. The simple sample preparation makes the method appropriate for analysis of the large number of samples obtained in pharmacokinetic studies in patients undergoing cytotoxic chemotherapy for malignant disease.

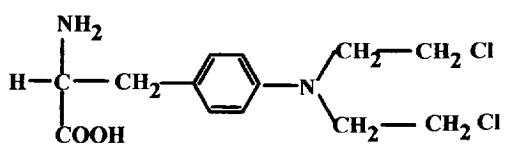
2. Experimental

2.1. Materials and reagents

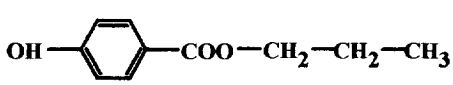
Melphalan (I) and mono- and dihydroxy of melphalan were obtained from Wellcome (London, UK). The internal standard (II), propylparaben, was obtained from Sigma (St. Louis, MO, USA) (Fig. 1). Methanol and acetic acid were of LC grade and were purchased from Prolabo (Paris, France).

Stock solutions of melphalan and internal standard were prepared in purified water (Laboratoires Fandre, Ludres, France) at concentrations of 5 and 0.01 mg/ml then stored at −20°C and 4°C, respectively. Standard solutions of melphalan were obtained from stock solution by dilution with purified water 10-, 100- and 1000-fold, extemporaneously. They were used to spike the plasma samples prior to extraction. An unextracted working standard solution (500 ng/ml) in purified water was prepared daily to check the resolution of the chromatographic system.

Pooled free plasma samples from healthy volunteers were used for the validation of the method.



Melphalan



Propylparaben

Fig. 1. Structural formulae of melphalan (I), and internal standard (II) used for the analytical method.

2.2. Instrumentation

The chromatographic system consisted of a Shimadzu Model LC9A pump (Kyoto, Japan), a Shimadzu Model SPD6A variable-wavelength UV absorbance detector, a Rheodyne loading valve (Model 7010) fitted with a 50-μl sample loop (Touzart and Matignon, Paris, France), a stainless-steel analytical column (250×4.6 mm I.D., Beckman, Paris, France) packed with Ultrasphere ODS (particle size, 5 μm), and a Shimadzu integrator Model C-R5A (chart speed, 0.5 mm/min).

2.3. Chromatographic conditions

The eluent mixture was comprised of methanol, purified water and acetic acid (49.5:49.5:1, v/v) and was deaerated by ultrasound prior to use. The mobile phase was pumped onto the column at 2 ml/min, which corresponds to a pressure of about 20.4 MPa. The volume injected was 50 μl. Chromatography was performed at ambient temperature. The analytes were detected at 261 nm.

2.4. Extraction procedure

In a 5-ml polypropylene centrifuge tube, to 0.5 ml of plasma spiked with internal standard solution (0.45 μg), 1 ml of absolute methanol (−80°C) was added and proteins were denatured during 30 s of full speed vortex mixing. Then all of the vials were centrifuged at 3000 *g* for 10 min at 4°C. A 50-μl aliquot of the supernatant was injected into the

column. As rapid degradation of melphalan occurred at room temperature, in the interval of two injections, extracts were stored at 0°C.

2.5. Instrument calibration

Calibration samples were prepared by spiking 0.5 ml of blank plasma with appropriate volumes of solutions of standards in order to obtain concentrations of 50, 100, 250, 500, 1000 and 2500 ng/ml. The volume added was always less than or equal to 2% of total volume of the samples, so that the integrity of the plasma was maintained.

2.6. Data analysis

The ratio of the peak area of melphalan to that of internal standard was used as the assay parameter. Peak-area ratios were plotted against theoretical concentrations.

Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data. The quality of fit was evaluated by comparing calculated concentrations to the nominal ones.

The linearity of the method was confirmed using the classical statistical tests; that is, comparison of intercept with zero and correlation coefficients.

2.7. Specificity

Several free human plasma samples from different healthy subjects were tested for the absence of interfering compounds. The retention times of endogenous compounds in plasma were compared with those of melphalan and internal standard.

The interference from the two hydrolysis products, mono- and dihydroxy of melphalan, was checked. The interferences from other drugs that could be co-administered was also studied using different plasma samples from treated patients assayed according to the extraction procedure and chromatographic conditions described above; consequently, the possible interference from metabolites of these drugs was also checked. The following drugs currently associated with melphalan in chemotherapy protocols were checked: ifosfamide, *cis*-dichlorodiaminoplatinum, carboplatine, etoposide, cyclophosphamide.

2.8. Recovery

The recovery of melphalan was determined by comparing peak areas from drug-free plasma spiked with known amounts of drug (80, 400 and 1500 ng/ml), processed according to the described method *versus* peak areas of the same concentrations prepared in purified water injected directly onto the analytical column. Each sample was determined in triplicate.

The extraction efficiency was also determined for the internal standard.

2.9. Precision and accuracy

The intra-day precision and accuracy of the method were evaluated by analysing on the same day six replicates of spiked samples at each of three concentrations (80, 400 and 1500 ng/ml) against a calibration curve.

Inter-day precision and accuracy were assessed by performing analyses of spiked samples at concentrations of 80, 400, 1000 and 1500 ng/ml against a calibration curve. The procedure was repeated on different days ($n=8$).

Accuracy was evaluated as percent error [(mean of measured − mean of added)/mean of added]×100, while the precision was given by the inter-day and intra-day coefficients of variation.

2.10. Determination of the limit of quantitation and of the limit of detection

The limit of quantitation (LOQ) was determined from the peak and the standard deviation of the noise level, S_N. The LOQ was defined as the sample concentration resulting in a peak area of 10 times S_N. The estimate of S_N was determined by extrapolation to zero. To determine accuracy and precision on the LOQ, spiked plasma samples were used ($n=6$). The

limit of detection (LOD) was defined as the simple concentration resulting in a peak area of 3 times S_N.

2.11. Stability studies

For stability studies, control human blood and plasma samples were spiked with 80, 400 and 1500 ng/ml of melphalan. Each determination was performed in triplicate.

The short-term stability in plasma was assessed at 0.5, 1, 2, 4 and 6 h at both ordinary laboratory conditions (20°C at daylight exposure) and at 4°C. The long-term stability of melphalan in frozen human plasma (−30°C) was determined by periodic analysis over a span of 3 months. Samples were analyzed immediately after preparation (reference values) and after storage. Prior to their analyses, samples were brought to room temperature and vortex-mixed well.

The freeze–thaw stability was also determined. Spiked plasma were analysed immediately after preparation and on a daily basis after repeated freezing–thawing cycles at −30°C on three consecutive days.

Moreover, the stability of melphalan in methanolic extract was inspected after 0.5, 1, 2, 4 and 6 h at 0 and 20°C.

In order to verify the stability of melphalan in blood before centrifugation, it was inspected after 30 min and 1 h of bench-top storage at room temperature (20°C).

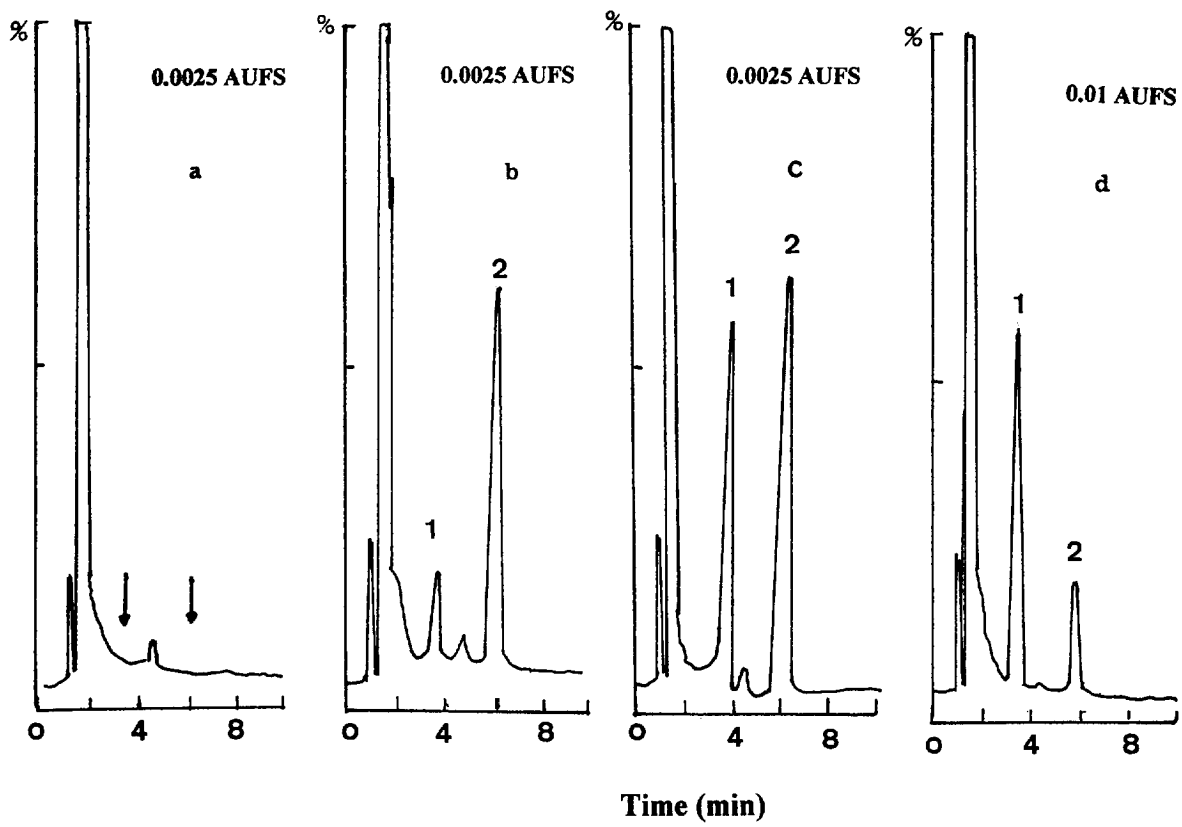


Fig. 2. Chromatograms of blank plasma (a) and of plasma spiked with 80 (b), 400 (c) and 1500 ng/ml (d) of melphalan. Peak 1 is the melphalan and peak 2 is the internal standard. For chromatographic conditions see text.

3. Results

3.1. Retention times

Observed retention times were 3.3 and 6 min for melphalan and internal standard, respectively. The capacity factors were 6.3 for melphalan and 12.3 for internal standard (Fig. 2).

3.2. Specificity

A representative chromatogram is shown in Fig. 2a. No interfering peaks at the retention times of melphalan or internal standard were detected. Likewise, no interference was found between the two hydrolysis products and the drugs tested that could be co-administered.

Table 1
Inter- and intra-assay reproducibilities of the HPLC analysis

Theoretical concentration (ng/ml)	Experimental concentration (mean±S.D.) (ng/ml)	C.V. (%)
Intra-day reproducibility (n=6)		
50	50.9±4.69	9.2
100	98.5±7.01	7.1
250	257.5±18.3	7.1
500	503.7±22.4	4.4
1000	992.9±46.6	4.7
2500	2500.5±18.0	0.72
Inter-day reproducibility (n=16)		
50	50.0±4.29	8.58
100	100.7±5.16	5.12
250	251.8±12.2	4.85
500	496.0±18.2	3.67
1000	1000.2±23.7	2.37
2500	2501.0±12.6	0.50

3.3. Linearity

In plasma the peak-area ratio of melphalan and the internal standard varied linearly with concentration over the 50 to 2500 ng/ml range. The correlation coefficients for calibration curves were equal to or better than 0.998.

Intra-assay reproducibility was determined for calibration curves prepared on the same day in replicate ($n=6$) using the same stock solutions. The intra-day average slope of the fitted straight lines was $0.00117\pm5.98\times10^{-5}$ ng^{-1} ml (C.V.=5.11%), the mean intercept of calibration curves was 6.83×10^{-3}. The corresponding mean (±S.D.) coefficient of the linear regression analysis was $0.9993\pm6.88\times10^{-4}$ (C.V.=0.069%).

For calibration curves prepared on different days ($n=16$), the average results were as follows: slope= $0.00112\pm9.22\times10^{-5}$ ng^{-1} ml (C.V.=8.23%), coefficient of the linear regression analysis= $0.9997\pm2.94\times10^{-4}$ (C.V.=0.0294%) and intercept= -2.54×10^{-4}.

Intra-day and inter-day variabilities at concentrations of calibration standards are given in Table 1. A linear regression of the calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to 0 (Student t-test).

The linearity of this method was confirmed statistically. For each calibration curve, the intercept was not statistically different from zero. Moreover, the residuals (difference between nominal and back-calculated concentrations) were normally distributed and centred around zero (Kolmogorov–Smirnov test).

3.4. Recovery

The mean recovery of melphalan averaged 91.0±4.3% ($n=9$). It is not statistically different over the range of concentrations studied.

The mean recovery of the internal standard averaged 93.3±2.0% ($n=3$).

3.5. Precision and accuracy

For concentrations of calibration standards ranging from 50 to 2500 ng/ml, the precision around the mean value not exceeded 10% coefficient of variation (Table 1).

Intra-day and inter-day precision and accuracy of the method, assessed by analysing quality control samples, are given in Table 2.

Table 2
Accuracy and precision of the HPLC method

Theoretical concentration (ng/ml)	Experimental concentration (mean±S.D.) (ng/ml)	C.V. (%)	Deviation from theoretical value (%)	Recovery (%)
Intra-day (n=6)				
80	75.6±5.33	7.05	5.5	94.5
400	387.3±26.3	6.79	3.2	96.8
1500	1466.5±88.1	6.01	2.2	97.8
Between-day (n=8)				
80	81.3±5.57	6.85	5.4	101.6
400	403.0±18.0	4.47	0.75	100.8
1000	991.5±62.6	6.31	0.85	99.2
1500	1451.3±46.0	3.17	3.25	96.8

3.6. Limit of quantitation and limit of detection

The limit of quantitation was 20 ng/ml. At this level, the mean concentration found was 21.2±2.76 ng/ml (C.V., 13.0%; accuracy, 106%).

The limit of detection was 8 ng/ml.

3.7. Stability studies

Stock solutions of melphalan (5 mg/ml) and internal standard (10 μg/ml) were stable for at least 3 and 6 months, respectively.

In plasma samples spiked with 80 ng/ml, melphalan concentration significantly decreased after 1 h at 4 and 20°C with losses averaging 40 and 50%, respectively. At concentrations of 400 and 1500 ng/ml, after bench-top storage at 20°C, melphalan was stable during 2 h (percent recoveries 92–105%; losses averaged 81.5±8.6% after 4 h) and 6 h (percent recoveries 94–98%), respectively. At 4°C, less than 10% degradation occurred over a span of 6 h for the concentrations of 400 (percent recoveries 95–103%) and 1500 ng/ml (percent recoveries 94–98%). Daylight did not modify the stability of melphalan.

The long-term freezer stability indicated that melphalan was stable during 3 months, the percent recovery averaged 98%. Compared to the reference values, no statistical difference appeared.

At room temperature, in methanolic extract, melphalan was stable during 30 min whatever the concentration studied. After 1 h, recoveries averaged 60, 93 and 96% for the concentrations of 80, 400 and 1500 ng/ml, respectively. Losses were decreased at 0°C; recoveries averaged 90% after 1 h for 80 ng/ml, and 90 and 93% after 6h for 400 and 1500 ng/ml, respectively.

At least three freeze–thaw cycles can be tolerated without losses higher than 10%.

In whole blood samples, melphalan was stable during 1 h whatever the concentration.

4. Discussion and conclusion

Melphalan is an instable drug with a very high solubility in water, together with a very unsatisfactory solvent extraction properties, owing to its amphoteric nature. Consequently, purification from the biological matrix was very difficult. The developed method proved to be useful and reliable for the determination of plasma concentrations of melphalan. The sample pre-treatment procedure, involving a direct deproteinization with methanol, is simple and rapid, avoiding degradation of the drug. Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. This method, validated for concentrations ranging from 50 to 2500 ng/ml, has a good reproducibility and accuracy and low limits of quantitation and detection compared to the most published HPLC methods. The limit of detection was of the same order of magnitude as that reported with the column-switching method [14] but higher than the LOD obtained with electrochemical detection [13]. How-

ever, the latter detection mode involved expensive equipment which is not readily available for routine analyses. The specificity of the present method with respect to endogenous substances and drugs that may be co-administered is satisfactory. With a run time of 8 min, this method is useful for clinical therapeutic drug monitoring. Stability studies carried out directly in plasma indicated that samples were stable for at least 3 months when stored at −30°C.

The method described was found to be suitable for the analysis of all samples collected during pharmacokinetic investigations in humans. It has been used for the determination of melphalan in plasma samples of 20 patients with advanced malignancies undergoing peripheral blood hematopoietic progenitor–cell transplantation. The pharmacokinetic parameters were determined following high-dose (140 mg/m^2) infusion (20 min). Plasma levels of melphalan declined in a biexponential fashion. The elimination half-life averaged 83±27 min. The total clearance was about 550±300 ml/min and the volume of distribution 1 l/kg. A representative plasma concentration–time curve is shown in Fig. 3. For concentrations higher than 2500 ng/ml plasma samples were adequately diluted before analysis.

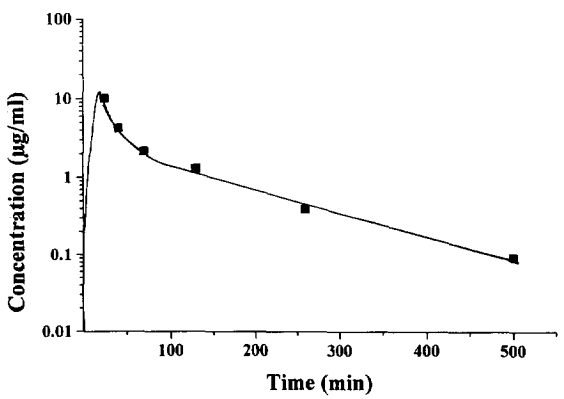


Fig. 3. Representative concentration–time curve following intravenous administration of melphalan (230 mg).

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