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Short Communication

Quantitation of melphalan in plasma of patients by reversed-phase high-performance liquid chromatography with electrochemical detection

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ABSTRACT

An analytical procedure for the separation and determination of melphalan in human plasma was carried out. A simple high-performance liquid chromatographic method with electrochemical detection was developed taking advantage of the high sensitivity of the electrode redox reaction. The sample pre-treatment consisted of a direct extraction of the interferents rather than of melphalan, owing to the difficulty of extraction of the drug, and was very simple, rapid and reproducible.

INTRODUCTION

Melphalan (Fig. 1) is an alkylating agent widely used in the chemotherapy of multiple myeloma and other neoplasias. Few pharmacokinetic studies have been carried out on this antineoplastic agent, and the analytical methods proposed were often quite complex and sometimes unsatisfactory.

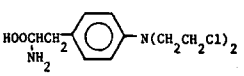

Fig. 1. Structure of melphalan.

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A method based on gas chromatography combined with chemical ionization mass spectrometry [1] provided good results, but it involved expensive equipment and time-consuming sample preparation. A high-performance liquid chromatographic (HPLC) determination with fluorimetric detection, exploiting the native fluorescence of melphalan, was carried out after separation from hydrolysis products in rat plasma, liver and kidney [2]. Melphalan was also determined in human plasma by liquid chromatography with fluorimetric detection after derivatization with N-acetylcysteine [3].

The main problems with these methods were (i) the poor recovery and reproducibility of the clean-up procedure, because of the difficulty of extraction of melphalan from biological matrices, (ii) the inefficiency of the derivatization reactions, and (iii) the high detection sensitivity necessary to carry out studies on human patients.

In order to develop a detailed pharmacokinetic study of this drug in human plasma, and simultaneously to overcome the problems mentioned above, we set out to develop a simpler HPLC method with electrochemical detection. We took advantage of the high sensitivity of the electrode redox reaction involving the amino groups present in the melphalan molécule, which allowed us to omit the derivatization and permitted the evaluation of blood levels in patient receiving conventional oral doses of the drug. The sample clean-up was very simple, rapid and reproducible.

EXPERIMENTAL

Instrumentation and materials

All measurements were performed on a two-pump Gilson HPLC system (pump 305 and pump 302 with manometric module 805 and dynamic mixer 811B, and a Rheodyne 7125 injection valve) equipped with an ESA Coulochem 5100A dual-electrode amperometric detector with an ESA 5011 analytical cell and a SpectraPhysics 4290 integrator.

The column used was a Phenyl Spheri 5 Brownlee (250 mm × 4.0 mm I.D., 5 μm particle size). Acetonitrile was HPLC grade (Fluka). The water used was purified using a Millipore Milli-Q system. Analytical-reagent grade NaH_2PO_4 and H_3PO_4 were used to buffer the aqueous component of the mobile phase, which was filtered on 0.22-μm cellulose filters (Millipore) before use. Melphalan was a pure standard from Sigma.

Blood sampling

To develop the method, pools of heparinized blood samples, obtained from healthy volunteers under no therapy, were used. To prepare spiked samples, 10 μl of a freshly prepared melphalan stock solution [1 mg/ml in acidified ethanol by addition of one drop of HCl (37%) to every 10 ml] were added to 1.0 ml of pooled drug-free plasma. Further dilution prepared the standard solutions for calibra-

tion in the range 5–1000 ng/ml. Then 100.0, 50.0, 10.0, 5.0, 1.0 and 0.5 μl of the previous 10 μg/ml plasma standard solution were added to 1.0-ml samples of human drug-free plasma.

Drug level data were obtained from five patients with multiple myeloma, beginning the first cycle of chemotherapy with melphalan (0.15 mg/kg). Samples were withdrawn by venipuncture just before melphalan administration, and then 20, 45 min, 1, 2, 4, 6, 8 and 24 h afterwards.

Blood samples (10.0 ml) were collected in glass tubes containing 300 I.U. of freeze-dried heparin. Plasma was separated immediately from each sample by centrifugation for 15 min at 600 *g* in a refrigerated (4°C) centrifuge (Hermle 320 K). Aliquots (1.5 ml) of each sample were immediately frozen at −80°C without further treatment. The remaining plasma was subjected to ultrafiltration by centrifugation in a cooled centrifuge (4°C, 30 min, 1200 *g* in a 60°C fixed-angle rotor) using an MPS 1 ultrafiltration system (Amicon) with YMT membranes (cut-off 1000 a.m.u.). Samples recovered after ultrafiltration were immediately frozen and stored at −80°C until analysis.

Sample preparation

After rapid thawing in water at 37°C, 1.0 ml of the frozen samples was acidified with 0.1 ml of perchloric acid (60%), vortex-mixed for 30 s, and then centrifuged at 4.0°C and 2000 *g* for 10 min; 0.7 ml of the supernatant was removed, 2.0 ml of chloroform were added, and the mixture was again vortex-mixed for 30 s and centrifuged at 4.0°C and 2000 *g* for 10 min; 0.3 ml of the supernatant was removed and directly injected onto the HPLC system.

HPLC assay

Isocratic elution conditions were adopted. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 4.5)–acetonitrile (88:12, v/v). The injection loop was 50 μl and the flow-rate 1.2 ml/min.

In order to improve the detector selectivity, taking into account the great complexity of the real sample matrix, we used the amperometric detector in the "screen mode". Once we had determined the potential corresponding to the limiting current wave of the melphalan by generation of a hydrodynamic voltammogram, we set the potential of the upstream electrode at a value (0.1 V) that was a few millivolts less than the rising portion of the voltammogram of the analyte. This stabilized the baseline and allowed better reproducibility of the chromatographic results. The second electrode was used to detect the analyte at its appropriate potential (0.6 V, corresponding to the limiting current wave).

Calibration curve

The quantitative determinations, in the absence of an internal standard whose behaviour during the clean-up procedure of the sample was reasonably comparable with that of melphalan, were calibrated by the external standard method.

Increasing amounts of melphalan (see *Blood sampling*) were added to aliquots of a sample of pooled human sera, which were treated according to the above procedure prior to injection into the HPLC system. A linear regression program was used to process the heights of the chromatographic peaks and the corresponding concentrations to build up the calibration plot.

To determine the clean-up efficiency, reproducibility and inter- and intra-assay variability, analyses were performed on drug standards in aqueous and spiked plasma samples.

RESULTS AND DISCUSSION

The instability and the very high solubility of melphalan in water, together with its very unsatisfactory solvent extraction properties, owing to its amphoteric nature, make its purification from the biological matrix very difficult.

As detailed in Experimental, we adopted the alternative procedure of precipitation and chloroform extraction of the interfering substances.

Fig. 2 shows the chromatograms obtained with (A) drug-free plasma samples and (B) plasma samples spiked with 125 ng/ml melphalan. The peak at *ca.* 15.0 min is satisfactorily separated from interfering coeluting substances.

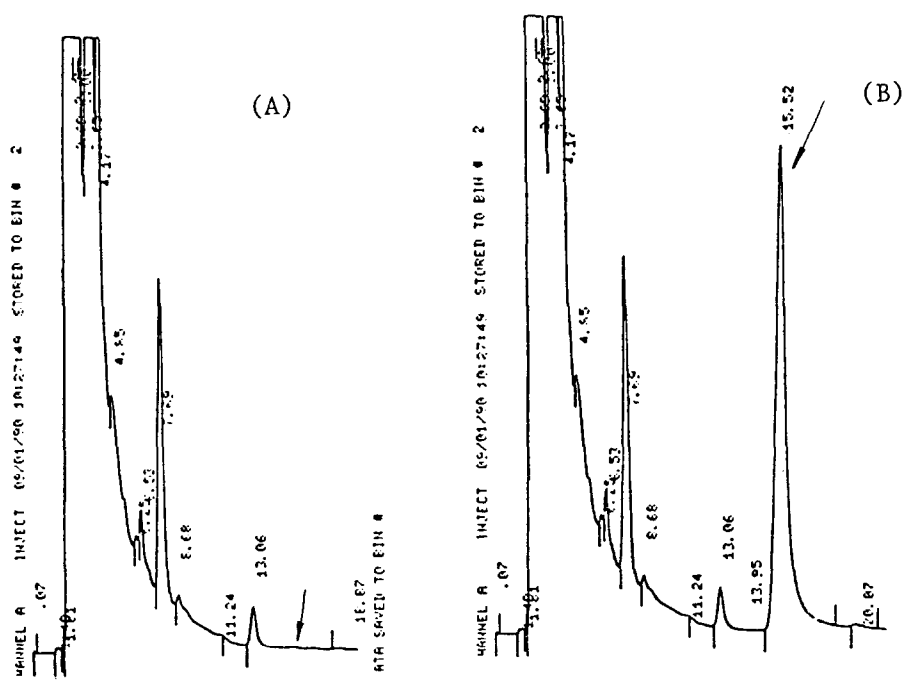

Fig. 2. (A) Chromatographic profile of a blood sample before any administration of melphalan. (B) Chromatographic profile of the same blood sample spiked with 125 ng/ml standard melphalan.

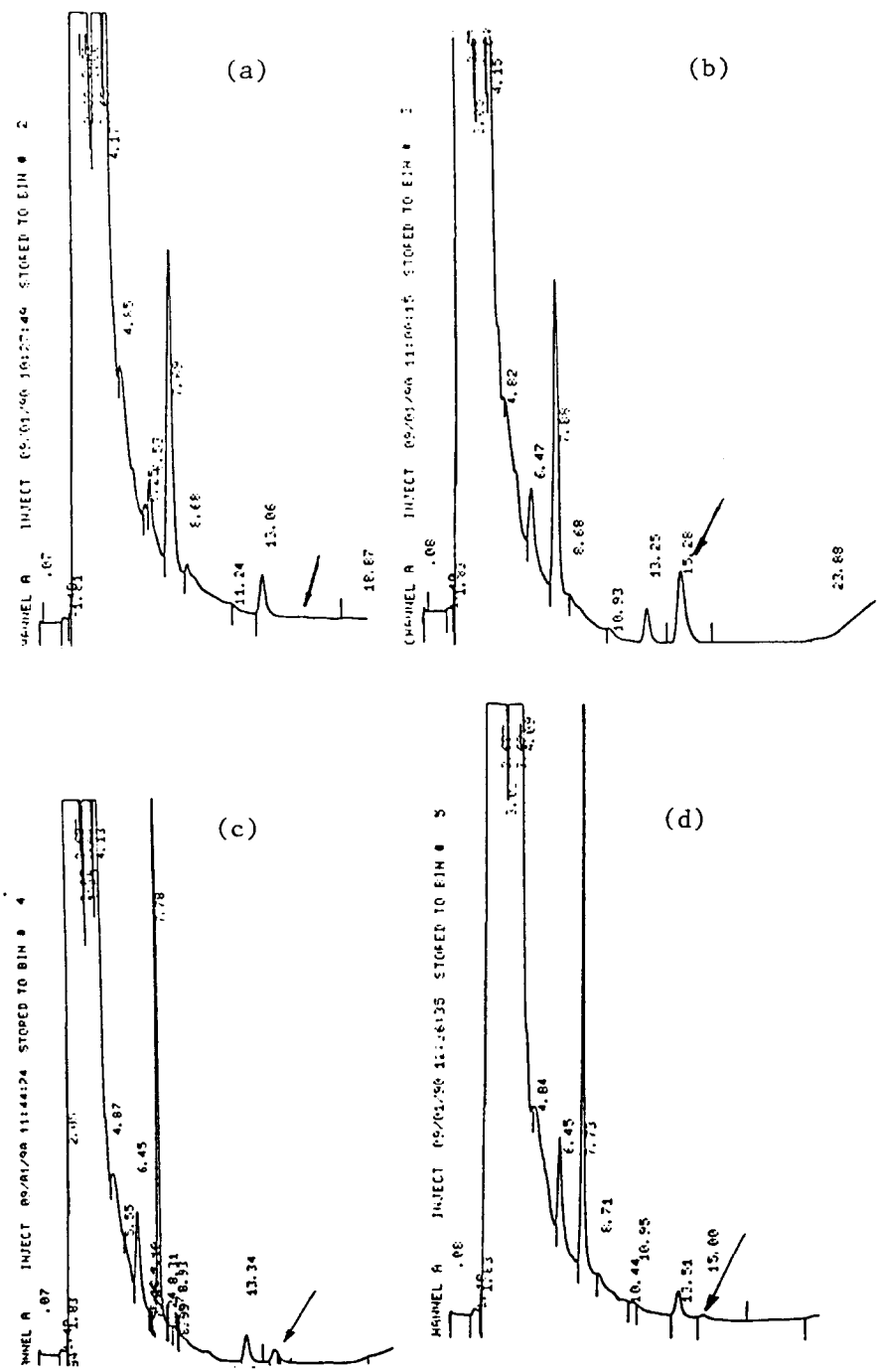

Fig. 3. Chromatograms of a sequence of blood samples taken at different times from a patient receiving oral doses of 0.15 mg/kg per day: (a) 20 min; (b) 1 h; (c) 2 h; (d) 4 h.

Fig. 3. shows a representative sequence of chromatographic analyses of the blood levels of melphalan in plasma of a patient receiving oral doses of 0.15 mg/kg.

In order to assay the plasma level of free melphalan (*i.e.* not bound to proteins), we ultrafiltered the plasma samples (see Experimental) before the clean-up procedure. Then an analogous chromatographic study was carried out. Fig. 4 shows the trends of plasma levels of total and free melphalan in the plasma of five patients receiving oral doses of 0.15 mg/kg. As can be seen, there is a large variation in the oral melphalan bioavailability, whereas the protein-binding properties of the drug are similar for all the patients. The results at 24 h have not been reported in the diagram because only one patient (B.A.) showed detectable levels of the drug at that time, 6.0 ng/ml total and 3.0 ng/ml free melphalan, respectively. The calibration curve (peak height *versus* concentration) was constructed by analysing six standard melphalan aqueous solutions of different concentration. The curve was linear in the range 5–1000 ng/ml (5, 10, 50, 100, 500, 1000 ng/ml, $r^2 = 0.9998$). Table I lists data for the recovery in plasma and aqueous samples spiked with different amounts of melphalan, corresponding to those used for the construction of the calibration curve. An average recovery of 96.8% (R.S.D. 3.1%) was obtained from the different concentrations tested. It is worth noticing that, even at the lower end of the concentration range examined, the individual recovery value was >90%.

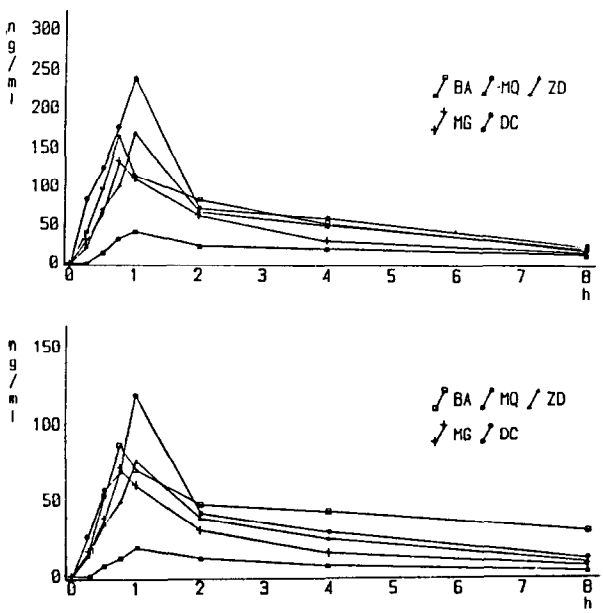

Fig. 4. (Top) Blood levels of total melphalan after oral administration of a single dose (0.15/mg/kg per day) to five patients. (Bottom) Blood levels of the unbound melphalan after oral administration of a single dose (0.15 mg/kg per day) to five patients (initials and corresponding symbols are indicated).

TABLE I

RECOVERIES OF MELPHALAN FROM SPIKED PLASMA SAMPLES AFTER CLEAN-UP

Concentration spiked (ng/ml)	Concentration recovered (mean ± S.D.) (ng/ml)		
	From water (*A*)[a]	From plasma (*B*)[b]	*B*/*A* (%)
1000.0	1000.5 ± 8.0	1005.0 ± 12.0	100.45
500.0	503.3 ± 6.7	500.0 ± 8.3	99.34
100.0	100.4 ± 1.1	98.3 ± 1.7	97.84
50.0	50.1 ± 1.2	48.4 ± 1.5	96.61
10.0	10.3 ± 0.5	9.8 ± 1.0	94.78
5.0	5.1 ± 0.6	4.7 ± 0.8	91.89

[a] Drug concentrations were evaluated from peak heights; values were estimated by linear regression calculations of the results obtained from three repeated analyses of the same sample.

[b] Drug concentrations evaluated from peak heights; data evaluated on regression line previously calculated from samples in water. For each concentration three repeated analyses were performed (three different preparations from the same spiked sample).

The detection limit, corresponding to a signal-to-noise ratio of 2, was *ca.* 100 pg injected, corresponding to a plasma sample concentration of 2 ng/ml.

As regards the reproducibility and intra- and inter-assay variability of the extraction method, the following results were obtained from plasma samples spiked with 100 or 10 ng/ml melphalan. The percentage standard deviations of the peak height of the drug, calculated on three repeated injections of the same supernatant from the clean-up procedure, were 0.6 and 0.7% of the mean peak height at 100 and 10 ng/ml, respectively. Three repeated injections of standard melphalan in water gave similar standard deviations (0.6% at 100 ng/ml and 0.65% at 10 ng/ml). The standard deviations, evaluated as before, from three chromatographic runs of separate preparations of the same sample, were 1.2% at 100 ng/ml and 2.1% at 10 ng/ml.

Two samples, spiked with 100 and 10 ng/ml melphalan, were divided into aliquots and frozen. The drug contents were then evaluated on different days using calibration curves established on the day of the analysis. Under these conditions, melphalan concentrations determined were 98.8 ± 1.1 and 9.5 ± 0.2 ng/ml, respectively.

The proposed method proved to be very useful and reliable for the determination of plasma concentrations of melphalan. What is more, the sample pre-treatment procedure is relatively simple and rapid, and the detection system is very sensitive. Application of the method to routine analyses can be recommended, particularly to examine the relationship between the bioavailability and the toxicity of the drug.

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