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Note

Modified extraction and chromatography for the measurement of plasma melphalan by ion-pair high-performance liquid chromatography

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Melphalan (Alkeran®) is currently used in the treatment of several malignant disorders, including multiple myeloma and carcinoma of the breast, ovary and testis [1]. Although the effectiveness of the drug in the therapeutic management of these patients is unquestionable, several potentially serious side effects, primarily related to bone marrow toxicity, may occur [2]. Consequently, the optimisation of melphalan therapy must take into account the predisposition of the patient towards side-effects [3]. Despite awareness of this problem for over 20 years, very little pharamacokinetic data are available on melphalan, primarily due to difficulties in measuring the low plasma concentrations of the drug following oral therapeutic doses (5—15 mg daily).

Recently, several methods have been described for the measurement of melphalan which provide acceptable chromatography and sensitivity at therapeutic levels [4—6]. Based on these reports we describe a modified assay which allows ultraviolet (UV) determination of the drug at concentrations (5 ng/ml) previously requiring fluorescence detection [4]. This procedure gives an acceptable resolution value with a relatively short retention time (9.5 min). Sample clean-up involves precipitation of plasma macromolecular components with concentrated perchloric acid [5] and extraction of drug from the supernatant by C_{18} Sep-Pak [4] which are straightforward in preparation and use.

MATERIALS AND METHODS

Instrumentation

High-performance liquid chromatography (HPLC) equipment from the Pye Unicam PU 4000 system (Cambridge, U.K.) incorporated a dual reciprocating pulseless pump and a variable-wavelength UV detector set to 260 nm (the mea-

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sured λ_{max} of melphalan in methanol). Detector sensitivity was set at 0.08 a.u.f.s. Chromatograms were recorded on a Pye CDP4 computing integrator with 100-mV input f.s.d. The column was Spherisorb ODS 5 μm particle size, 250 × 4.6 mm I.D., from Phase Separations (Queensferry, U.K.). Both column and Rheodyne 7125 injection valve (Cotati, CA, U.S.A.) with a 200-μl loop were mounted in a block heater (Jones Chromatography, Cardiff, U.K.) and maintained at 40°C. The guard column (3 cm) packed with LiChrosorb ODS (10 μm particle size) was also enclosed in the block heater.

Mobile phase

The mobile phase was a mixture of 80% methanol (Fisons, Loughborough, U.K.), 20% water and 0.0135% (w/v) sodium dodecyl sulphate (BDH, Poole, U.K.) as counter ion [6]. Mobile phase pH was adjusted to 3.11 using sulphuric acid. All materials and reagents were of HPLC grade and were filtered, when appropriate, with a 0.5-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to use. The flow-rate of the mobile phase was 1.3 ml/min.

Sample preparation and extraction

Whole blood (6 ml) was taken from a patient with an indwelling venous catheter and stored in blood collection tubes with lithium heparin anticoagulant. After centrifugation (1500 *g*; 10 min), 3-ml aliquots of plasma were removed and the macromolecular components precipitated with 132 μl [5] of cold concentrated perchloric acid (−20°C). The mixture was vortex-mixed for 5 min prior to centrifugation (−6°C; 1300 *g*; 15 min) in an MSE Coolspin (Fisons). The clear supernatant was removed and passed through a C_{18} reversed-phase Sep-Pak (Waters Assoc., Taunton, MA, U.S.A.) [4] which was subsequently washed with 10 ml of 15% methanol in water (4°C). The melphalan was eluted from the Sep-Pak with 2 ml of cold methanol (−20°C) [4]. The eluate was stored at −20°C prior to injection of 200-μl aliquots onto the column.

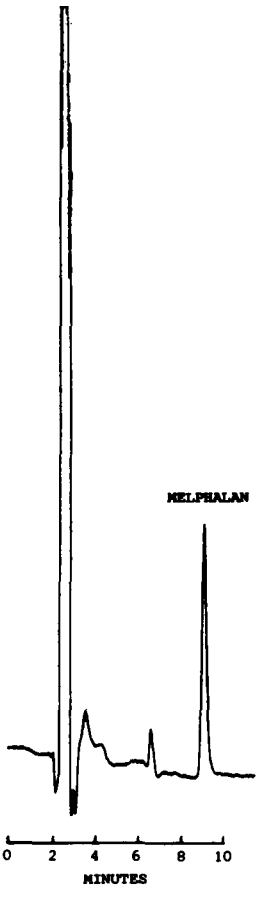
RESULTS AND DISCUSSION

Methanol has been used previously to precipitate the macromolecular components of plasma in an HPLC assay for melphalan, utilising fluorescence detection [4]. However, when this procedure was used in conjunction with UV detection, the method was unsatisfactory, due to superimposition of interfering peaks arising from plasma components onto the peak of interest. Precipitation with cold concentrated perchloric acid overcame this problem, in that most extraneous UV-absorbing material from the plasma was removed and resolution improved.

It was found that the packing material of the guard column had a significant effect on the separation of melphalan. A 5-cm Co:Pell ODS (30—38 μm particle size) guard column resulted in poor resolution. Increasing the pH of the mobile phase above 3.10 improved resolution somewhat but beyond pH 3.20 the retention time of the plasma components also lengthened. The best results were obtained using a 3-cm Pye LiChrosorb RP-18 (10 μm particle size) guard cartridge or a 5-cm Spherisorb ODS (10 μm particle size) guard column. Optimum retention times were in the region of 9.5 min. The measured resolution for

melphalan from the nearest plasma component was 2.15 (Figs. 1—4). Extraction of the drug with Sep-Pak cartridges [4] dispensed with the necessity to ultracentrifuge in order to remove solid material suspended in the eluate. The particulate material was either retained on the inlet plug of the Sep-Pak or removed by washing. This sample clean-up procedure is sufficiently consistent to allow the determination of sample concentrations from a standard addition calibration graph, using plasma spiked with 50, 100 and 150 ng/ml, each chromatographed in duplicate.

The coefficient of variation for ten concentration duplicates (10—2000 ng/ml) extracted from plasma was 2.67%. The correlation coefficient for the curve was 0.99. Within-batch variability for standard solutions of 20, 200 and 2000 ng/ml, each chromatographed ten times was 6.7%, 1.5% and 0.5%, respectively. Recovery of drug from plasma was in the order of 60% with a limit of detection of 5 ng/ml.



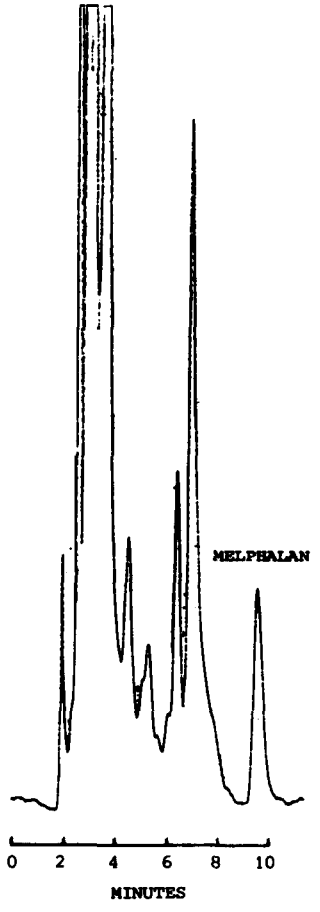


Fig. 1. Chromatography of 100 ng/ml melphalan in methanol.

Fig. 2. Extract of plasma spiked with 100 ng/ml melphalan. The anticoagulant citrate phosphate dextrose resulted in poorer resolution than lithium heparin, which was used in subsequent patient studies.

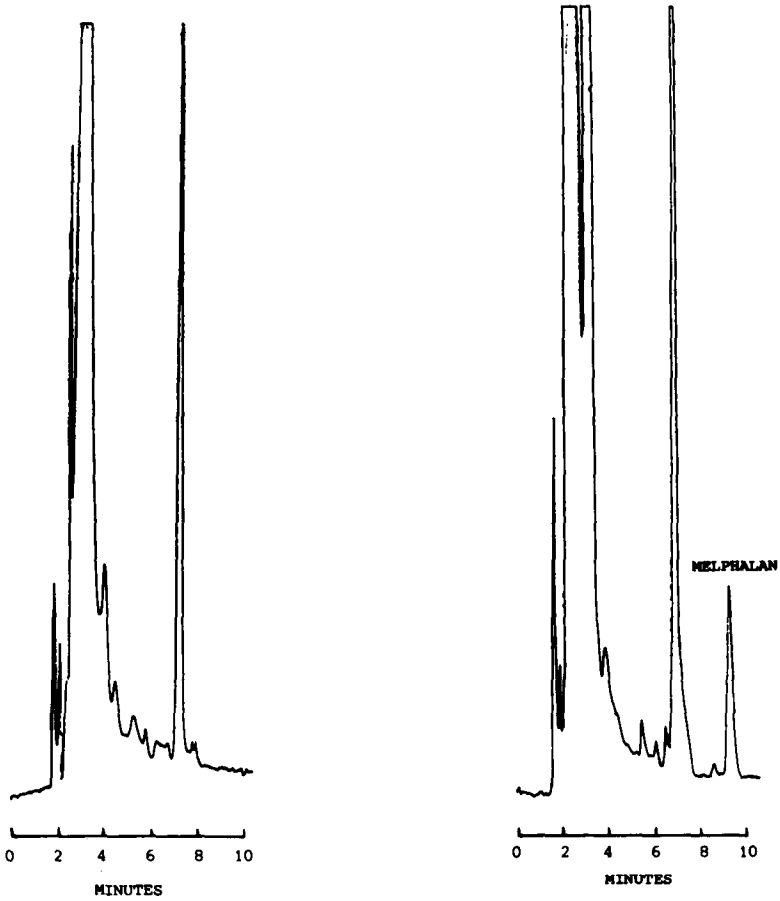


Fig. 3. Extract of a patient plasma. Sample before melphalan administration.

Fig. 4. Extract of a patient plasma sample taken 90 min after oral administration of 12 mg melphalan.

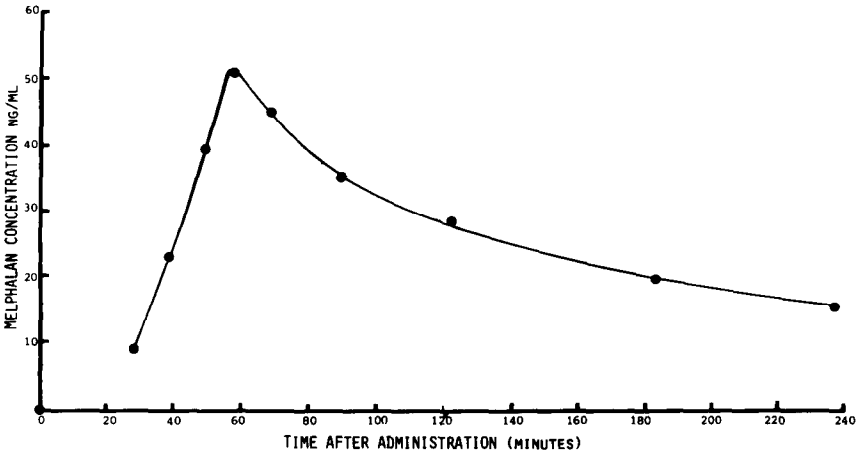


Fig. 5. Plasma levels of melphalan in a patient given 12 mg of oral melphalan. Patient samples were taken at intervals over a period of 4 h.

Using this method we have determined plasma levels of melphalan in six patients with multiple myeloma receiving 10 mg of oral melphalan daily for four days. Samples of peripheral blood taken at intervals over 4 h were stored in an ice bath to minimise drug degradation prior to analysis. Peak plasma concentrations of melphalan ranging from 34 to 190 ng/ml occurred 34—120 min after administration. Five patients exhibited normal time—concentration drug profiles similar to that shown in Fig. 5. Melphalan could not be detected in the plasma from one patient. Chromatography of methanolic solutions of prednisone and prednisolone confirmed that concurrent medication did not interfere with the assay for melphalan.

CONCLUSION

In this paper we have described a simple and rapid technique for the determination of melphalan in plasma. The use of UV spectrophotometry and a modified extraction technique enables a limit of detection (5 ng/ml based on four times baseline noise) which previously required fluorescence detection. We are currently using this assay to monitor patients with multiple myeloma receiving melphalan chemotherapy and for elucidating the pharmacokinetics of melphalan.

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