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MEASUREMENT OF PLASMA MELPHALAN AT THERAPEUTIC CONCENTRATIONS USING ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive isocratic high-performance liquid chromatographic (HPLC) method for the measurement of melphalan in plasma is presented. It requires an extraction step using columns of XAD-2 resin before injecting the clarified methanol eluate directly into the HPLC system. The HPLC system uses an isocratic mobile phase containing an ion-pair reagent, and a sensitive fixed-wavelength (254 nm) monitor with a noise specification of $<2 \cdot 10^{-5}$ absorbance units peak to peak.

The concentration of melphalan was followed in a patient with multiple myeloma on day 1 and day 4 of a four-day course of the drug. Little difference was detected between the two curves with terminal half-lives of 71 and 68 min respectively and areas under the curve of 1.08 and 1.15 min $\cdot \mu$g/ml $\cdot$ (mg dose)$^{-1}$.

INTRODUCTION

Since melphalan [4-bis(2-chloroethyl)amino-L-phenylalanine; L-phenylalanine mustard; L-PAM] was synthesised by Bergel and Stock [1], it has been used with considerable success in treating multiple myeloma [2, 3]. It has also been used in treating carcinoma of the ovary, breast and testis [4]. Only recently, however, have there been methods available to measure melphalan in biological samples after the administration of therapeutic doses. Mass spectrometry has been used by a number of groups [5—7] but this technique is not readily available for routine analysis.

High-performance liquid chromatography (HPLC) has been used to measure melphalan in the mouse and dog [8] and in man [9, 10]. However, none of

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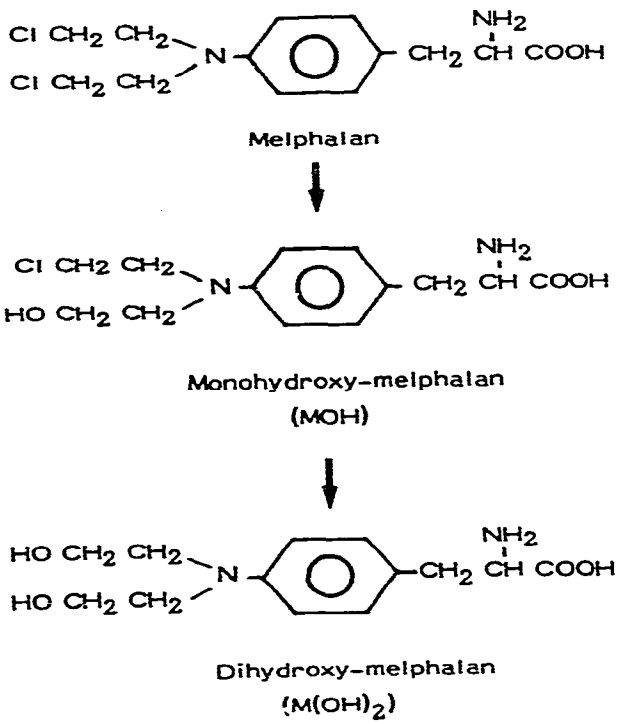

Fig. 1. Spontaneous degradation of melphalan in aqueous media.

these HPLC methods is capable of measuring plasma levels of the drug after normal oral therapeutic doses (5—15 mg) because peak plasma concentrations of melphalan are sometimes only 40—60 ng/ml [7, 11].

Despite the fact that a number of metabolites of melphalan have been identified in animals after intravenous and oral administration [12], none have been identified in man to date [5, 13, 14]. The drug does, however, degrade spontaneously yielding two hydrolysis products, monohydroxy-melphalan (MOH) and dihydroxy-melphalan [$M(OH)_2$; Fig. 1], although neither of these degradation products has cytotoxic activity [15].

This paper describes a sensitive assay for melphalan in plasma using a simple HPLC method. The separation of the biological material remaining after extraction from melphalan and an internal standard is accomplished by incorporating in the mobile phase a negatively charged ion-pair reagent (sodium dodecyl sulphate). This forms an ionic association (at low pH) with the positively charged amino group on melphalan giving a virtually hydrophobic ion-pair and the greater retention time necessary for separating it from the biological peaks. The method has been used to quantify the absorption of melphalan in multiple myeloma patients, after normal therapeutic doses were administered orally [11], and this work has led to the suggestion that melphalan should be taken after a meal to obtain greatest absorption [11].

EXPERIMENTAL

Materials

Melphalan was a generous gift from Burroughs Wellcome (Beckenham, Great Britain) and general labelled [^{3}H]melphalan was obtained from the Radiochemical Centre (Amersham, Great Britain). Sodium dodecyl sulphate and XAD-2 were purchased from BDH (Poole, Great Britain). Dns-arginine [DA, Nα(5-dimethylaminonaphthalene)-1-sulphonyl)-L-arginine], similar to Dns-proline [9] but eluting in a better position, was obtained from Sigma (Poole, Great Britain) and used as an internal standard. All materials were used as received except for the XAD-2 which was washed thoroughly with acetone and then with methanol until the absorbance (at 254 nm) of the washings was less than about 0.1 a.u. $M(OH)_2$ was prepared by the method of Furner et al. [8].

HPLC instrumentation

A dual reciprocating pulseless pump (Constametric IG, Laboratory Data Control (LDC), Stone, Great Britain) was used together with a Rheodyne injection valve (with a 200-μl loop), a 250 × 4.6 mm reversed-phase column packed with Spherisorb ODS (5 μm) and kept to 40 ± 1°C with a block heater (Jones Chromatography, Cardiff, Great Britain). A fixed-wavelength (254 nm) UV III monitor (LDC) with 0.002 absorbance units full scale (a.u.f.s.) maximum sensitivity was used rather than the possible alternative, the variable-wavelength Spectromonitor (LDC) set to a wavelength of 263 nm (melphalan's absorption maximum), due to the much reduced noise factor of the former. However, a new fixed-wavelength monitor did cause considerable problems for a time, when noise caused by a new type of digital display ruled out all sensitive work [16].

HPLC mobile phase

The mobile phase was made up by mixing one volume of 0.675 g/l solution of sodium dodecyl sulphate with four volumes of methanol and adjusting to approximately pH 3.0 with concentrated sulphuric acid (10—15 drops per 2.5 l; final concentration about 1.8 m*M* sulphuric acid). While the positions of the peaks seen in blank plasma extracts were virtually unaffected by pH, the positions of DA and melphalan were very dependent, and so final pH adjustment of the mobile phase was made by dropwise addition of acid. After each addition of acid, the mobile phase was used to chromatograph melphalan and DA to check whether the peaks were in satisfactory positions. The mobile phase was filtered through a 0.45-μm filter and degassed by ultrasonication prior to use.

Calibration

Every day the high-performance liquid chromatograph was calibrated with at least two 200-μl injections of a reference mixture of 0.4 μg/ml melphalan and 1.67 μg/ml DA in mobile phase. Any deviation from the expected peak heights and distances suggested faults in mobile phase or the chromatograph. The ratio (DA/melphalan) of the responses (measured in mm peak height at 0.002 a.u.f.s.

per ng material) was termed the response ratio.

The sample manipulation procedures were checked by extracting plasma samples containing known amounts of added melphalan and DA. Three standards were used with every extraction of one blank and eleven unknown plasma samples: 20 ng/ml melphalan, 200 ng/ml melphalan, and also a sample containing 200 ng/ml melphalan and 500 ng/ml DA to which no further DA was added. The results from these standards were used to calculate extraction efficiencies and thence a DA/melphalan extraction ratio.

Sample preparation

Patient management has been described previously [11], and plasma samples were stored at −40°C before analysis. As simple precipitation of protein [8, 9] was found to leave too much UV absorbing material in solution, extraction of the plasma was first carried out using XAD-2 resin. Columns of washed XAD-2 (25 × 5 mm, volume = 0.5 ml) were prepared in 5-ml pipettes and washed with 2 volumes (approx. 10 ml each) of acetone, 2 volumes of HPLC grade methanol and 2 volumes of degassed distilled water. DA (500 ng) was added to plasma samples (1.0 ml) and the mixtures applied to the columns. As soon as the plasma had run through, the column was washed with 1 volume of degassed distilled water, and the melphalan and DA eluted off with 1.5 ml methanol. Any slight precipitate was removed either by filtration through a 0.45-μm polytetrafluoroethylene filter or by microcentrifugation at 12,000 g for 3 min. The clarified eluate was stored at −40°C until 200-μl aliquots could be injected directly into the chromatograph.

Calculation

DA and melphalan peak heights were measured and a melphalan/DA peak height ratio (M/DA PHR) calculated. Melphalan concentrations were then calculated using the DA/melphalan response ratio (DA/M RR) and the DA/melphalan extraction ratio (DA/M ER; see Calibration) using the equation

$$\text{Concentration of melphalan (ng/ml)} = \frac{\text{M}}{\text{DA}}\ \text{PHR} \cdot \frac{\text{DA}}{\text{M}}\ \text{RR} \cdot \frac{\text{DA}}{\text{M}}\ \text{ER} \cdot \text{ng DA added per ml} \quad (1)$$

Data analysis

The data were then fitted to a biexponential equation of the form

$$C = -B(e^{-ka(t-t_o)} - e^{-\beta(t-t_o)}) \quad (2)$$

using the non-linear regression computer program NONLIN [11, 17] [where C = concentration of melphalan in plasma (ng/ml) at time t (min), B is a constant (ng/ml), β is an apparent first-order distribution rate constant measured in min^{-1}, ka is the apparent absorption rate constant (min^{-1}), and t_0 is the delay (min) before absorption starts].

Plasma half-lives ($t_{\frac{1}{2}}$; min) were calculated using the general formula:

$$t_{\frac{1}{2}}x = \frac{0.693}{x} \tag{3}$$

(where $x = \beta$ or ka), and the area under the curve [AUC; min·μg/ml·(mg dose)$^{-1}$] was calculated per mg dose of melphalan with the formula

$$\text{AUC} = \frac{10^{-3}}{\text{dose}} \cdot B\left(\frac{1}{\beta} - \frac{1}{ka}\right) \tag{4}$$

RESULTS

Fig. 2 shows the separation achieved between melphalan, Dns-arginine (the internal standard) and the two hydrolysis products of melphalan, MOH and $M(OH)_2$.

Fig. 3a shows the chromatogram of a blank plasma extract. The chromatograms shown in Fig. 3b and c are of extracts of a patient's plasma taken 237 and 15 min respectively after an intravenous dose of 25 mg of melphalan had been administered (the order was reversed so that the blank extract could more easily be compared to the sample containing low melphalan concentration). The plasma melphalan concentrations calculated from these chromatograms were 35 and 661 ng/ml respectively. $M(OH)_2$ and probably MOH are not extracted by this process.

Verification of the method was undertaken using both standard solutions of

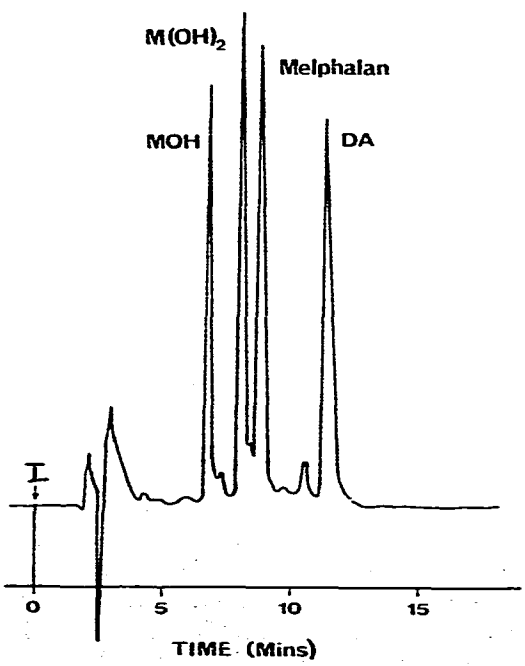

Fig. 2. Chromatogram showing the separation of approximately 400 ng DA, 200 ng melphalan, 400 ng $M(OH)_2$ and 200 ng MOH at 0.064 a.u.f.s.

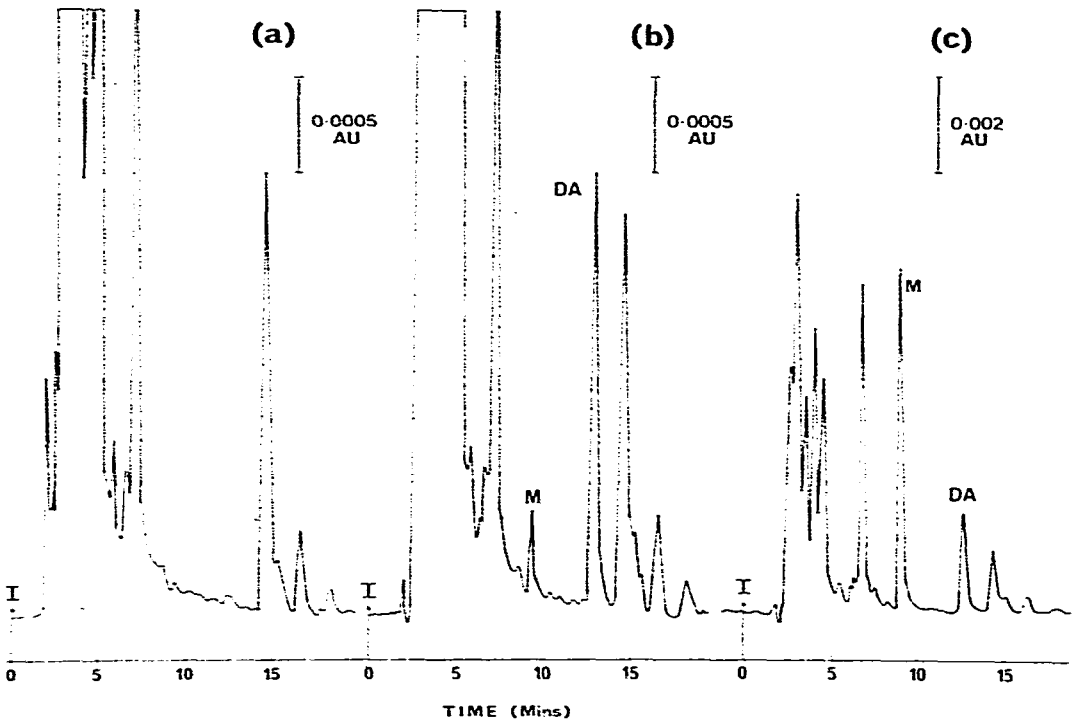

Fig. 3. Representative chromatograms of (a) a blank plasma extract at 0.002 a.u.f.s.; (b) an extract of plasma from a patient (adding DA at 500 ng per ml of plasma) 3 h 57 min after an intravenous dose of 25 mg melphalan (0.002 a.u.f.s.); and (c) identical extract of plasma taken 15 min after administration. Calculations from these chromatograms gave original plasma concentrations of melphalan of 35 and 661 ng/ml respectively. Peaks: M = melphalan; DA = internal standard; I = injection point.

melphalan and DA in mobile phase, and extracts of 1-ml blank plasma samples to which 6—1000 ng/ml melphalan had been added.

Linearity

The standard solutions of melphalan and DA gave excellent linearity with respect to peak height over 1—2000 ng per injection. A very good linearity was also seen between the melphalan/DA peak height ratio of extracted samples and melphalan concentration. The results of three separate experiments gave correlation coefficients of 1.000, 0.999 and 1.000.

Reproducibility

A reference mixture of melphalan (0.4 μg/ml) and DA (1.67 μg/ml) was always assayed, along with plasma extracts, to check the status of the HPLC system. Injections of 200 μl were made two to four times per day, and over a period of ten days the responses were for melphalan (measured as peak height) 14.73 ± 0.16 mm/ng at 0.002 a.u.f.s. [mean ± S.D.; $n = 9$; coefficient of variation (C.V.) = 1.07%], and for DA 5.43 ± 0.12 mm/ng (C.V. = 2.14%). The response ratio (DA/melphalan) calculated from these values was 0.3697 ± 0.0049 (C.V. = 1.32%).

Measurement of melphalan/DA ratios of five separate extractions on different days of 20 and 200 ng/ml plasma samples (adding 500 ng DA per ml of plasma) gave values of 0.167 ± 0.011 (C.V. = 6.2%) and 1.71 ± 0.11 (C.V. = 6.3%) respectively. The extraction efficiency for melphalan averaged 52.8%.

Sensitivity

The noise level of the high-performance liquid chromatograph, when pumping mobile phase and using the 5-sec time constant, was routinely about 10^{-5} a.u. However, this usually increased to two or three times this value around the area of the melphalan peak of a blank plasma extract (see Fig. 4a). Fig. 4b shows a melphalan peak just larger than the limits of detection, and from this the original plasma was calculated to contain 6 ng/ml melphalan. Thus the limit of detection is about 5 ng melphalan per ml of plasma, that is 5 ppb or $16 \cdot 10^{-9}$ M melphalan. The increased sensitivity of this method over other HPLC methods is partly due to an increased number of theoretical plates of the system, probably caused by the inclusion of an ion-pair reagent in the mobile phase. Figs. 2 and 3 show melphalan peaks with 4000—5000 plates ($N = 5.54 \cdot (d/w_{\frac{1}{2}})^2$ where N is the theoretical number of plates, d is the distance from the point of injection to the peak, and $w_{\frac{1}{2}}$ is the width of the peak at its half-height) and occasionally 8000—9000 plates are obtained. This compares favourably with the 1500—3500 plates of other published chromatograms [8—10].

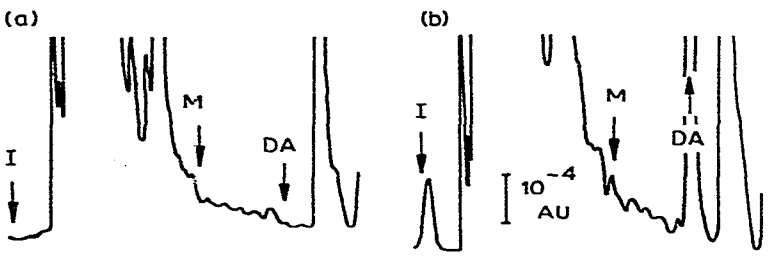

Fig. 4. Traces of (a) a blank plasma extract, and (b) a plasma sample that contained 6 ng melphalan per ml of plasma before extraction. These show the sensitivity that can be achieved with this method. Peaks: M = melphalan; DA = internal standard.

Stability of extracts

Plasma extracts were routinely kept at −40°C for up to 48 h before analysis by HPLC. Fifteen samples kept at −40°C for a further three months and analysed by HPLC a second time showed only minor differences when compared to the original chromatograms.

Melphalan concentrations in a patient

A patient who had been newly diagnosed as having multiple myeloma and had not received any prior chemotherapy was given 10 mg melphalan per day for a four-day course. On day 1 and day 4 he had a small breakfast of grapefruit, cereal and a drink before administration of melphalan. Blood

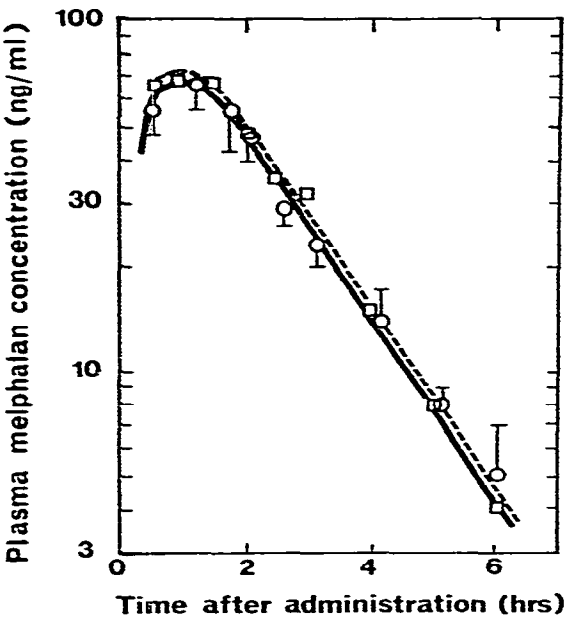

Fig. 5. Concentration of melphalan in the plasma of a patient after 10 mg melphalan given orally measured on day 1 (o—o) and day 4 (□– – –□) of a four-day course. The error bars on the day 1 points represent the S.D. of three separate determinations of melphalan concentration using the same original material.

samples were collected during day 1 and day 4, and Fig. 5 shows the concentration of melphalan in these samples. The error bars on the day 1 curve (continuous line) show the mean ± S.D. of three separate extractions of the same original material — the spread of results was probably due to a small percentage of the melphalan being converted to $M(OH)_2$ on each thawing of the plasma. The curves drawn through the points are the lines of best fit calculated by computer.

Table I gives the parameters calculated from the data in Fig. 5. The differences between the day 1 and day 4 values of t_0, B and $t_{\frac{1}{2}}ka$ are mainly due to

TABLE I

PARAMETERS CALCULATED FROM CURVES IN FIG. 5

The melphalan concentrations in the patient on day 1 (three separate extractions) and day 4 were fitted to biexponential curves. The parameters are calculated from the equations of these curves. The parameters for day 1 were determined for each extraction before a mean and S.D. were calculated.

	Units	Day 1	Day 4
Delay (t_0)	min	13.4 ± 0.7	9.2
Extrapolated intercept with Y axis (B)	ng/ml	130 ± 24	163
Half-life of absorption ($t_{\frac{1}{2}}ka$)	min	13.3 ± 0.6	19.4
Terminal half-life ($t_{\frac{1}{2}}\beta$)	min	70.7 ± 2.1	68.2
Area under the curve (AUC)	min·μg/ml·(mg dose)$^{-1}$	1.08 ± 0.15	1.15

the difficulty of fitting an equation to the absorption phase because the first sample was taken when the phase was nearly over. However, the terminal half-life is seen to be virtually identical on both days and the areas under the curves are not significantly different.

DISCUSSION

Recently, several groups have devised methods for the measurement of melphalan in biological samples [5—10]. However, none of the HPLC methods has the sensitivity to measure levels in human plasma samples after normal therapeutic doses of melphalan have been administered orally [8—10].

In this work we have been able to combine the sensitivity required with a simple isocratic HPLC system. The result is a method for the routine analysis of plasma melphalan that can be undertaken with equipment of relatively low cost. It has often been used in our laboratory to measure melphalan levels in solutions, and has also been used to follow the conversion of melphalan to MOH and to $M(OH)_2$ (data not shown). It has also been used to determine melphalan levels in cells in vitro at the end of drug uptake studies [18].

The sensitivity of this system has allowed us to obtain much useful information about melphalan levels in patients after they have taken normal therapeutic doses of oral melphalan (sometimes as little as 5 mg), as well as being able to follow the decay of the drug after intravenous administration [11]. The results from the patient in this study suggest that the administration of 10 mg melphalan over three days has not impaired the absorption of the drug on day 4 despite the sensitivity of the gastrointestinal tract to alkylating agents.

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