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Melphalan Estimation by Quantitative Thin-layer Chromatography

Observations on Melphalan Hydrolysis in vitro and Pharmacokinetics in Rabbits

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Summary. *A simple and specific quantitative high-performance thin-layer chromatographic (HPTLC) assay for melphalan in plasma is described. This assay was linear over the investigated range of 50–3,000 ng/ml, with a minimum level of detection of 20 ng/ml. Comparison with a high-pressure liquid chromatographic (HPLC) technique yielded similar estimates for melphalan concentrations in human plasma samples. The HPTLC method, unlike the HPLC technique, does not resolve monohydroxymelphalan satisfactorily. The HPTLC method was used to determine the activation energy for in vitro melphalan hydrolysis: this was 14.5 kcal/mole. The pharmacokinetics of melphalan in rabbits were also investigated. The mean* $t_{1/2}$ *in four animals was 32.6 ± 10.3 (S.D.) min and following IV administration to two animals the apparent volumes of distribution were 2.20 and 1.73 l/kg.*

Introduction

Although melphalan [p-di(2-chloroethyl)amino-L-phenylalanine] has been in use since the 1950s, little information regarding its pharmacokinetics is available, mainly because of the lack of specific and sensitive analytical methods and its rapid hydrolysis in solution. Melphalan estimation in biological materials has principally been carried out either with radiolabelled material [6, 9] or by high-pressure liquid chromatography [4, 7, 8]. The present work presents details of a rapid, accurate, and sensitive assay for melphalan by high-performance quantitative thin layer chromatography, and illustrates its use in the determination of melphalan kinetics in the rabbit and in vitro.

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Materials and Methods

Analytical. Methanol, ethyl acetate, ethyl alcohol, benzophenone, chloroform and glacial acetic acid were of Analar grade (BDH Ltd., Poole, Dorset) and were used as received. A stock solution of 10 mg melphalan (a gift of Dr. T. Priestman, Wellcome Research Institute, Beckenham, Kent) was prepared in a mixture of 98 ml methanol and 2 ml acetic acid. This was stored at −20° C and diluted appropriately before use.

a) High-performance Thin-layer Chromatography. One ml of plasma containing melphalan was placed in a centrifuge tube and 6 ml of a mixture of ethyl acetate containing 5% ethyl alcohol was added. The mixture was vortex-mixed for approximately 30 s and 5 ml of the organic phase was then quantitatively transferred to a clean centrifuge tube. This was evaporated to dryness under a stream of air, the residue redissolved in 30 µl methanol, and 25 µl spotted by hand onto the chromatography plate under a stream of cold air.

The plates used were of high-performance grade (HPTLC) silica gel 60 F_{254} fluorescent 10 × 20 cm plates (Merck: supplied by BDH Ltd., Poole, Dorset, Great Britain). They were developed in a glass tank equilibrated with the developing solvent which was chloroform: methanol: glacial acetic acid (35: 17: 3). This solvent system was prepared fresh on alternate days and then equilibrated in the tank, which was lined with filter paper (Whatman grade no. 1) for at least 3 h. The plate was developed over a distance of 9.8 cm and then air-dried for 10 min. They were scanned by means of a Vitatron TLC flying-spot densitometer (MSE Ltd., Crawley, Sussex), a mercury vapour lamp with a UVB blocking filter (which allows passage of light of wavelength less than 400 nm) being used in the incident beam. Plates were scanned in the direction of solvent flow.

b) High-pressure Liquid Chromatography. To provide comparison for the thin layer chromatographic technique, melphalan was also assayed by high-pressure liquid chromatography based on the methods published by Chang et al. [4] and Flora et al. [7]. Briefly, plasma samples were extracted as detailed for thin-layer chromatography following addition of the internal standard, benzophenone. Samples were introduced on to the column via a manual injection valve equipped with a 20-µl injection loop (Rheodyne model 7125). The column was a stainless steel tube (150 × 3 mm) packed with C_{18} Magnusphere (Magnus Scientific Ltd., Sandbach, Cheshire, Great Britain). The mobile phase consisted of a 60: 40 mixture of water and methanol delivered at a flow rate of 1.5 ml/min at a pressure of 2,000 p.s.i. by an Altex 110A pump.

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Melphalan and its metabolites were detected by a fixed wavelength (254 nm) ultraviolet detector (Applied Chromatography Systems Ltd., Luton, Bedfordshire, Great Britain). All separations were effected isocratically at ambient temperature. Quantitation was performed by constructing a standard curve of the ratio of the peak heights of melphalan to the internal standard versus melphalan concentration. This was linear up to at least 1,500 ng/ml, with a mean coefficient of variation of approximately 8% over the range 100–1,500 ng/ml.

In vitro Hydrolysis. Melphalan hydrolysis in plasma was studied by adding the pure substance to normal human plasma to yield a concentration of 5 μg/ml. This solution was maintained at a specified temperature in glass tubes in an electric heating block and samples were removed for analysis at designated intervals. The rate constant for hydrolysis was determined by plotting the log drug concentration against time (Fig. 2). The energy of activation, E, was estimated from the Arrhenius equation $\log k = \log A - E/2.303\ RT$, where k is the rate constant of the reaction, A the frequency factor, R the gas constant, and T the absolute temperature. Complete hydrolysis of melphalan was carried out by heating an aqueous solution at 60° C for 2 h. This material ('hydroxymelphalan') was used to determine the chromatographic behaviour of the more polar melphalan products (Fig. 1).

Pharmacokinetics in vivo. Four adult New Zealand white rabbits (weighing 1.25–2.35 kg) each received 8 mg/kg melphalan. This was administered to two by nasogastric tube and to two by IV injection. The injectable formulation (Alkeran, Wellcome) was used for both routes of administration. Venous blood samples (5 ml) were taken into lithium heparin tubes at 0, 5, 10, 15, 30, 60, 120, and 180 min after IV administration and 0, 15, 30, 60, 120, 180, and 240 min following PO administration. Plasma was separated by centrifugation and stored at −20° C pending analysis within a week. The first-order elimination rate constant k_e was estimated by a linear least-squares method from the log plasma concentration, time data, and the half-life determined from $0.693/k_e$. The area under the plasma concentration by time curve (AUC) was estimated by the trapezoidal rule and the clearance derived from Dose/AUC. The apparent volume of distribution was given by clearance/k_e.

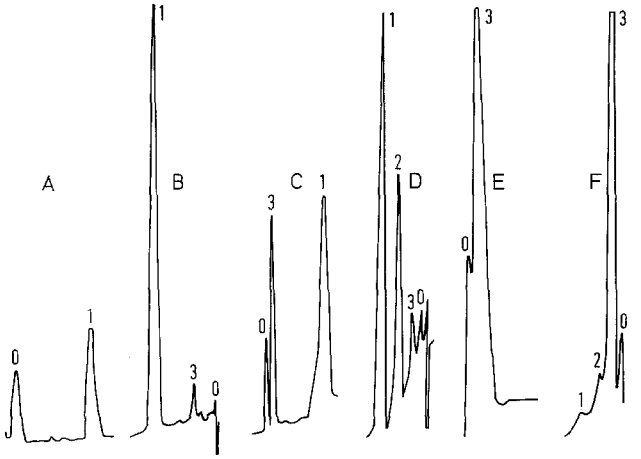

Fig. 1. A HPTLC of plasma extract spiked with melphalan (1); *B* HPLC of plasma extract containing melphalan (1) and dihydroxymelphalan (3); **C** HPTLC of extract of plasma containing melphalan following incubation at 60° C for 15 min; **D** HPLC of same extract as **C**; **E** HPTLC of extract of plasma containing melphalan following incubation at 60° C for 90 min; **F** HPLC of same extract as **E**. In all chromatograms 0 = origin of HPTLC or injection artifact of HPLC; 1 = melphalan; 2 = monohydroxymelphalan; 3 = dihydroxymelphalan

Results

Figure 1 shows representative thin-layer chromatographic scans. The R_f for melphalan was 0.51. No normal plasma constituents were found to give interfering peaks and the assay was found to be linear up to a concentration of 3,000 ng/ml, with a minimum level of detection of 20 ng/ml. The mean efficiency of extraction of melphalan from plasma was 86.6 ± 7.7% (S.D.) over this range of concentrations. The coefficient of variation within assays varied from 11.8% at 50 ng/ml to 4.2% at 3,000 ng/ml.

Figure 1 also shows comparable HPLC traces. It will be noted that HPLC distinguishes between mono- and dihydroxymelphalan but HPTLC does not yield a peak for the monohydroxy metabolite (Fig. 1C and D). Figure 2 gives a comparison of estimations of melphalan in authentic patient plasma samples by both methods. In this plot the regression line was determined by using the individual values of [log (HPLC estimate) − log (HPTLC estimate)] as estimates of log (slope of line of best fit). The slope was found by this method to be 1.009, with 95% confidence limits of 1.076–0.942. This suggests good agreement between the two methods of melphalan estimation and indicates that monohydroxymelphalan does not co-chromatograph with melphalan on the thin-layer system, thereby artifactually elevating the melphalan concentration estimated by this thechnique.

Figure 1 also indicates the hydrolysis of melphalan in vitro to initially the mono- and then the dihydroxylated compound. The in vitro degradation rate of melphalan is temperature-dependent (Fig. 3). The Arrhenius plot (Fig. 4) gives an energy of activation for its decomposition of 14,480 cals/mols.

Figure 5 shows the plasma concentration by time curves for 4 rabbits (nos. 1 and 3 given PO melphalan; nos. 4 and 6 given IV melphalan). The $t_{1/2}$ estimates were respectively 34.9, 44.4, 19.5, and 31.4 min. From the IV administrations the systemic clearances were estimated as 77.9 and 38.1 ml $\cdot min^{-1} \cdot kg^{-1}$ and the apparent volume of distribution as 2.20 and 1.73 l/kg. No major metabolites of melphalan were detected in the chromatograms of plasma taken during this study.

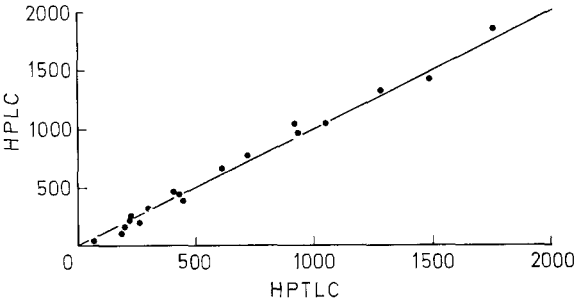

Fig. 2. Comparison of estimates of plasma melphalan concentration estimated by high-pressure liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC). Line of best fit determined as described in text

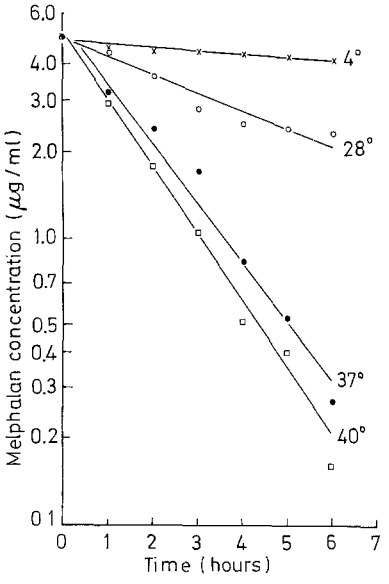

Fig. 3. Decay of melphalan in normal human plasma at different incubation temperatures

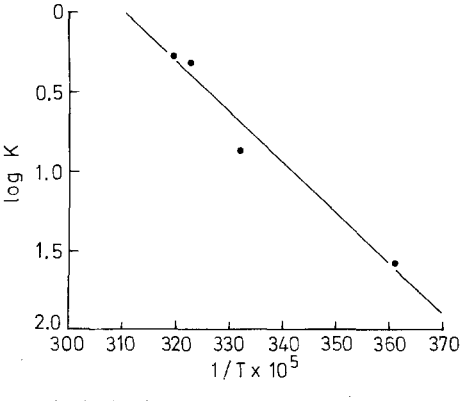

Fig. 4. Arrhenius plot for data shown in Fig. 3

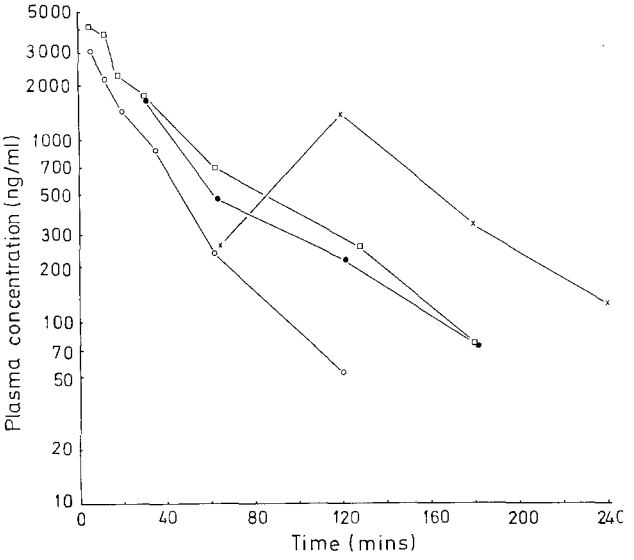

Fig. 5. Plasma concentration, time profiles for 4 rabbits given 8 mg melphalan/kg either IV (○, □) or PO (●, ×)

Discussion

A rapid and accurate quantitative HPTLC assay for melphalan in plasma has been developed, which may be compared with the HPLC assays previously described [4, 7, 8]. A comparison of the HPTLC and HPLC techniques shows that both yield estimates for the plasma melphalan concentration which are not significantly different (Fig. 2). Their specificity with regard to the hydroxylated melphalan metabolites is, however, different. From Fig. 1A and B it is seen that the HPTLC method is less sensitive than HPLC for the detection of dihydroxymelphalan. With controlled hydrolysis of melphalan first monohydroxy and then dihydroxymelphalan appears [8] (Fig. 1B, D, and F). This sequential change is not detected by the HPTLC technique, which does not resolve monohydroxymelphalan. In neither method, however, is there interference by these metabolites with quantitation of melphalan. There is no evidence that the hydroxylated metabolites of melphalan are cytotoxic and therefore either of the methods would be suitable for analysis of the parent compound in plasma.

The in vitro rate of melphalan disappearance in human plasma at 37° is comparable to the results detailed by Chang et al. [5]. The rapid rate of hydrolysis even at room temperature makes care necessary in plasma sample handling following withdrawal of blood, and rapid freezing and storage at low temperature is imperative if accurate estimates of

melphalan levels are to be obtained. Others have shown that bovine serum albumin exerts a retarding effect upon melphalan hydrolysis [5]. It has also been suggested that this spontaneous degradation, and not enzymatic metabolism, is the major determinant of melphalan pharmacokinetics in vivo [1].

Because of the problems associated with the estimation of melphalan in biological material, few data are available concerning its pharmacokinetics. In the mouse the half-life is reported as 41 min, and in the dog it is 50 min [8]. In man, following IV administration the half-life was found to be 108.0 ± 20.8 min [1] and 20.8 ± 5.6 min [3] by HPLC techniques. Radiolabel studies suggest a terminal phase half-life of 160 h [9]. Possibly this latter estimate results from binding of radiolabel to plasma protein. No major contribution from melphalan metabolites was noted in this study: similar findings have been reported in man [3, 9].

The absorption of melphalan after oral administration is reported as negligible [3] or else erratic, and less than that after intravenous administration [2]. Absorption in the rabbit after PO administration is sufficient to yield measurable plasma levels which in these experiments were only slightly lower than those following IV dosing (Fig. 5). Further work is required to determine the oral bioavailability of melphalan.

Melphalan is widely distributed in the body tissues of animals [8], and the apparent volume of distribution estimated in our rabbits would be consistent with this. In man the apparent volume of distribution was found to be on average somewhat less at 0.66 ± 0.21 l/kg [1], suggesting distribution in total body water.

The presently described HPTLC method for melphalan estimation is therefore adequate and accurate enough to undertake the study of the pharmacokinetics of melphalan in biological fluids from animals and man.

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