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Note

Method for the measurement of melphalan in biological samples by high-performance liquid chromatography with fluorescence detection

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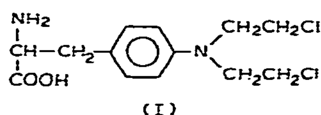
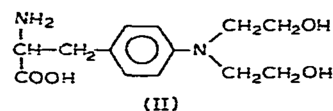
The alkylating agent melphalan (Alkeran; L-phenylalanine mustard) (I) was synthesized in the 1950s by Bergel and co-workers [1,2] and has been used clinically as an antitumour agent [3–5] and in the treatment of various neoplastic diseases [6,7].

Very few pharmacokinetic data on melphalan are available, despite the drug having been used for 20 years [8], because, in common with other alkylation agents, it is rapidly hydrolysed [9–11]. The main product of hydrolysis is the dihydroxy compound (II), but the intermediate monohydroxy compound (III) may also be detected as both are also formed metabolically.



(I)



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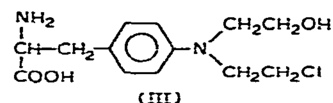

(III)

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Gas–liquid chromatographic [12] and high-performance liquid chromatographic (HPLC) [13,14] methods for the determination of melphalan have been described, in addition to a spectrophotofluorometric method [15]. This paper describes a sensitive method for measurement by reversed-phase HPLC using fluorescence detection, after separation from the hydrolysis products in rat plasma, liver and kidney.

EXPERIMENTAL

Materials

Methanol was of Distol grade (Fisons Scientific Apparatus, Loughborough, Great Britain). Water was glass-distilled. Melphalan (Wellcome Foundation, Dartford, Great Britain) was obtained in pure form. Standard solutions of melphalan were prepared in methanol and stored at −20°C.

Analytical methods

Extraction. Drug plus metabolites were extracted by a slight modification of published procedures [14]. To plasma or tissue homogenates (up to 3 ml) at 4°C were added two volumes of chilled methanol and the mixture vortex-mixed for 20 sec prior to centrifugation at 1500 *g* for 15 min. The clear methanolic supernatant was removed after centrifugation and the protein was re-suspended using a further volume of methanol. The two solvent extracts were pooled and stored at −20°C until processed further.

Separation of melphalan. The methanolic extract was added to four volumes of ice-cold water to produce a 15% methanol solution and passed through a reversed-phase C_{18} Sep-Pak cartridge (Waters Assoc., Stockport, Great Britain), where the melphalan was adsorbed. The eluent, which contained any dihydroxymelphalan present in the sample, was retained so that this metabolite could be measured later if required. The cartridge was washed with 10 ml of ice-cold 15% methanol and then the melphalan was eluted with 2 ml of methanol (the first 0.4 ml being discarded as the void volume) and stored at −20°C until assayed by HPLC. The fate of any monohydroxymelphalan present was not followed, but it was shown that this compound was eluted with the unretained peaks during the subsequent HPLC assay of melphalan and therefore did not interfere in the measurements.

HPLC assay method. A Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Winnersh, Great Britain) was attached to a Hitachi MPF2A fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, Great Britain). The fluorescence of melphalan was measured in a 20-µl flow cell (Hellma, Westcliff-on-Sea, Great Britain), employing excitation and emission wavelengths of 260 and 350 nm, respectively.

Chromatography was carried out isocratically at 60°C using 40% methanol in water at 3 ml/min as the mobile phase through a 250 × 4.9 mm I.D. stainless-steel column packed with 5-µm Spherisorb S5 ODS (Phase Separations, Queensferry, Great Britain). A run time of 15 min was satisfactory for all extracts.

Calibration method. Because of the instability of melphalan the chromatographic performance was checked by running a standard solution of melphalan before, during and after any run of samples.

RESULTS

Despite its lability at room temperature, melphalan is known to be stable in plasma at −20°C for at least 3 weeks [14]. Further, solutions in methanol were found to retain full integrity for more than 12 h at room temperature and for at least 4 weeks at −20°C.

Peak heights were linearly related to concentration for standard solutions of melphalan over the range 10 ng to 2.5 μg injected on to the column, as were tissue samples spiked at 250 ng/g to 12.5 μg/g (Fig. 1). The lower limit of detection (signal-to-noise ratio = 2) was about 500 pg injected.

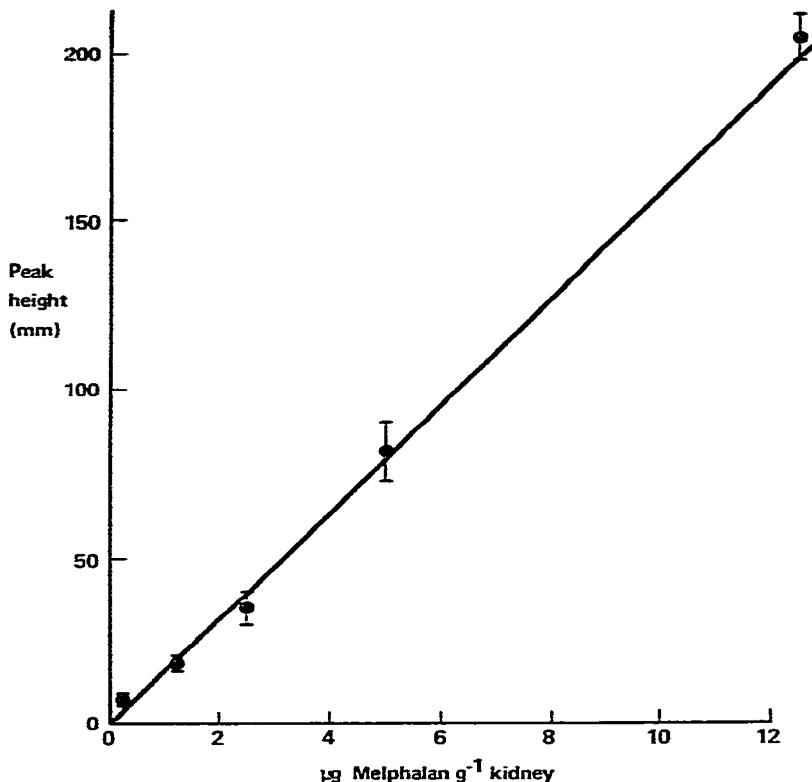

Fig. 1. Melphalan calibration graph for spiked kidney samples. Melphalan added to 3-ml samples of kidney homogenate then treated as described in the text. Final extract assayed by HPLC. For all concentrations $n = 4$.

Extracts from plasma, kidney and liver were sufficiently free from interfering substances to allow the melphalan to be measured directly. Representative chromatograms are shown in Fig. 2.

Following single oral doses to rats over a range from 0.5 to 5 mg/kg the concentrations of melphalan determined 1 h later were 0.14—1.9 μg/ml in plasma, 0.02—0.2 μg/g in liver and 0.17—4.8 μg/g in kidney.

The coefficient of variation of the extraction and assay of melphalan from

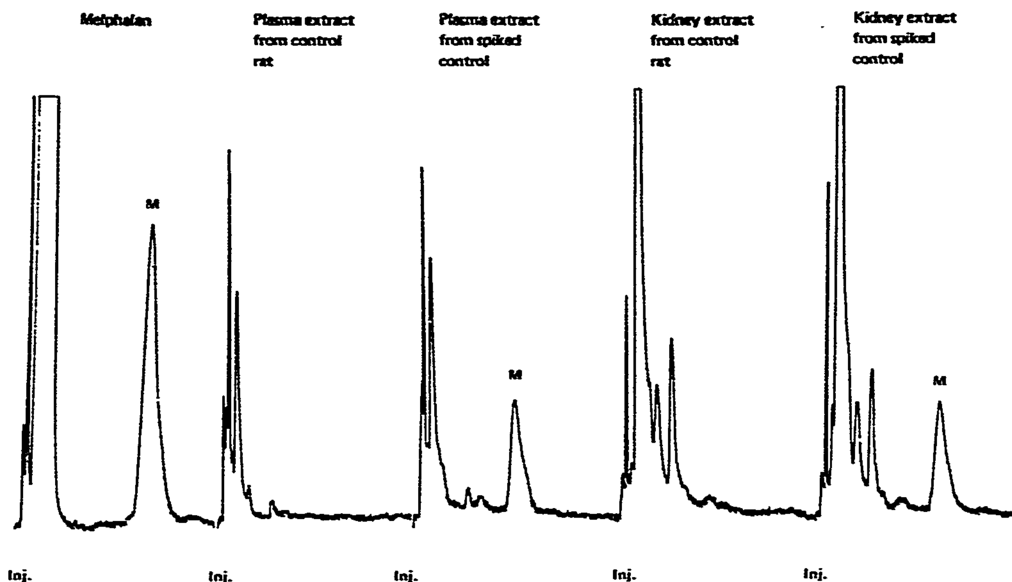

Fig. 2. Chromatograms of melphalan (M) in different samples.

spiked control plasma and tissues was consistently below ± 10%, usually below ± 5%. Standard melphalan solutions in methanol, when measured by HPLC, had coefficients of variation of less than ± 1.5% in all instances. The recovery of melphalan from plasma and tissues was 82% before and 62% after Sep-Pak separation, with a coefficient of variation of less than ± 7%.

DISCUSSION

The separation and measurement of melphalan is hampered by its instability and by the very high solubility of the compound in water. In addition, the amphoteric nature of the molecule makes solvent extraction unsatisfactory, and consequently previously described liquid chromatographic methods [13, 14,16] have depended on precipitation of the protein followed by direct measurement in the supernatant, which inevitably involves dilution of the original biological sample.

Measurements of substances eluted from the HPLC column have been carried out mainly with a UV detector [13,14,16], although Brox et al. [16] additionally used a fluorescence detector, but the advantages of fluorescence detection were not discussed.

In the present method, which depends on adsorption of melphalan on a Sep-Pak C_{18} cartridge and its subsequent elution, the volume of eluate is 1.6 ml regardless of the volume of the original sample, and consequently this step can lead to concentration rather than dilution of the drug. By separating melphalan from the major hydrolysis product before chromatography the drug was easily measured with a simple isocratic system. Dihydroxymelphalan separated by this method was available for subsequent assay without the use

of chromatographic gradients and in addition the background interference due to the biological samples was reduced.

By this means and by exploiting the native fluorescence of the drug, the sensitivity was improved over that of previously reported methods such that the lower limit of detection was well below 5 ng/ml in plasma.

The method, which was applied to rat plasma and tissues in this study, can also be used for human samples.

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REFERENCES

1 F. Bergel and J.A. Stock, J. Chem. Soc., (1954) 2409.
2 F. Bergel, V.C.E. Burnop and J.A. Stock, J. Chem. Soc., (1955) 1223.
3 J.P. Smith and F.N. Rutledge, Nat. Cancer Inst. Monogr., 42 (1974) 141.
4 R. Alexander, D.E. Bergsagel, P.J. Migliore, W.K. Vaughn and C.D. Howe, Blood, 31 (1968) 1.
5 B. Fisher, P. Carbone, S.G. Economou, R. Frelick, A. Glass, H. Lerner, D. Redmond, M. Zelen, P. Baird, D.L. Datrych, N. Wolmark and E.R. Fisher, N. Engl. J. Med., 292 (1975) 117.
6 T.H. Wasseman, R.L. Conis, M. Goldsmith, H. Handelmann, J.S. Penta, M. Slavick, W.R. Soper and S.K. Carter, Cancer Chemother. Rep., Part 3, 6 (1975) 399.
7 R.B. Livingstone and S.K. Carter, Single Agents in Cancer Chemotherapy, Plenum, New York, 1970, p. 99.
8 D. Alberts, S. Chang, L. Mernick, K. Himmelstein, P. Watson, J. Gross and S. Salmon, Proc. Amer. Ass. Cancer Res., 18 (1977) 128.
9 W.C.J. Ross, Biological Alkylating Agents, Butterworths, London, 1962, p. 19.
10 J.H. Linford, Biochem. Pharmacol., 8 (1961) 343.
11 O.M. Friedman and E. Boger, Anal. Chem., 33 (1961) 906.
12 J.T. Goras, J.B. Knight, R.H. Iwamoto and P. Liu, J. Pharm. Sci., 59 (1970) 561.
13 R.L. Furner, L.B. Mellett, R.K. Brown and G. Duncan, Drug Metab. Dispos., 4 (1976) 577.
14 S.Y. Chang, D.S. Alberts, L.R. Melnick, P.D. Watson and S.E. Salmon, J. Pharm. Sci., 67 (1978) 679.
15 M.A. Chingos and J.A.R. Mead, Anal. Biochem., 7 (1964) 259.
16 L. Brox, L. Birkett and A. Belch, Cancer Treat. Rev., 6, Suppl. (1979) 27.