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Note

High-performance liquid chromatographic analysis of melphalan in plasma, brain and peripheral tissue by *o*-phthalaldehyde derivatization and fluorescence detection

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Melphalan, 4-[bis(2-chloroethyl)amino]-L-phenylalanine, is used to treat multiple myeloma, ovarian carcinoma and in adjuvant therapy of breast cancer [1—4]. Although it has been in clinical use for some twenty years, it has only been with the relatively recent development of sensitive and specific high-performance liquid chromatographic (HPLC) assays that its pharmacokinetics have been analysed [5—8].

Several HPLC assays have been developed to quantify therapeutic concentrations of melphalan in blood; most have relied on detection of melphalan by UV absorbance [5—8]. Of these, all have been limited to the analysis of the drug in plasma and serum [6—8] or have lacked sufficient sensitivity for the analysis of other tissues [5]. Our attempts to adapt these assays to determine melphalan in other tissues have proved unsuccessful, due to the presence of interfering endogenous substances, particularly in brain where melphalan concentrations are low. Egan et al. [9] utilised the natural fluorescence of melphalan to determine its concentration. However, up to 3 ml of plasma or tissue homogenate were required. We therefore developed a new rapid and sensitive method for analysing melphalan in plasma and tissue samples by employing *o*-phthalaldehyde derivatization and fluorescence detection which yielded a product whose fluorescence was significantly greater than that of melphalan alone. In this paper we describe the technique.

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MATERIALS AND METHODS

Reagents

Melphalan was a gift from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). Methanol and acetonitrile were HPLC grade and were used as supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Sodium chloride, sodium monophosphate and sodium diphosphate, all reagent grade, were from Fisher (Fairlawn, NJ, U.S.A.). Ethanethiol, sodium acetate and acetic acid, all reagent grade, were from Kodak Chemical (Rochester, NY, U.S.A.). *o*-Phthalaldehyde was supplied by Pierce (Rockford, IL, U.S.A.).

Apparatus

HPLC was performed on a Waters Assoc. (Milford, MA, U.S.A.) system. This consisted of a Model 6000A solvent delivery pump, a Model 720 system controller, a WISP 710 B automatic injector and a Model 720 data module. The detector was a Kratos Model FS-950 Fluoromat fluorometer (Ramsey, NJ, U.S.A.) with a mercury lamp, an excitation filter of 365 nm and an emission UV cutoff filter of 426 nm. Separation was performed on a 10-μm Partisil 10 ODS 3 column (Whatman, Clifton, NJ, U.S.A.) with a guard column packed with pellicular C_{18} material.

Instrument conditions

Samples were eluted in a mobile phase of acetonitrile—15 m*M* phosphate buffer, pH 7.0 (36:64, v/v). The phosphate buffer was prepared from deionised, distilled water and was mixed with the acetonitrile. The mixture was degassed and filtered through a 0.22 μm pore diameter Millipore filter (Bedford, MA, U.S.A.) and allowed to equilibrate to room temperature. All columns were equilibrated to new solvents for at least 30 min. The flow-rate was maintained at 3 ml/min, which resulted in a column pressure of 170 bars.

Tissue preparation

Adult male Fischer 344 rats (Charles River, Wilmington, MA, U.S.A.), approximately 250 g weight, were anaesthetised (sodium pentobarbital 40 mg/kg, intraperitoneally) and injected intravenously (4 ml/kg) with melphalan (10 mg/kg) in 5% ethanol, 95% isotonic saline [0.9% (w/v) sodium chloride, pH 7.2]. At different times following melphalan administration, the animals were killed, blood was taken by cardiac puncture and the brain removed. The blood was placed in a heparinised tube and centrifuged at 7000 *g* for 1 min. The plasma was removed and together with the brain stored on dry ice. The brain was later thawed, dissected into regions and placed in preweighed vials, reweighed, and stored at −70° C.

Extractions

Plasma samples were thawed and placed in an ice bath. Then 200 μl of plasma were added to 400 μl of methanol. The mixture was vortexed for 20 sec and centrifuged at 7000 *g* for 1 min. A 200-μl aliquot of the supernatant was removed and stored at −70° C prior to analysis.

Tissue samples were similarly thawed and placed in an ice bath. A 5-μl

aliquot of methanol was added to every 1.5 mg of tissue. Each sample weighed approximately 150 mg. The tissue was sonicated (Ultrasonics, Plainview, NY, U.S.A.) for 45 sec and maintained at 0°C with an ice bath. The resulting suspension was centrifuged at 7000 *g* for 15 min at 4°C. A 200-μl aliquot of the supernatant was removed and stored at −70°C prior to analysis.

Derivatization

The methanolic solutions of extracted plasma and tissue were brought to room temperature immediately prior to HPLC analysis. To each 200-μl sample were added 50 μl of 0.5 *M* acetate buffer, pH 5.15, and 100 μl of methanol containing 20 mg/ml *o*-phthalaldehyde and 20 μl/ml ethanethiol. The mixture was vortexed for 20 sec, allowed to stand for 2 h at room temperature and injected onto the column.

Calibration and quantitation

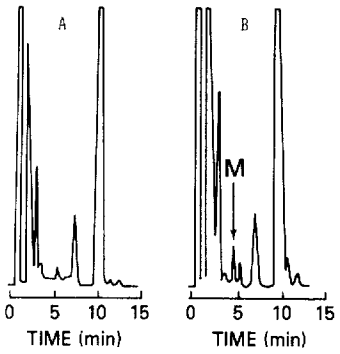
Melphalan plasma and tissue concentrations were quantified from calibration curves. Standard curves of six points, two samples per point, were run daily and intermixed with the unknown samples. Drug-free plasma (200 μl) or drug-free tissue (150 mg) was spiked with known and increasing amounts of freshly prepared melphalan. A least-squares linear regression between concentration and peak height was calculated for each preparation. The mean correlation coefficient of six curves run over a month period for plasma was 0.998 while that for brain was 0.999. The intercept was negligible for both. The plasma standard curves were linear over the range 10 ng to 1 μg and 1 μg to 50 μg. The brain standard curves were linear over the range 25 ng to 10 μg. Melphalan stability, tested by running identical samples at the beginning and end of each day, was maintained by keeping the samples at −70°C prior to derivatization.

RESULTS

The in vivo and in vitro breakdown of melphalan proceeds by hydrolysis to the monohydroxymelphalan and dihydroxymelphalan. We prepared these compounds by the method of Furner et al. [5] and obtained peaks at 1.24 min for dihydroxymelphalan, at 1.64 min for monohydroxymelphalan and 4.69 min for melphalan. Neither the mono- nor dihydroxy products, however, could be quantitated in biological samples because they co-eluted with the solvent front.

Fig. 1 shows the chromatograms of a blank plasma sample (A), and a plasma sample taken 30 min after the administration of melphalan (10 mg/kg, intravenously) (B) to a three-month-old male Fischer 344 rat. Fig. 2 shows chromatograms of a blank brain (A) and a brain sample taken at 30 min from the same animal as in Fig. 1B (B). Calculations against standards gave melphalan concentrations of 5.16 μg/ml and 0.82 μg/g for plasma and brain, respectively.

Sample stability was assessed by routinely analyzing, over a month period, single reference preparations of both plasma and brain stored at −70°C. No melphalan breakdown occurred in either case. Assay reproducibility was assessed by analyzing reference melphalan samples from six separate preparations on six different days over a period of one month. The coefficient of



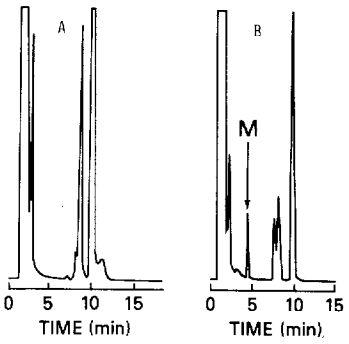


Fig. 1. Chromatograms of a 25-μl injection of (A) a blank plasma sample and (B) a plasma sample containing 5.16 μg/ml melphalan (M) taken at 30 min from a rat injected intravenously with 10 mg/kg melphalan.

Fig. 2. Chromatograms of a 25-μl injection of (A) a blank brain sample and (B) a brain sample containing 0.82 μg/g melphalan (M) taken at 30 min from a rat injected intravenously with 10 mg/kg melphalan.

variation for plasma (1 μg/ml) was 5.9% of the mean, and for brain (100 ng per 150 mg) was 5.3% of the mean.

DISCUSSION

HPLC with fluorescence detection, utilizing both pre- and post-column derivatization, has been widely used for the sensitive analysis of biogenic amines in body tissues. In particular, the fluorogenic reaction of *o*-phthalaldehyde with ethanethiol has been successfully employed for the separation and quantitation of amino acids and proteins in plasma and brain [10, 11]. Because melphalan is a primary amine and the nitrogen mustard derivative of the amino acid phenylalanine, it was thought that it also could be analysed by this technique.

For the analysis of amino acids, the reaction between *o*-phthalaldehyde, ethanethiol and the amines normally proceeds at a pH between 9 and 10.5. Attempts to derivatize melphalan at pH 10.4, however, caused its rapid hydrolysis to mono- and dihydroxy products. Buffering the reaction at pH 7.1 also caused hydrolysis but at a less rapid rate. An acetate buffer, pH 5.15, which was used eventually for the reaction, eliminated hydrolysis; however, it may also have decreased the fluorescence intensity and slowed the reaction rate [12]. Despite this the fluorescence was found to be approximately three times greater than that of the natural fluorescence of melphalan alone.

Fig. 3 relates the fluorescence intensity of the reaction to time up to 24 h, for a single sample of melphalan in brain and in plasma. In both tissues the reaction took 1 h to reach maximum intensity and then decayed at a rate that did not exceed 4% of the maximum intensity after 2 h. Samples therefore were injected onto the column 2 h after derivatization. At this time, fluorescence intensity was approximately 97% of maximum. The decay curve for melphalan was pseudo first-order and had a half-life of 20 h in plasma and 25 h in brain. Since no monohydroxymelphalan or dihydroxymelphalan could be detected

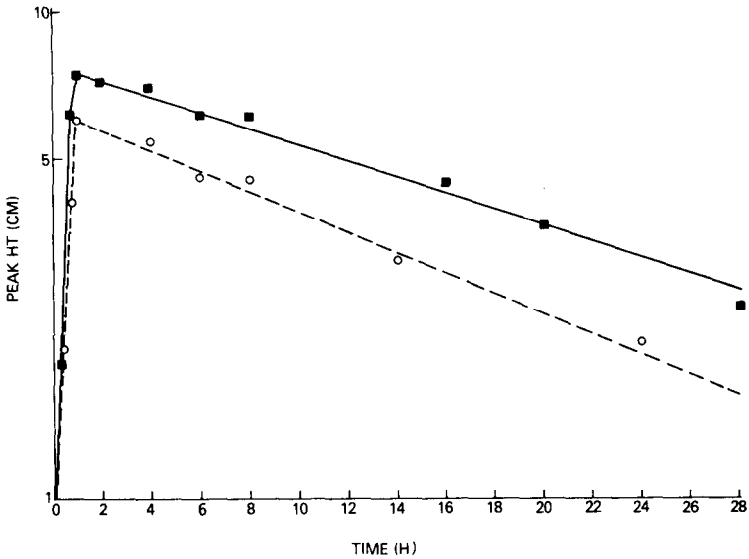


Fig. 3. Relationship between chromatogram peak height and time for a single sample of melphalan in plasma (■) and in brain (○).

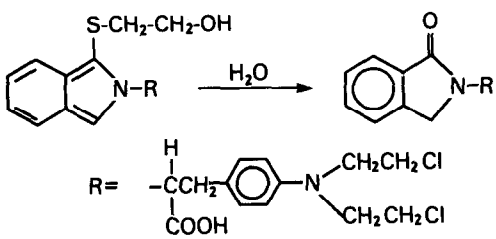


Fig. 4. Hydrolysis of the iso-indole product of the derivatization of melphalan (5 μg/ml) to the cyclic amide.

TABLE I

COMPARISON OF THE FLUORESCENCE ASSAY AND UV ABSORBANCE ASSAY FOR MELPHALAN IN PLASMA

UV absorbance assay: Chang et al. [6]; fluorescence detection assay: present paper. No significant difference between melphalan concentrations determined by either technique (Student's t-test: $p < 0.05$).

Plasma sample	Melphalan concentration (mean ± S.E.M., $n = 6$) (μg/ml)	
	UV detection technique	Fluorescence detection technique
1	0.953 ± 0.06	0.953 ± 0.04
2	0.546 ± 0.03	0.520 ± 0.03

in non-biological samples up to 24 h, it can be assumed that this fluorescence decay resulted from the hydrolysis of the iso-indole product to the non-fluorescent cyclic amide [13] (Fig. 4).

The fluorescence assay was compared to the UV absorbance melphalan assay

of Chang et al. [6] for the measurement of unknown plasma samples. There was no significant difference between the concentration determined by either technique (Table I). In addition, the fluorometric assay allows for the analysis of as little as 100 μl of plasma containing as little as 10 ng/ml melphalan. Further, for the first time, it provides a rapid and accurate analysis of as little as 25 ng/g melphalan in as little as 50 mg of tissue.

REFERENCES

1 R. Alexander, D. Bergsagel and P. Migilore, Blood, 31 (1968) 1.
2 O. McIntyre, L. Leone and T. Pajak, Blood, 52 (1978) 274.
3 J. Smith and F. Rutledge, Natl. Cancer Inst. Monogr., 42 (1974) 141.
4 B. Fisher, P. Carbone, S. Economon, R. Frelick, A. Glass, H. Lerner, C. Redmond, M. Zelen, P. Band, D. Katrych, N. Wolmark and E. Fisher, N. Engl. J. Med., 292 (1975) 117.
5 R. Furner, L. Mellet, R. Brown and G. Duncan, Drug Metab. Dispos., 4 (1976) 577.
6 S. Chang, D. Alberts, L. Melnick, P. Watson and S. Salmon, J. Pharm. Sci., 67 (1978) 679.
7 A. Ahmed and T. Hsu, J. Chromatogr., 222 (1981) 453.
8 A. Bosanquet and E. Gilby, J. Chromatogr., 232 (1982) 345.
9 C. Egan, C. Jones and M. McCluskey, J. Chromatogr., 224 (1981) 338.
10 B. Jones, S. Paabo and S. Stein, J. Liquid Chromatogr., 4 (1981) 565.
11 M. Fernstrom and J. Fernstrom, Life Sci., 29 (1981) 2119.
12 M. Roth, Anal. Chem., 43 (1971) 880.
13 S. Simons and D. Johnson, Anal. Biochem., 90 (1978) 705.