Blum, *ibid.*, **62**, 599 (1973).
(14) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 59.
(15) L. Snyder, *J. Chromatogr.*, **92**, 223 (1974).
(16) B. L. Karger, L. R. Snyder, and C. Horvath, "An Introduction to Separation Science," Wiley, New York, N.Y., 1973, pp. 146–150.

ACKNOWLEDGMENTS

Presented at the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, New York meeting, May 1977.

The authors acknowledge the technical assistance of W. Coldren and T. Bellnier.

High-Pressure Liquid Chromatographic Analysis of Melphalan in Plasma

S. Y. CHANG ˣ, D. S. ALBERTS, L. R. MELNICK, P. D. WALSON, and S. E. SALMON

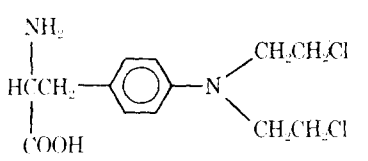
Received April 22, 1977, from the *Section of Hematology and Oncology, Departments of Internal Medicine and Pharmacology, and the Cancer Center, College of Medicine, University of Arizona, Tucson, AZ 85724.* Accepted for publication September 1, 1977.

Abstract □ A new sensitive and rapid high-pressure liquid chromatographic determination of melphalan in plasma was developed. Recovery of 1 μg added to 1 ml of plasma at 23° was 94% but was greatly reduced at higher temperature. The method has been applied to plasma determinations of melphalan in rats and humans and is currently being utilized for human pharmacokinetic studies.

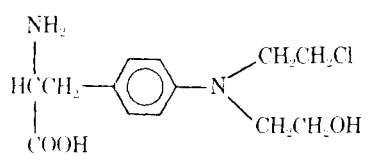
Keyphrases □ Melphalan—high-pressure liquid chromatographic analysis in plasma □ High-pressure liquid chromatography—analysis, melphalan in plasma □ Antineoplastic agents—melphalan, high-pressure liquid chromatographic analysis in plasma

Since melphalan [L-phenylalanine mustard, 4-bis(2-chloroethyl)amino-L-phenylalanine, NSC 8806] was synthesized (1), it has become an important drug for the treatment of various cancers, including multiple myeloma and ovarian and breast carcinoma (2–4). Little information is available concerning its pharmacokinetic properties in humans or animals because of its rapid hydrolytic degradation, which is common to alkylating agents (5–8).

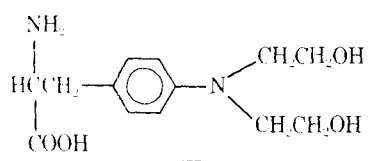
The colorimetric assays utilizing nitrobenzylpyridine (7, 9–11) and the spectrofluorometric method (8) for the determination of melphalan (I) in biological fluids are not sensitive enough for the study of its pharmacokinetic properties. The fluorometric method for biological samples is not practical because of background interference and its lengthy nature (8). These methods are not specific; they measure the total concentration of alkylating agents including one hydrolysis product of I. A GLC method for I (12) has not been applied to biological samples. Recently, Furner *et al.* (13) reported a high-pressure liquid chromatographic (HPLC) method for the determination of I in animal serum.



I



II



III

The present paper reports a sensitive and rapid HPLC method for the determination of I in biological fluids. Practical application of the developed method is demonstrated by *in vivo* determinations of the plasma I concentration in animals and humans. *In vitro* plasma recovery studies also are presented.

EXPERIMENTAL

Materials—Methanol[1] was used as received. Dansylproline[2] was used as an internal standard. The standard I[3] dissolved in methanol containing 2% acetic acid and the internal standard dissolved in methanol were stored at −20° at all times between usage. Aqueous solvents were filtered with a 0.45-μm cellulose acetate filter[4]. Methanol and other organic solvents were filtered with a fluoropore[4] prior to use.

Instrument Conditions—HPLC was performed with an apparatus consisting of two pumps[5], a solvent programmer[6], a detector[7], and a reversed-phase column[8]. An isocratic solvent system of water and methanol (1:1) with 1% acetic acid was delivered at the rate of 2 ml/min, and I was detected at 254 nm. The total analysis time per sample was approximately 20 min.

Extraction of I from Plasma—Five micrograms of internal standard was added to 1 ml of human plasma prior to extraction of I for *in vivo* studies. For *in vitro* studies, 2 μg of internal standard was added for every 1 μg of I. Two milliliters of methanol (0°) was added to 1 ml of plasma and mixed vigorously on a vortex mixer for 20 sec. The sample was then cooled at −60° (acetone and dry ice) for 3 min. The plasma–methanol mixture was centrifuged at 3000 rpm for 3 min on a clinical centrifuge. The clear methanolic solution, which contained the internal standard and I, was injected directly onto the column. Calculation of I was as follows:

[1] Burdick and Jackson Co., Muskegon, Mich.
[2] Pierce Chemical Co., Rockford, Ill.
[3] Obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[4] Millipore Corp., Bedford, Mass.
[5] Waters 6000, Waters Associates, Milford, Mass.
[6] Waters 600, Waters Associates, Milford, Mass.
[7] Waters 440, Waters Associates, Milford, Mass.
[8] Waters micro C_{18} column, Waters Associates, Milford, Mass.

Table I—Recovery of I from Plasma

Micrograms of I per Milliliter of Plasma	Recovery[a], % ± *SD*
1	93.5 ± 8.4
5	92.2 ± 3.5
10	89.9 ± 1.9
30	94.8 ± 2.6

[a] The immediate recovery of I at 23°.

$$\mu g \text{ of I} = \frac{\text{response of I sample}}{\text{response of internal standard sample}} \times \frac{\mu g \text{ of internal standard added}}{\text{relative weight response}} \quad \text{(Eq. 1)}$$

where:

$$\text{relative weight response} = \frac{\text{response of I}/\mu g}{\text{response of internal standard}/\mu g} \quad \text{(Eq. 2)}$$

Recovery—Various amounts of I (30, 10, 5, and 1 μg) and internal standard (twice the amount of I) were added to 1 ml of human plasma at 23°. The plasma was mixed well and then extracted for I as described. Experiments were run in quadruplicate.

Peak Identification—The I HPLC peak in plasma was identified as I by GLC–mass spectrometry[9]. Both trimethylsilyl and trifluoroacetyl *n*-butyl ester derivatives were used. Compound I in plasma was collected from the liquid chromatograph and then derivatized and introduced to a 3% OV-7 (0.5 m × 0.2 mm) column. The effluents were scanned for either an entire or a partial spectrum. The mass spectrometer was operated in the electron impact mode at 70 ev.

Stability of I in Plasma—The stability of I in plasma (30 μg/ml) was determined by the percent recovery of I after storing samples at −20° for 3 weeks or at −20° for 3 weeks followed by 3 weeks at 4°. All analyses were performed in triplicate. Stability also was determined at varying temperatures and incubation times, during which samples were taken at 1- or 2-hr intervals up to 12 hr. Plasma was maintained at 0, 23, and 37°. The 0° experiment was conducted over 24 hr.

Plasma I Concentration in Animals and Humans—Rats were given 6 mg/kg of I by intravenous injections over 1 min into a jugular venous catheter (14). Heparinized blood samples were taken at 5, 15, and 30 min and then hourly for 6 hr from a separate jugular catheter. Plasma levels of I were determined by HPLC.

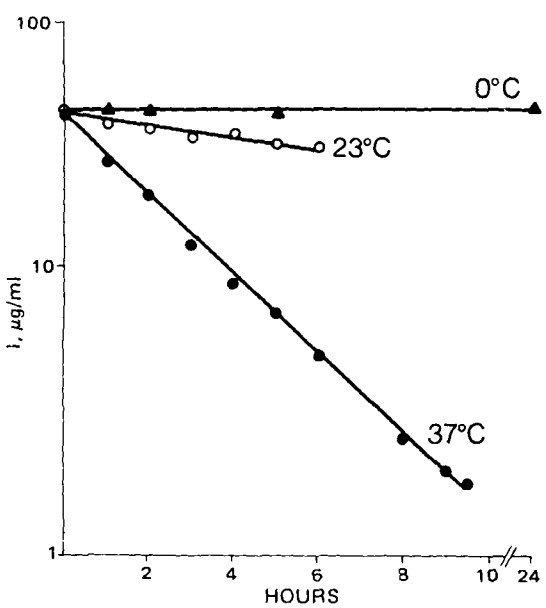


Figure 1—In vitro *recovery of I from human plasma as a function of temperature and incubation time. Compound I was added to human plasma in a concentration of 25 μg/ml and incubated at 0° (▲), 23° (○), and 37° (●). Aliquots were taken at designated time points and analyzed by HPLC.*

[9] Finnigan 3300-6100, Sunnyvale, Calif.

Table II—Stability of I

Age of Sample	Recovery, % ± *SD*	Remarks
0	94.8 ± 2.6	23°, immediate analysis
3 weeks	101.3 ± 5.2	−20° throughout
6 weeks	65.9 ± 3.5	3 weeks at −20° plus 3 weeks at 4°

Patients were given a 0.6-mg/kg dose of I by intravenous bolus injection or orally. Anticoagulated venous blood samples were drawn and quickly placed in ice. The plasma was separated by centrifugation and analyzed by HPLC as described.

RESULTS

Recovery—Recovery of 1, 5, 10, and 30 μg of I added to 1 ml of plasma at 23° is presented in Table I. The average recovery ranged from 90 to 95% and had greater standard deviation at lower concentrations.

Stability Studies—No change in the plasma I concentration was seen when 30 μg/ml was stored for 3 weeks at −20°. However, when the same sample was removed from −20° and placed at 4° for an additional 3 weeks, there was a 35% decrease in the plasma I concentration (Table II).

In contrast to the stability of I in plasma at low temperature, I was not stable in plasma at higher temperatures. The diminished recovery of I from human plasma at increasing temperature is shown in Fig. 1. Recovery of I was only 15% after 6 hr at 37°, as compared to 72% at 23° and over 95% at 4°. Similar diminution of recovery of I at higher temperature was observed in water. These results will be presented in detail elsewhere.

***In Vivo* Plasma Level Determination**—Figure 2 shows a standard chromatogram of 250 ng of I and 700 ng of internal standard. Figure 3 shows the human plasma background with added internal standard. A standard chromatogram of the HPLC assay of human plasma from a patient who had received an oral dose of I is shown in Fig. 4. The HPLC response indicates a human plasma concentration of 0.2 μg of I/ml. Serial concentrations of I in rat plasma after a dose of 6 mg/kg iv are shown in Fig. 5.

DISCUSSION

An accurate and sensitive determination of I in biological fluids is necessary for the study of its pharmacokinetics in animals and humans. An HPLC method (13) was reported that is far simpler and more sensitive than conventional colorimetric methods (7, 9–11). This HPLC method employs a solvent programming system requiring two pumps or a gradient generation system. The HPLC method in the present report utilizes an

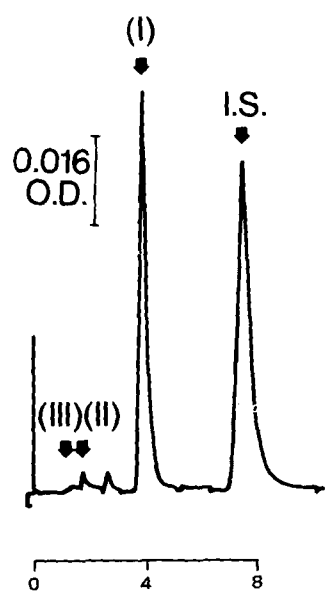


Figure 2—*Chromatogram of 250 μg of I and 700 μg of internal standard (I.S.) injected at 0.2 aufs.*

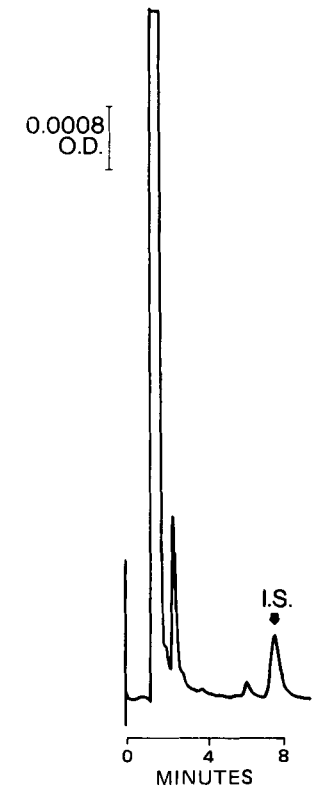


Figure 3—*Chromatogram of human plasma background. The equivalent to 10 μl of plasma and added internal standard (I.S.) were injected at 0.01 aufs.*

isocratic solvent system, and I is quantified using an internal standard. The internal standard, dansylproline, is added directly to plasma samples, after which I is extracted.

The statistics of recovery based on I *versus* the internal standard at 1, 5, 10, and 30 μg/ml indicate quantitative recovery of I. Furthermore, immediate extraction of I does not indicate any significant covalent binding to plasma proteins. When I was incubated in plasma at 37°, only 15% could be recovered after 6 hr. The diminished recovery at 37° may result from degradation or covalent plasma protein binding. The latter

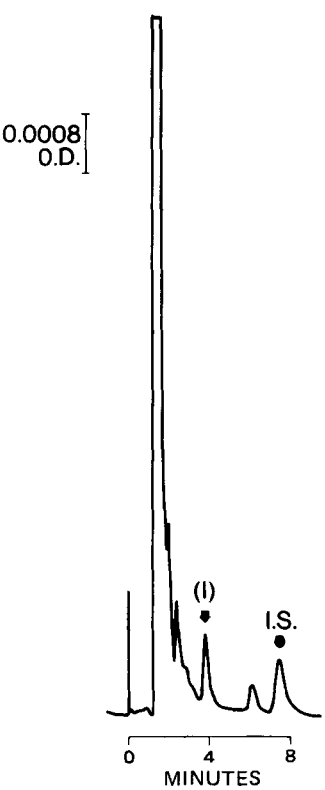


Figure 4—*Chromatogram of human plasma I concentration 4 hr after oral administration of 0.6 mg/kg. The final calculation indicates a concentration of 0.19 μg of I/ml of plasma.*

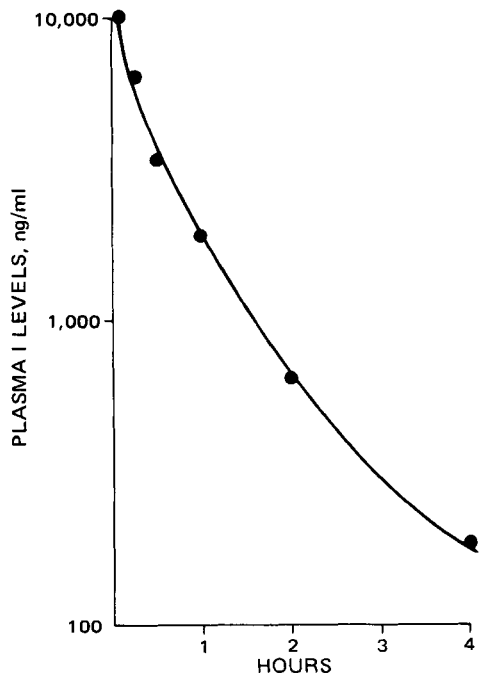


Figure 5—*Rat plasma I level. Compound I was administered to a rat at 6 mg/kg iv. Samples were drawn at the designated time points, and the plasma was analyzed with the HPLC method.*

is presumably not a major effect because similar degradation was observed in water (15).

The quantitative determination of II and III from biological samples is not possible because of background interference. Compounds II and III could be quantified in nonbiological fluids; however, to analyze these compounds in pharmacokinetic and biochemical pharmacology studies, a modification of the present method (*i.e.*, solvent programming) or other methods will be required.

The developed method is sensitive enough to measure the disappearance of I from human plasma after drug administration. Human pharmacokinetic studies are now underway. In preliminary studies, it was possible to measure plasma levels for as long as 13 hr after an intravenous dose of the drug. The minimum detectable level of I was 50 ng/ml of plasma. No important interfering peaks at the positions of I and the internal standard were observed.

The validity of the quantitative method has been confirmed by mass spectrometry. This HPLC method has been useful for the study of the degradation and protein binding properties of I (15).

REFERENCES

(1) F. Bergel and J. A. Stock, *J. Chem. Soc.*, **76**, 2409 (1954).

(2) R. B. Livingston and S. K. Carter, "Single Agents in Cancer Chemotherapy," Plenum, New York, N.Y., 1970, pp. 99–111.

(3) B. Clarkson, in "Antineoplastic and Immunosuppressive Agents 1," A. C. Sartorelli and D. G. Johns, Eds., Springer-Verlag, New York, N.Y., 1974, p. 172.

(4) R. Alexanenian, D. E. Bergsagel, P. J. Migliore, W. K. Vaughn, and C. D. Howe, *Blood*, **31**, 1 (1968).

(5) W. C. J. Ross, "Biological Alkylating Agents," Butterworths, London, England, 1962, pp. 19–31.

(6) J. H. Linford, *Biochem. Pharmacol.*, **8**, 343 (1961).

(7) O. M. Friedman and E. Boger, *Anal. Chem.*, **33**, 906 (1961).

(8) M. A. Chirigos and J. A. R. Mead, *Anal. Biochem.*, **7**, 259 (1964).

(9) O. Klatt, A. Griffin, and J. Stehlin, Jr., *Proc. Soc. Exp. Biol. Med.*, **104**, 629 (1960).

(10) J. Epstein, R. W. Rosenthal, and R. Ess, *Anal. Chem.*, **27**, 1435 (1955).

(11) H. Petering and G. Van Giessen, *J. Pharm. Sci.*, **52**, 1159 (1963).

(12) J. Goras, J. Knight, R. Iwamoto, and P. Liu, *ibid.*, **59**, 561 (1970).

(13) R. Furner, L. Mellett, R. Brown, and G. Duncan, *Drug. Metab.*

Dispos., **4**, 577 (1976).

(14) S. Nicolaidis, N. Rowland, M. Meile, P. Marfaing-Jallat, and A. Pesez, *Pharmacol. Biochem. Behav.*, **2**, 131 (1974).

(15) S. Y. Chang, D. S. Alberts, D. Farquhar, L. R. Melnick, P. D. Walson, and S. E. Salmon, *J. Pharm. Sci.*, **67**, 682 (1978).

ACKNOWLEDGMENTS

Supported in part by U.S. Public Health Service Grants CA-14102 and CA-17094 and research training Grant T22GM007103 from the National Institutes of Health, Bethesda, MD 20014.

Hydrolysis and Protein Binding of Melphalan

S. Y. CHANG ˣ, D. S. ALBERTS, D. FARQUHAR *, L. R. MELNICK, P. D. WALSON, and S. E. SALMON

Received April 22, 1977, from the *Section of Hematology and Oncology, Departments of Internal Medicine and Pharmacology, and the Cancer Center, College of Medicine, University of Arizona, Tucson, AZ 85724.* Accepted for publication September 1, 1977. *Department of Developmental Therapeutics, M. D. Anderson Hospital and Tumor Institute, Houston, TX 77025.

Abstract □ Melphalan (30 μg/ml) is completely hydrolyzed in water at 37° after 8 hr. At lower temperatures, hydrolysis proceeds at slower rates. The presence of bovine serum albumin retards hydrolysis of melphalan (30 μg/ml) in water. The melphalan hydrolysis rate is directly related to the bovine serum albumin concentration. At 37°, 8 g of bovine serum albumin/100 ml of water gives a recovery rate of melphalan similar to that of human plasma. *In vitro* alkylation of melphalan at 37° with human plasma containing 30 μg/ml, calculated by equilibrium dialysis, methanol extraction, and high-pressure liquid chromatographic analysis, is 30% after 8 hr.

Keyphrases □ Melphalan—hydrolysis, effect of bovine serum albumin, various temperatures □ Hydrolysis—melphalan, effect of bovine serum albumin, various temperatures □ Antineoplastic agents—melphalan, hydrolysis, effect of bovine serum albumin, various temperatures

The alkylation of nucleic acids and proteins by melphalan (I) has been studied using spectrophotometry, radiochemistry, and equilibrium dialysis (1, 2–4). Chirigos and Mead (2), using fluorometric detection and equilibrium dialysis, showed that 60% of I was covalently bound to plasma proteins at 26°. Other studies (1, 3, 4) also suggested covalent binding of I to proteins and nucleic acids.

It is necessary to understand the *in vitro* hydrolysis and alkylation of I to describe its pharmacokinetics in humans. This report documents the qualitative and quantitative aspects of adsorption, hydrolysis, and alkylation of I.

EXPERIMENTAL

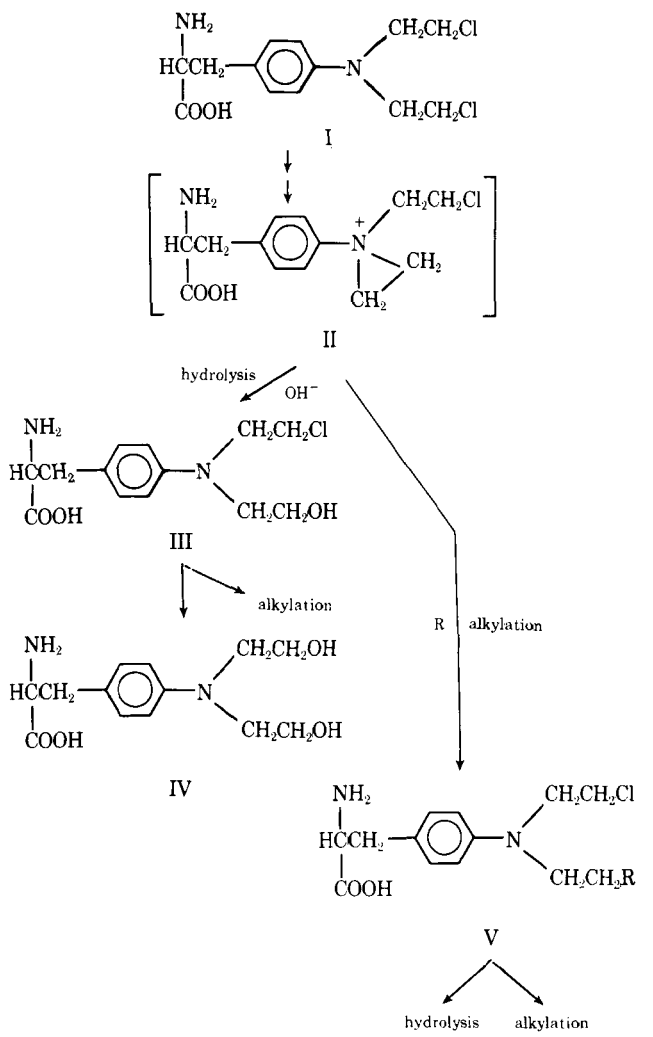
Materials—^{14}C-Melphalan (^{14}C-ethyl labeled) was synthesized according to the method of Bergel and Stock (5) and purified by high-pressure liquid chromatography (HPLC) (6). Dansylproline[1] was used as an internal standard for HPLC.

Assay of I—Compound I was extracted and analyzed using a previously described HPLC method (6).

Recovery Studies—The hydrolysis rate of I to III and IV in water (Scheme I) at 0, 23, and 37° was studied. Thirty-five micrograms of I and 70 μg of internal standard were added to 1 ml of water, and samples were taken at various times for HPLC analysis as previously described (6). The rate constants, k, were calculated according to $k = 2.3 \log ([I]_0/[I]_t)/t$, where $[I]_0$ and $[I]_t$ are the concentrations of I at times zero and t.

To study the effect of protein on the I hydrolysis rate in water, 30 μg of I and 70 μg of internal standard were added to increasing concentrations of bovine serum albumin[2] in water (*e.g.*, 8, 13, and 25 g/100 ml at 37°). At designated intervals, samples were taken for HPLC analysis.

The extractability of I from, and degradation in, human plasma was studied *in vitro* at 37°. Three hundred micrograms of I (1.2 μCi of carbon-14) and 700 μg of internal standard were added to 10 ml of human plasma, and 1-ml aliquots were extracted with methanol at given intervals



Scheme I

[1] Pierce Chemical Co., Rockford, Ill.

[2] Sigma Chemical Co., St. Louis, Mo.