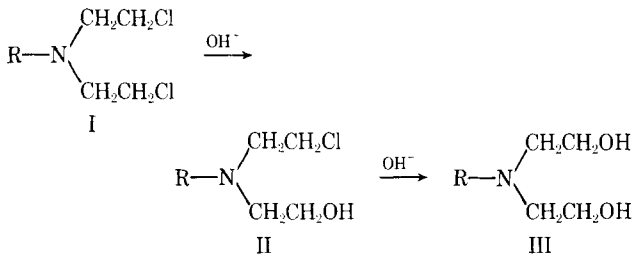
Gas-Liquid Chromatographic Determination of Melphalan

J. T. GORAS*, J. B. KNIGHT†, R. H. IWAMOTO†, and P. LIM†

Abstract □ The gas-liquid chromatography of melphalan and its decomposition products, determined as trimethylsilyl derivatives, is reported. The application of this procedure to the quantification of intact melphalan in a pharmaceutical preparation is described.

Keyphrases Melphalan and dosage forms—analysis □ GLC—analysis □ Chrysene—internal standard □ Mass spectroscopy—identification

The compound *p*-di(2-chloroethyl)amino-L-phenylalanine,[1] NSC 8806 (melphalan, L-sarcolysin, L-phenylalanine mustard), is a chemotherapeutic agent used in the treatment of various tumors (1–7). Because the drug is susceptible to hydrolytic degradation, an assay procedure that quantitatively measures intact melphalan in the presence of its hydrolysis products was desired. The hydrolytic degradation of melphalan (I) (8, 9) and related nitrogen mustards (10–12) has been reported as a replacement of one or both chlorine atoms by hydroxyl groups to give Forms II and III (see Scheme I).



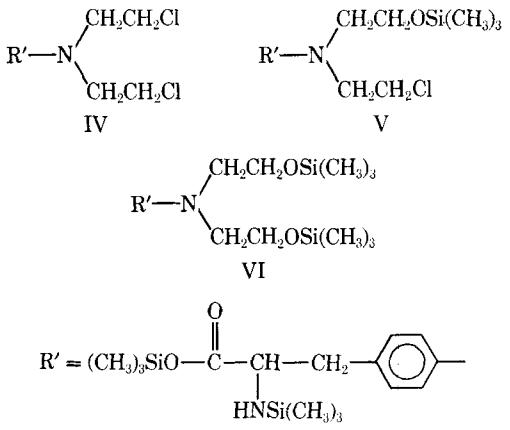
R = L-phenylalanine for melphalan

Scheme I

Quantitative procedures that have been applied to melphalan include direct UV spectrophotometry, ionic and total chlorine determination, spectrophotofluorimetry (8), and a colorimetric assay utilizing γ-(4-nitrobenzyl)pyridine (NBP) (12, 13). The first three procedures are inherently nonspecific in this application. The NBP assay has been employed by Friedman *et al.* (12) to study the hydrolysis of two simpler nitrogen mustards. However, it has been recently demonstrated that the NBP reagent is reactive with both Forms I and II of a mustard similar to melphalan and cannot be considered specific for the intact molecule (14).

Klebe *et al.* (15) have shown that, in the absence of moisture, many amino acids form stabile and volatile *N*- and *O*-bonded trimethylsilyl derivatives with bis(trimethylsilyl) acetamide (BSA) and may be assayed by GLC. Other silylating reagents have been used for quantitative preparations of trimethylsilyl derivatives of amino acids (16), but BSA was preferred for these experiments because of its higher reactivity as a silyl donor.

Quantitative conversion of amino acids to trimethylsilyl derivatives is accomplished simply by dissolving either the free amino acids or their hydrochlorides in an excess of BSA reagent. Degradation products II and III are capable of further reaction with BSA to form Compounds V and VI, respectively (see Scheme II).



Scheme II

Melphalan bulk, which served as a reference standard, was silylated with BSA and chromatographed as the trimethylsilyl derivative of the intact compound. A mixture of hydrolytic decomposition products was prepared, silylated, and chromatographed under the same conditions. Peaks having retention times of 1.1 and 1.3 relative to the intact melphalan derivative (IV) were recorded and identified as trimethylsilyl derivatives V and VI, respectively. The separation permitted the quantitative determination of intact melphalan in a freeze-dried dosage formulation containing mannitol. With the use of an internal standard, the assay is accurate as well as rapid and specific.

EXPERIMENTAL

Reagents and Materials—Melphalan bulk used in this study was provided by the Drug Distribution Unit of Cancer Chemotherapy National Service Center, National Institutes of Health. A sterile, freeze-dried intravenous dosage formulation of melphalan dihydrochloride was prepared from bulk material by this laboratory. Each vial contained 50 mg. of melphalan and 100 mg. of mannitol. A purified grade of BSA[2] in septum-stoppered vials was obtained for the experiment. A solution of chrysene[3] in chloroform (2.5 mg./ml.) was prepared fresh daily for use as an internal standard. The column packing employed for GLC was a laboratory preparation consisting of 2.5% (w/w) of a silicone polymer[4] on acid-washed,

[1] Manufactured as Alkeran by Burroughs Wellcome and Co.

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

[3] Aldrich Chemical Co., Milwaukee, Wis.

[4] SE-54, General Electric Co., Waterford, N. Y.

Table I—Mass Spectral Analysis of Fractions

Compound	Retention Time, min.	—Molecular Weight— Theory	Found
IV	12.6	448	447
V	15.2	502	502
VI	17.5	556	556

Table II—Retention Times

Compound	Relative Retention Time
IV	1.0
V	1.1
VI	1.3
Mannitol-TMS	0.13
Chrysene	0.66

silanized, 80/100 mesh diatomaceous earth.[5] All solvents were reagent grade quality and used as supplied.

Instrumentation—A gas chromatograph[6] equipped with a flame-ionization detector and an all-glass column (1.8 m. long, 3 mm. i.d.) was employed for quantitative work. Prepurified nitrogen was used as the carrier gas at a flow rate of 30 ml./min. The column oven was operated isothermally at 210°, the injector at 250°, and the detector at 215°. A similar gas chromatography system[7] was used to obtain fractions for mass spectrometry analysis. The mass spectral data were obtained from a modified mass spectrometer[8] with an upper limit of *m/e* 500 and with resolution capabilities of 1/1000.

Procedures—Derivatives were prepared for qualitative GLC analysis by reacting approximately 10 mg. of dry sample with 0.5 ml. of BSA. The reactions were carried out in 1-ml. septum-stoppered vials, and BSA was introduced with a hypodermic syringe to exclude atmospheric moisture. The reactions were complete in 15 min. at room temperature, and 3-μl. portions of the solutions were injected onto the column with a microliter syringe.[9] This procedure was followed using samples of melphalan bulk, melphalan freeze-dried dosage formulation, and hydrolytically degraded melphalan. The latter material was obtained by heating an aqueous solution of bulk melphalan at 40° and carefully monitoring the extent of the hydrolysis by measuring the amount of ionic chlorine liberated. When the concentration of ionic chlorine represented one-half of the total chlorine, the solution was cooled and lyophilized.

The quantification of intact melphalan in the freeze-dried dosage formulation was accomplished with the aid of an internal standard. Exactly 10.0 ml. of internal standard solution was added to the contents of each vial. The vials were fitted with septum-stoppered caps and 2.5 ml. of BSA was introduced with a hypodermic syringe. The vials were shaken and allowed to stand at room temperature for 1 hr. A syringe was used to withdraw 4-μl. portions of the solutions for injection onto the column. A standard solution was prepared for each series of samples by following the same procedure with an accurately weighed quantity of melphalan bulk approximately equivalent to that in the samples. The initial injection was used only to condition the column and the associated chromatogram was disregarded. Samples and standard solution were alternately chromatographed.

Calculations—Peak heights were measured from adjusted baselines and the peak height ratios of intact melphalan to chrysene were determined. The quantity of intact melphalan in the samples was determined by direct comparison of peak height ratios between samples and standard solution.

Identification of Derivatives—Samples of the trimethylsilyl derivatives were collected at the exit port of the gas chromatograph for identification. The collected fractions were protected from atmospheric moisture and immediately analyzed by mass spectrometry. The mass spectral data are summarized in Table I. In the case of the fraction representing the derivative of melphalan reference material, sufficient sample was isolated to allow recovery of the parent compound by hydrolysis of the trimethylsilyl groups. The resulting product, melphalan, was identified by IR spectroscopy and paper chromatography.

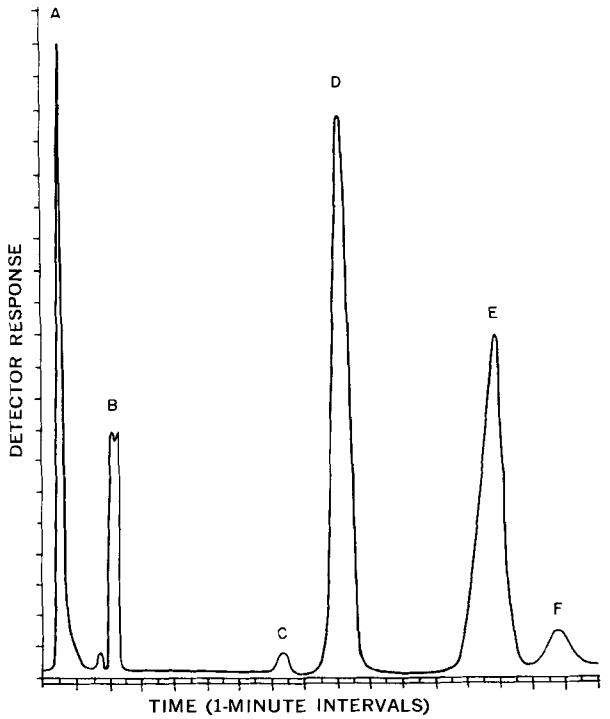


Figure 1—*Chromatogram of an assay preparation from freeze-dried dosage formulation: A = solvent, B = mannitol-TMS, C = impurity in chrysene, D = chrysene, E = melphalan-TMS (IV), F = decomposition product-TMS (V).*

RESULTS AND DISCUSSION

Qualitative Determinations—The silylated melphalan reference sample chromatographed as a single component. Three components, the first of which corresponded to the reference sample, were obtained when partially hydrolyzed material was silylated and chromatographed. Fragments common to *N,O*-bis(trimethylsilyl) derivatives of amino acids (17) and aryl amino acids (18) were prominent in the mass spectrum of each fraction. Molecular weights determined from the mass spectral data were in agreement with those calculated for IV, V, and VI. In addition, paper chromatographic and IR spectroscopic analyses established that the parent compound of the reference sample derivative was intact melphalan.

GLC analysis of silylated dosage formulation revealed the presence of V. Mannitol in the formulation eluted early as a silylated product and posed no problem. Relative retention times are given in Table II.

The same elution order was found when the derivatives were chromatographed using a relatively less polar liquid phase;[10] however, the order was reversed when a relatively more polar phase[11] was employed.

Quantification of Intact Melphalan in Dosage Formulation—A typical chromatogram for the assay of a freeze-dried dosage formulation preparation is shown in Fig. 1 (in this particular assay, Compound VI is not present). Chrysene was selected for the internal standard because it is nonreactive with BSA and no decomposition products elute at or near its retention time (0.66 relative to intact melphalan).

[5] Chromosorb W, Johns-Manville Corp., New York, N. Y.
[6] Perkin-Elmer model 801.
[7] Beckman model GC-5.
[8] Consolidated Electrodynamics Corp. model CEC-105.
[9] Hamilton Co., Whittier, Calif.
[10] SE-30, General Electric Co.
[11] OV-17, Applied Science Labs., Inc., State College, Pa.

The average of five replicate determinations for percent intact melphalan in a single vial of dosage formulation was 88.7% with a standard deviation of 1 (range = 2.5%).

Variations of less than 3% were found in the assay of several vials from the same production lot. The samples and reference solutions may be used for quantitative work up to 24 hr. after preparation.

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Synthesis of 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric Acid and Its Behavior in the 4-(4-Nitrobenzyl)pyridine Assay Procedure

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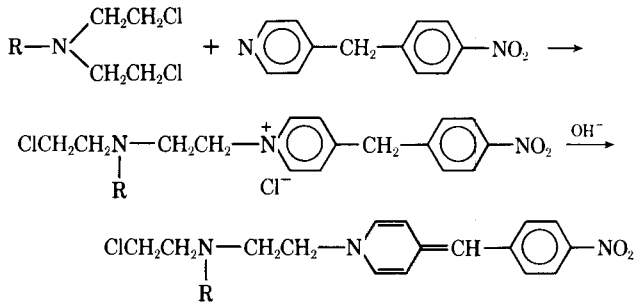
Abstract □ The synthesis of 4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)-amino]phenyl}butyric acid is reported. Investigation of the behavior of this compound in the NBP assay procedure showed that it alkylates NBP in a manner similar to chlorambucil. Therefore the results obtained from the assay of chlorambucil and similar aryl nitrogen mustards by the NBP method must be interpreted with due caution.

Keyphrases □ 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]-phenyl}butyric acid—synthesis □ 4-(4-Nitrobenzyl)pyridine alkylation—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}-butyric acid □ Chlorambucil analysis—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric acid interference □ Colorimetric analysis—spectrophotometer □ TLC—separation, identification □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The compound 4-(4-nitrobenzyl)pyridine (NBP) has been used as an analytical reagent for alkylating agents, among which are the nitrogen mustards such as melphalan,[1] chlorambucil,[2] and uracil mustard.[3] The general method involves the alkylation of NBP; subsequent basification results in the formation of a chromophore whose intensity can be measured photometrically.

The following reaction sequence has been suggested by Petering and Van Giessen (1) (Scheme I).



Scheme I

Holtzman (2) reportedly was able to isolate the product obtained from the reaction of mustard gas with NBP; he identified it as the mono-NBP product.

The NBP procedure has been considered in the authors' laboratories for the assay of chlorambucil. One

[1] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl-L-alanine.

[2] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl butyric acid was furnished by Cancer Chemotherapy National Service Center.

[3] 5-[Bis(2-chloroethyl)amino]uracil.