

Identification of Phosphorodiamidic Acid Mustard as a Human Metabolite of Cyclophosphamide¹

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SUMMARY

An active metabolite of cyclophosphamide, *N,N*-bis(2-chloroethyl)phosphorodiamidic acid, has been confirmed as a circulating and excreted metabolite in patients receiving the drug in therapy, by selected ion monitoring on a gas chromatograph mass spectrometer.

INTRODUCTION

We have recently reported the identification of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid as a metabolite isolated from the incubation *in vitro* of cyclophosphamide and mouse liver microsomes (2). This compound has been shown to be active against many experimental tumors, both *in vitro* and *in vivo* (7). It was of immediate importance to ascertain whether or not this compound is also formed *in vivo* in patients receiving the drug therapeutically. Our efforts to identify *N,N*-bis(2-chloroethyl)phosphorodiamidic acid in blood and urine of patients are reported here.

The amounts of material available to us from patients were much smaller than those from the *in vitro* study. Thus we decided to use the gas chromatograph mass spectrometer in its more sensitive multiple-ion-monitoring mode to search for *N,N*-bis(2-chloroethyl)phosphorodiamidic acid. Multiple-ion monitoring is a relatively new technique (4, 11) whereby preselected ions are monitored continuously by the mass spectrometer throughout a gas chromatographic separation. A signal is detected only when material is eluted from the gas chromatograph which fragments to the ions being monitored. Care must be taken to select peaks for monitoring that are not only intense but also highly characteristic of the compound.

MATERIALS AND METHODS

Clinical Samples. Blood was drawn from a patient 1 hr after the administration of a single dose of cyclophosphamide, 60 mg/kg (4800 mg). After addition of heparin, the sample was centrifuged and the plasma was decanted and stored at 0°. Urine samples from this patient and 1

additional patient were collected during the 4 hr following administration of the drug. Ten ml of plasma and 1 ml of urine were percolated at 4° through prewashed columns of Amberlite XAD-2 (Rohm and Haas, Philadelphia, Pa.) to remove the drug metabolites and other organic compounds from aqueous solution. The columns were washed with water, and then adsorbed material was recovered in methanol. Spectrophotometric assays of the alkylation of nitrobenzylpyridine (5) confirmed that greater than 90% of the alkylating activity applied to the column in urine was recovered in the methanol wash. The methanol solutions were dried and evaporated, and the residue was treated for 15 min at 0° with ethereal diazomethane prepared by the directions in the Aldrich Chemical Co. catalog.

This derivatized mixture was subjected to thin-layer chromatography on 250- μ m Silica GF plates (Analtech, Newark, Del.) using chloroform:methanol:ammonium hydroxide (132:15:0.7). Standard material spotted on the same plate was visualized with nitrobenzylpyridine to guide extraction of the appropriate band with methanol. This system does not separate the mono-, di-, and trimethyl derivatives of phosphorodiamidic acid mustard from each other, but it separates them as a group from methylcarboxycyclophosphamide and cyclophosphamide.

Synthetic *N,N*-Bis(2-chloroethyl)phosphorodiamidic Acid and Carboxyphosphamide. These were gifts from Dr. O. M. Friedman, Collaborative Research Corp., Waltham, Mass.

Gas Chromatography-Mass spectrometry. Analyses were carried out on a DuPont 491 mass spectrometer coupled with a glass jet molecular separator to a Varian 2700 gas chromatograph. The multiple-ion-monitoring unit is also a commercial unit (E. I. DuPont de Nemours & Co., Wilmington, Del.) which permits 4 ions to be monitored simultaneously through a 60% mass range. All 4 ions were measured once/sec. Plasma samples were analyzed on 3% OV-17 on Supelcoport and urine samples were analyzed on 3% Dexsil 300 on Supelcoport, both in 6-ft glass columns with the oven temperature programmed from 125° at 6°/min. The interface line was maintained at 250°, and the source was kept at 250°. Isobutane gas was used for chemical ionization.

RESULTS AND DISCUSSION

In both the *in vitro* study and the present *in vivo* study, we treated the extracts with diazomethane to produce deriva-

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tives suitable for gas chromatographic purification and mass spectral analysis. The reaction leads to a mixture of 3 products, carrying 1, 2, and 3 methyl groups, respectively. The major peaks found in the electron impact mass spectra (Charts 1 to 3) of all of these derivatives represent ions formed by cleavage in the C—C bond once removed from nitrogen. In each case the resulting species contains a chlorine atom (Scheme 1). Consequently, a pair of ions is detected differing by 2 mass units, the relative intensities of which are approximately 3:1, reflecting the natural abundance of ^{35}Cl and ^{37}Cl . These pairs of chlorine-containing ions were selected for monitoring because they offered the best combination of sensitivity and structural specificity. Other details of spectral interpretation have been published in our earlier study (2).

The multiple-ion monitor traces of ions of masses 199 and 201 (dimethyl derivative) and 213 and 215 (trimethyl derivative) are shown in Chart 4A, obtained with a sample of synthetic phosphoramidate mustard treated with diazomethane. A peak occurs whenever material that comes out of the gas chromatograph fragments in the mass spectrometer to give ions with one of the masses being monitored. The dimethyl and trimethyl derivatives are characterized by their retention times, by the formation of characteristic

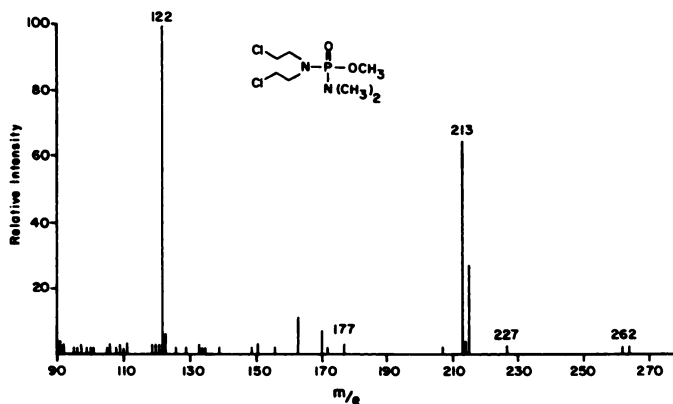


Chart 1. Electron impact mass spectrum of the trimethyl derivative of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid.

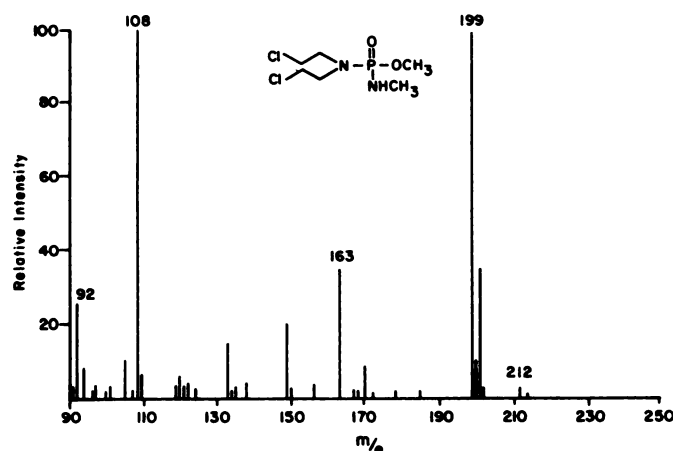


Chart 2. Electron impact mass spectrum of the dimethyl derivative of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid.

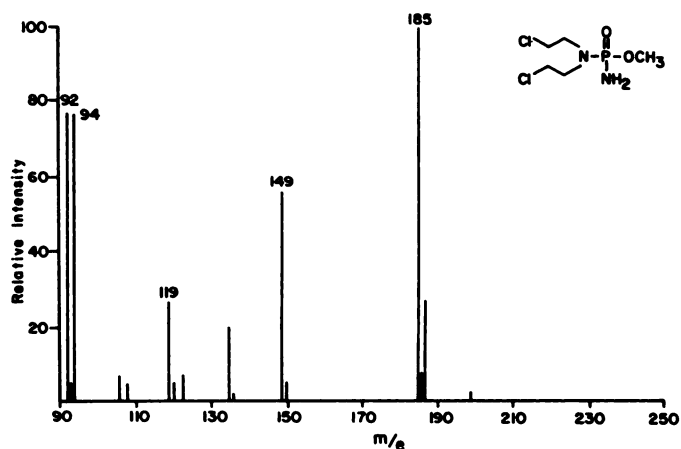
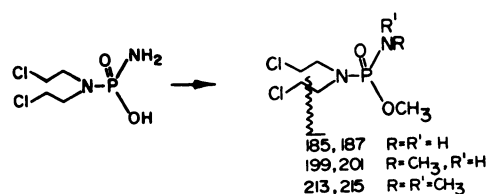


Chart 3. Electron impact mass spectrum of the monomethyl derivative of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid.



Scheme 1.

fragment ions, and by the relative intensities of the 2 chlorine isotopes.

Chart 4B shows the ion profiles of those same ions formed from components of an extract of 1 ml of human urine that has been treated with CH_2N_2 and separated on the gas chromatograph. The urine had been collected from a patient receiving cyclophosphamide in therapy. Although the sample is impure (as reflected to some extent in the ion profiles), it contains the dimethyl and trimethyl derivatives of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid. The monomethyl derivative was identified in a separate run where ions of masses 185 and 187 were monitored. Thus the cytotoxic *N,N*-bis(2-chloroethyl)phosphorodiamidic acid is identified as an excreted human metabolite of cyclophosphamide on the basis of the chemical history of the sample, the gc retention time, the formation of characteristic ions on electron impact, the occurrence of these ions with intensity ratios of about 3:1, and of course the characterization of all 3 derivatives.

Urine from a 2nd patient receiving cyclophosphamide was processed as described above, except that it was not treated with diazomethane. In this instance no peaks corresponding to methylated *N,N*-bis(2-chloroethyl)phosphorodiamidic acid were observed. When the remaining sample was then treated with diazomethane, the methylated derivatives were detected as described above. On the basis of these experiments it was concluded that these derivatives were produced by the chemical reaction and were not themselves metabolites.

In a 2nd control experiment 70 mg of the parent drug cyclophosphamide were added directly to 25 ml of urine from a patient who had not received the drug. Analysis of

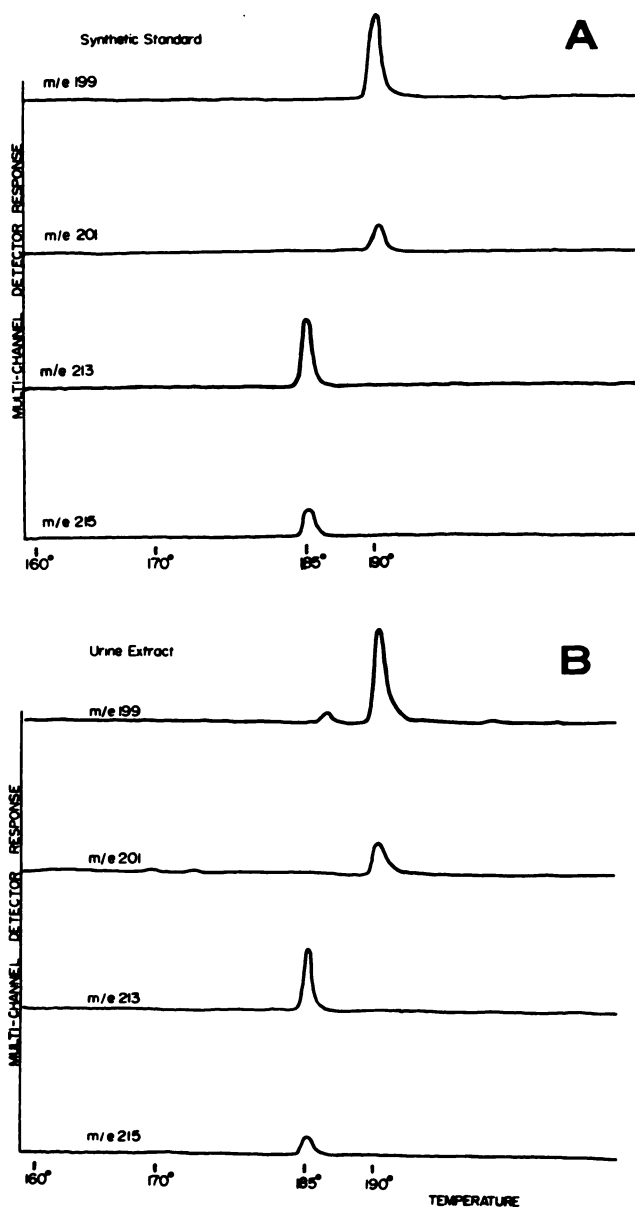


Chart 4. A, selected ion profiles of synthetic *N,N*-bis(2-chloroethyl)-phosphorodiamidic acid treated with diazomethane; B, selected ion profiles of urine extract treated with diazomethane.

this urine by the above procedure revealed no evidence of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid.

Similarly, carboxyphosphamide, the major urinary metabolite of cyclophosphamide (10), was tested to see whether its spontaneous degradation might produce *N,N*-bis(2-chloroethyl)phosphorodiamidic acid under the conditions of our sample preparation. A solution of 5 mg of carboxyphosphamide was run through the XAD column and treated by the procedure described above. Indeed, the monomethyl derivative of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid was detected with the electron multiplier of the mass spectrometer set at high gain. However, comparison of ion intensities in the selected ion profiles of material derived from the synthetic carboxyphosphamide standard and from

the original patient's urine indicated that concentrations of carboxyphosphamide in urine in excess of 15 mg/ml would be required to produce by decomposition the amount of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid detected in the patient's urine. Similarly, serum levels in excess of 4 mg/ml would be required to produce through decomposition the amount of *N,N*-bis(chloroethyl)phosphorodiamidic acid detected in the patient's serum. These levels are so high as to be incompatible with the dose administered to the patient (4800 mg).

The sensitivity and selectivity of multiple-ion monitoring can be illustrated by examining the conventional flame ionization detection gas chromatogram (Chart 5) obtained from this urine extract. The 2 arrows indicate the points in the gas chromatogram at which the multiple-ion monitor indicates that the dimethyl and trimethyl derivatives of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid are eluted. The mass spectrum scanned at the 1st arrow of material on the trailing edge of the 2nd large peak in the chromatogram, is shown in Chart 6. This is the spectrum of a diester of

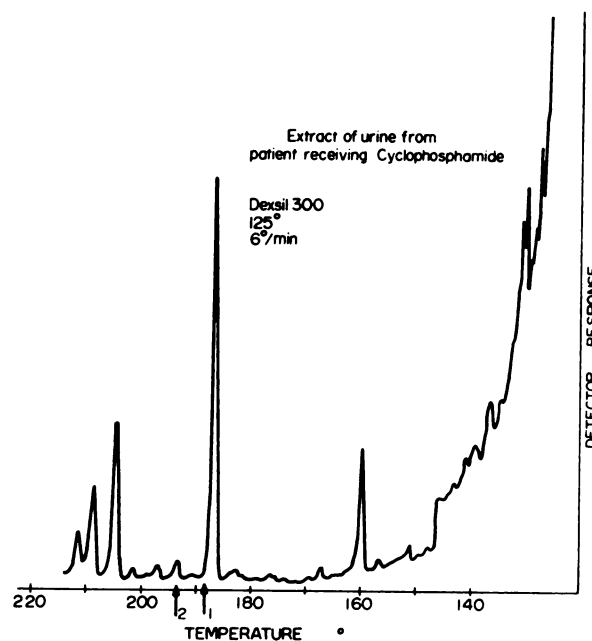


Chart 5. Gas chromatogram of derivatized urine extract traced by the flame ionization detector. Arrows, elution points of 2 metabolite derivatives.

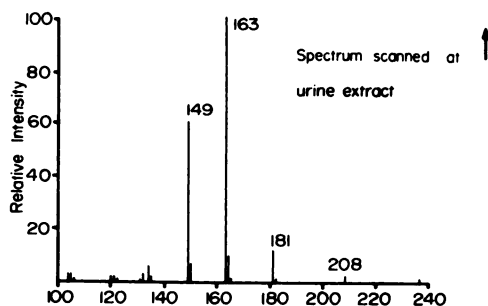


Chart 6. Electron impact mass spectrum scanned at the 1st arrow in Chart 5.

phthalic acid, a family of plasticizers often encountered as contaminants in biological samples. Despite the fact that the metabolite derivative is also eluted at this point, none of the ions formed from the metabolite show up with intensities as high as 1% relative to the ions of mass 163 formed from the plasticizer. Thus, it would have been impossible to identify the metabolite from the scanned spectrum. However, the multiple-ion monitor did not detect the plasticizer, since the plasticizer did not form ions with the masses being monitored. Rather, the multiple-ion monitor selectively identified the minor component of interest.

The sample extracted from plasma was similarly treated with diazomethane and analyzed using the multiple-ion monitor. Chart 7 shows the ion profiles measured using electron impact of the pairs of peaks expected from the monomethyl and dimethyl derivatives of the active metabolite. The monomethyl derivative usually comprises more than 80% of the mixture of products from the diazomethane reaction and should thus be the easiest compound to detect. Nonetheless, there is a great deal of interference in the selected ion profiles from other lipophilic material present in blood in concentrations comparable to that of any metabolite present. We decided to use chemical ionization (8) in the mass spectrometer in the hopes of simplifying spectra of the contaminating compounds and minimizing their contributions to the selected ion profiles.

The chemical-ionization mass spectrum of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid monomethyl ester is shown in Chart 8. The most intense peaks in the spectrum are seen to be the trio of $M + H$ peaks which represent ions containing the 3 possible combinations of 2 chlorine isotopes in the approximate intensity ratio of 9:6:1. These 3 ions were chosen for monitoring.

The ion profiles obtained with chemical ionization from a sample of synthetic *N,N*-bis(2-chloroethyl)phosphorodiamidic acid that had been treated with diazomethane are

shown in Chart 9A. The ion profiles for the derivatized extract of plasma from a patient receiving cyclophosphamide are shown in Chart 9B, and Chart 9C shows the ion profiles for a control sample extracted from blood of an untreated volunteer. The use of chemical ionization does simplify the selected ion profiles sufficiently to permit the identification of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid in plasma extract (as its monomethyl ester).

This work identifies *N,N*-bis(2-chloroethyl)phosphorodiamidic acid as a circulating human metabolite of cyclophosphamide on the basis of the chemical history of the sample, gc retention time, formation of characteristic ions, and the relative intensities of these ions.

N,N-Bis(2-chloroethyl)phosphorodiamidic acid is postulated to arise from the spontaneous (or possibly enzymatic) decomposition (2, 3) of an initial microsomal hydroxylation product, 4-hydroxycyclophosphamide (6, 12). Tautomerization of the 4-hydroxycyclophosphamide to 2-formylethyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (aldophosphamide) (6,9) and elimination of acrolein (1, 13) would yield phosphorodiamidic acid mustard. The relative

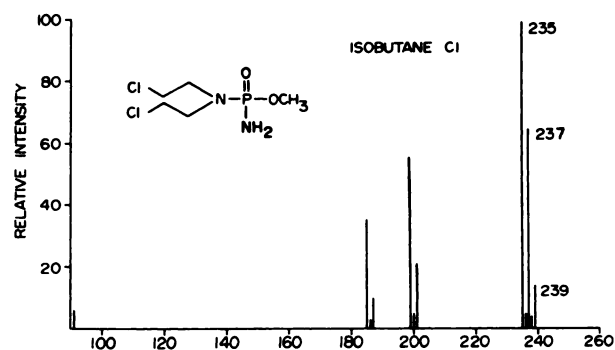
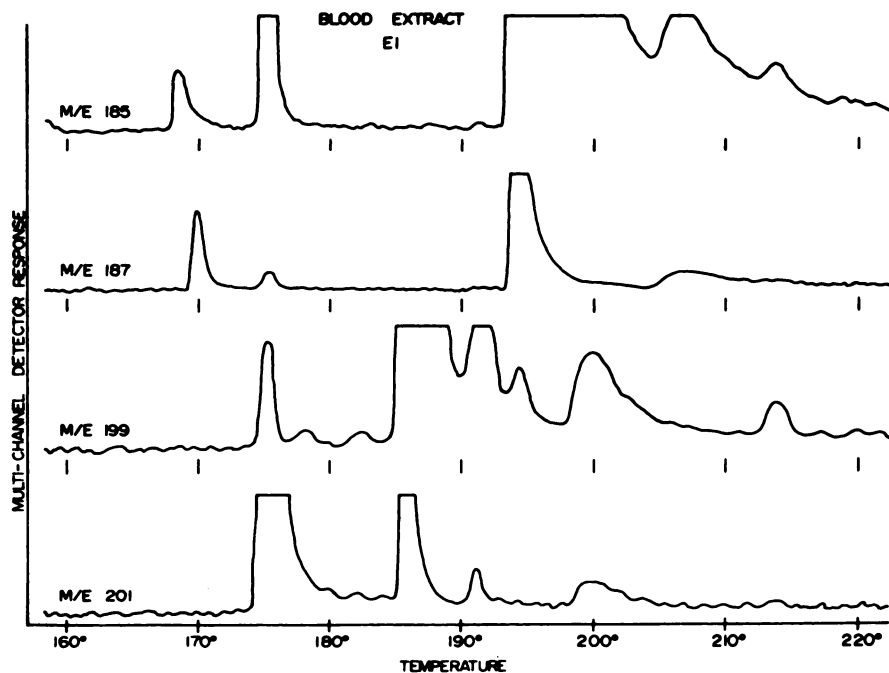


Chart 8. Isobutane chemical ionization mass spectrum of the monomethyl derivative of *N,N*-bis(2-chloroethyl)phosphorodiamidic acid.

Chart 7. Selected ion profiles measured by electron impact of serum extract treated with diazomethane.



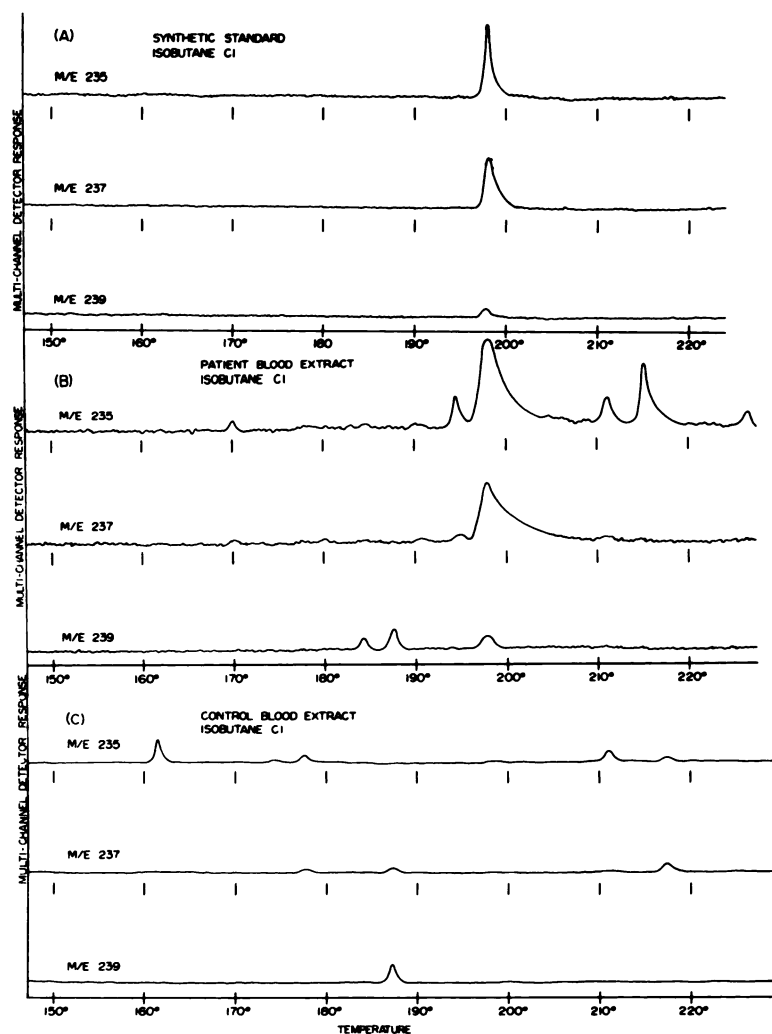


Chart 9. *A*, selected ion profiles of synthetic *N,N*-bis(2-chloroethyl)phosphorodiamidic acid treated with diazomethane. *B*, selected ion profiles of plasma extract treated with diazomethane. *C*, selected ion profiles of control plasma extract treated with diazomethane.

role of each compound in this sequence in producing the pharmacological effects of cyclophosphamide is uncertain. We believe that the measurement of these compounds in biological fluids after administration of cyclophosphamide is important to the understanding of the pharmacology of cyclophosphamide. The selected ion profiles are quantifiable, and we are now addressing our efforts to pharmacokinetic studies of this metabolite and to the identification of other circulating metabolites.

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