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# Gas chromatographic–mass spectrometric assay for N-2-chloroethylaziridine, a volatile cytotoxic metabolite of cyclophosphamide, in rat plasma

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**Abstract**

A sensitive and specific method for the quantitative analysis of N-2-chloroethylaziridine (CEA), a volatile cytotoxic metabolite of cyclophosphamide, has been developed using gas chromatography–mass spectrometry and stable isotope dilution techniques. The high volatility problem of CEA during isolation procedure was overcome by the combined use of a deuterium-labeled analog as the internal standard and a Snyder column–concentrator assembly. The assay was found to be linear from 16.7 to 2667 ng/ml in rat plasma with a routine detection limit of 5 ng/ml. The within-run precision at 33, 333 and 1333 ng/ml ($n = 6$) was found to be 4.8, 4.9, and 6.1%, respectively. The between-run precision was 6.4% ($n = 6$). The dichloromethane extraction recoveries at 33, 333, and 1333 ng/ml were found to be 101, 98, and 91%, respectively (all at $n = 6$). However, the overall recovery through extraction and evaporation was only 18.3, 15.2, and 27.7% at 33, 333, and 1333 ng/ml levels, respectively. The analytical method was used to evaluate the generation of CEA from its precursors in sodium phosphate buffer, in cell culture media, and the degradation of CEA in these media. In pH 7.4, 0.067 *M* sodium phosphate buffer at 37°C, both phosphoramide mustard (PM) and nornitrogen mustard (NNM) were degraded in an apparent first-order fashion with half-lives of 24.8 and 14.5 min, respectively. The generated CEA was rather stable in this buffer and degraded with a half-life of 20 h. It was found that 32% PM and 91% NNM were converted to CEA in pH 7.4, 0.067 *M* sodium phosphate buffer at 37°C, respectively, and 41% PM was transformed into CEA in RPMI 1640 tissue culture media containing 10% FBS at 37°C. The generated CEA was very stable in the culture media with a degradation half-life of 265 h.

*Keywords:* N-2-Chloroethylaziridine; Cyclophosphamide

## 1. Introduction

As early as 1968, N-2-chloroethylaziridine (CEA) was found to be a volatile metabolite of cyclophosphamide (CP), a widely used anticancer alkylating agent, and about 2% of the administered dose was excreted as CEA in the expired air of rats [1]. Little subsequent information was available until recently. Chan et al. [2] found that CEA was the major in vitro

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breakdown product of phosphoramide mustard (PM) and CEA was highly cytotoxic in cell culture system. Therefore, the study on the in vivo CEA disposition becomes important. However, no suitable assay method for its quantitation was available. The only described method was the 4-(*p*-nitrobenzyl)pyridine (NBP) assay [1] which was insensitive and nonspecific. Thus, we describe in this report the development of a GC–MS assay method for the analysis of CEA in plasma and other biological fluids using a stable isotope dilution technique.

## 2. Experimental

### 2.1. Chemicals and apparatus

PM as its cyclohexylamine salt (PM) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, the National Cancer Institute (Bethesda, MD, USA). [$\beta$-$^2H_4$]PM was synthesized in this laboratory [3]. $C_{18}$ Reversed-phase resin (Bondsil, Preparative grade, 40 $\mu$m) was purchased from Analytichem International (Harbor City, CA, USA). N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, USA). HPLC-grade methanol and methylene chloride were obtained from Curtin Matheson Scientific (Brea, CA, USA). Kuderna-Danish (K-D) concentrator tube (5ml capacity) and micro Snyder column (three bulbs) were purchased from ACE Glass (Louisville, KY, USA). Sodium metal, bis(2-chloroethyl)amine hydrochloride (nor-nitrogen mustard hydrochloride, NNM) and ethyleneglycol were obtained from Aldrich(Milwaukee, WI, USA).

### 2.2. Synthesis of CEA and [$\beta$-$^2H_4$]CEA

CEA was synthesized by the method of Avetyan et al. [4]. Sodium ethyleneglycolate was prepared by first dissolving sodium metal (0.7 g, 30 mmol) in 10 g ethyleneglycol in a 50-ml round-bottom flask immersed in an ice bath. Then, to the flask was added NNM hydrochloride (1.8 g, 10 mmol) and the mixture was heated to 90°C in an oil bath while maintaining under an $N_2$ atmosphere. The generated CEA was immediately distilled over to a collector immersed in dry-ice at a pressure of 70 mmHg. The reaction product obtained was found to be pure by TLC (silica gel, acetone–dichloromethane, 1:4, iodine vapor visualization) and GC–MS (*m/z* 106 and 108, corresponding to the $MH^+$ and its chlorine isotope peak, respectively) and the yield was 98%. [$\beta$-$^2H_4$]CEA (CEA-$d_4$) was synthesized by the same method using deuterium-labeled starting material, [$\beta$-$^2H_4$]bis(2-chloroethyl) amine hydrochloride synthesized previously in this laboratory [3].

### 2.3. Extraction and concentration

Appropriate amounts of CEA and 100 ng internal standard CEA-$d_4$ in 0.3 ml plasma were extracted once with 3 ml of methylene chloride. After centrifugation, the organic phase was removed and placed in a tube containing 0.5 g anhydrous sodium sulfate. The mixture was allowed to stand at room temperature for 5 min and the solution was transfered into a K-D tube coupled to a Snyder column. The two units were held together via two springs. The methylene chloride in the Snyder column-concentrator was evaporated to about 10 $\mu$l in a 90°C water bath. A 2-$\mu$l aliquot was injected into the GC–MS.

### 2.4. Gas chromatography–mass spectrometry

GC–MS analysis of CEA and its deuterium-labeled analog was carried out on a Finnigan ITS40 ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) directly coupled to a 3300/3400 Varian gas chromatograph (Walnut Creek, CA, USA) via a capillary splitless injector. The mixture was separated on a DB-5 fused-silica capillary column (30 m × 0.25 mm I.D.) bonded with a 0.25 $\mu$m film thickness of 5% methylsilicone (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas with a head pressure set at 15 p.s.i. (ca. $10^5$ Pa). Chemical ionization mode was used with ammonia as the reagent gas. The temperatures of the injection port, transfer line and source were set at

160, 220, and 230°C, respectively. The initial oven temperature was maintained at 60°C for 4 min and increased to 150°C at a rate of 10°C/min. Under these conditions, the retention times of CEA and CEA-$d_4$ were found to be 3.58, and 3.48 min, respectively. Quantitation was performed by measuring the area ratios between ions at $m/z$ 106 and 110 corresponding to CEA and CEA-$d_4$, respectively.

### 2.5. Calibration curve

Appropriate amounts of CEA ranging from 5 to 800 ng were each placed into nine test tubes, followed by addition of 100 ng of CEA-$d_4$ and 0.3 ml of rat plasma. After extraction and evaporation, samples were analyzed on the GC–MS. A standard curve was obtained by plotting the peak-area ratios of analyte to the internal standard against amounts of CEA.

### 2.6. Within- and between-run precisions

Within-run precision of this method was evaluated at 10, 100, and 400 ng levels in 0.3 ml rat plasma. Six replicate samples were determined at each level. The between-run precision was evaluated by estimation of the coefficient of variation of slopes of standard curves at six different days.

### 2.7. Recovery

The CEA extraction recovery at 10, 100, and 400 ng per 0.3 ml was evaluated. Six replicates at each drug level without the internal standard were extracted with 3 ml dichloromethane. Then, to each of these tubes containing the organic solution was added 100 ng of the internal standard. After evaporation, CEA in the residue was analyzed by the GC–MS. At the same time, six control samples in dichloromethane containing the same amount of CEA and the internal standard without extraction were evaporated and analyzed. The average value of the ion intensity ratios between CEA and the internal standard in the control was used to calculate the recovery.

### 2.8. Production of CEA from PM in pH 7.4, 37°C sodium phosphate buffer

PM in 0.067 *M* pH 7.4 sodium phosphate buffer at 0.14 m*M* was incubated in a 37°C water bath. At a predetermined time schedule of 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 270, 360, 460, 600, 840, 1500, 1770, 2940, 3180, 4350, and 4620 min following incubation, a 50-$\mu$l aliquot each was removed for PM and CEA analysis. A duplicate sample was removed at each time point. The concentrations of CEA in the PM solution were followed by the previously described assay procedure. The decay of PM concentrations was measured by the method of Watson et al. [5] using PM-$d_4$ as the internal standard. The trimethylsilyl derivatives of the dechlorinated PM and its $d_4$ analog were eluted at 10.1 and 10.0 min, respectively. Ions at $m/z$ 329 and 333 were selectively monitored.

### 2.9. Production of CEA from PM in tissue culture medium

PM in RPMI 1640 containing 10% fetal bovine serum at 0.13 m*M* was incubated at 37°C. A 50-$\mu$l aliquot each was removed at 0, 5, 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 300, 360, 795, 1460, 2110, 2950, 3190, 3550, and 4510 min from the start of incubation for PM and CEA analysis. The experiment was done in duplicate and CEA and PM concentrations were measured as before.

### 2.10. Production of CEA from NNM in pH 7.4, 37°C sodium phosphate buffer

NNM at 0.030 m*M* in 0.067 M, pH 7.4 sodium phosphate buffer was incubated in a 37°C water bath. For NNM and CEA analysis, a 50-$\mu$l aliquot each was removed at 0, 5, 10, 20, 30, 45, 60, 90, 120, 190, 315, 422, 645, 1500, 1680, 3105, 3315, 4335 and 4695 min from the start of incubation. The analytical method for NNM in sodium phosphate buffer, including extraction, evaporation, and chromatography conditions, was the same as for CEA except for using [$\alpha,\beta$-$^2H_8$]NNM as the internal standard. The CEA

and NNM concentrations were determined at the same time. A calibration curve of NNM in the buffer was constructed to measure NNM concentration. The ions monitored were those at $m/z$ 142 and 150 for NNM and NNM-$d_8$, respectively. The respective retention times for NNM and NNM-$d_8$ were 8.3 and 8.2 min under the same chromatographic conditions as used for CEA.

### 2.11. Data analysis

Regression analysis of the PM, NNM, and CEA concentration–time data was accomplished using PCNONLIN (Statistical Consultants, Lexington, KY, USA) on an IBM PC.

## 3. Results and discussion

### 3.1. GC–MS assay of CEA in rat plasma

The GC–MS chromatograms of blank plasma and plasma spiked with 500 ng CEA and 100 ng of internal standard CEA-$d_4$ are shown in Fig. 1. As shown, no interference in the region of interest was found in blank plasma. The assay was found to be a linear from 16.7 to 2667 ng/ml in rat plasma. The routine assay detection limit was found to be 5.0 ng/ml. The within-run precisions at 33, 333, and 1333 ng/ml on six replicate determinations at each level were found to be 4.8, 4.9, and 6.1%, respectively. The between-run precision was found to be 6.4% ($n$ = 6). The extraction recoveries from dichloromethane at 33,333, and 1333 ng/ml were found to be 101, 98, and 91% (all $n$ = 6). The accuracy of the assay at 33, 333, and 1333 ng/ml was found to be 102, 110, and 95.8%, respectively.

CEA is a highly volatile compound and readily polymerizes in pure form. Following solvent extraction, evaporation and concentration step by $N_2$ stream cannot be used because of excess sample loss. The use of a K-D concentrator and Synder column assembly alleviated this problem and greatly reduced the CEA loss. This technique has been successfully used for the assay of highly volatile nitrosoamines [6,7]. It should be emphasized that the extraction solvent used in this method should be more volatile than the analyte of interest. Even so, the recovery of CEA using the K-D tube assembly was unexpectedly low, yielding only $18.3 \pm 6.8$, $15.2 \pm 7.4$, and $27.7 \pm 10.7$% at 33, 333, and 1333 ng/ml levels, respectively. However, Sen and Seaman [7] obtained about 90% recovery for nitrosamines using the same method. Thus, the observed low recovery might be due to polymerization degradation of CEA. The use of a stable isotopically labeled internal standard, however, provided satisfactory results, despite of the low recovery.

### 3.2. Production and stability of CEA

The degradation profile of PM in pH 7.4, 0.067 $M$ sodium phosphate buffer at 37°C is shown in Fig. 2. As shown, PM degraded in an apparent first-order manner with a half-life of 24.8 min. CEA was found to be the major degradation product, which was similar to results previously obtained in this laboratory [2], CEA concentrations were found to peak at 176 min and decline significantly slower than those of PM with an apparent half-life of 1210 min. No NNM was detected. It has been shown previously that the major hydrolytic pathway of PM is via the direct P–N bond cleavage at pH values below the p$K_a$ of PM or via the formation of aziridinium ion, followed by a nucleophilic attack on the aziridine ring at pH values above the p$K_a$ of PM [5]. The recent finding [2] and the current data indicated that the P–N bond cleavage from the aziridinium ion to generate CEA also occurred to a large extent. Therefore, the proposed overall hydrolysis pathways of PM in vitro is shown in Fig. 3. in this report. The fraction of PM that is transformed into CEA in pH 7.4 sodium phosphate buffer, fd, can be calculated by using following equation.

$$\mathrm{fd} = \mathrm{AUC}_{\mathrm{CEA}} / (C_0 / K_{\mathrm{CEA}})$$

where $\mathrm{AUC}_{\mathrm{CEA}}$ is the area under the concentration-time curves of generated CEA. $C_0$ is the initial concentration of PM in buffer and $K_{\mathrm{CEA}}$ is the apparent first-order degradation rate con-

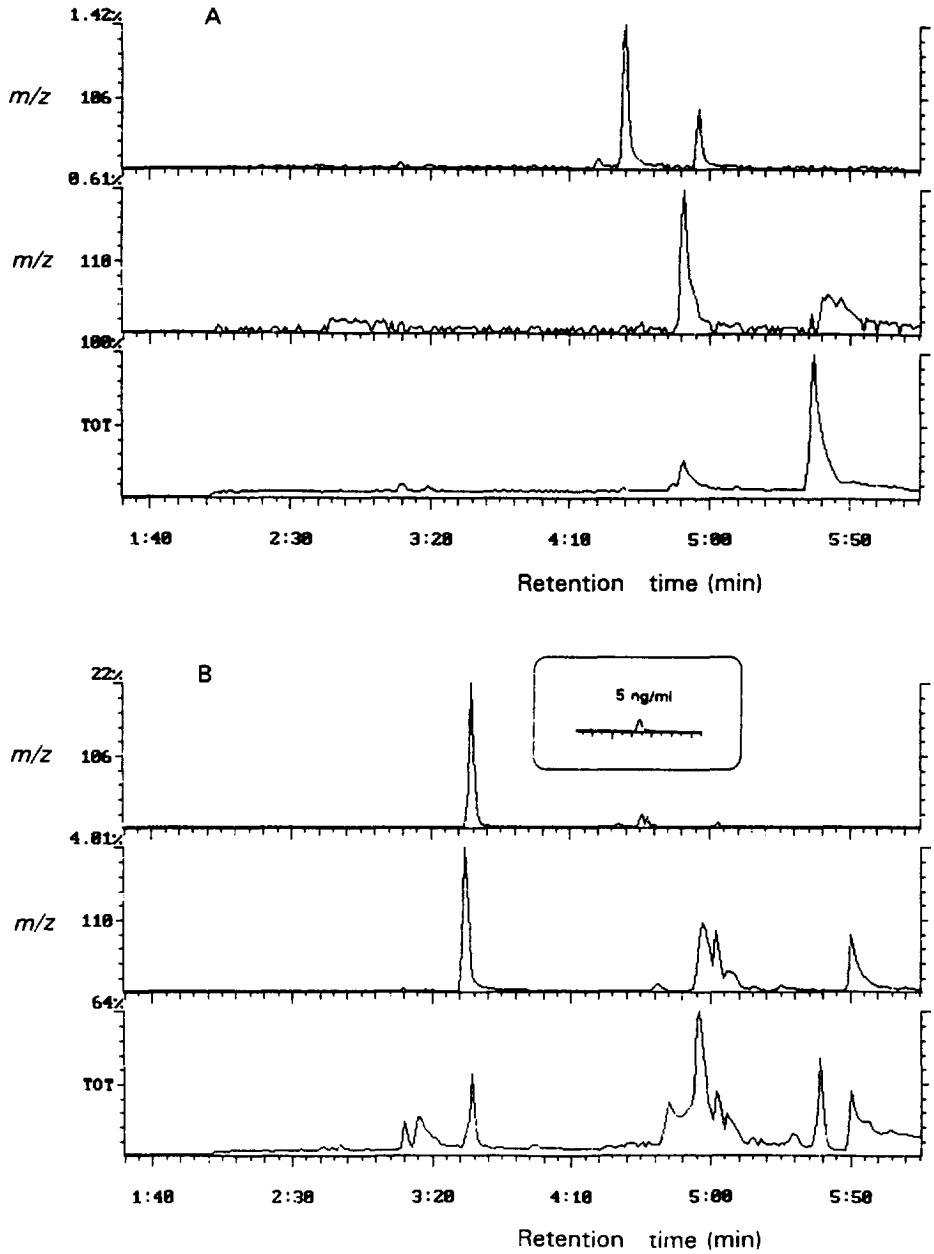


Fig. 1. Typical GC–mass ion chromatograms of (A) blank rat plasma and (B) those spiked with CEA, CEA-$d_4$; $m/z$ 106, $MH^+$ for CEA and $m/z$ 110, $MH^+$ for CEA-$d_4$.

stant of generated CEA in buffer. The calculation indicated that 32% of PM was converted to CEA in pH 7.4 sodium phosphate buffer.

The degradation profile of PM in tissue culture media is shown in Fig. 4. As shown, the degradation of PM follows an apparent first-order process with a half-life of 37.1 min. Again, CEA was found to be the major degradation product and its concentrations peaked at 230 min and declined slowly with an apparent half-life of 265

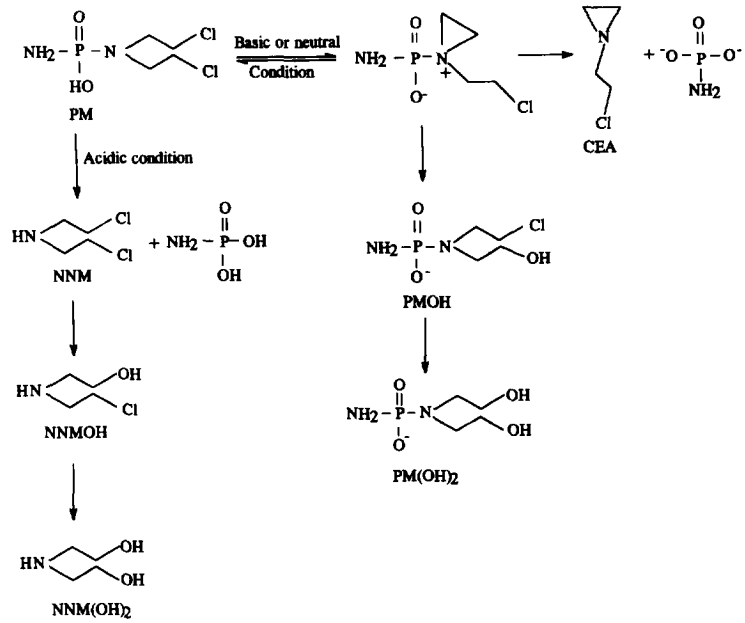


Fig. 3. Hydrolytic pathways of PM in vitro.

h, which indicated that CEA was extremely stable in tissue culture media. About 41% of PM was degraded into CEA as estimated by the AUC method described as before.

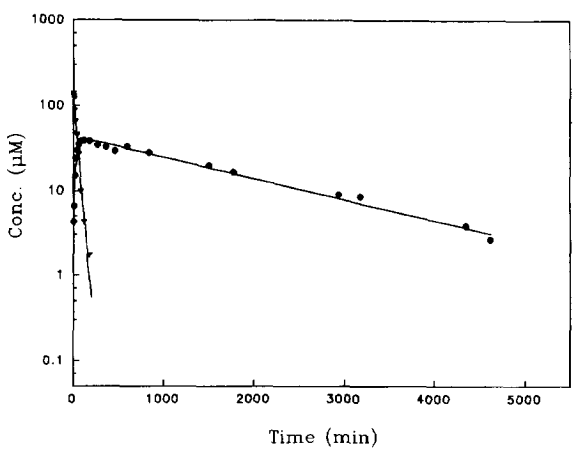


Fig. 2. The PM (▼) and CEA (●) concentration–time profiles, following incubation of 0.14 m*M* PM in pH 7.4, 0.067 *M* sodium phosphate buffer at 37°C. The curves are regressed to equations: $C_{PM} = C_0\, e^{-K_f t}$ and $C_{CEA} = A\,(e^{-K_{CEA} t} - e^{-K_f t})$, where $C_0$ is initial concentration of PM, $A = C_0^{K_f/(K_f - K_{CEA})}$. $K_f$ and $K_{CEA}$ are apparent first-order formation and degradation rate constants of CEA, respectively. $K$ is degradation rate constant of PM.

The degradation profile of NNM in pH 7.4, 0.067 *M* sodium phosphate buffer at 37°C is shown in Fig. 5. NNM degraded monoexponentially with a half-life of 14.5 min. The major product CEA concentrations peaked at 81.1 min and declined much slowly with an apparent half-life of 1187 min. About 91% of NNM was

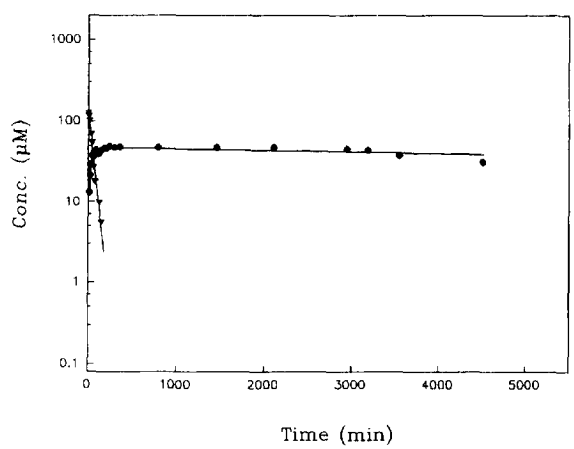


Fig. 4. PM (▼) and CEA (●) concentration–time profiles after incubation of 0.13 m*M* PM in RPMI 1640 tissue culture media containing 10% of fetal bovine serum at 37°C. The curves are best fitting by using the same method as Fig. 2.

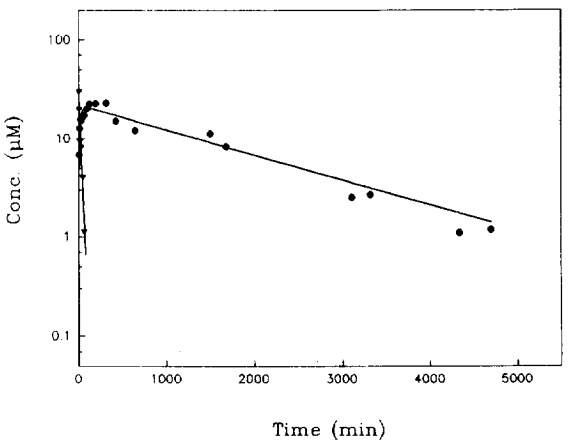


Fig. 5. NNM (▼) and CEA (●) concentration–time profiles after incubation of 30 $\mu M$ NNM in pH 7.4, 0.067 $M$ sodium phosphate buffer at 37°C. The curves are best fitting by using the same method as Fig. 2.

estimated to be converted to CEA. These results suggest that CEA formation from NNM is a favorable process under a slightly basic condition.

## 4. Conclusion

A GC–MS method for the quantitative determination of CEA in aqueous media has been developed. This method demonstrated that the combined use of a stable isotopically labeled internal standard and a Kuderna-Danish concentrator–evaporator could minimize evaporation loss for volatile analytes. Using this method, quantitative data for a newly described major degradation pathway of PM in aqueous media to generate CEA is presented. This information may be important to relate to the antitumor activity of PM.

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