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## Short Communication

# Determination of nor-nitrogen mustard hydrochloride using gas chromatography with flame ionization detection

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ABSTRACT

A megabore gas chromatographic method with flame ionization detection was developed for the determination of nor-nitrogen mustard hydrochloride (C.A. No. 821-48-7) in toluene. The method is based on a derivatization procedure with trifluoroacetic anhydride. Benzylmethylamine was used as an internal standard. The calibration graph was linear within the range investigated (2–1050 μg/ml in toluene) with a correlation coefficient of 0.9999. The relative overall recovery was 96 ± 1.5% for a concentration of 20 μg of nor-nitrogen mustard hydrochloride per ml of toluene and 100 ± 3% for 200 μg/ml. The minimum detectable concentration in toluene was less than 0.5 μg/ml. The method is applicable to the determination of nor-nitrogen mustard hydrochloride in industrial working environments to establish occupational exposure to the compound.

## INTRODUCTION

Nor-nitrogen mustard hydrochloride [bis-(2-chloroethyl)amine hydrochloride] is a commercially important amine used as a raw material in the production of various cytostatic drugs such as cyclophosphamide [1] and estramustine disodium phosphate.

Nor-nitrogen mustard is a mutagenic substance and its genetic toxicology has been reviewed in detail by Fox and Scott [2]. The compound has been shown to induce mutations in *Salmonella his* [3] and chromosomal damage in Chinese hamster cells [4]. Cytogenetic experiments with human lymphocytes have revealed that nor-nitrogen mustard induces a dose-dependent increase in chromosomal aberrations and sister chromatic exchanges [5].

Nor-nitrogen mustard hydrochloride is a white crystalline powder. In the production of cytostatic drugs it is manually handled during weighing and charging operations. Occupational exposure can arise from airborn dust or contaminated surfaces and therefore the monitoring of this substance in working environments is of

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major importance. The monitoring consists both of sampling and analytical procedures. The sampling procedures will be described in a separate paper [6].

The direct gas chromatographic (GC) analysis of free nor-nitrogen mustard in a solvent is impaired by peak taling. To overcome this tailing effect, trifluoroacetic anhydride (TFAA) derivatizations have been used in gas–liquid chromatography [7]. This method has been used to determine nor-nitrogen mustard in human serum and urine using gas chromatography–mass spectrometry (GC–MS) in the chemical ionization mode with tetradeuterated nor-nitrogen mustard as the internal standard [8,9].

The aim of this study was to develop an accessible and simple method for the determination of nor-nitrogen mustard hydrochloride which is applicable for the levels found in industrial working environments.

## EXPERIMENTAL

### *Apparatus*

A Varian 3400 gas chromatograph equipped with an on-column injector and a flame ionization detector was used. The column temperature was maintained at 120°C, the injection port and detector temperatures were 230°C. Nitrogen was used as the carrier gas at a flow-rate of 15 ml/min and as the make-up gas at a flow-rate of 20 ml/min. The gas chromatograph was equipped with a recorder and an integrator, which was used for peak evaluation. A 0.5-$\mu$l volume was injected using a Hamilton syringe.

A Finnigan MAT TSQ 70 mass spectrometer connected to a Hewlett-Packard 5890 A gas chromatograph was used for the identification of the derivatives. The mass spectrometer was used in the electron-impact mode with the ionization energy set at 70 eV.

$^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC-250 NMR instrument with tetramethylsilane as the internal standard deuterochloroform ($C^{2}HCl_3$) solution.

A Rotavapor R (Büchi) was used for the evaporation of toluene containing the synthesis standards of TFAA derivatives of nor-nitrogen mustard and benzylmethylamine (BMA). For the enrichment of samples the toluene, containing trifluoroacetic anhydride derivatives, was evaporated in a stream of nitrogen.

### *Columns*

A fused-silica megabore column with a chemically bonded stationary phase, DB-1 (J&W Scientific), 30 m × 0.53 mm I.D., with a film thickness of 1.5 $\mu$m was used.

For the GC–MS determinations a capillary column with cross-linked methylsilicone gum, HP 1 (Hewlett-Packard), 12 m × 0.2 mm I.D., with a film thickness of 0.33 $\mu$m was used.

### *Chemicals*

The chemicals used were nor-nitrogen mustard hydrochloride (synthesized at Kabi Pharmacia (Helsingborg, Sweden), and acetone, chloroform, ethyl acetate and toluene from BDH (Poole, UK). Phosphate buffer (pH 7.0), diisobutylamine and

TFAA from Merck (Darmstadt, Germany), BMA, di-*n*-butylamine and thionyl chloride from Janssen (Beerse, Belgium), diethanolamine from Aldrich (Steinheim, Germany), ethanol from Kemetyl (Stockholm, Sweden) and diethyl ether from Lab-Scan (Dublin, Ireland) were also used.

*Synthesis of nor-nitrogen mustard hydrochloride*

In a 250-ml three necked, round-bottomed flask equipped with a stirrer, reflux condenser and a dropping funnel, 10.5 g (0.1 mol) of diethanolamine were dissolved in 50 ml of chloroform.

The mixture was heated to near reflux and a solution of 28.3 ml (0.4 mol) of thionyl chloride in 50 ml of chloroform was added dropwise with stirring. Reflux was maintained during the addition and for another 2 h. After reflux, about 50 ml of the chloroform were distilled off. The reaction mixture was cooled, transferred into a glass beaker and diethyl ether was added to precipitate the hydrochloride.

The precipitate was filtered off, washed with diethyl ether and suspended in 200 ml of acetone. The mixture was heated and ethanol was added cautiously until a clear solution was obtained. The nor-nitrogen mustard hydrochloride crystallized on cooling and was filtered, washed with acetone and dried under vacuum at room temperature.

*Synthesis of TFAA derivatives of nor-nitrogen mustard and BMA*

A 1-ml (7 mmol) volume of TFAA was slowly added to a solution containing 0.5 g (2.8 mmol) of nor-nitrogen mustard hydrochloride in 50 ml of toluene. The mixture was heated to 70°C for 20 min and then extracted with phosphate buffer (pH 7.0) to remove any excess of the reagent and acid formed. The organic phase was separated and the toluene was evaporated on a Rotavapor evaporator. The TFAA derivative of the internal standard (BMA) was synthesized in a similar manner. The two derivatives are liquid at room temperature.

*Preparation of standard solutions*

Standard solutions of the derivatives of TFAA were prepared by dissolving accurately weighed amounts of each derivative in toluene. The solutions were further diluted in toluene to the appropriate concentrations. Standard solutions of the derivatives of TFAA were also prepared using the work-up procedure.

The stability of the standard in light is good and they may be kept in the laboratory at room temperature for months without degradation. The recovery after 2 months for the investigated concentration range (2–300 μg/ml) was 97.6 ± 1.9% ($n$ = 9). The values are given with a 95% confidence limit (Student's $t$-distribution [10]).

The internal standard used was BMA.

*Work-up procedure*

To a 25-ml Erlenmeyer flask 5 ml of toluene, 6 mg of nor-nitrogen mustard hydrochloride, 1 μl of BMA and 0.2 ml of TFAA were added. The mixture was immediately shaken for 5 min. The excess of anhydride reagent and acid formed were removed by extraction with 5 ml of phosphate buffer solution (pH 7.0). The toluene layer containing the derivatives was then transferred into a calibrated flask and further diluted with toluene to the appropriate concentrations.

*Enrichment*

For enrichment the solvent was evaporated under a low flow-rate of nitrogen. The volume was reduced from 1 ml to *ca.* 100 μl or to dryness. The dry residue was dissolved in 100 μl of ethyl acetate.

## RESULTS AND DISCUSSION

*Standards*

The identity of the nor-nitrogen mustard hydrochloride was confirmed by NMR and the purity by titration with perchloric acid. The purity was 99%.

The identities of the TFAA derivatives prepared were confirmed by GC–MS (Fig. 1 and 2) and NMR. The purity was determined using megabore GC–flame ionization detection (FID). The purity was greater than 99%. The purity was further examined by elemental analysis and the percentage composition of carbon, hydrogen and nitrogen are shown in Table I.

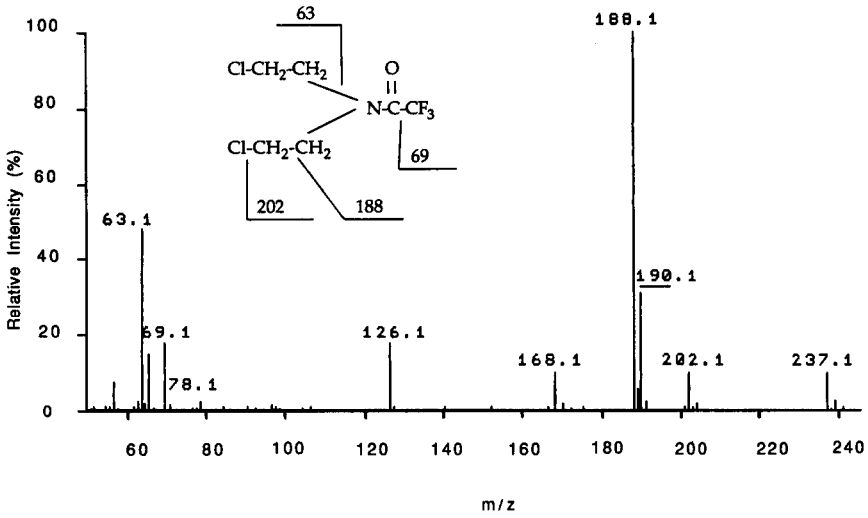


Fig. 1. Mass spectrum of TFAA derivative of nor-nitrogen mustard obtained in the electron-impact mode at an ionization potential of 70 eV.

*Internal standard.* BMA, di-isobutylamine and di-*n*-butylamine were tested for their suitability as internal standards. Di-isobutylamine and di-*n*-butylamine gave unsatisfactory results. The di-isobutylamine derivative performed differently to nor-nitrogen mustard during the enrichment procedure and the di-*n*-butylamine derivative was not separated from the nor-nitrogen mustard derivative in the GC analysis.

BMA was the best choice as it gave a similar performance to nor-nitrogen mustard hydrochloride during the work-up procedure. In addition, it is not expected to be present in the same working environment as the compound under investigation.

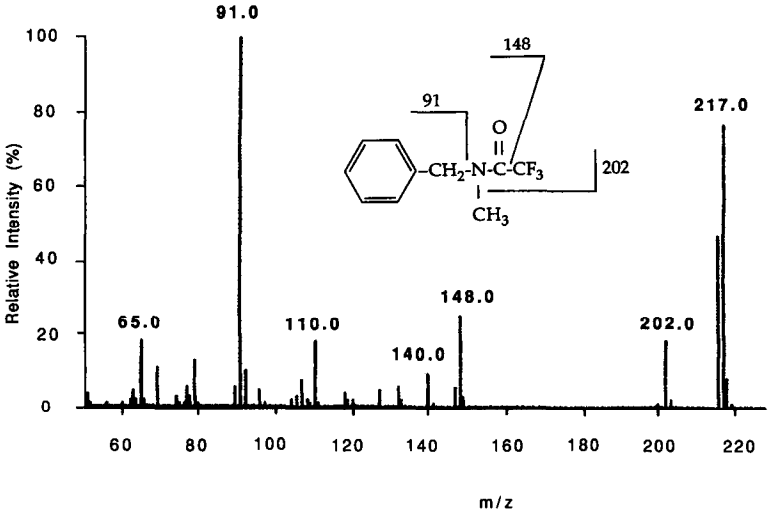


Fig. 2. Mass spectrum of TFAA derivative of BMA obtained in the electron-impact mode at an ionization potential of 70 eV.

TABLE I

ELEMENTAL ANALYSIS OF THE TFAA DERIVATIVES

| Agent | | C (%) | H (%) | N (%) |
|---|---|---|---|---|
| Nor-nitrogen mustard derivative | Found | 29.77 | 3.38 | 5.44 |
| | Calculated | 30.27 | 3.39 | 5.88 |
| BMA derivative | Found | 54.53 | 4.64 | 6.20 |
| | Calculated | 55.30 | 4.64 | 6.45 |

*Work-up procedure*

*Derivatization.* The derivatization reactions with TFAA in toluene were completed within 2 min at room temperature.

*Extraction.* Excess reagent and liberated acid were removed by extraction with phosphate buffer (pH 7.0). The removal of the excess reagent and acid in the sample increased the lifetime of the megabore column.

The losses during the extraction procedure were studied by comparing the amount of derivatives in toluene before and after the extraction of 1-ml toluene solutions with 1 ml of phosphate buffer. The results at a 95% degree of confidence (Student's *t*-distribution [10]) are shown in Table II.

*Enrichment.* The derivatives were enriched ten-fold. Evaporation of the solvent to dryness with a gentle flow of nitrogen gave losses of 5–35%. The losses of the nor-nitrogen mustard derivative were the same as for the BMA derivative. To decrease losses, especially for low concentrations, it is better to only reduce the volume, not to evaporate to dryness.

TABLE II

CONCENTRATION OF DERIVATIVES IN TOLUENE BEFORE AND AFTER EXTRACTION WITH PHOSPHATE BUFFER (pH 7.0) AT A 95% DEGREE OF CONFIDENCE

In all cases, $n = 6$.

| Agent | Before phopshate buffer extraction ($\mu$g/ml) | After phosphate buffer extraction $\mu$m/ml) |
|---|---|---|
| Nor-nitrogen mustard | 1.32 ± 0.04 | 1.29 ± 0.02 |
| BMA | 0.94 ± 0.02 | 0.92 ± 0.01 |
| Nor-nitrogen mustard | 16.8 ± 0.4 | 16.5 ± 0.3 |
| BMA | 7.6 ± 0.1 | 7.4 ± 0.1 |
| Nor-nitrogen mustard | 168.4 ± 0.7 | 168.5 ± 1.0 |
| BMA | 75.6 ± 0.5 | 75.5 ± 0.5 |

The accuracy was good after correction for losses with the aid of the internal standard (BMA). For a concentration of 168.4 ± 0.7 $\mu$g of nor-nitrogen mustard hydrochloride per ml, a result of 166.6 ± 0.5 $\mu$g/ml ($n = 6$) was obtained; for 16.8 ± 0.4 $\mu$g/ml, 16.6 ± 0.4 $\mu$g/ml ($n = 5$) was obtained; and for 1.32 ± 0.04 $\mu$g/ml, 1.49 ± 0.02 $\mu$g/ml ($n = 6$) was obtained. The values are given with a 95% confidence range (Student's $t$-distribution [10]).

*Chromatography*

The TFAA dervatives of nor-nitrogen mustard and BMA were studied. Both compounds showed excellent chromatographic behaviour using GC–FID. Baseline separation of the two derivatives was evident (Fig. 3). Peaks from impurities in the

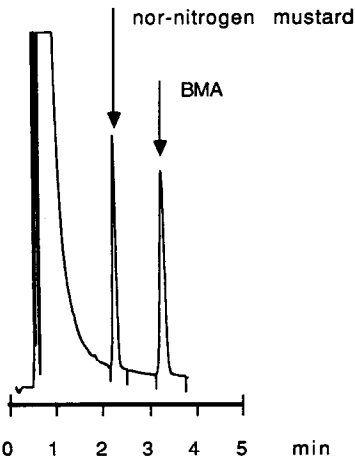


Fig. 3. Gas chromatogram of TFAA derivatives of nor-nitrogen mustard and BMA (injected amounts: nor-nitrogen mustard, 85 ng; BMA, 40 ng). The chromatogram was performed using GC–FID with the on-column injection of 0.5 $\mu$l of toluene solution. Megabore column: J&W fused silica coated with DB-1 bonded stationary phase (30 m × 0.53 mm I.D.), film thickness 1.5 $\mu$m. Isothermal at 120°C. Carrier gas (nitrogen) at a flow-rate of 15 ml/min. Make-up gas (nitrogen) at a flow-rate of 20 ml/min.

toluene interfered in the determination of low concentrations after the enrichment procedure.

*Quantitative analysis*

*Recovery.* The relative overall recovery was studied by spiking toluene solutions and performing the derivatization and work-up procedures as described. The peak areas were corrected with the aid of the internal standard and compared to those of standards using GC–FID. The relative recovery was 96 ± 1.5% ($n$ = 5) for a concentration of 20 μg/ml nor-nitrogen mustard hydrochloride and 100 ± 3% ($n$ = 6) for 200 μg/ml). The values are given with a 95% confidence range (Student's $t$-distribution [10]).

*Calibration graphs.* The calibration graph for the nor-nitrogen mustard derivative of TFAA for the concentration range 2–1050 μg/ml in toluene was linear and gave a correlation coefficient of 0.9999 ($n$ = 10 ; $y$ = 2.0863$x$ + 3.627) for a plot of the peak area ratio for ten concentrations.

*Detection limit.* Using GC–FID the detection limit, calculated as three times the noise level, for the derivative of nor-nitrogen mustard in ethyl acetate where the chromatograms were free from interfering peaks, was less than 0.05 ng of the injected amount. This corresponds to a concentration of 0.1 μg/ml. Small interfering peaks appeared in toluene, giving a detection limit of the order of 0.4 μg/ml.

Preliminary studies have shown that GC with nitrogen–phosphorus detection gives a detection limit in toluene of less than 0.0025 ng of the injected amount. This corresponds to a concentration of 0.005 μg/ml.

The detection limit can be lowered further by enrichment, and an enrichment factor of up to 10 is possible.

*Precision.* When calculating the precision of procedures involving extraction and enrichment, the addition of an internal standard to the samples before the analysis is strongly recommended. In this study, the internal standard chosen behaves in a similar manner to nor-nitrogen mustard throughout the analytical procedure. The overall precision (relative standard deviation) with the work-up procedure (including weighing, derivatization, extraction and the enrichment procedure) and GC analysis, using BMA as the internal standard, was 1.3% ($n$ = 5) for 20 μg/ml nor-nitrogen mustard hydrochloride spiked in toluene, and 2.8% ($n$ = 6) for 200 μg/ml.

## CONCLUSIONS

A GC–FID method has been demonstrated for the determination of nor-nitrogen mustard in toluene or ethyl acetate. The method is based on a derivatization procedure with TFAA. The internal standard (BMA) has the same performance during the analytical procedure as the nor-nitrogen mustard, which results in accurate and precise determinations. The method is sensitive enough for the determination of nor-nitrogen mustard hydrochloride in industrial working environments, *e.g.* in air using a filter sample technique and on surfaces using a wipe sample technique. Further details about the sampling procedures and workplace monitoring are described in a separate paper [6].

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