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Characterization and study of piperazinium salts, degradation products of nitrogen mustards by nuclear magnetic resonance spectroscopy and liquid chromatography–mass spectrometry

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ABSTRACT

We synthesized and analyzed the degradation products, piperazinium salts from bis(2-chloroethyl)methylamine (HN2) and bis(2-chloroethyl)ethylamine (HN1) using ^{1}H nuclear magnetic resonance (NMR) and liquid chromatography–mass spectrometry (LC–MS). Piperazinium salt is the major degradation product of HN2, not *N*-methyldiethanolamine above a concentration of 0.01 M in water and is a non-scheduled chemical that may be generally assumed relevant to the Chemical Weapons Convention (CWC) within the context of the Organization for the Prohibition of Chemical Weapons (OPCW) proficiency test. In verification analysis, ^{1}H NMR offers real-time information about degradation pathway of nitrogen mustards and LC–MS is expected to play an increasing role in the analysis of environmental samples for the degradation products of chemical warfare agents.

1. Introduction

Nitrogen mustards, bis(2-chloroethyl)ethylamine (HN1), bis(2-chloroethyl)methylamine (HN2) and tris(2-chloroethyl)amine (HN3) are blistering agents, and belong to the same class of chemical agents with vesicant properties as lewisites and sulfur mustards [1,2]. Nitrogen mustards were developed in the 1930s and stockpiled during World War II for military use. However, the use of such chemical warfare agents (CWA) is now prohibited by Chemical Weapons Convention (CWC). According to this treaty, the development, production, stockpiling and use of chemical weapons are prohibited [3–5]. In 1995, AUM Shinrikyo terrorists released the nerve agent sarin in the Tokyo subway system [6]. This real incident points to the great threat of terrorism by chemical warfare. In addition, the disarmament of stockpiled chemical weapons is now an urgent issue. In both chemical warfare terrorism and disarmament, the detection and identification of CWA is important for verifying their presence and possible exposure to casualties.

The transformations undergone by the nitrogen mustards in water were chosen as the first point of attack since a detailed knowledge of these reactions seemed essential to an understanding of the general chemistry of the nitrogen mustards. Furthermore, the transformations of the nitrogen mustards in water are of interest from the biochemical point of view, since water is a major constituent of all biological systems. Finally, the possibility that the nitrogen mustards might be employed in chemical warfare as water contaminants made it imperative to study the chemical reactions exhaustively undergone by these agents in water.

Nitrogen mustards usually undergo hydrolysis in the presence of water to form relevant ethanolamines. *N*-ethyldiethanolamine (EDEA), *N*-methyldiethanolamine (MDEA) and triethanolamine (TEA), are produced from bis(2-chloroethyl)ethylamine (HN1), bis(2-chloroethyl)methylamine (HN2) and tris(2-chloroethyl)amine (HN3), respectively. Piperazinium salts are also degradation products of the nitrogen mustards and non-scheduled chemicals that may be generally assumed relevant to the CWC within the context of the OPCW proficiency test [7–9].

Surprisingly few attempts have been made to characterize and study piperazinium salts in solution. Golding and Kebbell reported the study of HN2 nitrogen mustard by NMR [10]. In this paper as an extension of this work, we have investigated piperazinium salts, degradation products of the nitrogen mustards (HN1, HN2) via ^{1}H nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography–mass spectrometry (LC–MS). NMR spectroscopy offers real-time information of degradation products of nitrogen mustards and LC–MS is being increasingly applied to the analysis

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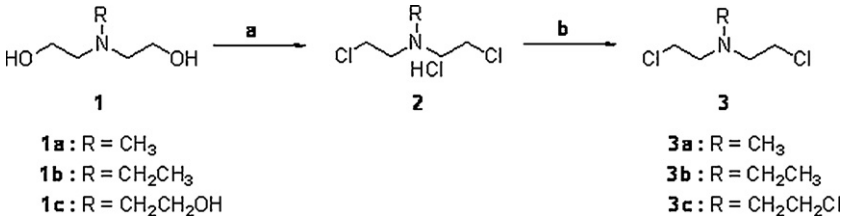

Fig. 1. The preparation of compound **3**: (a) $SOCl_2$/benzene; (b) Na_2CO_3/diethylether.

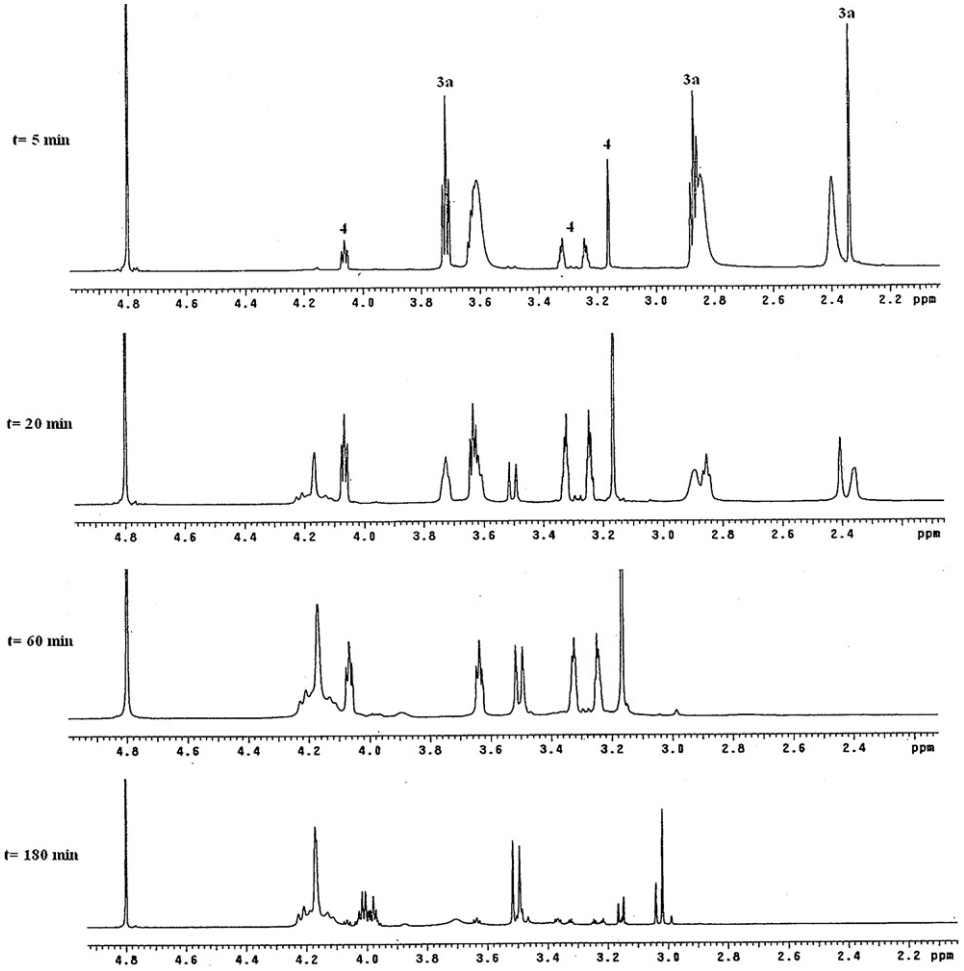

Fig. 2. The change of ^{1}H NMR spectra of bis(2-chloroethyl)methylamine (HN2) **3a** in D_2O. (a) t = 5 min, (b) t = 20 min, (c) t = 60 min, and (d) t = 180 min.

of CW agent residues using atmospheric pressure ionization techniques [11–14]. In addition to advantages with regard to sample preparation, LC–MS may identify polar degradation products that are not seen using GC–MS analysis [11,13].

2. Experimental

2.1. Reagents and materials

N-ethyldiethanolamine (EDEA) was obtained from the Tokyo Chemical Industry (Tokyo, Japan). *N*-methyldiethanolamine (MDEA) and triethanolamine (TEA) were obtained from Wako Pure Chemical (Osaka, Japan). Reagents for synthesis were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gradient-grade solvents (benzene, diethylether) were purchased from Merck (Darmstadt, Germany).

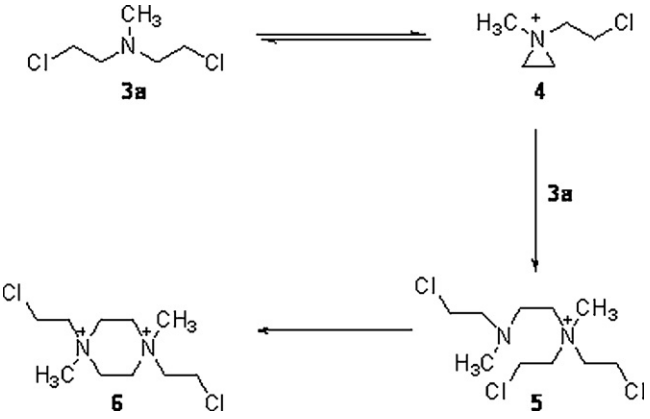

Fig. 3. The plausible reaction mechanism of bis(2-chloroethyl)methylamine (HN2) **3a**.

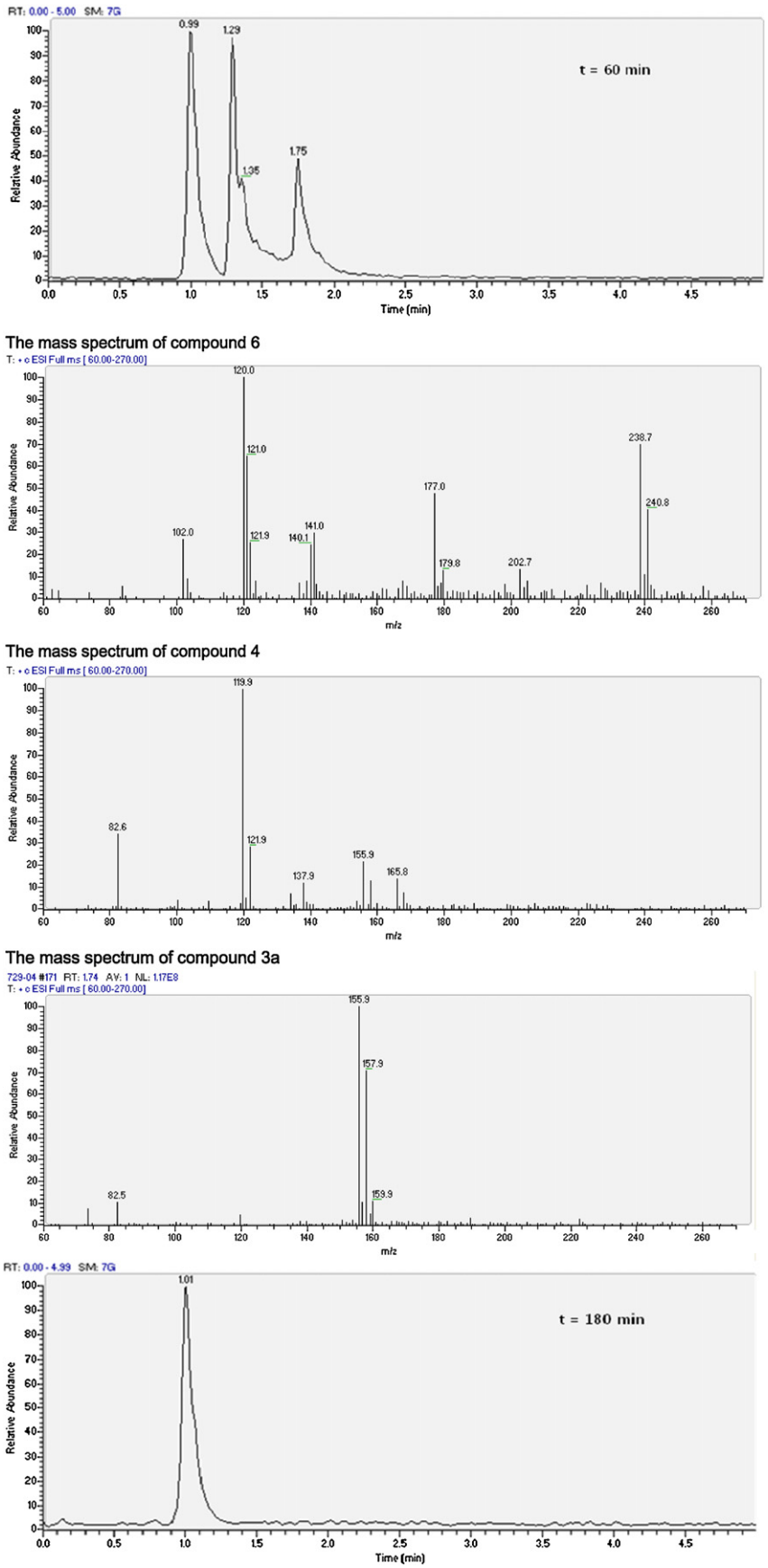

Fig. 4. Time profile of bis(2-chloroethyl)methylamine (HN2) **3a** in water for 3 h. (a) Total ion chromatogram $t = 60$ min, (b) mass spectrum of compound **6**, (c) mass spectrum of compound 4, (d) mass spectrum of compound **3a**, and (e) total ion chromatogram $t = 180$ min.

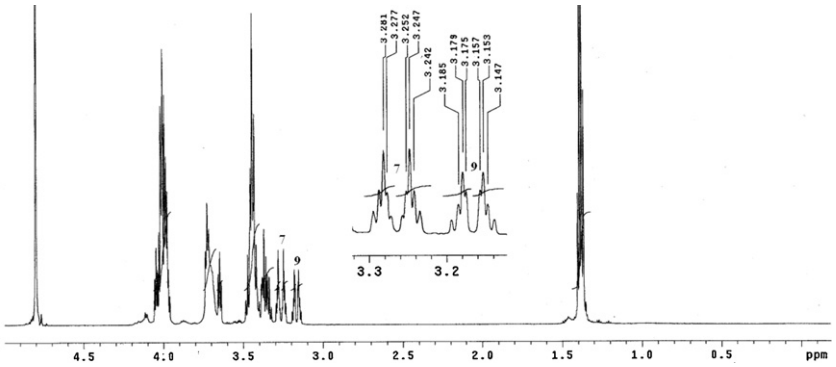

Fig. 5. The change of ^{1}H NMR spectra of bis(2-chloroethyl)ethylamine (HN1) **3b** in D_2O.

2.2. Synthesis of nitrogen mustards

The bis(2-chloroethyl)methylamine (HN2) **3a** was easily prepared from commercially available *N*-methyldiethanolamine **1a** by two-step sequence (Fig. 1). Thionyl chloride was added to a stirred solution of *N*-methyldiethanolamine **1a** in benzene at room temperature. After refluxing for 2 h, the reaction mixture was cooled to yield a mass of white crystals. The bis(2-chloroethyl)methylamine hydrochloride **2a** was turned into a free amine by using sodium carbonate in aqueous solution [10].

2.3. Instruments

2.3.1. Liquid chromatography–mass spectrometry

LC–MS analyses were performed on Thermo Finnigan Surveyor (San Jose, CA, USA). The system was fitted with a 150 mm × 2.1 mm Aquasil C18 column (Thermo Electron Co., USA), with 3 μm particle size and 100 Å pore size. The mobile phase at isocratic elution consisted of 0.1% formic acid in water at a flow rate of 0.2 mL/min. Injections (10 μL) were made using the autosampler.

The column effluent was introduced into a LCQ Deca XP max mass spectrometer (Thermo Finnigan, San Jose, CA, USA) via an atmospheric pressure ionization source/interface operated in electrospray ionization (ESI) mode. Capillary and tube lens voltages were optimized to give maximum response to *m*/*z* 156 $[M+H]^+$ from bis(2-chloroethyl)methylamine (HN2). This ensured that the $[M+H]^+$ ions were prominent in the spectra. ESI conditions were as follows: capillary temperature 275 °C, spray voltage 5 kV, sheath gas nitrogen (Air Products, Singapore) at 90 psi. The mass scan range was *m*/*z* 60–*m*/*z* 300. CID product ion spectra were obtained from the protonated molecules of the compound using the LC conditions described above. Argon was used as collision gas, collision offset −35 V and Q^0 offset −5 V.

2.3.2. Nuclear Magnetic Resonance spectroscopy

^{1}H NMR spectra were acquired on a Varian 600 MHz-Premium compact NMR spectrometer (Varian Inc., Palo Alto, CA, USA) and processed using the Varian VNMR software. The ^{1}H NMR experiments were conducted at 25 °C with a pulse repetition delay of 4 s. Prior to the NMR study, the proton line shape, sensitivity and pulse lengths were checked for the probe head. The magnetic field homogeneity adjustment was performed carefully, and, always before final accumulation, trial spectra with a few scans were acquired to check the line shape and line width.

3. Results and discussion

3.1. ^{1}H NMR and LC–MS study of bis(2-chloroethyl)methylamine (HN2) in D_2O

As a first step, ^{1}H NMR spectrum of bis(2-chloroethyl)methylamine (HN2) **3a** in D_2O solution (10 mg/mL) was studied and the change of proton signals was monitored according to reaction time (Fig. 2).

As shown in Fig. 2, a new set of signals appeared and showed the development of new species within 5 min at room temperature. Resonances observed at δ 3.16 (s, Me), 3.2–3.3 (m, 2× aziridinium CH_2), 3.64 (t, CH_2N) and 4.06 (t, CH_2Cl) are assigned to the aziridinium ion **4** [15]. After 20 min, the proton signals of piperazinium salt **6** started to appear at δ 3.49, 3.51 (2× NMe), and 4.16 (methylene protons) and those of aziridinium ion **4** completely disappeared within 180 min. This indicated that the aziridinium ion **4** is stable in an aqueous solution and reacts with bis(2-chloroethyl)methylamine (HN2) **3a**, which arises by reversion of **4** to **3a** to give piperazinium dimers **6** (cis:trans = 1:2) after intramolecular cyclization (Fig. 3).

The degradation pathway of bis(2-chloroethyl)methylamine (HN2) **3a** in aqueous solution was studied using LC–MS. To confirm the formation of piperazinium salt from the bis(2-chloroethyl)methylamine (HN2) **3a** in water solution, deionized water (1 mL) was spiked with 10 mg of **3a** at a concentration of 0.06 M. The reaction samples were diluted at a concentration of 20 μg/mL before analysing by LC–MS. The degradation of bis(2-chloroethyl)methylamine (HN2) **3a** in water was shown in Fig. 4. After 1 h, piperazinium salt **6** (RT = 0.99 min) was the major degradation product of **3a** and similar amount of aziridinium ion **4** (RT = 1.29 min) and unchanged **3a** (RT = 1.75 min) were also detected. Bis(2-chloroethyl)methylamine (HN2) **3a** was fully converted to piperazinium salt **6** after 3 h. The pH 7 of reaction solution was not changed. After concentrating the H_2O solution, the piperazinium salt **6** was purified by precipitation from aqueous solution by the addition of acetone and isolated in 82% yield.

3.2. ^{1}H NMR and LC–MS study of bis(2-chloroethyl)ethylamine (HN1) in D_2O

The transformations of bis(2-chloroethyl)ethylamine (HN1) **3b** in H_2O was studied. As may be expected from its close structural similarity to bis(2-chloroethyl)methylamine (HN2) **3a**, we thought the behavior of bis(2-chloroethyl)ethylamine (HN1) **3b**, in many respects, would be similar to that of its homolog, bis(2-chloroethyl)methylamine (HN2) **3a**. However, in the ^{1}H NMR spectrum, two types of methylene protons of aziridinium ions **7** and **9** were detected at 3.14–3.18 ppm and 3.24–3.28 ppm (Fig. 5).

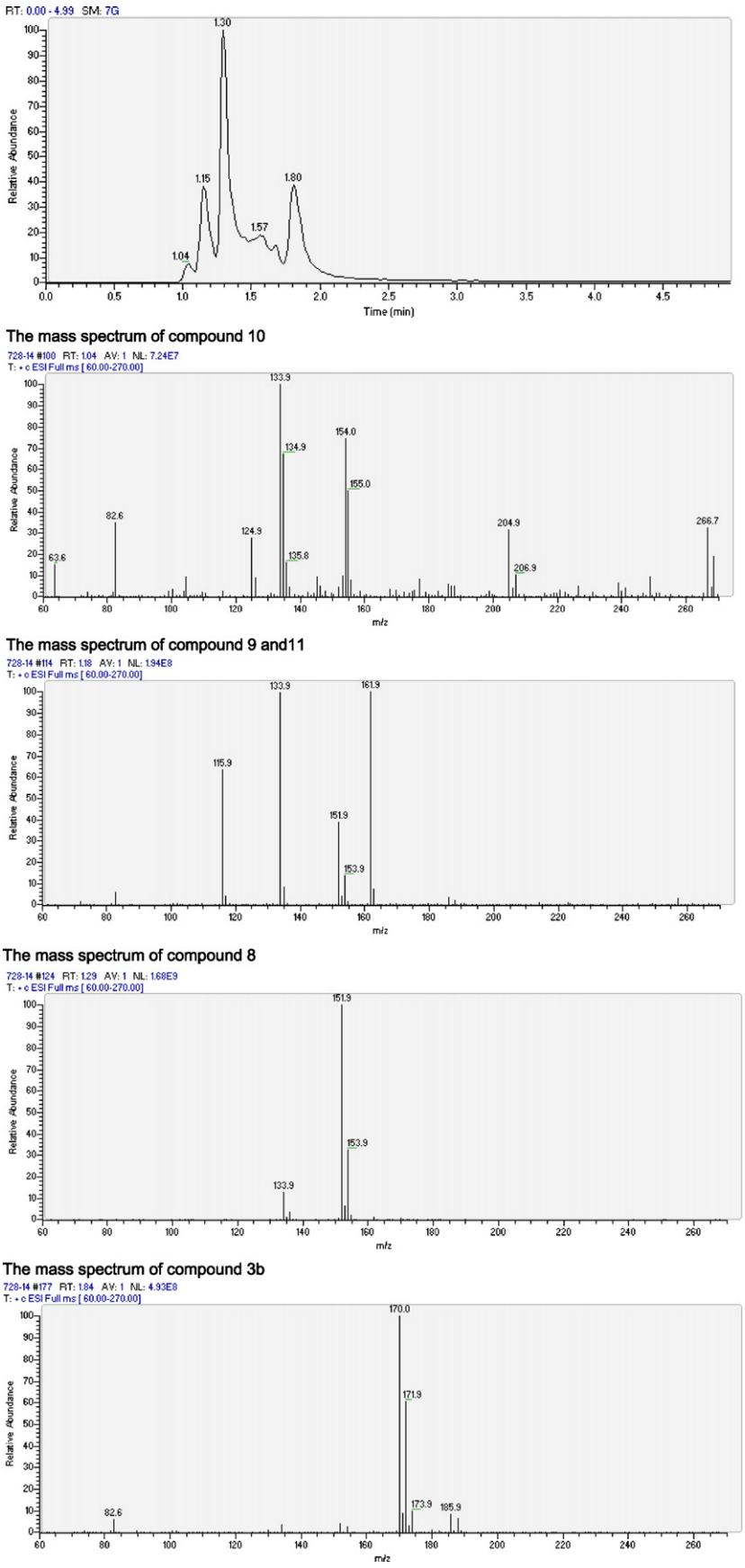

Fig. 6. Time profile of bis(2-chloroethyl)ethylamine (HN1) **3b** in water for 24 h.

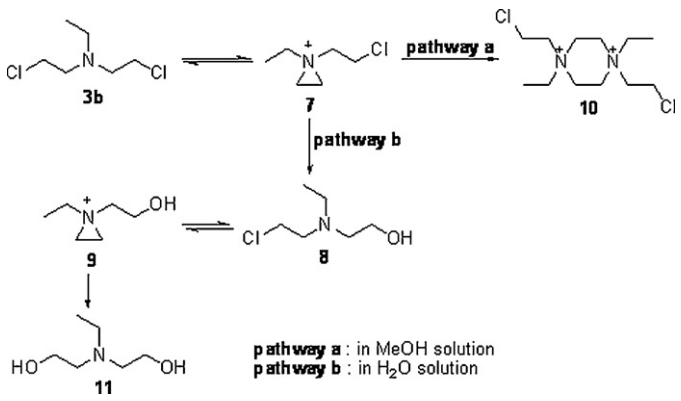

Fig. 7. The plausible reaction mechanism of bis(2-chloroethyl)ethylamine (HN1) **3b**.

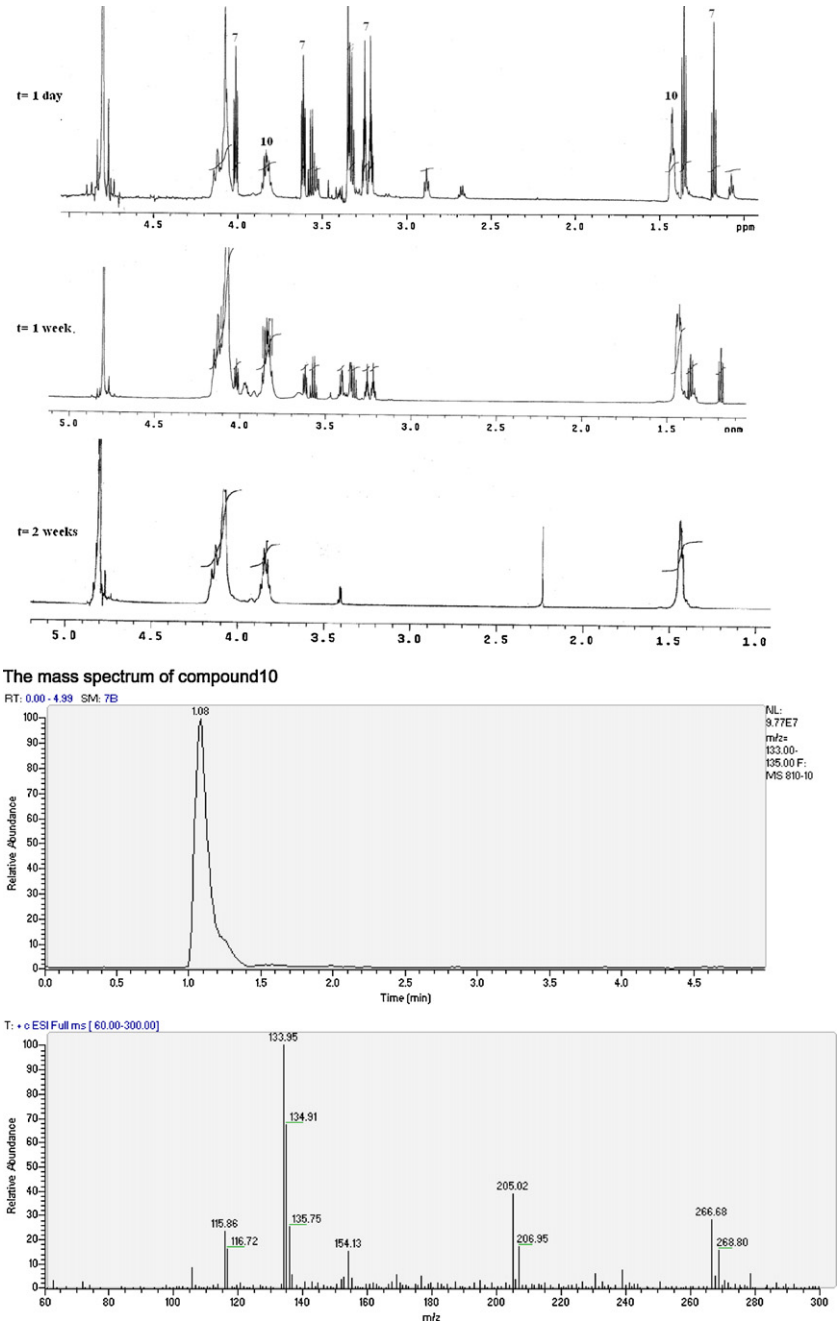

Fig. 8. The change of ^{1}H NMR spectra of bis(2-chloroethyl)ethylamine (HN1) **3b** in MeOH and LC–MS data.

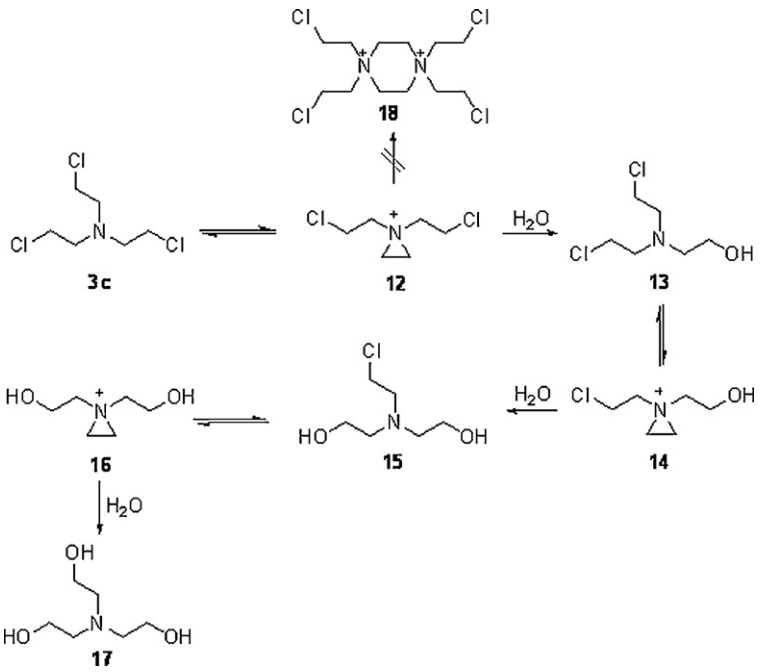

Fig. 9. The plausible reaction mechanism of tris(2-chloroethyl)amine (HN3) **3c**.

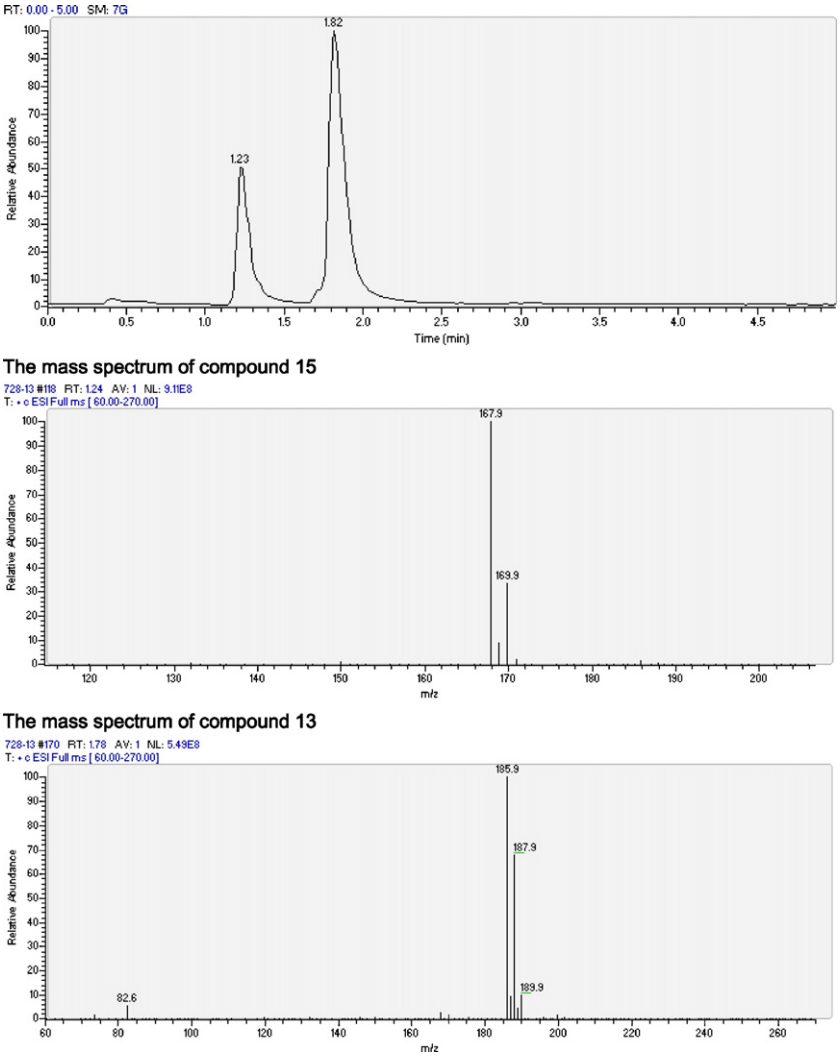

Fig. 10. Time profile of tris(2-chloroethyl)amine (HN3) **3c** in water for 72 h.

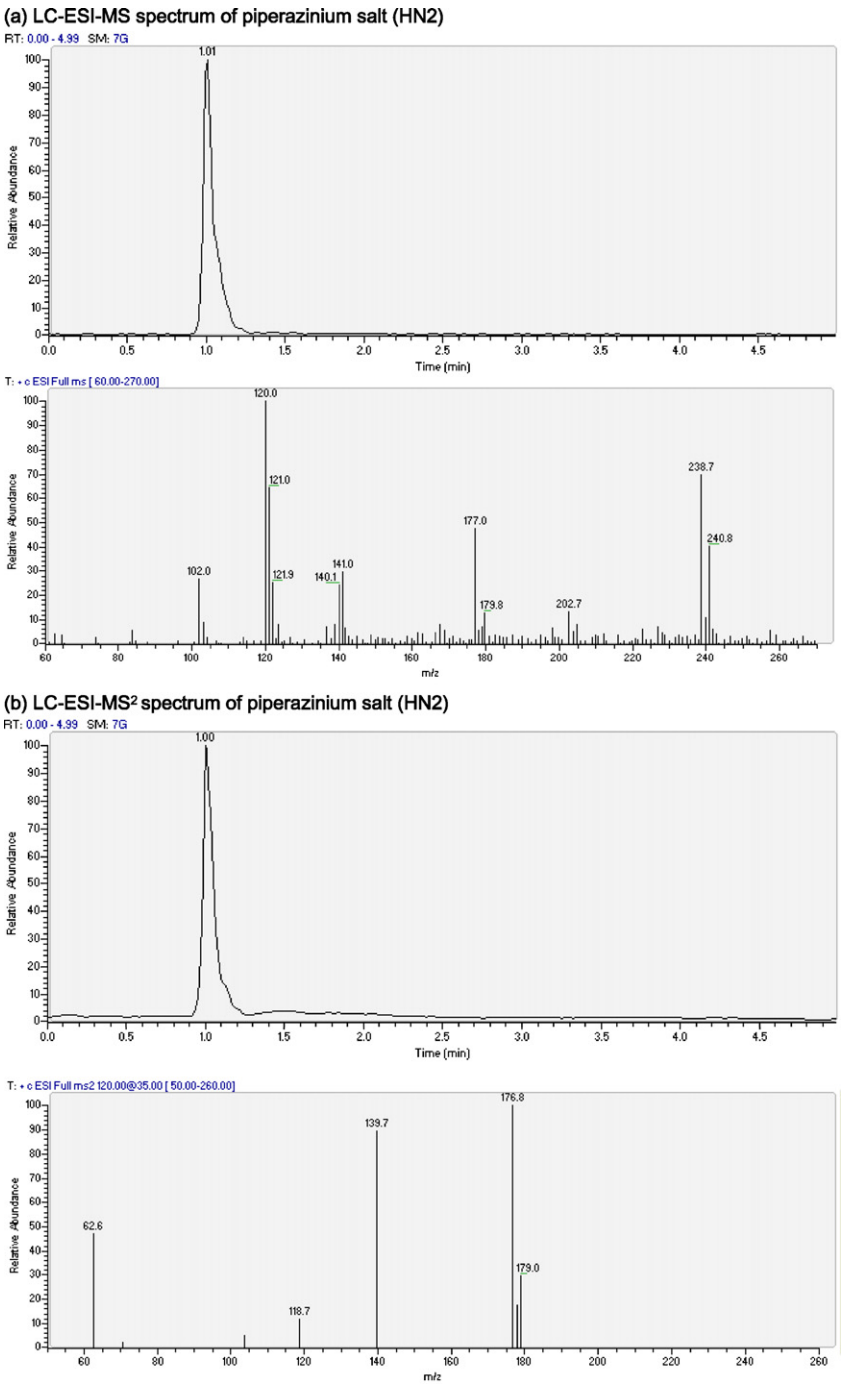

Fig. 11. LC–ESI-MS, ESI-MS^2 data of piperazinium salt **6**.

To confirm this result as above, the time profile study was performed to ascertain the degradation pathway of bis(2-chloroethyl)ethylamine (HN1) **3b** by LC–MS. Fig. 6 shows the degradation of bis(2-chloroethyl)ethylamine (HN1) **3b** in water. After 24 h, ethanolamine **8** (RT = 1.30 min), aziridinium ion **9** and ethyldiethanolamine **11** (RT = 1.15 min) were the major degradation products and unchanged **3b** (RT = 1.80 min) remained. Also, the small amount of piperazinium salt **10** (RT = 1.04 min) was detected.

These results indicated that aziridinium ion **7** from bis(2-chloroethyl)ethylamine (HN1) **3b** is less stable compared to aziridinium ion **4** from bis(2-chloroethyl)methylamine (HN2) **3a** and has less a tendency to form dimeric products than **3a** in an aqueous solution does (Fig. 7).

According to Price et al., piperazinium salts could also be made in an alcoholic solution. The formation of piperazinium salts is dependent upon the concentration of bis(2-chloroethyl)ethylamine (HN1) **3b** and bis(2-chloroethyl)methylamine (HN2) **3a**. As the concentration of bis(2-chloroethyl)ethylamine (HN1) **3b** is raised, the formation of piperazinium salt is favored [16,17].

As shown in Fig. 7, the reaction condition and concentration of solvent is important for the formation of piperazinium salts. In the aqueous solution, bis(2-chloroethyl)ethylamine (HN1) **3b** was rapidly hydrolyzed, producing hydrolytic products that were mainly ethanolamine **8**, aziridinium ion **9** and ethyldiethanolamine **11** with the corresponding pH change found to drop from 7 to 5. However, unlike in the H_2O, piperazinium salt **10** was the main

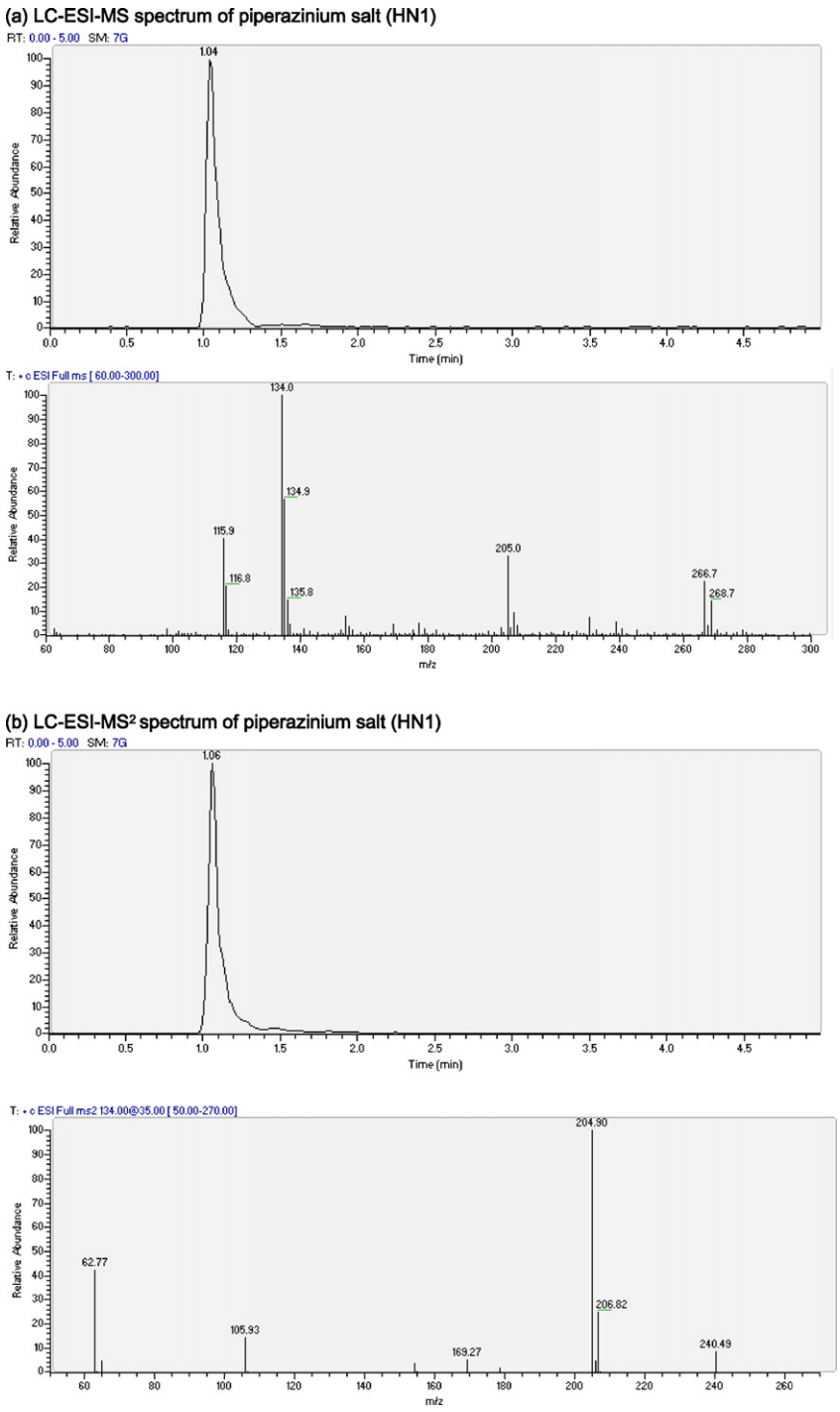

Fig. 12. LC–ESI-MS, ESI-MS^2 data of piperazinium salt **10**.

degradation product in the alcoholic solution. The ^{1}H NMR showed the only formation of aziridinium ion **7** and piperazinium salt **10** similar to degradation pathway of bis(2-chloroethyl)methylamine (HN2) **3a** and LC–MS data supported this result (Fig. 8). Thus, the piperazinium salt **10** was obtained in methanolic solution and recrystallized by the addition of acetone in 75% yield.

3.3. ^{1}H NMR and LC–MS study of tris(2-chloroethyl)amine (HN3) in D_2O

The reaction mechanism of tris(2-chloroethyl)amine (HN3) **3c** was more complicated compared to bis(2-chloroethyl)methylamine (HN2) **3a** and bis(2-chloroethyl)ethylamine (HN1) **3b**. In the ^{1}H NMR of tris(2-chloroethyl)amine (HN3) **3c**, the methylene ($-CH_2$) peaks of aziridinium ions **12**, **14** and **16** were not detected during the reaction (Fig. 9). When we investigated the degradation process of tris(2-chloroethyl)amine (HN3) **3c** by LC–MS, the piperazinium salt **18** was not formed and two ethanolamines **13** (RT = 1.82 min), **15** (RT = 1.23 min) were the major degradation products of the reaction of tris(2-chloroethyl)amine (HN3) **3c** (Fig. 10). In contrast to bis(2-chloroethyl)ethylamine (HN1) **3b** and bis(2-chloroethyl)methylamine (HN2) **3a**, the pH dramatically dropped from pH 7 to pH 2 over the course of the experiment.

This result indicated that aziridinium ions **12** and **14** were very unstable and not long lived in an aqueous solution. Thus, the hydrolysis reaction occurred via these aziridinium ions, producing hydrolytic products that were mainly ethanolamines with either the bis(chloroethyl) **13** or chloroethyl **15** group attached (Fig. 10).

3.4. Limit of concentration for the formation of piperazinium salts

As shown in the above result, the concentration of a reaction solution is important for the formation of piperazinium salts in water. We investigated the effect of concentration for the formation of piperazinium salts. The bis(2-chloroethyl)methylamine (HN2) **3a** was rapidly converted into its corresponding piperazinium salt **6** at a concentration of 0.06 M. When the initial concentration of **3a** (HN2) is decreased from 0.06 M to 0.01 M, the peak of *N*-methyldiethanolamine appeared in the LC–MS. Under a concentration of 0.001 M, *N*-methyldiethanolamine was the major product in the water. In case of bis(2-chloroethyl)ethylamine (HN1) **3b**, the ethanolamine **8** was a major degradation product and the small amount of piperazinium salt **10** was detected even above a concentration of 0.06 M in water. However, piperazinium salt **10** was slowly formed in the methanol solution at a concentration of 0.06 M.

3.5. Analysis of piperazinium salts by LC–MS

Our next attention was given to analyze and confirm the piperazinium salts of bis(2-chloroethyl)ethylamine (HN1) **3b** and bis(2-chloroethyl)methylamine (HN2) **3a** by LC–MS. GC–MS is suitable only for ethanolamine degradation products after derivatization. LC–MS methods are now of similar sensitivity and should be the preferred method for analyzing for degradation products. For the analysis of the hydrolysis or degradation products, GC–MS may be difficult and time consuming since these products tend to be highly polar. Thus, determination by liquid chromatography coupled to mass spectrometry would be suited for analysis of the piperazinium salts, degradation products of nitrogen mustards.

We purified and recrystallized the piperazinium salts of bis(2-chloroethyl)ethylamine (HN1) **3b** and bis(2-chloroethyl)methylamine (HN2) **3a**. Deionized water (1 mL) was spiked with 1 mg of piperazinium salt of **3a** (HN2) at a concentration of 1 mg/mL. A final concentration was 20 μg/mL. Fig. 11 showed the total ion chromatogram of LC–ESI-MS and LC–ESI-MS^2 of the piperazinium salt of bis(2-chloroethyl)methylamine (HN2) **3a**.

The piperazinium salt **6** of bis(2-chloroethyl)methylamine (HN2) **3a** is M^{2+} ion and its molecular mass is 240. In Fig. 11(a), the ion at *m*/*z* 120 showed a dominant quasimolecular ion $M^{2+}/2 = 120$ for M^{2+} ion. Also, in the mass spectra of chlorinated derivatives, the corresponding intensity of isotopic peak $[M+2]^{2+}/2$ at *m*/*z* 121 was clearly seen. In the LC–ESI-MS^2, the ion at *m*/*z* 120 was chosen as a parent ion at a collision cell offset voltage of −35 V. The LC–ESI-MS^2 spectra of piperazinium salts **6** showed that the fragmentation ions at *m*/*z* 177 represent the loss of chloroethane ($\Delta m/z = 63$) from the molecular ion (Fig. 11).

The piperazinium salt **10** of bis(2-chloroethyl)ethylamine (HN1) **3b** is also M^{2+} ion and its molecular mass is 268. Fig. 12(a) showed a quasimolecular ion $M^{2+}/2$ at *m*/*z* 134 and the corresponding isotopic peak at *m*/*z* 135. In the LC–ESI-MS^2, the ion at *m*/*z* 134 was chosen as a parent ion at a collision cell offset voltage of −35 V and the fragmentation ion at *m*/*z* 205 represents the loss of chloroethane ($\Delta m/z = 63$) from the molecular ion (Fig. 12).

3.6. Stability study and limit of detection of piperazinium salts

We investigated the stability and limit of detection of piperazinium salts in aqueous solution. To determine the thermal stability of the piperazinium salts **6** and **10**, the water (1 mL) was spiked with 1 mg of piperazinium salts of bis(2-chloroethyl)methylamine (HN2) **3a** and bis(2-chloroethyl)ethylamine (HN1) **3b** at a concentration of 1 mg/mL and the samples were stored in the oven at 100 °C under an air atmosphere. After two weeks, the samples were cooled at room temperature and analyzed by NMR and LC–MS. The ^{1}H NMR spectra of piperazinium salts **6** and **10** had not changed and hydrolysis products were not detected. As a consequence, piperazinium salts are stable even under severe condition. Limit of detection (LOD) for the piperazinium salts **6** and **10** reached the values of 2 μg/mL in the LC–ESI-MS full scan mode and 10 ng/mL in the LC–ESI-MS^2 (parent ion of *m*/*z* 120 and 134 and product ion of *m*/*z* 63 at a collision cell offset voltage of −35 V) SRM scan mode.

4. Conclusion

In summary, we analyzed and studied the formation of degradation products, piperazinium salts from bis(2-chloroethyl)methylamine (HN2) **3a** and bis(2-chloroethyl) ethylamine (HN1) **3b** by ^{1}H NMR and LC–MS. This study gave information about degradation of nitrogen mustards in aqueous solution. First, the stability of aziridinium ions is an important factor for the formation of piperazinium salts from nitrogen mustards. Second, the formation of piperazinium salt is dependent upon the concentration of nitrogen mustards and reaction solvent. Third, surprisingly, piperazinium salt is the major degradation product of HN2, not *N*-methyldiethanolamine above a concentration of 0.01 M and is non-scheduled chemical that may be generally assumed relevant to the Chemical Weapons Convention (CWC) within the context of the OPCW proficiency test. In verification analysis, ^{1}H NMR offers real-time information about degradation pathway of nitrogen mustards and LC–MS is expected to play an increasing role in the analysis of environmental samples for the degradation products of chemical warfare agents.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.107.

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