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Analysis of the nitrogen mustard mechlorethamine in topical pharmaceutical preparations by high-performance liquid chromatography

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Abstract

Mechlorethamine in topical pharmaceutical formulations was derivatized with benzenethiol to form the disubstitution product and analyzed by normal-phase HPLC on silica gel using dibutyl phthalate as an internal standard. The derivatization reaction, purification, and isolation were conveniently performed in a single test tube. Analyses were successfully performed on three types of ointment formulations: anhydrous hydrophobic petrolatum-based ointments, anhydrous hydrophilic ointments, and hydrous hydrophilic ointments. Precision for the analysis of mechlorethamine standard or mechlorethamine in ointments ranged from 0.08 to 0.52% RSD ($n = 6$). Recoveries from ointments spiked with 0.02% mechlorethamine hydrochloride were 98.4–100.4%. The chromatograms were clean, showing minimal or no interference from ointment excipients or reagents.
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Keywords: Nitrogen mustard; Mechlorethamine; Ointments; HPLC; Topical pharmaceutical

1. Introduction

Nitrogen mustards, the oldest class of synthetic compounds shown to possess anticancer activity in man, have been used for the treatment of Hodgkin's disease, non-Hodgkin's lymphoma, leukemia, and multiple myeloma [1,2]. The nitrogen mustards are powerful bi- or tri-functional alkylating agents, which can alkylate a DNA base or crosslink between two bases of a DNA helix [1–7]. Such actions are usually cytotoxic, leading to cell death as desired for cancer cells, but may also be mutagenic, carcinogenic, or teratogenic [2,5,6].

Mechlorethamine was the first nitrogen mustard to be used therapeutically. While it has largely been supplanted by less reactive nitrogen mustards, it remains in use today for specific therapeutic applications. The drug is used in a combination chemotherapy regimen consisting of mechlorethamine, oncovin (vincristine), procarbazine, and prednisone (MOPP) for the treatment of Hodgkin's disease [2,5] and has been particularly successful in the topical treatment of mycosis fungoides, a form of cutaneous T-cell lymphoma [2,4,6–11]. In the latter case, mechlorethamine hydrochloride (HCl) is administered topically as a solution or as an ointment formulation [2,6,10,12–15]. Due to the high reactivity of mechlorethamine, aqueous solutions of the drug are unstable, particularly under alkaline conditions. When mechlorethamine is dissolved in water, it rapidly cyclizes intramolecularly to an aziridinium chloride, which is more slowly converted to a host of other products, including 1,4-dimethyl-1,4-bis(2-chloroethyl)piperazinium chloride (a dichloro cyclic dimer), 2-chloroethyl-2-hydroxyethylmethylamine (the monosubstitution product with water), and bis(2-hydroxyethyl)methylamine (the disubstitution product with water) [16–19]. The rate of degradation of mechlorethamine in aqueous solutions is dependent upon pH, temperature, the concentration of mechlorethamine, and

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the concentration and nature of bases or other components in solution. When an unbuffered solution of mechlorethamine was stored for 48 h at room temperature, only 11% of the mechlorethamine was recovered unchanged [18]. Therefore, if aqueous solutions of mechlorethamine are prepared for topical therapeutic use, the solution must be used once and discarded [12].

In lieu of aqueous solutions of mechlorethamine, the drug may be freshly compounded by a pharmacist as an ointment formulation to extend the shelf life of the drug product. Typically, an absolute ethanol solution of mechlorethamine HCl, prepared from mechlorethamine HCl for injection (Trituration of Mustargen®, each vial of which contains 10 mg mechlorethamine HCl and sodium chloride q.s. 100 mg, Merck & Co., Inc., Whitehouse Station, NJ, USA), is mixed with an ointment base, such as Aquabase™, Aquaphor®, hydrophilic petrolatum, white soft paraffin, or a 50/50 mixture of liquid paraffin–white soft paraffin, to give a final concentration of 0.01–0.04% mechlorethamine HCl [2,6,10,12,13,15].

There are few analytical methods reported for mechlorethamine, perhaps because its high chemical reactivity makes it difficult to trap the compound as a derivative without unwanted degradation. Mechlorethamine has been assayed colorimetrically by reaction with nitrobenzyl pyridine [20,21], but this reagent is neither sensitive nor selective, and it reacts with mechlorethamine degradation products making it unsuitable for the measurement of the active drug in the presence of its degradation products, and thus, unsuitable for stability studies. Cummings et al., developed an HPLC method for the analysis of mechlorethamine in plasma [9] and mechlorethamine in ointment preparations [12]. With this method, mechlorethamine is treated with diethyldithiocarbamic acid (DDTC) to form the disubstituted derivative, which is analyzed by HPLC using a UV detector. This HPLC method [12] yielded a reported recovery from a formulated mechlorethamine ointment of 76.1% with a 10.4% RSD.

While using this method in our laboratory to analyze for mechlorethamine in topical pharmaceutical preparations, we found numerous peaks in the chromatograms, most of which came from the diethyldithiocarbamic acid (DDTC) reagent itself or its decomposition products formed during the derivatization reaction. This was verified by running blanks using DDTC without mechlorethamine. Diethyldithiocarbamic acid purchased from a second manufacturer gave the same results. Often, the DDTC–mechlorethamine derivative co-eluted with some of these extraneous peaks in the chromatogram making it necessary to adjust the mobile phase gradient to achieve separation.

The current study describes a method for the analysis of mechlorethamine in topical pharmaceutical formulations by derivatization with benzenethiol and analysis by normal-phase silica gel HPLC. This method offers an improvement in sensitivity, recovery, precision, and analysis time, and the chromatograms have minimal or no extraneous peaks from ointment excipients or reagents.

2. Experimental

2.1. Safety and handling

Mechlorethamine is a highly toxic nitrogen mustard that is carcinogenic, mutagenic, teratogenic, and corrosive [2,5,6]. Protective clothing, including gloves, lab coat, mask, and safety glasses were worn when handling the drug. Mechlorethamine HCl samples were weighed in a glove box and handled in a hood. Waste mechlorethamine and any spills were decomposed with an aqueous solution of 5% sodium bicarbonate and 5% sodium thiosulfate. Benzenethiol has a stench and was handled in a hood.

2.2. Chemicals

Mechlorethamine hydrochloride, *tert*-butylamine (TBA), and benzenethiol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Reagent grade *n*-butyl phthalate (dibutyl phthalate) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade Omni-Solve acetonitrile (MeCN), methanol, isopropanol, heptane, methyl *t*-butyl ether (MTBE), *n*-butanol, dimethylformamide (DMF), and reagent grade concentrated ammonia and hydrochloric acid were from EMD Chemicals (Gibbstown, NJ, USA). Reagent grade 50% sodium hydroxide was from Taylor Chemical (St. Louis, MO). Water was purified to 18 MΩ cm using a Milli-Q Water System (Millipore, Bedford, MA, USA).

2.3. Derivatization procedure

2.3.1. Solutions

A standard solution of mechlorethamine hydrochloride was prepared at a concentration of 0.4 mg ml^{-1} in DMF (STD solution A). This solution was diluted 1:10 with DMF (STD solution B, 40 μg ml^{-1}). Benzenethiol reagent consisting of benzenethiol–DMF–TBA (2:5:1 parts by volume) was prepared by mixing benzenethiol with DMF, then adding TBA and mixing; it must be freshly prepared daily. The sample solvent consisted of *n*-butanol–heptane (1:100). The internal standard (ISTD) solution was prepared by diluting 2 ml dibutyl phthalate to 200 ml with the sample solvent and diluting 10 ml of this solution to 250 ml with sample solvent, giving a final concentration of 0.04% dibutyl phthalate (ISTD solution).

2.3.2. Reaction and liquid–liquid extraction

The synthesis, liquid–liquid extraction, and back extraction of the derivative were all performed in a single Pyrex test tube, 16 mm × 125 mm, tightly sealed with a PTFE-lined screw cap. A PTFE-coated magnetic stir bar with dimensions of 20 mm × 8 mm without a pivot ring fits into the screw-cap glass test tube and stirs the reaction mixture quite effectively with the stir bar oriented in a vertical position. During the workup procedure, shaking and centrifugation were performed with stir bars in the test tubes with no prob-

lems. A bench-top centrifuge was used (Centrifig, model 228, 3300 rpm maximum speed, Fisher Scientific, Pittsburgh, PA, USA).

2.3.2.1. Sample preparation. A portion of an ointment or other pharmaceutical formulation equivalent to 40 μg of mechlorethamine hydrochloride (e.g., 200 mg of an ointment labeled to contain 0.02% mechlorethamine hydrochloride) was placed in a test tube. A PTFE-coated magnetic stir bar was added to the tube. One milliliter of benzenethiol reagent, 1 ml DMF, and 1 ml heptane were added. The tube was tightly capped, then stirred and heated at 50 °C for 2 h in a Reacti-Therm III heating/stirring module (Pierce, Rockford, IL, USA). The reaction solution was chilled in a refrigerator, then 3 ml 1 M HCl, 1 ml methanol, and 5 ml heptane were added. The mixture was shaken, centrifuged for 1 min, and the upper heptane layer was removed and discarded (the upper layer was rapidly and conveniently drawn off using a 22.5 cm (9 in.) Pasteur pipet connected to a trap and a water-circulating aspirator pump), leaving a small amount of upper layer to avoid the removal of any of the lower layer. If a solid or gelatinous material were suspended in the heptane or on the sides of the tube in the heptane layer (possible ointment excipients), it was removed along with the heptane. Gentle tapping on the tube helped to disperse a solid at the interface into the heptane layer for its removal. The solution was washed two more times with 5 ml of heptane in the same manner. A 2.00 ml portion of internal standard solution and 3 ml of 2 M NaOH were added. The mixture was shaken, centrifuged for 5 min, and ∼1.5 ml of the upper layer was transferred to a vial for analysis by HPLC.

2.3.2.2. Standard preparation. The same procedure that was used for the sample preparation was applied for standard preparation except no ointment was used and 1.00 ml of STD solution B, equivalent to 40 μg of mechlorethamine hydrochloride, was placed into the tube instead of 1 ml DMF.

2.3.2.3. Blank preparation. Blank samples were analyzed to demonstrate that there were no components from the ointment formulation or the benzenethiol reagent that co-eluted with the ISTD or analyte during HPLC. The same procedure that was used for the sample preparation was applied for the blank preparation except the topical formulation without mechlorethamine HCl was used and 2 ml of the sample solvent were used in place of 2 ml of the ISTD solution.

2.4. HPLC system

Analyses were performed on an Agilent 1100 liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostatted autosampler, a thermostatted column compartment, and a variable wavelength detector using ChemStation software, version A.08.03. During the development and evaluation of the method, three silica gel HPLC columns were employed: (1) Lichrosorb 5 SIL, 60 Å silica gel, 5 μm particle size, 150 mm × 4.6 mm (Phenomenex, Torrance, CA, USA), (2) YMC-Pack SIL, 120 Å silica gel, 5 μm particle size, 150 mm × 4.6 mm (Waters, Milford, MA, USA), and (3) Spherisorb silica gel, 3 μm particle size, 50 mm × 4.6 mm (Phenomenex, Torrance, CA, USA). A BrownLee NewGuard column, silica, spherical, 300A, 7 μm particle size, 3.2 mm × 15 mm (Applied Biosystems, Foster City, CA, USA) was used with the 150 mm columns, but none was used with the 50 mm analytical column. While all three columns performed well, ultimately, the YMC 150 mm column was used for the analysis. The mobile phase, consisting of H_2O–MeCN–MTBE–heptane (0.01:2:8:100), was prepared by placing 10 ml MeCN, 50 μl H_2O, and 40 ml MTBE in a flask, stirring briefly with a magnetic stir bar, then adding 500 ml heptane. The flask was capped and the mixture was stirred rapidly for 2 min. The HPLC system was operated isocratically at a flow rate of 1.0 ml min^{-1} and a run time of ∼8 min. The column oven temperature was maintained at 30 °C. These conditions gave a system back pressure of 35×10^5 Pa (35 bar). The detector wavelength was set at 256 nm, the λ_{max} for the benzenethiol-mechlorethamine derivative. Injector volume was 25 μl.

2.5. MS analysis

The target compound in the derivatization reaction was bis(2-phenylthioethyl)methylamine, the disubstitution product resulting from the displacement of the two reactive chlorine atoms with benzenethiol (Fig. 1). To confirm this structure, the reaction product was analyzed by electrospray

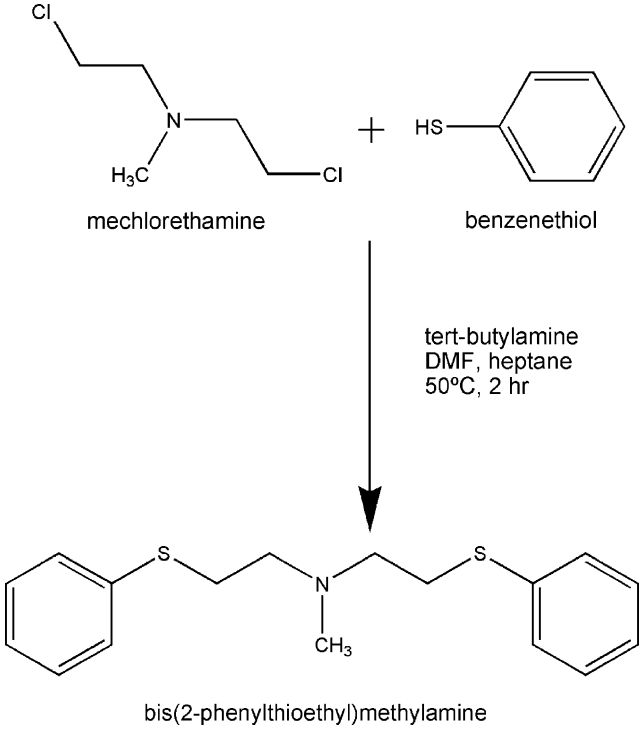

Fig. 1. Synthesis of the benzenethiol derivative of mechlorethamine.

ionization MS. Purified samples of the analyte were obtained by two procedures: (1) the component corresponding to the analyte peak was collected during HPLC analysis, and (2) the synthesis was performed as described above on the mechlorethamine standard, but the 2 ml of ISTD solution was replaced with 2 ml of the sample solvent, thus eliminating the ISTD in the final product. The solvent from each of these two procedures was evaporated and the residues were dissolved in 200 μl MeOH containing HOAc (one drop of HOAc in 5 ml MeOH). These samples were analyzed on an LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corporation, San Jose, California, USA) operated in electrospray ionization positive ion mode with direct infusion at 3 μl min^{-1}. The spray needle voltage was 5.0 kV and the nitrogen sheath gas flow rate was maintained at 5 units (an arbitrary value to the LCQ instrument). The ion transfer capillary temperature was 250 °C and the capillary voltage was 7 V. Helium was used in the ion trap as a buffer gas to improve trapping efficiency and as a collision gas for collision induced dissociation (CID) experiments. During CID analysis, the parent ion at 304 amu was fragmented with the collision energy optimized at 26%.

3. Results and discussion

3.1. Derivative formation

Mechlorethamine reacts with benzenethiol through a displacement of the two chlorine atoms to give the disubstitution product, bis(2-phenylthioethyl)methylamine as shown in Fig. 1. Benzenethiol was chosen as the derivatizing reagent because it is a strong nucleophile, particularly in its thiophenolate anion form, and it provides a sensitive chromophore for UV detection. The precursor, mechlorethamine, has no suitable chromophore and is highly reactive, making it poorly suitable for HPLC analysis. Dimethylformamide (DMF) was selected as the reaction solvent because (1) it has no OH group available for reaction with mechlorethamine, and thus, it does not compete with benzenethiol to reduce the reaction yield or generate side products, and (2) it is a polar aprotic solvent which favors bimolecular nucleophilic substitution (S_N2) displacement reactions. The reaction mechanism for alkylation with a nitrogen mustard is reported to involve two steps: (1) an intramolecular cyclization to an aziridinium ion, and (2) an S_N2 reaction between the aziridinium ion and a nucleophile [22]. In the case of mechlorethamine, step 1 is faster than step 2, thus, the S_N2 reaction is the rate limiting step. *tert*-Butylamine (TBA) is a sufficiently strong base to form the nucleophilic thiophenolate anion, which significantly enhances the rate of the reaction. Heptane (1 ml) was added to the DMF (∼2 ml) in the reaction solution to solubilize ointment preparations. Since heptane and DMF are immiscible in these proportions, the reaction mixture is biphasic and both polar and non-polar materials are dissolved. With all materials in solution (with the possible exception of a small amount of flocculent material from some topical formulations) mechlorethamine or its derivative cannot be occluded in a solid matrix, and therefore, high recoveries are attained. The amounts of benzenethiol, TBA, DMF, and heptane were optimized for the reaction. During the purification steps, the product was extracted as an amine salt into aqueous HCl, washed with heptane to remove non-polar reagents and ointment excipients, neutralized to the free amine, and back extracted into the predominately heptane ISTD solution. With some formulations (e.g., Aquaphilic® ointment), a gelatinous precipitate forms in the final extract which significantly lowers recovery. Addition of 1 ml of MeOH during the extraction process prevents the formation of this precipitate and yields high recoveries. However, addition of excess MeOH (>2 ml) during the reaction workup reduces the recovery of both the ISTD and the analyte.

The rate of formation of the benzenethiol–mechlorethamine disubstitution product, observed by conducting a series of reactions on mechlorethamine standard under identical conditions for different lengths of time, is shown in the upper trace of Fig. 2. The reaction reaches 94% completion in 30 min and close to 100% in 60 min. When the reaction was continued for 1.5, 2, 4, 8, and 24 h, the yield was consistently 100%. Thus, the reaction reaches completion in about 1 h and the disubstituted benzenethiol product is stable under the reaction conditions. Reactions on mechlorethamine standard that were not stirred proceeded slightly slower than stirred reactions, gave yields that were 1–2% lower, and had slightly greater variations in assay values (stirred: 0.2% RSD, $n = 6$; not stirred: 0.7% RSD, $n = 6$). Stirring was preferred for these reasons and to promote mixing of the ointment preparations.

During early stages of the reaction, a peak is seen in the chromatogram that appears between the ISTD and the disubstitution product peaks. This peak is due to the intermediate monosubstitution product. Fig. 2 shows that this compound is at its highest concentration within the first few minutes, then declines with time as the disubstitution product increases. In

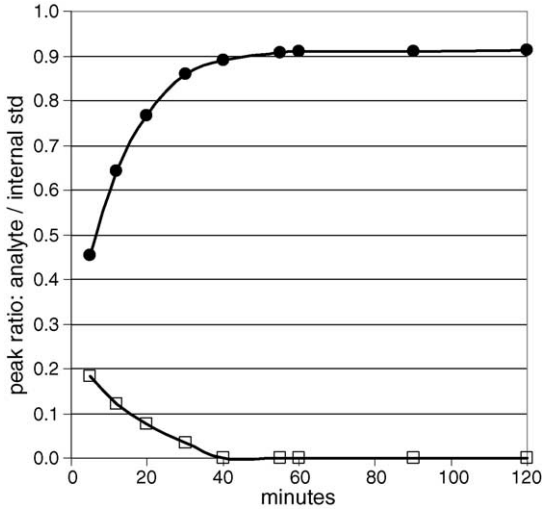

Fig. 2. Rate of reaction of benzenethiol with mechlorethamine at 50 °C in DMF. Key: (●) di-substitution product and (□) mono-substitution product.

order to confirm the structure of this compound, a synthesis was stopped after 3 min at 50 °C, worked up in the usual manner except the sample solvent was added instead of the ISTD solution. When analyzed by HPLC, two peaks were seen in the chromatogram, one corresponding to the usual disubstitution product and a peak at shorter retention time for the suspected monosubstitution product. The early eluting component was collected from the column effluent and its structure was confirmed by MS and UV spectrophotometry (see discussion below).

Bis(2-phenylthioethyl)methylamine has been synthesized previously [23] by the reaction of benzenethiol with mechlorethamine in the presence of KOH in refluxing ethanol. In that study, the compound was evaluated as a potential Ag(I) complexing reagent. The product obtained from the synthesis reported there, as from the synthesis reported here, is the disubstitution compound. To demonstrate that the same product was obtained under these two different conditions, the earlier reported synthesis was repeated here on an analytical scale, mimicking the synthetic procedure described above, using KOH in absolute ethanol in place of TBA in DMF, with and without the addition of heptane, at 50 and 78 °C (the boiling point of absolute ethanol). The products from the two syntheses are the same by HPLC analysis. After 2 h at 50 °C, the KOH/ethanol reaction reached 85% completion, and after 2 h at 78 °C or 6 h at 50 °C the reaction was complete. No decomposition of the product occurred in the KOH/ethanol reaction solution at 78 °C for 22 h. While both reactions gave high yields, the KOH/ethanol reaction gave yields that were about 2–5% below those from the TBA/DMF reaction and showed greater variation in results. The MS, MS product ion spectra, and the UV spectra are identical for the products of the two reactions (see data below).

3.2. MS analysis

Analysis of the product of the reaction of mechlorethamine with benzenethiol by positive ion electrospray ionization MS yields the pseudo molecular ion for the disubstituted benzenethiol derivative at *m*/*z* 304 amu (MH^+, Fig. 3a). CID of the pseudo molecular ion at 304 amu generates a fragment at *m*/*z* 137 due to fragmentation of the N–CH_2 bond with the positive charge residing on the phenylthioethyl fragment (Fig. 3b). The MS and CID analysis of the disubstitution product from the synthesis using KOH/EtOH as previously reported yields identical results, thus, confirming the forma-

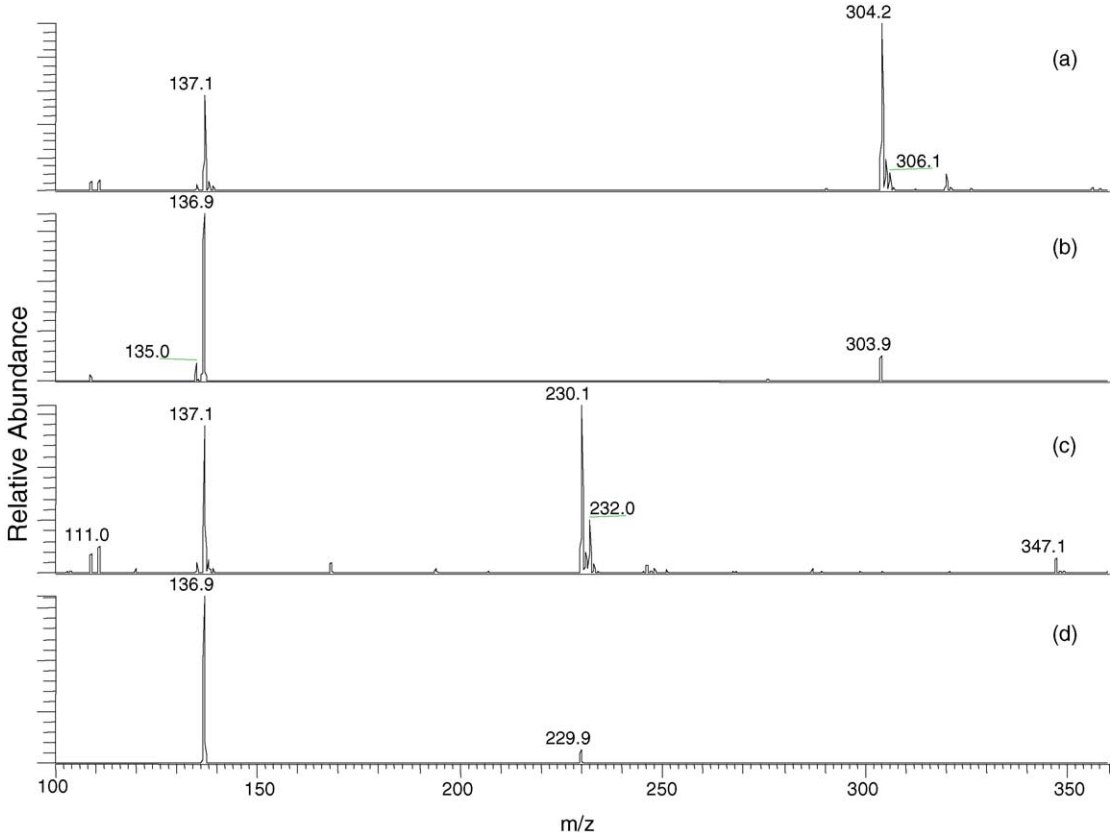

Fig. 3. (a) Electrospray ionization (ESI) MS of the benzenethiol derivative of mechlorethamine showing the pseudo molecular ion (MH^+) at *m*/*z* 304 amu, (b) CID of the parent ion (*m*/*z* 304) using a collision energy of 26% to generate the daughter ion at *m*/*z* 137, (c) ESI MS of the monosubstitution product of benzenethiol with mechlorethamine obtained during early stages of the reaction showing the pseudo molecular ion (MH^+) at *m*/*z* 230 amu with a $P+2$ peak at *m*/*z* 232, and (d) CID of the monosubstitution product parent ion (*m*/*z* 230) using a collision energy of 23% to generate the daughter ion at *m*/*z* 137, the same fragment obtained from the disubstitution product.

tion of the disubstitution product in the synthesis reported here.

When the synthesis was conducted at a shorter reaction time of 3 min or at a lower reaction temperature of 30 °C, a second compound was formed. This compound, suspected to be the monosubstitution product, was collected from the HPLC effluent and analyzed by MS, which gave the pseudo molecular ion at *m/z* 230 amu (MH^+, Fig. 3c) with a *P* + 2 peak at *m/z* 232 corresponding the ^{37}Cl isotope, and a fragment at *m/z* 137, the same fragment as that from the disubstitution product. CID experiments on the parent peak at *m/z* 230 generates the fragment at *m/z* 137 (Fig. 3d), the same fragment observed for the CID analysis of the disubstituted compound described above.

3.3. UV spectrophotometry

A UV spectrum of the analyte, recorded during HPLC analysis using a photodiode array detector, is shown in Fig. 4. The compound has a maximum absorbance at 256 nm. This is in agreement with the λ_{max} 256 nm for phenyl ethyl sulfide [24], a compound that contains the same chromophore as the analyte. To achieve optimal response for the analyte, a wavelength of 256 nm was selected for the HPLC analysis.

The UV spectrum of the monosubstitution product gave a λ_{max} at 256 nm and was superimposable with spectrum of the disubstitution product, as expected since these two compounds have the phenylthioethyl chromophore in common. The UV spectrum of the disubstitution product from the KOH/EtOH reaction was also superimposable with the product prepared via the method described here.

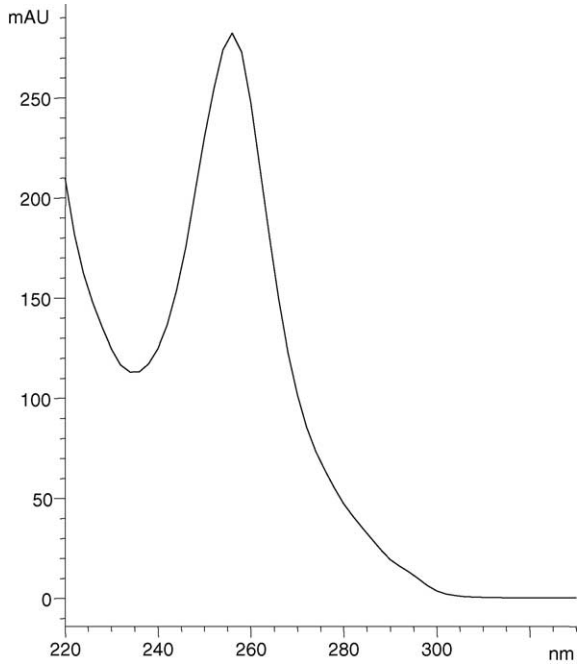

Fig. 4. Ultraviolet (UV) spectrum of the benzenethiol derivative of mechlorethamine in the mobile solvent (H_2O–MeCN–MTBE–heptane, 0.01:2:8:100) recorded during the analysis by HPLC.

3.4. HPLC

A typical chromatogram showing the analysis of mechlorethamine in Aquaphor® ointment is shown in Fig. 5. The chromatogram is clean, showing only peaks due to dibutyl phthalate (ISTD), the analyte bis(2-phenylthioethyl)methylamine, and a peak which elutes near the solvent front. Minor peaks of relatively insignificant intensity can be seen when the chromatographic scale is expanded. The peak near the solvent front is more intense for samples that were extracted fewer than three times with heptane during the purification step. Chromatograms of derivatized mechlorethamine standard or mechlorethamine in Aquaphor® ointment, Aquaphilic® ointment, white petrolatum, or 50/50 white petrolatum/liquid petrolatum are almost identical. Ointment excipient peaks are absent from the chromatograms.

Retention times of the ISTD and the analyte on the silica column can be controlled by adjusting the concentration of MeCN and MTBE. MeCN has a stronger effect on the retention of the analyte than on the retention of the ISTD. Thus, by increasing the MeCN content (e.g., from 1.5 to 2%), the retention of the analyte is reduced more than that of the ISTD, and the two peaks will elute closer together. On the other hand, increasing the concentration of MTBE reduces

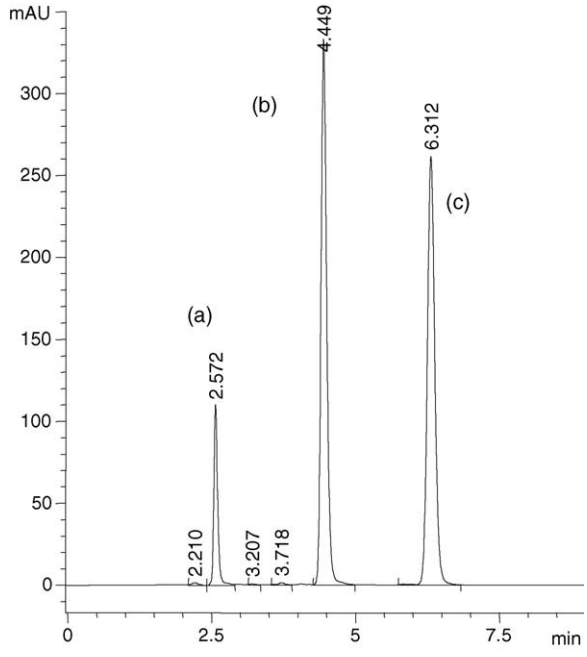

Fig. 5. A typical chromatogram for the analysis of 0.02% mechlorethamine in Aquaphor® ointment on a YMC silica gel column, 5 μm particle size, 150 mm × 4.6 mm, using a mobile phase consisting of H_2O–MeCN–MTBE–heptane (0.01:2:8:100). Key: (a) solvent front, (b) *n*-butyl phthalate (internal standard), and (c) benzenethiol disubstitution product of mechlorethamine (analyte).

the retention of both compounds similarly. Changing the ratio of MTBE-heptane from 8:92 to 10:90 or from 8:92 to 7:93 results in changes in retention times of less than 0.4 min for both of the two peaks on both 150 mm silica columns. The higher the ratio of MTBE to heptane, the shorter the retention times. A small amount of water (0.009%) was included in the mobile solvent to control the activity of the silica gel, and thus, minimize retention time drift once the column has equilibrated. The analyte peak requires a longer initial column equilibration time and is subject to more retention time drift than the ISTD peak. However, since the mobile phase is delivered under isocratic conditions, once equilibration is established, the retention times and area ratios are quite reproducible even though there may be a small drift in retention time from run to run. One sample solution derived from the assay of 0.02% mechlorethamine in Aquaphor® ointment, injected repeatedly ($n = 10$) to test the reproducibility of the HPLC system, gave the following results: retention time of ISTD: 0.04% RSD; retention time of analyte: 0.19% RSD; area of ISTD: 0.07% RSD; area of analyte: 0.06% RSD; area of analyte/area of ISTD: 0.05% RSD.

3.5. Linearity, limit of quantitation, limit of detection, precision, recovery

The assay procedure requires a number of steps: synthesis of the benzenethiol derivative, purification by extraction into aqueous acid and washing with heptane, isolation by back extraction from aqueous base into the ISTD solution (primarily heptane), and analysis by HPLC. Each of these steps has an associated error, and the error of the total assay procedure is the sum of the errors of these individual steps. In the method discussed in this paper, both the synthetic yields and recovery from ointments are high, and this helps in attaining a high degree of precision. The precision of the assay for mechlorethamine HCl standard and the precision and recovery of the assay for mechlorethamine HCl in various ointment preparations when carried through all the steps of the method are given in Table 1.

The assay method was evaluated using various types of ointment preparations. White soft paraffin (white petrolatum) and 50/50 white soft paraffin/liquid petrolatum are anhydrous, hydrophobic petrolatum-based ointments. Aquaphor® (Beiersdorf, Inc., Wilton, CT, USA) ointment is an anhydrous, hydrophilic ointment which contains white petrolatum and other components and has the ability to absorb water. Aquaphilic® ointment (Medco Labs, Inc., Sioux City, IA, USA) is a hydrous, hydrophilic ointment which contains ∼50% water, ∼20% white petrolatum, ∼20% stearyl alcohol, and other components. Table 1 shows that the recovery of mechlorethamine from these ointments is in the range of 98.4–100.4%. Thus, the recovery and precision for this method in which a benzenethiol derivative is formed is a significant improvement over the recovery (76.1%) and precision (10.4% RSD) reported for the assay of mechlorethamine by formation of a diethyldithiocarbamate derivative [12].

Solutions containing 5, 10, 20, 40, 60, and 80 μg of mechlorethamine were derivatized and analyzed by HPLC. A plot of micrograms of mechlorethamine HCl versus signal response gave a linear plot with $r^2 = 0.99999$. While an amount equivalent to 40 μg of mechlorethamine HCl (an amount found in 200 mg of an ointment containing 0.02% mechlorethamine HCl) is used normally in the analysis, the limit of detection is 0.004 μg (signal-to-noise = 4) and the limit of quantitation (requiring a maximum RSD of 5%) is 0.02 μg (4.6% RSD, $n = 6$; signal-to-noise = 20).

3.6. Stability of mechlorethamine and the benzenethiol–mechlorethamine derivative in solution

Stock solutions of mechlorethamine in DMF were prepared at higher concentrations (e.g., 0.4 mg ml^{-1}) for spiking experiments during method development and at lower concentrations (e.g., 0.04 mg ml^{-1}) for use as standard solutions and stored at room temperature in the dark. These solutions were assayed by the procedure described in this paper to detect any loss of mechlorethamine after several days of storage. Two concentrated solutions showed a loss of 1.1 and 1.6% in 18 days, and a third concentrated solution showed a loss of 2.8% in 32 days. Two solutions at 0.04 mg ml^{-1} showed losses of 4.2 and 4.8% in 13 days. Thus, more concentrated solutions are more stable than dilute solutions. Due to the sensitivity of mechlorethamine to moisture, we suspect that these losses are due to hydrolysis, although it is possible

Table 1
Precision for the HPLC assay of mechlorethamine HCl standard and precision and recovery for the HPLC assay of mechlorethamine HCl in ointment preparations

Preparation	μg[a]	Precision (% RSD)[b]	Recovery (%)
Mechlorethamine HCl standard, day 1	40	0.24	–
Mechlorethamine HCl standard, day 2	40	0.21	–
Mechlorethamine HCl standard, day 3	40	0.15	–
Mechlorethamine HCl standard	4	0.14	–
Aquaphor ointment	40	0.52	100.4
Aquaphor ointment	4	0.23	99.5
Aquaphilic ointment	40	0.08	98.4
White soft paraffin	40	0.13	98.8
50/50 white soft parafin/liquid paraffin	40	0.45	99.4

[a] Amount in μg of mechlorethamine HCl as standard or amount used to spike 200 mg ointment.
[b] % Relative standard deviation ($n = 6$) for the assay method (includes all steps: derivatization through HPLC).

that the losses or part of the losses are due to adsorption of the analyte onto the glass surface. These decreasing concentrations of mechlorethamine in DMF during storage should be considered when preparing standard solutions because fresh solutions improve the assay accuracy over aged solutions. The more dilute solutions (40 $\mu g\,ml^{-1}$) should be freshly prepared each day.

Four samples of benzenethiol-mechlorethamine derivative in dibutyl phthalate ISTD solution were analyzed on days 0, 3, and 10. There was no detectable degradation of the benzenethiol–mechlorethamine product or the dibutyl phthalate ISTD in the HPLC sample solvent at room temperature over 10 days. In addition, the mobile solvent was still good after storage in a tightly sealed bottle at room temperature for 10 days.

As reported above, the benzenethiol–mechlorethamine derivative showed no decomposition under the derivatization reaction conditions for 24 h.

4. Conclusions

This report presents an assay for mechlorethamine in topical pharmaceutical preparations by derivatization with benzenethiol followed by normal-phase silica gel HPLC. The benzenethiol converts the highly reactive mechlorethamine to a stable compound and adds a strong chromophore visible to a UV detector. The method is sensitive, specific, and precise, has a short analysis time using an isocratic mobile phase, gives high recovery from topical pharmaceutical formulations, and has minimal or no chromatographic interference from excipients or reagents.

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