HPLC-UV DETERMINATION OF A MECHLORETHAMINE CROSSLINK IN A DNA DUPLEX CONTAINING A CYTOSINE-CYTOSINE MISMATCH PAIR

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ABSTRACT: Mechlorethamine is a nitrogen mustard that can form a crosslink at a C-C mismatch pair in a DNA duplex. These pairs may occur in DNA structures linked with Fragile X syndrome, a triplet repeat expansion disease, and mechlorethamine may be a useful probe for these structures. The molecular structure of the mechlorethamine C-C crosslink has not been determined, in part due to the poor recovery of the crosslinked duplex following gel electrophoretic separation. Here, we report the development of a simple high-performance liquid chromatographic (HPLC) method for isolation of the mechlorethamine crosslink in a DNA duplex containing a C-C mismatch pair. The mechlorethamine crosslink was prepared by incubating a DNA duplex d[CTCACACCGTGGTTC]•d[GAACCACCGTGTGAG] (underlined bases are a C-C mismatch pair) with mechlorethamine at room temperature for 2 hours. Isolation of the crosslinked duplex was most efficient using a Biobasic-C4 column with gradient elution of 5-15% acetonitrile in 100 mM TEAA and 0.1 mM EDTA over 60 min at a flow rate of 1 ml/min, a column temperature at 33°C, and UV detection at 260 nm. The crosslinked duplex (25% of the total DNA) eluted at 40.54 min, while the unreacted DNA strands eluted at 28.02 and 25.81 min. Thus, the HPLC method gave good separation between the crosslink and the unreacted DNA. The percentage of crosslink out of the total DNA detected by this method was about 25%, in good agreement with our previous results for this reaction based on detection by gel electrophoresis. Therefore, we conclude that the HPLC method can be used for preparation of purified mechlorethamine-crosslinked C-C mismatch DNA for further structural characterization.

Keywords: DNA crosslink, Mechlorethamine, HPLC, C-C mismatch pair

INTRODUCTION: Mechlorethamine (Figure 1A) is a nitrogen mustard that reacts at nucleophilic sites of DNA, including N7 of guanine[1,2] and N3 of adenine[3-5]. The antitumor activity of mechlorethamine is partly due to interstrand DNA crosslink formation between two guanine bases at d[G_1XC]•d[G_3YC] duplex fragments, where X-Y is a Watson-Crick pair[6-10] (Figure 1B). This reaction results in crosslinking of the G_1-G_3 bases with mechlorethamine in the major groove of the duplex. We have shown that mechlorethamine can also form an interstrand DNA crosslink at a cytosine-cytosine (C-C) mismatch pair[11] (Figure 1C). This reaction seems to occur in the DNA minor groove through N3 of each cytosine, but the structure of the mechlorethamine C-C crosslink has not been determined.

The instability of C-C mismatch pairs allows DNA containing this pair to adopt different conformations, including those found in unusual structures formed by the d[CCG]$_n$ strand of the trinucleotide repeat sequence d[CGG]$_n$•d[CCG]$_n$, which is associated with Fragile X syndrome[12-16]. The discovery of the C-C crosslinking reaction

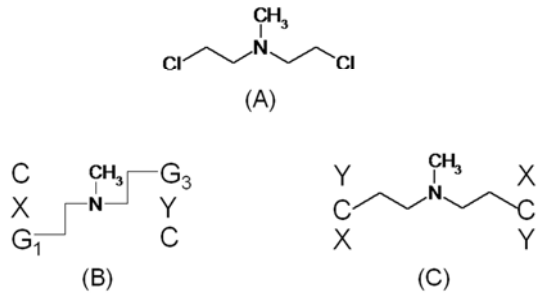


Figure 1 Mechlorethamine (A) and representation of DNA interstrand crosslinks induced by mechlorethamine at (B) a G_1-G_3 guanine bases and (C) a C-C mismatch pair; where X-Y is a Watson-Crick pair.

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suggests that mechlorethamine may be used to probe structures formed by d[CCG]$_n$. It would be of benefit to determine the structure of the mechlorethamine C-C crosslink for development of the probing assay, and this requires initial purification of a large amount of crosslinked DNA.

Polyacrylamide gel electrophoresis (PAGE) techniques have been widely used for the isolation and analysis of synthetic oligonucleotides, DNA restriction fragments, polymerase chain reaction (PCR) products, and plasmids. These methods have also been developed for detection and separation of crosslinked DNA, including mechlorethamine-crosslinked DNA[11,15-18]. However, only a low yield is obtained with PAGE, and an alternative method for preparation and purification of the mechlorethamine-crosslinked C-C mismatch is needed for further structural characterization. One potential technique is high performance liquid chromatography (HPLC), since this facilitates collection of large quantities of a highly purified sample. Here, we report the development of a simple HPLC method for isolation of the mechlorethamine crosslink in a DNA duplex containing a C-C mismatch pair.

MATERIALS AND METHODS:

Chemicals

Synthetic 15-mer oligodeoxynucleotides d[CTC ACACCGTGGTTC] (top-strand DNA) and d[GA ACCACCGTGTGAG] (bottom-strand DNA) were purchased from Sigma-Proligo (St. Louis, MO, USA). Mechlorethamine hydrochloride, ethylene-diaminetetraacetic acid (EDTA) disodium salt, dimethylsulfoxide, tris(hydroxylmethyl)amino methane (Tris) and triethylamine were purchased from Sigma (St. Louis, MO, USA). Acetonitrile was purchased from Lab-Scan (Samutsakorn, Thailand) and glacial acetic acid was purchased from Carlo Erba (Bangkok, Thailand). All reagents were of AR or HPLC grade.

HPLC Instrumentation

HPLC analysis was performed using a Shimadzu-VP system (Shimadzu, Japan) consisting of an SCL-10A VP system controller, an LC-10AD VP pump, an SIL-10AD VP auto-injector, a DGU-14A degasser, an SPD-10A VP UV-VIS detector and LC Solution Software.

Preparation of Mechlorethamine-Crosslinked DNA

Stock solutions of top- and bottom-strand DNA were separately prepared in purified water at 100 µM. The stock solutions of top- and bottom-strand DNA were mixed in 0.1 M sodium chloride and 0.05 M Tris buffer pH 7.5 to obtain a DNA duplex solution at 10 µM. The solution was heated at 90ºC for 2 min and then slowly cooled to room temperature to allow annealing of the DNA to form a duplex d[CTCACAC<u>C</u>GTGGTTC]• d[GAACCAC<u>C</u>GTGTGAG] (underlined bases are a C-C mismatch pair). The duplex solution (100 µl) was incubated with 0.1 M mechlorethamine in dimethylsulfoxide (1 µl) for 2 hours at room temperature, and the resulting reaction mixture was subjected to HPLC determination.

Determination of Mechlorethamine-Crosslinked DNA

The mechlorethamine-crosslinked DNA duplex was determined by reverse-phase HPLC using a Biobasic-C4 column (4.6×250 mm i.d., 5µm) (Thermo Electron, Waltham, MA) with a column temperature of 60ºC, 33ºC or 25-30ºC (ambient temperature). Gradient elution was performed with 5-15% acetonitrile in 100 mM triethylamine acetate (TEAA) and 0.1 mM EDTA over 60 min at a flow rate of 1 ml/min with UV detection at 260 nm. The injection volume was 20 µl. The percentage of DNA crosslinked by mechloreth-amine was calculated from the peak area of the crosslink divided by the sum of all peaks in the chromatogram, multiplied by 100. Control experiments were performed under the same HPLC conditions by injecting top-strand DNA, bottom-strand DNA, or annealed DNA duplex.

RESULTS:

HPLC Chromatograms

HPLC chromatograms obtained using a column at 60ºC, 33ºC or ambient temperature are shown in Figures 2-4, respectively. The retention times of the top-strand DNA, bottom-strand DNA, annealed duplex, and crosslinked DNA are shown in Table 1. The chromatograms of the top- and bottom-strand DNA gave single peaks (Figures 2-4A&B) while the chromatogram of the annealed duplex gave two peaks due to denaturation of the

duplex on the column (Figures 2-4C). These two peaks corresponded to bottom- and top-strand DNA, respectively. The chromatogram of the cross-linked DNA showed three peaks (Figures 2-4D). The first two of these peaks corresponded to bottom- and top-strand DNA, respectively, while the third peak was assigned to the mechlore-thamine-crosslinked DNA duplex.

Determination of Mechlorethamine-Crosslinked DNA

Mechlorethamine-crosslinked DNA accounted for 19%, 25% and 22% of the total DNA in HPLC assays using columns at 60°C, 33°C and ambient temperature, respectively (Table 1).

DISCUSSION: The control sample containing the annealed DNA duplex without mechlorethamine gave two peaks on the chromatogram (Figure 2-4C). The first peak, identified as the bottom-strand DNA, is consistent with the peak observed in the control sample containing only the bottom-strand DNA (Figure 2-4B). Similarly, the second peak, identified as the top-strand DNA, is consistent with the peak observed in the control sample containing only the top-strand DNA (Figure 2-4A). The observation of two peaks corresponding to the top- and bottom-strand DNA, without a duplex peak, suggested that the duplex is denatured under the HPLC conditions

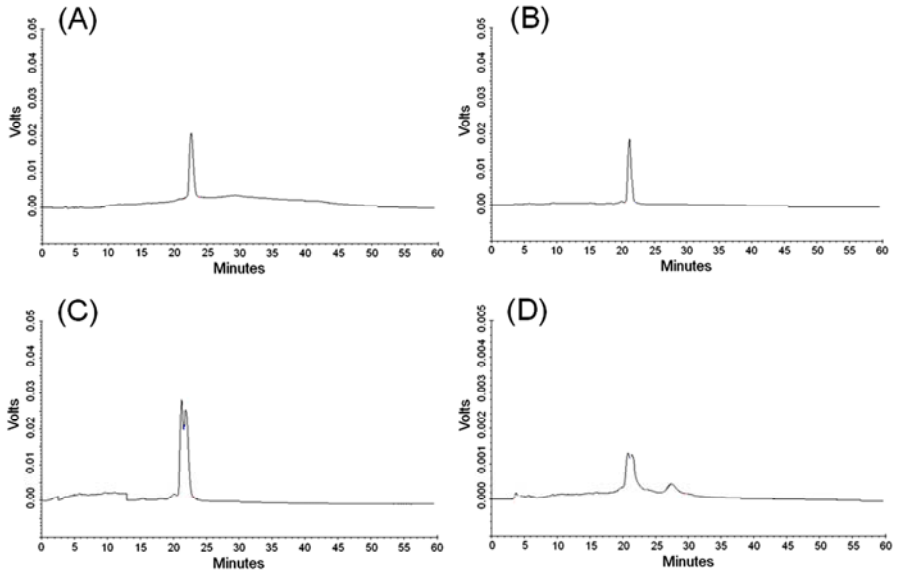


Figure 2 HPLC-UV chromatograms using a column temperature of 60°C

(A) Top-strand DNA,
(B) bottom-strand DNA,
(C) DNA duplex, and
(D) the mechlorethamine-crosslinked DNA duplex

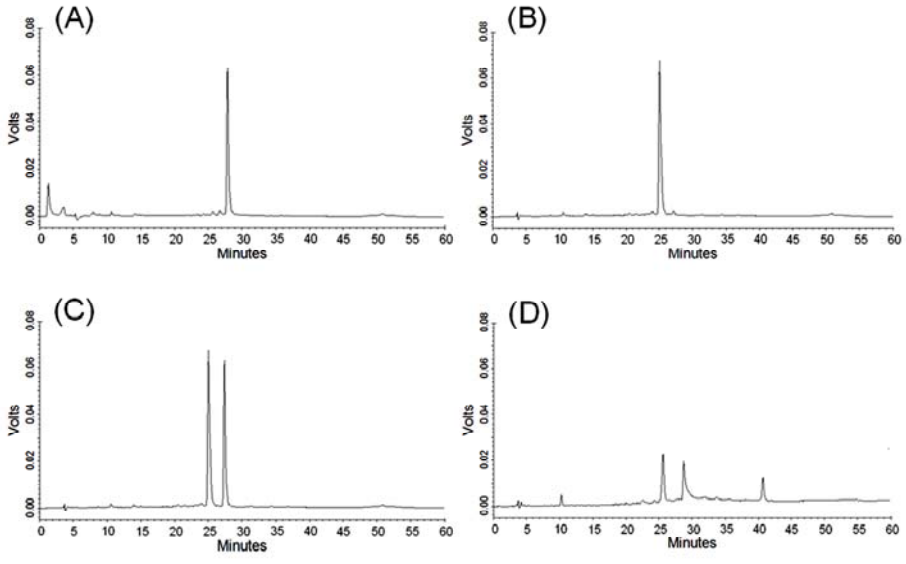


Figure 3 HPLC-UV chromatograms using a column temperature of 33°C

(A) Top-strand DNA,
(B) bottom-strand DNA,
(C) DNA duplex, and
(D) the mechlorethamine-crosslinked DNA duplex

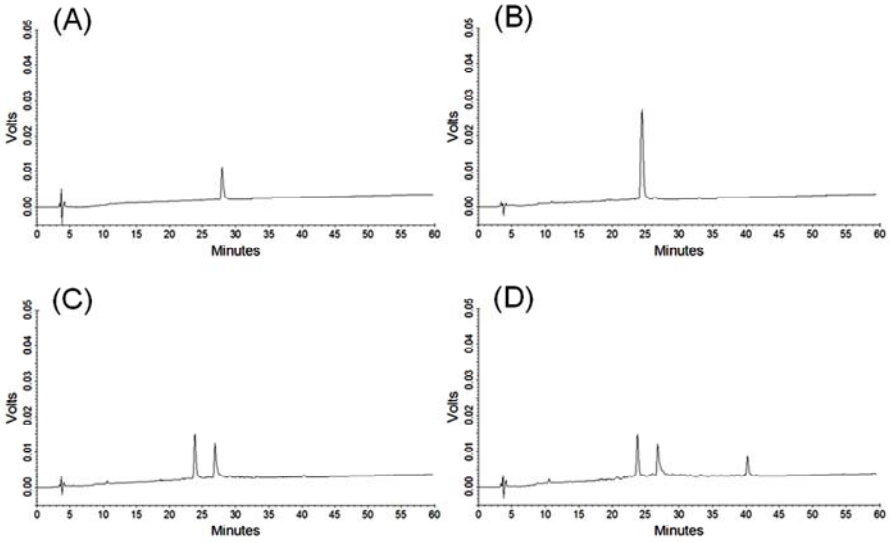


Figure 4 HPLC-UV chromatograms using a column at ambient temperature (~25ºC-30ºC)

(A) Top-strand DNA,
(B) bottom-strand DNA,
(C) DNA duplex, and
(D) the mechlorethamine-crosslinked DNA duplex

Table 1 Retention times of top-strand DNA, bottom-strand DNA, and mechlorethamine-crosslinked C-C mismatch DNA, and the amount of crosslinked DNA as a percentage of the total DNA

Samples	Retention Time (min)			Crosslink*
	Top-Strand	Bottom-Strand	Crosslink	(%)
Column temperature at 60ºC				
Top-Strand DNA	22.64	-	-	
Bottom-Strand DNA	-	21.18	-	
Duplex DNA	21.91	21.28	-	
Mechlorethamine-DNA Crosslink	21.39	20.80	27.41	19
Column temperature at 33ºC				
Top-Strand DNA	27.87	-	-	
Bottom-Strand DNA	-	25.07	-	
Duplex DNA	27.93	25.56	-	
Mechlorethamine-DNA Crosslink	28.02	25.81	40.54	25
Column at ambient temperature (about 25-30ºC)				
Top-Strand DNA	27.98	-	-	
Bottom-Strand DNA	-	24.47	-	
Duplex DNA	26.91	23.88	-	
Mechlorethamine-DNA Crosslink	26.91	23.81	40.30	22

$$*\%\ \text{Crosslink} = \frac{(\text{Crosslink}) \times 100}{[(\text{unreacted top strand}) + (\text{unreacted bottom strand}) + (\text{Crosslink})]}$$

used. In the presence of mechlorethamine, the duplex was crosslinked and the chromatogram of the reaction mixture showed three peaks (Figure 2-4D). The first two peaks were identified as the bottom- and top-strand DNA, respectively, based on the chromatograms of the control experiments (Figures 2-4A, 2-4B and 2-4C). The third peak eluted after the single strands, indicating a product with increased molecular weight. This suggests that the third peak is due to the mechlorethamine-crosslinked duplex.

In the HPLC analysis, we slowly increased the amount of acetonitrile over 60 min to ensure complete separation of the mechlorethamine-crosslinked DNA from unreacted strand DNA. The HPLC analysis was performed at 60ºC, 33ºC and ambient temperature (about 25-30ºC). All temperatures used in this study in conjunction with the presence of acetonitrile are sufficient to denature the duplex, since the C-C mismatch causes a low melting temperature (Tm). An advantage of having the DNA duplex denature during the HPLC separation is the avoidance of interference of the duplex peak with the mechlorethamine-crosslink peak. However, the temperature at 60ºC gave poor resolution of the top- and bottom-strand DNA, while the ambient temperature may reduce the precision for the retention time. Therefore, the HPLC analysis should be performed at a column temperature of 33°C. This column temperature is low enough to ensure resolution of the single strands while giving good precision for the retention time.

The amount of mechlorethamine-crosslinked DNA as a percentage of the total DNA was in the range of 19-25%. The differences in the percentage crosslink could be due to the poorer resolution of the top- and bottom-strand DNA at higher temperature and poor precision at lower temperature. Thus, the peak areas of the top- and bottom-strand DNA might not be accurate due to the poor resolution at 60ºC and the low precision at ambient temperature. The amount of mechlor-ethamine-crosslinked DNA determined by gel electrophoresis was about 25-27%[16], and HPLC analysis with a column temperature of 33ºC gave a percentage of mechlorethamine-crosslinked DNA of about 25%. This result supports the conclusion that HPLC using a column at 33ºC provides the optimum conditions for isolation of the crosslink species.

CONCLUSION: In this work, we established a method for preparation and HPLC isolation of a mechlorethamine-crosslinked DNA duplex containing a cytosine-cytosine mismatch pair. The HPLC method was simple and demonstrated good separation between the methlorethamine C-C crosslink and unreacted DNA. Therefore, the method can be used for preparation of purified mechlorethamine-crosslinked C-C mismatch DNA with high recovery of the crosslink compared to gel electrophoresis. We are currently characterizing the connectivity of the crosslink species using mass spectrometry.

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