Research Article

Rapid Communications in Mass Spectrometry

Received: 27 February 2011 Revised: 17 April 2011 Accepted: 17 April 2011 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2011**, *25*, 2027–2034
(wileyonlinelibrary.com) DOI: 10.1002/rcm.5064

Quantification of meCCNU-induced dG-dC crosslinks in oligonucleotide duplexes by liquid chromatography/electrospray ionization tandem mass spectrometry

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Chloroethynitrosoureas (CENUs) are important alkylating agents widely used in the treatment of cancers. Decomposition of CENUs generates active electrophilic ions that damage DNA, including the formation of dG-dC crosslinks which represents the most important cytotoxic mechanism of CENUs. In this work, a high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) method was employed to analyze the dG-dC crosslinks induced by 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (meCCNU, Semustine). The direct quantitation of dG-dC crosslinks in oligonucleotide duplexes was achieved by the selected reaction monitoring (SRM) mode using synthesized $^{15}N_3$-labeled dG-dC as an internal standard. Methods of enzymatic digestion and HPLC separation were developed for obtaining separation and reproducibility of the dG-dC peak in chromatograms. The limit-of-detection (LOD) was determined to be 0.08 nM and the limit-of-quantification (LOQ) was determined to be 0.16 nM. The linearity of the calibration curve was 0.9997 over the range of 0.08 to 32 nM. The precision and accuracy of the method ranged from 1.1 to 6.6% and 96 to 109%, respectively. The recovery of the dG-dC crosslink in the enzymatic hydrolysates from the oligonucleotide duplex was determined to be from 91 to 106%. The results of the validation study indicate that the method is suitable for quantifying dG-dC crosslinks in DNA. Consequently, this method was used to determine meCCNU-induced dG-dC crosslinks in four duplexes with different GC contents. The results showed that the crosslinking fraction (CF) increased as the GC content in the duplex increased, and a relatively low CF was observed in the early period of the reaction.

Chloroethynitrosoureas (CENUs), such as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (meCCNU), have been widely used as chemotherapeutics for the inhibition of leukemia, Hodgkin's disease and various solid tumors.[1–4] CENUs are unstable in aqueous media and spontaneously decompose to yield chloroethyldiazohydroxides, which give rise to diazonium ions, and perhaps other carbocations.[5–10] These electrophilic ions damage DNA by alkylation of bases, single/double strand breakages and interstrand crosslinks (ISC).[10–12] Significant effort has been devoted to the investigation of DNA damage by CENUs. These studies demonstrated that DNA ISC were the most important type of DNA modification leading to cytotoxicity by CENUs. This is because such DNA modifications prevented the separation of DNA double strands in the replication process.[13,14] Bodell *et al.*[15,16] compared the levels of DNA alkylation products in human glioma cell lines after treatment with ^{3}H-CENUs using high-performance liquid chromatography (HPLC). They demonstrated that there was a significant correlation between LD_{10} of CENUs and the levels of dG-dC crosslink, which suggested that the levels of dG-dC crosslink could be used as molecular dosimeters of therapeutic response following treatment with CENUs. Electrophoretic and fluorescence assays were performed for the quantification of ISC induced by CENUs. Chen *et al.*[17] investigated the effect of buffers on DNA adduction, crosslinks and cytotoxicity induced by CENUs using polyacrylamide gel electrophoresis. Their results demonstrated that Tris increased the yield of not only the monofunctional adducts, but also the bifunctional adducts, such as the formation of the interstrand dG-dC crosslinks. The agarose gel electrophoresis assay of ISC showed a correlation between the time of exposure to Fotemustine and Semustine (meCCNU) and the level of ISC,[18] which indicated increasing exposure time led to increases in the amount of ISC formed. Penketh *et al.*[19] investigated the ISC induced by Cloretazine, which is a relatively new prodrug with a similar anticancer mechanism to CENUs, using a fluorescence assay with the Hoechst 33258 dye. Using an ethidium bromide fluorescence assay, Ueda-Kawamitsu *et al.*[20] measured the time course of DNA ISC in L1210 cells treated with BCNU. The results showed that the percentage of crosslinks reached the maximum after 6 h exposure to BCNU and subsequently decreased presumably because of DNA repair. As hypothesized in our previous research,[21,22] the distance between a pair of negative atoms in DNA base pairs spatially matches with

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the $–CH_2–CH_2–$ ethylene bridge. Consequently, the N3 position of cytosine is inferred to be the favorable site for the second alkylation and therefore the crosslinks form between the N1 atom of guanine and the N3 atom of cytosine. By modeling a crosslinked 13-mer B-DNA, the crosslink between N1 of guanine and N3 of cytosine was demonstrated to be the most energetically preferred structure when compared with the crosslinks of G(O^6)-C(N^4), G(N^2)-C(O^2) and A(N^6)-T(O^4).[23] The works mentioned above reported a series of methods for the quantitation of DNA ISC induced by CENUs. However, these methods were relatively non-specific for the detection of dG-dC crosslinks and validation of the methods was insufficient.

In this work, a high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) method was employed to analyze the dG-dC crosslinks induced by meCCNU. The direct quantitation of dG-dC crosslinks was achieved by selected reaction monitoring (SRM) mode following enzymatic digestion and HPLC separation. The method was applied to determine the crosslinking fractions of four oligonucleotide duplexes with different sequences. This work is expected to shed light on *in vitro* and *in vivo* investigations examining the formation of DNA adducts induced by anticancer agents, carcinogens and related chemicals. Based on the sensitivity of the assay, only several micrograms of DNA are required for analysis of animal or human samples.

EXPERIMENTAL

Materials

MeCCNU, acetonitrile (HPLC grade), 2′-deoxyguanosine, 2′-deoxycytidine and phosphodiesterase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nuclease S1, alkaline phosphatase (CIAP) and deoxyribonuclease I were obtained from TaKaRa (TaKaRa Biotechnology, Japan). $^{15}N_3$-2′-Deoxycytidine was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The synthetic oligonucleotides were acquired from SBS (SBS Genetech Co., Ltd., China). Microcon YM-10 centrifugal columns were purchased from Millipore (Billerica, MA, USA). All other chemicals, reagents and solvents were purchased from Sigma-Aldrich. Deionized water was purified using a PALL deionizer (NYSE, PALL, USA).

Standards

The synthesis of the dG-dC standard, 1-[N^3-deoxycytidyl]-2-[N^1-deoxyguanosyl]ethane, was carried out according to previously reported procedures.[15,24,25] N^2,3′,5′-Triacetyl-2′-deoxyguanosine was initially synthesized and was used as the material for the subsequent synthesis of O^6-(2-fluoroethyl)-2′-deoxyguanosine (O^6-FEtdG). O^6-FEtdG (10 mg, 32 μmol) was reacted with 2′-deoxycytidine (20 mg, 88 μmol) in dimethyl sulfoxide (DMSO) (200 μL) at 55 °C for 20 days. The synthesized dG-dC was isolated by HPLC using a ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm particle size). The mobile phase was 2 mM ammonium acetate (0.1% acetic acid, pH 6.8) (A) and acetonitrile (B) with a gradient of 5 to 10% of buffer B for 20 min followed by a gradient to 30% buffer B over 10 min. An isocratic wash was then used at 30% buffer B for 3 min and this was followed by a gradient down to 5% buffer B over 2 min. The flow rate of the mobile phase was 1 mL/min and the UV detector was set at 258 nm.

The synthesis of the internal standard, isotope-labeled $^{15}N_3$-dG-dC, was carried out using the same procedure of unlabeled dG-dC except $^{15}N_3$-2′-deoxycytidine was used in the final step of the synthesis. Isotope-labeled $^{15}N_3$-2′-deoxycytidine (5 mg, 22 μmol) was reacted with O^6-(2-fluoroethyl)-2′-deoxyguanosine (5 mg, 16 μmol) in DMSO (50 μL) at 55 °C for 20 days. The final products and intermediates were characterized by NMR, MS/MS, IR and UV spectroscopy, and the data were consistent with results obtained previously.[15,24–26]

Incubation of oligonucleotide duplexes with meCCNU

The commercially purchased synthetic oligonucleotides were annealed to form duplexes. Sixty OD (2 mg) of oligonucleotide was freshly dissolved in annealing buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.6). The oligonucleotides were then annealed by heating the solution to 95°C and cooling slowly to room temperature. This annealing step generated duplexes with different sequences (see Table 1), which were diluted using deionized water to a concentration of 0.25 mg/mL. meCCNU (4 mg) freshly dissolved in 25 μL of alcohol was added into the duplex solution and the final concentration of meCCNU in the reaction system was 2 mM. The reaction mixture was incubated at 37°C for 10 h in the dark. At 2 h intervals, 200 μL was removed from reaction mixture. The sample solutions were stored at −20°C until enzyme hydrolysis was performed.

Enzymatic digestion of the oligonucleotide duplexes

To facilitate the release of dG-dC from the DNA duplexes, four digestion enzymes were used. Every sample solution (200 μL, containing about 50 μg of the oligonucleotides) was firstly hydrolyzed using 100 units of DNase I (30 μL, buffered in CH_3COONa 20 mM, NaCl 150 mM, pH 5.0) and 170 units of nuclease S1 (30 μL, buffered in CH_3COONa 10 mM, NaCl 150 mM, $ZnSO_4$ 0.05 mM, pH4.6). After incubation at 37 °C for 3 h, the oligonucleotides were further digested by the addition of 17 units (50 μL) of alkaline phosphatase and 3 milliunits of phosphodiesterase I (5 μL) buffered in Tris-HCl 500 mM, $MgCl_2$ 10 mM (pH 9.0), and incubated at 37 °C for 3 h. After enzymatic digestion, the samples were filtered using Microcon YM-10 centrifugal filters to remove the enzymes, and ~315 μL of filtrate was obtained from each sample which was used for HPLC/ESI-MS/MS analysis.

HPLC/ESI-MS/MS conditions

The resulting mixtures of nucleosides containing dG-dC crosslinks were analyzed by reversed-phase HPLC/ESI-MS/MS with a Thermo TSQ QUANTUM Discovery MAX triple quadrupole tandem mass spectrometer equipped with a ThermoFinnigan HPLC system (ThermoFinnigan, San Jose, CA, USA). The instrument was operated using Xcalibur 1.4 software. A ZORBAX SB-C18 column (2.1 × 150 mm, 5 μm particle size; Agilent Technologies, Palo Alto, CA, USA) was used for the separation of dG-dC from the digestion mixture. Solutions of 2 mM ammonium acetate containing 0.1% acetic

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Table 1. The sequences and GC contents of the four 26-mer oligonucleotide duplexes treated with meCCNU for the analysis of dG-dC crosslinks

Name	Sequences	Number of GC pairs	Content of GC pairs (%)	Molecular weight of duplexes
A	5′-TTAATAAATAAAATATATAAATATTA-3′ 3′-AATTATTTATTTTATATATTTATAAT-5′	0	0	15929.0
B	5′-TTAATGGGCGCAATACGCGAATATTA-3′ 3′-AATTACCCGCGTTATGCGCTTATAAT-5′	10	38	15938.6
C	5′-TTAATGGGCGCCATACGCGGGCATTA-3′ 3′-AATTACCCGCGGTATGCGCCCGTAAT-5′	14	54	15942.5
D	5′-TGGCGGGCGCCAATACGCGGGCGCGA-3′ 3′-ACCGCCCGCGGTTATGCGCCCGCGCT-5′	20	77	15948.3

acid (pH 6.8) (solution A) and acetonitrile (solution B) were used as the mobile phase. The mobile phase gradient started from 5% buffer B and linearly increased over 25 min to 15% buffer B where it was held for 2 min. The percentage of buffer B was then reduced to 5% in 1 min followed by an equilibration time of 7 min. All calibration standards and enzymatic digestion samples were introduced to the column via a 25 μL sample loop. The sample components from the HPLC column were eluted into the mass spectrometer.

Mass spectrometric detection was performed in the positive mode with an electrospray ionization (ESI) source. The parameters of the ESI source were optimized using a dG-dC standard solution and was set as follows: spray voltage 4000 V; sheath gas (nitrogen) pressure 50 psi; aux gas (nitrogen) pressure 15 psi; capillary temperature 300°C; and tube lens offset 141 V. Argon was used as the collision gas for tandem mass spectrometric analysis and the collision gas pressure was 1.0 mTorr. The collision energy was set to 20 V. Positive ions were acquired in the selected reaction monitoring (SRM) mode. The signal of dG-dC was monitored by the transition *m/z* 521 to 289, and the signal of the internal standard $^{15}N_3$-dG-dC was monitored by the transition *m/z* 524 to 292. The product ion spectra of dG-dC and $^{15}N_3$-dG-dC are presented in Fig. 1.

Calibration standards

A stock solution of dG-dC and the internal standard stock of $^{15}N_3$-dG-dC were prepared at 1.6 and 0.53 mM in deionized water, respectively. The calibration working standards of dG-dC with a fixed amount of $^{15}N_3$-dG-dC were prepared by diluting the corresponding appropriate amounts of dG-dC and $^{15}N_3$-dG-dC initial stock solutions with deionized water. The stock solution of dG-dC was serially diluted with deionized water to obtain a series of lower calibration working solutions of 0.08, 0.16, 0.32, 0.8, 1.6, 3.2, 8, 16 and 32 nM, and the $^{15}N_3$-dG-dC working solution (5.3 nM) was prepared by gradient dilutions of the internal standard stock solution with deionized water. All solutions were stored at −20°C.

The calibration curves were obtained by plotting the SRM peak area ratios of dG-dC to $^{15}N_3$-dG-dC versus the concentration of dG-dC. The linearity of the calibration curves was evaluated by the values of the correlation coefficients (R^2). The concentration of dG-dC in the enzymatic digestion samples was calculated from their peak area ratios by using the calibration equations.

Method validation

Quality control (QC) samples were prepared independently by diluting appropriate amounts of dG-dC and $^{15}N_3$-dG-dC stock solutions in deionized water. The concentrations of the QC samples were calculated 0.32, 3.2, 16 and 32 nM of dG-dC with 5.3 nM of $^{15}N_3$-dG-dC. The precision and accuracy of intra-day and inter-day assays[27–31] were determined by replicate analyses of QC samples at the four concentration levels. The intra-day assay precision and accuracy were obtained by repeated analyses (n = 6) of the QC samples within one assay, and the inter-day assay precision and

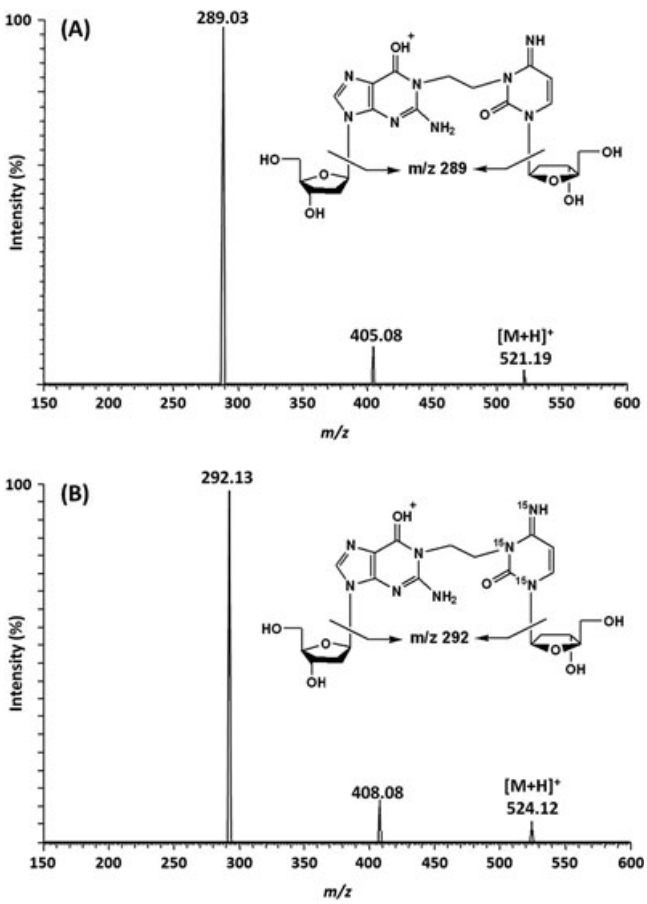

Figure 1. Positive ESI product ion spectrum of $[M + H]^+$ ions of (A) dG-dC and (B) $^{15}N_3$-dG-dC.

accuracy were determined by repeated analysis of the QC samples over three consecutive days.

To test the recovery of the dG-dC crosslinks in the enzymatic digestion samples, a series of control samples were spiked with standard dG-dC at concentrations of 0.32, 3.2, 16 and 32 nM with a fixed $^{15}N_3$-dG-dC concentration of 5.3 nM. The concentrations of dG-dC in every sample were analyzed six times. The recovery was calculated as the formula:

$$\text{Recovery} = \text{mean of the determined concentration} / \text{added concentration} \times 100\%.$$

The stability of the 1.6 mM dG-dC and 0.53 mM $^{15}N_3$-dG-dC stock solutions was determined after storage at −20°C for 3 months with intermittent thawing and freezing. The response was compared to a freshly prepared stock solution and stability was expressed as the percentage recovery of the stored solution relative to the fresh solution. The stability of dG-dC in the enzymatic digestion mixtures during sample preparation was evaluated by assaying samples after 24 h of storage at room temperature and 4°C.

Data analysis

HPLC/ESI-MS/MS chromatograms were integrated using analysis software Xcalibur 1.4. Calibration curves in the concentration range of 0.08–32 nM were constructed by plotting the SRM peak area ratios of dG-dC/$^{15}N_3$-dG-dC versus the corresponding dG-dC concentrations. The concentrations of dG-dC in the mixtures were determined according to the calibration equation. The obtained concentrations of dG-dC were used to calculate the crosslinking fractions (CF) of the oligonucleotide duplexes with the following formula:

$$CF = \left(C \times V \times 10^7\right)/(26 \times C_0 \times V_0/M_0) \quad (1)$$

Because the oligonucleotides were completely hydrolyzed to free nucleosides by the enzymatic digestion, the values of CF indicated the number of crosslinked GC base pairs in every 10^7 base pairs. In the formula for CF, C refers to the determined concentrations of dG-dC crosslinks in the samples (in nM); V refers to the volume of the enzymatic digestion solution, which was ~315 μL; C_0 is concentration of the oligonucleotide duplexes, which was 0.25 mg/mL; V_0 is the volume of the sample withdrawn from the reaction solution in 2 h intervals, which was 200 μL; and M_0 represents the molecular weight of the oligonucleotide duplex.

RESULTS AND DISCUSSION

Development of HPLC/ESI-MS/MS analysis

Because *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was demonstrated to have activity against LI210 cells in 1959,[32,33] significant effort has been made to clarify the anticancer mechanism of CENUs. DNA ISC induced by the active electrophilic species yielded from the decomposition of CENUs was considered to play a predominant role in the anticancer activity of CENUs. In order to obtain a better understanding of the relationship between DNA ISC and the anticancer activity of CENUs, qualitative and quantitative assays of DNA ISC have been performed by various analytical techniques. Using an alkaline elution technique, Erickson *et al.*[34] demonstrated a correlation between DNA ISC and cytotoxicity, and Aida *et al.*[35] obtained a crosslinking index for rat brain tumor cells treated with BCNU. Polyacrylamide gel electrophoresis has been applied to evaluate the buffer effects on DNA adduction, crosslinking and cytotoxicity induced by CENUs.[17] Penketh *et al.*[19,36,37] reported a fluorescence method for the quantitation of DNA ISC in T7 DNA using the H33258 molecular probe. The values of the crosslinking fraction (CF), which were represented by the fraction of DNA molecules containing one or more crosslinks per molecule, were employed to compare the crosslinking activity of anticancer agents. The above research provided suitable protocols for the analysis of DNA ISC; however, these methods did not directly quantify the number of crosslinked base pairs or confirm the position of the crosslinking sites in DNA. Bodell *et al.*[11,15,16,38,39] reported a series of direct quantifications of dG-dC crosslinks in purified calf thymus DNA and in glioma cell lines using HPLC. Fischhaber *et al.*[40] performed direct quantitation of dG-dC crosslinks in synthesized DNA duplexes by HPLC/ESI-MS. We recently investigated dG-dC crosslinks in calf thymus DNA treated with BCNU using HPLC/ESI-MS/MS.[26] These research efforts achieved direct quantitation of dG-dC crosslinks in DNA or cell lines; however, these methods either had relatively low specificity and sensitivity or did not indicate the validation of the assay, such as the precision, accuracy and recovery.

In this work, an analytical method with high selectivity and sensitivity was developed for the quantitation of dG-dC crosslinks induced by meCCNU. The dG-dC standard was synthesized and used to optimize the ESI-MS/MS conditions. The optimum instrumental parameters were obtained as described in the Experimental section, including spray voltage, gas pressure, capillary temperature, tube lens offset and collision energy. The standard solution was repeatedly introduced into the mass spectrometer to confirm the optimum SRM precursor/fragment ion transition for dG-dC. The product ion spectrum of the protonated dG-dC is presented in Fig. 1(A). The $[M+H]^+$ ion of dG-dC (*m/z* 521) yields two fragment ions at *m/z* 405 and 289, which respectively correspond to the loss of one or two ribosyl moieties because of the cleavage of the glucosidic bond. Because the signal of *m/z* 289 is much stronger than the signal for the *m/z* 405 species, the positive SRM quantitation for dG-dC was performed at the *m/z* 521 → 289 transition. Stable isotopically labeled $^{15}N_3$-dG-dC was used as the internal standard to avoid possible measurement error from sample preparation and instrument response. The tandem mass spectrum of $^{15}N_3$-dG-dC is shown in Fig. 1(B). The $[M+H]^+$ ion of dG-dC (*m/z* 524) also yields two fragment ions at *m/z* 408 and 292, which are produced from the same fragmentation mechanism as dG-dC. The precursor/fragment ion transition at *m/z* 524 → 292 was therefore employed as the SRM quantitation of $^{15}N_3$-dG-dC.

A number of protocols for HPLC separation were tested to obtain satisfactory separation and peak shape of dG-dC from the mixture of the hydrolysates. Various combinations of the inorganic mobile phase (deionized water or ammonium acetate solution with a concentration range of 1–10 mM) and organic mobile phase (methanol or acetonitrile) were used for the separation. The optimal mobile phase selected was 2 mM

ammonium acetate (A) and acetonitrile (B) because these reagents gave good performance in both HPLC and MS. Acetic acid with a concentration of 0.1% was added into the mobile phase A to enhance the ionization of the analytes. The optimum gradient was 5–15% buffer B for 25 min followed by an isocratic elution for 2 min. The percentage of buffer B was then decreased to 5% in 1 min followed by an equilibration time of 7 min. Figure 2 shows the SRM chromatograms of dG-dC (Fig. 2(A)) and $^{15}N_3$-dG-dC (Fig. 2(B)) in the enzymatic hydrolysates from the oligonucleotides treated with meCCNU, the SRM chromatogram of dG-dC in the control samples (Fig. 2(C)) and the HPLC/UV chromatogram of the enzymatic digestion samples (Fig. 2(D)). The retention time for dG-dC in the digestion mixture is 22.2 min, and its corresponding isotope-labeled standard, $^{15}N_3$-dG-dC, has the same retention time. Figure 2(C) indicates that there is no signal detected with the SRM transition for dG-dC in the hydrolysates of the control samples. Figure 2(D) shows that the nucleosides arising from the digest of the duplexes have essentially distinguishable retention times from the analytes of interest.

Precision, accuracy, recovery and stability studies

HPLC/ESI-MS/MS calibration curves were established by a series of standards each containing varying amounts of synthetically prepared dG-dC which were compared with the $^{15}N_3$-labeled internal standard. Each calibration point was determined in triplicate. The calibration curve was linear over the range from 0.08 to 32 nM with a correlation coefficient (R^2) of 0.9997. The calibration curve and corresponding equation are shown in Fig. 3. The accuracy and precision were determined by analyzing replicates of the standards at 0.32, 3.2, 16 and 32 nM concentrations. As listed in Table 2, the accuracy of the quantitative method is in the range of 98 to 106% for the intra-day assay and 96 to 109% for the inter-day assay. The precision represented by the relative standard deviation (RSD) ranges from 1.2 to 4.7% and 1.1 to

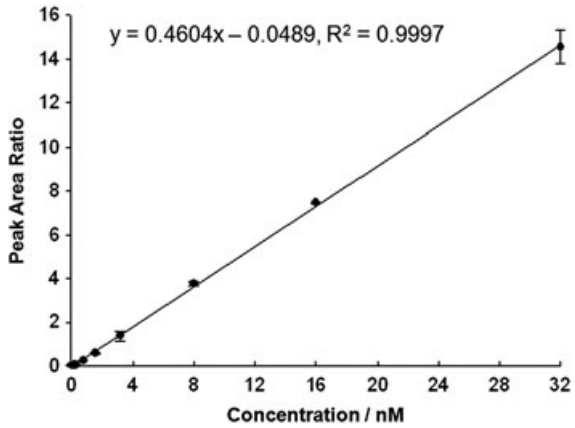

Figure 3. The calibration curve of dG-dC which was constructed by plotting SRM peak area ratios between dG-dC and $^{15}N_3$-dG-dC versus the concentration of dG-dC.

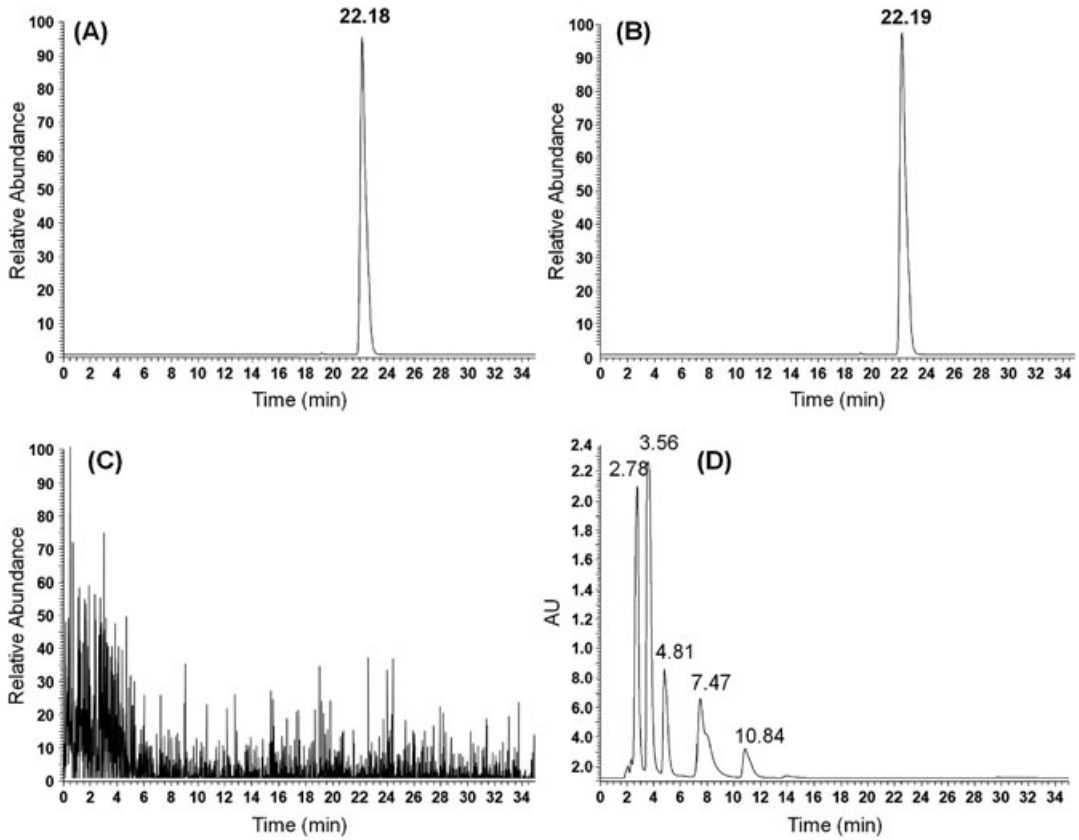

Figure 2. SRM chromatograms of (A) dG-dC and (B) $^{15}N_3$-dG-dC in the enzymatic digestion mixture of the DNA duplexes treated with meCCNU with the addition of the internal standard. (C) SRM chromatograms of the enzymatic digestion mixture of the control duplex. (D) HPLC/UV chromatogram at 258 nm of the hydrolysates from the duplexes treated with meCCNU.

Table 2. Precision and accuracy of the assay of dG-dC crosslinks determined by replicate analyses of QC samples at four concentration levels

Concentration of QC samples (nM)	Intra-day assay precision and accuracy (n = 6)			Inter-day assay precision and accuracy (n = 18)		
	Mean (nM)	RSD (%)	Accuracy (%)	Mean (nM)	RSD (%)	Accuracy (%)
0.32	0.34	3.1	106	0.35	3.6	109
3.2	3.14	4.7	98	3.08	6.6	96
16	15.9	1.5	99	16.4	2.3	103
32	31.6	1.2	99	32.0	1.1	100

6.6% for the intra-day and inter-day assay, respectively. The accuracy and precision results indicate that the presented quantitation method gives good reproducibility. The limits-of-detection (LODs) of dG-dC, with a signal-to-noise (S/N) ratio higher than five, was determined to be 0.08 nM (0.042 ng/mL). The limit-of-quantification (LOQ) of dG-dC with an S/N ratio >20 was determined to be 0.16 nM (0.083 ng/mL).

In order to evaluate the influence of sample preparation and matrix effects on the measured results, the recovery of dG-dC in the hydrolysates was studied with a series of control samples spiked with dG-dC (0.32, 3.2, 16 and 32 nM) and $^{15}N_3$-dG-dC (5.3 nM). As summarized in Table 3, an acceptable recovery can be obtained ranging from 91 to 106%, which indicates that the influence of sample preparation and matrix effects is negligible on the obtained results of the dG-dC concentration.

Stability studies were performed using a dG-dC stock solution, a $^{15}N_3$-dG-dC stock solution and the enzymatic digestion solution. Considering the comparatively long period of storage of the synthesized standards, the stock solutions were assayed after storage at −20 °C for 3 months with intermittent thawing and freezing. The stability of dG-dC in the enzymatic digestion mixtures was also examined to determine whether the sample preparation and matrix have influence on the stability of the analyte. The results showed that dG-dC was very stable in both stock and enzymatic digestion solutions. Therefore, the standard stock solution can be conserved at −20 °C for several months, and the sample preparation and matrix have no influence on the stability of dG-dC.

Table 3. Recovery studies for dG-dC crosslinks in the enzymatic digestion mixtures of the oligonucleotide duplexes

Added concentrations (nM)	Determined concentrations (nM)	Recovery (%)
0.32	0.29	91
3.2	3.40	106
16	15.8	99
32	31.4	98

Quantitation of dG-dC in the hydrolysates from oligonucleotide duplexes

Using the quantitative method, the dG-dC crosslinks in the hydrolysates of the four oligonucleotides that have different GC contents were assayed. Table 4 lists the observed concentrations of dG-dC in the samples withdrawn from the reaction mixture at various times. The crosslink fractions for every sample were calculated according to Eqn. [1] (see Data analysis in the Experimental section). The CF for the four oligos treated with 2 mM meCCNU at 37 °C for 0, 2, 4, 6, 8 and 10 h is plotted in Fig. 4. Except for oligo A, which is composed of only AT base pairs, the concentrations of dG-dC in the other three oligos have a maximum value of 7.72 nM, which corresponds to a maximum CF of 298 dG-dC crosslinks in every 10^7 base pairs. Clearly the CF value increases as both the reaction time and the GC content increase. Moreover, examination of the CF values as a function of time indicates that the dG-dC crosslinks formed more slowly in the early period of the reaction (about 2 h) than in the longer reaction time points. This result provides rational evidence for the supposed mechanism of DNA ISC induced by CENUs, which postulates that guanine is initially alkylated by chloroethyl cations arising from the decomposition of CENUs and then the dG-dC crosslink is formed via the second alkylation of the complimentary cytosine base.

Table 4. The determined concentrations of dG-dC in the enzymatic hydrolysates from the four duplexes treated with 2 mM meCCNU

Oligos	Concentrations of dG-dC (nM)					
	T = 0 h	T = 2 h	T = 4 h	T = 6 h	T = 8 h	T = 10 h
A	0	0	0	0	0	0
B	0.01	0.07	0.36	0.82	1.43	1.69
C	0.02	0.14	0.60	1.28	2.36	3.30
D	0.02	0.29	1.23	2.98	5.57	7.72

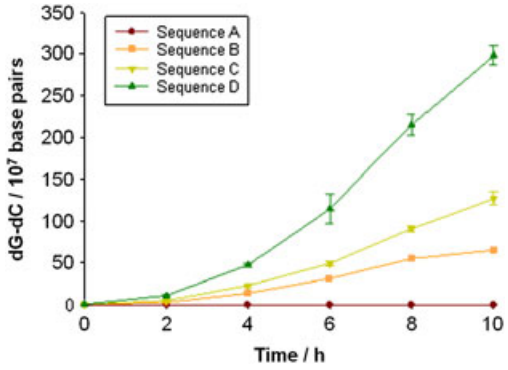

Figure 4. The crosslinking fractions in the four oligonucleotide duplexes with different sequences treated with 2 mM meCCNU.

CONCLUSIONS

A new method for the direct quantitation of dG-dC interstrand crosslinks in DNA was established by using HPLC/ESI-MS/MS. Validation of the method was demonstrated to have satisfactory precision, accuracy and recovery. Using this method, quantitative analysis of dG-dC crosslinks in synthesized oligonucleotides was performed. The observed CF values indicated that as the GC content in DNA increased a greater number of dG-dC crosslinks formed. Our results confirm the earlier report of Fischhaber and coworkers,[40] which noted that the yield of crosslinks depended on the number of deoxyguanosines in the oligos, and that there appeared to be little sequence specificity with respect to crosslink formation. This quantitation method should be suitable for examining the pharmacology and pharmacokinetics of anticancer alkylating agents or the carcinogenic mechanisms of chemical carcinogens.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 20907002), the Beijing Nova Program (No. 2009B08) and the Natural Science Foundation of Beijing Municipality (No. 8113031).

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