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Specific Method for the Determination of Genomic DNA Methylation by Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry

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Herein we report a novel method for determining genomic DNA methylation that utilizes liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to measure 5-methyl-2′-deoxycytidine levels following enzymatic hydrolysis of genomic DNA. LC separation of 5-methyl-2′-deoxycytidine from the four deoxyribonucleosides, the four ribonucleosides, and 5-methyl-2′-cytidine, a RNA methylation product, has been achieved within 15 min. In combination with ESI-MS/MS detection, the reported method is highly specific and extremely sensitive with a limit of detection (LOD) of 0.2 fmol and a quantification linearity range from 1 fmol to 20 pmol. Genomic DNA methylation was expressed as the ratio of 5-methyl-2′-deoxycytidine to 2′-deoxyguanosine and was determined directly using 2′-deoxyguanosine as the internal standard. Because deoxycytidine methylation typically ranges from 2 to 6% in mammalian genomes, and pharmacological or genetic manipulations have not achieved levels lower than 0.1%, we validated the assay for methylation levels ranging from 0.05 to 10%. Importantly, both RNA contamination and incomplete DNA hydrolysis had no appreciable effect on 5-methyl-2′-deoxycytidine quantification. LOD studies indicate that only 4 ng of DNA is required for this assay. This LOD should permit the use of this method for applications having limiting amounts of DNA that were not previously candidates for global genomic DNA methylation analysis, e.g., clinical trial samples, or cells collected by laser capture microdissection.

Modification of the 5 position of the cytosine ring of 2′-deoxycytidine by covalent attachment of a methyl group is a central mammalian epigenetic control mechanism that impacts gene expression, genome stability, genetic imprinting, and cellular differentiation.[1] In addition, alterations in DNA methylation make a major contribution to oncogenesis, and a primary mechanism of this is the global loss of 5-methyl-2′-deoxycytidine (5mdC) from genomic DNA in cancer cells.[2] These observations have provoked great interest in developing sensitive analytical methods suitable for determining 5mdC concentrations in mammalian DNA samples. A number of different methods have been developed for this task, and these approaches are classified as either gene-specific or nonspecific (or global) analyses.[3−6] Gene-specific methods, which interrogate the methylation patterns of specific genetic loci, are typically based on the use of methylation-sensitive restriction enzymes, sodium bisulfite modification, or both.[4,6] Gene-specific assays are crucial for integrating information about DNA methylation patterns with gene expression, chromatin modifications, and assembly of transcription factors at gene promoters.[7] In contrast, nonspecific (or global) measurements of DNA methylation provide an overall picture of cellular DNA methylation levels and are crucial for understanding the relationship between genomewide alterations in DNA methylation, gene specific methylation patterns, and genome stability [2].

The earliest recognition of DNA methylation defects in human cancer was the discovery of global DNA hypomethylation in various human cancers over 20 years ago.[8,9] In contrast to these initial observations, in recent years, most studies of cancer-specific alterations in DNA methylation have focused on gene- and promoter-specific changes.[10] However, global measurements of DNA methylation remain a valuable tool for understanding the molecular pathology of human cancer, for measuring the potential effect of tumor preventative or promoting compounds, and for monitoring therapeutic responses to hypomethylating agents undergoing evaluation in human clinical trials. In many instances, sample size is limited, particularly in clinical samples, archived tumor specimens, and samples derived from laser capture micro-

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dissection. Thus, there remains a significant impetus to develop highly sensitive and quantitative methods for measuring genomic DNA methylation.

Reported methods for the determination of genomic DNA methylation include chromatographic techniques such as thin-layer chromatography (TLC),[11−13] gas chromatography,[14,15] liquid chromatography (LC),[16−28] and micellar electrokinetic capillary chromatography (MECC).[29−33] These techniques rely on the release of DNA components such as deoxyribonucleotides,[11−13,18,25−27,33] deoxyribonucleosides,[12,13,16,17,21,22,24,31] or bases[14,15,19,20,23,29,30,32] by means of enzymatic/chemical treatment, followed by chromatographic separation and detection of the corresponding components. The components that have been measured include 5-methyl-2′-deoxycytidine monophosphate and 2′-deoxycytidine monophosphate,[11−13,18,25−27,33] 5mdC and 2′-deoxycytidine (dC),[12,13,15−17,21,22,24,31] and 5-methylcytosine and cytosine.[14,15,19,20,23,29,30,32] A number of these methods can provide quantification with high reproducibility, and among them, the LC methods are the best and most widely accepted. TLC has the advantages of simple instrumentation, large screening scales, and low cost, with the disadvantage being reduced accuracy.[11−13] GC is as accurate as LC but requires conversion of the DNA bases to volatile derivatives for the separation. The MECC technique was initially reported in 1984[34] and has also been used for global DNA methylation analysis more recently.[29−33] Although MECC provides faster separation and higher separation efficiency than traditional LC, sample loading volume is usually limited and separation reproducibility can be affected by slight alterations in the sample matrix.[35]

UV detection has often been utilized for the LC and MECC methods[16,17,19−24,26,27,29−31] due to its simplicity, low cost, and reproducibility. UV detection can provide a limit of detection (LOD) as low as 400 fmol of 5mdC.[5] In contrast, fluorescence detection can show a 10-fold higher sensitivity but has drawbacks including tedious labeling procedures and possible side reaction products.[25,33] TLC methods use radioactive ^{32}P labeling of deoxyribonucleotides, and a LOD of ∼20 fmol has been achieved.[5] The TLC method was successfully used for the detection of DNA methylation in *Drosophila melanogaster*[12] and *Aspergillus flavus,*[13] which were the initial reports of methylation in these organisms. More recently, both electrochemical detection (with MECC) by Chen et al.[32] and electrospray ionization-mass spectrometry (ESI-MS) (with LC) by Friso et al.[28] have been described.

An inherent weakness of the UV detection method is that specificity is dependent on the chromatographic separation. While the majority of reported methods provided good separation of the five DNA components, RNA contamination of the DNA sample could cause the quantification to fail. In fact, the only study to achieve good separation of all 10 DNA and RNA components was reported by Gehrke et al.,[22] and this method required long separation times of ∼40 min. Even with the recently reported LC-ESI-MS assay of Friso et al.,[28] this problem remained because the method was incapable of distinguishing 5mdC from 5-methyl-2′-cytidine (5mC).

The speed of the methods utilized is also a consideration. In these cases, speed is primarily a function of the chromatographic separation. Reported LC separation times have ranged from times as long as 60[16,18,21] to 40[22,25,27] and 15−20 min.[17,19,23,26] In contrast to LC, MECC separations are generally faster and they can be finished in ∼10 min.[30,31]

In addition to chromatographic techniques, other methods developed to determine genomic DNA methylation levels include the SssI acceptance assay,[37] chloroacetaldehyde assay,[38] and immunochemical analysis.[39] These methods are dependent on one or two specific reactions to selectively label and detect 5-methylcytosine. However, the sensitivity and reproducibility of the SssI acceptance assay is poor, the speed of the chloroacetaldehyde assay is slow, and immunochemical analyses are not well suited for quantitative studies.

Here we report the use of liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for the determination of genomic DNA methylation. Excellent LC separation of the five DNA and five RNA components was achieved within 15 min, using a novel separation column. Furthermore, the ESI-MS/MS detection method reported here easily distinguished 5mdC from 5mC. Together, our data indicate that the LC-ESI-MS/MS method provides unambiguous quantification of 5mdC with high reproducibility and extremely high sensitivity (LOD 0.2 fmol). This LOD is compatible with a requirement for as little as

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4 ng of DNA for measurement of samples with deoxycytidine methylation levels as low as 0.05%.

EXPERIMENTAL SECTION

Reagents. The five deoxyribonucleosides, 5mdC, dC, 2′-deoxyguanosine (dG) monohydrate, 2′-deoxyadenosine (dA) monohydrate, and thymidine (T), and the five ribonucleosides, 5mC, cytidine (C), guanosine (G), adenosine (A), and uridine (U), were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium acetate, ammonium bicarbonate, formic acid, water, and methanol were purchased from VWR international, Inc. (West Chester, PA).

Apparatus. The LC system consisted of an Agilent (Palo Alto, CA) 1100 HPLC system with a vacuum degasser (model G1322A), binary pump (model G1312A), and microautosampler (model G1313A). A Waters (Milford, MA) Atlantis dC_{18} 2.1 × 150 mm column (5-μm particle size) protected by a 2.1 × 20 mm guard column (5-μm particle size) was used for the separation. An Applied Biosystems MDS Sciex API 3000 triple quadrupole mass spectrometer (Concord, ON, Canada) was coupled with the LC system through a TurboIonSpray ion source interface for the detection of nucleosides. The LC/MS/MS system was controlled by Analyst 1.3 software (Applied Biosystems).

Cell Culture and DNA isolation. The human colon adenocarcinoma cell line HCT116 and the HCT116 derivative line DKO, in which the DNA methyltransferase genes *DNMT1* and *DNMT3b* are disrupted by homologous recombination,[40] were obtained from Dr. Bert Vogelstein (Johns Hopkins University School of Medicine). Cells were cultured at 37 °C in a 5% CO_2 incubator using McCoy's 5A medium (VWR International, Inc. West Chester, PA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Genomic DNA was isolated from HCT116 and DKO cells using the Puregene Genomic DNA purification kit (Gentra Systems, Minneapolis, MN), according to the instructions of the manufacturer. Briefly, cells suspended in culture medium were pelleted and resuspended in cell lysis solution (Tris EDTA, SDS). The resulting cell lysate was incubated at 37 °C with RNase A solution for 2 h, followed by vigorous vortexing with protein precipitation solution (ammonium acetate) at room temperature. The cell lysate was then pelleted by centrifugation, and the supernatant was transferred to a clean tube containing 100% 2-propanol. Following precipitation and centrifugation, the DNA pellet was washed with 70% ethanol, air-dried, and rehydrated in hydration solution (10 mM Tris, 1 mM EDTA) by first incubating at 65 °C for 1 h, followed by an overnight incubation at room temperature. Alternatively, genomic DNA was purified by a standard phenol/chloroform extraction followed by precipitation with two volumes of 100% ethanol.

DNA Hydrolysis. DNA hydrolysis was performed as previously described by Crain.[41] Briefly, 1 μg of genomic DNA was first denatured by heating at 100 °C for 3 min and then chilling on ice. After adding a 1/10 volume of 0.1 M ammonium acetate (pH 5.3) and 2 units of nuclease P1 (Roche Molecular Biochemicals Indianapolis, IN), the mixture was incubated at 45 °C for 2 h. A 1/10 volume of 1 M ammonium bicarbonate and 0.002 unit of venom phosphodiesterase I (Sigma Chemical Co.) were subsequently added to the mixture, and the incubation was continued at 37 °C for 2 h. Next, 0.5 unit of alkaline phosphatase (Fermentas, Hanover, MD) was added, and the mixture was incubated at 37 °C for 1 h.

LC-ESI-MS/MS Procedure. LC separation was performed at a flow rate of 220 μL/min. Two buffers, 0.1% formic acid in methanol and 0.1% formic acid in water, were used, with a linear gradient increase of 1.5%/min of the organic buffer from 0 to 22.5%, for the elution of nucleosides. The injection volume was 20 μL. ESI conditions were optimized by infusion of 10 μg/mL 5mdC in a buffer containing 5% methanol and 0.1% formic acid at a flow rate of 220 μL/min. The optimized ESI conditions were as follows: nebulizer gas flow, 8; curtain gas flow, 8; collision-activated dissociation (CAD) gas flow, 4; TurboIonSpray voltage, + 4000 V; heater gas flow, 7 L/min; turboprobe temperature, 400 °C; turboprobe position, H7, L4; declustering potential (DP) voltage, 22 V; focusing potential (FP) voltage, 98 V; entrance potential (EP) voltage, 3.8 V; collision energy (CE), 13 V; collision cell exit potential (CXP), 9 V. Quantification was accomplished in multiple reaction monitoring (MRM) mode by monitoring a transition pair of m/z 242.1 (molecular ion)/126.3 (fragment ion) for 5mdC and m/z 268.1/152.3 for dG, which was used as an internal standard for the measurement, with a scan time of 180 ms for each pair. Alternatively, qualitative LC-ESI-MS/MS chromatograms were acquired in MRM mode by monitoring 10 transition pairs of m/z 242.1/126.3, 228.2/112.2, 268.1/152.3, 252.3/135.9, 243.3/127.2, 258.3/126.1, 244.1/112.2, 284.1/152.3, 268.1/136.1, and 245.1/112.9 for 5mdC, dC, dG, dA, T, 5mC, C, G, A, and U, respectively, with a scan time of 45 ms for each pair.

RESULTS AND DISCUSSION

Figure 1A reports a full-scan spectrum (ESI-MS spectrum) of 5mdC. As shown in the figure, the $[M + H]^+$ adduct appears at m/z 242.4. Also present, however, is the $[M + Na]^+$ adduct at m/z 264.3, the $[2M + H]^+$ adduct at m/z 483.5, and the $[2M + Na]^+$ adduct at m/z 505.5. Sodium adducts are frequently observed in ESI mass spectra of organic compounds. Armentano et al. recently reported the formation of dimers of cytosine nucleosides in the gas phase under ESI conditions, and three hydrogen bonds were concluded to form between two nucleoside molecules.[42] Figure 1B reports a product ion scan spectrum (ESI-MS/MS spectrum) of 5mdC (m/z 242.4). After fragmentation, the $[M + H]^+$ adduct of 5-methylcytosine appears at m/z 126.1. However, the $[M + H]^+$ adduct of β-D-2-deoxyribofuranose appears at m/z 117.2 with extremely low intensity, presumably due to its low proton affinity. By comparing Figure 1A and 1B, it was concluded that in-source CAD occurred under our ESI conditions, due to the presence of m/z 126.1 in Figure 1A.

To achieve high-sensitivity ESI-MS/MS measurements, the ESI-MS conditions were first optimized to maximize the intensity of the $[M + H]^+$ adduct of 5mdC, as shown in Figure 1A. While the formation of dimers, sodium adducts, and in-source CAD fragment ions could not be completely eliminated, they were controlled to the lowest possible level using an Applied Biosystems MDS Sciex API 3000 triple quadrupole mass spectrometer with a TurboIonSpray ion source. The absence of a glass capillary in the

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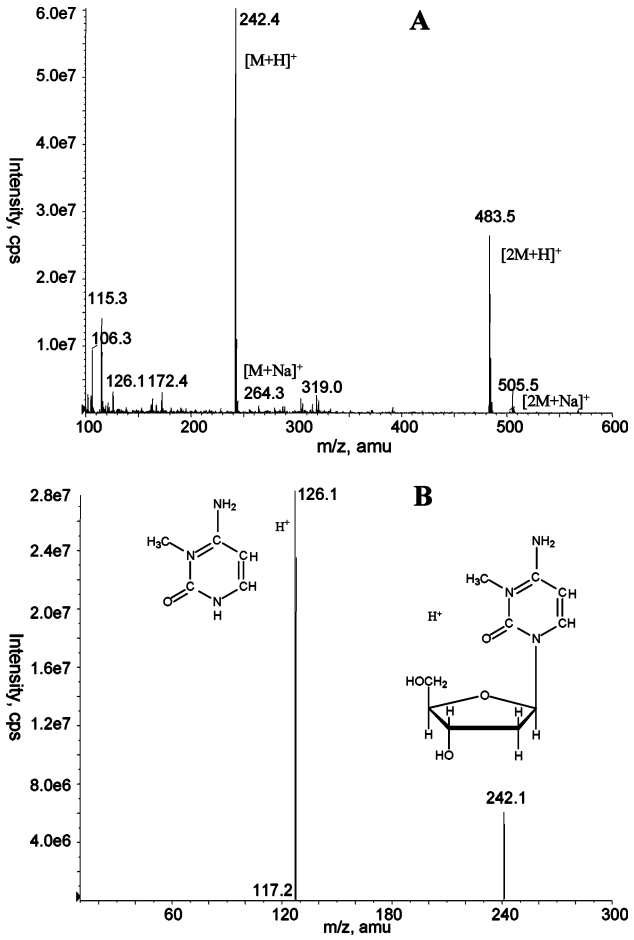

Figure 1. Full-scan (A) and product ion (B) spectra of 10 μg/mL 5mdC in a buffer containing 5% methanol and 0.1% formic acid. Ten scans were summed together, and the scan time was 1 s. The infusion flow rate was 220 μL/min, and ESI conditions were optimized as described in the Experimental Section.

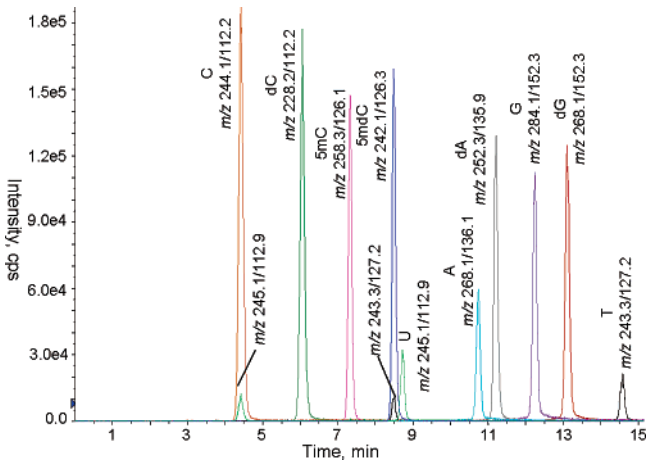

Figure 2. LC-ESI-MS/MS (MRM mode with the monitor of 10 transition pairs and a scan time of 45 ms for each pair) chromatogram of 10 standard nucleosides with a loading amount of 1 ng each. The LC-ESI-MS/MS conditions are described in the Experimental Section. C was detected by monitoring both *m*/*z* 244.1/112.2 and 245.1/112.9; 5mdC was detected by monitoring both *m*/*z* 242.1/126.3 and 243.3/127.2, due to the isotopic peaks of the molecular ions and the use of unit resolution.

ion path of this mass spectrometer significantly reduced the potential for sodium adduct formation. The TurboIonSpray ion source consisted of an IonSprayProbe used in conjunction with a heated TurboProbe. The TurboProbe directed a jet of heated dry gas up to ~550 °C at the spray produced by the IonSprayProbe, and therefore increased the rate of droplet evaporation, resulting in an increased ion signal. The TurboIonSpray ion source was especially suited for this assay because of the high content of aqueous buffer used for LC separation. In addition, the heated dry gas was probably also advantageous to limit the formation of dimers, by breaking the hydrogen bonds between two monomers. Our data indicate that 5mdC is a fragile molecule that tends to lose its β-D-2-deoxyribofuranose moiety to become 5-methylcytosine during ion transfer from the ion source to Q0. Low DP, FP, and EP potential of 22, 98, and 3.8 V, respectively, were therefore selected to reduce the incidence of in-source CAD.

Under the optimized ESI-MS conditions shown in Figure 1A, the intensity of *m*/*z* 242.4 ($I_{242.4}$) versus the total ion intensity of *m*/*z* 483.5, 242.4 and 126.1 ($I_{483.5+242.4+126.1}$) is ~2/3. ESI mass spectrometry of 5mdC was also investigated by Zambonin et al..[43] They also observed sodium adduct ions, dimer ions, and in-source CAD fragment ions, using a Finnigan MAT (San Jose, CA) LCQ ion trap mass spectrometer. The $I_{242.4}/I_{483.5+242.4+126.1}$ ratio seen in their study is ~2/7, based on the reported spectrum.[43] Recently, Friso et al. reported a LC-ESI-MS method for the assessment of genomic DNA methylation using a Bruker Daltonics (Billerica, MA) Esquire ion trap mass spectrometer.[28] In this study, because $I_{126.1} > I_{242.4}$, 5-methylcytosine was chosen for LC-ESI-MS quantification of DNA methylation, resulting in nonspecific detection of 5mdC versus 5mC.[28] In contrast, the $I_{126.1}/I_{242.4}$ ratio can be estimated to be ~0.5 in the report by Zambonin et al.,[43] and 0.007 in our study, as shown in Figure 1A. Therefore, we achieved more efficient ESI-MS conditions to generate the $[M + H]^+$ adduct of 5mdC than has been reported previously,[28,43] which enabled a more specific and sensitive ESI-MS/MS detection of 5mdC. ESI-MS/MS conditions, including CAD gas flow, CE, and CXP, were optimized to achieve the highest signal of *m*/*z* 126.1, i.e., the $[M + H]^+$ adduct of 5-methylcytosine, as shown in Figure 1B. In addition, a 3-fold increase in sensitivity was achieved by using a buffer containing 0.1% formic acid rather than 5 or 7 mM ammonium acetate pH 6.7, which were used previously.[28,43] The buffer selection was based on the assumption that formic acid is a better proton donor for positive ESI. Significant changes of the full-scan and product ion scan spectra between the two buffers were not observed under our ESI conditions.

Full-scan and product ion scan spectra of the five deoxyribonucleosides (5mdC, dC, dG, dA, T) and the five ribonucleosides (5mC, C, G, A, U) were acquired, and the spectra showed very similar patterns. Therefore, the transition pairs of *m*/*z* 242.1/126.3, 228.2/112.2, 268.1/152.3, 252.3/135.9, 243.3/127.2, 258.3/126.1, 244.1/112.2, 284.1/152.3, 268.1/136.1, and 245.1/112.9 at unit resolution (FWHM 0.6−0.8 amu) were chosen for the detection of 5mdC, dC, dG, dA, T, 5mC, C, G, A, and U, respectively, with a scan time of 45 ms for each pair, to monitor LC separation in MRM mode. Figure 2 reports a LC-ESI-MS/MS chromatogram of the 10 nucleosides with a loading amount of 1 ng each. The LC elution conditions were optimized by using two buffers: 0.1%

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formic acid in methanol and 0.1% formic acid in water. A linear gradient of 1.5%/min increase of the organic buffer from 0 to 22.5% was selected because it provided the shortest separation time and the highest separation efficiency. Figure 2 shows that the detection of T and U was less sensitive than other nucleosides, which may be attributed to their weaker proton affinity.[44]

As shown in Figure 2, C was detected by monitoring *m/z* 244.1/112.2. However, the monitor of *m/z* 245.1/112.9 also detected C with less sensitivity. This was because C had isotopic peak at *m/z* 245.1 and MRM acquisition used unit resolution (FWHM 0.6−0.8 amu). U was detected by monitoring *m/z* 245.1/112.9 and the monitor of *m/z* 244.1/112.2 did not detect U as a less intense peak. This was because U had no isotopic peak at *m/z* 244.1. The detection of 5mdC and T by monitoring *m/z* 242.1/126.3 and 243.3/127.2, respectively, showed results similar to the detection of C and U. Therefore, Figure 2 demonstrates complete separation of all 10 nucleosides within 15 min.

A potential problem in the measurement of genomic DNA methylation is interference from RNA contamination. Most chromatography-based methods depend on a sufficient DNA purification procedure to eliminate RNA interference. Although a total separation of the 10 nucleoside components from both DNA and RNA would also solve the problem, only Gehrke et al.[22] achieved it, but only by using a lengthy separation time of ~40 min. Our complete separation, as shown in Figure 2, was achieved using a newly developed Atlantis dC18 column (see Experimental Section). This column has the advantage of resisting sudden loss in retention in 100% aqueous mobile phase, is ideal for low-pH reversed-phase separation of amine-containing compounds, and is fully end capped for superior peak shape of amine-containing compounds. As shown in Figure 2, the separation gave very sharp peaks with a separation efficiency of ~145 000 theoretical plate numbers/meter for 5mdC, which in turn allowed the separation to be accomplished within 15 min.

By combining a specific detection method (ESI-MS/MS) with a complete separation of the 10 nucleosides from both DNA and RNA, we were able to achieve a rapid method for the accurate determination of genomic DNA methylation. Most reported methods have expressed global DNA methylation as [5mdC]/([5mdC] + [dC]), where the concentration of 5mdC and dC are determined using external[17−19,21−23,25−27,30−33] or internal standard[14,15,28] calibration. So far, possibly the most accurate measurement of 5mdC and dC was achieved by using isotope-labeled internal standards, i.e., (methyl-d_3,ring-6-d_1)5-methyl-2′-deoxycytidine and [$^{15}N_3$]2′-deoxycytidine, as reported by Friso et al.[28] However, in this assay, the internal standards could only be incorporated in the LC-ESI-MS step; i.e., they could not be incorporated in sample preparation steps such as DNA hydrolysis. Therefore, insufficient DNA hydrolysis could still impair accurate 5mdC determination. In addition, the accuracy of the formula [5mdC]/([5mdC] + [dC]) to calculate genomic DNA methylation can be significantly reduced by experimental errors present in the measurement of either 5mdC or dC. To circumvent these disadvantages, and to avoid the use of expensive isotope-labeled standards, we utilized dG as the internal standard, based on the assumption that [dG] = [5mdC] + [dC] in genomic DNA.

(44) Fu, Y.; Sharma, S.; Lee, J. K. Acidity and proton affinity of the pyrimidine-base nucleobases: thymine and cytosine, *52nd Annual ASMS Conference Proceedings*, Nashville, 2004; poster MPQ278.

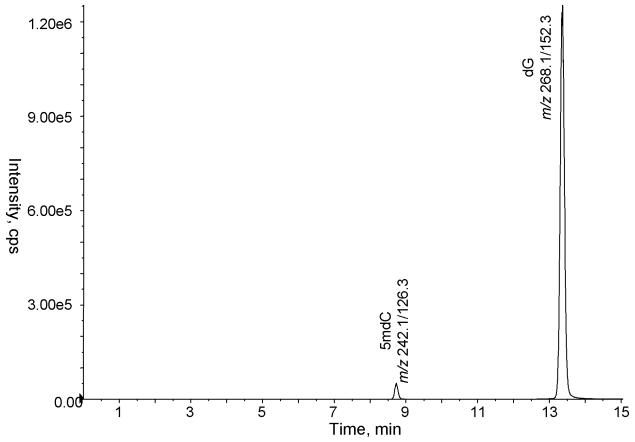

Figure 3. LC-ESI-MS/MS chromatogram of a calibration standard mixture containing 40 pmol of dG, 5mdC + dC, dA, and T with 2.5% [5mdC]/[dG]. In contrast to Figure 2, MRM mode with the monitor of two transition pairs and a scan time of 180 ms for each pair was employed to enhance the sensitivity. Other LC-ESI-MS/MS conditions were the same as used in Figure 2.

Although DNA damage can cause dG modifications, which may make this equation less accurate, dG modifications are far less prevalent in genomic DNA as compared to dC methylation.[45] Therefore, we expressed genomic DNA methylation as [5mdC]/[dG] and quantified this value using a calibration curve between the peak area ratio of 5mdC to dG (A_{5mdC}/A_{dG}) versus [5mdC]/[dG]. Because ESI-MS/MS can provide a linear calibration range of over 3 orders of magnitude in MRM mode, we have not found the higher concentration of dG as compared to 5mdC to be a problem for the quantification.

We injected 80 ng of DNA hydrolysis products, corresponding to ~40 pmol of dG, for LC-ESI-MS/MS analysis. Calibration standard mixtures were prepared to imitate DNA hydrolysis products with 40 pmol of dG, 5mdC + dC, dA, and T. [5mdC]/[dG] was prepared at 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.0%, as dC methylation typically ranges from 2 to 6% in mammalian genomes,[36] and pharmacological or genetic manipulations of DNA methylation have not achieved levels lower than 0.1%.[40] MRM transition pairs of *m/z* 242.1/126.3 and 268.1/152.3 were monitored for the detection of 5mdC and dG, respectively, with a scan time of 180 ms for each pair. Figure 3 reports a typical LC-ESI-MS/MS chromatogram of a calibration standard mixture containing 40 pmol of dG, 5mdC + dC, dA, and T with 2.5% [5mdC]/[dG].

Validation of the method was accomplished using two DNA samples as quality controls (QC): the colon cancer cell line HCT116 (high QC) and the HCT116 derivative cell line DKO (low QC).[40] Table 1 reports the accuracy and precision of the LC-ESI-MS/MS method over 5 days of genomic DNA methylation determination. During the first 3 days, a calibration curve was generated with calibration standard mixtures for each set of QC analysis. We observed that the calibration curves were linear over the 0.05−10% range with correlation coefficients higher than 0.999. In addition, the calibration equation was nearly identical on the same day. Therefore, during the last 2 days of measurement, only one calibration curve was generated for the three sets of QC

(45) Dawidzik, J. B.; Patrzyc, H. B.; Iijima, H.; Budzinski, E. E.; Higbee, A. J.; Cheng, H.-C.; Box, H. C. *Biochim. Biophys. Acta* **2003**, *1621*, 211−217.

Table 1. Accuracy and Precision of the LC-ESI-MS/MS Method for the Determination of Genomic DNA Methylation

	nominal [5mdC]/[dG] (%)	0.05	0.10	0.25	0.50	1.00	2.50	5.00	10.0	low QC	high QC
day 1 n=3	calcd mean [5mdC]/[dG] (%)	0.048	0.089	0.242	0.492	0.969	2.60	4.96	9.83	1.45	4.18
	RSD[a] (%)	17.9	11.5	1.65	0.82	2.79	2.04	0.42	1.23	4.17	0.91
	RE[b] (%)	−3.73	−10.9	−3.20	−1.53	−3.1	4.00	−0.87	−1.67		
day 2 n=3	calcd mean [5mdC]/[dG] (%)	0.049	0.089	0.239	0.493	0.994	2.57	4.97	10.0	1.39	4.12
	RSD (%)	16.3	17.3	4.20	2.05	2.42	2.02	0.47	2.00	2.99	0.42
	RE (%)	−1.20	−1.14	−4.53	−1.47	−6.33	2.80	−6.67	0.00		
day 3 n = 3	calcd mean [5mdC]/[dG] (%)	0.043	0.086	0.235	0.485	0.998	2.61	4.93	10.0	1.42	4.13
	RSD (%)	4.00	8.61	1.72	1.86	2.21	0.38	0.73	0.68	1.86	1.46
	RE (%)	−1.39	−1.41	−6.13	−3.07	−2.33	4.40	−1.40	2.33		
day 4 n = 3, 1[c]	calcd mean [5mdC]/[dG] (%)	0.051	0.083	0.250	0.481	1.02	2.54	4.98	10.0	1.44	4.18
	RSD (%)	na[d]	na	na	na	na	na	na	na	2.17	0.78
	RE (%)	4.00	−1.69	0.00	−3.80	2.00	1.60	−4.00	0.00		
day 5 n = 3, 1	calcd mean [5mdC]/[dG] (%)	0.045	0.094	0.239	0.484	0.976	2.60	4.96	9.97	1.41	4.12
	RSD (%)	na	na	na	na	na	na	na	na	2.68	0.92
	RE (%)	−1.08	−5.60	−4.40	−3.20	−2.40	4.00	−8.00	−3.00		
overall	calcd mean [5mdC]/[dG] (%)	0.047	0.088	0.240	0.489	0.989	2.59	4.95	9.96	1.41	4.14
	RSD (%)	12.9	10.6	2.85	1.60	2.52	1.57	0.55	1.37	3.03	0.97
	RE (%)	−6.07	−1.20	−4.18	−2.29	−1.12	3.56	−0.91	−0.41		

[a] Relative standard deviation. [b] Relative error. [c] Calibration standard mixtures, n = 1; QCs, n = 3. [d] Only one calibration curve was generated.

analyses. Table 1 illustrates that the LC-ESI-MS/MS method is very consistent and reliable with overall low relative standard deviations (RSDs) and relative errors (REs). As shown in Table 1, the proportion of dC methylation for HCT116 and DKO DNA samples was 4.14 and 1.41%, respectively, which is similar to that reported previously using HPLC.[40]

We observed that loading of 80 ng of DNA hydrolysis products from HCT116 and DKO DNA samples resulted in dG peak heights of approximately 1.24 and 1.56 × 10^6 counts/s, respectively. Loading of 40-pmol dG calibration standard mixtures resulted in dG peak heights of ~1.25 × 10^6 counts/s, as shown in Figure 3. Therefore, 80 ng of DNA hydrolysis products contained dG amounts very close to 40 pmol. Additional experiments indicated that the calibration curves did not change significantly for dG loading amounts ranging from 2 to 200 pmol, giving the method great flexibility.

The specificity of the method was also assessed by the addition of purified human total RNA extract into the QC DNA samples by a weight proportion of 50% prior to hydrolysis. This test was performed on validation day 2, and the proportion of dC methylation for HCT116 and DKO DNA samples was determined to be 4.30 and 1.39%, which did not significantly differ from the results without RNA addition: 4.12 and 1.39% with RSD 0.42 and 2.99% (n = 3), respectively. Figure 4 reports LC-ESI-MS/MS chromatograms of 80 ng of HCT116 DNA plus 40 ng of purified HCT116 total RNA (A) as compared to 80 ng of HCT116 DNA alone (B). Figure 4A shows a substantial amount of ribonucleosides existing in the sample, and RNA cytosine methylation was detected by the appearance of a 5mC peak. Surprisingly, Figure 4B indicates low-level RNA contamination of the HCT116 DNA sample, despite the fact that the purification procedure incorporated an RNAse A step (see Experimental Section). At this level of contamination, no obvious RNA methylation was detected, as indicated by the nonexistence of a 5mC peak. These data suggest that, for analytical methods that do not discriminate between 5mC and 5mdC, extreme care must be taken in the DNA isolation step to completely eliminate RNA contamination. However, for the method reported here, RNA contamination is not a concern, as it provides unambiguous detection of 5mdC. The specificity of the method was further tested by decreasing the units of enzymes

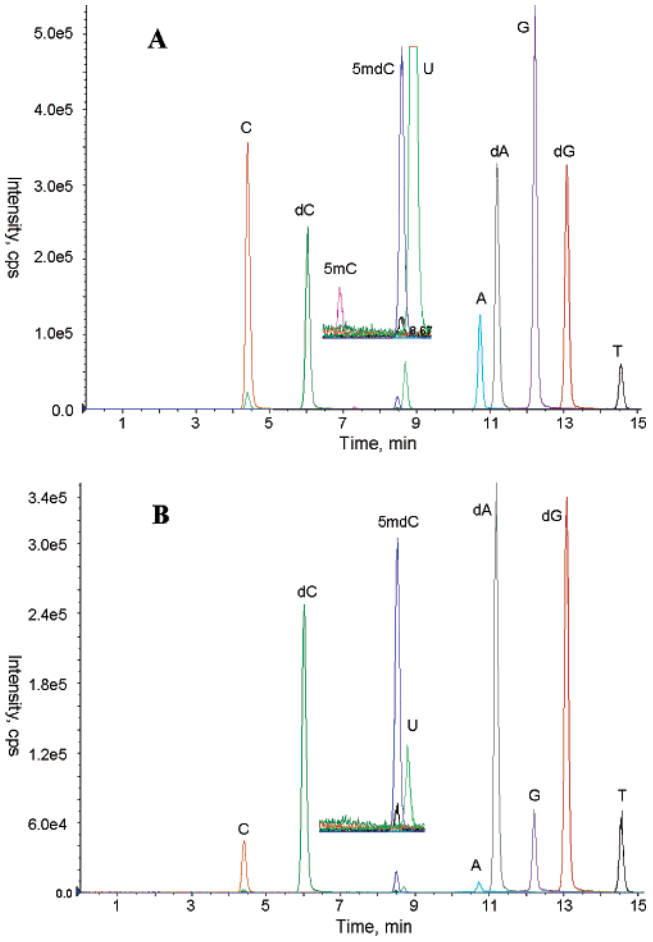

Figure 4. LC-ESI-MS/MS chromatogram of (A) 80 ng of HCT116 DNA plus 40 ng of purified human RNA and (B) 80 ng of HCT116 DNA alone. The insets are enlargements of the separation of 5mC, 5mdC, and U. The LC-ESI-MS/MS conditions were the same as used in Figure 2.

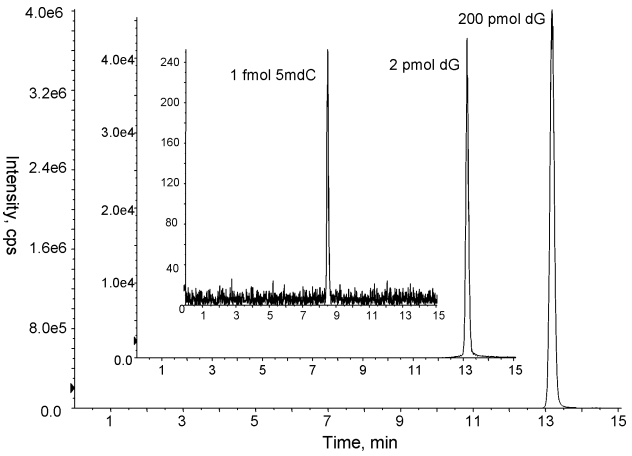

Figure 5. Extracted ion LC-ESI-MS/MS chromatogram of 1 fmol of 5mdC, 2 pmol of dG, and 200 pmol of dG, showing the detection of 1 fmol of 5mdC in DNA hydrolysis mixtures containing either 2 or 200 pmol of dG. The LC-ESI-MS/MS conditions were the same as used in Figure 3. The detection of 5mdC with a transition pair of *m*/*z* 242.1/126.3 generated very low background noise (~20 counts/s), resulting in a LOD of 0.2 fmol.

used for the DNA hydrolysis (described in Experimental Section) by a factor of 5. This test was also performed on validation day 2, and the proportion of dC methylation for HCT116 and DKO DNA samples was determined to be 4.26 and 1.38%, respectively, which did not differ significantly from the results with complete DNA hydrolysis: 4.12 and 1.39% with RSD 0.42 and 2.99% ($n = 3$), respectively. These data indicate that experimental conditions that prevent complete DNA hydrolysis, e.g., the presence of inhibitors in the source sample, should not reduce the accuracy of the reported method.

Based on a series of experiments with calibration standard mixtures, the limit of detection (LOD) of 5mdC was determined to be ~0.2 fmol (S/N > 2), with a linearity range of 1 fmol to 20 pmol. The low LOD is illustrated by Figure 5, which reports the detection of 1 fmol of 5mdC in DNA hydrolysis mixtures containing either 2 or 200 pmol of dG. The low LOD of the reported method permits the use of only 4 ng of digested DNA, corresponding to ~2 pmol of dG, for the measurement of dC methylation ratios ranging from 0.05 to 10%. For the measurement of even lower methylation ratios, as much as 400 ng of digested DNA, corresponding to ~200 pmol of dG, can be loaded onto the LC column, and a methylation ratio as low as 5 ppm can be quantified.

We also made an estimate of the LOD by varying the input amounts of DNA into the DNA hydrolysis step. Hydrolysis of HCT116 genomic DNA was performed as described in the Experimental Section, with 5-, 10-, 20-, 40-, 80-, and 160-ng DNA amounts in a final volume of 25 μL. A 20-μL aliquot of each digest was injected for analysis, and the peak height of 5mdC (H_{5mdC}) was found to have a linear relationship with DNA amount (A): $H_{5mdC} = 455A + 2.81 \times 10^3$ (correlation coefficient 0.9992). This relationship suggests that the recovery of nucleosides from DNA digestion remains constant even when the DNA amount is as small as 5 ng. Based on our analysis of 5 ng of DNA, we estimate the peak height of 5mdC to be 5.09×10^3 counts/s, according to the equation. Because the dC methylation ratio of HCT116 DNA was 4.14% (Table 1), we estimate that 5-ng DNA input should allow the measurement of dC methylation ratios as low as 0.04% (S/N > 2, $N = 20$ counts/s).

Finally, the potential impact of the DNA isolation method on the performance of the described assay was tested. Our standard DNA isolation procedure utilized a widely used commercial DNA isolation kit (see Experimental Section). For comparison, genomic DNA isolated by a conventional phenol/chloroform extraction followed by ethanol precipitation was utilized. We found that the LC-ESI-MS/MS assay is fully compatible with genomic DNA purified by phenol/chloroform extraction and that the calculated 5mdC/dG ratio of HCT116 cell DNA using the two methods did not differ significantly (data not shown).

CONCLUSION

In comparison with previously reported methods for the determination of genomic DNA methylation, the LC-ESI-MS/MS method described here has superior speed, specificity, and sensitivity. Efficient separation of 10 standard nucleosides resulting from DNA and RNA hydrolysis was achieved within 15 min. The high specificity of the method is provided by efficient LC separation followed by ESI-MS/MS detection. This method allows accurate measurement of DNA methylation even if RNA contaminates the DNA sample or if the hydrolysis of the DNA sample is incomplete. To our knowledge, this method provides the best sensitivity reported to date, with a LOD of 0.2 fmol, which permits the use of only 4 ng of DNA for the measurement of dC methylation ratios ranging from 0.05 to 10%. The extremely high sensitivity has been achieved due to more efficient ESI, less in-source fragmentation, more sensitive MRM acquisition, and sharper LC peaks. The use of dG as an internal standard allows an easier and more accurate determination of dC methylation and avoids the use of expensive isotope-labeled internal standards. These characteristics should enable the use of this method in applications in which DNA samples are limited, as is often the case for clinical samples or for cells collected by laser capture microdissection. In addition, the speed of this method should enable applications in which high throughput is critical, e.g., population-based studies examining the impact of dietary folate on genomic DNA methylation.

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