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A Method to Assess Genomic DNA Methylation Using High-Performance Liquid Chromatography/ Electrospray Ionization Mass Spectrometry

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Eukaryotic DNA is methylated at some cytosine residues, and this epigenetic feature performs critical functions. We developed a method for quantitative determination of 5-methyl-2′-deoxycytidine in human DNA using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). The DNA was enzymatically hydrolyzed by sequential digestion with three enzymes. DNA hydrolyzates were subsequently separated by reversed-phase high-performance liquid chromatography in isocratic mode. The four major DNA bases and 5-methyl-2′-deoxycytidine were resolved and eluted in 13 min. Identification of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine was obtained by combined diode array UV spectra analysis and mass spectra of chromatographic peaks. The isotopomers [$^{15}N_3$]-2′-deoxycytidine and (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine were used as internal standards. Ions of *m*/*z* 126 and 130 were used to detect 5-methyl-2′-deoxycytidine and its isotopomer, and ions of *m*/*z* 112 and 115 were used to detect 2′-deoxycytidine and its stable isotopomer, respectively. The DNA methylation status was calculated on the basis of the amount of 5-methyl-2′-deoxycytidine per microgram of DNA with percent relative standard deviations (%RSD) for a method precision of 7.1 (within-day) and 5.7 (day-to-day). This method also allows the measurement of 5-methyl-2′-deoxycytidine expressed as a percentage of total deoxycytidine residues in genomic DNA with %RSD for method precision of 1.9 (within-day) and 1.7 (day-to-day). This LC/MS method for quantitative determination of genomic DNA methylation status is rapid, sensitive, selective, and precise.

A characteristic feature of many eukaryotic genomes is methylation of cytosine at the carbon 5′ position of CpG dinucleotides.[1] Typically, the methylation of cytosine occurs predominantly in CpG-rich regions, the so-called "CpG islands" that are largely localized in gene promoter regions or in the initial exons of genes.[2] DNA methylation is a fundamental mechanism for epigenetic control of gene expression and the maintenance of genomic integrity.[3−5] Therefore, evaluation of genomic DNA methylation status is critical for the study of cell growth regulation, tissue specific differentiation,[1,2,6] and carcinogenesis.[5]

The two most widely used methods for assessing genomic DNA methylation status are a Southern blot technique that follows digestion with methylation-sensitive restriction endonucleases[1,7−10] and a radioassay that utilizes a bacterial DNA methyltransferase to catalyze the de novo methylation of the cytosine-guanine doublet sites with a radioactive methyl donor in vitro.[11−15] Recently, another method based on methylation-sensitive endonucleases followed by single nucleotide extension with radiolabeled [^{3}H]-dCTP[16] was described. Generally, these methods have wide variations in precision as a result of inconsistencies in the activity of methyl-sensitive endonucleases and the instability of methyltransferase activity.[17]

Chromatography has been used for the separation of purines and pyrimidines[18] as well as for the identification of modified deoxyribonucleosides.[19−23] Reversed-phase high performance liquid chromatography (HPLC) methods have been also applied for

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genome-wide methylation analysis. These HPLC methods, however, require the availability of microgram quantities of genomic DNA (5−50 μg) and synthesis of ^{32}P-labeled deoxyribonucleosides as well as a relatively long run time.[19,21]

In 1976, a mass spectrometry technique was described to obtain higher sensitivity in the detection and identification of 5-methylated cytosine residues from intact underivatized DNA.[24] Thereafter, in the 1980s, a derivatization method using *N*-methyl-*N*-(*tert*-butyl)dimethylsilyl trifluoroacetamide for the detection of 5-methylcytosine by stable-isotope-dilution GC/MS using deuterium-labeled 5-methylcytosine was described for the analysis of modified bases in calf thymus DNA.[25,26] Separation and detection of 5-methylcytosine was also obtained after derivatization with trifluoroacetic anhydride.[27] A further study described the use of HPLC combined with GC/MS[28] for the achievement of better specificity for the identification of the free DNA bases following derivatization with bis(trimethylsilyl) trifluoroacetamide.[28,29] The application of GC/MS to DNA hydrolyzates has been limited, however, because of the intrinsic polarity of these compounds.[30]

The development of electrospray ionization (ESI) enabled liquid chromatography/mass spectrometry (LC/MS) to be utilized for the quantitative determination and structural characterization of a great number of polar/ionic molecules, such as nucleic acids,[31−39] in biological samples. The use of LC/MS was suggested recently for the assessment of genomic methylation in DNA purified from a green alga.[40] This procedure, however, requires the availability of a rather large amount of DNA (25 μg), ^{32}P labeling of individual nucleosides, and uses an off-line combination of HPLC and MS,[40] in which the HPLC is used to separate and identify the deoxyribonucleosides by retention times and ultraviolet (UV) absorbance, and the ESI-MS [34] is used to analyze individually the compounds collected as HPLC fractions.[40]

We describe here a new, on-line LC/MS method for the measurement of methylated cytosine residues in 1 μg of genomic DNA. This LC/MS method allows the quantitative determination of genomic DNA methylation status. The technique relies on the quantitative hydrolysis of DNA and the complete removal of potential residual RNA and enables the separation and identification of the DNA bases and 5-methyl-2′-deoxycitidine by ESI-MS.

EXPERIMENTAL SECTION

Apparatus. Experiments were performed using a Hewlett-Packard/Bruker Esquire-LC ion trap liquid chromatograph/mass spectrometer (Billerica, MA). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (DAD) (model G1315A). A Suplex p*K*b 100 analytical column (25 cm × 2.1 mm) protected by a 5-μm Suplex p*K*b 100 precolumn (2 cm × 2.1 mm) (Supelco, Bellefonte, PA) was used. The HPLC system was controlled by HP ChemStation software. The mass spectrometer, from Bruker Daltonik (Bremen, Germany), was equipped with an electrospray ionization (ESI) source. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.0. Both software packages were run on an HP Kayak XA PC under the Microsoft Windows NT, version 4.0, operating system.

Reagents. The mobile phase consisted of 7 mM ammonium acetate pH 6.7/methanol 5% (v/v) and was prepared using HPLC-grade water, methanol (both from J. T. Baker, Philipsburg, NJ), and ammonium acetate (Aldrich, Milwakee, WI). The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfield, IL) before use. The stable isotope-labeled compounds [$^{15}N_3$]2′-deoxycytidine and the custom-made (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine (both from Cambridge Isotopes Laboratories, Inc., Andover, MA) were used as internal standards for 2′-deoxycytidine and 5-methyl-2′-deoxycytidine residues, respectively. Quality control data showed a chemical purity of 98% for [$^{15}N_3$]2′-deoxycytidine and 95%+ for (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine and a 98%+ isotopic enrichment for both compounds.

Mixtures of 5-methyl-2′-deoxycytidine and 2′-deoxycytidine (Sigma, St. Louis, MO) were used as external standards, to create a calibration curve, to evaluate the sensitivity of the technique for these compounds, and to determine the limit of detection.

CpGenome universal enzymatically methylated human male genomic DNA was used (Intergen Company, Purchase, NY) to evaluate different amounts of 5-methyl-2′-deoxycytidine in human DNA.

DNA Extraction and Hydrolysis. Genomic DNA was extracted from the buffy coat of human blood using a classical phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] protocol. Residual RNA was treated with both RNase A and T_1 (Invitrogen, Carlsbad, CA) to a final concentration of 10 units/mL at 37 °C for 1 h. The DNA was reprecipitated with 7.5 M ammonium acetate (1:1/2, v/v) and ethanol 100% (1:2, v/v) and dissolved in TE buffer (10 mM tris−HCl, 1 mM EDTA pH 8.0). DNA was dialyzed on 0.025-μm filter paper (Millipore, Bedford, MA) and stored at −70 °C until analysis. The procedure of DNA hydrolysis was performed as described by Crain.[33] Briefly, 1 μg of DNA was denatured by heating at 100 °C for 3 min and subsequently chilled in ice slush. One-tenth volume of 0.1 M ammonium acetate (pH 5.3) and 2 units of nuclease P1 (Roche Molecular Biochemicals, Mannheim, Germany) were added. The mixture was then incubated at 45 °C for 2 h. To the solution were subsequently added 1/10 volume of

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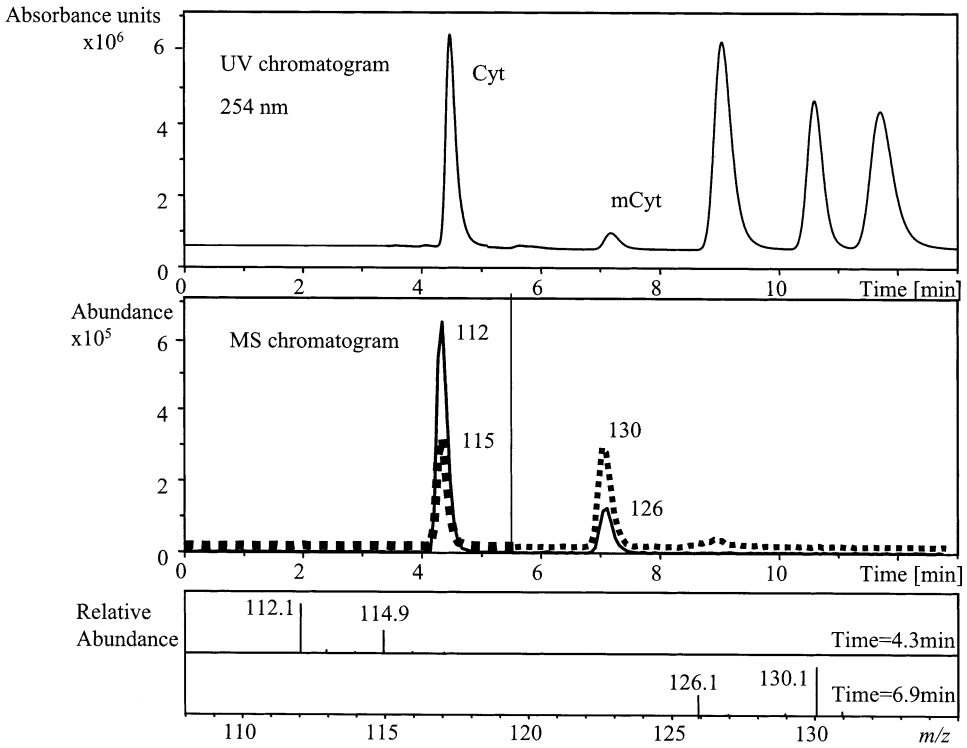


Figure 1. Typical LC/MS chromatogram of DNA digests. In the top panel is represented the UV chromatogram obtained after complete enzymatic hydrolysis of human PBMC DNA and detected at 254 nm. The first peak eluting after 4.5 ± 0.5 min corresponded to cytosine (Cyt), and the second peak eluting after 6.5 ± 0.5 min corresponded to 5-methylcytosine (mCyt). In the middle panel is represented a typical MS chromatogram. The first two MS peaks corresponded to Cyt (m/z = 112) and its stable isotope (m/z = 115), and the second two peaks corresponded to mCyt (m/z = 126) and its stable isotope (m/z = 130), as indicated in the bottom panel.

1 M ammonium bicarbonate (Sigma, St. Louis, MO) and 0.002 units of venom phosphodiesterase I (Sigma, St. Louis, MO). The incubation was continued for an additional 2 h at 37 °C. Thereafter, the mixture was incubated for 1 h at 37 °C with 0.5 units alkaline phosphatase (Sigma, St. Louis, MO). The stable isotopes [$^{15}N_3$]-2′-deoxycytidine and (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine were then added to the samples to reach a final concentration of 1 and 0.5 ng/μL, respectively, in a total volume of 35 μL.

LC/ESI/MS Procedure. Prior to its use, the instrument was checked to meet the sensitivity defined by the manufacturer. The DAD was calibrated and tested using the DAD diagnosis procedure of the ChemStation software for HP1100 system. The HP1100 MSD was calibrated with ESI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specifications and sensitivity were achieved over the entire mass range.

A 20-μL portion of the hydrolyzed-DNA solution was injected onto the analytical column thermostated at 21 °C. The separation of the four major DNA bases as well as that of 5-methyl-2′-deoxycytidine was obtained by isocratic elution. The mobile-phase flow rate was 0.3 mL/min, and the run time was 13 min. Electrospray source conditions were capillary, 30 nA and nitrogen drying gas, 9.0 L/min, with auxiliary 40.0 psi gas to assist with nebulization and drying temperature of 350 °C. The mass spectrometer was operated at a capillary voltage of 2500 V, and spectra were collected in positive ion mode. The electrospray needle was maintained at ground. The ion trap mass range was set to evaluate 2′-deoxycytidine from m/z 110 to 118 and 5-methyl-2′-deoxycytidine from m/z 125 to 132 in two different time windows. The time window for 2′-deoxycytidine was from 0 to 5.5 min, and the time-run window for 5-methyl-2′-deoxycytidine was from 5.5 to 13 min (shown in Figure 1). The acquisitions were typically 16 time points/min. Sixty scans were obtained per time point, and they were averaged before being stored in the database. Identification of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine was obtained by combined UV detection at 254 and 280 nm, and MS analysis of the chromatographic peaks eluting after 4.5 ± 0.5 and 6.5 ± 0.5 min, respectively.

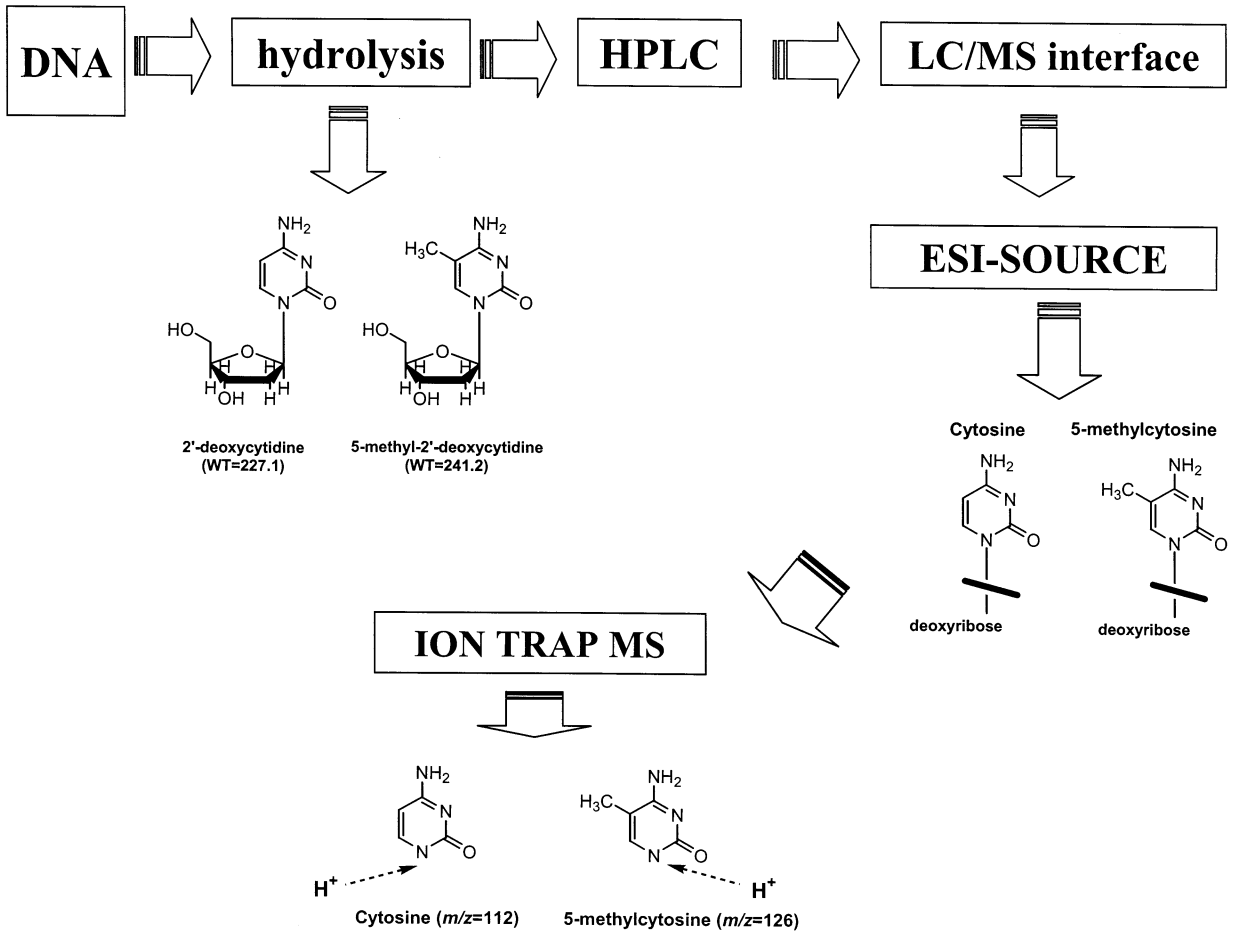
RESULTS AND DISCUSSION

The separation of the four bases and 5-methyl-2′-deoxycytidine residues was obtained using a reversed-phase column as described by Zambonin et al.[31] This method was modified using isocratic elution of a mobile phase that consisted of 7 mM ammonium acetate pH 6.7/5%methanol. The time run was 13 min.

As shown in Scheme 1, the electrospray ionization conditions caused the loss of the 2′-deoxyribose moiety in both 2′-deoxycytidine and 5-methyl-2′-deoxycytidine and the production of fragment ions whose molecular weight was consistent with the addition of a proton to the pyrimidine ring for both DNA bases (m/z 112 and 126, respectively).

As expected, the ESI source, with the mass spectrometer in positive ion detection mode, gave protonated molecules and also fragment ions for both 2′-deoxycytidine and 5-methyl-2′-deoxycytidine. We chose to monitor the fragment ions of the compounds of interest rather than the protonated molecules, because they provided more signal and better quantification results.

Scheme 1. Simplified Representation of the LC/MS Method for the Assessment of DNA Methylation Status[a]



[a] After the separation of DNA bases, the ESI conditions caused the separation of the pentose moiety from the pyrimidine ring of both 2′-deoxycytidine (wt = 227.1) and 5-methyl-2′-deoxycytidine (wt = 241.2) and resulted in the production of cytosine (wt = 111.10) and 5-methylcytosine (wt = 125.13), respectively. The molecular weight of the final fragment ions was consistent with the addition of a proton to the pyrimidine rings of both DNA bases (cytosine, m/z = 112; 5-methylcytosine m/z = 126).

Figure 1 shows a typical LC/MS chromatogram for a sample of purified and completely hydrolyzed peripheral blood mononuclear cell (PBMC) DNA. The top panel shows a UV chromatogram obtained at 254 nm. The middle panel shows a set of mass chromatograms. The HPLC peak eluting after 4.5 ± 0.5 min and at m/z 112 corresponded to 2′-deoxycytidine, and the HPLC peak eluting after 6.5 ± 0.5 min, and at m/z 126 corresponded to 5-methyl-2′-deoxycytidine.

The UV chromatogram was used to verify the complete digestion of the DNA samples as well as the absence of RNA residues. As previously described by Crain et al.,[26] an incomplete digestion of DNA due to the aberrant function of one or more enzymes could adversely affect the results, particularly in view of the very small amounts of 5-methyl-2′-deoxycytidine present in DNA.[33] The presence of RNA can interfere with the determination of the methylated cytosine residues in DNA, since both tRNA and rRNA contain 5-methylcytidine.[41,42] Because of the loss of the pentose moiety during the ESI procedure, the attribution of 5-methylcytosine exclusively to DNA is not possible. Therefore, we attempted to minimize the possible RNA contamination by using two different RNases and cleaning up the DNA by reprecipitation. In the case of RNA contamination, the chromatogram shows the presence of an extra peak eluting at 3.5 ± 0.5 min. As detailed in the Experimental Section, the ESI source with the mass spectrometer in positive ion detection mode, gave protonated molecules as well as fragment ions for both 2′-deoxycytidine and 5-methyl-2′-deoxycytidine, the molecular weight of which was consistent with the addition of a proton to the pyrimidine ring for both DNA bases (Figure 1). The molecular weight of the molecule eluting at 3.5 ± 0.5 min was consistent with the addition of a proton to the cytidine residue (m/z 244). All of the DNA samples were treated to avoid the presence of residual RNA, as described above, and no extra peaks were detected.

As shown in Figure 1, the ion at m/z 115 corresponded to the isotope labeled [$^{15}N_3$]2′-deoxycytidine, and the ion at m/z 130 corresponded to the isotope labeled (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine. The signal-to-noise ratio for a 20-μL injection of a 5 pg/μL concentrated sample of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine was 8.5 and 12.4, respectively.

The correlation between the MS signal and concentrations of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine was linear for scalar

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Table 1. Precision of the LC/MS Method for the Determination of 5-Methyl-2′-deoxycytidine

	absolute amount of 5-methyl-2′-deoxycytidine[a] (ng mCyt/μg DNA)	RSD[b] (%, $n = 9$)	percentage of 5-methyl-2′-deoxycytidine[a,c]	RSD (%, $n = 9$)
within-day variation	4.8 ± 0.3	7.1	5.8 ± 0.1	1.9
day to day variation	4.3 ± 0.2	5.7	5.4 ± 0.1	1.7

[a] From samples of human PBMC DNA. Values are expressed as means ± SD. [b] Relative standard deviation. [c] Equation to calculate the percentage of 5-methyl-2′-deoxycytidine (mCyt) of the total 2′-deoxycytidine: (Cyt) = (mCyt/mCyt + Cyt) × 100.

concentrations of both external standards ranging from 0.1 to 10 ng/μL, the usual working range. The Pearson's correlation coefficient was 0.991 for 2′-deoxycytidine and 0.994 for 5-methyl-2′-deoxycytidine.

The ratios between each of the external standards and the corresponding stable isotopomer remained constant when scalar concentration of the external standards for 2′-deoxycytidine and 5-methyl-2′-deoxycytidine were compared with a fixed concentration of the corresponding stable isotopomer. The concentrations of 2′-deoxycytidine ranged from 0.0175 to 3.5 ng/μL, and the concentration of [$^{15}N_3$]2′-deoxycytidine was 3.5 ng/μL. The Pearson's correlation coefficient of expected and observed ratios between scalar concentrations of 2′-deoxycytidine and its isotopomer [$^{15}N_3$]2′-deoxycytidine was 0.999 with $P = 0.002$.

The correlation of expected and observed ratios between scalar concentration of the external standard 5-methyl-2′-deoxycytidine (from 0.0175 to 3.5 ng/μL) and fixed concentrations of its corresponding isotope-labeled (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine (1.75 ng/μL) showed a Pearson's correlation coefficient of 0.999 and a P-value of <0.0001. The concentration of the stable isotopes was chosen for both internal standards on the basis of values obtained from a series of experiments from biological samples that gave the best percent relative standard deviation (usually <5%). These concentrations were 3.5 ng/μL for [$^{15}N_3$]2′-deoxycytidine and 1.75 ng/μL for (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine.

With this method, DNA methylation status can be expressed either by the absolute amount of 5-methyl-2′-deoxycytidine (i.e., ng/μg DNA) or by the relative amount of 5-methyl-2′-deoxycytidine compared to total cytosine residues (i.e. % of total cytosines).

The absolute amount of 5-methyl-2′-deoxycytidine per microgram of DNA was calculated using the abundance ratio between the 5-methyl-2′-deoxycytidine and (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine. To express the amount of 5-methyl-2′-deoxycytidine as a percentage, the following equation was used:

$$\frac{\text{5-methyl-2′-deoxycytidine}}{\text{(5-methyl-2′-deoxycytidine + 2′-deoxycytidine)}} \times 100$$

To determine the precision of the method, we performed a series of experiments using PBMC DNA samples. For quantification of both 2′-deoxycytidine and 5-methyl-2′-deoxycytidine, we compared the values obtained from biological samples with those of the internal standards of known concentration.

To determine the within-day percent relative standard deviation (%RSD) we ran nine samples of DNA hydrolyzates from the same DNA aliquot. As shown in Table 1 the within-day %RSD for data expressed as the absolute amount of 5-methyl-2′-deoxycytidine was 7.1. In Table 1 is shown the within-day %RSD for data expressed as 5-methyl-2′-deoxycytidine percentage of total 2′-deoxycytidines (1.9).

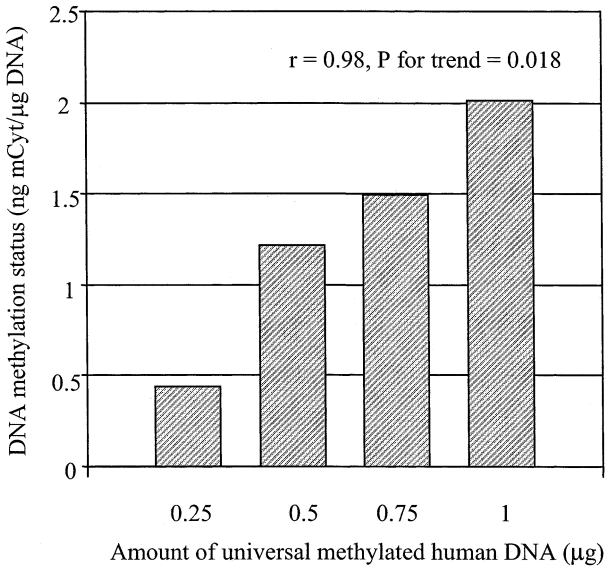

Figure 2. DNA methylation status expressed as an absolute amount of 5-methylcytosine (ng mCyt/μg DNA) in samples of scalar concentrations of universal methylated human DNA. *P* for trend = 0.018 with 0.98 of Pearson's correlation coefficient.

To evaluate the precision of an experiment using the same DNA sample performed in different days, nine aliquots from the same human PBMC DNA sample were run for 3 days in a row. As shown in Table 1, the day-to-day %RSD was 5.7 for data expressed as an absolute amount of 5-methyl-2′-deoxycytidine. The day-to-day %RSD was 1.7 for data expressed as the 5-methylcytosine percentage of total cytosines.

As shown in Table 1, the proportion of methylcytosine residues in human genomic DNA was ~5%, which is similar to that previously described by others.[1]

To verify the method's ability to detect different quantities of 5-methyl-2′-deoxycytidine in a proportional manner, we also performed experiments with scalar amounts of universal methylated human genomic DNA, as represented in Figure 2. There was a linear correlation between concentration of universal methylated

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DNA and the absolute amount of 5-methyl-2′-deoxycytidine (r = 0.98, P for trend = 0.018).

CONCLUSIONS

The LC/MS method described here allows accurate measurement of the absolute amount of 5-methyl-2′-deoxycytidine in DNA by utilizing a newly synthesized (methyl-d_3,ring-6-d_1)-5-methyl-2′-deoxycytidine stable isotopomer. The use of the isotope-labeled compound [$^{15}N_3$]2′-deoxycytidine as an internal standard for 2′-deoxycytidine also allows the assessment of the amount of 5-methyl-2′-deoxycytidine relative to the total amount of cytosine residues. Therefore, either parameter can be used for the particular research questions at hand. Furthermore, the quantity of DNA utilized in this method is relatively low and the on-line LC/MS method has a shorter run time for each sample, as compared to previously described methodologies. As we recently reported,[43] these characteristics enable this method to be used in large sample sets that could not be confidently analyzed with previous methods.[17] Since the determination of DNA methylation is crucial for the study of gene expression and stability, the applicability of this method extends to a wide field of biochemistry and molecular biology studies.

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