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Short communication

Determination of 5-methyl-2′-deoxycytidine in genomic DNA using high performance liquid chromatography-ultraviolet detection

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ABSTRACT

The formation of 5-methyl-2′-deoxycytidine (5-MedC) following methylation of the C-5 position of cytosine in genomic DNA provides an epigenetic mechanism for the regulation of gene expression and cellular differentiation. We describe the development of a method using HPLC-ultraviolet (UV) detection for the accurate determination of 5-MedC in DNA. Genomic DNA was obtained from HeLa cells and rat liver tissue using an optimised anion-exchange column DNA extraction procedure incorporating a ribonuclease incubation step to remove any potential interference from RNA. Following extraction the DNA samples were enzymatically hydrolysed to 2′-deoxynucleosides using a combination of an endo-exonuclease plus 5′-exonuclease together with a 3′-nucleotidase. The hydrolysed DNA samples (10 μg on column) were analysed using narrow-bore reverse phase HPLC-UV detection. The level of 5-MedC in the DNA samples was expressed as a percentage of the level of 2′-deoxycytidine (dC) determined from calibration lines constructed using authentic standards for 5-MedC and dC. The percentage 5-MedC level determined for commercially available calf thymus DNA was 6.26%, for HeLa cell DNA was 3.02% and for rat liver DNA was 3.55%.

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1. Introduction

The methylation of the C-5 position of cytosine in genomic DNA resulting in the formation of 5-methyl-2′-deoxycytidine (5-MedC) attracts major research interest from all areas of the biological scientific community [1–5]. This epigenetic alteration plays an important role in many biological processes such as cellular differentiation, gene expression and maintaining genome integrity [4,5]. Furthermore aberrant methylation has been found to be associated with carcinogenesis [2,3,6]. In particular global hypomethylation has been observed in various human cancers [4,7]. In mammalian cells the majority of 5-MedC is located within 5′-CpG-3′ dinucleotide containing sequences [1,4,5].

The importance of 5-MedC has resulted in the development of numerous methods for the assessment of global methylation and also in site-specific sequences. Non-chromatographic methods used for the assessment of genomic DNA methylation include: the preferential conversion of unmethylated cytosine to uracil by sodium bisulphite and subsequent determination by PCR amplification and sequencing [2,8]; a radioactive assay which involves the de novo transfer of a radioactive methyl donor by DNA methyltransferase to 2′-deoxycytidine (dC) sites within the DNA [9]; a Southern blot assay that utilises DNA digestion with restriction endonucleases that are sensitive to methylated sites [2,10]; an assay involving single nucleotide extension with radiolabelled [^{3}H]dCTP following cleavage of DNA with methylation-sensitive restriction endonucleases [11]. Chromatographic approaches include methods such as thin layer chromatography (following ^{32}P-postlabelling) [12,13], high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) and mass spectrometric [14–17] detection, gas chromatography [18,19] and capillary electrophoresis [20–24].

For the determination of 5-MedC in DNA by HPLC-UV detection the analyses are performed at the 2′-deoxynucleoside [25–30] or 2′-deoxynucleotide [31,32] level following enzymatic hydrolysis or at the nucleobase level following acid hydrolysis [33–36]. The latter is not a preferred option due to the potential of contamination from 5-methylcytosine derived from RNA that may be present in the DNA sample which is also released by acid hydrolysis.

We describe the development of a HPLC-UV detection method for the determination of 5-MedC which has been applied to

Abbreviations: 5-MedC, 5-methyl-2′-deoxycytidine; dC, 2′-deoxycytidine; dG, 2′-deoxyguanosine; T, Thymidine; dA, 2′-deoxyadenosine; G, Guanosine.

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the analysis of 5-MedC content in calf thymus DNA, HeLa cell DNA and rat liver DNA following enzymatic hydrolysis to 2′-deoxynucleosides.

2. Materials and methods

2.1. Chemicals

5-MedC was purchased from Chemos GmbH (Regenstauf, Germany). Calf thymus DNA, 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG), thymidine (T), 2′-deoxyadenosine (dA), guanosine (G), micrococcal nuclease (dissolved in HPLC grade water), nuclease P1 (dissolved in 0.28 M sodium acetate, 0.5 mM zinc chloride, pH 5.0) were purchased from Sigma (Poole, UK). Calf spleen phosphodiesterase (prepared by dialysis against HPLC grade water) was purchased from Calbiochem (Nottingham, UK). RNase A (heated at 80 °C for 10 min to inactivate DNAses) was purchased from Novagen (Nottingham, UK). RNase T_1 and Proteinase K were purchased from Roche (Hertfordshire, UK). Ammonium formate was purchased from BDH (Poole, UK). Methanol (HPLC fluorescence grade) was purchased from Fisher Scientific (Loughborough, UK) HPLC grade water, 18.2 MΩ cm output quality was obtained from Maxima purification equipment (Elga, High Wycombe, UK).

2.2. Preparation of 2′-deoxynucleoside standard stock solutions

The 5-MedC and dC standard stock solutions were prepared and dissolved in HPLC grade water. The concentration of each solution was calculated using the extinction coefficient following determination of the UV absorbance, for 5-MedC $\varepsilon = 8500\ M^{-1}\ cm^{-1}$ at λ_{max} 277 nm and dC $\varepsilon = 9000\ M^{-1}\ cm^{-1}$ at λ_{max} 271 nm (U-3010 spectrophotometer, Hitachi, Tokyo, Japan) [37].

2.3. HeLa cells

HeLa cells were cultured in Dulbecco's Minimum Eagle Medium (DMEM), supplemented with 10% foetal calf serum. The cells were harvested using trypsin following the attainment of 70% confluency (approximately 7×10^6 cells). The harvested cells were washed twice with PBS and then centrifuged for 5 min at 200 × *g* and 4 °C. Following removal of the supernatant, the cell pellet was stored at −20 °C overnight.

2.4. Animals

Male Wistar rats (8–10 weeks old, 350–475 g) were purchased from Charles River Laboratories (Margate, UK). The rats were control animals (administered corn oil by gavage) from a study previously conducted in our laboratory. The animals were culled 48 h following dosing with corn oil by exsanguination under halothane anaesthesia and the liver tissue was immediately frozen in liquid nitrogen following resection.

2.5. DNA extraction from HeLa cells

The DNA was extracted from the HeLa cells using the Qiagen kit (Qiagen Ltd., West Sussex, UK) which utilises an anion-exchange column procedure, as described in the instructions for the kit but with a few modifications. The cells were suspended in ice-cold buffer C1 (2 mL) and ice-cold HPLC grade water (6 mL) and mixed by inversion followed by incubation on ice for 10 min. The lysed cells were centrifuged for 15 min at 1800 × *g* and 4 °C. The supernatant was discarded and the pelleted nuclei resuspended in ice-cold buffer C1 (1 mL) plus ice-cold HPLC grade water (3 mL) by vortex mixing. Following centrifugation for 15 min at 1800 × *g* and 4 °C, the supernatant was again discarded. The nuclei were resuspended by the addition of buffer G2 (5 mL) and vortexing for 30 s. RNA contamination was removed by the addition of RNase A (2.5 mg) and RNase T_1 (100 U) at 37 °C for 30 min which was followed by a further incubation at 37 °C with 500 μL of proteinase K (25 mg/mL) for 2.5 h. The DNA was purified using the Midi Qiagen genomic tips (100/G) equilibrated with 4 mL of buffer QBT by gravity flow. The sample mixture was vortexed for 30 s and applied to the equilibrated Qiagen column which was then washed twice with 7.5 mL of buffer QC. The DNA was eluted from the column with 5 mL of buffer QF (maintained at 37 °C). Ice-cold isopropanol (0.7, v) was then added to the solution, mixed well by inversion and left overnight at −20 °C. The solution was centrifuged at 3200 × *g* and 4 °C for 25 min. The DNA pellet was washed with 0.5 mL of ethanol, centrifuged at 17,500 × *g* for 10 min, then washed again with 0.5 mL ethanol/HPLC grade water (70:30, v/v) and centrifuged at 17,500 × *g* for 10 min. The pellet was air dried and dissolved in 0.5 mL of HPLC grade water. The concentration of each DNA sample was calculated by determining the absorbance at 260 nm (GeneQuant spectrophotometer, Biochrom, Cambridge, UK) assuming that one absorbance unit equals 50 μg/mL for double stranded DNA. The samples were stored at −80 °C.

2.6. DNA extraction from animal tissue

The DNA was extracted from rat liver tissue using the Qiagen kit as described in the instructions for the kit but with a few modifications. Approximately 500 mg of tissue was homogenised using a Dounce homogeniser following suspension in 19 mL of G2 buffer solution. RNA contamination was removed by the addition of RNase A (2.5 mg) and RNase T_1 (100 U) at 37 °C for 30 min which was followed by a further incubation at 37 °C with 500 μL of proteinase K (25 mg/mL) for 2.5 h. The DNA was purified using the Maxi Qiagen genomic tips (500/G) equilibrated with 10 mL of buffer QBT by gravity flow. The homogenate mixture was vortexed for 30 s and applied to the equilibrated Qiagen column which was then washed twice with 15 mL of buffer QC. The DNA was eluted from the column with 15 mL of buffer QF (maintained at 37 °C). Ice-cold isopropanol (0.7, v) was then added to the solution and mixed well by inversion until the DNA was visible and then centrifuged at 3200 × *g* and 4 °C for 25 min. The DNA pellet was washed with 1.0 mL of ethanol, centrifuged at 17,500 × *g* for 10 min, then washed again with 1.0 mL ethanol/HPLC grade water (70:30, v/v) and centrifuged at 17,500 × *g* for 10 min. The pellet was air dried and dissolved in 1.0 mL of HPLC grade water. The concentration of each DNA sample was calculated by determining the absorbance at 260 nm assuming that one absorbance unit equals 50 μg/mL for double stranded DNA. The samples were stored at −80 °C.

2.7. Enzymatic hydrolysis of DNA

DNA samples (20–50 μg) were evaporated to dryness using a centrifugal vacuum evaporator (Speed vac plus SC210A, Savant, Farmingdale, US). The dried DNA samples were dissolved in 10 μL of digestion buffer, 100 mM sodium succinate, 50 mM calcium chloride, pH 6.0 and incubated with 5 μL of micrococcal nuclease (0.4 U/μL) and 35 μL of calf spleen phosphodiesterase (0.001 U/μL) at 37 °C overnight. The samples were then incubated with 10 μL of nuclease P1 (2 U/μL) at 37 °C for 4 h. Following centrifugation for 20 min at 17,500 × *g* the supernatants were transferred to new tubes and evaporated to dryness using a centrifugal vacuum evaporator. The samples were reconstituted with HPLC grade water to give a final concentration of 1 μg of hydrolysed DNA per μL and transferred to HPLC vials containing low volume inserts for analysis by HPLC-UV detection.

2.8. HPLC-UV detection

The hydrolysed DNA samples were analysed by injecting a 10 μL aliquot (equivalent to 10 μg of hydrolysed DNA) onto a Waters HPLC system consisting of Alliance 2690 separations module and 2487 UV detector (Waters Ltd., Hertfordshire, UK) connected to a Synergi Fusion-RP 80A C_{18} (4 μm, 250 mm × 2.0 mm) column (Phenomenex, Macclesfield, UK) attached to a Synergi Fusion-RP 80A C_{18} (4 μm, 4.0 mm × 2.0 mm) guard column and KrudKatcher (Phenomenex) disposable pre-column (0.5 μm) filter. The column was eluted using a gradient with mobile phase solvent A, 0.05 M ammonium formate, pH 5.4 and solvent B, methanol at flow rate of 0.2 mL/min. The following gradient was used: 0 min – 2%B, 18 min – 10%B, 30 min – 25%B, 35 min – 2%B and 40 min – 2%B. The UV absorbance was monitored at 277 nm.

2.9. Calibration lines for 5-MedC and dC

The calibration lines were constructed by the dilution of the stock standard solutions for 5-MedC (0.0625–10 nmol on column) and dC (0.125–25 nmol on column) which were used to determine level of 5-MedC and dC in the hydrolysed DNA samples.

2.10. Calculation of the percentage 5-MedC in DNA

The level of 5-MedC present in the DNA samples was expressed as a percentage of the level of dC which was calculated using the following equation:

$$\%\,5\text{-MedC} = \left[\frac{5\text{-MedC (nmol)}}{\text{dC (nmol)} + 5\text{-MedC (nmol)}}\right] \times 100$$

3. Results and discussion

For the precise assessment of global 5-MedC in DNA the majority of non-chromatographic methods are dependent on the consistent activity of methyl-sensitive restriction endonucleases, which if inconsistent may lead to ambiguities in the reported levels of 5-MedC. We have developed a relatively straightforward and cost effective HPLC-UV method when compared to mass spectrometric approaches for the accurate determination of 5-MedC following the enzymatic hydrolysis of DNA to 2′-deoxynucleosides. The HPLC-UV method was applied to the determination of 5-MedC levels in DNA samples from three different sources; commercially available calf thymus DNA, HeLa cell and rat liver DNA. DNA was obtained from HeLa cells and rat liver using an optimised anion-exchange DNA extraction procedure incorporating a ribonuclease incubation step to remove RNA contamination. The removal of RNA from the DNA sample is critical to prevent the generation of ribonucleosides following the enzymatic hydrolysis step, which could potentially lead to interference with the subsequent HPLC-UV analysis by co-elution with the 2′-deoxynucleosides if they are inadequately resolved. A combination of two enzymes, RNAse A hydrolysing at pyrimidine residues and RNase T_1 hydrolysing at guanine residues, was used to hydrolyse the RNA [29,38].

The DNA samples were initially hydrolysed to 2′-deoxynucleoside 3′-monophosphates by an overnight incubation with a combination of an endo-exonuclease (micrococcal nuclease) and 5′-exonuclease (calf spleen phosphodiesterase). The 2′-deoxynucleosides were generated by cleavage of the phosphate group following incubation with a 3′-nucleotidase (nuclease P1) for 4 h. The hydrolysed DNA samples were analysed using reverse phase HPLC-UV detection that allowed for the separation of 5-MedC and dC from the remaining three 2′-deoxynucleosides present in DNA. In principle the HPLC-UV approach used was similar to previously published methods [25–29]. Since both 5-MedC and dC are detected in the same chromatogram internal standardisation is not required. The HPLC-UV method required 10 μg of hydrolysed DNA on column for the determination of 5-MedC. A narrow-bore column (2.0 mm diameter) with a flow rate of 0.2 mL/min was used for the separation of the 2′-deoxynucleosides. The limits of detection for 5-MedC and dC were 0.007 nmol (S/N = 3.5) and 0.015 nmol (S/N = 4.4) on column, respectively. The lower limits of quantitation for 5-MedC and dC were 0.015 nmol (S/N = 11.2) and 0.030 nmol (S/N = 12.8) on column, respectively. The amount of 5-MedC and dC present in the DNA samples was determined from calibration lines constructed using authentic standards for 5-MedC and dC. A linear response was observed for the determination of 5-MedC from 0.0625 to 10 nmol on column ($y = 2341376.9x$, $r = 0.9999$) and for dC from 0.125 to 25 nmol on column ($y = 2203737.2x$, $r = 0.9999$) following HPLC-UV analysis.

Typical HPLC-UV chromatograms for the analysis of enzymatically hydrolysed calf thymus DNA, HeLa cell DNA and rat liver DNA are shown in Fig. 1A–C, respectively. The identity of the peaks was confirmed by co-elution with the corresponding authentic 2′-deoxynucleoside standards. The identity of the 5-MedC peak was

Table 1
Intra- and inter-assay variation for the determination of 5-MedC in calf thymus DNA, HeLa cell DNA and rat liver DNA.

DNA sample	% 5-MedC				Inter-assay mean
	Intra-assay mean				
	Day 1	Day 2	Day 3	Day 4	
Calf thymus DNA	6.29 ± 0.02 CV 0.29% n = 4	6.21 ± 0.11 CV 1.79% n = 4	6.24 ± 0.20 CV 3.19% n = 4	6.28 ± 0.06 CV 0.99% n = 4	6.26 ± 0.04 CV 0.57% n = 4
HeLa cell DNA	2.98 ± 0.16 CV 5.47% n = 4	3.02 ± 0.09 CV 3.02% n = 4	3.08 ± 0.08 CV 2.56% n = 4	3.00 ± 0.09 CV 2.95% n = 4	3.02 ± 0.04 CV 1.41% n = 4
Rat liver DNA					
Animal 1	3.44 ± 0.08 CV 2.47% n = 4	3.50 ± 0.01 CV 0.23% n = 4	3.50 ± 0.01 CV 0.23% n = 4	3.51 ± 0.01 CV 0.14% n = 4	3.49 ± 0.03 CV 0.90% n = 4
Animal 2	3.63 ± 0.11 CV 3.06% n = 4	3.71 ± 0.02 CV 0.52% n = 4	3.59 ± 0.18 CV 4.90% n = 4	3.47 ± 0.01 CV 0.36% n = 4	3.60 ± 0.10 CV 2.69% n = 4
Animal 3	3.54 ± 0.01 CV 0.16% n = 4	3.56 ± 0.01 CV 0.16% n = 4	3.57 ± 0.005 CV 0.14% n = 4	3.56 ± 0.01 CV 0.23% n = 4	3.55 ± 0.01 CV 0.39% n = 4

Values shown are mean ± standard deviation for the percentage of 5-MedC ([5-MedC/(dC + 5-MedC)] × 100) (CV = coefficient of variation).

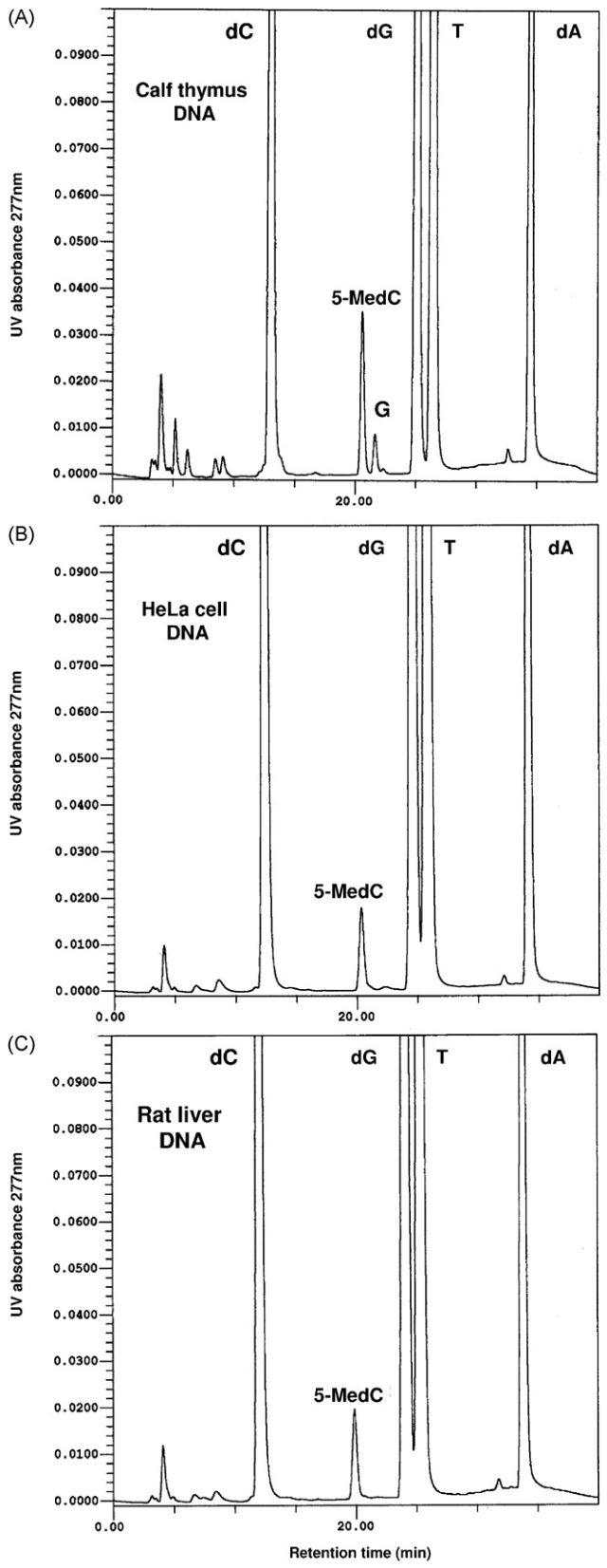

Fig. 1. Typical HPLC-UV chromatograms for the detection of 5-MedC in enzymatically hydrolysed (A) calf thymus DNA, (B) HeLa cell DNA and (C) rat liver DNA.

further confirmed by positive electrospray ionisation mass spectrometric analysis of pooled HPLC fractions that were collected and evaporated to dryness. An ion at *m/z* 242 consistent with the expected $[M+H]^+$ ion for 5-MedC was observed. Further confirmation of the structural identity of the peak was obtained by collison induced dissociation tandem mass spectrometric analysis of the 242 *m/z* $[M+H]^+$ ion. A product ion at *m/z* 126 was observed consistent with the $[Base+H_2]^+$ ion for 5-methylcytosine which was formed by the cleavage of the glycosidic bond and the accompanied hydrogen atom transfer from the 2′-deoxyribose moiety (data not shown). The identity of the guanosine peak in the calf thymus DNA sample was similarly confirmed by collison induced dissociation tandem mass spectrometric analysis of pooled HPLC fractions. An ion at *m/z* 284 consistent with the expected $[M+H]^+$ ion for guanosine was observed which resulted in a product ion at *m/z* 152 consistent with the $[Base+H_2]^+$ ion for guanine formed by the cleavage of the glycosidic bond and loss of the ribose moiety (data not shown). The elution of the guanosine peak in close proximity to the 5-MedC peak in the chromatogram for the hydrolysed calf thymus DNA (Fig. 1A) sample highlights the importance of the removal of RNA contamination by incubation with RNAse A and T_1 during DNA extraction which was applied to the HeLa cell and rat liver DNA (Fig. 1B and C). The typical retention times for the peaks detected were: dC, 12.0 ± 1.0 min; 5-MedC, 19.7 ± 1.3 min; G, 21.4 ± 0.7 min; dG, 23.9 ± 1.5 min; T, 25.1 ± 1.5 min; dA, 33.6 ± 1.0 min.

The levels of 5-MedC in the DNA samples were expressed as a percentage of the level of dC. Actual nmol amounts of 5-MedC and dC as determined from the calibration lines rather than HPLC peak areas were used to calculate the percentage 5-MedC since absorbance measurements are dependent on the value of the molar extinction coefficient for each 2′-deoxynucleosides at the detection wavelength [25]. The values obtained for the percentage level of 5-MedC determined in calf thymus DNA, HeLa cell DNA and rat liver DNA are shown in Table 1. The average intra-assay coefficients of variation (CV) for the determination of percentage 5-MedC in the different DNA samples were for calf thymus DNA, 1.56% ($n=4$), HeLa cell DNA, 3.50% ($n=4$) and rat liver DNA, 1.05% ($n=12$). The inter-assay CVs for the determination of percentage 5-MedC in calf thymus DNA and HeLa cell DNA were 0.57% and 1.41%, respectively. The average percentage 5-MedC determined for the three rat liver DNA samples was 3.55 ± 0.06% and the inter-assay CV ranged from 0.39% to 2.69%. The typical reported values in the literature for the percentage 5-MedC levels in DNA from various cancer cell lines range from 3.53% to 4.65%, for calf thymus DNA range from 4.75% to 8.10% and for rat liver DNA range from 3.33% to 4.91% [15,21,39,40]. The percentage 5-MedC values obtained for the three different DNA samples analysed in this study were consistent with previously published values in the literature [15,21,22,26,27,32,39,40].

4. Conclusion

The development of a relatively straightforward and cost effective HPLC-UV detection method has been described for the accurate assessment of 5-MedC content in genomic DNA.

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