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Simultaneous determination of 8-hydroxy-2′-deoxyguanosine and 5-methyl-2′-deoxycytidine in DNA sample by high performance liquid chromatography/positive electrospray ionization tandem mass spectrometry

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ABSTRACT

8-Hydroxy-2′-deoxyguanosine (8-OHdG) and 5-methyl-2′-deoxycytidine (5-mdC) are utilized as useful biomarkers not only for early diagnosis but also for the detection and assessment of high-risk individuals. In the present study, a sensitive and specific method was developed for simultaneous determination of 8-OHdG and 5-mdC in DNA by high performance liquid chromatography/positive electrospray ionization tandem mass spectrometry. The limits of quantification for 8-OHdG and 5-mdC were 80 and 40 pg/ml, respectively. The calibration curves of 8-OHdG and 5-mdC were linear over the concentration range of 0.02–100 ng/ml and the correlation coefficients were higher than 0.9990. The intra-day and inter-day relative standard derivative values were in the range of 0.70–7.47% for 8-OHdG and 1.07–7.06% for 5-mdC, respectively. The recoveries were 93.4–108.5% for 8-OHdG and 87.4–104.9% for 5-mdC, respectively. This method was validated by determination of the background levels of 8-OHdG and 5-mdC in calf thymus DNA, and satisfactory results were obtained.

1. Introduction

Reactive oxygen species (ROS) such as superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are formed continuously in living cells of aerobic organisms as part of the physiological process, metabolic and other biochemical reactions [1]. However, these endogenously produced ROS might cause oxidative damage to DNA molecule and generate a multitude of lesions [2]. 8-Hydroxy-2′-deoxyguanosine (8-OHdG), a prevalent form of oxidized nucleoside of DNA, is one of the most frequently detected and studied biomarkers of DNA oxidative damage [3]. Epidemiological and experimental studies suggest that 8-OHdG may induce carcinogenesis via both genotoxic and non-genotoxic mechanisms. Generally, genotoxic mechanism involves a mutation resulting in G:T transversion whereas non-genotoxic mechanism involves the decrease of binding affinity between DNA methyltransferases and DNA duplex by 8-OHdG [4–6].

DNA methylation is a major epigenetic modification in diverse organisms from bacteria to humans. This chemical modification is formed by a family of DNA methyltransferases to catalyze the transfer of a methyl group from the s-adenosyl-L-methionin to the 5-position of cytosine. 5-Methyl-2′-deoxycytidine (5-mdC), primarily found in CpG dinucleotides, represents 2–5% of all the 2′-deoxycytidine in mammalian genomes [7]. DNA methylation plays an important role in the normal development and functioning of organisms such as transcriptional regulation, X chromosome inactivation and genomic imprint [8]. Abnormal DNA methylation has been considered to be related to the aging and other developmental diseases. Global genomic hypomethylation can result in oncogene expression, genomic instability, and loss of imprinting [9]. Hypermethylation of promoter regions can lead to the silence of tumor suppressor gene by interfering with the binding of transcriptional factors or by recruiting a transcriptional corepressor complexes [10].

There is growing evidence that oxidative DNA damage and aberrant DNA methylation are associated with the pathogenesis of various diseases, especially aging and carcinogenesis [11,12]. And also many investigations have demonstrated that several classes of environmental factors such as irradiation, tobacco smoke, metals and air pollutants can trigger oxidative DNA damage and change DNA methylation patterns [13,14]. Thus, oxidative DNA damage and DNA methylation are often used as useful biomarkers not only for early diagnosis but also for the detection and assessment of high-risk individuals [15]. In addition, recent reports indicated that the presence of 8-OHdG at or adjacent to the recognition site could profoundly reduce the binding affinity of DNA methyltransferases and methyl-CpG binding proteins to oligonucleotide duplex and

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dramatically inhibit methylation [5,6,16]. On the contrary, it has been shown that DNA hypermethylation could also induce the silence of anti-oxidant genes, such as glutathione *S*-transferase P1 gene and manganese superoxide dismutase gene [17,18]. Therefore, in order to better understand the roles of 8-OHdG and 5-mdC in the development of human cancers and tumors and how oxidative DNA damage and DNA methylation interact with each other, it would be very helpful to develop simple and efficient methods to simultaneously identify and determine the contents of 8-OHdG and 5-mdC in DNA sample.

A variety of chromatography-based methods have been developed for the detection of 8-OHdG or 5-mdC, such as thin layer chromatography (TLC), GC/MS, high performance capillary electrophoresis (HPCE), HPLC/UV, HPLC/ECD, LC/MS and LC/MS/MS [19–26]. Among these methods, TLC needs simple instrument but has low accuracy. HPCE, HPLC/UV and HPLC/ECD can provide quantification with high reproducibility and sensitivity, but it lacks analyte specificity and must depend on chromatographic separation. GC/MS method can provide highly sensitive, but sample preparation requires tedious derivatization steps with its possible "artefactual" oxidation of nucleosides. The analytical approaches used to detect 8-OHdG or 5-mdC have been comprehensively reviewed by Peoples et al. and Dahl et al. [27,28], respectively. However, these methods are specified only for either 8-OHdG or 5-mdC. To our knowledge, simultaneous determination of 8-OHdG and 5-mdC in DNA sample has not been reported by far in literatures.

Mass spectrometric technology combined with high performance liquid chromatography is an alternative method to detect 8-OHdG and 5-mdC. The use of mass spectrometer under multiple reaction monitoring (MRM) mode can provide high sensitivity and selectivity. Here, we report a sensitive and specific method for simultaneous quantification of 8-OHdG and 5-mdC in DNA using high performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI/MS/MS) under MRM mode. And this method was applied to the determination of the levels of 8-OHdG and 5-mdC in calf thymus DNA.

2. Experimental

2.1. Chemicals

HPLC-grade methanol and acetonitrile were purchased from Merk (Darmstadt, Germany). Formic acid (HPLC grade), sodium acetate and zinc butter were obtained from Dupont (Delaware, USA). 8-OHdG, 5-mdC, nuclease P1 (from *Penicillium citrinum*), snake venom phosphodiesterase (phosphodiesterase I from *Crotalus adamanteus*), lambda DNA and calf thymus DNA were purchased from Sigma (St. Louis, MO). Calf intestine alkaline phosphatase and 10× incubation buffer were obtained from New England Biolabs (Beverly, MA). 2′-Deoxycytidine (dC), 2′-deoxyguanosine (dG), 2′-deoxyadenosine (dA) and thymidine (T), cytidine (C), guanosine (G), adenosine (A), uridine (U) were purchased from Amresco. Other chemicals were all of analytical grade.

Deionized water was prepared using a Direct-Q water purification system (Millipore, Bedford, MA, USA).

2.2. DNA enzymatic hydrolysis

Enzymatic hydrolysis of DNA was performed by the combined use of three major enzymes (nuclease P1, phosphodiesterase I and calf intestine alkaline phosphatase) as described previously by Taghizadeh et al. [29]. Briefly, about 100 μg of DNA was dissolved in deionized water, and then 3/10 volume of 30 mM sodium acetate (pH 6.8), 1/10 volume of 10 mM zinc butter, 3/100 volume of 50 mM deferoxamine and 8 units of nuclease P1 were added. The mixture was incubated at 37 °C for 3 h. Then 4/10 volume of 30 mM sodium acetate (pH 7.8), 200 units of snake venom phosphodiesterase I and 36 units of calf intestine alkaline phosphatase were subsequently added to the mixture, and then the incubation was continued overnight at 37 °C.

The final DNA hydrolysate was filtrated by Microcon centrifugal filter device (Millipore, Bedford, MA, USA; YM-10, MW cut-off 10,000) to remove protein. The spin filter were firstly pre-rinsed with deionized water (300 μl) to remove glycerol, and then an aliquot (100 μl) of the DNA digest was transferred to Microcon centrifugal filter and centrifuged at 12,000 × *g* at 4 °C for 30 min.

Table 1
Working parameters for mass spectrometry.

Parameter	Value
Scan type	MRM
Ionization mode	Positive
Nebulizer pressure (psi)	40
Drying gas temperature (°C)	350
Dry gas flow (l/min)	12
Dwell time per transition (ms)	100
Capillary voltage (V)	4500
Resolution	Unit
Fragmentor voltage (V)	75 (8-OHdG) and 40 (5-mdC)
Collision energy (ev)	11 (8-OHdG) and 8 (5-mdC)
Ion transition for 8-OHdG (*m*/*z*)	284.2 > 168.0
Ion transition for 5-mdC (*m*/*z*)	242.2 > 126.1

2.3. HPLC/ESI/MS/MS conditions

LC analysis was performed with an Agilent 1200 high performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA). The liquid chromatography was equipped with a vacuum degasser, an autosampler, a rapid resolution binary pump, a diode array detector and a thermostated column compartment. A Waters (Milford, MA) Atlantis dC18 2.1 mm × 150 mm column (5-μm particle size) protected by a Phenomenex dC18 2.1 mm × 20 mm guard column (5-μm particle size) was used for the chromatographic separation. 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) were used as mobile phases, and the flow rate was set at 0.22 ml/min. The linear gradient elution was 0–18% of solvent B in 24 min. The sample injection volume was 10 μl. The detection wavelength for dG and dC was set at 284 nm.

Mass spectrometric detection was performed on an Agilent 6410 triple quadrupole tandem mass spectrometer coupled with an electrospray ionization source. Optimized ESI source parameters were shown in Table 1. Quantitative determination of 8-OHdG and 5-mdC were performed under multiple reaction monitoring (MRM) mode and MS data were collected during two MS segments (7–12 min and 18–24 min) corresponding to transitions *m*/*z* 284.2–168.0 for 8-OHdG and *m*/*z* 242.2–126.1 for 5-mdC, respectively. Agilent Mass Hunter workstation software (version B.01.03) was used for data acquisition and processing.

2.4. Preparation of stock solutions, calibration standard solutions and quality control samples

Stock solutions of 8-OHdG and 5-mdC were prepared at 1 mg/ml in deionized water. Standard working solutions of 8-OHdG and 5-mdC (1, 10, 100, 1000 ng/ml) were obtained by further dilution of stock solutions with deionized water. Calibration standard solutions of 8-OHdG and 5-mdC (0.02, 0.04, 0.2, 0.5, 2.5, 5, 10, 20, 100 ng/ml) were prepared by further diluting the standard working solutions with deionized water.

Quality control (QC) samples were prepared by diluting standard working solutions in deionized water. The final concentrations of 8-OHdG and 5-mdC were 0.1, 1, 10 and 100 ng/ml, respectively.

All the above solutions were stored at −20 °C and allowed to equilibrate at room temperature before use.

2.5. Recovery

Aliquots (180 μl) of an enzymatic hydrolysate of 100 μg lambda DNA were spiked with 20 μl of one of the four standard working solutions, so that the concentrations of 8-OHdG and 5-mdC were 0.1, 1, 10, 100 ng/ml. After ultrafiltration through a Microcon centrifugal filter at 12,000 × *g* at 4 °C for 30 min (Millipore, Bedford, MA, USA; YM-10, MW cut-off 10,000), the filtrates were analyzed by HPLC/ESI/MS/MS system to determine the concentrations of 8-OHdG and 5-mdC. The final concentration calculation was based on subtraction of the background level of 8-OHdG and 5-mdC in lambda DNA hydrolysate.

3. Results and discussion

3.1. Optimization of HPLC/ESI/MS/MS conditions

Originally, both positive and negative modes were tested for the LC/ESI/MS/MS analysis of 8-OHdG and 5-mdC and the results indicated that the positive mode was more sensitive than the negative mode (data not shown). The full scan of ESI mass spectra of 8-OHdG and 5-mdC were obtained by direct infusion of a standard working solution into the mass spectrometer. The data indicated that protonated 8-OHdG ($[M+H]^+$, *m*/*z* 284.2) and 5-mdC ($[M+H]^+$, *m*/*z* 242.2) were the predominant ion species formed by ESI. The product ion spectra of 8-OHdG and 5-mdC were shown in Fig. 1, and it can be found that the main product ion of 8-OHdG was *m*/*z* 168.0 whereas the main product ion of 5-mdC was *m*/*z* 126.1. These product ions were the results of the cleavage of the *N*-glycoside bond accompanied by transfer of a hydrogen atom from the sugar moiety. Thus, the precursor/product ion pairs of *m*/*z* 284.2/168.0 for 8-OHdG and *m*/*z* 242.2/126.1 for 5-mdC were used as MRM transitions. By applying automatic optimization procedures, other working parameters for mass spectrometry such as collision energy, fragmentor voltage, and capillary voltage were obtained and shown in Table 1.

A potential problem which may affect the accuracy of 8-OHdG and 5-mdC detection is the interference of RNA contamination in DNA sample. In previous chromatography-based studies, two schemes were often adopted. Firstly, RNA contamination was sufficiently eliminated from DNA sample during DNA isolation and purification procedures. Secondly, a total chromatographic separation method of all the nucleosides was developed [30]. In our method, for the purpose to eliminate RNA interference in the measurement of 8-OHdG and 5-mdC, different mobile phase conditions consisting of water–methanol were investigated for total chromatographic separation of normal and modified nucleosides (dC, dG, dA, T, C, G, A, U, 8-OHdG, 5-mdC). The best sensitivity and selec-

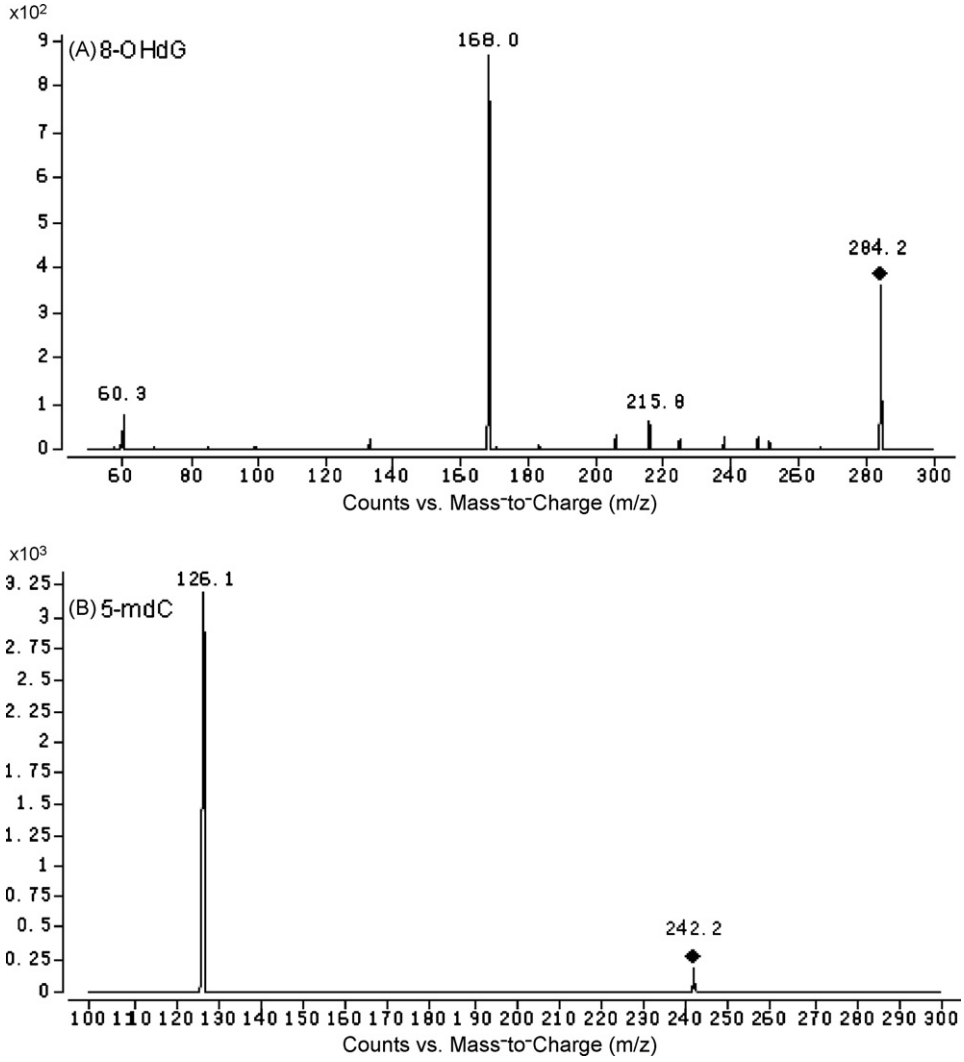

Fig. 1. Positive ESI product ion spectra of 8-OHdG (A) and 5-mdC (B).

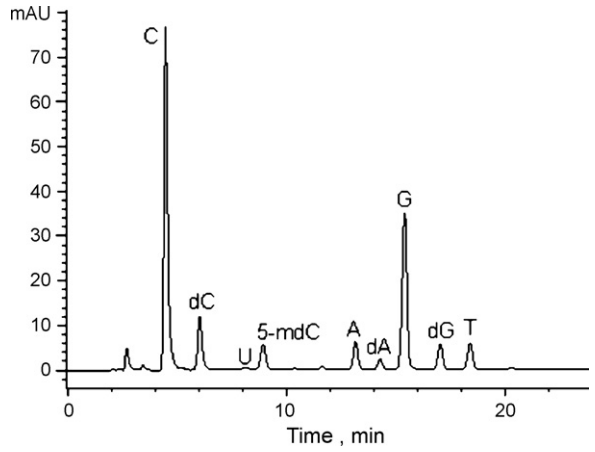

Fig. 2. LC/UV chromatogram of normal and modified nucleosides at 284 nm.

tivity, with good separation of normal and modified nucleosides, was achieved using gradient elution conditions of 0.1% formic acid in water and 0.1% formic acid in methanol. Fig. 2 showed the LC/UV chromatogram of normal and modified nucleosides. It was obvious that the nucleosides were completely separated from each other and the retention times for dC, dA, dG, T, C, U, A, G, 5-mdC were 6.3, 14.3, 17.4, 18.8, 4.7, 8.6, 13.4, 15.8 and 9.1 min, respectively. Typical MRM ion chromatograms for 8-OHdG and 5-mdC of hydrolyzed calf thymus DNA were shown in Fig. 3. From Figs. 2 and 3, it can be clearly seen that the detection of 8-OHdG and 5-mdC would not be influenced by other nucleosides.

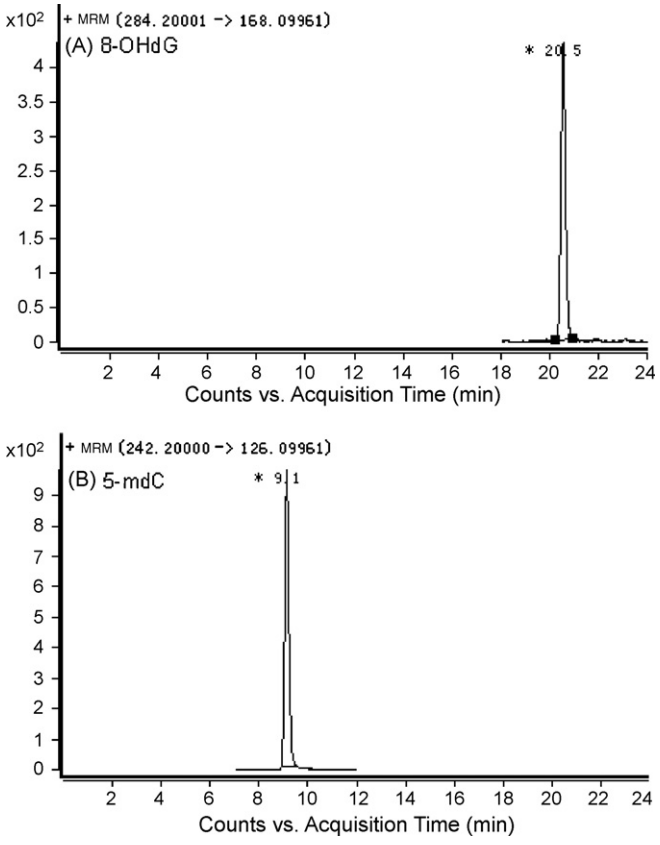

Fig. 3. Typical MRM chromatograms resulting from the hydrolyzed calf thymus DNA sample: (A) 8-OHdG (RT 20.5 min) and (B) 5-mdC (RT 9.1 min).

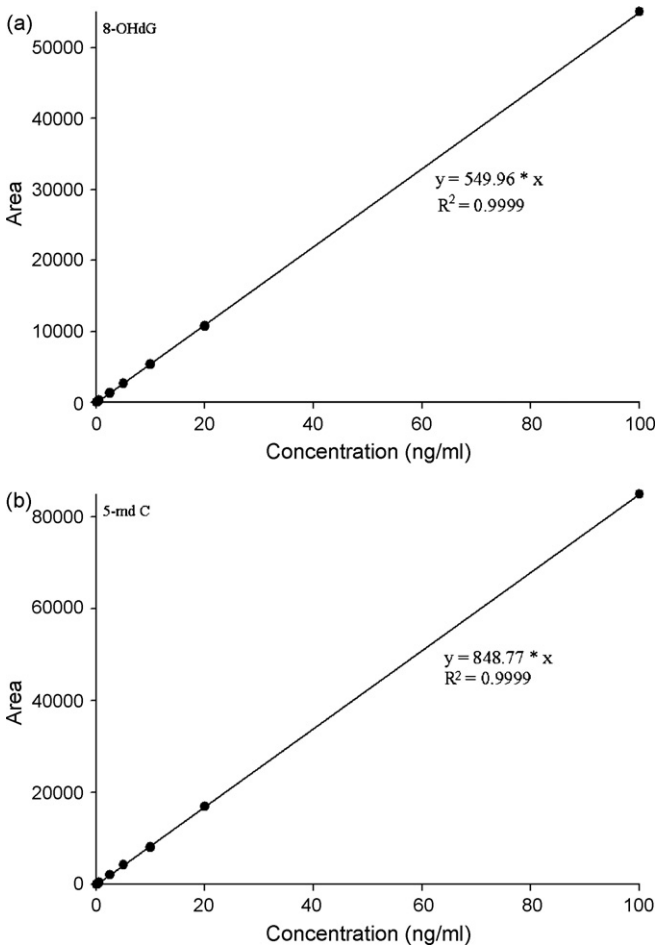

Fig. 4. Calibration curves for the positive ion electrospray LC/ESI/MS/MS measurement of 8-OHdG (A) and 5-mdC (B).

3.2. Calibration curves and sensitivity

Calibration curves of 8-OHdG and 5-mdC obtained on consecutive five days were linear over the concentration ranges of 0.02–100 ng/ml, with the correlation coefficient R^2 over 0.9990 (Fig. 4).

The limits of detection (LOD) of 8-OHdG and 5-mdC ($S/N = 3$) were determined to be 40 pg/ml (1.41 fmol) and 20 pg/ml (0.83 fmol), respectively. The limits of quantification (LOQ) of 8-OHdG and 5-mdC ($S/N = 10$) were determined to be 80 pg/ml (2.82 fmol) and 40 pg/ml (1.65 fmol), respectively.

3.3. Precision

To determine the precision of the present method, QC samples of 8-OHdG and 5-mdC were prepared and the concentrations were 0.1, 1, 10 and 100 ng/ml. Intra-day precision was evaluated by analysis of QC samples at different times during the same day ($n = 3$). Inter-day precision was determined by repeated analysis of QC samples three times a day for five consecutive days ($n = 15$). The concentration of each sample was determined using calibration standards prepared on the same day. Precision was expressed as the relative standard deviation (RSD, %) of concentrations calculated for QC samples, and the accepted criteria for each quality control was that the RSD value should not exceed 15%. The intra-day precision values were in the range of 0.70–5.69% for 8-OHdG and 1.07–3.05% for 5-mdC, respectively. The inter-day precision values were in the range of 3.57–7.47% for 8-OHdG and 3.32–7.06%

Table 2
Intra- and inter-assay relative standard derivatives of 8-OHdG and 5-mdC.

Concentration (ng/ml)	Intra-day (RSD, %)		Inter-day (RSD, %)	
	8-OHdG	5-mdC	8-OHdG	5-mdC
0.1	5.69	3.05	7.47	3.32
1	1.60	1.07	3.60	7.06
10	1.42	2.53	5.23	6.14
100	0.70	1.10	3.57	4.46

Table 3
Recoveries of 8-OHdG and 5-mdC.[a]

Analyte	Concentration added (ng/ml)	Recovery (%)	RSD (%)
8-OHdG	0.5	93.4	1.01
	5	102.8	0.41
	50	108.5	0.51
	100	98.5	4.24
5-mdC	0.5	88.1	4.07
	5	103.1	1.12
	50	87.4	0.25
	100	104.9	0.15

[a] Each recovery value represents the average of triplicate samples.

for 5-mdC, respectively (Table 2). These results suggested that the present method had very good precision.

3.4. Recovery

In the present study, lambda DNA was used as the matrix to measure the recovery of the present method. Our analytic data indicated that the background levels of 8-OHdG and 5-mdC in lambda DNA were below the detection limit of our method (data not shown). The recoveries for 8-OHdG and 5-mdC at different concentrations were summarized in Table 3. It can be seen that satisfactory recoveries were obtained for both 8-OHdG (93.4–108.5%) and 5-mdC (87.4–104.9%).

3.5. Application assay

To assess the feasibility of this optimized HPLC/ESI/MS/MS method, calf thymus DNA was hydrolyzed by nuclease P1 system (see Section 2). After molecular weight cut-off ultracentrifugation, the levels of 8-OHdG and 5-mdC were determined. The background level of 8-OHdG in calf thymus DNA was determined to be 44.10 ± 5.95 adducts per 10^6 2′-deoxyguanosine. In literatures this value ranged from 28.8 adducts per 10^6 2′-deoxyguanosine determined by immunoaffinity column purification followed by LC/MS/MS method [31] to 120 adducts per 10^6 2′-deoxyguanosine determined by HPLC/APCI/MS/MS method [26]. The relative abundance of 5-mdC in DNA is expressed as the ratio of 5-mdC to the sum of 5-mdC and 2′-deoxycytidine (%). The global DNA methylation level in calf thymus DNA obtained by our method is 6.74 ± 0.20%, and this value is very close to those detected by LC/ESI/MS/MS, LC/UV and GC/MS methods [32–34]. So, this method developed in the present study is effective for simultaneous determination of 8-OHdG and 5-mdC in DNA sample.

4. Conclusions

A sensitive and reliable HPLC/ESI/MS/MS method for the simultaneous detection of 8-OHdG and 5-mdC in DNA has been established for the first time in the present study. Under optimized conditions, the method showed satisfactory sensitivity, precision and recovery. The limits of quantification of this method were 80 pg/ml for 8-OHdG and 40 pg/ml for 5-mdC, respectively. The intra- and inter-day relative standard derivatives of 8-OHdG and 5-mdC were in the range of 0.70–7.47 and 1.07–7.06%, respectively. This study provides an alternative method to simultaneously determine the levels of 8-OHdG and 5-mdC in DNA and it would be helpful in future studies to explore the roles of 8-OHdG and 5-mdC and the relationship between them in the process of aging and carcinogenesis.

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