Supporting Information

for

Existence of 5-Hydroxymethylcytosine and 5-Formylcytosine in both DNA and RNA in Mammals

Hao-Ying Zhang,† Jun Xiong,† Bao-Ling Qi, Yu-Qi Feng,* Bi-Feng Yuan *

Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Wuhan 430072, China.

† These authors contributed equally to this work.

* To whom correspondence should be addressed. Tel. +86-27-68755595; fax. +86-27-68755595. E-mail address: bfyuan@whu.edu.cn; yqfeng@whu.edu.cn

Chemicals and Reagents

2'-deoxycytidine (dC), 2'-deoxyguanosine (dG), 2'-deoxyadenosine (dA), thymidine (T), cytidine (rC), guanosine (rG), adenosine (rA), uridine (rU), 5-methyl-2′-deoxycytidine (5-mdC), manganese dioxide (MnO_2), ascorbic acid, and phosphodiesterase I were purchased from Sigma-Aldrich (Beijing, China). 5-hydroxymethyl-2'-deoxycytidine (5-hmdC), 5-hydroxymethylcytidine (5-hmrC), 5-formyl-2'-deoxycytidine (5-fodC), and 5-formylcytidine (5-forC) were purchased from Berry & Associates (Dexter, MI). S1 nuclease and alkaline phosphatase were from Takara Biotechnology Co., Ltd (Dalian, China). Dansylhydrazine (DNSH) were purchased from J&K Co., Ltd (Beijing, China). Hydroquinone were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Chromatographic grade methanol and acetonitrile (ACN) were purchased from Tedia Co. Inc. (Fairfield, OH). All other solvents and chemicals used were of analytical grade. Water used throughout all experiments was purified by a Milli-Q water purification apparatus (Millipore, Bedford, MA).

Cell Culture and Treatment

HeLa (human cervical carcinoma) and 293T cell lines were obtained from the China Center for Type Culture Collection (CCTCC). HeLa and 293T cells were maintained in DMEM medium at 37°C under 5% CO_2 atmosphere. The media was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Ascorbic acid and hydroquinone were added to medium at the concentration of 100 μM and cells were harvested after 24 h treatment.

DNA and RNA Extraction and Enzymatic Digestion

Genomic DNA and total RNA were extracted from the cultured cells using E.Z.N.A.™ DNA Kit (Omega Bio-Tek Inc., Norcross, GA) and E.Z.N.A.™ Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA), respectively, according to the manufacture's recommended protocol. The concentration of the extracted DNA and RNA were determined using B-500 spectrophotometer (Metash Instruments Co., Ltd., Shanghai, China).

The enzymatic digestion of DNA and RNA was performed according to the previously described method with slight modification.[1] Briefly, the mixture of 5 μg DNA and 5 μg RNA in 16 μL H_2O was denatured by heating at 95ºC for 5 min, and then chilled on ice for 2 min. After adding 2 μL of S1 nuclease buffer (30 mmol/L CH_3COONa, pH 4.6, 280 mmol/L NaCl, 1 mmol/L $ZnSO_4$) and 100 units (0.5 μL) of S1 nuclease, the mixture (20 μL) was incubated at 37ºC for 16 h. Then 66 μL H_2O, 10 μL alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 0.005 units (3 μL) of venom phosphodiesterase, and 30 units (1 μL) of alkaline phosphatase were subsequently added. The mixture was incubated at 37ºC for an additional 4 h. After adding 200 μL H_2O, the resulting solution was extracted with chloroform twice to obtain the digested nucleosides.

MnO_2 Oxidation

MnO_2 was used to oxidize 5-hmdC and 5-hmrC to 5-fodC and 5-forC, respectively, followed by centrifugation to remove MnO_2. To achieve the best oxidation efficiency, we optimized the oxidization conditions, including reaction temperature and time, and amounts of MnO_2.

We first investigated MnO_2 amounts ranging from 2 - 50 mg. Generally, 200 pmol of 5-hmdC and 5-hmrC were dissolved in 360 μL ACN, 20 μL formic acid, 3 μL 50 % glycerol, 2 μL S1 nuclease buffer, 10 μL alkaline phosphatase buffer and 5 μL H_2O. Then MnO_2 was added followed by incubation at 40°C for 2 h. Next, we optimized the reaction temperature ranging from 30°C to 70°C. Finally, the reactions time was optimized ranging from 15 min to 4 h. 5-mdC and rG were used as internal standards for the quantification of the oxidation products of 5-hmdC and 5-hmrC, respectively.

The oxidation products were examined on a Shimadzu LC-20A HPLC (Tokyo, Japan) equipped with two LC-20A pumps, a CTO-20A thermostatted column compartment, and a SPD-20A UV/VIS detector. A Hisep C18 column (150 mm × 4.6 mm i.d., 5 μm, Weltech Co., 180 Ltd., Wuhan, China) was used for the separation. The column temperature was set at 35°C and the wavelength was set at 280 nm. Ammonium acetate in water (5 mM, solvent A) and methanol (solvent B) were employed as mobile phase. An isocratic elution with 95% A and 5% B was used, and the flow rate was set at 0.8 mL/min.

Dansylhydrazine Derivatization

In this study, dansylhydrazine (DNSH) was used to label the oxidation products of 5-hmdC and 5-hmrC (i.e., 5-fodC and 5-forC). To achieve the best derivatization efficiency, we optimized the derivatization conditions, including reaction temperature and time, and concentration of DNSH.

We first optimized the reaction temperature ranging from 30°C to 70°C. The reactions were incubated with 0.4 mM of DNSH for 100 min. The concentration of DNSH was also optimized ranging from 0.02 to 1 mM, and the reactions were incubated at 40°C for 100 min. Finally, the derivatization time was optimized ranging from 5 min to 100 min, and the reactions were performed at 40°C with 0.4 mM of DNSH.

The DNSH labeled products were examined on a on a Shimadzu LC-20A HPLC (Tokyo, Japan) equipped with two LC-20A pumps, a CTO-20A thermostatted column compartment, and a SPD-20A UV/VIS detector. A Hisep C18-T column (250 mm × 4.6 mm i.d., 5 μm, Weltech Co., 180 Ltd., Wuhan, China) was used for the separation. The column temperature was set at 35°C and the wavelength was set at 280 nm. Water containing 0.1% formic acid (v/v, solvent A) and ACN (solvent B) were employed as mobile phase. A gradient of 15 min of 30 – 60% B was used. The flow rate of mobile phase was set at 0.8 mL/min.

Analysis of the 5-hmdC, 5-hmrC, 5-fodC and 5-forC in Mammalian Cell

The nucleoside mixture from DNA and RNA digestion was oxidized in 360 μL of ACN, 20 μL of H_2O, and 20 μL of formic acid using 40 mg of MnO_2 at 40°C for 1 h. The mixture was then passed through a graphitized carbon black-SPE (Weltech Co., Ltd, Wuhan, China) to remove salt followed with derivatization by DNSH. The derivatization was performed in 0.4 mM DHSN, 100 μL of ACN, 5 μL of HAc at 40°C for 1 h followed by drying with nitrogen gas at 37°C. The derivatized products were then reconstituted in 50 μL of water and subjected to LC-ESI-MS/MS analysis.

Analysis of nucleosides was performed on the LC-ESI-MS/MS system consisting of an AB 3200 QTRAP LC-MS/MS (Applied Biosystems, Foster City, CA) with an electrospray

ionization source (Turbo Ionspray) and a Shimadzu LC-20AD HPLC (Tokyo, Japan) with two LC-20AD pumps, a SIL-20A autosampler, a CTO-20AC thermostatted column compartment, and a DGU-20A3 degasser. Data acquisition and processing were performed using AB SCIEX Analyst 1.5 Software (Applied Biosystems, Foster City, CA). The LC separation was performed on a Hisep C18-T column (150 mm × 2.1 mm i.d., 5μm, Weltech Co., Ltd., Wuhan, China) with a flow rate of 0.2 mL/min at 35°C. Water (solvent A) and methanol (solvent B) were employed as mobile phase. A gradient of 5 min 10% B, 5 min 10 - 20% B, 5 min 20 – 43% B, and 25 min 43% B was used.

The mass spectrometry detection was performed under positive electrospray ionization (ESI) mode. The target nucleosides were monitored by multiple reaction monitoring (MRM) mode and the mass transitions are listed in Table S4. The MRM parameters of all nucleosides were optimized to achieve maximal detection sensitivity.

High resolution mass spectrometry experiments was performed on the LC-QTOF-MS system consisting of a MicrOTOF-Q orthogonal-accelerated TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with an ESI source (Turbo Ionspray) and a Shimadzu LC-20AB binary pump HPLC (Tokyo, Japan), a SIL-20AC auto sampler, and a DGU-20A3 degasser. Data acquisition and processing were performed using Bruker Daltonics Control 3.4 and Bruker Daltonics Data analysis 4.0 software.

Optimization of MnO_2 Oxidation Conditions

Here we used MnO_2 to oxidize 5-hmdC and 5-hmrC, and the formed oxidation products (5-fodC and 5-forC) were examined by tandem mass spectrometry analysis. The results showed that 5-hmdC and 5-hmrC can be efficiently oxidized to 5-fodC and 5-forC by MnO_2 (Figure S1A and S1B).

To obtain a good oxidation efficiencies of 5-hmdC and 5-hmrC, we optimized the oxidization conditions, including reaction temperature and time, and amounts of MnO_2. Our result showed that 40°C was appropriate for the reaction (Figure S1C). As for the oxidation time, the result showed that 1 h was sufficient (Figure S1D). In addition, the optimal amount of MnO_2 was investigated. The result showed that the peak areas of the oxidation products of 5-hmdC and 5-hmrC reached a plateau when the amount of MnO_2 was 40 mg (Figure S1E).

Taken together, the optimized oxidation conditions for 5-hmdC and 5-hmrC by MnO_2 were under 40°C for 1 h using 40 mg MnO_2. Under the optimized conditions, more than 99% of 5-hmdC and 5-hmrC can be oxidized to 5-fodC and 5-forC, and the other normal nucleosides still kept intact (Data not shown).

Optimization of Derivatization Conditions

DNSH harbors hydrazide moiety that can readily react with aldehyde to give hydrazone derivatives with easily chargeable moiety. Therefore, here we chose DNSH to derivatize the oxidation products of 5-hmdC and 5-forC (i.e., 5-fodC and 5-forC). The product ion spectrum showed that *m/z* 503.3 and 387.2, which represent the parent ion of the 5-fodC derivative and its product ion, were observed after DNSH derivatization (Figure S5A). Similarly, the product ion spectrum showed that *m/z* 519.2 and 387.1, which represent the parent ion of the 5-forC derivative and its product ion, were also observed after DNSH derivatization (Figure S5B).

The results demonstrated that the expected products were obtained by DNSH derivatization.

To achieve good derivatization efficiencies of 5-fodC and 5-forC, we optimized the derivatization conditions, including reaction temperature and time, and concentration of DNSH. All the derivatization reactions were performed in 100 μL of ACN, 5 μL of HAc, 200 pmol of 5-fodC and 5-forC. As for reaction temperature, our result showed that 40°C was suitable to obtain good derivatization (Figure S2A). As for the derivatization time, the result showed that 1 h was sufficient for the derivatization reaction by DNSH (Figure S2B). Finally, we optimized the concentration of DNSH, and the result showed that 0.4 mM DNSH was sufficient for the derivatization (Figure S2C).

Taken together, the optimized derivatization conditions for 5-fodC and 5-forC by DNSH were under 40°C for 60 min with 0.4 mM DNSH. Shown in Figure S2D and S2E are the typical chromatograms of 5-fodC and 5-forC and their derivatives. Under the optimized conditions, the derivatization efficiency by DNSH can reach over 95%.

Method Validation

The calibration curves of 5-hmdC and 5-hmrC were constructed by plotting the mean peak area ratio of 5-hmdC/10^6 dG and 5-hmrC/10^6 rG versus the mean molar ratio of 5-hmdC/10^6 dG and 5-hmrC/10^6 rG based on the data obtained from triplicate measurements of the oxidation-derivatization products. The results showed that good linearities within the range of 1-1000 of 5-hmdC/10^6 dG and 0.5-500 of 5-hmrC/10^6 rG were obtained with the coefficient of determination (R^2) being great than 0.99 (Table S5). In addition, to quantify the endogenous 5-fodC and 5-forC in DNA and RNA in cells, we also constructed the calibration curve of 5-fodC and 5-forC by plotting the mean peak area ratio of 5-fodC/10^6 dG and 5-forC/10^6 rG versus the mean molar ratio of 5-fodC/10^6 dG and 5-forC/10^6 rG based on the data obtained from triplicate measurements of the derivatization products (Table S5).

The reproducibility of the developed method was evaluated by the measurement of intra- and inter-day precisions. The intra- and inter-day RSDs were calculated with different amounts of 5-hmdC and 5-hmrC spiked in nucleosides mixture. Three parallel treatments of samples over a day gave the intra-day RSDs, and the inter-day RSDs were determined by treating samples independently for three consecutive days. The results showed that the intra- and inter-day RSDs were less than 12.0% and 15.1%, respectively (Table S6), demonstrating that good reproducibility was achieved. The results also showed that good accuracy was achieved, which are manifested by the relative errors (RE) being less than 13.5% for 5-hmdC and 16.0% for 5-hmrC, respectively (Table S6). The result indicated that the ODMS method was reliable for the sensitive and simultaneous quantification of 5-hmdC and 5-hmrC in DNA and RNA.

Statistical Analysis

The statistical data were processed with SPSS 19.0 software (SPSS Inc.). The independent *t* test was perform to evaluate the differences of cytosine modifications upon ascorbic acid and hydroquinone treatment. All *p* values were two-sided, and *p* values < 0.05 were considered to have statistical significance.

Table S1. Comparison of the developed ODMS method with other methods for the detection of 5-hmdC and 5-hmrC.

Detection Methods	Samples analyzed	Analytes	LOD (fmol)	Reference	Year
LC-ESI-MS/MS	DNA from 293T cells, mouse embryonic stem cells and mouse tissues	5-hmdC	2.5	(2)	2011
LC-ESI-QTOF-MS	DNA from hepatocellular carcinoma tissues	5-hmdC	0.19	(3)	2013
SPE-LC-MS/MS	DNA from three human cell lines and seven yeast strains	5-hmdC	1.5	(4)	2013
LC-MS/MS	DNA from HEK293A cells	5-hmdC	7.8	(5)	2014
Stable isotope dilution LC-MS/MS	Human urine samples	5-hmdC	0.25	(6)	2015
ODMS	DNA from 293T cells and HeLa cells	5-hmdC 5-hmrC	0.04 0.03	Current study	2015

Table S2. Limits of detection of 5-hmdC and 5-hmrC without and with ODMS method.

	LOD (fmol)	
	5-hmdC	5-hmrC
Analysis by LC-ESI-MS/MS without ODMS	14.5	11.4
Analysis by ODMS method	0.04	0.03
Detection sensitivity increased folds	363	380

Table S3. Measured contents of 5-hmdC, 5-hmrC, 5-fodC and 5-forC in human HeLa and 293T cells.

Cells	Treatment	5-hmdC/10^6 dG	5-hmrC/10^6 rG	5-fodC/10^6 dG	5-forC/10^6 rG
HeLa cells	Control	68.4 ± 10.1	3.1 ± 0.4	4.5 ± 0.6	9.0 ± 1.2
	Ascorbic acid	165.5 ± 20.3	14.3 ± 1.5	13.8 ± 2.6	24.3 ± 3.9
	Hydroquinone	117.8 ± 15.3	7.1 ± 0.5	18.3 ± 0.4	17.7 ± 3.2
293T cells	Control	109.1 ± 14.4	6.9 ± 0.7	19.1 ± 3.3	8.5 ± 1.4
	Ascorbic acid	299.1 ± 31.1	22.6 ± 3.7	56.2 ± 10.2	22.1 ± 2.8
	Hydroquinone	145.7 ± 7.3	11.3 ± 2.0	41.1 ± 6.7	19.4 ± 1.2

Table S4. The mass transitions in the analysis of targets analytes by multiple reaction monitoring (MRM) mode.

Analytes	Mass transitions
rA	268.1 → 136.1
rU	245.1 → 113.0
rC	244.1 → 112.1
rG	284.1 → 152.1
dA	252.1 → 136.1
T	243.1 → 127.1
dC	228.1 → 112.1
dG	268.1 → 152.1
5-hmdC	258.1 →142.1
5-hmrC	274.1 → 142.1
5-fodC	256.1 → 140.0
5-forC	272.1 → 140.0
5-fodC derivative	503.2 → 387.1
5-forC derivative	519.2 → 387.1

Table S5. Linearities of 5-hmdC and 5-hmrC obtained by ODMS strategy and 5-fodC and 5-forC obtained by DNSH derivatization combined with LC-ESI-MS/MS analysis.

Compounds molar ratio	Linear range	Calibration curve data				
		Slope	Intercept	R^2 value	LOD (fmol)	LOQ (fmol)
5-hmdC/dG	$1/10^6$ - $1000/10^6$	34.90	0.000138	0.992	0.04	0.13
5-hmrC/rG	$0.5/10^6$ - $500/10^6$	9.95	0.000133	0.996	0.03	0.10
5-fodC/dG	$0.5/10^6$ - $250/10^6$	4.07	0.0000181	0.997	0.04	0.13
5-forC/rG	$0.1/10^6$ - $250/10^6$	9.91	0.0000214	0.991	0.03	0.10

Table S6. Precision (intra- and inter-day) and accuracy for the determination of 5-hmdC and 5-hmrC by ODMS method.

QC	Theoretical values	Measured values	Relative error	Intra-day (RSD%, n=3)	Inter-day (RSD%, n=3)
5-hmdC/10^5 dG	2.0 (Low)	2.2	10.5%	2.2	15.1
	20.0 (Medium)	22.7	13.5%	12.0	13.5
	100.0 (High)	91.0	-9.0%	9.4	6.6
5-hmrC/10^6 dG	2.0 (Low)	1.8	-8.0%	3.4	10.9
	20.0 (Medium)	23.2	16.0%	6.5	3.7
	100.0 (High)	92.4	-7.6%	6.2	7.2

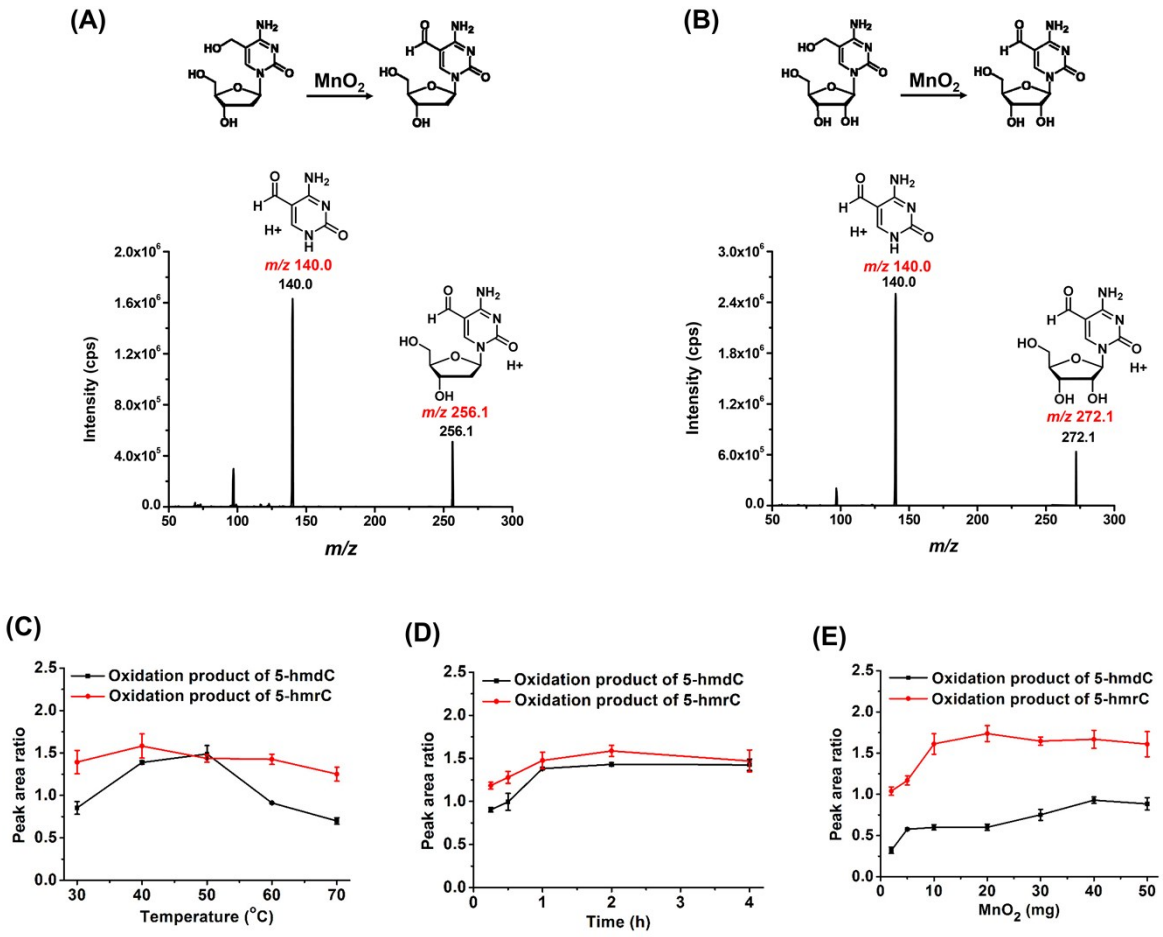

Figure S1. Optimization of the oxidation conditions for 5-hmdC and 5-hmrC by MnO_2. (A) Product ion spectrum of the oxidation product of 5-hmdC. (B) Product ion spectrum of the oxidation product of 5-hmrC. (C) Optimization of the reaction temperature. (D) Optimization of the reaction time. (E) Optimization of the amounts of MnO_2.

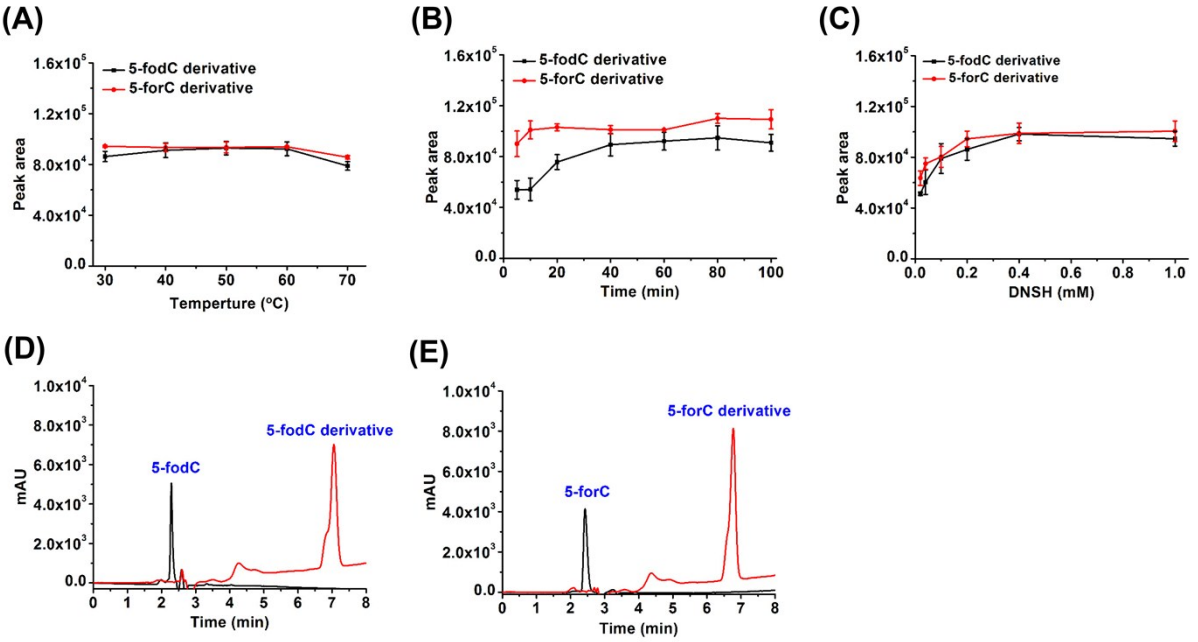

Figure S2. Optimization of the derivatization conditions for 5-fodC and 5-forC by DNSH. (A) Optimization of the reaction temperature. (B) Optimization of the reaction time. (C) Optimization of the concentrations of DNSH. (D) HPLC chromatograms of 5-fodC and 5-fodC derivative under optimized conditions. (E) HPLC chromatograms of 5-forC and 5-forC derivative under optimized conditions.

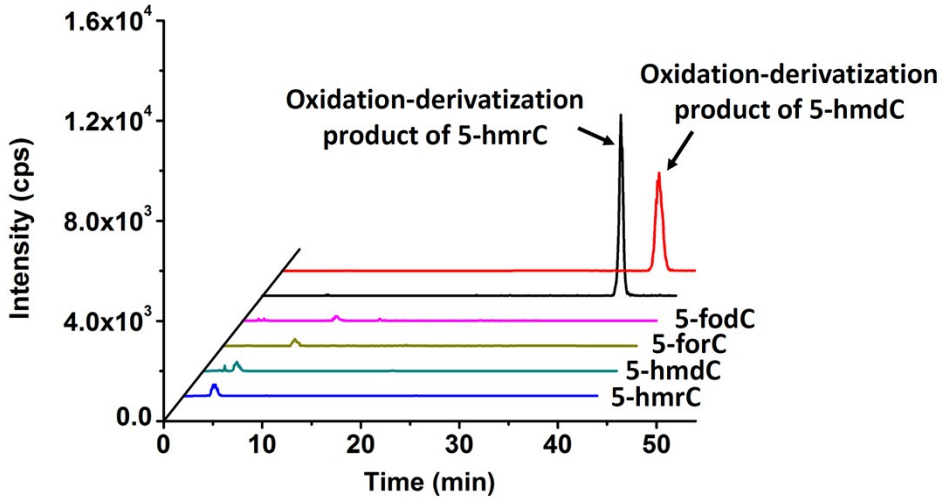

Figure S3. MRM chromatograms of 5-hmdC, 5-hmrC, 5-fodC, 5-forC and the oxidation-derivatization products of 5-hmdC and 5-hmrC.

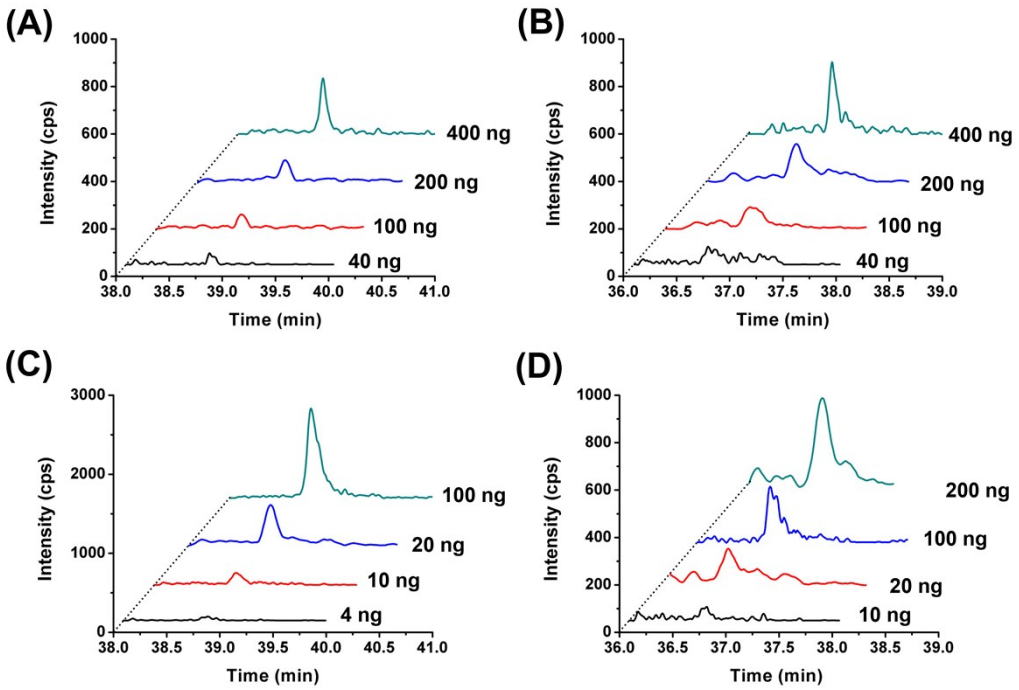

Figure S4. MRM chromatograms of modified nucleosides. (A) Detected endogenous 5-fodC using different amounts of DNA by DNSH derivatization. (B) Detected endogenous 5-forC using different amounts of RNA by DNSH derivatization. (C) Detected 5-hmdC using different amounts of DNA by ODMS method. (D) Detected 5-hmrC using different amounts of RNA by ODMS method.

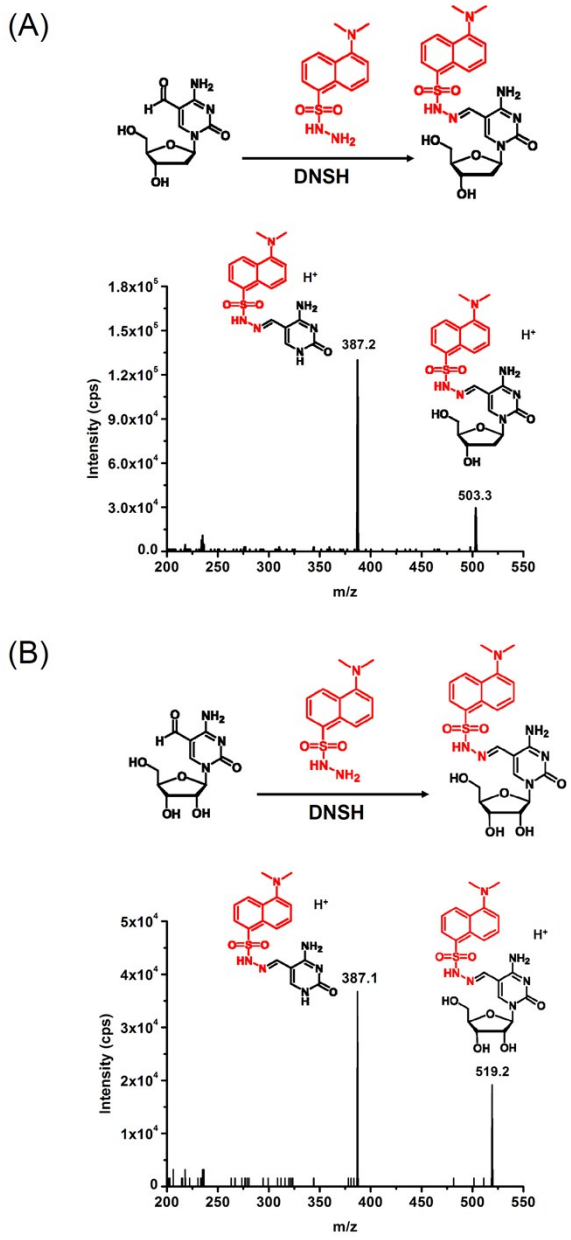

Figure S5. Derivatization of 5-fodC and 5-forC by DNSH. (A) Product ion spectrum of 5-fodC derivative. (B) Product ion spectrum of 5-forC derivative.

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