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Ultraperformance Liquid Chromatography Tandem Mass Spectrometry Assay of DNA Cytosine Methylation Excretion from Biological Systems

Jing Qu, Avinash Kumar, Yi-Ming Liu,* Oluwatoyin V. Odubanjo, Felicite K. Noubissi, Yixin Hu, and Hankun Hu*

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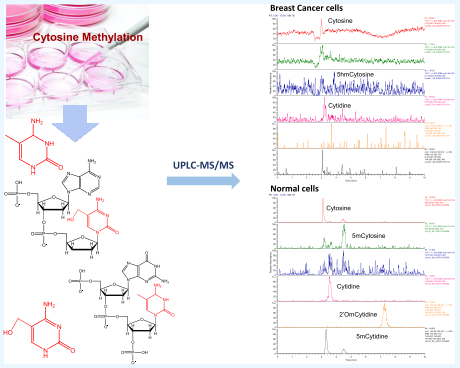
ABSTRACT: Measuring DNA cytosine methylation excretion presents challenges because methylated cytosine species are released in various forms including free molecules and those bound in DNA fragments. Herein, we report a novel UPLC-MS/MS method that allows the quantification of both free and DNA fragment-bound forms of methylated cytosine species excreted, providing total amounts for each. Cell culture medium and genomic DNA isolated from cells are analyzed to quantify methylated cytosine species. In genomic DNA isolated from MDA-MB-231 breast cancer cells, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) are detected at 5.1% and 0.07% of total cytosine residues, respectively. In the cell culture medium, only 5hmC is detected at a low level (ca. 7 nM). However, in two normal cell lines (i.e., primary mouse lung epithelial cells and HEK293 kidney cells) 5mC, 5-methylcytidine, and 2′-oxymethylcytidine (but no 5hmC) are found present in cell culture medium at concentrations ranging from 10 to 320 nM. Further, it is observed for the first time that treating MDA-MB-231 cells with carboplatin significantly increases the 5hmC level in the culture medium, indicating a carboplatin-boosted DNA cytosine methylation excretion from cancer cells.



INTRODUCTION

DNA cytosine methylation involves covalently adding a methyl group to the fifth position on the cytosine ring of CpG dinucleotides by DNA methyltransferase enzymes.[1−3] Alterations in genomic DNA methylation status are associated with various diseases, such as cardiovascular diseases,[4] neurodegenerative diseases,[5] and notably cancers.[6,7] Global DNA hypomethylation, for example, is strongly linked to various cancers as it can induce genomic instability and heighten cellular susceptibility to genetic alterations.[8−10] In addition to 5-methylcytosine (5mC), detection of its oxidized derivatives such as 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5carC) in DNA was reported.[11−13] Analytical methods based on various techniques were developed for assessing DNA methylation, including bisulfite conversion-PCR.[14−18] Recently, assays based on quantification of nucleobases using GC-MS and HPLC-MS were developed.[19−21] These instrumental techniques have been shown to be very useful for detection of modified nucleobases, nucleosides, and nucleotides in biological samples.[22−24]

Previous studies have shown that DNA demethylation can occur both actively and passively. The active mechanism involves specific enzymatic processes that remove DNA methylation marks. For example, TET enzymes (Ten-Eleven Translocation) oxidize 5-methylcytosine, and cells package DNA fragments along with other molecules into extracellular vesicles or exosomes. The passive mechanism is through a gradual loss of DNA methylation marks over time. This process mainly occurs during DNA replication when methylated or modified marks are not fully restored to the newly synthesized DNA strand, for example, if DNA methyltransferases are inactive or absent during replication. Together, passive and active release mechanisms ensure that cells can dynamically and adaptively regulate gene expression through the controlled loss of the marks.[25−27] Detection of excreted methylated cytosine species in biofluids may make them valuable for noninvasive diagnostic and treatment evaluation purposes. In this work, a UPLC-MS/MS method was developed for sensitive quantification of methylated cytosine species. Since it has a unique capability to quantify both free and DNA fragment-bound forms of methylated cytosine species, it is expected to be particularly useful for

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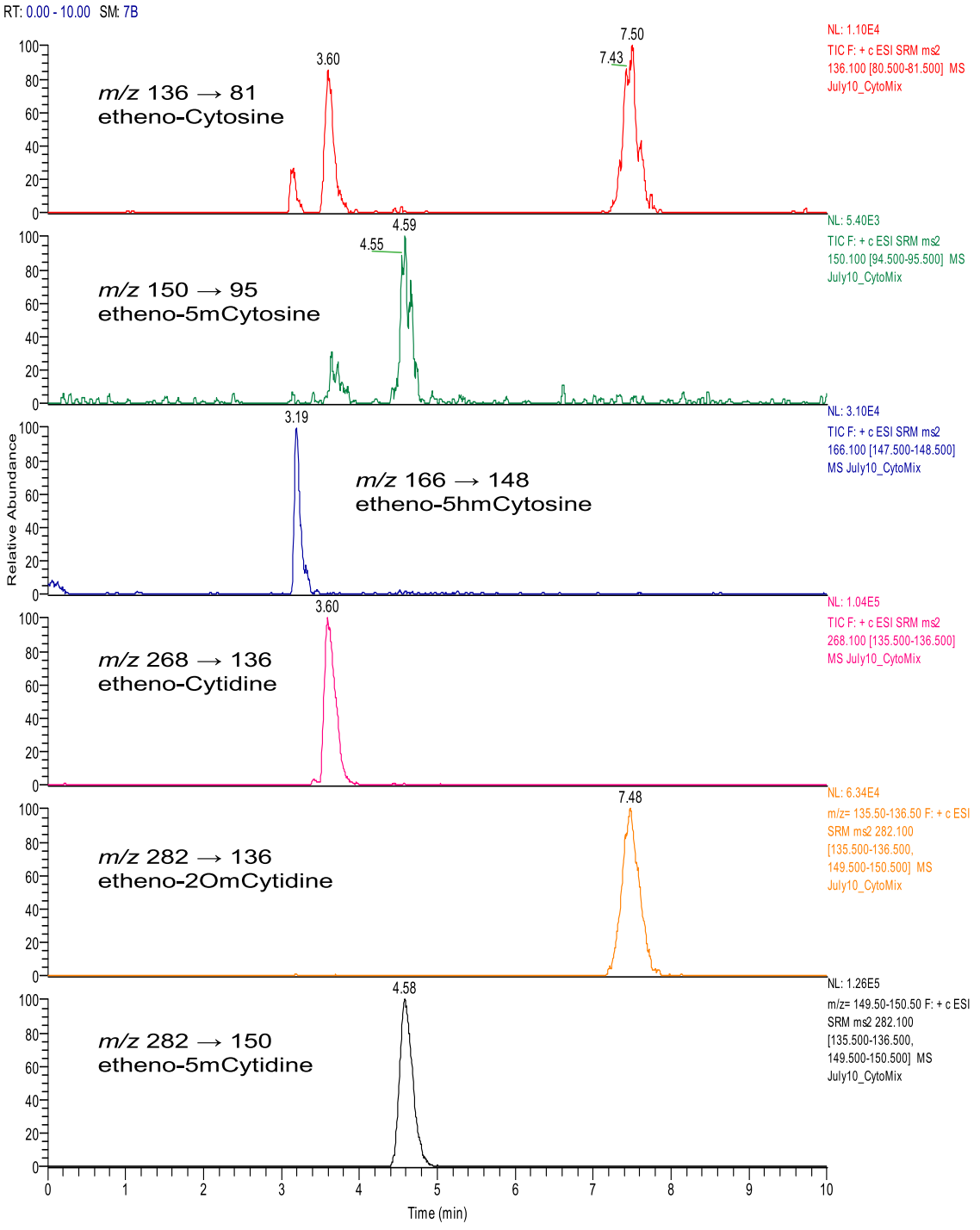

Figure 1. Chromatograms from separating a standard mixture of etheno-cytosine derivatives.

studying the excretion of DNA cytosine methylation from biological systems.

MATERIALS AND METHODS

Online SPE-UPLC-MS/MS Analysis. The analysis was conducted using a Shimadzu LC system equipped with an LC-40B X3 binary pump and a SIL-40CXS autosampler (Shimadzu, Kyoto, Japan). Sample injection volume was 10 μL. For online solid-phase extraction (SPE), a C_{18} guard column cartridge (5 × 2.1 mm, 2.7 μm, Restek) was employed. LC separation was performed on a biphenyl column (50 mm × 2.1 mm, 1.8 μm, Restek Force) with a mobile phase of 15% methanol in water containing 0.1% formic acid. Mass spectrometry was carried out with a TSQ Quantum triple quadrupole mass spectrometer (ThermoFinnigan) equipped with a heated electrospray ionization probe (HESI-II). MS data acquisition and processing were handled by using Xcalibur software (ThermoFinnigan). The detection conditions were optimized for positive mode with the following parameters: ESI voltage was set to +3.4 kV, vaporizer temperature was maintained at 320 °C, sheath gas pressure was set to 30 psi, ion sweep gas pressure was set to 0 psi, aux gas pressure was set to 10 psi, and capillary temperature was maintained at 350 °C.

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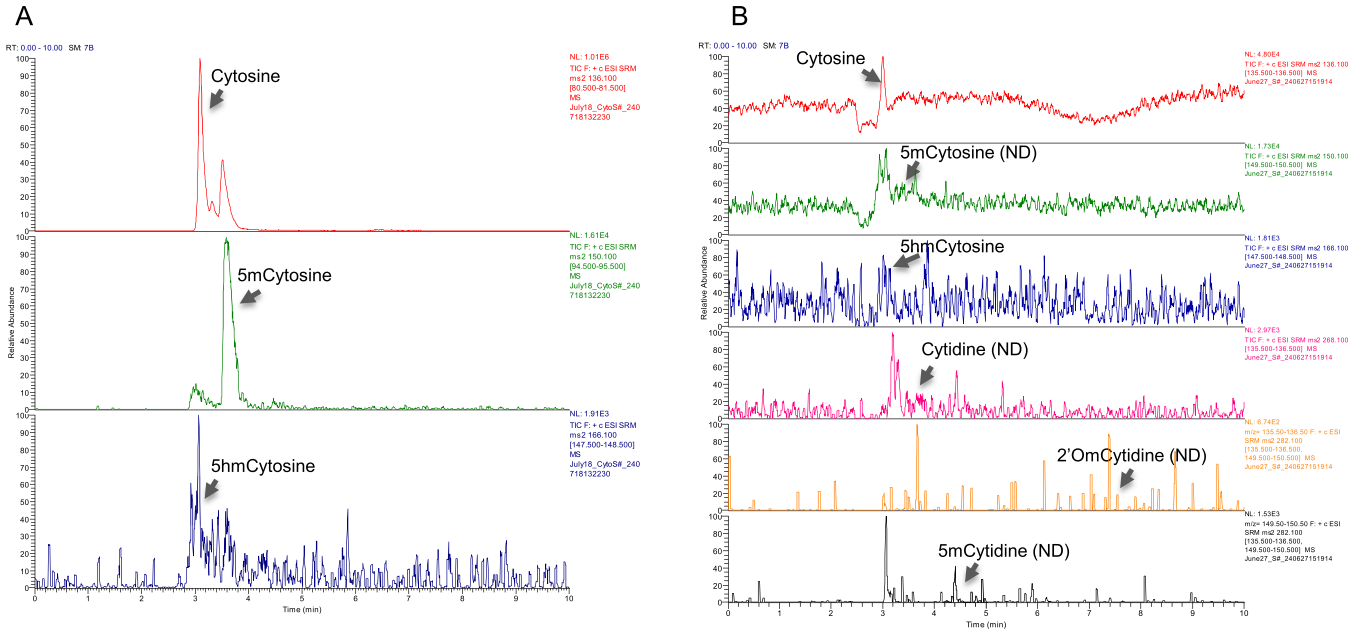

Figure 2. Chromatograms obtained from analysis of a genomic DNA sample (A) and a culture medium sample (B) from MDA-MB-231 cells ("ND" stands for "not detected").

The relative collision energy was set to 20%, and the isolation width was 0.7 m/z.

Online SPE was performed by loading and washing the sample with a mobile phase of water at a flow rate of 0.12 mL/min. Isocratic elution was carried out with a mobile phase of 15% methanol in water containing 0.1% formic acid at a flow rate of 0.28 mL/min. In each run, sample injection and SPE were performed on the precolumn in the first 2 min followed by an 8 min isocratic elution. Transition from online SPE to UPLC separation was easily realized by turning the 6-port valve attached to the mass spectrometer from position "Load" to "Injection" 2 min after the run started, which was set up by Xcalibur software.

RESULTS AND DISCUSSION

Online SPE-UPLC-MS/MS Analysis of Etheno-Cytosine Derivatives. Chloroacetaldehyde (CAA) is known for long to bind to nucleobases in DNA, forming etheno derivatives and causing depurination and depyrimidination.[28−30] In our study, it was noted that etheno-cytosine was produced from CAA-DNA reaction, while etheno-cytidine was produced from CAA-RNA reaction. This is likely because the 2′- hydroxyl group in ribonucleosides plays a role in preventing them from undergoing a glycosidic bond cleavage when reacting with CAA. These etheno-derivatives can be conveniently distinguished by mass spectrometry. The UPLC-MS/MS assay proposed in this study employs CAA-based precolumn derivatization with two primary objectives. First, CAA derivatization converts both free and DNA-bound nucleobases into their corresponding etheno derivatives. Second, the tagging-induced increase in structural aromaticity significantly enhances the separability of these etheno derivatives on reversed-phase columns, while also enabling efficient and cost-effective recovery through online SPE. To achieve MS/MS detection of etheno-derivatives, etheno- cytosine (C), 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), cytidine, 2′-*O*-methylcytidine (2'OmCytid), and 5-methylcytidine (5mCytidine) were prepared from standard solutions treated with CAA. MS^2 spectra of the etheno-derivatives were acquired by using the UPLC-MS/MS technique. Figure S1 shows the MS spectral results along with the respective chemical structures. As the results indicate, the chemical structure of each of the etheno-derivatives tested is consistent with the respective MS^2 spectrum. It is worth noting that due to a rigid and stable chemical structure, fragmentation of these etheno-derivatives in MS/MS analysis is limited, resulting in a single product ion that is very helpful for achieving a good MS/MS detection sensitivity. Based on these results, ion transitions: m/z 136.2 → 81 for C, m/z 150.2 → 95 for 5mC, m/z 166.1 → 148.21 for 5hmC, m/z 268.2 → 136 for Cytid, m/z 282.2 → 136 for 2'OmCytid, and m/z 282.2 → 150 for 5mCytid were selected for MRM detection.

Liquid chromatographic separation of etheno-cytosine derivatives was investigated with various mobile phases and columns, including C_{18}, phenyl, and biphenyl columns. The best separation was achieved using a methanol/water mobile phase on a biphenyl reversed phase column. As compared on a C_{18} column, all the test compounds showed much longer retention times likely because of the strong conjugated $\pi-\pi$ interaction between etheno-derivative molecules and the biphenyl moiety in the stationary phase. Figure 1 shows the ion extracted chromatograms obtained from separating a mixture of etheno-derivatives. As shown, the retention times are 3.07 min for etheno-cytosine, 3.26 min for etheno-5mC, 3.19 min for etheno-5hmC, 3.60 min for etheno-cytidine, 7.48 min for etheno-2'OmCytidine, and 4.58 min for etheno-5mCytidine.

Under the conditions selected, analytical figures of merit for the online SPE-UPLC-MS/MS method were studied by performing simultaneous quantifications of C, 5mC, 5hmC, Cytid, 5mCytid, and 2'OmCytid. Five-point calibration curves were prepared with CAA-standard solutions at concentrations ranging from 0.010 to 1.50 μM in 1x PBS. Triplicate analyses were carried out for each solution. Peak heights were used for the calculation. Linear regression analysis on peak height/analyte concentration data yielded linear calibration curves for

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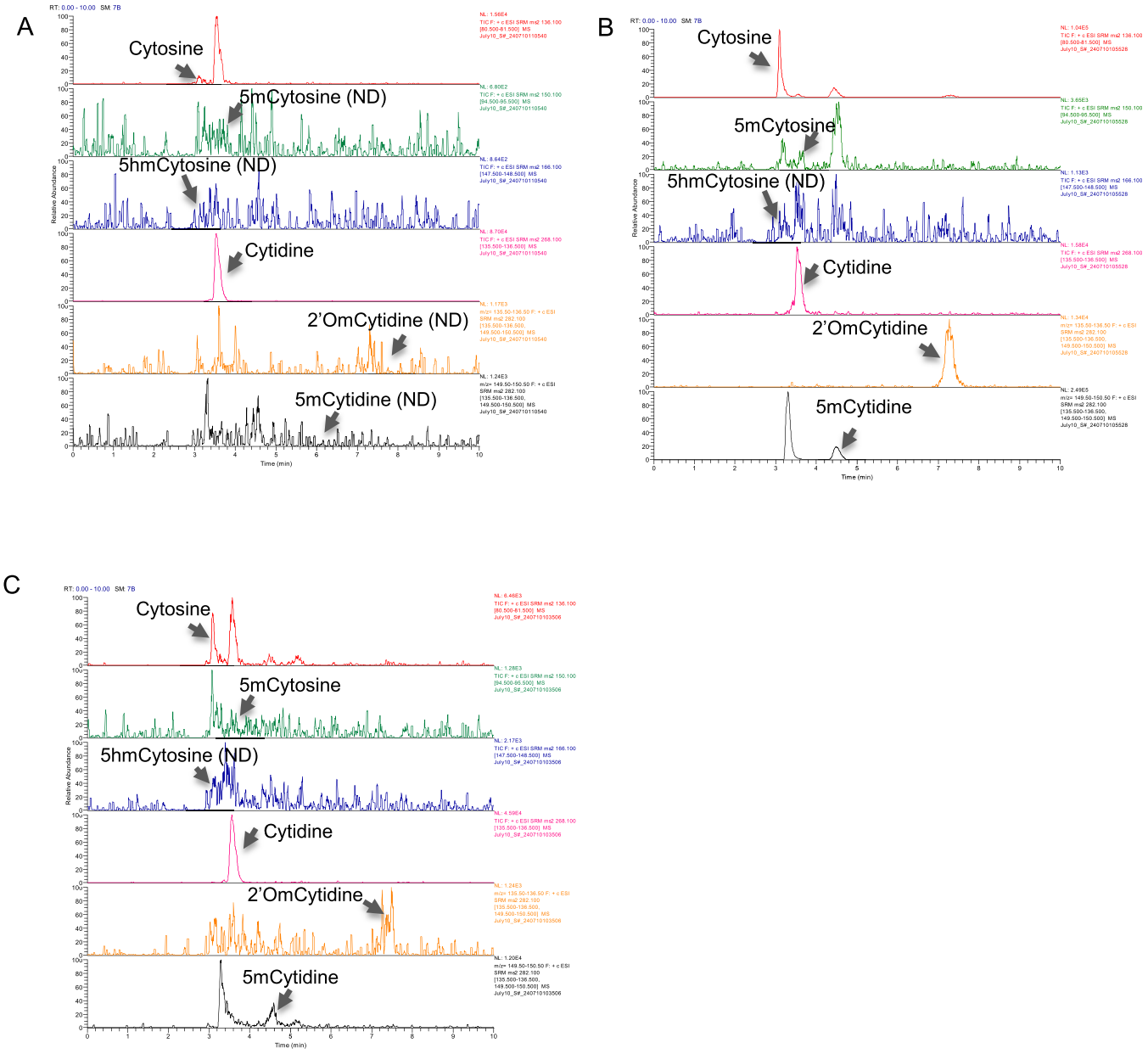

Figure 3. Typical chromatograms obtained from analysis of a neat RPMI medium sample (A), HEK293 cell culture medium sample (B), and PMLEC cell culture medium sample (C). ("ND" stands for "not detected").

the compounds tested. Taking 5mCytosine as an example, the linear regression equation is $Y = 76.70X + 2.46$ with $R^2 = 0.995$ was obtained, where X was the analyte concentration in μM and Y was peak height after being reduced by a factor of $1 \times e$.[4] Limits of detection were estimated to be in the range from 1.3 nM for C to 3.3 nM for 2'OmCytid (signal/noise = 3). To assess the assay repeatability, two standard mixtures of the six ethanol-derivatives (at 15.0 nM each and 0.500 μM each, respectively) were analyzed 5 times each. Repeatability (RSD) of peak height was calculated to be in the range from 0.37% (for etheno-cytidine at 0.500 μM) to 4.2% (for 2'OmCytid at 15.0 nM). The above results indicate that the online SPE-UPLC-MS/MS method is very sensitive and offers good assay repeatability. In addition, it is easy to carry out and cost-effective.

Quantitative Analysis of Cytosine Methylation in Cellular Models. Although cytosine methylation in DNA has been extensively studied, study of excretion of the epigenetic marks from biological systems has been very limited so far. Established cellular models have been utilized as dependable and versatile biological systems for biomedical research. However, to the best of our knowledge, there have been no reports on measuring cytosine methylation in a cell culture medium, which is an effective way to reveal the secretion profiles of DNA epigenetic marks from cells. This may be because the most popular qPCR-based assays are not useful for quantification of free or DNA fragment-bond-methylated cytosines excreted from cells. In this work, comparatively studying cytosine methylation across normal and cancer cell lines (i.e., HEK293 embryonic kidney cells, primary mouse lung epithelial cells, and MDA-MB-231 breast cancer cells) was carried out. Cell culture medium samples were collected and analyzed to determine methylated cytosine species by using the proposed online SPE-UPLC-MS/MS method. Figure 2A

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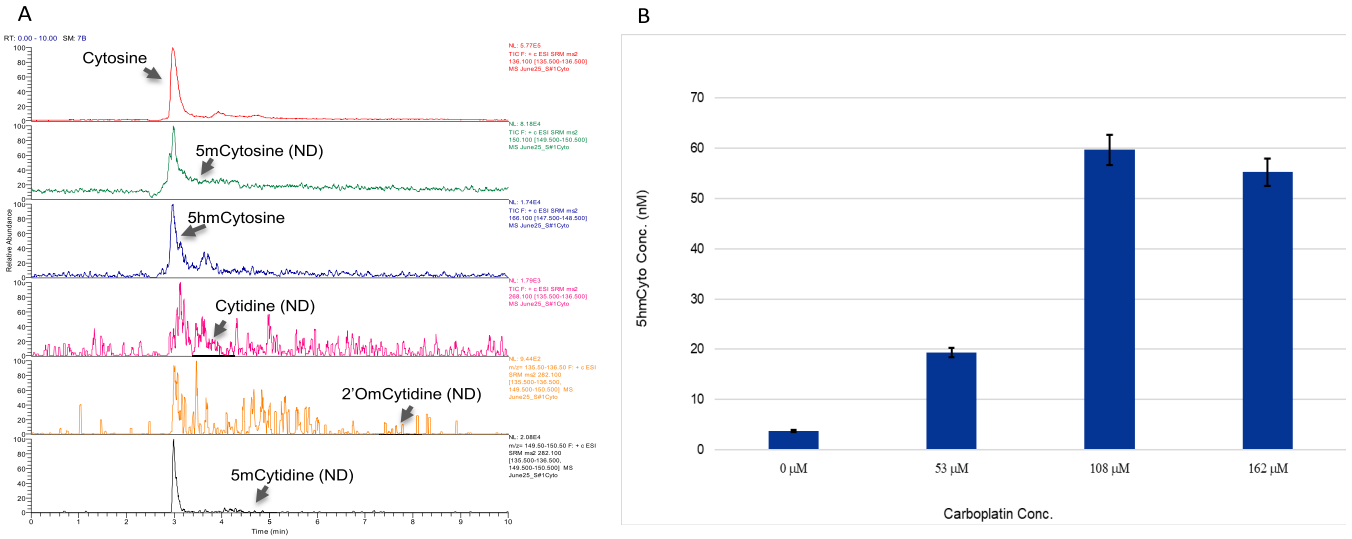

Figure 4. (A) Ion extracted chromatograms from analysis of a culture medium sample and (B) 5hmC concentrations found in the culture medium of MDA-MB-231 cells treated with carboplatin at different doses. ("ND" stands for "not detected".)

shows a typical chromatogram obtained from the analysis of genomic DNA isolated from MDA-MB-231 breast cancer cells. Two epigenetic marks, i.e., 5mC and 5hmC, were detected at 5.1% and 0.07% of total cytosine residues, respectively. These results are in consistency with those found in the literature, showing that 5mC constitutes about 2−6% of total cytosine residues and 5hmC represents approximately 0.1% of total cytosine residues in DNA.[31−34] In the culture medium collected from MDA-MB-231 breast cancer cell culture, C and 5hmC were detected as shown in Figure 2B. It is worth noting that 5mC was not detected. 5hmC was found to be at a low level (∼ 7 nM). Previous studies have shown that 5hmC is a major oxidation product of 5mC, mediating DNA demethylation.[35,36] Based on these results, the excretion of epigenetic marks from the cancer cells tested is limited. In the search for support for this conclusion, cell culture medium samples collected from two normal cell lines were analyzed to quantify methylated cytosine species.

Figure 3 shows typical chromatograms obtained from the analysis of culture medium samples collected from HEK293 embryonic kidney cells and primary mouse lung epithelial cells. C and Cytid were detected at 0.67 ± 0.08 and 0.33 ± 0.04 μM (n = 3), respectively, in neat RPMI medium that was used for culturing HEK293 kidney cells. As can be seen by comparing the results from medium control (Figure 3A) and those from cell culture medium (Figure 3B), three epigenetic marks, i.e., 5mC, 2'OmCytid, and 5mCytid, were detected in the cell culture medium.

Their concentrations in the medium were found to be 0.32 ± 0.05, 0.08 ± 0.01, and 0.16 ± 0.03 μM (n = 3). A similar profile of cytosine methylation was observed in the culture medium collected from primary mouse lung epithelial cells (Figure 3C). 5mC, 2'OmCytid, and 5mCytid were detected at 0.007 ± 0.005, 0.008 ± 0.003, and 0.01 ± 0.003 μM (n = 3). It was noted that 5hmC was detected in the culture medium of MDA-MB-231 breast cancer cells but not in the culture medium samples collected from HEK293 kidney cells and primary mouse lung epithelial cells. From the perspective of cellular biological characteristics, both HEK293 kidney and primary mouse lung epithelial cells are identified as normal cells. These results suggest that MDA-MB-231 breast cancer cells exhibit metabolic abnormalities compared with normal cells. These abnormalities could be attributed to the unique metabolic pathways or regulatory mechanisms active in cancer cells.[37−39] Moreover, the cancer cells release 5hmC into the culture medium, but the normal cells do not. Previous studies showed that the active DNA demethylation process may be linked to aberrant methylation and involved in cancer development. Loss of 5-hmC in the epigenome is an epigenetic hallmark of certain cancers.[40,41] Excretion of methylated cytosines from biological systems can be through active secretion mechanisms. For example, methylated cytosines within DNA fragments are encapsulated in exosomes and released into extracellular space.[25−27,42,43] It is worth mentioning that the 5hmC amount measured by the UPLC-MS/MS assay in this work is the total 5hmC amount, including both free and DNA fragment-bound 5hmC. This is because the sample treatment with CAA-PBS causes a complete depyrimidination from DNAs or oligodeoxynucleotides. This is a significant advantage of the UPLC-MS/MS assay proposed herein. Quantification of both free and DNA fragment-bound methylated cytosines at the same time is critical in studying the excretion of methylated cytosines from biological systems.

After observing 5hmC secretion from MDA-MB-231 cells, we further studied the implications of drug treatments on the secretion. MDA-MB-231 cells were treated with carboplatin, a chemotherapy drug used for human breast cancer treatment[44] at 53, 108, and 162 μM. Culture medium samples were collected on the fifth day of drug exposure. Cell viability was checked at the time of medium sample collection and found to be >86% in all cases. Figure 4A shows the chromatograms from analysis of a cell culture medium samples. As shown, treatment with carboplatin significantly increased the 5hmC concentration in the culture medium (for comparison, see the results obtained from untreated cells shown in Figure 2B). Further, it was found that the 5hmC level in the culture medium increased with increasing carboplatin concentration. Figure 4B illustrates the dose dependence. 5hmC concentration in the medium increases from ∼7 nM in control to >55 nM with carboplatin at 108 μM or above. It is evidenced herein for the first time that carboplatin treatment boosts 5hmC secretion from MDA-MB-231 breast cancer cells. These results indicate

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that 5hmC may serve as signaling molecules or biomarkers of decease progression and treatment evaluation.[45,46]

CONCLUSIONS

A UPLC-MS/MS assay is proposed for quantitatively assessing the excretion of DNA cytosine methylation from cellular models. The proposed assay has a unique capability to quantify both free and DNA fragment-bound forms of methylated cytosines, providing the total amounts for each. By using the UPLC-MS/MS assay, cytosine methylation excretion in three cell lines, including one breast cancer and two normal cell lines, was studied for the first time. A distinct excretion profile was observed for MDA-MB-231 breast cancer cells. Three methylated cytosine species (i.e., 5mC, 2'OmCytid, and 5mCytid) were detected in the culture medium of normal cells (i.e., HEK293 kidney cells and primary mouse lung endothelial cells), while only 5hmC was detected in the culture medium of the breast cancer cells. These results suggest that the cancer cells exhibit a metabolic abnormality, implicating cytosine methylation. Also interestingly, our study found that treating the cancer cells with carboplatin (a chemotherapeutic agent) substantially boosted 5hmC excretion. Whether passively excreted or actively secreted from the cells, 5hmC may serve as a biomarker of disease progression and treatment evaluation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c11277.

The Supporting Information is available free of charge on the ACS Publications Web site at DOI: Materials; Cellular sample preparation; MS^2 spectrum of CAA derivatives of methylated cytosines (PDF)

AUTHOR INFORMATION

Corresponding Authors

Yi-Ming Liu − *Jackson State University, Department of Chemistry, Physics and Atmospheric Science, Jackson, Mississippi 39217, United States;* orcid.org/0000-0002-7760-9565; Phone: +1-601-9793491; Email: yiming.liu@jsums.edu

Hankun Hu − *Department of Pharmacy, Zhongnan Hospital of Wuhan University, School of Pharmaceutical Sciences, Wuhan University, Wuhan, Hubei 430071, China*; Phone: +86-13971431601; Email: hankunhu@whu.edu.cn

Authors

Jing Qu − *Jackson State University, Department of Chemistry, Physics and Atmospheric Science, Jackson, Mississippi 39217, United States*

Avinash Kumar − *Jackson State University, Department of Chemistry, Physics and Atmospheric Science, Jackson, Mississippi 39217, United States*

Oluwatoyin V. Odubanjo − *Department of Biology, Jackson State University, Jackson, Mississippi 39217, United States*

Felicite K. Noubissi − *Department of Biology, Jackson State University, Jackson, Mississippi 39217, United States*

Yixin Hu − *Department of Pharmacy, Zhongnan Hospital of Wuhan University, School of Pharmaceutical Sciences, Wuhan University, Wuhan, Hubei 430071, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c11277

Notes

The authors declare no competing financial interest.

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