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Simultaneous Quantification of Nucleosides and Nucleotides from Biological Samples

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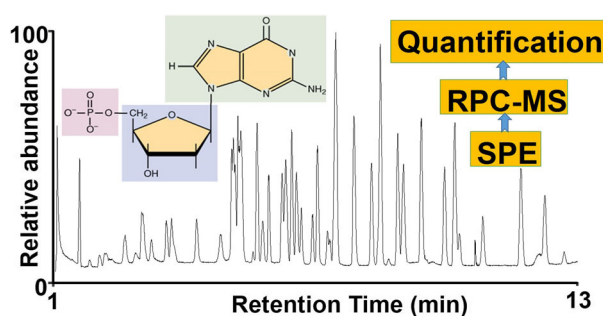
Abstract. We report a reverse phase chromatography mass spectrometry (LC-MS) method for simultaneous quantification of nucleosides and nucleotides from biological samples, where compound identification was achieved by a tier-wise approach and compound quantification was achieved via external calibration. A total of 65 authentic standards of nucleosides and nucleotides were used for the platform development. The limit of detection (LOD) of those compounds ranged from 0.05 nmol/L to 1.25 μmol/L, and their limit of quantification (LOQ) ranged from 0.10 nmol/L to 2.50 μmol/L. Using the developed method, nucleosides and nucleotides from human plasma, human urine, and rat liver were quantified. Seventy-nine nucleosides and nucleotides were identified from human urine and 28 of them were quantified with concentrations of 13.0 nmol/L–151 μmol/L. Fifty-five nucleosides and nucleotides were identified from human plasma and 22 of them were quantified with concentrations of 1.21 nmol/L–8.54 μmol/L. Fifty-one nucleosides and nucleotides were identified from rat liver and 23 were quantified with concentrations of 1.03 nmol/L–31.7 μmol/L. These results demonstrate that the developed method can be used to investigate the concentration change of nucleosides and nucleotides in biological samples for the purposes of biomarker discovery or elucidation of disease mechanisms.

Keywords: Quantification, Nucleosides, Nucleotides, LC-MS

Introduction

Nucleosides and nucleotides are two groups of molecules in an organism with important biological functions. A nucleoside consists of a nitrogenous base (e.g., adenine, cytosine, guanine, thymine, or uracil) covalently attached to a deoxyribose or ribose moiety without a phosphate group. A nucleotide consists of a nitrogenous base, a deoxyribose, or ribose moiety, and one to three phosphate groups. Nucleosides and

nucleotides are interchangeable in a biological system. Nucleosides can be converted into nucleotides by specific kinases through phosphorylation, while nucleotides can be broken down into nucleosides and phosphates by hydrolytic enzymes called nucleotidases.

Nucleosides participate in many biochemical reactions in a biological system, including acetylation, cyclization, deamination, hydroxylation, isomerization, methylation, selenylation, and reduction, as well as conjugating with sugars and amino acids [1]. Ribonucleosides are the primary composition of ribonucleic acids (RNAs). In the nucleolus or mitochondria, an RNA can be modified by nuclear-encoded RNA-modifying enzymes [2]. Some modifications are required for RNAs to function properly, and the absence of those modifications can cause pathological consequences [3]. On the other hand, some RNA modifications can affect RNA activity, localization, stability, and even contribute to human disease development/progression [4]. In the cytoplasm, modified RNAs can be degraded into unmodified nucleosides and modified nucleosides. The unmodified nucleosides (i.e., adenosine, cytidine, guanosine, and uridine) can be either excreted or recycled into the nucleus. The modified nucleosides are excreted into urine as metabolic end products. Nucleosides are natural metabolic activators and contribute to the regulation of some cellular events [5–7]. The abundance levels of nucleosides in urine are considered as biomarkers for the whole-body turnover of RNAs [8, 9]. Previous studies also showed that the abundance levels of nucleosides are positively correlated with cancer status [10–12]. Profiling of urinary ribonucleosides demonstrated that ribonucleosides can potentially be used as clinical biomarkers for cancers and other diseases [8, 12–15].

Nucleotides play a vital role in cellular metabolism. For instance, nucleoside triphosphates, including adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP), provide chemical energy in metabolism. Nucleotides such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) contribute to cell signaling as cofactors of enzymatic reactions. In addition to functioning as an energy driver for anabolic metabolism, ATP could also function as an inhibitor of arylamine N-acetyltransferase 1 activity by post-transcriptionally regulating the acetylation of Lys100 to affect multiple intracellular pathways [16]. ATP release is required for toll-like receptor-induced monocyte/macrophage activation, inflammasome signaling, interleukin-1β production, and the host immune response to infection [3]. Moreover, ATP, NAD+, and nucleotide hexoses are respectively substrates for protein post-translational modifications. Nucleotides are the building blocks of nucleic acid polymers deoxyribonucleic acids (DNAs) and RNAs.

Liquid chromatography (LC) with ultraviolet detection has been used to analyze nucleosides and nucleotides [4, 5]. Recently, nucleosides and nucleotides were analyzed by liquid chromatography mass spectrometry (LC-MS) or capillary electrophoresis mass spectrometry (CE-MS) [6–12]. Sakaguchi et al. compared the separation of nucleosides on a hydrophilic interaction chromatography (HILIC) column and a reverse phase chromatography (RPC) column, and showed that HILIC can profile modified nucleosides [13]. HILIC or RPC coupled with triple-quadrupole mass spectrometry was used for the simultaneous analysis of nucleosides and nucleotides [14, 15]. According to a study by Neubauer et al., RPC could cover more compounds for nucleotide, nucleoside, and nucleobase detection [16]. A comprehensive two-dimensional liquid chromatography mass spectrometry (2DLC-MS) method was used to analyze modified nucleosides from biological samples [17].

While many methods have been reported for analysis of nucleosides and/or nucleotides, compound coverage in those methods was very low, e.g., less than 10 nucleosides and/or nucleotides [6, 8, 12]. Moreover, Sakaguchi and coworkers identified 34 nucleosides by HILIC-MS, but those compounds were identified only by their m/z and retention time [13] that might have a high rate of false identifications in analysis of biological samples. Low sensitivity is another important factor in the analysis of nucleosides and nucleotides. The LOD was 5–10 μg/mL in Inoue's study [12] and 0.7 μg/mL in Gill's study [15]. Though Neubauer et al. lowered the LOD to 0.3–38 nmol/L, they only investigated 18 nucleosides and nucleotides [16].

The objective of this work was to develop a LC-MS method to simultaneously quantify nucleosides and nucleotides from biological samples with increased molecular coverage and high sensitivity for biomedical studies. We developed a LC-MS method for simultaneous quantification of nucleosides and nucleotides via external calibration. A tier-wise compound identification method was also developed for high confidence of nucleoside and nucleotide identification. The developed method was validated by quantifying nucleosides and nucleotides from human plasma, human urine, and rat liver.

Materials and Methods

Chemicals and Reagents

Authentic standards of 50 nucleosides and 15 nucleotides were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), Carbosynth (San Diego, CA, USA), Santa Cruz Biotechnology (Dallas, TX, USA), and Cayman Chemical (Ann Arbor, MI, USA). Methanol (LC grade) and formic acid were purchased from Sigma-Aldrich Corp. Acetonitrile (LC grade) was purchased from Thermo Fisher Scientific International, Inc. (Pittsburgh, PA, USA).

Preparation of Standard Solutions and Calibration Curves

Stock solutions of the nucleoside and nucleotide standards were prepared at concentrations of 5–100 mmol/L in water or dimethyl sulfoxide (DMSO), depending on the solubility of the compounds. The stock solutions were kept in the dark at − 80 °C until use. A total of 19 calibration solutions were prepared using the stock solutions. The concentrations of all compounds

in a calibration solution were identical with the following values: 0.049, 0.098, 0.12, 0.24, 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5, 625, 1250, 2500, 5000, 10,000, 20,000, 40,000, and 80,000 nmol/L. All calibration solutions were prepared in diH$_2$O prepared from a Millipore synergy system (Burlington, MA, USA).

Biological Samples

Male weanling Sprague-Dawley rats ($n = 6$) were purchased from Envigo (Indianapolis, IN, USA) and group housed in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle. Animals had free access to rodent chow diet (LabDiet, Cat no. 5010) and tap water. The procedures of animal care were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. Liver samples were collected under anesthesia with ketamine/xylazine (100/10 mg/kg i.p.) after the rats were fed for 9 weeks. All samples were snap frozen in liquid nitrogen and stored in − 80 °C until use.

For human samples, peripheral blood (60 mL) from healthy human donors was collected into multiple 4.5-mL Vacutainer Buffered Sodium Citrate Blood Collection Tubes (Becton Dickinson, Cat no. 369714). Blood was diluted 2.5 times with phosphate-buffered saline (Corning, Cat no. 21-040-CM), carefully layered onto Ficoll Paque PLUS (GE Healthcare, Cat no. 17-1440-02) and centrifuged at 1600 rpm for 40 min at room temperature. The plasma (top layer) was transferred into fresh tubes. Six plasma samples ($n = 6$) were used in this study. Spontaneous morning urine samples ($n = 5$) were collected from another group of 25–40-year-old male volunteers. After sample collection, all samples were immediately stored in − 80 °C until use. All samples were collected after informed consent was obtained, and all procedures were approved by the University of Louisville IRB.

Nucleoside and Nucleotide Extraction and Purification

Rat liver samples were thawed at room temperature. About 10 mg of liver was weighed and ground in a glass vial after adding water at a ratio of 1:10 (mg liver:mL solution). One hundred microliters of homogenized liver was transferred to a 2-mL tube. After adjusting the pH of the sample to 8.5 using ammonium in methanol, methanol was added at the ratio of 1:4 (v:v). The mixture was centrifuged at 14,000 rpm for 20 min at 4 °C after being vortexed for 3 min. The supernatant was collected and evaporated under a nitrogen gas stream. Each dried sample was then dissolved in 100 µL water.

To process the human plasma samples, each sample was thawed at room temperature. Five hundred microliters of plasma was transferred to a 5-mL tube. The pH adjustment and methanol-water extraction method were identical to those used in processing rat liver samples. Human urine samples were processed without methanol-water extraction, i.e., each sample was centrifuged at 14,000 rpm for 20 min at 4 °C immediately after adjusting the pH to 8.5 using ammonium in methanol. Supernatant was collected for purifying nucleosides and nucleotides.

To purify extracted nucleosides and nucleotides, each sample was loaded onto an OASIS HLB cartridge (Waters Corp., Milford, MA, USA) that was previously activated for three times with water and methanol in the order of water-methanol-water. After loading the sample, the cis-diol compounds were eluted with 100 µL 2.8% ammonium hydroxide (NH$_4$OH) in methanol for three times. The eluate was dried under nitrogen flow. The residue was reconstructed in 100 µL water and centrifuged at 14,000 rpm for 10 min at 4 °C. The upper clear solution was transferred to a LC vial for LC-MS analysis.

A pooled sample was also prepared for each type of biological sample by mixing a small portion of the upper clear solution from each sample. All samples were processed only once by solid phase extraction.

LC-MS/MS Analysis

All samples were analyzed on a Thermo DIONEX UltiMate 3000 HPLC system hyphenated with a Thermo Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The UltiMate 3000 HPLC system was equipped with an ACQUITY UPLC HSS T3 column (150 × 2.1 mm i.d., 1.8 µm) purchased from Waters Corp. (Milford, MA, USA). The temperature of the column was set as 40 °C. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 60% acetonitrile with 0.1% formic acid. The gradient was as follows: 0 min, 0% B; 0 to 2.5 min, 4.8% B; 2.5 to 20 min, 4.8 to 30% B; 20 to 28 min, 30 to 50% B; 28 to 30 min, 50 to 100% B; 30 to 34 min, 100% B; 34 to 34.1 min, 100 to 0% B. The flow rate was 0.2 mL/min.

The mass spectrometry parameters were as follows: electrospray ionization probe was fixed at level C, auxiliary gas 15 arbitrary units, auxiliary gas heater temperature 450 °C, capillary temperature 320 °C, sheath gas 55 arbitrary units, spray voltage 3.5 kV, sweep gas 3 arbitrary units, S-lens RF level 65.0, full scan range 140 to 1300 (m/z), resolution 30,000, maximum injection time 50 ms, and automatic gain control (AGC) 10^6 ions.

Each biological sample was analyzed by LC-MS in positive mode to obtain the full MS data of each compound. The pooled sample was analyzed after analysis every five or six biological samples, and the full MS data of each pooled sample were used for quality control and quality assessment of the LC-MS system. Each pooled sample was also analyzed by LC-MS/MS in positive mode at different collision energies (i.e., 10, 20, 40, and 60 eV) to acquire MS/MS spectra for compound identification. All samples were analyzed in a random order to avoid systems bias.

Data Analysis

After LC-MS data acquisition, all LC-MS data were converted into mzXML format. MetSign software was used for spectrum

deconvolution and cross-sample peak list alignment [18–20]. An in-house database containing parent ion m/z, retention time, and/or MS/MS spectra of each nucleoside or nucleotide was used for compound identification with thresholds of m/z variation ≤ 5 ppm, retention time difference < 0.1 min, and MS/MS spectral similarity score ≥ 0.4.

To calculate the MS/MS spectrum similarity score between a query MS/MS spectrum S_q and an MS/MS spectrum S_{db} in the in-house database, spatial clustering of applications with noise (DBSCAN) [21] was used to cluster the m/z values of S_q and S_{db} so that the same fragment ions from the two MS/MS spectra can be paired without a user-defined m/z variation window [19]. To do this, all m/z values in S_q and S_{db} were merged into a vector X. DBSCAN then clustered those merged m/z values using two parameters, P_{min} and E_{ps}, based on the density distribution of those m/z values. P_{min} is the minimum number of m/z values in a cluster C. E_{ps} is the maximum distance between two closest m/z values in a cluster C, and it was estimated as follows [22]:

$$E_{ps} = \left(\frac{P_{min} * (X_{max} - X_{min}) * \Gamma(0.5 * d + 1)}{N * \sqrt{\pi^d}} \right)^{\frac{1}{d}} \quad (1)$$

where X_{max} and X_{min} are the maximum and the minimum of m/z values in X, Γ is the gamma function, N is the size of X, and d is the dimensionality of X. In this study, the distance between two m/z values l and m was measured as $E_{lm} = |(m/z)_l - (m/z)_m|$. We also set $P_{min} = 1$ and $d = 1$.

After DBSCAN clustering, each cluster was further processed. For a fragment ion in S_{db} that did not have a matched fragment ion from S_q in a cluster C, an artificial ion was added to C. The m/z value of the added artificial ion equals to that of the ion in S_{db} and intensity equals to zero. In case that a cluster C only has a fragment ion from S_q, i.e., it does not have a matched ion from S_{db}, the cluster C was deleted. The spectrum similarity score was then calculated using cosine similarity as follows:

$$S_C(X, Y) = \frac{X \circ Y}{\|X\| \cdot \|Y\|} \quad (2)$$

where $X = (x_1, x_2, \ldots, x_n)$, $Y = (y_1, y_2, \ldots, y_n)$, and x_i and y_i are the intensities of the ith fragment ions in S_q and S_{db}, respectively. The inner product $X \circ Y = \sum_{i=1}^{n} x_i \cdot y_i$ and the norm $\|X\| = \left(\sum_{i=1}^{n} x_i^2 \right)^{1/2}$.

Results and Discussion

Method Optimization

To optimize the sample processing method, randomly selected authentic standards of 21 nucleosides and nucleotides were mixed, and the mixture was used for testing. Figure S1 depicts the experiments designed to optimize sample processing

methods, where a human urine sample was used and its pH was adjusted to 2–3 or 8.5 as reported previously [23, 24]. After centrifugation at 14,000 rpm for 20 min at 4 °C, the clear upper layer liquid was divided into eight aliquots. Four aliquots were directly uploaded onto the cartridges, two of which were eluted respectively by 80% methanol or 2.8% NH_4OH in methanol, and the other two aliquots were first washed by 100 μL of 2% formic acid in water three times, then respectively eluted by 80% methanol or 2.8% NH_4OH in methanol. The remaining four aliquots were extracted by 80% methanol and then purified by cartridges using the abovementioned methods.

The eluate from the cartridge by each of the eight examined methods was then analyzed by LC-MS. The results showed that extraction with 80% methanol (Figure S2A) and washing with 2% formic acid in water (Figure S2B) decreased the intensity of all nucleosides and nucleotides. The elution solvents, i.e., 80% methanol or 2.8% NH_4OH in methanol, did not affect the intensity of nucleosides and nucleotides (Figure S2C). However, the intensities of 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 5-methyluridine (m^5U), and N6-methyladenosine (m^6A) were notably increased in samples with pH 8.5 (Figure S2D). In summary, the sample processing method labeled as Method-12 in Figure S1 was the best.

To optimize the LC conditions, we first tried a SeQuant®-cHILIC column (150 × 2.1 mm i.d., 3 μm) purchased from Millipore (Burlington, MA, USA) for separation. The results showed that most of the compounds co-eluted together and gave a very bad separation (data not shown). We then chose a reverse phase column, i.e., an ACQUITY UPLC HSS T3 column, for LC separation. The LC performance was tested using different mobile phases and gradient rates. The mobile phase A was 25 mM ammonium acetate (pH 6.0) or 0.1% formic acid in water, while the mobile phase B was 0.1% formic acid in 60% or 100% acetonitrile. The results showed that 0.1% formic acid in water as mobile phase A produced higher sensitivity for analysis of the mixture, and 0.1% formic acid in 60% acetonitrile as the mobile phase B gave a better separation. While several gradient rates were tested, a 30-min gradient with 1.44% B per min from 2.5 to 20 min gave the best separation (Figure 1a). Although multiple compounds co-eluted from the RPC column, the m/z values of those compounds are different (Figure 1a, inserts). Therefore, those co-eluting compounds do not cause any problems for compound quantification. However, some co-eluting compounds such as 3-methylcytidine (m^3C) and N4-methylcytidine (m^4C) have identical m/z values. MetSign software was able to deconvolute those overlapping peaks into two chromatographic peaks with $t_R = 5.09$ min and $t_R = 5.13$ min, respectively (Figure 1b, table insert).

We then analyzed the mixture by LC-MS in positive and negative modes, respectively. Compared with the data acquired in negative mode, the LC-MS data acquired in positive mode had higher instrument signals (data not shown). Therefore, all biological samples were only analyzed in positive mode.
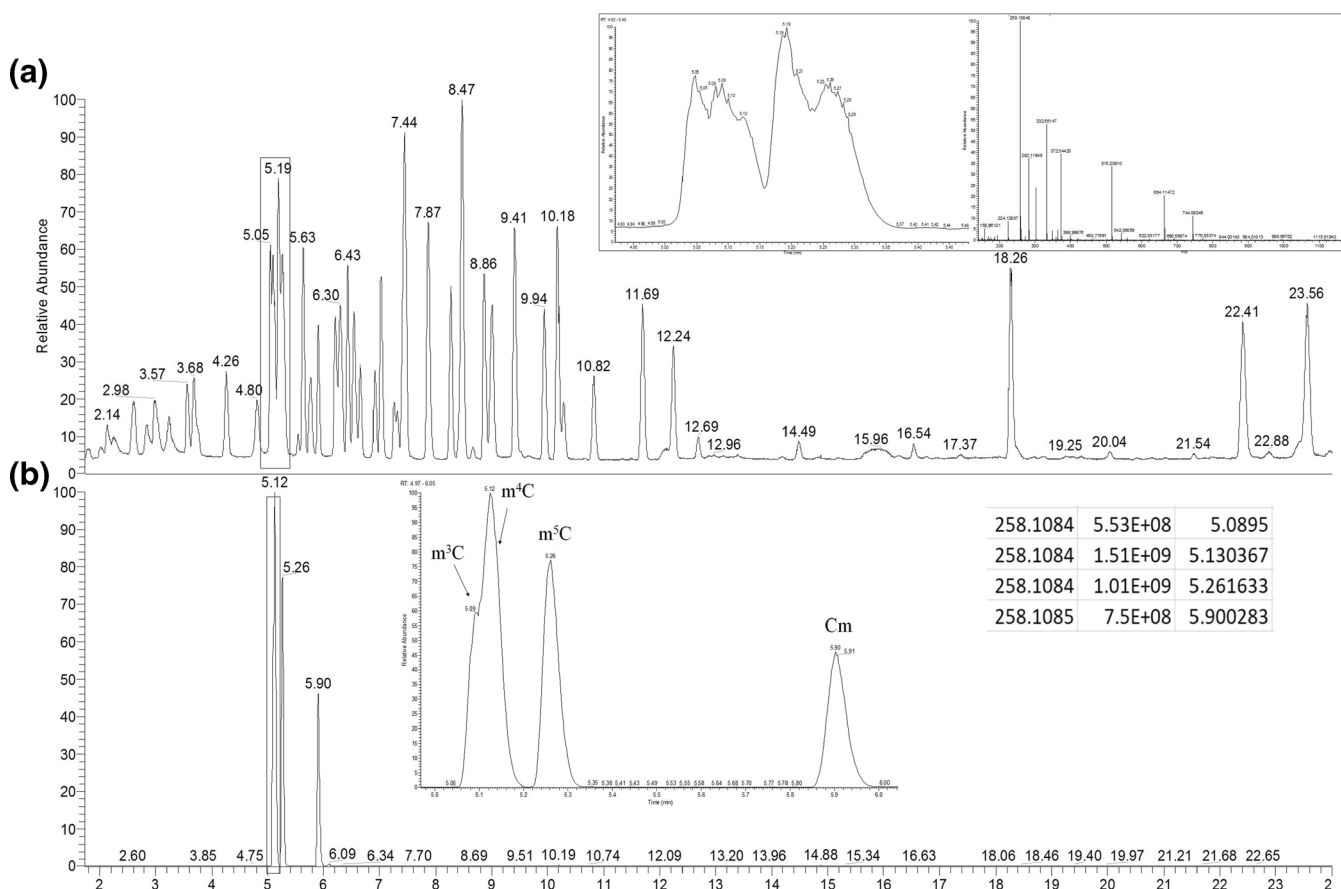
Figure 1. Separation and identification of nucleosides and nucleotides. (**a**) is a total ion chromatogram of a mixture of 65 authentic standards of nucleosides and nucleotides. The concentration of each molecule in the mixture was 5 μmol/L. The two inserts show that six compounds were co-eluted in the range of retention time 5.05–5.29 min, but those compounds had different *m/z* values. (**b**) is an extracted ion chromatogram (XIC) of molecules with *m/z* value between 258.10742 and 258.10948, where four compounds were detected. The right insert is zoom in of the XIC and the result of compound identification by MetSign software. The left insert displays that the two compounds co-eluted from LC were deconvoluted into two chromatographic peaks with retention time of 5.09 min and 5.13 min

Compound Identification

A total of 175 nucleosides and nucleotides were recorded in our in-house database. We categorized those compounds into three tiers (Tier I, Tier II, and Tier III) based on the available information of their chemical characteristics, including parent ion *m/z*, retention time, and MS/MS spectrum. A Tier I compound has a parent ion *m/z*, a retention time, and multiple MS/MS spectra acquired under different collision energies. A Tier II compound has a parent ion *m/z* and one or multiple predicted or experimental MS/MS spectra extracted from public databases. A Tier III compound has only its parent ion *m/z*. This tier-wise identification method is well aligned with the minimum reporting standards recommended by the members of Metabolomics Society that have four levels [25]. The Tier I is equivalent to the Level 1, while Tier II and Tier III are equivalent to the Level 2 and Level 3, respectively.

To obtain the information for each Tier I compound, we purchased authentic standards of 65 nucleosides and nucleotides that were commercially available. Those compounds were mixed into nine mixtures, where the compounds in each mixture had different molecular weights. Each mixture was first analyzed by LC-MS in positive mode to obtain the retention time and parent ion *m/z* for each compound. Then the mixture was analyzed by LC-MS/MS in positive mode to obtain the MS/MS spectra of each compound under different collision energies, i.e., 10, 20, 40, and 60 eV. The parent ion *m/z*, retention time, and MS/MS spectra of each compound were extracted from the experimental data and recorded in our in-house database. The MS/MS spectra of Tier II compounds were extracted from ChemSpider (http://www.chemspider.com/) and PubChem (https://pubchem.ncbi.nlm.nih.gov/). The parent ion *m/z* of Tier II and Tier III compounds were calculated from their molecular formula assuming a single charge and appropriate adducts, i.e., [M+H]$^+$ in positive mode.

Among the 175 nucleosides and nucleotides recorded in our in-house database, 65 compounds were Tier I compounds, 10 were Tier II compounds, and 100 were Tier III compounds. To identify a compound, the experimental information of a compound obtained from LC-MS/MS was first matched to the Tier

I compounds. The data that did not have a match with the Tier I compounds were then matched to the Tier II compounds. The remaining data that still did not have a match to the Tier II compounds were matched to the Tier III compounds.

Figure 2 depicts a sample identification result from human urine. A total of four compounds were detected with m/z between 298.11341 and 298.11579 (Figure 2a). Two compounds eluted at 8.28 min and 8.50 min and were respectively identified as the Tier I compounds, 2′-o-methylguanosine (Gm) and N2-methylguanosine (m²G), based on their parent ion m/z, retention time, and MS/MS spectral similarity. The two compounds eluted at 7.25 min and 7.95 min were not identified as Tier I compounds because of large retention time difference. However, it is interesting that the MS/MS spectra of those two compounds were very similar to the MS/MS spectra of 7-methylguanosine (m⁷G) and m²G (Figure S3). Based on the high similarity of MS/MS spectra, we strongly believe that the compounds eluted at 7.25 min and 7.95 min are two isomers of m⁷G and m²G. A similar observation was found with a potential isomer of m⁵U (Figure S4).

Construction of Calibration Curves

To determine the LODs and the LOQs of nucleosides and nucleotides by the developed LC-MS system, 19 calibration solutions were prepared. Table 1 lists the information of the calibration curves of 64 nucleoside and nucleotides, including

calibration equation, linear range, LOD, LOQ, retention time, and theoretical m/z value. The calibration curve of nucleoside 2-methylthio-N6-(dimethylallyl) adenosine (ms²i⁶A) was not constructed because this compound co-eluted with a column background peak. The linearity of calibration curves for those compounds were excellent, with $R^2 \geq 0.995$, except for ATP whose $R^2 = 0.98$ partially owing to its broad chromatographic peak (Figure S5).

The LODs of the 64 nucleosides and nucleotides were in the sub-nmol/L range, except for ATP that was 1.25 μmol/L. The LODs of 34 nucleosides were less than 0.05 nmol/L, and 15 nucleosides were between 0.24 and 4.88 nmol/L (Table 1). Our method lowered the LODs at least 1000-fold compared to those reported in references [11, 14, 15], and 2–13-fold compared to those reported in reference [16]. For nucleotide detection, LODs for adenosine-5′-monophosphate (AMP), guanosine-5′-monophosphate (GMP), inosine-5′-monophosphate (IMP), thymidine-5′-monophosphate (TMP), and uridine-5′-monophosphate (UMP) were ≤ 0.05 nmol/L, which were 5.6–52-fold lower than those reported in reference [16], and more than 100 times lower than those reported in other studies [12, 14]. Furthermore, only monophosphates and a few modified nucleosides were analyzed in the previous studies. Our method was able to detect diphosphates, triphosphates, and many modified nucleosides. The LODs for adenosine-5′-diphosphate (ADP), cytidine-5′-monophosphate (CMP), and cytidine-5′-diphosphate (CDP) were ≤ 0.12 nmol/L, LOD for
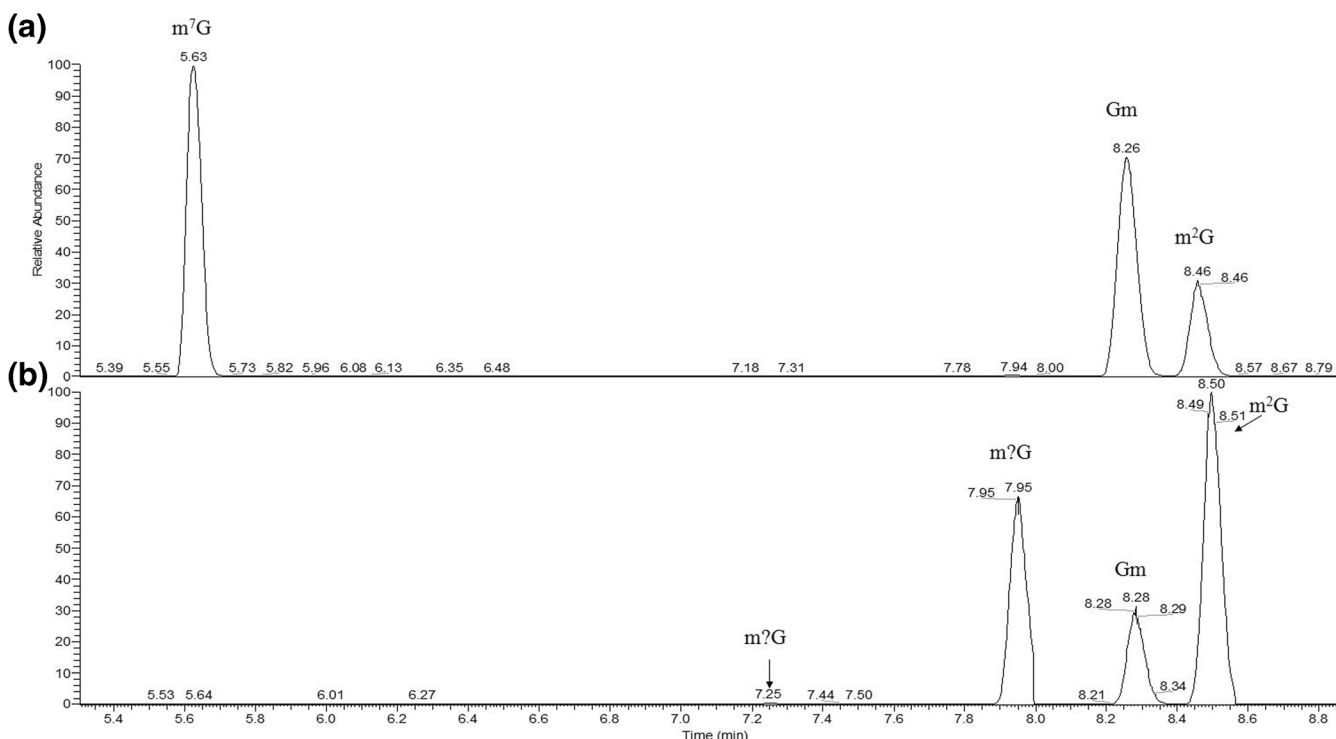
Figure 2. A sample of compound identification in human urine samples. (**a**) is an extracted ion chromatogram (XIC) of the 65 authentic standards of nucleosides and nucleotides with m/z values between 298.11341 and 298.11579. (**b**) is an XIC of human urine data with m/z values between 298.11341 and 298.11579. Two of four XIC peaks were identified as Gm and m2G based on parent ion m/z, retention time, and MS/MS spectrum similarity. The other two peaks eluted at 7.25 min and 7.95 min could not be identified because of the large retention time variation

Table 1. Calibration Curves for Quantification for 64 Nucleosides and Nucleotides

No.	Abbreviation	Full name	LOD (nmol/L)	Calibration equation[a]	LOQ (nmol/L)	Linear range (nmol/L)	R^{2b}	t_R (min)[c]	$[M+H]^{+d}$
1	A	Adenosine	< 0.05	$y = 370,390.94x + 22,918,829.9$	39.06	39.06–2500	0.9995	6.22	268.104
2	m⁶A	N6-Methyladenosine	< 0.05	$y = 1,473,671.22x + 3,504,913.69$	0.24	0.24–312.5	0.9998	8.48	282.12
3	m²A	2-Methyladenosine	< 0.05	$y = 911,129.92x + 10,011,403.60$	0.12	0.12–1250	0.9986	7.05	282.12
4	Am	2′-O-Methyladenosine	< 0.05	$y = 615,985.71x + 12,120,338.00$	0.24	0.24–2500	0.9994	7.88	282.12
5	ac⁶A	N6-Acetyladenosine	< 0.05	$y = 649,793.42x + 13,075,982.21$	0.05	0.05–2500	0.9979	9.41	310.115
6	ms²m⁶A	2-Methylthio-N6-methyladenosine	< 0.05	$y = 850,798.97x + 25,264,057.23$	0.12	0.12–2500	0.9979	22.43	328.107
7	m¹A	1-Methyladenosine	< 0.05	$y = 361,304.46x + 2,276,518.56$	0.12	0.12–1250	0.9993	5.22	282.12
8	i⁶A	N6-Isopentenyladenosine	< 0.05	$y = 745,481.25x + 3,266,388.45$	0.10	0.10–2500	0.9998	23.57	336.167
9	ms²t⁶A	2-Methylthio-N6-threonylcarbamoyladenosine	< 0.05	$y = 853,677.80x + 20,460,878.59$	0.10	0.10–2500	0.9978	18.27	459.129
10	U	Uridine	< 0.05	$y = 24,651.54x + 3,157,951.20$	39.06	39.06–10,000	0.9986	5.54	245.077
11	Y	Pseudouridine	0.05	$y = 37,314.06x + 3,658,795.29$	0.12	0.12–10,000	0.9981	3.71	245.077
12	Um	2′-O-Methyluridine	4.88	$y = 25,342.68x + 753,349.36$	9.77	9.77–10,000	0.9992	5.88	259.092
13	s⁴U	4-Thiouridine	4.88	$y = 21,906.74x + 904,383.72$	19.53	19.53–10,000	0.9985	8.67	261.054
14	m³Y	3-Methylpseudouridine	< 0.05	$y = 234,848.72x + 1,821,813.62$	0.24	0.24–2500	0.9995	6.32	259.092
15	m⁵s²U	5-Methyl-2-thiouridine	4.88	$y = 70,827.11x + 2,556,849.16$	4.88	4.88–10,000	0.9989	10.29	275.07
16	mo⁵U	5-Methoxyuridine	4.88	$y = 59,762.81x + 2,085,116.55$	4.88	4.88–10,000	0.9995	7.26	275.087
17	ncm⁵U	5-Carbamoylmethyluridine	< 0.05	$y = 175,248.72x + 261,672.48$	4.88	4.88–2500	0.9996	5.29	302.098
18	mcm⁵s²U	5-Methoxycarbonylmethyl-2-thiouridine	< 0.05	$y = 259,310.91x + 433,605.18$	0.05	0.05–2500	0.9998	12.24	333.075
19	mcm⁵U	5-Methoxycarbonylmethyluridine	0.24	$y = 180,427.28x + 1,871,234.36$	0.24	0.24–2500	0.9997	9.00	317.098
20	m⁵U	5-Methyluridine	0.24	$y = 74,677.73x + 4,185,158.55$	19.53	19.53–10,000	0.9993	7.33	259.092
21	cm⁵U	5-Carboxymethyluridine	4.88	$y = 90,844.50x + 6,243,036.78$	4.88	4.88–10,000	0.9987	6.32	303.082
22	s²U	2-Thiouridine	4.88	$y = 28,334.03x + 1,968,151.15$	9.77	9.77–10,000	0.997	7.87	261.054
23	ho⁵U	5-Hydroxyuridine	4.88	$y = 37,807.69x - 282,123.91$	19.53	19.53–2500	0.9997	5.08	261.072
24	cmo⁵U	Uridine 5-oxyacetic acid	0.24	$y = 116,322.03x + 4,632,978.57$	0.24	0.24–10,000	0.9997	6.65	319.077
25	mcmo⁵U	Uridine 5-oxyacetic acid methyl ester	< 0.05	$y = 232,947.73x + 13,057,273.48$	0.24	0.24–10,000	0.9988	9.94	333.093
26	m¹Y	1-Methylpseudouridine	< 0.05	$y = 128,869.62x + 2,203,305.95$	0.24	0.24–2500	0.999	5.67	259.092
27	C	Cytidine	< 0.05	$y = 179,327.67x + 11,531,759.17$	39.06	39.06–1250	0.9954	3.57	244.093
28	m⁵C	5-Methylcytidine	< 0.05	$y = 288,276.77x + 5,764,094.20$	0.24	0.24–1250	0.9972	5.26	258.108
29	m³C	3-Methylcytidine	< 0.05	$y = 179,926.68x + 1,589,835.80$	0.10	0.10–1250	0.9986	5.09	258.108
30	m⁴C	N4-Methylcytidine	< 0.05	$y = 448,137.34x + 4,193,651.37$	9.76	9.76–1250	0.9955	5.12	258.108
31	s²C	2-Thiocytidine	4.88	$y = 121,375.73x + 1,651,276.15$	19.53	19.53–10,000	0.9994	5.78	260.07
32	m⁴₂Cm	N4,N4,2′-O-Trimethylcytidine	< 0.05	$y = 2,149,655.50x + 3,460,739.89$	0.12	0.12–312.5	0.9998	7.45	286.14
33	ac⁴C	N4-Acetylcytidine	< 0.05	$y = 308,375.50x + 5,625,348.25$	0.12	0.12–1250	0.996	9.02	286.103
34	f⁵C	5-Formylcytidine	4.88	$y = 135,049.19x + 4,064,140.16$	4.88	4.88–2500	0.9978	6.95	272.088
35	ac⁴Cm	N4-Acetyl-2′-O-methylcytidine	< 0.05		0.24	0.24–156.25	0.9969	11.69	300.119

Table 1 (continued)

No.	Abbreviation	Full name	LOD (nmol/L)	Calibration equation[a]	LOQ (nmol/L)	Linear range (nmol/L)	R^{2b}	t_R (min)[c]	$[M+H]^{+d}$
36	hm^{5}C	5-Hydroxymethylcytidine	4.88	$y = 909{,}322.17x + 930{,}371.89$ $y = 221{,}181.06x + 1{,}766{,}580.49$	4.88	4.88–2500	0.9994	3.68	274.103
37	m^{4}Cm	N4,2′-O-Dimethylcytidine	< 0.05	$y = 1{,}069{,}415.24x + 3{,}433{,}740.32$	0.24	0.24–156.25	0.9991	6.44	272.124
38	Cm	2′-O-Methylcytidine	< 0.05	$y = 370{,}188.06x + 2{,}708{,}083.38$	0.24	0.24–312.5	0.996	5.91	258.108
39	G	Guanosine	< 0.05	$y = 245{,}773.93x + 32{,}007{,}088.63$	39.06	39.06–2500	0.9974	6.57	284.099
40	m^{7}G	7-Methylguanosine	< 0.05	$y = 437{,}315.38x + 13{,}374{,}536.40$	0.24	0.24–2500	0.9981	5.63	298.115
41	m^{2,2,7}G	N2,N2,7-Trimethylguanosine	< 0.05	$y = 719{,}875.63x + 10{,}183{,}854.84$	0.24	0.24–2500	0.9979	8.86	326.146
42	m^{2,7}G	N2,7-Dimethylguanosine	< 0.05	$y = 394{,}519.67x + 6{,}084{,}067.12$	0.05	0.05–1250	0.9956	7.42	312.13
43	m$^2_2$G	N2,N2-Dimethylguanosine	< 0.05	$y = 1{,}287{,}360.79x + 4{,}765{,}262.07$	0.24	0.24–312.5	0.998	10.28	312.13
44	Gm	2′-O-Methylguanosine	< 0.05	$y = 450{,}858.75x + 11{,}796{,}667.27$	0.24	0.24–1250	0.9966	8.27	298.115
45	imG-14	4-Demethylwyosine	4.88	$y = 56{,}419.47x + 6{,}145{,}361.93$	19.53	19.53–10,000	0.9975	12.68	322.115
46	m^{2}G	N2-Methylguanosine	< 0.05	$y = 229{,}591.38x + 677{,}525.90$	0.12	0.12–312.5	0.9982	8.43	298.115
47	preQ0	7-Cyano-7-deazaguanosine	0.24	$y = 212{,}451.27x + 1{,}086{,}390.40$	0.24	0.24–2500	0.9987	10.82	308.099
48	I	Inosine	< 0.05	$y = 119{,}627.76x + 7{,}748{,}498.53$	19.53	19.53–1250	0.9956	6.59	269.088
49	Im	2′-O-Methylinosine	< 0.05	$y = 238{,}451.03x + 288{,}454.34$	0.10	0.10–156.25	0.9979	8.38	283.104
50	AMP	Adenosine 5′-monophosphate	0.05	$y = 212{,}211.30x + 8{,}503{,}382.05$	0.24	0.24–10,000	0.9997	4.26	348.07
51	ADP	Adenosine 5′-diphosphate	0.12	$y = 94{,}757.55x - 24{,}448{,}944.97$	156.25	156.25–10,000	0.9959	2.85	428.037
52	ATP	Adenosine 5′-triphosphate	1250.00	$y = 4652.02x - 10{,}562{,}983.86$	2500.00	2500–40,000	0.9816	2.90	508.003
53	m^{6}AMP	N6-Methyladensine-5′-monophosphate	156.25	$y = 1726.67x - 226{,}641.49$	156.25	156.25–10,000	0.9988	5.48	362.086
54	UMP	Uridine 5′-monophosphate	0.05	$y = 127{,}636.38x + 5{,}366{,}028.00$	19.53	19.53–2500	0.9985	3.22	325.043
55	UDP	Uridine 5′-diphosphate	78.13	$y = 25{,}915.76x - 6{,}004{,}370.55$	78.13	78.13–40,000	0.9985	2.26	405.009
56	CMP	Cytidine-5′-monophosphate	0.12	$y = 193{,}394.53x + 2{,}873{,}252.40$	0.24	0.24–2500	0.9995	2.59	324.059
57	CDP	Cytidine 5′-diphosphate	0.12	$y = 73{,}834.40x + 4{,}201{,}076.94$	19.53	19.53–10,000	0.9994	2.14	404.025
58	CTP	Cytidine 5′-triphosphate	156.25	$y = 20{,}936.65x - 7{,}584{,}428.71$	156.25	156.25–20,000	0.9969	2.14	483.992
59	GMP	Guanosine 5′-monophosphate	0.05	$y = 169{,}056.37x + 2{,}538{,}462.28$	39.06	39.06–10,000	0.9991	4.81	364.065
60	GDP	Guanosine 5′-diphosphate	156.25	$y = 51{,}185.54x - 6{,}039{,}185.84$	156.25	156.25–40,000	0.9996	3.30	444.032
61	IMP	Inosine 5′-monophosphate	0.05	$y = 46{,}750.55x + 3{,}032{,}839.82$	4.88	4.88–2500	0.9983	4.78	349.054
62	IDP	Inosine 5′-diphosphate	156.25	$y = 43{,}586.71x - 24{,}858{,}422.81$	156.25	156.25–40,000	0.9989	2.97	429.021
63	TMP	Thymidine 5′-monophosphate	0.05	$y = 54{,}889.46x + 7{,}451{,}844.48$	39.06	39.06–10,000	0.9975	5.81	323.064
64	NADH	Nicotinamide adenine dinucleotide (reduced)	19.53	$y = 38{,}923.17x - 652{,}947.85$	19.53	19.53–1250	0.9943	6.27	666.132

[a] x is concentration (nmol/L); y is peak area
[b] R^2 is regression coefficient
[c] t_R is retention time
[d] $[M+H]^+$ is the theoretical m/z of a compound

nicotinamide adenine dinucleotide (NADH) was less than 20 nmol/L, LOD for uridine 5′-diphosphate (UDP) was 78.13 nmol/L, and LODs were 156 nmol/L for cytidine 5′-triphosphate (CTP), guanosine 5′-diphosphate (GDP), inosine

Table 2. Intraday Variation of the Pooled Sample

Name	Peak area						Concentration (nM)					
	Urine	RSD (%)	Plasma	RSD (%)	Liver	RSD (%)	Urine	RSD (%)	Plasma	RSD (%)	Liver	RSD (%)
A	4.76E+09	8.1E+00	2.50E+08	5.8E+00	4.90E+09	5.7E+00	12,777.30	8.1E+00	1533.47	6.4E+00	13,171.98	5.7E+00
ac^{4}C	1.28E+09	8.1E+00	6.67E+06	1.9E+01	4.07E+06	8.2E+00	4131.38	8.1E+00	8.43	1.2E+02	–	–
ac^{6}A	1.63E+05	9.4E+01			9.17E+04	7.8E+01	–	–			–	–
ADP			3.75E+06	3.9E+01	2.02E+06	3.3E+01			743.93	5.2E+00	279.33	2.5E+00
Am	3.52E+07	7.8E+01			3.08E+06	9.4E+00	65.94	1.4E+01	–	–	–	–
AMP	4.04E+06	1.6E+00	1.80E+07	2.8E+00	9.11E+07	9.0E+00	–	–	112.10	5.3E+00	389.07	1.0E+01
C	3.91E+08	9.7E+00	6.75E+06	4.2E+00	1.25E+09	6.9E+00	2116.12	1.0E+01	–	–	6888.21	7.0E+00
Cm	4.56E+08	8.5E+00	8.31E+06	8.7E+00	6.21E+06	7.7E+00	1224.33	8.6E+00	37.80	1.3E+01	9.45	1.4E+01
CMP					7.28E+07	2.5E+01					361.48	2.6E+01
G	1.11E+08	8.8E+00	7.23E+06	3.2E+00	2.37E+08	7.5E+00	322.61	1.2E+01	–	–	832.59	8.7E+00
GMP			2.51E+06	2.6E+00	8.74E+06	1.5E+01	–	–	–	–	36.66	2.2E+01
I	7.35E+07	1.2E+01	5.62E+07	2.9E+00	2.37E+09	2.0E+01	549.35	1.3E+01	1013.44	3.4E+00	19,736.29	2.0E+01
Im	4.05E+07	2.7E+01	8.44E+05	8.2E+00	1.41E+06	8.4E+00	168.55	2.7E+01	5.82	1.2E+01	4.71	1.1E+01
imG-14			1.03E+07	2.8E+01			–	–	184.64	6.9E+01		
IMP			1.84E+05	7.7E+01	4.34E+05	7.8E+01			–	–	–	–
m^{1}A	1.24E+10	1.1E+01	9.49E+07	1.6E+01	1.40E+08	8.1E+00	34,345.60	1.1E+01	641.10	1.6E+01	380.37	8.2E+00
m^{1}Y	1.63E+07	7.7E+00	1.34E+06	3.7E+00			109.20	9.0E+00	–	–		
m^{2,2,7}G	1.56E+09	8.2E+00	5.33E+06	2.0E+01	8.71E+06	8.5E+00	2148.85	8.3E+00	–	–	–	–
m$^2_2$G	6.84E+09	7.8E+01	7.56E+07	1.3E+01	1.03E+08	7.4E+00	5310.24	7.8E+01	137.47	1.4E+01	76.56	7.8E+00
m^{2}A	4.18E+06	1.3E+01					–					
m^{2}G	2.49E+09	8.8E+00	8.98E+06	1.4E+01	2.89E+07	6.6E+00	10,854.12	8.8E+00	90.44	1.5E+01	123.12	6.7E+00
m^{3}C	3.86E+07	1.1E+01	9.99E+05	7.1E+00	1.44E+07	8.3E+00	205.67	1.2E+01	–	–	71.47	9.3E+00
Gm	2.97E+08	8.4E+00	2.18E+06	5.1E+00	5.57E+06	8.5E+00	631.95	8.8E+00	–	–	–	–
m^{5}C	7.73E+08	8.2E+00	4.57E+06	1.6E+01	3.67E+07	7.2E+00	2660.29	8.2E+00	–	–	107.43	8.6E+00
m^{3}Y	5.58E+07	7.9E+00	7.75E+05	6.0E+00			229.91	8.1E+00	–	–		
m^{4}Cm	1.09E+06	8.8E+00			1.12E+06	5.9E+00	–	–			–	–
m^{5}U	4.85E+06	4.4E+00	5.53E+06	4.6E+00	3.94E+05	1.3E+01	8.86	3.2E+01	45.09	1.9E+01	–	–
m^{6}A	1.11E+09	6.8E+00	2.57E+06	1.9E+01	1.38E+07	8.2E+00	752.29	6.8E+00	–	–	7.00	1.1E+01
m^{7}G	4.81E+07	9.2E+00	2.12E+05	8.7E+01	2.19E+05	8.2E+01	79.45	1.3E+01	–	–	–	–
mcm^{5}s^{2}U	3.88E+08	7.7E+01	6.04E+06	7.7E+01	7.31E+06	8.1E+00	1494.00	7.8E+01	54.02	8.3E+01	26.53	8.6E+00
mcm^{5}U	3.07E+06	1.2E+01	3.52E+05	7.8E+01			6.67	3.0E+01	–	–		
ncm^{5}U	1.26E+08	4.3E+00	1.32E+06	1.1E+01	7.97E+05	2.0E+01	716.41	4.3E+00	15.04	1.4E+01	3.06	3.0E+01
U	1.36E+07	5.5E+00	4.09E+07	1.8E+00	6.95E+08	4.5E+00	423.69	7.2E+00	3827.01	1.9E+00	28,059.01	4.5E+00
Um	1.82E+07	9.7E+00			2.55E+05	1.0E+01	687.07	1.0E+01			–	–
UMP			8.20E+04	7.9E+01	3.58E+06	2.9E+00			–	–	–	–
Y	6.41E+09	4.9E+00	1.16E+08	6.6E+00	8.00E+07	6.1E+00	171,592.30	4.9E+00	7534.82	6.8E+00	2044.93	6.4E+00

– refers to a compound that was detected but not quantified because its concentration was out of the linear range of the calibration curve. A blank in the table means that a compound was not detected

5′-diphosphate (IDP), and N6-methyladensine-5′-monophosphate (m6AMP). However, the LOD for ATP was much higher as 1250 nmol/L.

The LOQs for 12 nucleosides detected in reference [21] were 200 nmol/L and the LOQ for pseudouridine (Ψ) was 10 μmol/L. LOQs of those nucleosides were all improved to ≤ 39.1 nmol/L in this study. Compared to those reported in reference [16], the LOQs for adenosine (A), uridine (U), cytidine (C), and guanosine (G) were not improved in this study. However, those four compounds have very high concentrations in biological samples, and the LOQs of those compounds obtained by the current method are good enough to quantify those compounds from biological samples. Moreover, the LOQs of some modified nucleosides were in sub-nmol/L range in this study. For instance, the LOQs of 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U), 2′-O-methylinosine (Im), N4-acetylcytidine (ac^{4}C), 2′-O-methylcytidine (Cm), 3-methylpseudouridine (m^{3}Ψ), and N2,N2,7-trimethylguanosine (m^{2,2,7}G) were 0.05 nmol/L, 0.1 nmol/L, 0.12 nmol/L, 0.24 nmol/L, 0.24 nmol/L, and 0.24 nmol/L, respectively.

For nucleotides, the LOQs for monophosphates were larger than 1000 nmol/L in a previous study [15], but were lowered to ≤ 39.1 nmol/L in this study. The LOQs of AMP, IMP, and CMP in the current study were respectively 12.9-, 1.2-, and 36.3-fold lower than those reported in reference [16]. However, the LOQs for UMP and GMP in this study (i.e., 19.5 nmol/L for UMP, 39.1 nmol/L for GMP) were higher than those in reference [16] (i.e., 3.1 nmol/L for UMP, 4.3 nmol/L for GMP). Overall, the method developed in this study offers better sensitivity and could detect a much greater number of nucleosides and nucleotides in one analysis.

Analysis of Biological Samples

To determine if the current method could be applied to biological samples, we quantified nucleosides and nucleotides from human urine, human plasma, and rat liver samples. While 175 nucleosides and nucleotides have been reported in the literature, many of them are not commercially available. Therefore, a majority of the nucleosides and nucleotides were considered as

Table 3. Interday Variation of the Pooled Sample

Name	Peak area						Concentration (nM)					
	Urine	RSD (%)	Plasma	RSD (%)	Liver	RSD (%)	Urine	RSD (%)	Plasma	RSD (%)	Liver	RSD (%)
A	5.41E+09	9.0E+00	2.43E+08	3.7E+01	5.14E+09	5.7E+00	14,551.96	9.0E+00	1488.81	4.0E+01	13,808.79	5.8E+00
ac^4C	1.48E+09	1.3E+01	8.94E+06	8.2E+01	6.50E+06	4.0E+01	4767.03	1.3E+01	11.29	1.0E+02	10.80	3.4E+01
ac^6A	2.36E+05	9.6E+01			3.13E+06	9.6E+01	–	–			–	–
ADP	3.17E+06	7.4E+01	4.51E+06	4.6E+01	2.04E+06	4.7E+01	291.44	8.4E+00	764.07	7.2E+00	279.59	3.6E+00
Am	6.01E+07	1.3E+01			9.63E+06	7.0E+01	77.97	1.6E+01			–	–
AMP	5.16E+06	2.4E+01	1.91E+07	1.4E+01	9.72E+07	4.4E+00	–	–	125.24	2.6E+01	417.91	4.9E+00
C	4.39E+08	1.3E+01	1.03E+07	3.3E+01	1.24E+09	6.0E+00	2381.31	1.4E+01	–	–	6862.74	6.0E+00
Cm	5.09E+08	1.2E+01	8.35E+06	5.3E+01	9.54E+06	3.5E+01	1367.45	1.2E+01	37.98	6.3E+01	18.45	4.8E+01
CMP	1.12E+06	5.9E+01	1.36E+05	1.1E+02	6.62E+07	1.2E+01	–	–	–	–	327.37	1.2E+01
G	1.22E+08	1.1E+01	3.89E+06	8.8E+01	2.37E+08	9.3E+00	366.51	1.5E+01	–	–	832.92	1.1E+01
Gm	3.19E+08	1.2E+01	4.46E+06	6.3E+01	6.61E+06	3.0E+01	681.48	1.2E+01	–	–	–	–
GMP	1.14E+06	6.8E+01	2.68E+06	2.1E+01	1.13E+07	1.4E+01	–	–	6.40	–	51.56	1.9E+01
ho^5U	4.07E+06	5.9E+01					115.19	5.5E+01				
I	8.71E+07	1.2E+01	7.22E+07	3.2E+01	2.70E+09	1.3E+01	663.69	1.4E+01	1121.76	3.5E+01	22,510.86	1.3E+01
Im	4.12E+07	3.2E+01	7.71E+05	3.5E+01	1.98E+06	4.5E+01	171.66	3.2E+01	5.32	4.9E+01	7.11	5.2E+01
imG-14	1.44E+07	2.0E+01	1.19E+07	1.3E+01			146.28	3.5E+01	87.70	1.4E+01		
IMP					5.65E+05	2.5E+01					–	–
m^1A	1.33E+10	5.7E+00	9.57E+07	7.8E+01	1.92E+08	2.4E+01	36,845.47	5.7E+00	646.50	7.8E+01	525.26	2.5E+01
m^1Y	1.76E+07	1.1E+01	1.51E+06	2.0E+01	1.16E+06	2.1E+01	119.10	1.3E+01	–	–	–	–
$m^{2,2,7}G$	1.98E+09	1.6E+01	1.04E+07	8.8E+01	1.57E+07	4.6E+01	2733.78	1.6E+01	40.23	9.3E+01	16.85	3.3E+01
m^2_2G	1.06E+10	1.4E+01	7.67E+07	8.1E+01	1.27E+08	2.6E+01	8251.79	1.4E+01	139.47	8.2E+01	95.11	2.7E+01
m^2A	4.52E+06	8.2E+00			4.37E+06	1.1E+02	–	–			–	–
m^2G	2.69E+09	8.9E+00	9.01E+06	7.9E+01	3.33E+07	2.1E+01	11,692.48	8.9E+00	96.28	8.1E+01	141.87	2.1E+01
m^3C	5.42E+07	2.6E+01	1.63E+06	4.0E+01	1.40E+07	5.8E+01	292.54	2.7E+01	8.18	8.8E+01	69.03	2.5E+01
m^3Y	6.03E+07	1.0E+01	1.39E+06	4.7E+01	9.30E+05	7.7E+01	249.07	1.1E+01	–	–	–	–
m^4_2Cm	1.61E+06	2.0E+01			9.35E+06	1.0E+02	–	–			6.83	2.9E+01
m^4C	7.66E+08	9.1E+00	1.91E+06	7.3E+01	3.66E+07	4.3E+00	1699.03	9.1E+00	1.33	9.8E+01	72.22	4.9E+00
m^4Cm	9.23E+05	5.9E+01			4.26E+06	7.4E+01	–	–			3.47	3.8E+01
m^5C	5.61E+06	3.7E+01	6.91E+05	3.5E+01	3.96E+07	1.3E+01	–	–	–	–	117.30	1.5E+01
m^5U	5.25E+06	1.4E+01	5.44E+06	1.4E+01	8.00E+05	4.6E+01	14.23	6.7E+01	42.04	6.2E+01	–	–
m^6A	1.27E+09	1.0E+01	4.54E+06	8.8E+01	2.50E+07	5.1E+01	861.12	1.0E+01	9.42	8.7E+01	14.55	6.0E+01
m^7G	4.67E+07	9.4E+00			1.86E+05	1.0E+02	76.18	1.3E+01			–	–
mcm^5s^2U	6.11E+08	9.4E+00	5.83E+06	4.4E+01	8.44E+06	2.4E+01	2354.76	9.4E+00	54.55	4.6E+01	30.87	2.6E+01
mcm^5U	3.28E+07	1.9E+01	9.02E+05	4.6E+01	1.09E+06	9.4E+01	171.56	2.0E+01	–	–	–	–
ms^2t^6A	5.50E+08	1.2E+01			5.02E+06	7.3E+01	620.00	1.2E+01			–	–
ncm^5U	9.05E+07	5.5E+01	1.22E+06	5.0E+01	1.20E+06	3.3E+01	514.81	5.5E+01	15.49	5.6E-01	5.37	4.2E+01
preQ0					1.34E+06	4.2E+01					2.52	6.3E+01
U	1.53E+07	1.5E+01	4.21E+07	8.2E+00	6.93E+08	8.8E+00	493.72	1.9E+01	3948.09	8.9E+00	27,982.97	8.8E+00
UDP					6.84E+05	1.1E+02					258.09	1.1E+01
Um	2.02E+07	1.1E+01			3.90E+05	3.5E+01	766.37	1.2E+01			–	–
UMP			4.90E+05	2.9E+01	4.63E+06	1.9E+01			–	–	–	–
Y	6.93E+09	1.0E+01	1.43E+08	4.9E+01	9.47E+07	1.4E+01	185,749.41	1.0E+01	9415.42	5.0E+01	2441.14	1.4E+01

– refers to a compound that was detected but not quantified because its concentration was out of the linear range of its calibration curve. A blank in the table means that a compound was not detected

Tier II (with parent ion m/z and predicted MS/MS spectra) or Tier III compounds (with parent ion m/z only) in our in-house database. A total of 79, 55, and 51 nucleosides and nucleotides were respectively identified from human urine, human plasma, and rat liver, of which 34, 31, and 34 compounds were respectively identified as Tier I compounds in these three types of samples. Two and 43 compounds were respectively identified as Tier II and Tier III compounds from human urine. Two and 22 compounds were respectively identified as Tier II and Tier III compounds from human plasma. Two and 15 compounds were respectively identified as Tier II and Tier III compounds from rat liver.

The intraday and interday variations of compound quantification were also studied. For each type of biological samples, 40 μL of each sample was mixed to make a pooled sample for intraday and interday analysis. Each pooled sample was detected for three consecutive injections for intraday detection. For interday variation study, each pooled sample was analyzed by three consecutive injections each day and continued for 4 days (day 1, day 2, day 3, and day 7). The results showed that the relative standard deviation (RSD) of detected intensity for intraday and interday variation was between 2–94 and 4–110%, respectively. The quantification variation was in the range of 1.9–123% and 0.6–100.5%, respectively (Tables 2 and 3). The large RSD values were mainly caused by the low instrument response (i.e., peak area) or low concentrations. For instance, three compounds have a RSD larger than 100% in rat liver with an average RSD of peak area 105.3%. The average peak area of these compounds is only 2×10^6, which is 711 times less than the average peak area of the top six compounds that have RSDs less than 10%.

Table 4. Nucleosides and Nucleotides Quantified in Biological Samples by LC-MS/MS Analysis

No.	Abbreviation	Peak area			Concentration (nM)[a]			RT (min)
		Urine	Plasma	Liver	Urine	Plasma	Liver	
1	A	4.71E+09	2.59E+08	4.19E+09	12,664.73 ± 2946.69	1591.84 ± 68.51	11,257.30 ± 2354.80	6.22
2	ac^{4}C	1.29E+09	5.87E+06	2.55E+06	4166.49 ± 1049.67	6.33 ± 1.18	–	9.02
3	ADP	1.28E+06	6.18E+06	6.79E+06	271.49	808.02 ± 18.89	329.65 ± 40.35	2.85
4	Am	5.19E+07		2.74E+06	64.54 ± 19.72		–	7.88
5	AMP	4.61E+06	2.12E+07	9.78E+07	–	149.18 ± 24.39	420.98 ± 36.30	4.26
6	C	3.49E+08	1.49E+07	1.23E+09	1879.77 ± 187.36	47.16 ± 11.59	6798.18 ± 494.33	3.57
7	Cm	4.89E+08	9.79E+06	5.93E+06	1314.65 ± 175.85	47.85 ± 4.18	8.72 ± 2.67	5.91
8	CMP	1.45E+06		1.01E+08	–		508.93 ± 43.45	2.59
9	f^{5}C	2.64E+06			–			6.95
10	G	1.09E+08	7.93E+06	2.47E+08	311.34 ± 126.58	–	875.56 ± 99.36	6.57
11	GDP		4.73E+05			318.05 ± 2.36		3.30
12	Gm	2.85E+08	2.43E+06	5.71E+06	606.25 ± 149.33	–	–	8.27
13	GMP		3.06E+06	1.13E+07		7.75 ± 3.38	51.84 ± 3.54	4.81
14	ho^{5}U	9.83E+06			267.38 ± 62.51			5.08
15	I	7.69E+07	6.50E+07	2.47E+09	577.88 ± 167.41	1195.53 ± 73.31	20,549.59 ± 1643.31	6.59
16	i^{6}A			9.41E+05			–	23.57
17	Im	2.42E+09	7.77E+05	1.21E+06	10,163.35 ± 1103.46	5.12 ± 0.42	3.85 ± 0.88	8.38
18	IMP		5.23E+05	8.41E+05		–	–	4.78
19	m^{1}A	1.27E+10	8.89E+07	1.25E+08	35,261.55 ± 4485.76	599.50 ± 34.35	339.68 ± 48.82	5.22
20	m^{1}Y		3.68E+06	7.93E+05		28.60 ± 3.03	–	5.67
21	m^{2,2,7}G	1.86E+09	4.19E+06	1.09E+07	2571.15 ± 497.55	–	2.26 ± 0.62	8.86
22	m^{2,7}G			2.06E+06			–	7.42
23	m^{22}G	9.57E+09	6.75E+07	9.41E+07	7428.88 ± 1630.73	121.92 ± 9.22	69.36 ± 17.67	10.28
24	m^{2}A	5.50E+06			–			7.05
25	m^{2}G	2.38E+09	8.35E+06	2.80E+07	10,363.53 ± 2465.55	83.53 ± 6.90	118.98 ± 15.03	8.43
26	m^{3}C	3.98E+07	1.21E+06	1.40E+07	212.55 ± 65.17	–	69.08 ± 6.28	5.09
27	m^{3}Y		1.94E+06			1.21 ± 0.43		6.32
28	m^{4}Cm	1.69E+05	1.63E+05	4.53E+06	–	–	1.03 ± 0.69	6.44
29	m^{5}C	7.16E+08	5.04E+06	3.84E+07	2464.37 ± 244.05	4.74 ± 0.83	113.15 ± 8.08	5.26
30	m^{5}U	5.16E+06	6.20E+06	4.29E+05	13.02 ± 2.69	67.30 ± 27.45	–	7.33
31	m^{6}A	1.18E+09	2.34E+06	1.39E+07	800.45 ± 81.28	–	7.09 ± 1.07	8.48
32	m^{6}AMP		4.43E+05	5.46E+07		969.29 ± 76.85	31,729.03 ± 7327.03	5.48
33	m^{7}G	4.28E+07			67.37 ± 21.93			5.63
34	mcm^{5}s^{2}U	5.29E+08	7.95E+06	1.14E+07	2037.26 ± 712.45	72.42 ± 3.16	42.29 ± 4.50	12.24
35	mcm^{5}U	3.16E+07	4.83E+05		164.74 ± 37.78	–		9.00
36	ms^{2}m^{6}A	6.47E+06			–			22.43
37	ms^{2}t^{6}A	5.20E+08	2.32E+06	2.66E+06	585.35 ± 122.13	–	–	18.27
38	ncm^{5}U	1.09E+08	1.55E+06	8.58E+05	620.87 ± 112.57	18.44 ± 2.05	–	5.29
39	s^{2}C	3.64E+06			16.41			5.78
40	U	1.28E+07	4.58E+08	6.57E+08	393.00 ± 133.41	4322.30 ± 191.47	26,539.24 ± 1936.13	5.54
41	Um	1.92E+07		3.26E+05	728.07 ± 19.32		–	7.88
42	UMP			8.26E+06			22.70 ± 9.64	3.22
43	Y	5.64E+09	1.31E+08	7.93E+07	150,986.02 ± 21,860.35	8536.37 ± 711.37	2028.16 ± 354.89	3.71
44	3,4-PCNR/2,5-PCNR	3.87E+09	3.13E+07	6.01E+07				7.33
45	3,4-PCNR/2,5-PCNR	3.31E+08	3.78E+06	3.73E+07				6.43
46	AICAR	3.81E+08	1.90E+06					5.45
47	cAMP (3'5')	7.96E+08	1.97E+06	2.33E+06				6.78
48	cmnm^{5}Um/acp^{3}Y/acp^{3}U	4.30E+08		6.59E+06				5.36
49	D	1.40E+09	2.51E+07	1.00E+07				3.71
50	DHU	3.30E+08	9.66E+06					2.27
51	DHU	8.03E+07						5.02
52	DHU	9.21E+07						2.06
53	io^{6}A	6.51E+07						27.71
54[b]	m$^?$G	3.84E+09		3.96E+07				7.95
55[b]	m$^?$G	1.14E+08						7.26
56[b]	m$^?$U	2.07E+08		1.16E+06				8.47
57	m^{1}acp^{3}Y	1.06E+08						5.42
58	m^{1}I	4.26E+07	1.50E+07	2.66E+07				8.66
59	m$^2_2$Gm	1.86E+09		6.98E+06				9.24
60	m^{4,4}Cm	7.38E+07						5.24
61	m^{4,4}Cm	7.86E+07						3.93
62	m^{4,4}Cm	1.15E+08						12.40
63	m^{5}D	5.02E+08	2.71E+06					2.29
64	m^{5}Um/m^{3}Um	5.46E+07						2.98
65	m^{5}Um/m^{3}Um	2.03E+07						5.08
66	m^{5}Um/m^{3}Um	4.08E+07	6.60E+05					10.58
67	m$^6_2$A/m^{6}Am/m^{1}Am/m^{2,8}A/m^{6,6}A	1.25E+08	3.87E+06	5.44E+06				10.97
68	m$^6_2$A/m^{6}Am/m^{1}Am/m^{2,8}A/m^{6,6}A	4.46E+07	2.49E+06	7.18E+06				9.46

Table 4 (continued)

No.	Abbreviation	Peak area			Concentration (nM)[a]			RT (min)
		Urine	Plasma	Liver	Urine	Plasma	Liver	
69	m^6t^6A/hn^6A	4.12E+08	2.60E+06					17.73
70	mcm^5Um	8.92E+06	1.23E+06					10.31
71	mimG	7.49E+07						10.64
72	mnm^5U	1.63E+08						1.81
73	mnm^5U	4.53E+08	2.67E+06					5.81
74	ms^2io^6A	5.34E+07						9.87
75	ms^2io^6A	7.38E+07	8.12E+05					26.97
76	MTA	1.18E+09	9.77E+06	4.61E+07				12.45
77	MTA		1.04E+07					12.66
78	MTA-SO	2.12E+08	6.55E+05					11.26
79	MTA-SO		1.64E+06	2.04E+06				6.05
80	N6-SAR	4.68E+08	7.67E+06	3.80E+07				10.49
81	NA-R	3.16E+08						3.70
82	NA-R	2.11E+08	9.32E+05					4.04
83	nm^5ges^2U	7.72E+07						28.42
84	o^2yW	2.12E+07						3.45
85	OHyW	2.53E+08						18.31
86	s^2Um	6.18E+07						3.19
87	T(6)A	2.18E+09	1.74E+07	2.58E+07				14.72
88	$Ym/m^3U/IAA$-R	2.15E+08		9.69E+06				4.91
89	$Ym/m^3U/IAA$-R	1.13E+08						5.94
90	$Ym/m^3U/IAA$-R	5.82E+07						6.31
91	k^2C		5.90E+06					17.48
92	$mchm^5U$		2.02E+05	1.62E+07				6.50

– refers to a compound that was detected but not quantified because its concentration was out of the linear range of its calibration curve. A blank in the table means that a compound was not detected

[a]Results are expressed as means ± SE. SE stands for standard error. A concentration without ± SE means the compound was just detected in one sample

[b]Those compounds were tentatively identified because of their high similarity of MS/MS spectra with the corresponding isomers in our in-house database

Table 4 shows the detected intensities and calculated concentrations of those nucleosides and nucleotides. It has been reported that the abundance levels of C, m^3C, m^5C, G, m^2G, m^6A, Ψ, 1-methylinosine (m^1I), and inosine (I) are much higher in breast cancer patients than those in the normal controls, and those molecules might be potential tumor markers in human cancers [23, 26]. All those nucleosides were detected from human urine, human plasma, and rat liver in the present study. It should be noted that the concentrations of nucleosides $m^3Ψ$, Im, and ac^4C were respectively 1.21, 5.12, and 6.33 nmol/L in human plasma, while the concentrations of m^4Cm, $m^{2,2,7}G$, and Im were respectively 1.03, 2.26, and 3.85 nmol/L in rat liver. To our knowledge, those nucleosides were quantified for the first time from human plasma and rat liver.

Among the 65 nucleosides and nucleotides with authentic standards, we identified 34, 31, and 34 nucleosides and nucleotides as the Tier I compounds from human urine, human plasma, and rat liver, respectively. Among the identified Tier I compounds, 28, 22, and 23 compounds were respectively quantified from human urine, human plasma, and rat liver. Some nucleosides and nucleotides were not quantified in the current study because their concentrations were not covered by the linear range of the corresponding calibration curves. That means the LOQs of the developed method need to be further improved in future studies.

A total of 45, 24, and 17 compounds were also identified as Tier II and Tier III compounds based on parent ion m/z with or without predicted MS/MS spectra from human urine, human plasma, and rat liver, respectively. It is likely some of those identifications were false positives. Therefore, the identification of those compounds needs to be further confirmed by other methods. While the concentrations of those Tier II and Tier III compounds could not be determined owing to the lack of authentic standards, the relative change of their abundance levels between groups (e.g., disease vs control) can be analyzed by statistical significance tests [18, 27]. For this reason, the developed method can be also used in metabolomics for relative quantification of nucleosides and nucleotides in biological samples with much increased coverage of nucleosides and nucleotides.

Conclusions

A reverse phase chromatography mass spectrometry method was developed for simultaneous quantification of nucleosides and nucleotides from biological samples. Compound quantification was achieved via external calibration with intraday and interday variation of 1.9–123% and 0.6–100.5%, respectively. A three-tier compound identification method was also developed based on experimental information of a compound of interest. A total of 65 authentic standards of nucleosides and

nucleotides were considered as the Tier I compounds because of the availability of their parent ion *m/z*, retention time, and MS/MS spectra. The LODs of those nucleosides were between 0.05 and 4.88 nmol/L and LOQs were 0.05 and 39.1 nmol/L. The LODs of nucleotides were between 0.05 and 1.25 μmol/L and LOQs were 0.24 and 2.50 μmol/L. The developed method was applied to quantify the Tier I nucleosides and nucleotides from human urine, human plasma, and rat liver. Thirty-four nucleosides and nucleotides were identified from human urine, of which 28 were quantified with concentration ranged from 13.0 nmol/L to 151 μmol/L. Thirty-one nucleosides and nucleotides were identified as Tier I compounds from human plasma and 22 were quantified with a concentration range of 1.21 nmol/L to 8.54 μmol/L. Thirty-four nucleosides and nucleotides were identified as Tier I compounds from rat liver and 23 were quantified with concentration range of 1.03 nmol/L to 31.7 μmol/L.

Acknowledgements

Compliance with Ethical Standards

The procedures of animal care were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. All samples were collected after informed consent was obtained, and all procedures were approved by the University of Louisville IRB.

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