



Original article

MTBSTFA derivatization-LC-MS/MS approach for the quantitative analysis of endogenous nucleotides in human colorectal carcinoma cells

Huixia Zhang¹, Yan Li¹, Zheng Li, Christopher Wai-Kei Lam, Peng Zhu, Caiyun Wang, Hua Zhou^{**}, Wei Zhang^{*}

State Key Laboratory of Quality Research in Chinese Medicines, Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Taipa, Macau, China

ARTICLE INFO

Article history:

Received 7 June 2020

Received in revised form

28 October 2020

Accepted 14 January 2021

Available online 22 January 2021

Keywords:

Ribonucleotides

Deoxyribonucleotides

Derivatization

N-(t-butyltrimethylsilyl)-N-

methyltrifluoroacetamide

High-performance liquid chromatography

tandem mass spectrometry

ABSTRACT

Endogenous ribonucleotides (RNs) and deoxyribonucleotides (dRNs) are important metabolites related to the pathogenesis of many diseases. In light of their physiological and pathological significances, a novel and sensitive pre-column derivatization method with N-(t-butyltrimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was developed to determine RNs and dRNs in human cells using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). A one-step extraction of cells with 85% methanol followed by a simple derivatization reaction within 5 min at room temperature contributed to shortened analysis time. The derivatives of 22 nucleoside mono-, di- and triphosphates were retained on the typical C₁₈ column and eluted by ammonium acetate and acetonitrile in 9 min. Under these optimal conditions, good linearity was achieved in the tested calibration ranges. The lower limit of quantitation (LLOQ) was determined to be 0.1–0.4 μM for the tested RNs and 0.001–0.1 μM for dRNs. In addition, the precision (CV) was <15% and the RSD of stability was lower than 10.4%. Furthermore, this method was applied to quantify the endogenous nucleotides in human colorectal carcinoma cell lines HCT 116 exposed to 10-hydroxycamptothecin. In conclusion, our method has proven to be simple, rapid, sensitive, and reliable. It may be used for specific expanded studies on intracellular pharmacology *in vitro*.

© 2021 Xi'an Jiaotong University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Endogenous ribonucleotides (RNs) and deoxyribonucleotides (dRNs) are essential for life process and related to the pathogenesis of many diseases such as neurodegeneration [1], cancer [2], and mitochondrial depletion syndrome [3,4]. Deoxy- and ribonucleoside triphosphates (dNTPs and NTPs) are the precursors for DNA and RNA synthesis, and source energy providers such as adenosine triphosphate (ATP) as well as guanosine triphosphate (GTP). Nucleoside mono- and diphosphates are also involved in several physiological processes including signal transduction [5] and

nutrient metabolism. Adenosine monophosphate (AMP) can initiate the AMP-activated protein kinase (AMPK) which plays a role in cellular energy homeostasis to activate glucose and fatty acid uptake and oxidation when cellular energy is low [6,7]. Perturbations of RNs and dRNs pools can cause serious diseases either by decreased level of one of the nucleotides or by an imbalance among them. For instance, the balance of nucleotides pool is critical for the high fidelity of DNA replication as the imbalance of the dNTP/NTP ratios will increase the possibility of incorrectly incorporating NTPs into DNA and result in DNA instability [8]. These considerations underscore the potential utility of a rapid and convenient assay for quantification of different phosphorylated forms of intracellular RNs and dRNs.

Several analytical methods have been established to quantify nucleotides, including radioimmunoassay [9], aptamer-modified gold nanoparticles [10,11], and high-performance liquid chromatography (HPLC) or capillary electrophoresis [12] with tandem mass spectrometry (MS/MS) detection. Although the sensitivity of

Peer review under responsibility of Xi'an Jiaotong University.

* Corresponding author.

** Corresponding author.

E-mail addresses: hzhou@must.edu.mo (H. Zhou), wzhang@must.edu.mo (W. Zhang).

¹ These authors contributed equally to this work.

<https://doi.org/10.1016/j.jpha.2021.01.001>

2095-1779/© 2021 Xi'an Jiaotong University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

radioimmunoassay and aptamer-modified gold nanoparticles for the determination of RNs and dRN has been reported previously, these approaches are hindered by the lack of specific antibodies or aptamers directed against the monophosphate and diphosphate nucleotides. Capillary zone electrophoresis (CZE) [13] and micellar electrokinetic chromatography (MEKC) [14] have a unique advantage in separating high-charged analytes. However, the incompatibility of the mobile phase containing large amounts of non-volatile buffers with mass spectrometry limits the application of MEKC. The poor precision resulting from poor injection repeatability is also a major obstacle for the widespread use of CZE in routine analysis. The addition of ion-pair reagents into mobile phase is another conventional strategy to enhance the separation of RNs and dRN in reversed-phase liquid chromatography (RPLC) because these molecules are extremely polar due to their polyanionic phosphate groups [15–17]. However, there is no doubt that ion-pair reagents can cause ionization suppression of the analytes and contaminate the ion source as well as the HPLC systems. During the last decade, the analysis of nucleotides has evolved from ion-pairing chromatography to a novel, more compatible hydrophilic interaction chromatography (HILIC), which has a good separation performance against highly hydrophilic analytes and is compatible with MS using ion-pair free agents [8,18–20]. Although HILIC has been broadly used to quantify polar compounds, severe peak tailing and poor peak shape have always been problematic. Kong et al. [8] developed a ZIC-cHILIC method to quantify eight NTPs and dNTPs. However, it remains uncertain whether this strategy can be used to accurately measure deoxyribonucleoside monophosphates and diphosphates levels because their amounts were much lower than those of the respective triphosphate metabolites. Moreover, positive electrospray ionization was employed in this methodology, which would significantly decrease its sensitivity due to numerous hydroxyl groups of RNs and dRN. So far, the simultaneous separation of RNs and dRN is still a highly complex issue.

Derivatization is a powerful tool used to increase the volatility and chromatographic mobility of polar and unstable organic compounds [21]. Several derivatization methods using chloroacetaldehyde, propionyl or benzoyl acid anhydride, hexamethylenimine, etc. have been reported in the literature for the quantification of nucleotides in biological samples with the aid of LC-MS/MS. Zhang et al. [22] synthesized chloroacetaldehyde derivatives of adenosine 3',5'-cyclic monophosphate (cAMP) for LC fluorescence analysis by derivatizing the pyrimidine base with 2-chloroacetaldehyde at 80 °C for 30 min, which is likely to result in degradation of nucleoside di- and triphosphate. Nordström et al. [23] used propionyl or benzoyl derivatives to increase hydrophobicity and electrospray ionization (ESI) response of AMP, ADP and ATP. The derivatization was performed in acetonitrile and N-methylimidazole at 37 °C for 30 min. However, these methods are useable only for the trace amount of NTPs and dNTPs because of their poor solubility in these relatively hydrophobic solvents. Flarakos et al. [24] then developed a method for the synthesis of dGMP derivatives utilizing coupling reagents typically employed in peptide synthesis. The hydrophobic derivatives showed increased ionization efficiency and improved peak shape in an LC-MS/MS method. Unfortunately, this method is complex and time-consuming. Silylation is another derivatization reagent implemented to address the lack of volatility and chromatographic retention. For this method, N-(*t*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) is preferred for polar and unstable organic compounds according to substituting the labile hydrogen with *t*-butyldimethylsilyl on the polar function groups such as –COOH, –OH, and –NH [25]. The derivatives could possess higher molecular weights and slightly stronger resistance to moisture. Up to date, it is mainly used in gas chromatography and few

approaches are reported to determine nucleotides with liquid chromatography.

In this study, a simple, rapid and sensitive MTBSTFA derivatization method has been developed to quantify 22 nucleotides in human cells. Moreover, a detailed study containing optimization of derivatization conditions, selectivity of preferred extraction reagents, and the optimal liquid chromatography gradient was carried out. This derivatization approach was also applied to determine the concentrations of RNs and dRN in HCT 116 cells treated with 10-hydroxycamptothecin in order to prove the versatility for this method.

2. Materials and methods

2.1. Chemicals and reagents

Adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), deoxyadenosine monophosphate (dAMP), deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), deoxyguanosine monophosphate (dGMP), thymidine monophosphate (dTMP), thymidine diphosphate (dTDP), thymidine triphosphate (dTTP), stable isotope labeled adenosine-¹³C₁₀,¹⁵N₅-triphosphate (ATP-¹³C₁₀,¹⁵N₅), adenosine-¹³C₁₀,¹⁵N₅-monophosphate (AMP-¹³C₁₀,¹⁵N₅), and ammonium acetate (NH₄OAc) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). HPLC-mass grade methanol and acetonitrile were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). N-(*t*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, 95%) was provided by Dieckmann Chemical Industry Company Ltd. (Hong Kong, China). Ultra-pure water was produced from a deionized water system Milli-Q (Millipore Corporation, Burlington, MA, USA) and used throughout the study.

For cell culture, phosphate buffer saline (PBS), McCoy's 5A medium, penicillin-streptomycin solution and fetal bovine serum (FBS) were obtained from GIBCO Invitrogen Co. (Carlsbad, CA, USA). Human colon cancer epithelial cell lines (HCT 116) were supplied by American Type Culture Collection (ATCC; Rockville, MD, USA).

2.2. Stock solutions, internal standards and calibration curve standards

Initial stock solutions at 400 μM (dATP, dCTP and dTTP), 500 μM (dAMP, dCMP, dGMP dTMP, dADP, dCDP and dTDP), 1 mM (ADP, CDP, GDP, UDP, ATP, CTP, GTP and UTP), 2 mM (AMP, CMP, GMP and UMP) in 50% methanol were prepared and stored at –20 °C in brown glass bottles until use. The isotope-labeled internal standard (IS) solutions of AMP-¹³C₁₀,¹⁵N₅ and ATP-¹³C₁₀,¹⁵N₅ were diluted to a concentration of 20 μM in 85% methanol respectively and stored as above. Calibration curve standards and quality control (QC) samples were obtained by mixing and diluting the corresponding stock solutions and IS solutions to appropriate concentrations with analyte-free cell matrix, which was the cell lysate extraction with 85% methanol, and stripped from endogenous interference with activated carbon at 60 mg/mL [26,27].

2.3. Sample preparation and derivatization

HCT 116 cells were maintained with McCoy's 5A medium containing 10% (V/V) FBS, 100 U/mL penicillin and 100 μg/mL

streptomycin in a 37 °C humidified incubator with 5% CO₂. After incubation, cells were washed with ice-cold PBS once and then treated with trypsin. Each digested sample was re-suspended in 10 mL of ice-cold PBS, followed by centrifugation for 5 min (300 g, 4 °C); then the pellets were stored at –80 °C until use. Cell pellets were treated with cold 85% methanol (2×10^6 cells/100 μ L) containing 2 μ M AMP-¹³C₁₀, ¹⁵N₅ and 2 μ M ATP-¹³C₁₀, ¹⁵N₅, then vortexed for 1 min and placed on ice for 10 min. After centrifugation (13,000 g) at 4 °C for 15 min, the supernatant was transferred into another tube. The derivatization reaction was initiated by adding 75 μ L of derivatization reagent MTBSTFA into 200 μ L of cell lysis with 85% methanol and completed over 5 min with consistent vortex. Then derivatization samples were centrifuged (13,000 g) at 4 °C for 10 min, and 25 μ L of the supernatant was injected into LC-MS/MS system for analysis.

2.4. LC-MS/MS analysis

2.4.1. Instrumentation

All experiments were carried out on a Thermo Fisher TSQ LC-MS/MS system (Thermo Fisher Scientific Co., San Jose, CA, USA) consisting of an Accela Autosampler, an Accela pump, and a Quantum Access triple quadrupole mass spectrometer. All system control, data acquisition, mass spectral data evaluation, and data processing were performed using XCalibur software version 2.0.7 (Thermo Fischer, San Jose, CA, USA) and Thermo LCquan software version 2.5.6 (Thermo Fischer).

2.4.2. LC-MS/MS conditions

Chromatographic separation was performed on a Sepax GP-C₁₈ column (2.1 mm \times 150 mm, 1.8 μ m; Sepax Technologies, Inc., Newark, DE, USA) with a mobile phase consisting of 5 mM ammonium acetate aqueous solution (A) and acetonitrile (B). The column was maintained at 35 °C and the HPLC system was set up to operate at a flow rate of 0.2 mL/min under the following linear gradient conditions: 0–2 min, 5 %–16% B; 2–7 min, 16 %–30% B; 7–9 min, 30 %–55% B; 9–9.2 min, 55 %–5% B; 9.2–20 min, 5% B. MS conditions were as follows: ESI in negative mode, vaporizer temperature 290 °C, capillary temperature 320 °C, sheath gas pressure 50 psi, auxiliary gas pressure 20 psi and spray voltage 2600 V. Full scan and product ion modes with the range of m/z 100–900 were performed, and the quantitative data were acquired in multiple reactions monitoring (MRM) mode.

2.5. Method development and validation

The method was validated according to the FDA Guidance for Industry: Bioanalytical Method Validation (FDA, 2018) [28]. In brief, selectivity was assessed by analyzing the analyte-free cell samples that were pre-treated with activated carbon and lower limit of quantification (LLOQs) from six different sources, and comparing their peak areas. The peak areas of co-eluting interfering peaks or background noise should be <20% and <5% that of analytes and IS, respectively, at the LLOQs. Linearity was evaluated by calibration standard assays in duplicate on three consecutive days. The calibration curves consisting of seven calibrator levels were constructed using least-squares linear regression analysis with $1/x^2$ weighting by plotting the peak area ratios of the analyte to the corresponding IS against the concentrations. ATP-¹³C₁₀, ¹⁵N₅ was used as IS for nucleoside triphosphates and AMP-¹³C₁₀, ¹⁵N₅ was used as IS for nucleoside mono- and diphosphates, respectively. Each calibration curve with a correlation coefficient (r^2) higher than 0.990 was acceptable. Accuracy and precision were investigated by calculating the relative error (RE) and the coefficients of variation (CV%) of QCs at three concentration levels (low, medium and high),

which were assayed in five replicates on three consecutive days. The accuracy and intra- and inter-batch precision were considered acceptable if RE and CV were within $\pm 15\%$. Autosampler stability was evaluated at 4 °C for 24 h by measuring replicates ($n=5$) of LQC, MQC, and HQC samples. Analytes were considered stable when the calculated concentration was within $\pm 15\%$ of the theoretical value. The matrix effect was evaluated by analyzing two sets of samples at LQC, MQC, and HQC levels: (A) QC samples with analyte-free cell matrix from five lots and (B) neat solution containing the same amount of nucleotides as QC samples, both of which were processed according to the established derivatization procedures. Matrix factor (MF) is the ratio of peak area in the presence of matrix (solution A) to the peak area in absence of matrix (solution B), and was calculated for each lot of matrix. Then, the IS normalized MF (IMF) was calculated by dividing the MF of the analyte by the MF of the IS. The relative standard deviation (RSD) of IMF calculated from the five lots of matrix should be <15%.

2.6. Effect of 10-hydroxycamptothecin on nucleotides and cell cycle distribution analysis

HCT 116 cells were also cultured as mentioned above. After the treatment with 10-hydroxycamptothecin for 24 h, all the samples were harvested and washed with ice-cold PBS and trypsinized. Then they were divided into two equal parts and centrifuged at 1,500 g for 5 min. One of them was applied to determine the concentrations of RNs and dRN pools. The other was suspended to be fixed with cold 70% (V/V) ethanol and stored overnight at –20 °C for cell cycle distribution analysis. Subsequently, the fixed cells were collected, re-suspended in cold PBS and incubated with propidium iodide (PI, Sigma Aldrich Chemical Co., St. Louis, MO, USA) containing 0.05% RNase A (Sigma Aldrich) at room temperature for 30 min in the dark. Last, cell cycle distribution profile after the staining treatment was assessed by a flow cytometer (Muse™ cell analyzer, Merck Millipore, Darmstadt, Germany). The percentages of cells in G0/G1, S and G2/M phases were analyzed by using MODFIT software (Verity Software House, Topsham, ME, USA).

3. Results and discussion

3.1. MTBSTFA derivatization of nucleotides

Nucleotides are a kind of high polar components with three molecular fragments including a nitrogenous base (either a purine or a pyrimidine), a five carbon sugar and one or more phosphate groups [29]. Hence, they are weakly retained on an RPLC column. Silylation is a powerful tool to enhance highly polar compounds retention on RPLC, as well as improving ESI response in the mass spectrometry. In this study, silylation based on MTBSTFA was used to modify the nucleotide compounds for the sake of generating new products with better chromatographic properties. This derivatization reaction was operated under mild reaction conditions and quantitatively completed with good yields.

Fig. 1 shows the full scan and product ion mass spectra of silyl derivatives of the representative cytidine phosphates (CMP, CDP and CTP). In the precursor ion full scan spectra, the $[M - H]^-$ precursor ions were used for target compounds. From this figure, CMP (m/z 322) could be completely derivatized by MTBSTFA and derivatization product (m/z 436) after the introduction of a *t*-butyldimethylsilyl with increasing mass of 114 was obviously observed, indicating that the derivatization reaction could fully completed with a good yield. The same results were confirmed in other nucleotide derivatives based on the above pattern. In secondary ion mass spectra, m/z 211 and 273 were the most abundant fragments for MTBSTFA derivatives. The possible fragment

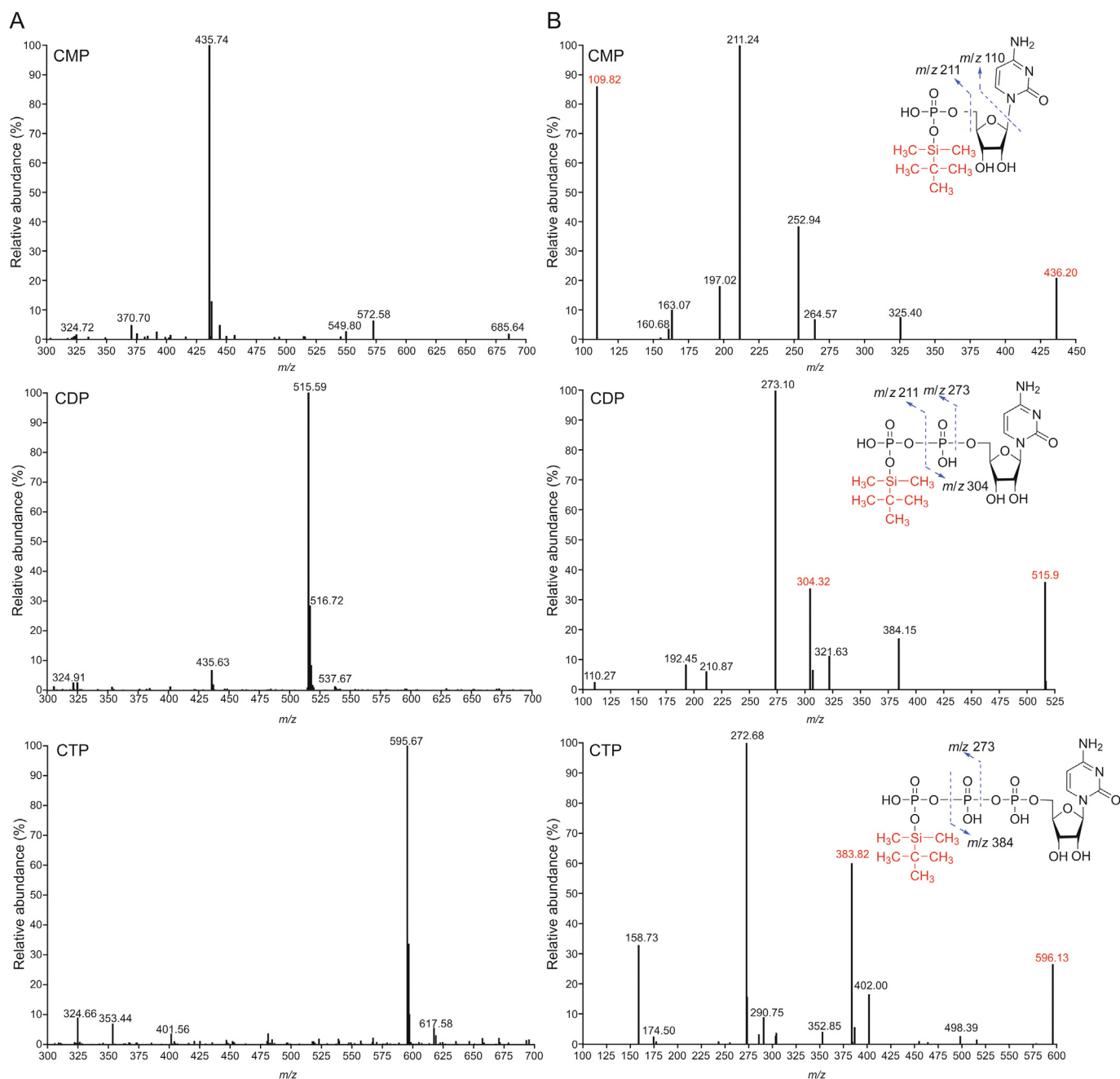


Fig. 1. (A) Full scan and (B) product ion mass spectra of silyl derivatives of the representative cytidine phosphates.

pathways are provided in Fig. 1B. Based on the secondary mass spectral fragmentation pattern, we confirmed that the silyl group was bonded with the phosphate groups. Also, each of these derivatives presented some other characteristic fragments for the analyzed compounds, such as the ions of base, base combined with pentose or nucleoside combined with a phosphate group, which made identification very reliable.

3.2. Optimization of the MTBSTFA derivatization

3.2.1. Effects of time and temperature

It is well known that reaction time can directly affect the derivatization reaction equilibrium and further exerts a great influence on the analysis sensitivity of analytes [30]. The reactions

were performed at 5, 10, 20 and 40 min, respectively. The MTBSTFA derivatization reaction could be rapidly completed in 5 min and no extra contribution to the analytical response of the derivatives was observed with increasing reaction time. Finally, 5 min was selected as the total reaction time because long reaction time may lead to degradation of RNs and dRN.

Temperature is another important factor that can affect the MTBSTFA derivatization [31]. In order to investigate the impact of temperature on MTBSTFA derivatization, the reaction was conducted at room temperature (about 25 °C), 37 °C, and 45 °C. It was found that reaction temperature had a significant effect on the complexity of derivatization products. Briefly, the vice-products with more than one t-butyldimethylsilyl group turned out to be larger after increasing the reaction temperature. However, the ratio

was still lower than 50% even at 45 °C for 40 min, which demonstrated that it was hard to completely convert more than one silyl group on the analytes due to its potential sterically hindered effect [30]. Consequently, reaction at room temperature was optimal for the stability of derivatives with only one silyl group.

3.2.2. Effect of water content

Apart from the reaction time and temperature, the extraction solvent can also influence the derivatization reaction [32]. It is known that water has an adverse effect on both the reaction and the stability of the derivatives for silylation [21,30,33]. It is ideal to conduct MTBSTFA derivatization reaction without water; however, preparing nucleotides solutions in pure methanol is impossible, especially for nucleoside di- and triphosphates. Therefore, the effect of water was evaluated by preparing standard solutions in methanol containing 5%, 10%, 15%, 20%, and 25% (V/V) of water in order to optimize the suitable reaction solvent. The obtained chromatographic peak areas of RNs in each sample were compared respectively. The peak area of each analyte with methanol at 5% water was used as the standard and the relative response of each analyte with different water content was compared with the standard (Fig. 2). The yields for most of the derivatization compounds slightly decreased with increasing water content from 5% to 15%. Comparatively, it exhibited a dramatically decreasing trend when the percentage of water in reaction mixture was above 20% and the relative responses were almost lower than half of the initial methanol solution at 5% water especially for di- and triphosphate. This may be caused by more competition between the analytes and water for reacting with MTBSTFA. In short, traces of water up to 15% have similar effects on the derivatization reaction. However, the response declined significantly when the aqueous content was over 15%. Hence, 15% water content was finally selected.

3.2.3. Sample preparation

Sample preparation was probably the major critical step for the determination of endogenous nucleotides in biological samples that could affect the analysis efficiency and the accuracy of results significantly. According to a former study on determination of intracellular nucleotides [34], the first step of sample pre-treatment was to lyse the cells or organelles and block the metabolism of nucleotides immediately by inactivation of the enzymes. A precipitation step constituted the identical procedure common to all methods previously described. Perchloric acid (PCA), trichloroacetic acid (TCA), methanol, acetonitrile or a mixture of methanol or acetonitrile with different proportions of water were the most commonly used protein precipitation and extraction reagents [35]. PCA and TCA deproteinizations were time-consuming for the

subsequent requirement of neutralization using basic solutions and the finally extracted samples contained too much water that would deplete the derivatization reagent. Although acetonitrile was commonly used as a reaction solvent in silylation, its poor dissolving capability of nucleotides resulted in the disability of extraction from cell lysis. Therefore, methanol and a series of methanol/water mixtures were investigated to obtain good extraction efficiency and derivatization yield. Methanol was firstly employed to lyse cells and extract nucleotides. However, only nucleoside monophosphates and a part of nucleoside diphosphates could be detected, and there was just extremely low detection signal of nucleoside triphosphates. Considering the low solubility in methanol, we speculated that there were few di- and triphosphates in the cell extraction. So different proportions of water contents were evaluated and optimized: 5, 10, 15, 20 and 25% (V/V). Finally, 85% methanol (with 15% water) was selected to precipitate the protein which was excellent for interference removal and recovery of all the analytes.

Recently, solid phase extraction (SPE) after protein precipitation has been more and more applied in cellular nucleotides determination [8,16] to enrich analytes and reduce endogenous interference. We also evaluated the effect of SPE. However, the complex elution solution inhibited the derivatization reaction and no expected products were observed.

3.3. Chromatographic separation and mass spectral characteristics of derivatized compounds

Generally, the endogenous RNs and dRNAs are too polar to be retained on the RPLC column under conventional chromatographic conditions. Hence, RNs and dRNAs including mono-, di- and triphosphate nucleotides were reacted with MTBSTFA. In our study, several elution solvents were tried, including methanol, acetonitrile, water, water containing 0.1% formic acid, and 5 mM ammonium acetate (pH 6.8). Acetonitrile and 5 mM ammonium acetate were selected as gradient elution solutions because of their better separation and peak shapes. Finally, an LC-MS/MS based assay was developed for quantitatively profiling these endogenous molecules in a 9 min run with good reproducibility as well as sensitivity. Since the main derivatives of nucleotides with MTBSTFA could only occupy one hydroxyl group of phosphonates, the polarity of nucleoside triphosphates was higher than that of diphosphates, then monophosphates. The MRM chromatograms of derivatives of all the analytes are presented in Fig. 3. As shown in this figure, the triphosphate nucleotides mainly appeared with the retention time in the range of 3.9–4.4 min and the diphosphate nucleotides were eluted during 5.3–6.1 min. Finally, the monophosphate nucleotides were eluted in less than 9 min. The order of these characteristic

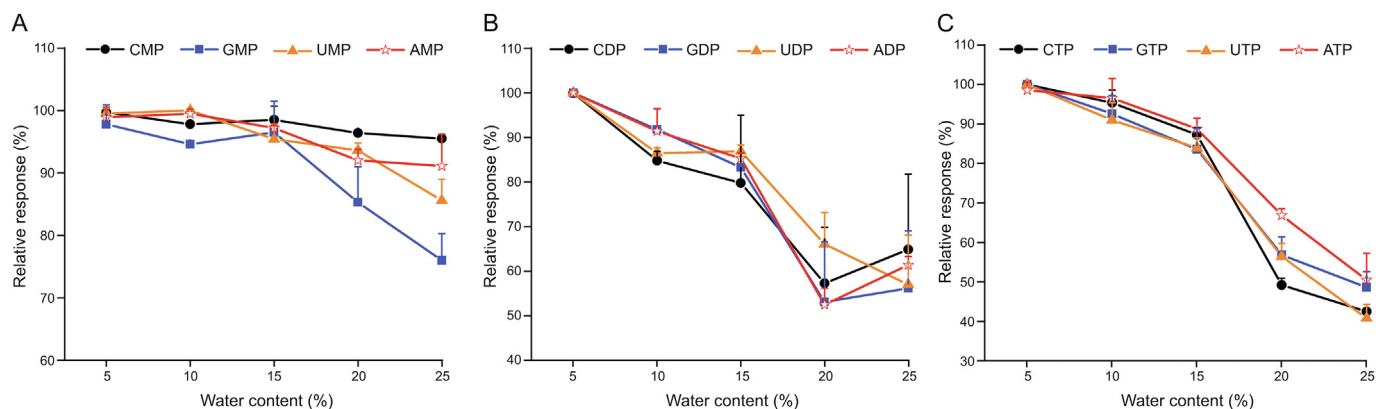


Fig. 2. Effects of water content on nucleoside mono- (A), di- (B) and tri- (C) phosphates standard solutions.

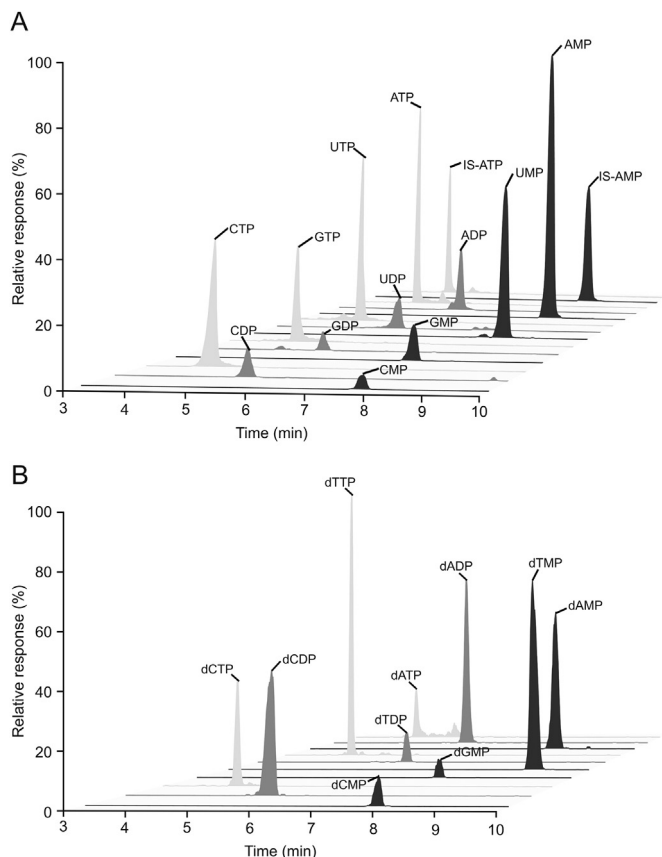


Fig. 3. Selected reconstructed MRM chromatograms of derivatives of (A) RNs and (B) dRN using the established LC-MS/MS method.

peaks may attribute to their polar difference. The completely chromatographic separation among the mon-, di- and triphosphate

Table 1

Retention time, mass spectrometric parameters, and linear range or the quantification of each nucleotide derivative and internal standard in negative ion mode in the LC-MS/MS method.

Analyte	Retention time (min)	Prototype [M – H] [–] (m/z)	MTBSTFA derivative				
			Precursor ion → Product ion (m/z)	Tube lens (V)	Collision energy (eV)	Linear range (μM)	LLOQ (μM)
AMP	8.23	346	460 → 134	157	26	0.2–20	0.2
ADP	5.78	426	540 → 328	129	15	0.2–20	0.2
ATP	4.37	506	620 → 408	116	18	0.4–40	0.4
CMP	7.77	322	436 → 110	176	17	0.2–20	0.2
CDP	5.37	402	516 → 304	95	18	0.2–20	0.2
CTP	3.94	482	596 → 384	99	20	0.4–40	0.4
GMP	7.46	362	476 → 150	176	27	0.1–10	0.1
GDP	5.38	442	556 → 344	130	16	0.2–20	0.2
GTP	4.29	522	636 → 424	118	16	0.4–40	0.4
UMP	8.20	323	437 → 111	127	21	0.2–20	0.2
UDP	5.57	403	517 → 305	110	13	0.2–20	0.2
UTP	4.29	483	597 → 385	95	19	0.4–40	0.4
dAMP	8.33	330	444 → 134	150	33	0.004–0.4	0.004
dADP	5.96	410	524 → 273	141	21	0.1–10	0.1
dATP	4.38	490	604 → 273	132	21	0.02–2	0.02
dCMP	7.87	306	420 → 211	160	23	0.004–0.4	0.004
dCDP	5.52	386	500 → 273	134	22	0.1–10	0.1
dCTP	4.33	466	580 → 273	122	21	0.02–2	0.02
dGMP	7.55	346	460 → 150	109	20	0.001–0.1	0.001
dTMP	8.93	321	435 → 125	133	24	0.004–0.4	0.004
dTDP	6.01	401	515 → 273	150	22	0.1–10	0.1
dTTP	4.37	481	595 → 273	134	24	0.02–2	0.02
IS-AMP	8.20	361	475 → 144	214	33	–	–
IS-ATP	4.37	521	635 → 273	108	28	–	–

nucleotides not only indicated their detectable polarity differences, but also enhanced the specificity via removing mis-annotation of in-source fragmentation [36]. Both positive and negative ionization modes were compared for these analytes, and the negative mode was more selective and sensitive than that of the positive mode due to the high polarity and the acidic nature of the target analytes. To optimize the MS responses, some instrument parameters including tube lens and collision energy were optimized and the settings of the mass spectrometric parameters are listed in Table 1.

3.4. Method validation

In this work, activated charcoal was used to prepare the analyte-free surrogate matrix for method validation according to previous study [27]. Briefly, activated charcoal was added to the broken cells with the ratio of 60 mg/mL. The cell suspension containing activated charcoal was shaken for 4 h. Analyte-free matrices were collected after centrifugation (12,700 r/min) for 40 min at 4 °C, and stored at –80 °C until utilized. The analyte-free matrix showed no obvious interfering peaks for any of the analytes when determined by the established LC-MS/MS method, indicating that activated charcoal could efficiently remove nucleotides RNs and dRN from cell lysis. The validation data are summarized in Tables 1 and 2. All the calibration curves exhibited good linearities with correlation coefficients (r^2) > 0.99. The LLOQ for the tested RNs and dRN were in the range of 0.1–0.4 and 0.001–0.1 μM, respectively. The intra-day precision (CV) of the low, medium and high QC levels was respectively in the range of 3.2%–12.9%, 1.3%–14.4%, and 3.1%–12.4%. The inter-day precision (CV) of the three spiked concentrations was 6.3% to 13.3%, 3.4%–13.3%, and 4.7%–14.8%, separately. The intra-day and inter-day accuracy (RE) was respectively in the range of –10.8% to 9.4% and –10.4% to 11.2%. No significant matrix effect was observed within the range from 91.1% to 113.9%. The tested RNs and dRN derivatives were confirmed to be stable in the autosampler at 4 °C for 24 h with RE in the range of 86.4%–112.2% and RSD less than 10.4%. Therefore, it is suggested that this method can be used to quantify endogenous RNs and dRN in a sensitive and reproducible manner.

Table 2
Intra- and inter-day precision, accuracy, stability and matrix effect of the developed method.

Analyte	Added (μM)	Precision		Accuracy		Stability (n=5)		Matrix effect (n=5)		
		Intra-day CV (%)	Inter-day CV (%)	Intra-day RE (%)	Inter-day RE (%)	Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RE (%)	RSD (%)
AMP	0.6	6.2	10.2	3.4	4.8	110.7 ± 2.5	2.2	104.9 ± 3.3	4.9	3.1
	3.0	7.6	9.2	6.6	6.7	110.6 ± 1.7	1.5	109.5 ± 1.1	9.5	1.0
	15.0	4.2	6.3	-1.6	-2.8	99.4 ± 4.9	5.0	107.9 ± 2.8	7.9	2.6
ADP	0.6	3.6	9.7	-0.5	-2.2	109.0 ± 3.3	3.1	112.4 ± 4.6	12.4	4.1
	3.0	10.2	9.5	-0.3	-0.2	107.9 ± 2.8	2.6	95.4 ± 3.7	-4.6	3.8
	15.0	10.3	8.4	-1.8	0.7	105.5 ± 4.4	4.2	101.1 ± 8.7	1.1	8.6
ATP	1.2	8.9	11.2	4.3	7.4	104.3 ± 4.9	4.7	100.9 ± 5.1	0.9	5.0
	6.0	5.6	9.5	3.1	6.2	103.1 ± 2.6	2.5	104.8 ± 3.6	4.8	3.5
	30.0	4.3	4.7	0.9	0.6	100.9 ± 4.4	4.3	99.5 ± 4.9	-0.5	4.9
GMP	0.3	12.7	11.1	-7.1	2.2	95.3 ± 7.2	7.6	106.6 ± 6.3	6.6	5.9
	1.5	14.4	10.7	-10.8	1.5	86.4 ± 1.9	2.2	104.3 ± 4.0	4.3	3.8
	7.5	6.2	9.0	0.1	-2.1	96.0 ± 6.7	7.0	98.8 ± 2.0	-1.2	2.1
GDP	0.6	4.4	8.5	0.1	-2.2	102.8 ± 4.8	4.6	98.9 ± 6.7	-1.1	6.7
	3.0	4.5	6.2	-1.0	-0.1	109.4 ± 1.3	1.2	95.0 ± 3.8	-5.0	4.0
	15.0	4.1	6.5	-0.5	0.8	94.9 ± 9.9	10.4	104.6 ± 9.7	4.6	9.2
GTP	1.2	5.4	10.1	2.3	-4.1	102.3 ± 3.2	3.1	107.9 ± 6.3	7.9	5.8
	6.0	5.3	9.7	-3.1	-4.5	96.9 ± 2.1	2.2	96.0 ± 4.1	-4.0	4.3
	30.0	4.5	5.7	-0.4	-0.5	99.6 ± 4.1	4.1	101.3 ± 2.3	1.3	2.2
CMP	0.6	12.9	12.1	3.1	0.8	111.1 ± 1.4	1.2	96.6 ± 6.9	-3.4	7.1
	3.0	14.1	10.9	7.8	4.5	112.2 ± 1.5	1.3	107.9 ± 3.1	7.9	2.9
	15.0	3.2	11.6	1.0	-5.3	104.3 ± 7.1	6.8	100.8 ± 2.3	0.8	2.2
CDP	0.6	6.9	8.1	-2.6	-3.0	104.4 ± 7.0	6.7	108.5 ± 2.6	8.5	2.4
	3.0	5.4	8.2	1.8	0.3	109.9 ± 2.4	2.2	91.4 ± 3.8	-8.6	4.2
	15.0	3.8	9.2	0.1	1.6	92.6 ± 1.7	1.8	105.4 ± 9.4	5.4	8.9
CTP	1.2	8.4	9.9	5.1	6.6	105.1 ± 3.5	3.3	102.0 ± 4.6	2.0	4.6
	6.0	1.3	3.4	-0.1	0.6	99.9 ± 1.2	1.2	101.0 ± 4.0	1.0	4.0
	30.0	5.9	7.1	2.9	-2.0	102.9 ± 3.2	3.1	106.6 ± 1.0	6.6	0.9
UMP	0.6	6.6	11.7	3.9	5.4	108.1 ± 2.3	2.1	102.2 ± 3.2	2.2	3.2
	3.0	4.4	7.4	2.7	4.3	109.1 ± 1.6	1.4	110.3 ± 1.9	10.3	1.7
	15.0	5.1	8.0	-3.3	-4.6	99.6 ± 5.8	5.8	102.4 ± 1.8	2.4	1.8
UDP	0.6	5.5	6.3	0.2	1.3	98.9 ± 2.4	2.4	91.1 ± 7.3	-8.9	8.0
	3.0	5.7	5.6	0.6	-0.4	99.2 ± 1.4	1.4	110.0 ± 4.1	10.0	3.8
	15.0	7.8	7.0	-1.2	-1.1	100.6 ± 9.3	9.2	95.5 ± 4.4	-4.5	4.7
UTP	1.2	4.1	13.3	-0.1	-2.8	99.9 ± 4.0	4.0	104.8 ± 5.5	4.8	5.2
	6.0	5.0	13.3	2.8	-4.8	102.8 ± 2.3	2.2	105.4 ± 8.2	5.4	7.8
	30.0	4.9	11.6	3.7	-4.0	103.7 ± 1.2	1.2	97.4 ± 1.9	-2.6	2.0
dAMP	0.012	11.8	12.6	4.7	4.5	112.0 ± 2.4	2.1	109.2 ± 10.3	9.2	9.5
	0.06	4.7	10.0	2.3	5.5	112.0 ± 2.1	1.8	112.2 ± 2.9	12.2	2.6
	0.3	7.3	6.9	-3.5	-3.7	98.3 ± 4.8	4.9	104.0 ± 4.0	4.0	3.8
dADP	0.3	8.0	10.4	3.8	-3.3	107.8 ± 5.7	5.3	104.3 ± 3.3	4.3	3.2
	1.5	10.6	8.8	-5.4	-2.0	110.7 ± 1.8	1.6	91.5 ± 4.3	-8.5	4.7
	7.5	9.3	10.2	3.8	4.6	110.7 ± 4.0	3.6	99.0 ± 8.7	-1.0	8.7
dATP	0.12	7.7	11.5	5.3	1.6	93.4 ± 6.1	6.6	99.0 ± 1.5	-1.0	1.5
	0.6	10.0	12.3	-4.5	4.4	103.3 ± 5.3	5.1	108.0 ± 5.9	8.0	5.5
	3.0	6.1	12.0	-1.1	4.9	110.6 ± 3.7	3.4	107.4 ± 5.4	7.4	5.1
dGMP	0.003	12.3	10.0	2.2	1.1	90.9 ± 5.1	5.6	107.5 ± 9.9	7.5	9.2
	0.015	5.8	9.9	-1.1	3.4	93.6 ± 2.6	2.8	104.8 ± 12.5	4.8	11.9
	0.075	9.8	12.4	4.8	7.0	97.8 ± 6.5	6.6	113.9 ± 1.6	13.9	1.4
dCMP	0.012	9.1	11.7	3.3	6.4	108.7 ± 4.4	4.0	97.9 ± 8.6	-2.1	8.8
	0.06	9.2	11.5	1.7	4.7	107.0 ± 7.1	6.6	102.5 ± 9.0	2.5	8.7
	0.3	3.1	9.9	-1.3	3.0	105.8 ± 5.0	4.7	96.1 ± 1.8	-3.9	1.8
dCDP	0.3	5.6	12.1	1.1	-3.4	99.2 ± 7.6	7.7	102.8 ± 7.2	2.8	7.0
	1.5	6.7	11.1	3.2	4.7	99.9 ± 1.1	1.1	101.4 ± 4.8	1.4	4.7
	7.5	7.8	9.0	3.9	-0.1	102.1 ± 4.1	4.0	103.0 ± 9.9	3.0	9.6

(continued on next page)

Table 2 (continued)

Analyte	Added (μM)	Precision		Accuracy		Stability ($n=5$)		Matrix effect ($n=5$)		
		Intra-day CV (%)	Inter-day CV (%)	Intra-day RE (%)	Inter-day RE (%)	Mean \pm SD (%)	RSD (%)	Mean \pm SD (%)	RE (%)	RSD (%)
dCTP	0.12	5.7	10.3	3.1	2.2	103.1 \pm 2.7	2.6	103.7 \pm 0.6	3.7	0.5
	0.6	6.6	8.8	2.1	-1.7	102.1 \pm 4.6	4.5	108.1 \pm 7.6	8.1	7.1
	3.0	10.4	14.8	-7.8	-10.4	92.2 \pm 2.3	2.5	103.6 \pm 3.2	3.6	3.1
dTMP	0.012	8.1	9.7	5.4	2.7	98.8 \pm 6.9	7.0	105.7 \pm 10.0	5.7	9.5
	0.06	4.3	9.1	-0.6	1.8	102.7 \pm 1.9	1.9	111.6 \pm 4.3	11.6	3.8
	0.3	12.4	14.4	-9.4	-7.2	88.4 \pm 3.6	4.0	102.0 \pm 3.7	2.0	3.6
dTDP	0.3	8.3	6.4	-4.0	-1.4	100.3 \pm 6.1	6.1	106.1 \pm 3.2	6.1	3.0
	1.5	4.5	8.5	-0.2	-2.0	101.6 \pm 5.7	5.6	100.1 \pm 6.3	0.1	6.3
	7.5	8.3	8.8	3.3	4.3	106.0 \pm 2.2	2.0	98.8 \pm 8.4	-1.2	8.5
dTTP	0.12	3.2	9.4	-0.3	2.9	99.7 \pm 2.9	2.9	106.2 \pm 8.0	6.2	7.5
	0.6	9.9	12.8	-3.2	-7.0	92.5 \pm 2.2	2.4	104.8 \pm 4.1	4.8	3.9
	3.0	12.3	13.6	9.4	11.2	109.4 \pm 3.6	3.3	108.2 \pm 4.5	8.2	4.1

Note: CV, coefficients of variation; RE: relative error; SD: standard deviation; RSD: relative standard deviation.

Table 3

Amounts of RNs and dRN pools in HCT 116 cells treated with different dosages of 10-hydroxycamptothecin (pmol/ 10^6 cells, $n=3$).

Analyte	Control	0.1 μM	1.0 μM
AMP	1174.4 \pm 77.8	3134.9 \pm 159.0**	52.8 \pm 2.7**
ADP	3088.3 \pm 107.4	7838.3 \pm 348.9**	710.2 \pm 129.1**
ATP	3937.3 \pm 99.3	6728.8 \pm 184.8**	6855.5 \pm 92.2**
GMP	141.9 \pm 17.4	266.3 \pm 42.3**	26.7 \pm 1.3**
GDP	233.2 \pm 16.1	856.8 \pm 70.7**	54.5 \pm 8.5**
GTP	212.8 \pm 22.7	621.3 \pm 41.9**	361.3 \pm 6.5**
CMP	214.8 \pm 14.1	575.6 \pm 19.8**	140.7 \pm 3.1**
CDP	300.3 \pm 23.7	864.2 \pm 70.5**	69.2 \pm 3.8**
CTP	230.4 \pm 24.4	621.4 \pm 84.3**	624.0 \pm 45.2**
UMP	775.2 \pm 12.4	1648.8 \pm 66.1**	118.9 \pm 8.5**
UDP	444.1 \pm 25.7	1206.4 \pm 66.1**	134.6 \pm 8.5**
UTP	500.3 \pm 23.4	950.0 \pm 110.2**	1671.2 \pm 36.2**
dAMP	0.4 \pm 0.1	2.7 \pm 0.1**	0.3 \pm 0.0*
dADP	11.2 \pm 0.9	36.3 \pm 6.7**	UDL
dATP	5.9 \pm 2.5	26.6 \pm 8.2**	7.1 \pm 2.1
dGMP	0.5 \pm 0.0	1.4 \pm 0.1**	0.2 \pm 0.0**
dCMP	3.2 \pm 0.4	29.0 \pm 2.0**	8.6 \pm 0.2**
dCDP	7.3 \pm 1.8	35.3 \pm 1.1**	UDL
dCTP	8.1 \pm 1.5	27.1 \pm 2.9**	13.2 \pm 0.9**
dTMP	1.5 \pm 0.4	4.8 \pm 0.6**	0.3 \pm 0.0**
dTDP	67.3 \pm 6.3	207.3 \pm 13.4**	10.7 \pm 18.5**
dTTP	33.1 \pm 9.2	82.7 \pm 3.9**	65.9 \pm 1.5**

Note: The result is expressed as mean \pm standard deviation (SD) ($n=3$); * $P < 0.05$, ** $P < 0.01$, compared with control group; UDL under detected limit of assay.

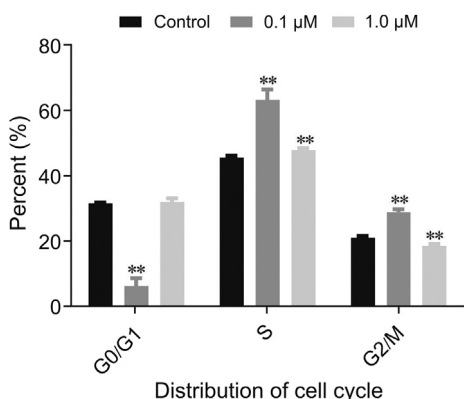


Fig. 4. The statistical analysis of cell cycle distribution in HCT 116 cells upon the exposure of 10-hydroxycamptothecin. ** $P < 0.01$, compared with control group.

As reported by Kuskovsky et al. [36], it is critical to choose suitable ISs for the quantification of nucleotides; thus the stable

isotope labeled ISs are optimal for their identity with the analytes in terms of structure, chemical property, and the obstacles during detection, such as in-source fragmentation and matrix effect. Practically, the application of isotope labeled ISs for every analytes was limited because of the requirements of multiple isotope labeled compounds, their commercial unavailability, the potential safety hazards and high cost. Thus, we just chose two readily-accessible ATP- $^{13}\text{C}_{10}$, $^{15}\text{N}_5$ and AMP- $^{13}\text{C}_{10}$, $^{15}\text{N}_5$ as ISs for nucleoside triphosphates, and for nucleoside mono- and diphosphates, respectively.

3.5. Determination of RNs and dRN of the selected HCT 116 cells

10-hydroxycamptothecin is a DNA-topoisomerase I inhibitor and it could restrain the proliferation of many tumor cells on the basis of inhibiting the synthesis of DNA. The investigation of cellular RNs and dRN could facilitate the understanding of underlying mechanism of this compound. The described novel method was applied for the determination of RNs and dRN concentrations in HCT 116 cells treated with 10-hydroxycamptothecin. The amounts of intracellular RNs and dRN pools as expressed in pmol/ 10^6 cell are shown in Table 3. These intracellular metabolic levels obviously varied in different groups. Briefly, the contents of all the RNs were much higher than those of the dRN. In addition, the adenosine ribonucleotides and thymidine deoxyribonucleotides contained the highest portion of RNs and dRN, respectively. After the incubation of 0.1 μM 10-hydroxycamptothecin, all the RNs and dRN pools in HCT 116 cells increased extremely compared with those of the control group ($P < 0.01$). By contrast, after the incubation of 1.0 μM 10-hydroxycamptothecine, the mono- and diphosphates mainly presented a significant decreasing tendency ($P < 0.01$) except dCMP. Interestingly, the contents of all the triphosphates were still higher than those of the control group ($P < 0.01$). The levels of RNs and dRN in eukaryotic cells vary on the basis of the cell cycle. In this study, cell cycle profiles were performed by using flow cytometry analysis at the same time. From the results in Fig. 4, the reduction of cell population in G0/G1 phase was accompanied by a concomitant increase in S phase when cells exposed to 0.1 μM 10-hydroxycamptothecine which was consistent with the previous study reported by Shao et al. [37]. Cellular RNs and dRN are dynamic during the progression of cell cycle and this process is related to the enzyme involved in their synthesis, especially ribonucleotide reductase (RR) [38,39]. Mammalian RR is composed of three sub-units, namely, RRM1, RRM2 and p53R2, which are expressed in a cell cycle-dependent manner [40]. In cycling cells, the RRM1

protein is metabolically stable throughout the cell cycle whereas the expression and degradation of RRM2 protein limit the S-phase-dependent activity of RR complex, leading to the high cellular dNTPs pools at S phase and low dNTPs pools outside S phase [41]. It could be supposed that 10-hydroxycamptothecin may have the effect of these enzymes and such hypothesis needs to be further studied.

4. Conclusions

An optimized LC-MS/MS method for the simultaneous quantitative of 22 endogenous RNs and dRN in cancer cells has been established after pre-column MTBSTFA derivatization. A one-step extraction of cells with 85% methanol followed by a simple derivatization reaction within 5 min at room temperature contributed to shortened analysis time. The derivatives were retained on the typical C₁₈ column and eluted by ammonium acetate and acetonitrile in 9 min. This approach has been validated and successfully applied to the analysis of nucleotides in HCT 116 cells exposed to 10-hydroxycamptothecin. It can be concluded that this newly described LC-MS/MS method for nucleotides detection and quantification is simple, rapid, specific and efficient. Further, it provides a new insight into the quantification of the metabolites of nucleoside analogs and other phosphonate-containing drugs like bisphosphonates.

CRedit author statement

Huixia Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original draft preparation; **Yan Li:** Methodology, Validation, Investigation, Writing - Original draft preparation; **Zheng Li:** Conceptualization, Formal analysis; **Christopher Wai-Kei Lam:** Writing - Reviewing and Editing; **Peng Zhu:** Resources; **Caiyun Wang:** Visualization; **Hua Zhou:** Writing - Reviewing and Editing; **Wei Zhang:** Writing - Reviewing and Editing, Supervision, Funding acquisition.

Declaration of competing interest

Authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by Science and Technology Development Fund, Macau SAR (File Nos.: 0052/2018/A2 and 0077/2019/A2) and the National Natural Science Foundation of China (Grant No.: 61827819).

References

- [1] M. Fasullo, L. Endres, Nucleotide salvage deficiencies, DNA damage and neurodegeneration, *Int. J. Mol. Sci.* 16 (2015) 9431–9449.
- [2] H. Gad, T. Koolmeister, A.-S. Jemth, et al., MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool, *Nature* 508 (2014) 215–221.
- [3] A. Suomalainen, B.J. Battersby, Mitochondrial diseases: the contribution of organelle stress responses to pathology, *Nat. Rev. Mol. Cell Biol.* 19 (2018) 77–92.
- [4] A.-K. Berglund, C. Navarrete, M.K. Engqvist, et al., Nucleotide pools dictate the identity and frequency of ribonucleotide incorporation in mitochondrial DNA, *PLoS Genet.* 13 (2017), e1006628.
- [5] A.M. Spiegel, Signal transduction by guanine nucleotide binding proteins, *Mol. Cell Endocrinol.* 49 (1987) 1–16.
- [6] B.B. Kahn, T. Alquier, D. Carling, et al., AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism, *Cell Metab.* 1 (2005) 15–25.
- [7] D. Garcia, R.J. Shaw, AMPK: mechanisms of cellular energy sensing and restoration of metabolic balance, *Mol. Cell* 66 (2017) 789–800.
- [8] Z. Kong, S. Jia, A.L. Chabes, et al., Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in biological samples by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry, *Nucleic Acids Res.* 46 (2018), e66.
- [9] E.M. Pfall, G.W. Aherne, V. Marks, The quantitative determination of 2'-deoxycytidine-5'-triphosphate in cell extracts by radioimmunoassay, *Anal. Biochem.* 154 (1986) 276–281.
- [10] Y. Huo, L. Qi, X.-J. Lv, et al., A sensitive aptasensor for colorimetric detection of adenosine triphosphate based on the protective effect of ATP-aptamer complexes on unmodified gold nanoparticles, *Biosens. Bioelectron.* 78 (2016) 315–320.
- [11] Y. Guo, S. Li, J. Liu, et al., Double functional aptamer switch probes based on gold nanorods for intracellular ATP detection and targeted drugs transportation, *Sens. Actuators B Chem.* 235 (2016) 655–662.
- [12] M. Mateos-Vivas, J. Domínguez-Álvarez, E. Rodríguez-Gonzalo, et al., Capillary electrophoresis coupled to mass spectrometry employing hexafluoro-2-propanol for the determination of nucleosides and nucleotide mono-, di- and tri-phosphates in baby foods, *Food Chem.* 233 (2017) 38–44.
- [13] C. Zhao, Y. Yang, L. Wei, et al., Simultaneous determination of intracellular nucleotides and coenzymes in *Yarrowia lipolytica* producing lipid and lycopen by capillary zone electrophoresis, *J. Chromatogr. A* 1514 (2017) 120–126.
- [14] A.H. Rageh, U. Pyell, Imidazolium-based ionic liquid-type surfactant as pseudostationary phase in micellar electrokinetic chromatography of highly hydrophilic urinary nucleosides, *J. Chromatogr. A* 1316 (2013) 135–146.
- [15] E. Fromentin, C. Gavegnano, A. Obikhod, et al., Simultaneous quantification of intracellular natural and antiretroviral nucleosides and nucleotides by liquid chromatography-tandem mass spectrometry, *Anal. Chem.* 82 (2010) 1982–1989.
- [16] T. Kamčeva, T. Bjānes, A. Svardal, et al., Liquid chromatography/tandem mass spectrometry method for simultaneous quantification of eight endogenous nucleotides and the intracellular gemcitabine metabolite dFdCTP in human peripheral blood mononuclear cells, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 1001 (2015) 212–220.
- [17] G. Zhang, A.D. Walker, Z. Lin, et al., Strategies for quantitation of endogenous adenine nucleotides in human plasma using novel ion-pair hydrophilic interaction chromatography coupled with tandem mass spectrometry, *J. Chromatogr. A* 1325 (2014) 129–136.
- [18] N.L. Padivitage, M.K. Dissanayake, D.W. Armstrong, Separation of nucleotides by hydrophilic interaction chromatography using the FRULIC-N column, *Anal. Bioanal. Chem.* 405 (2013) 8837–8848.
- [19] K. Inoue, R. Obara, T. Hino, et al., Development and application of an HILIC-MS/MS method for the quantitation of nucleotides in infant formula, *J. Agric. Food Chem.* 58 (2010) 9918–9924.
- [20] E. Johnsen, S.R. Wilson, I. Odsbu, et al., Hydrophilic interaction chromatography of nucleoside triphosphates with temperature as a separation parameter, *J. Chromatogr. A* 1218 (2011) 5981–5986.
- [21] J.M. Halket, V.G. Zaikin, Derivatization in mass spectrometry-1. Silylation, *Eur. J. Mass Spectrom.* 9 (2003) 1–21.
- [22] L. Zhang, H. Zhang, J. Wang, et al., Determination of trace level of cAMP in *Locusta migratoria manilensis* Meyen by HPLC with fluorescence derivation, *Int. J. Mol. Sci.* 7 (2006) 266–273.
- [23] A. Nordström, P. Tarkowski, D. Tarkowska, et al., Derivatization for LC-electrospray ionization-MS: a tool for improving reversed-phase separation and ESI responses of bases, ribosides, and intact nucleotides, *Anal. Chem.* 76 (2004) 2869–2877.
- [24] J. Flarakos, W. Xiong, J. Glick, et al., A deoxynucleotide derivatization methodology for improving LC-ESI-MS detection, *Anal. Chem.* 77 (2005) 2373–2380.
- [25] H.-X. Zhang, Y. Li, Z. Li, et al., Rapid and sensitive determination of four bisphosphonates in rat plasma after MTBSTFA derivatization using liquid chromatography-mass spectrometry, *J. Pharm. Biomed. Anal.* 190 (2020), 113579.
- [26] B. Zhu, H. Wei, Q. Wang, et al., A simultaneously quantitative method to profiling twenty endogenous nucleosides and nucleotides in cancer cells using UHPLC-MS/MS, *Talanta* 179 (2018) 615–623.
- [27] C. Hellmuth, M. Weber, B. Koletzko, et al., Nonesterified fatty acid determination for functional lipidomics: comprehensive ultrahigh performance liquid chromatography-tandem mass spectrometry quantitation, qualification, and parameter prediction, *Anal. Chem.* 84 (2012) 1483–1490.
- [28] U.S. Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry>. (Accessed 24 May 2018).
- [29] F.B. Rudolph, The biochemistry and physiology of nucleotides, *J. Nutr.* 124 (1994) 1245–1275.
- [30] C. Schummer, O. Delhomme, B.M. Appenzeller, et al., Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis, *Talanta* 77 (2009) 1473–1482.
- [31] H.G. Mol, S. Sunarto, O.M. Steijger, Determination of endocrine disruptors in water after derivatization with N-methyl-N-(tert-butyl)dimethyltrifluoroacetamide using gas chromatography with mass spectrometric detection, *J. Chromatogr. A* 879 (2000) 97–112.
- [32] H.-Y. Zhang, P.-P. Zhang, X.-X. Tan, et al., Derivatization method for the quantification of lactic acid in cell culture media via gas chromatography and applications in the study of cell glycometabolism, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 1090 (2018) 1–6.

- [33] T.G. Sobolevsky, A.I. Revelsky, B. Miller, et al., Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids, *J. Separ. Sci.* 26 (2003) 1474–1478.
- [34] S. Cohen, L.P. Jordheim, M. Megherbi, et al., Liquid chromatographic methods for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy: a review, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 878 (2010) 1912–1928.
- [35] C. Polson, P. Sarkar, B. Incedon, et al., Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 785 (2003) 263–275.
- [36] R. Kuskovsky, R. Buj, P. Xu, et al., Simultaneous isotope dilution quantification and metabolic tracing of deoxyribonucleotides by liquid chromatography high resolution mass spectrometry, *Anal. Biochem.* 568 (2019) 65–72.
- [37] S.L. Shao, W.W. Zhang, W. Zhao, et al., Apoptosis of human leukemia cell line K562 cells induced by hydroxycamptothecine, *Adv. Mater. Res.* 535-537 (2012) 2420–2424.
- [38] B. Liu, J. Großhans, The role of dNTP metabolites in control of the embryonic cell cycle, *Cell Cycle* 18 (2019) 2817–2827.
- [39] C.K. Mathews, DNA precursor metabolism and genomic stability, *Faseb. J.* 20 (2006) 1300–1314.
- [40] B. Yousefi, N. Samadi, Y. Ahmadi, Akt and p53R2, partners that dictate the progression and invasiveness of cancer, *DNA Repair (Amst)* 22 (2014) 24–29.
- [41] Y. Engström, S. Eriksson, I. Jildevik, et al., Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits, *J. Biol. Chem.* 260 (1985) 9114–9116.