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Rapid and selective simultaneous quantitative analysis of modified nucleosides using multi-column liquid chromatography-tandem mass spectrometry

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Abstract

Background: The profiles of modified nucleosides could act as useful biomarkers for cancer and cellular stress-induced diseases. However, there are no reports of high throughput and simultaneous quantitative methods for using biomarker evaluation and discovery at the bedside.

Methods: Modified nucleosides were separated on two CAPCELL PAK ADME S3 (100 mm × 2.1 mm i.d.; 3-μm particle size) analytical columns coupled with a CAPCELL PAK ADME cartridge (10 mm × 2 mm i.d.; 3-μm particle size) guard column. Both columns were used in tandem during multi-column LC analysis to reduce analysis time. Two mobile phases were used, including 20 mM ammonium acetate adjusted to pH 5.3 using acetic acid and 1.0 M ammonium acetate/acetonitrile/water/acetic acid (1/95/5/0.03, *v/v/v/v*), with the post-column addition of methanol to enhance ionization efficiency. Tandem mass spectrometry detection was performed using a triple quadrupole mass spectrometer equipped with a heated electrospray ionization source in selected reaction monitoring mode.

Results: Four major nucleosides and 11 modified nucleosides, including guanosine, adenosine, uridine (U), cytidine, inosine, 1-methyladenosine, 5-methylcytidine, 2′-O-methylcytidine, 3-methylcytidine, 7-methylguanosine (m^7G), 5-methyluridine (m^5U), pseudouridine, 2-thiocytidine, N^2-methylguanosine (m^2G), N^2,N^2-dimethylguanosine, 2-fluoro-2′-deoxyadenosine as an internal standard, and its isotopic isomers were separated within 7 min and analyzed within 10 min. This resulted in limits of quantitation of 0.50–5.00 ng mL^{-1}, except for m^2G (10.0 ng mL^{-1}), m^7G (12.5 ng mL^{-1}), U (12.5 ng mL^{-1}), and m^5U (50.0 ng mL^{-1}). This method provides a wide range of linearity, with correlation coefficients greater than 0.99 for all nucleosides. Both the accuracy and precision of this method satisfied criteria of <15% for higher concentrations and <20% for the lowest concentrations.

Conclusions: In this study, we describe a rapid and selective method that uses multi-column liquid chromatography with tandem mass spectrometry (LC-MS/MS) to simultaneously quantify modified nucleosides. This global analysis will be useful for evaluating modifications in RNA.

Keywords: Modified nucleoside, LC-MS/MS, Quantification, Multiple columns, Rapid analysis, tRNA

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Background

Modified nucleosides are mainly included in transfer RNA (tRNA) (Agris 2004), where tRNA modifications increase conformational stability (Anderson et al. 1998), identification (Shin et al. 2011), and translational fidelity (Crick 1966; Gerber and Keller 1999; Agris 2015). For example, 1-methyadenosine (m^1A) is necessary for conformational stability (Anderson et al. 1998), where the 2-thiocytidine (s^2C) occurring in the anticodon loop interferes with the inosine–adenosine interaction (Agris 2015). As such, tRNA modification has been considered essential for tRNA maturation and stability. Modified nucleosides contained in most tRNA have been associated with turnover (Topp et al. 1993; Nakano et al. 1993; Sander et al. 1986); therefore, modified nucleosides, such as m^1A, 2-methylguanosine (m^2G), and pseudouridine (Ψ), have been reported to be cancer biomarkers (Itoh et al. 1992; Jeng et al. 2009; Djukovic et al. 2010; Cho et al. 2009). Recent studies have shown that altering the tRNA modification profile plays a major role in the cellular stress response (Chan et al. 2010, 2012, 2015; Endres et al. 2015), where 5-methylcytidine (m^5C), 2′-O-methylcytidine (Cm), and N2,N2-dimethylguanosine (m^2_2G) reportedly increase under oxidative conditions created by H_2O_2; other oxidative stress reagents do not influence these modified nucleosides (Chan et al. 2010, 2012). Alkylating stress affects 3-methylcytidine (m^3C), where increasing m^3C promoted the translation of ACC- and ACT-rich messenger RNA (Chan et al. 2015). In addition, inosine (I), 5-methyluridine (m^5U), m^5C, Cm, 7-methylguanosine (m^7G), and m^1A were altered by replication stress (Endres et al. 2015). These cellular stress response markers may be candidate biomarkers of cellular stress-induced diseases. The profile of modified nucleosides could act as sensitive biomarkers for cancer and be useful as potential biomarkers for investigating cellular stress-induced diseases (Table 1).

A simultaneous quantitative analysis method is necessary to confirm the profile of modified nucleosides. This is because there are many structural isomers, regioisomers, and isotopic isomers in modified nucleosides, where adequate chromatographic separation is the key to simultaneous analysis of modified nucleosides. On the other hand, one of the goals of these analytical methods is their use as biomarkers for diagnoses at the bedside on a daily basis. In addition, biomarker evaluation and discovery necessitates the analysis of large sample sizes to ensure statistical reliability (Koulman et al. 2009); thus, the analytical methods applied must have a high throughput. Analytical methods for modified nucleosides include liquid chromatography (LC), ultra-performance LC (UPLC), gas chromatography, or capillary electrophoresis combined with ultraviolet or mass spectrometry (MS) (Russell and Limbach 2013; Struck et al. 2011; Lee et al. 2004; Basanta-Sanchez et al. 2016). Of these methods, LC-tandem MS (LC-MS/MS) analysis is the most frequently used for modified nucleoside determination due to its high selectivity and quantitative performance. Several studies have reported good chromatographic separation; however, these methods require long times to separate nucleosides (Djukovic et al. 2010; Cho et al. 2009; Struck et al. 2011; Lee et al. 2004). Rodríguez-Gonzalo et al. (2016) reported an analytical method that quickly and simultaneously determined nucleosides; however, this method has not been used to study significant cancer biomarkers or cellular stress markers, such as m^5C, Cm, and m^2_2G.

Table 1 RNA modification associated with disease and cellular dysfunction

Modified nucleoside	Related disease and cellular dysfunction	Reference
m^1A	Leukemia, lymphoma, breast cancer, replication stress, abnormal conformation of tRNA	(Anderson et al. 1998; Itoh et al. 1992; Cho et al. 2009)
I	Hepatocellular carcinoma, replication stress	(Jeng et al. 2009)
m^2G	Esophageal adenocarcinoma	(Djukovic et al. 2010)
m^7G	Replication stress	(Endres et al. 2015)
m^2_2G	Gastric cancer, intestinal cancer, lung cancer, esophageal adenocarcinoma, breast cancer, oxidative stress	(Nakano et al. 1993; Djukovic et al. 2010; Cho et al. 2009; Chan et al. 2010)
m^3C	Alkylating stress	(Chan et al. 2015)
m^5C	Oxidative stress, replication stress	(Chan et al. 2010, 2012)
Cm	Oxidative stress, replication stress	(Chan et al. 2010)
s^2C	Interference with codon—anti-codon interaction	(Agris 2015)
m^5U	Replication stress	(Endres et al. 2015)
Ψ	Gastric cancer, intestinal cancer, lung cancer, AIDS, leukemia, lymphoma	(Nakano et al. 1993; Itoh et al. 1992)

Basanta-Sanchez et al. (2016) demonstrated a highly sensitive, accurate, quantitative UPLC-MS/MS nucleosides analysis method; however, this method applies to cellular RNA modifications and is thus unsuitable for human biological samples, such as blood and urine, due to its narrow calibration range. Several studies have attempted to increase LC analysis throughput, while also showing the utility of the multiple column LC method (Korfmacher et al. 1999; Cass et al. 2001; Oertel et al. 2002; Orton et al. 2013; Lee et al. 2015; Patel et al. 2016). This approach is helpful for reducing the total run time without sacrificing chromatographic separation, as using multiple columns reduces separation and wash times. In this study, we developed a rapid, selective method that simultaneously quantifies modified nucleosides through a multi-column LC-MS/MS system.

Methods

Chemicals

A nucleoside test mix, purchased from Sigma-Aldrich (St. Louis, MO, USA), containing 50 μg/mL of cytidine (C), 25 μg/mL of guanosine (G), 25 μg/mL of I, 25 μg/mL of m^1A, 50 μg/mL of m^5C, 20 μg/mL of Cm, 100 μg/mL of m^3C methosulfate, 25 μg/mL of m^7G, 100 μg/mL of m^5U, 25 μg/mL of Ψ, 10 μg/mL of s^2C dehydrate, and 25 μg/mL of uridine (U) was used. Adenosine (A) and 2-fluoro-2′-deoxyadenosine (f^2dA) were purchased from Sigma-Aldrich. m^2G was purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). m_2^2G was purchased from Toronto Research Chemicals (Toronto, Canada). Methanol (MeOH) and acetonitrile (MeCN) of LC/MS grade were obtained from Kanto Chemical (Tokyo, Japan). LC/MS-grade ammonium acetate (CH_3COONH_4) and acetic acid (CH_3COOH) were obtained from Wako Pure Chemical Industries. Ultrapure-grade water was prepared using a PURELAB Ultra from Organo (Tokyo, Japan).

Standard solutions

Stock standard solutions of 100 μg mL^{-1} for A, m^2G, m_2^2G, m^3C, m^5U, and f^2dA; at 50.0 μg mL^{-1} for C and m^5C; at 25.0 μg mL^{-1} for m^1A, I, G, m^7G, U, and Ψ; at 20.0 μg mL^{-1} for Cm; and at 10.0 μg mL^{-1} for s^2C were prepared in 20.0 mM CH_3COONH_4, adjusted to a pH of 5.3 using CH_3COOH. These stock solutions were stored at −30 °C in brown glass bottles. Working solutions were prepared by appropriate dilution of these stock solutions.

LC-MS/MS conditions

The LC system was based on a NANOSPACESI-2 LC system (Shiseido, Tokyo, Japan), with a CAPCELL PAK ADME S3 (100 mm × 2.1 mm i.d.; 3-μm particle size; Shiseido, Tokyo, Japan) analytical column, coupled with a CAPCELL PAK ADME cartridge (10 mm × 2 mm i.d.; 3-μm particle size; Shiseido, Tokyo, Japan) guard column. Our multiple column LC system is composed of one auto sampler, two six-port valves, four pumps, and two analytical columns coupled with a guard column; this system selects the column in line with the mass spectrometer. Both columns are used in parallel during multi-column LC analysis. One column is used as an analytical column, while the other is washed and prepared for the next injection. Figure 1 shows flow diagrams and valve positions of this multiple column analysis setup, where method A is separated by column A and method B is separated by column B. Valve A was activated to switch between the two columns when the sample was injected. Valve B (the MS divert valve) was switched to MS/MS after 0.5 min of injection. All systems were controlled via the autosampler trigger signal. Two mobile phases were used, including 20 mM CH_3COONH_4 adjusted to pH 5.3 using CH_3COOH (mobile phase A) and 1.0 M CH_3COONH_4/MeCN/H_2O/CH_3COOH (1/95/5/0.03, *v*/*v*/*v*/*v*) (mobile phase B), with the post-column addition of MeOH to enhance ionization efficiency. Other LC condition parameters are summarized in Table 2.

MS/MS detection was performed using a triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source (TSQ Quantum Ultra, Thermo Fisher Scientific, San Jose, CA, USA). This system operated under the following conditions: positive mode ionization, spray voltage 4.0 kV, vaporizer temperature 450 °C, sheath gas pressure 60 psi, auxiliary pressure 40 psi, capillary temperature 300 °C, collision gas pressure 1.0 mTorr, tube lens offset 90 V, and collision energy 20 eV. Descriptions of selected reaction monitoring (SRM) transitions can be found in previous studies and are summarized in Table 3. (Lee et al. 2004; Su et al. 2014; Fan et al. 2006) The LC-MS/MS system was controlled by Xcalibur software (Thermo Fisher Scientific), which was also used to collect data.

Calibration

All peaks were automatically integrated using the Xcalibur software, and compound concentrations were calculated from calibration curves describing the relationship between peak ratios and areas, using f^2dA as an internal standard (IS). Calibration curves for system A ranged from 1.00–200 ng mL^{-1}; specifically, m^1A ranged from 1.25–500 ng mL^{-1}; I, G, C from 2.50–500 ng mL^{-1}; m^2G from 10.0–2000 ng mL^{-1};

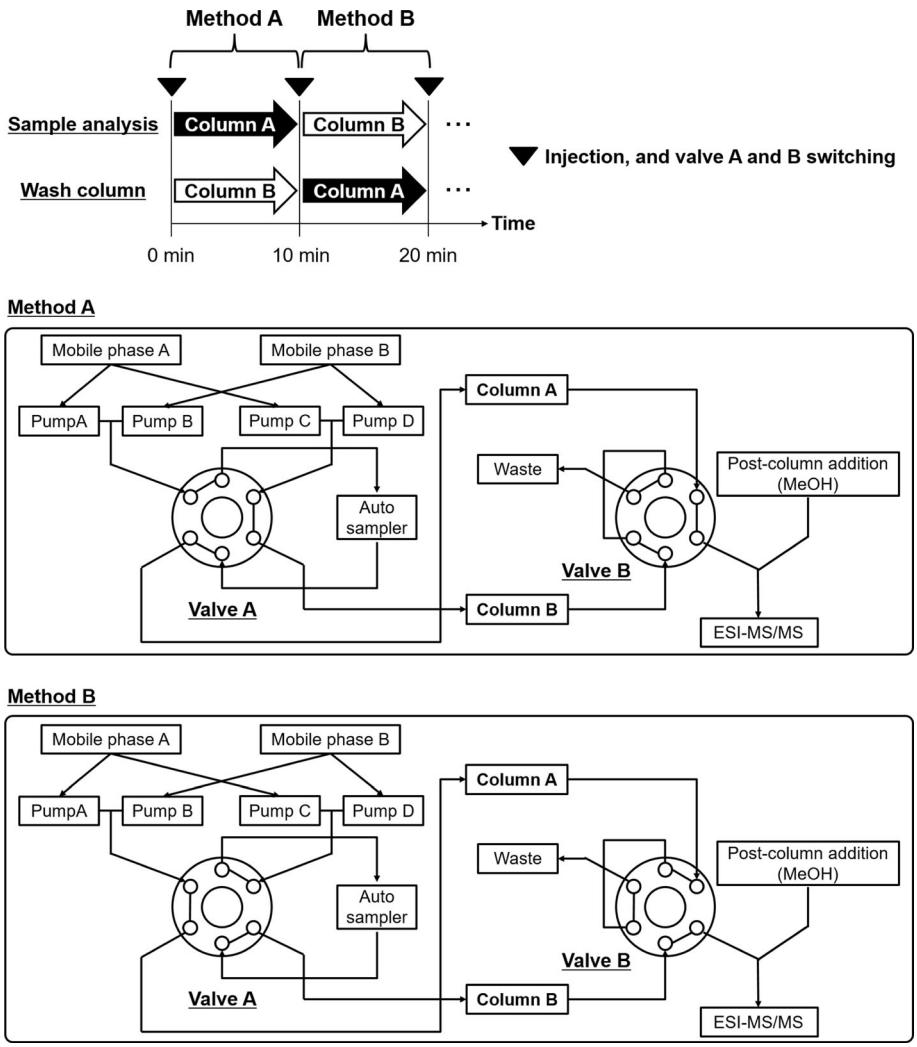

Fig. 1 Flow diagram outlining multi-column analysis. Method A is separated by column A, and method B is separated by column B. One column is used as the analytical column, while the other column is washed and prepared for the next injection

m^5C from 0.50–500 ng mL^{-1}; m^7G, U from 12.5–500 ng mL^{-1}; m_2^2G from 1.00–2000 ng mL^{-1}; m^5U from 50.0–2000 ng mL^{-1}; m^3C from 1.39–696 ng mL^{-1}; Cm from 1.00–400 ng mL^{-1}; s^2C from 4.39–175 ng mL^{-1}; and Ψ from 5.00–500 ng mL^{-1} (including an IS of 200 ng mL^{-1}). Calibration curve linearity was evaluated over at least six concentrations.

Precision and accuracy

To evaluate accuracy and precision, we analyzed four standard solution concentrations consisting of five samples on three separate days. Accuracy was calculated from the percentage deviation of the mean from the true value, and the precision was expressed as the relative error and coefficient of variation (CV, %).

Results and discussion

Chromatographic separation

Our MS/MS system was used to detect four major nucleosides and 11 modified nucleosides and f^2dA (IS), which were separated by HPLC in <10 min using a CAPCELL PAK ADME column (Fig. 2). The resultant retention times using the two-column, parallel system were as follows: C (2.30 min), U (2.71 min), Ψ (1.56 min), m^5C (2.27 min), m^3C (3.63 min), Cm (3.79 min), m^5U (3.96 min), s^2C (3.28 min), A (5.15 min), I (3.61 min), m^1A (2.99 min), G (3.75 min), m^7G (4.69 min), m^2G (3.39 min), m_2^2G (5.41 min), and f^2dA (IS) (6.16 min). The 10-min analytical time is half the previously reported time of 20 min (Djukovic et al. 2010; Cho et al. 2009; Russell and Limbach 2013; Struck et al. 2011; Lee et al. 2004;

Table 2 Optimal conditions for LC analysis of nucleosides

LC system	NANOSPACE SI-2 (SHISEIDO)
Analytical column	CAPCELL PAK ADME
	(100 × 2.1 mm i.d., 3-μm particle size, Shiseido)
Mobile phase	A, 20 mM CH_3COONH_4/H_2O
	(Adjusted to pH 5.3 using CH_3COOH)
	B, 1.0 M CH_3COONH_4/MeCN/H_2O/CH_3COOH
	=1:95:5:0.03 (*v/v/v/v*)
Analytical gradient	0.0–1.0 min; A/B = 100.0/0.0
	1.0–4.0 min; A/B = 100.0/0.0 → 91.4/8.6
	4.0–5.0 min; A/B = 91.4/8.6 → 88.6/11.4
	5.0–8.0 min; A/B = 88.6/11.4 → 79.9/20.1
	8.0–10.0 min; A/B = 79.9/20.1 → 0.0/100.0
Analytical flow rate	0.0–10.0 min; 400 μL/min
Wash gradient	0.0–4.0 min; A/B = 0.0/100.0
	4.0–5.0 min; A/B = 0.0/100.0 → 100.0/0.0
	5.0–10.0 min; A/B = 100.0/0.0
Wash flow rate	0.0–1.0 min; 400 μL/min → 700 μL/min
	1.0–4.0 min: 700 μL/min
	4.0–5.0 min; 700 μL/min → 400 μL/min
	5.0–10.0 min; 400 μL/min
Post-column addition	MeOH; 400 μL/min
MS divert valve	0.0–0.5 min; waste
	0.5–10.0 min; detector
Oven temperature	40 °C
Injection volume	3 μL

Table 3 LC-MS/MS and calibration curve parameters for nucleosides

Compound	t_R (min)	Transition (*m/z*)	Calibration range (ng mL^{-1})	Correlation coefficient
A	5.13	268.1 → 136.1	1.00–200	0.9993
m^1A	3.01	282.1 → 150.1	1.25–500	0.9983
I	3.63	269.1 → 137.1	2.50–500	0.9987
G	3.76	284.1 → 152.1	2.50–500	0.9989
m^2G	3.48	298.1 → 166.1	10.0–2000	0.9998
m^7G	4.71	298.1 → 166.1	12.5–500	0.9983
m_2^2G	5.47	312.1 → 180.1	1.00–2000	0.9999
C	2.27	244.1 → 112.1	2.50–500	0.9994
m^3C	3.62	258.1 → 126.1	1.39–696[a]	0.9999
m^5C	2.33	258.1 → 126.1	0.50–500	0.9999
Cm	3.80	258.1 → 112.1	1.00–400	0.9984
s^2C	3.28	260.1 → 128.1	4.39–175[b]	0.9977
U	2.74	245.1 → 113.1	12.5–500	0.9979
m^5U	3.99	259.1 → 127.1	50–2000	0.9973
Ψ	1.56	245.1 → 209.0	5.00–500	0.9994
f^2dA (IS)	6.24	270.2 → 154.2	–	–

[a]Nucleoside test mix contained 100 μg mL^{-1} of 3-methylcytidine methosulfate, and 69.6 μg mL^{-1} of m^3C

[b]Nucleoside test mix contained 10 μg mL^{-1} of 2-thiocytidine dihydrate and 8.77 μg mL^{-1} of s^2C

Basanta-Sanchez et al. 2016) Longer re-equilibration time allows reproducibility on column separation. However, one-third of the total analysis time was spent on re-equilibration. To circumvent this problem, we separated re-equilibration time from total analysis time using the multiple column system. It is important to note that our LC system consists of the least equipment to perform the analytical and wash gradient compared to previous reports (Korfmacher et al. 1999; Cass et al. 2001; Oertel et al. 2002; Orton et al. 2013; Lee et al. 2015; Patel et al. 2016). This is simple and easy-to-use for most LC/MS users. We also note that the peak at 2.30 min in the chromatogram of U corresponds to the C + 1 isotope peak, where our method can differentiate between the U and C + 1 isotope peak. Additionally, this method can differentiate between m^5U for $m^5C + 1$ and $m^3C + 1$, m^5U for $m^5C + 2$ and $m^3C + 2$, and I for A + 1. Typical chromatograms for the standard solutions are shown in Fig. 2 and demonstrate sufficient separation using an ADME column. Simultaneous quantitative analysis of modified nucleosides was previously performed using the ODS column (Djukovic et al. 2010; Russell and Limbach 2013; Lee et al. 2004; Basanta-Sanchez et al. 2016; Rodríguez-Gonzalo et al. 2016). Chromatographic separation on the ODS column depends on the hydrophobic interaction, which enables separation by the number of methyl groups (Núñez et al. 2007). However, the ODS column is generally unsuitable for the separation of hydrophilic compounds, such as nucleosides, because of the weak interaction due to low surface polarity. An extended time is required for sufficient separation of nucleosides on the ODS column. An ADME column is composed of an adamantyl functional group with polymer-coated silica. While retaining the hydrophobic interaction similarly to the ODS column, it also has higher surface polarity. Therefore, the ADME column provides good separation of hydrophilic compounds. In fact, separation of hydrophilic compounds, such as amino acids, carboxylic acids, and other hydrophilic metabolites on ADME columns has been reported (Mochizuki et al. 2015; Uno et al. 2015; Song et al. 2016). Using this unique surface property of the ADME column, we achieved good chromatographic separation of nucleosides and rapid analysis. Tubercidin was previously used as an internal standard (Mishima et al. 2014). However, we used f^2dA because it was not

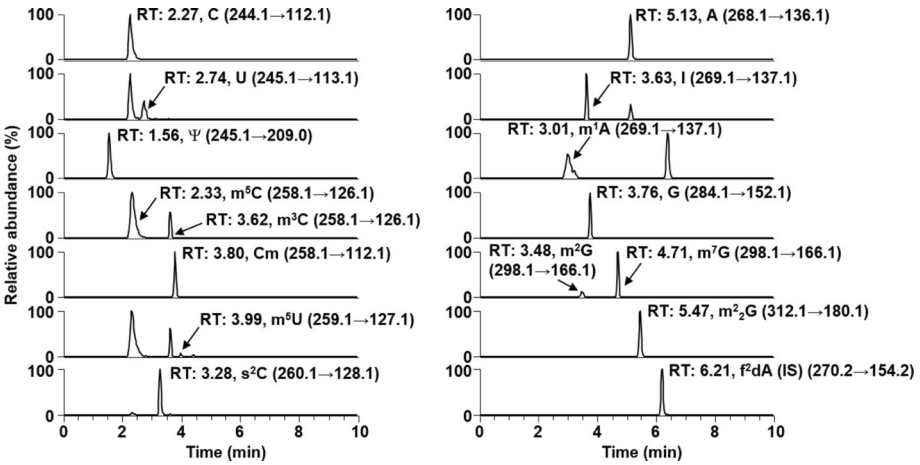

Fig. 2 SRM product ion chromatograms of modified nucleoside standards in HESI positive ionization mode. *SRM* selected reaction monitoring, *HESI* heated electrospray ionization

separated from other nucleosides under this chromatographic condition.

Comparison of peak area ratio using multiple column LC mode

As this method uses multiple LC columns to reduce runtime, we evaluated column identity by investigating the ratio between target peak areas and IS area using the two columns. No differences in area ratios for the nucleosides using either column were detected (Fig. 3), proving that our method can be used in multi-column LC mode.

Limit of quantification (LOQ) and linearity

The LOQ corresponds to the lowest concentration that is quantifiable given accuracy criteria of < ±15% and a signal-to-noise ratio >10 AU. The LOQ for our system was measured as 1.00–5.00 ng mL^{-1}, except for m^2G (10.0 ng mL^{-1}), m^7G (12.5 ng mL^{-1}), U (12.5 ng mL^{-1}), and m^5U (50.0 ng mL^{-1}).

Linearity

Calibration curves for all nucleosides are summarized in Table 3. Correlation coefficients were greater than 0.99 for all nucleosides, such that the calibration curves showed good linearity within the quantification range. In comparison with previous studies (Djukovic et al. 2010; Cho et al. 2009; Lee et al. 2004; Rodríguez-Gonzalo et al. 2016), these results suggest that our method is applicable to concentrations of modified nucleosides found within biological samples.

Accuracy and precision

The accuracy ranged from 85.05–114.7% for the highest three concentrations and 80.03–119.6% for the lowest concentrations across all nucleosides (Table 4). Precision ranged from 1.073–14.84% for the highest concentrations of all compounds and 1.731–19.92% for the lowest concentrations of nucleosides (Table 4). These results demonstrate that the method presented has both good reliability and repeatability, where data satisfied criteria of <15% within higher concentrations and <20% within the lowest concentrations.

Conclusions

This study describes a selective, high-throughput method to simultaneously quantify modified nucleosides using a novel multi-column LC-MS/MS analysis. This method selectivity separated modified nucleosides and

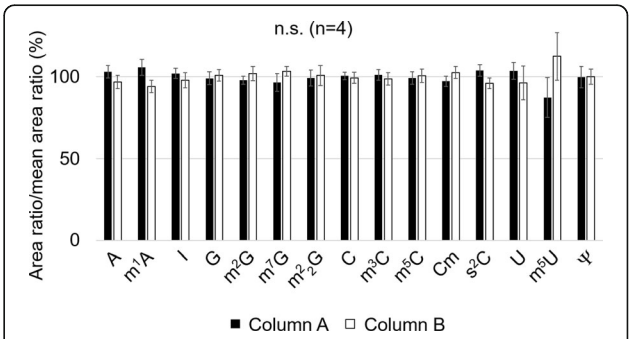

Fig. 3 Mean ± SE area ratio/mean area ratio (%) obtained via multiple column LC mode. The mean area ratio was calculated from an average of the column A and column B area ratios for each nucleoside. *SE* standard error; *LC*: liquid chromatography

Table 4 Accuracy and precision of nucleosides determination

Compound	Nominal conc.	Accuracy (%, $n = 5$)			Precision (CV %, $n = 5$)		
	(ng mL^{-1})	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
A	1.00	80.89	115.1	90.92	8.695	18.96	18.77
	2.00	99.87	100.4	101.6	11.39	14.22	13.4
	100	103.4	106.4	98.96	9.693	13.86	4.715
	200	94.53	103.4	87.53	7.254	4.434	9.463
m^1A	1.25	112	99.07	92.01	17.5	17.31	18.39
	2.50	98.75	104.2	89.22	14.8	13.63	6.688
	250	104.7	96.29	107.4	2.781	14.01	5.248
	500	105.9	99.13	100.7	5.26	12.35	3.37
I	2.50	80.03	101.2	89.46	17.17	11.15	3.536
	5.00	106	95.87	104.1	14.76	11.5	11.21
	250	98.16	105.8	101.3	5.065	12.14	4.513
	500	85.72	89.05	98.19	4.975	4.251	9.22
G	2.50	96.91	82.1	91.72	11.76	19.92	13.07
	5.00	87.73	104	104.8	13.68	8.85	13.57
	250	107.2	113.4	107.5	5.877	6.036	3.147
	500	98.92	109.1	101.5	6.147	4.587	4.092
m^2G	10.0	112.5	104.1	106.3	14.64	14.14	6.845
	20.0	91.89	102.6	104.1	7.714	13	7.213
	1000	108.8	107.1	105.8	4.05	4.05	3.614
	2000	101.5	100.1	97.28	8.088	8.087	4.625
m^7G	12.5	101	105.7	113.6	19.92	9.69	16.52
	25.0	96.96	93.67	106.4	13.45	8.244	13.57
	250	101.4	112.2	96.06	5.524	4.694	7.28
	500	102.9	103.3	99.69	7.926	6.845	4.815
m_2^2G	1.00	119.6	119.1	115.9	13.68	14.68	7.302
	2.00	86.04	93.41	86.09	9.516	11.8	12.52
	1000	109	103.7	112.2	6.465	5.908	10.27
	2000	99.07	96.76	101.6	3.59	2.827	3.27
C	2.50	82.88	106.6	117.5	18.67	17.03	7.551
	5.00	114.7	94.29	107.9	14.71	8.268	14.39
	250	102.4	109.3	97.02	5.971	4.065	12.05
	500	96.1	102.9	95.4	1.073	2.961	9.001
m^3C	1.39	84.14	111.2	106.4	19.35	13.05	8.499
	3.48	104.8	113	96.75	14.17	13.6	6.68
	348	105.9	104	100.4	1.999	5.678	2.158
	696	92.01	95.75	94.3	3.692	12.98	4.793
m^5C	0.50	81.32	106.6	117	15.81	7.609	2.149
	1.00	94.27	110.2	101.8	8.507	9.095	1.588
	250	93.69	95.94	93.64	3.874	5.248	6.573
	500	89.01	85.05	89.83	4.385	3.218	3.484

Table 4 Accuracy and precision of nucleosides determination *(Continued)*

Cm	1.00	86.72	119	93.69	13.98	14.89	17.82
	2.00	92.96	86.7	99.21	14.84	13.67	9.533
	200	103.1	113.6	111.1	5.603	7.017	3.349
	400	95.17	106.6	100.6	3.041	4.291	6.287
s^2C	4.39	102.8	119.5	107	18.05	13.98	7.756
	8.77	98.38	91.49	95.23	12.52	10.3	13.62
	87.7	106.7	109.8	111.6	9.275	4.069	5.446
	175	108.2	90.96	99.43	10.06	9.576	3.505
U	12.5	98.98	106.7	103.2	18.14	7.711	1.731
	25.0	102.3	99.52	94.26	12.13	7.518	10.73
	250	102.7	98.59	105.6	9.811	9.06	2.607
	500	108.8	105.6	103.7	10.47	6.208	5.767
m^5U	50.0	106.7	109.8	117.6	15.21	3.571	6.369
	100	91.63	89.08	94.54	10.2	4.907	11.57
	1000	111.1	102.4	109.5	5.757	5.617	4.958
	2000	104	110.3	112.1	8.855	5.899	2.927
Ψ	5.00	97.95	108.5	99.8	17.19	9.301	17.63
	12.5	98.89	95.34	99.66	12.16	7.573	9.511
	250	108.8	99.72	94.49	6.829	6.079	5.563
	500	96.65	95.83	101.5	7.376	4.857	2.329

their isotopic isomers quickly (within 7 min), analyzing them within 10 min using a unique multiple-column setup, whose linearity spans a wide concentration range. Both values of accuracy and precision satisfied criteria of <15% for higher concentrations and <20% for the lowest concentration. This unique method provides a fast, reliable means of analyzing the profile of modified nucleosides, which could be used to diagnose at bedside on a daily basis.

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Authors' contributions
DJ performed experimental and analytical work and prepared an initial draft of the article. KS and SN assisted method development and analysis. YK, HT, YM, TA, and YT reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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