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Determination of urinary nucleosides by direct injection and coupled-column high-performance liquid chromatography

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Abstract

A coupled-column liquid chromatographic method for the direct analysis of 14 urinary nucleosides is described. Efficient on-line clean-up and concentration of 14 nucleosides from urine samples were obtained by using a boronic acid-substituted silica column (40 mm × 4.0 mm I.D.) as the first column (Col-1) and a Hypersil ODS_2 column (250 mm × 4.6 mm I.D.) as the second column (Col-2). The mobile phases applied consisted of 0.25 mol/L ammonium acetate (pH 8.5) on Col-1, and of 25 mmol/L potassium dihydrogen phosphate (pH 4.5) on Col-2, respectively. Determination of urinary nucleosides was performed on Col-2 column by using a linear gradient elution comprising 25 mmol/L potassium dihydrogen phosphate (pH 4.5) and methanol–water (60:40, v/v) with UV detection at 260 nm. Urinary nucleosides analysis can be carried out by this procedure in 50 min requiring only pH adjustment and the protein precipitation by centrifugation of urine samples. Calibration plots of 14 standard nucleosides showed excellent linearity ($r > 0.995$) and the limits of detection were at micromolar levels. Both of intra- and inter-day precisions of the method were better than 6.6% for direct determination of 14 nucleosides. The validated method was applied to quantify 14 nucleosides in 20 normal urines to establish reference ranges.

Keywords: Coupled-column; Urinary nucleosides; High-performance liquid chromatography

1. Introduction

Modified nucleosides are formed at the post-transcriptional level by chemical modifications of normal nucleosides within the RNA molecules. Because, there are no specific enzyme systems to incorporate the modified nucleosides into the macromolecular nucleic acids, they cannot be reutilized and further degraded, but are either metabolized or excreted intact in urine during the process of tRNA turnover [1,2]. Consequently, the levels of urinary nucleosides reflect RNA degradation in the organism. Therefore, any disorder of RNA turnover alters the levels of modified nucleosides. A number of studies have examined their biomedical significance as possible biomarkers for cancer [3–9], AIDS [10–12] and for the whole-body turnover of RNAs [13,14]. In addition, urinary nucleosides also have been used to monitor progress of disease and response to therapy [4,15–18].

Several methodologies, including gas chromatography (GC) [3], reversed-phase high-performance liquid chromatography (RP-HPLC) [4,5,9,15–17,19–21], capillary electrophoresis (CE) [7–9,22,23] and immunoassays [6,24–26] have been used for the analysis of urinary nucleosides. Although immunoassays allow rapid analysis of hundreds of samples a day, the lack of specificity is of concern in evaluating the accuracy of the quantitative data. With GC, a time-consuming laborious sample pretreatment is required. Usually, after extraction, solvent evaporation and derivatization cannot be avoided owing to the rather non-volatile nature of nucleosides. Both RP-HPLC and CE when applied to the determination of urinary nucleosides, still require manual enrichment and clean-up steps, performed by solid-phase extraction in affinity mode. Recently, Weimann et al. [27] adopted the mode of the low-temperature reversed-phase liquid chromatography, which performed at

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1 °C on a single ODS column for separation of six types of 8-hydroxylated guanine modifications in urine without any sample pre-treatment. Actually, coupled-column HPLC is a very adaptable strategy for the direct analysis of target compounds in biological fluids, as indicated by studies [28,29], including also the analysis of purine nucleic acid components [30,31]. In this study, we described a fully automated method for the determination of 14 urinary nucleosides by coupled-column liquid chromatography with UV detection. In addition, the developed method was used to the analysis of urines from healthy volunteers to establish reference ranges.

2. Experimental

2.1. Chemicals and reagents

The following 14 nucleoside standards, including the internal standard 8-bromoguanosine hydrate (Br8G) were obtained from Sigma (St. Louis, MO, USA): pseudouridine (Pseu), cytidine (C), uridine (U), 1-methyladenosine (m1A), inosine (I), 5-methyluridine (m5U), guanosine (G), 1-methylinosine (m1I), 1-methylguanosine (m1G), N^4-acetylcytidine (ac4C), N^2-methylguanosine (m2G), adenosine (A), N^2,N^2-methylguanosine (m22G) and N^6-methyladenosine (m6A). Methanol (MeOH) was HPLC-grade purchased from Tedia (Fairfield, OH, USA). Ammonium acetate (NH_4AC), ammonia ($NH_3{\cdot}H_2O$) and potassium dihydrogenphosphate (KH_2PO_4) were all analytical reagents obtained from Shenyang Federation Reagent Factory (Shenyang, Liaoning, China). Water was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Equipment

The HPLC system consisted of three Shimadzu LC-10ATVP pumps (Kyoto, Japan), an autoinjector model SIL 10ADVP, a SPD-10AVP UV–vis detector, set at 260 nm and a SCL 10AVP interface. An electric six-port valve (Rheodyne, USA) was used for the automated column switching. Valve switching and data acquisition were done on Shimadzu Class-VP version 6.10 software. The column 1 (40 mm × 4.0 mm I.D.) was a laboratory-prepared boronic acid-substituted silica column. It has binding capacity of 0.18 mmol nucleoside per gram dry weight and tolerates pH values from 2 to 12 as well as the usual organic solvents. The more details of its preparation and physicochemical properties have been described by Hagemeier et al. [30]. The column 2 (250 mm × 4.6 mm I.D.) was packed with 5 μm Hypersil ODS_2 (Elite, Dalian, China). For efficient on-line clean-up and concentration of 14 nucleosides from urine samples, the mobile phases applied consisted of 0.25 mol/L ammonium acetate (pH 8.5) on column 1, and of 25 mmol/L potassium dihydrogen phosphate (pH 4.5) on column 2, respectively. Determination of urinary nucleosides was performed on column 2 by using a linear gradient elution comprising 25 mmol/L potassium dihydrogen phosphate (pH 4.5) and methanol–water (60:40, v/v).

2.3. Sample preparation

A mixed working standard solution (MWS), containing 14 nucleosides was prepared by diluting and mixing the concentrated stock aqueous solution of individual nucleosides. The final concentrations of the mixed working standard solution were: 1.28 mmol/L for Pseu, 0.016 mmol/L for C, 0.032 mmol/L for U, 0.16 mmol/L for m1A, 0.016 mmol/L for I, m5U and G, 0.064 mmol/L for m1I and m1G, 0.042 mmol/L for ac4C, 0.032 mmol/L for m2G and A, 0.08 mmol/L for m22G, 0.008 mmol/L for m6A. Br8G was prepared at a concentration of 0.30 mmol/L in Milli-Q water. All the nucleoside solutions were kept frozen at −20 °C. Stock solutions were stable for 2 months when stored at −20 °C and no evidence of degradation of the analyte was observed on the chromatograms during this period.

Spontaneous urine samples were collected from 20 healthy adults from author's institute (age range, 25–66 years). After collection the samples were frozen immediately and stored at –20 °C. Prior to analysis, the samples were thawed at room temperature and adjusted to pH 8.5 with 5% $NH_3{\cdot}H_2O$ (v/v), followed by vortex for 3 min at 5000 r/min. Aliquots of 1 ml centrifuged urine, containing 30 μl of Br8G (0.30 mmol/L) were transferred to autosampler vials and samples of 150 μl were injected to a coupled-column HPLC system.

2.4. Analytical procedure

Column 1 was equilibrated for 5 min with 0.25 mol/L ammonium acetate (pH 8.5) delivered by pump 1. After sample injection (150 μl urine), column 1 was washed for 7 min with the same buffer. During that time, nucleosides were selectively retarded on the column 1 and the sample matrix was discharged. At the same time, column 2 was conditioned with 25 mmol/L potassium dihydrogen phosphate (pH 4.5) delivered by pump 2 (position 1; Fig. 1). After this clean-up step, column 1 was series-connected in front of column 2. The group-specifically bound nucleosides on the column 1 were then eluted and concentrated on top of the column 2 over a period of 3 min (position 2; Fig. 1). Separation of nucleosides on the column 2 was carried out with a linear gradient elution program over 35 min, while the column 1 was regenerated for a new extraction cycle (position 1; Fig. 1). The nucleosides were detected by 260 nm and quantified using the internal standard method. Table 1 shows the time events used for the analytical procedure.

Peak identification was made on the basis of retention times. The MWS was run daily before and after the samples to monitor reproducibility of retention times. The standard addition method was also used to confirm peak identification. The levels of the urinary nucleosides were calculated by the calibration curves, and then were transformed into

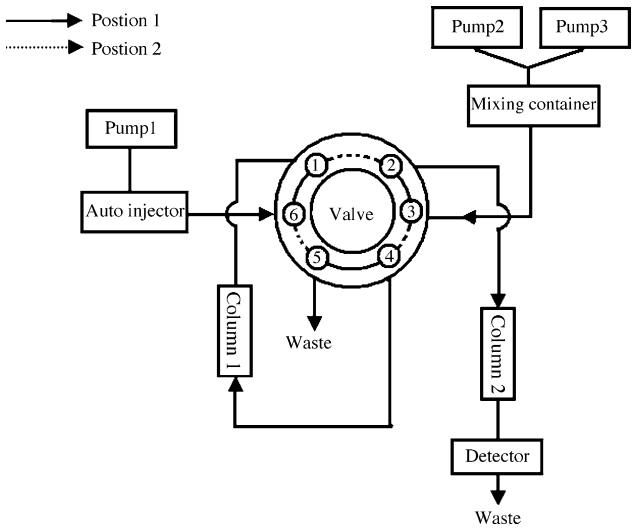

Fig. 1. Schematic diagram of the coupled-column HPLC system.

nmol/μmol creatinine. Urinary creatinine levels were determined as described by Zheng et al. [22].

2.5. Method validation

For the calibration, six different volumes (0.05 ml, 0.1 ml, 0.2 ml, 0.4 ml, 0.6 ml, 1.0 ml) of MWS, containing 14 nucleosides were diluted with water to 1 ml and mixed with 30 μl of Br8G (0.30 mmol/L), then samples of 150 μl were injected separately to a coupled-column HPLC system. With these six standard samples, 14 calibration curves for nucleosides detected at 260 nm were established.

The recoveries of 14 nucleosides from human urine were determined by analyzing three different volumes (0.08 ml, 0.30 ml, 0.8 ml) of MWS. The peak area ratios of three-extracted samples at each volume were compared with those of three injections of standard solutions to derive a percent recovery.

Intra- and inter-day precisions of the method were evaluated by replicate analysis by the same three different levels of the MWS that were used for the recovery experiments. Five samples of each concentration of these three different levels of MWS were prepared and analyzed on 3 non-consecutive days.

The acceptance criteria for the limit of quantification were that the precision and accuracy for three-extracted sample were under 20% variability, while the limit of detection was calculated taking a signal-to-noise ratio of 3.

3. Results and discussion

Methodically in most of the related studies the nucleosides are isolated from the urine specimen by phenylboronate

Table 1
Time events for the switching of column and of mobile phase

Time (min)	Pump (eluent)	Event	Valve position
0.00æ7.00	Pump 1 (eluent A) Pump 2 (eluent B)	Sample matrix are discharged by column 1 Conditioning of column 2	1
7.00æ10.00	Pump 2 (eluent B)	Analytes are transferred from column 1 to column 2	2
10.00æ50.00	Pumps 2 and 3 (eluents B and C) Pump 1 (eluent A)	Analysis of nucleosides on column 2 by using a linear gradient elution program Conditioning of column 1	1

Eluents: eluent A, 0.25 mol/L NH_4AC (pH 8.5); eluent B, 25 mmol/L KH_2PO_4 (pH 4.5); eluent C, methanol–water (3:2, v/v). Flow rate: pump 1, 0.2 ml/min; pump 2, 1.2 ml/min. Detection wavelength: 260 nm.

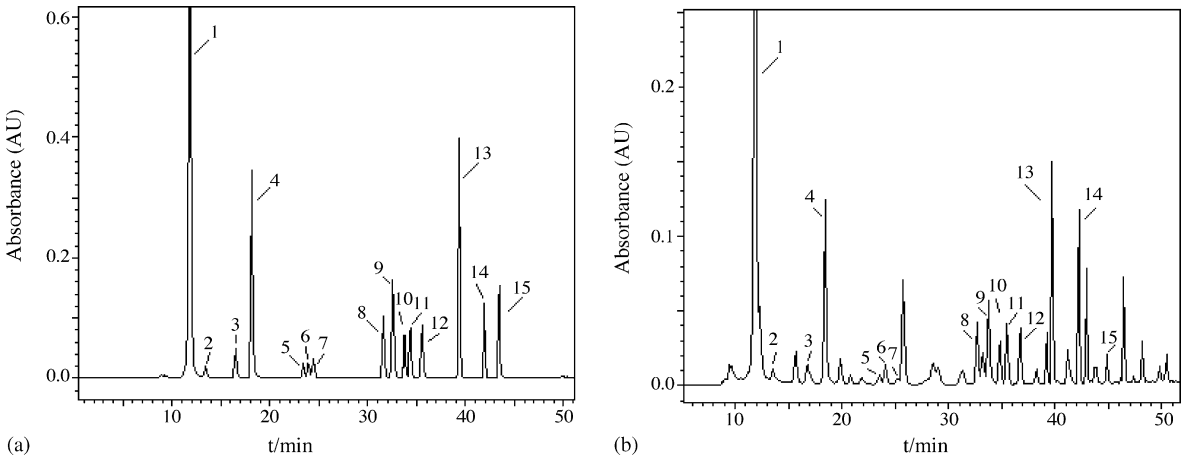

Fig. 2. Typical chromatograms of (a) 14 standard nucleoside mixtures (b) nucleosides extracted from a spontaneous urine obtained under the established conditions. Coupled-column HPLC conditions as Table 1. Peak identification: (1) Pseu; (2) C; (3) U; (4) m1A; (5) I; (6) m5U; (7) G; (8) m1I; (9) m1G; (10) ac4C; (11) m2G; (12) A; (13) m22G; (14) Br8G; (15) m6A.

Table 2
Analytical characteristics of the method and extraction recoveries of 14 nucleosides from human urine

Compound	Linear correlation[a]	Correlation coefficient	LOD[b] (μmol/l)	LOQ[c]	Recovery (%)[d]		
					Run 1	Run 2	Run 3
Pseu	$y = 0.3222x - 0.0308$	0.9995	0.56	2.22	95.3	100.1	98.6
C	$y = 0.5175x + 0.0047$	0.9991	0.51	2.03	110.0	115.5	105.8
U	$y = 0.5353x - 0.0020$	0.9997	0.37	1.50	89.5	94.0	86.1
m1A	$y = 0.5759x - 0.0009$	0.9997	0.26	0.80	86.2	90.5	94.5
I	$y = 0.4776x - 0.0005$	0.9998	0.40	1.59	91.9	96.5	92.5
m5U	$y = 0.5170x + 0.0097$	0.9977	0.38	1.52	82.0	86.1	90.4
G	$y = 0.7293x - 0.0002$	0.9998	0.30	1.22	87.2	91.6	94.2
m1I	$y = 0.3631x + 0.0032$	0.9999	0.36	1.07	91.7	96.3	95.6
m1G	$y = 0.8130x - 0.0005$	0.9999	0.22	0.66	91.8	96.4	88.3
ac4C	$y = 0.5213x + 0.0015$	0.9998	0.34	1.01	82.8	86.9	91.4
m2G	$y = 0.7989x - 0.0014$	0.9999	0.22	0.78	90.3	94.8	86.8
A	$y = 0.9114x - 0.0027$	0.9997	0.19	0.58	98.3	103.2	94.5
m22G	$y = 1.0271x - 0.0118$	0.9991	0.10	0.30	90.9	95.4	88.6
m6A	$y = 3.7856x + 0.0267$	0.9952	0.03	0.12	115.0	118.7	110.6

[a] The linear correlations were obtained by determining the six standard nucleoside aqueous solutions with different concentrations on the coupled-column HPLC, where y is the relative peak area of nucleosides to internal standard, and x the relative concentration of nucleosides to the internal standard.
[b] Calculated based on three signal-to-noise ratios.
[c] LOQ, limit of quantification.
[d] Recoveries were obtaining by adding different levels of MWS to 1 ml urine. Run1, 0.08 ml of MWS; run 2, 0.30 ml of MWS; run 3, 0.80 ml of MWS.

affinity gel chromatography and separated by RP-HPLC [4,5,9,15–17,19–21]. These clean-up procedures for urinary nucleosides need manual operation, resulting in artificial error and time consuming. Hagemeier et al. [30,31] successfully prepared a boronic acid-substituted silica material, which was used for on-line sample processing and analyzed the purine nucleic acid components, but Pseu, a important RNA excretory product, is not quantitatively recovered at that operational condition.

Here, the employed coupled-column HPLC system is illustrated in Fig. 1. The time schedule for the switching events is given in the experimental section. In order to determine the elution profile and retention times of the nucleosides in the urine matrix, the column 1 was directly connected to the UV detector. At first, the influence of the pH of 0.25 mol/L NH_4AC as the mobile phase was investigated for the efficient sample clean-up. Varying the buffer pH from 6.0 to 8.0, the nucleosides fraction gave a good elution profile. Unfortunately, it had same retention time with the sample matrix. The increase of pH value from 9.0 to 10.0 widened the elution profile of the nucleosides fraction, which might lead to elute difficultly in the follow step. Under the condition of the

Table 3
Intra- ($n = 5$) and inter-day ($n = 3$) precisions of retention times for the assay of three different levels of mixed working standard solution (MWS)

Compound	R.S.D. (%)					
	MWS 1[a]		MWS 2[b]		MWS 3[c]	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Pseu	0.33	1.38	0.10	1.21	0.30	1.60
C	0.73	2.63	0.39	1.53	0.76	2.26
U	0.99	3.51	0.68	1.78	1.17	2.68
m1A	0.41	2.35	0.26	1.27	0.86	2.01
I	0.95	4.10	1.39	3.25	1.54	3.29
m5U	0.60	2.67	0.94	2.42	1.04	3.11
G	1.13	4.50	1.63	3.77	1.70	3.51
m1I	0.84	2.82	1.01	2.57	0.82	2.71
m1G	1.05	3.53	1.27	3.03	1.05	2.92
ac4C	0.78	2.76	1.03	2.54	0.83	2.71
m2G	0.70	2.62	1.01	2.51	0.78	2.48
A	0.86	2.35	1.21	2.83	0.99	2.89
m22G	0.39	1.59	0.21	1.08	0.40	1.07
m6A	0.75	2.05	0.12	1.34	0.20	1.81

[a] 0.08 ml of MWS was diluted with water to 1 ml, followed by mixed with 30 μl of Br8G (0.30 mmol/L).
[b] 0.30 ml of MWS was diluted with water to 1 ml, followed by mixed with 30 μl of Br8G (0.30 mmol/L).
[c] 0.80 ml of MWS was diluted with water to 1 ml, followed by mixed with 30 μl of Br8G (0.30 mmol/L).

Table 4
Intra- ($n = 5$) and inter-day ($n = 3$) precisions of peak areas relative to the internal standard (Br8G) for the assay of three different levels of MWS

Compound	R.S.D. (%)					
	MWS 1[a]		MWS 2[b]		MWS 3[c]	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Pseu	1.72	2.41	2.10	4.81	1.56	2.64
C	0.06	0.27	1.98	5.70	2.22	5.41
U	1.55	2.05	0.58	2.98	0.76	3.01
m1A	0.66	1.33	1.11	2.21	0.75	1.45
I	1.94	2.89	1.26	2.68	0.87	2.12
m5U	1.43	1.98	0.72	2.01	1.29	2.41
G	2.22	2.87	2.53	2.95	0.97	2.15
m1I	2.26	3.84	2.38	4.04	2.25	3.84
m1G	1.63	2.01	1.02	3.06	1.21	2.35
ac4C	2.02	4.01	1.84	3.77	1.15	2.81
m2G	1.31	3.28	1.06	2.97	1.07	2.26
A	1.54	1.68	0.98	2.04	1.99	2.48
m22G	1.74	2.75	1.43	2.63	1.60	3.63
m6A	1.13	5.01	3.06	4.98	1.34	6.58

[a] 0.08 ml of MWS was diluted with water to 1 ml, followed by mixed with 30 μl of Br8G (0.30 mmol/L).
[b] 0.30 ml of MWS was diluted with water to 1 ml, followed by mixed with 30 μl of Br8G (0.30 mmol/L).
[c] 0.80 ml of MWS was diluted with water to 1 ml, followed by mixed with 30 μl of Br8G (0.30 mmol/L).

buffer pH 8.5, the nucleosides fraction not only had the good elution profile, but also was retained on the column 1 without any leakage and without overlapping with the sample matrix. So, the column-switching time for the sample clean-up step was set at 7 min by using 0.25 mol/L NH_4AC (pH 8.5) as the mobile phase. Secondly, in order to keep the elution volume of the nucleosides fraction from the column 1 to column 2 at a minimum, 25 mmol/L KH_2PO_4 (pH 4.5), free of organic solvents, was selected as the mobile phase. It was found that the nucleosides fraction could be transferred to the top of column 2 in a shorter time (3 min). Finally, to get the optimal separation on column 2, the linear gradient elution program was well adjusted. The injection volume was 150 μl to satisfy the desired sensitivity. The chromatographic performance of columns 1 and 2 was maintained with over 100 injections of 150 μl of urine, but the upper limit has not yet been determined.

Fig. 2 shows typical chromatograms of the analysis of 14 standard nucleosides as well as the internal standard Br8G and that of 150 μl of human urine at the established conditions. Table 2 shows the analytical characteristics of the method developed. The good recoveries obtained by analyzing the three different levels of MWS are also given in Table 2. The intra- and inter-day precisions of the method were determined by analyzing five replicates three different levels of MWS on 3 non-consecutive days. The relative standard deviations (R.S.D.) of retention times were better than 1.8% (intra-day) and 4.5% (inter-day), and those of peak areas relative to the internal standard (Br8G) were better than 3.1% (intra-day) and 6.6% (inter-day). These results are shown in Tables 3 and 4.

To avoid the inconvenience of 24-h collection for urine samples, spontaneous urine samples have been used and the excretion values of urinary nucleosides were expressed as nmol/μmol creatinine [32,33]. In this study, spontaneous urine samples were also used instead of 24-h collection. To check the reliability of the method, we compared the results of 14 nucleoside levels in normal human urine determined by coupled-column HPLC system with the conventional off-line RP-HPLC method. The results obtained by both methods show good agreement and correlation (data shown in Fig. 3 for m1I as an example). The mean levels of 14 nucleosides in normal urines have been established (Table 5). The results were well correlated with those obtained by other authors [14,18,19,23].

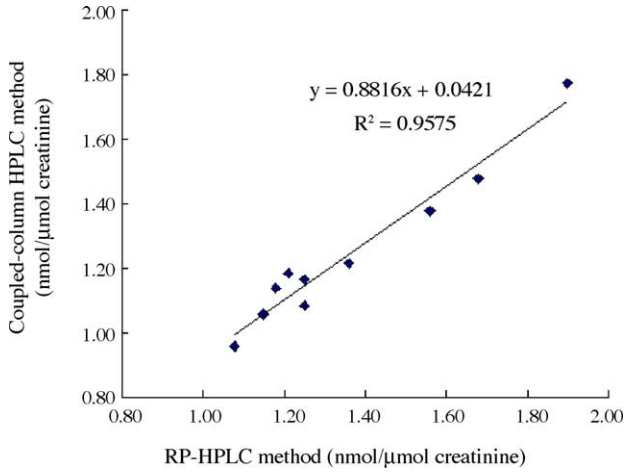

Fig. 3. Correlation between the coupled-column HPLC method and the conventional RP-HPLC method for the determination of m1I in 10 different urines.

Table 5
Mean nucleoside concentrations in 20 normal urine samples (nmol/μmol creatinine)

Compound	Concentration	S.D.	Concentration $_{min}$	Concentration $_{max}$
Pseu	22.89	5.51	8.97	29.17
C	0.14	0.13	0.04	0.40
U	0.28	0.17	0.12	0.74
m1A	1.74	0.51	1.43	2.59
I	0.32	0.12	0.15	0.49
m5U	0.06	0.04	0.03	0.12
G	0.10	0.03	0.06	0.15
m1I	1.18	0.21	0.86	1.77
m1G	0.97	0.20	0.67	1.59
ac4C	0.62	0.08	0.47	0.80
m2G	0.45	0.11	0.31	0.73
A	0.44	0.13	0.18	0.63
m22G	1.16	0.18	0.88	1.58
m6A	0.05	0.03	0.01	0.10

4. Conclusions

An automated method for the determination urinary nucleosides by coupled-column HPLC was developed with success. The method is simple and rapid, requiring a total analysis time of 50 min per sample by direct injection of 150 μl urine samples. The good linearity, precision, sensitivity and selectivity obtained with this method allow its use for routine clinical analysis.

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