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Quantitation of urinary nucleosides by high-performance liquid chromatography

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Abstract

It is known that some modified, especially methylated, nucleosides originating from RNA degradation are excreted in abnormal levels in the urine of patients with malignant tumours and they have been proposed as tumour markers. Their measurement could provide a non-invasive diagnostic method, be helpful in the identification of different cancers and in the monitoring of therapeutic effects. In this study, we developed and optimized an analytical procedure to isolate and quantify normal and modified ribonucleosides. The extraction of urinary nucleosides was performed by affinity chromatography on a phenylboronic acid column prior to separation. The reversed-phase high-performance liquid chromatography method allowed a complete separation of sixteen urinary ribonucleosides. The recoveries for the different nucleosides ranged from 83 to 100%, except for xanthosine (66%) and pseudouridine (74%). In normal 24 h urine, the mean levels of thirteen nucleosides (in nmol of nucleoside/μmol of creatinine) were found to be as follows: dihydrouridine (6.37), pseudouridine (25.52), cytidine (0.07), uridine (0.21), 1-methyladenosine (2.19), inosine (0.30), guanosine (0.06), xanthosine (0.59), 3-methyluridine (0.11), 1-methylinosine (1.13), 1-methylguanosine (0.74), adenosine (0.21) and 5′-deoxy-5′-methylthioadenosine (0.12). The first results concerning two kinds of tumours, i.e. breast and floor of mouth tumours, showed some abnormal levels of ribonucleosides. Further experiments are now in progress to measure the modified nucleosides in urine of patients with different forms of cancer.

Keywords: Nucleosides

1. Introduction

Modified nucleosides are formed post-transcriptionally in RNA by a number of modification enzymes. During RNA turnover, free modified nucleosides are created. They circulate in the blood stream and are excreted in the urine. It has been shown that their urinary levels are useful parameters for studies on RNA turnover [1,2] and protein metabolism [3] and, in many instances, they have been proposed as tumour markers due to their increased urinary excretion in conjunction with altered RNA turnover in carcinogenesis. Several modified nucleosides, e.g. pseudouridine and 1-methyladenosine have been shown to be elevated in urine from patients with leukemia [4,5], lung cancer [4], tumours of the brain [1], gastrointestinal cancer [2] and other carcinomas [1].

For some nucleosides, attempts have been made to develop immunoassays for their quantification [6]. However, so far the most reliable method for the analysis of urinary nucleosides, especially when the broad spectrum of modified nucleosides is studied [7], is reversed-phase high-performance liquid chromatography (RP-HPLC). The measurement of urinary nucleosides could provide a non-invasive diagnostic tool and be helpful in the identification of different cancers. This paper describes a method that

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could be usefully applied in the clinical chemical laboratory.

2. Experimental

2.1. Preparation of urine samples

Urines are extracted by affinity chromatography using a phenylboronic acid gel [8] (Affigel 601; Bio-Rad; 500 mg) in a glass column (140×14 mm). After activation of the gel [8] and equilibration with 35 ml of 0.25 *M* NH_4OAc, pH 8.5, 10 ml of centrifuged urine containing 0.5 ml of an aqueous solution of the internal standard, isoguanosine (0.25 m*M*), are applied to the column. Then the gel is washed with 20 ml of 0.25 *M* NH_4OAc, twice with 3 ml of methanol–water (1:1, v/v) and the nucleosides are eluted with 25 ml of 0.1 *M* HCOOH in methanol–water (1:1, v/v). After evaporation, the residue is dissolved in 1 ml of 25 m*M* KH_2PO_4. Before re-use, the gel is regenerated with 25 ml of 0.1 *M* HCOOH in methanol–water (1:1, v/v) and 25 ml of methanol–water (1:1, v/v). Between two extractions, 45 ml of 0.25 *M* NH_4OAc, pH 8.5, are used for equilibration of the gel. The same treatment is applied for the standard solution containing the fifteen nucleosides and the internal standard used for

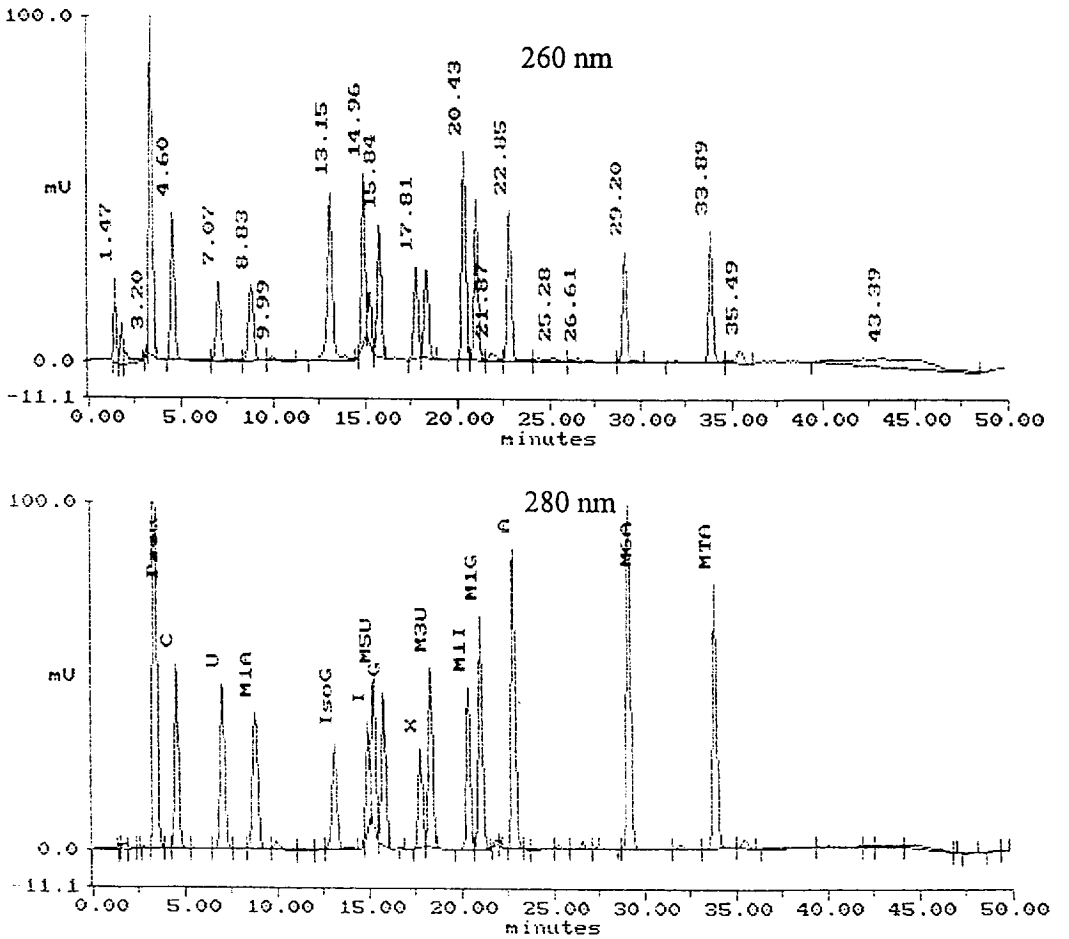

Fig. 1. Chromatograms of the HPLC analysis of ribonucleosides in the standard solution (DHU, dihydrouridine is detected at 260 nm after 3.20 min; Pseu, pseudouridine; C, cytidine; U, uridine; M1A, 1-methyladenosine; IsoG, isoguanosine; I, inosine; M5U, 5-methyluridine; G, guanosine; X, xanthosine; M3U, 3-methyluridine; M1I, 1-methylinosine; M1G, 1-methylguanosine; A, adenosine; M6A, N^6-methyladenosine; MTA, 5′-deoxy-5′-methylthioadenosine).

the calibration of the HPLC method. Under these conditions, the gel can be used for fifteen extractions.

2.2. Reversed-phase high-performance liquid chromatography

The HPLC apparatus (Merck-Hitachi) is composed of an L-6200 pump, an L-3000 photo diode array detector, a 655A-40 column oven and a D-6000 interface.

The isolated nucleosides, contained in 1 ml of 25 m*M* KH_2PO_4 buffer, pH 4.7, are separated on a 250×4 mm, 5 μm LiChrospher 100 C_{18} column (Merck) at 30°C and with a gradient beginning with 100% 25 m*M* KH_2PO_4m pH 4.7, and changing to 60% methanol–water (3:2, v/v) over 40 min. The nucleosides are detected by measuring the UV absorbance at 260 and 280 nm and quantified using the internal standard method.

For the calibration, four different volumes of a standard stock solution containing the fifteen nucleosides, to each of which was added 0.5 ml of the internal standard (0.25 m*M* isoguanosine), are treated separately on the affinity chromatography gel. With these four standard samples, we established fifteen calibration curves at 260 and 280 nm. The HPLC analysis allows the determination of the concentration of the nucleosides (nmol/ml) in urine, which

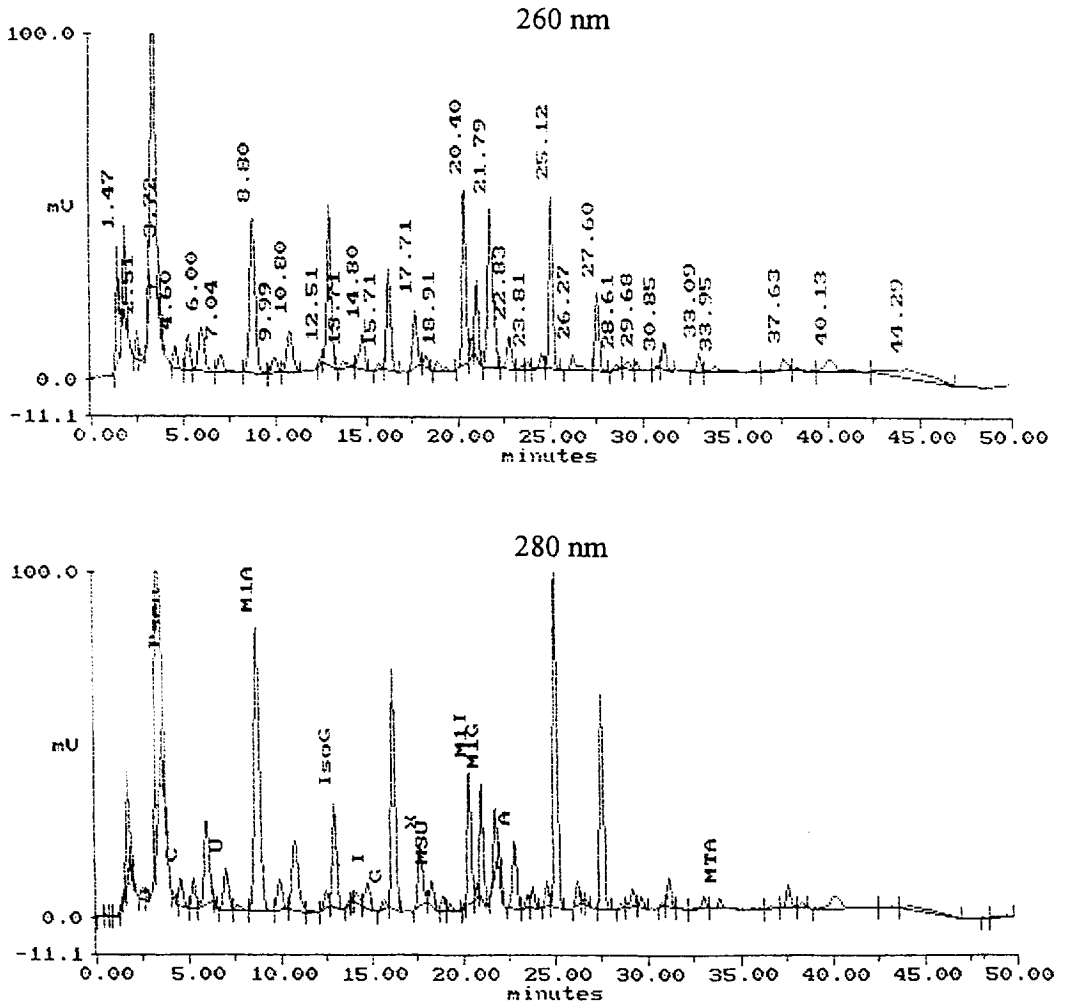

Fig. 2. Chromatograms of the HPLC analysis of ribonucleosides in normal urine (M5U and M6A do not seem to be present in normal urine).

Table 1
Levels of ribonucleosides (nmol nucleoside/μmol creatinine) in normal urine

	nmol/μmol Creatinine					
	Females		Males		29 Normal adults	
	Average	SD	Average	SD	Average	SD
DHU	7.03	1.31	5.83	1.22	6.37	1.38
Pseu	26.02	4.62	25.11	5.01	25.52	4.82
C	0.07	0.05	0.08	0.06	0.07	0.05
U	0.21	0.09	0.22	0.07	0.21	0.08
M1A	2.37	0.37	2.03	0.20	2.19	0.33
I	0.28	0.15	0.31	0.17	0.30	0.16
G	0.06	0.02	0.05	0.02	0.06	0.02
X	0.59	0.36	0.58	0.23	0.59	0.30
M3U	0.09	0.04	0.12	0.07	0.11	0.06
M1I	1.13	0.15	1.13	0.20	1.13	0.18
M1G	0.74	0.24	0.75	0.16	0.74	0.19
A	0.27	0.14	0.17	0.08	0.21	0.13
MTA	0.13	0.11	0.12	0.05	0.12	0.08

SD=standard deviation.

are then expressed as nmol of nucleoside/μmol of creatinine. The concentrations of creatinine in the urine samples are determined using a modified Jaffé method [9].

3. Results and discussion

Under our conditions, sixteen normal and modified ribonucleosides, including the internal standard, isoguanosine, were separated and identified (Figs. 1 and 2). Dihydrouridine is only detected at 260 nm. The recovery rates range from 83 to 100%, except for xanthosine (66%) and pseudouridine (74%).

The calibration curves for the fifteen nucleosides are linear through zero. The mean correlation coefficient for each nucleoside is generally 0.99.

The ribonucleoside extraction was realized for 29 normal 24 h urine samples (thirteen females and sixteen males). The mean levels (in nmol of nucleoside/μmol of creatinine) and the standard deviation for females and males were calculated separately (Table 1). The results show no significant variation based on gender.

The determination of the levels of ribonucleosides (in nmol/μmol creatinine) in spontaneous urine or in 24 h urine are comparable for the same patient.

The relative standard deviation in series has been determined using a normal pool urine sample. It has been extracted ten times on the affinity chromatography gel and these ten samples have been analysed with our HPLC method. The results are shown in Table 2.

Our first results concerning the urinary levels of nucleosides in two kinds of tumours, i.e. breast and floor of mouth tumours, show differences compared to normal values (Table 3). 1-Methyladenosine and 1-methylguanosine are present in higher levels in two breast tumour patients. In floor of mouth cancer,

Table 2
Relative standard deviation in series, determined in a normal pool urine

	at 260 nm			at 280 nm		
	Average (nmol/ml)	SD (nmol/ml)	R.S.D. (%)	Average (nmol/ml)	SD (nmol/ml)	R.S.D. (%)
DHU	60.16	2.43	4.03			
Pseu	255.54	10.79	4.22	221.36	7.13	3.22
C	1.21	0.09	7.45	1.11	0.11	10.38
U	4.10	0.13	3.09	3.02	0.14	4.65
m1A	21.36	0.76	3.54	20.47	0.19	0.91
I	0.73	0.09	11.76	0.80	0.09	11.29
G	0.59	0.04	6.16	0.86	0.05	5.73
X	5.89	0.40	6.74	4.88	0.30	6.11
m3U	0.64	0.08	12.77	0.68	0.12	17.71
m1I	14.65	0.39	2.67	13.16	1.47	11.17
m1G	6.94	0.32	4.58	7.86	0.92	11.64
A	2.82	0.11	3.85	2.84	0.09	3.26
MTA	1.46	0.20	13.96	1.25	0.25	19.64

SD=standard deviation; R.S.D.=relative standard deviation.

Table 3
Levels of nucleosides (nmol nucleoside/μmol creatinine) in urines from patients with cancer

	nmol/μmol Creatinine					
	Breast tumour		Floor of mouth tumour		Normal adults	
	Patient 1	Patient 2	Patient 1	Patient 2	Patient 1	Patient 2
DHU	12.70	3.11	7.04	14.39	6.37	1.38
Pseu	62.45	13.74	52.38	39.64	25.52	4.82
C	0.10	0.04	0.05	0.58	0.07	0.05
U	0.54	0.14	0.22	0.45	0.21	0.08
M1A	7.69	9.94	2.85	4.67	2.19	0.33
I	1.90	0.08	0.46	0.29	0.30	0.16
G	0.27	0.23	0.06	0.11	0.06	0.02
X	1.33	0.65	1.21	1.06	0.59	0.30
M3U	0.18	0.12	0.20	0.20	0.11	0.06
M1I	3.14	2.04	1.30	2.97	1.13	0.18
M1G	2.00	2.10	0.24	1.53	0.74	0.19
A	0.51	1.24	0.24	0.92	0.21	0.13
MTA	0.33	0.75	0.54	1.15	0.12	0.08

the values of pseudouridine and 5′-deoxy-5′-methylthioadenosine are increased in the two cases studied.

The method appears to be suitable for routine application. The analysis of a single sample or a series of samples can be completed in one day.

Further studies are now in progress on other patients with breast and floor of mouth tumours as well as on patients with other forms of cancer, to evaluate the diagnostic relevance of nucleosides in the identification of specific forms of cancer and to monitor pre- and postsurgery states.

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