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Note

Improved and rapid method for quantitation of modified nucleosides in urine and sera with Radial-Pak cartridge

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Patients with cancer excrete in their urine elevated levels of modified nucleosides, most of which stem from the breakdown of transfer RNA [1–4]. A possible mechanism of the origin of these elevated nucleosides in the urine has been indicated by finding very high rates of turnover of transfer RNA in tumor tissues [1]. A number of investigators are exploring the usefulness of the determination of nucleoside markers in the urine as a non-invasive method for determining precancerous conditions and, perhaps more importantly, for monitoring the effectiveness of therapy of cancers because the marker levels return to normal soon after effective therapy [1—4]. Elevated levels of pseudouridine [5, 6], N^2,N^2-dimethylguanosine [5], N^2-methylguanosine [7] and 1-methylinosine [7] have been found in the sera of some patients with cancer. Owing to the presence of relatively smaller amounts of modified nucleosides in sera compared to urine, the diagnostic and prognostic potential of altered modified nucleosides in sera has not been fully explored. Methods for modified nucleoside analysis by high-performance liquid chromatography (HPLC) have been developed by Gehrke and co-workers [8—10] and Schöch et al. [11]. We describe here a rapid and more sensitive method for determining picomole quantities of modified nucleosides in sera and urine using a C_{18} Radial-Pak cartridge.

EXPERIMENTAL

Apparatus

All chromatographic studies were conducted on an HPLC system consisting

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of two Beckman 100A pumps controlled by a 420 microprocessor controller/programmer and a Beckman gradient mixer (Beckman Instruments, Palo Alto, CA, U.S.A.), and a Waters 710A WISP automatic injector (Waters Assoc., Milford, MA, U.S.A.). Detection was carried out with a Waters 440 UV—VIS absorbance detector at 254 and 280 nm. A Spectrum 1021A noise filter and amplifier (Spectrum Scientific, Newark, DE, U.S.A.) was used to cut down on inherent detector noise and also to increase the sensitivity of the detector. Peak areas, retention times, and concentrations based on an internal standard method were calculated with a Perkin-Elmer Σ-10 data station.

Chemicals and reagents

Boronic acid gel (Affi-gel 601, 75—150 μm diameter) was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). HPLC-grade ammonium acetate was from Fisher Scientific (Fairlawn, NJ, U.S.A.). Ammonium phosphate monobasic crystal (HPLC grade), formic acid, absolute methanol and dibutylamine (5.94 *M*) were all Baker-analyzed reagent grade and purchased from J.T. Baker (Phillipsburgh, NJ, U.S.A.). The modified nucleosides used in this study were obtained from the following sources: pseudouridine (Ψ), N^4-acetylcytidine (ac^4C), 1-methylguanosine (m^1G), 8-bromoguanosine (Br^8G), 7-methylguanosine (m^7G) from Sigma (St. Louis, MO, U.S.A.); N^2-methylguanosine (m^2G), N^2,N^2-dimethylguanosine (m^2_2G), 1-methyladenosine (m^1A), 6-methyladenosine (m^6A) and 1-methylinosine (m^1I) from Vega Biochemicals (Tucson, AZ, U.S.A.). N^6-Threonyladenosine (t^6A) was kindly provided by Dr. Barbara Vold, SRI International (Menlo Park, CA, U.S.A.).

HPLC-grade ultrapure water (conductivity > 16 mΩ) generated by a water-purification system (Millipore, Milford, MA, U.S.A.) consisting of a 1-μm particulate filter, one organic filter and two ion-exchange filters connected in series, was used in the preparation of solutions. All solutions were filtered through a 0.45-μm filter (Magna Nylon 66, Fisher Scientific). Buffers were degassed before use.

Isolation of ribonucleosides from urine

The method of sample clean-up and the reagents used were essentially those described by Gehrke and co-workers [8—10] and Uziel et al. [12] with some modifications. The sample was thawed and shaken thoroughly to ensure homogeneity. An aliquot (0.5 ml) was transferred to a 1.5-ml Eppendorf microfuge tube, 300 μl of 2.5 *M* ammonium acetate buffer (pH 9.5) were added and the sample was mixed intermittently for 5 min with a Vortex mixer. The sample was centrifuged 12 000 *g* for 5 min, then the supernatant was transferred onto a phenylboronate affinity column (4.5 × 0.7 cm), equilibrated with 0.25 *M* ammonium acetate buffer (pH 8.8). The pellet was reextracted with 1 ml of 0.25 *M* ammonium acetate (pH 8.8) buffer, centrifuged and the supernatant was added to the phenylboronate column.

The affinity column was washed with two 4-ml aliquots of 0.25 *M* ammonium acetate (pH 8.8). The nucleosides were eluted with 10 ml of 0.1 *M* formic acid. The eluate was transferred to glass tubes and lyophilized. The lyophilized material was reconstituted in 2.0 ml of Br^8G (12.5 nmol/ml) solution.

Isolation of ribonucleosides from serum

The method was essentially the same as described for urine with minor modifications. A 1.0-ml volume of the serum was transferred to a 1.5-ml Eppendorf microfuge tube, 400 μl of 2.5 *M* ammonium acetate buffer (pH 9.5) were added and the sample was treated as described for urine. The lyophilized material after affinity chromatography was redissolved in 2 ml of water and transferred to a 100 × 13 mm Pyrex tube and lyophilized again. The residual material was reconstituted in 0.5 ml of Br^8G (12.5 nmol/ml) solution. After elution of the nucleosides from the boronate gel, the columns were washed with 20 ml of 0.1 *M* sodium chloride to remove pigmented material and stored in this solution. Prior to reuse the columns were washed with 20 ml of 0.1 *M* formic acid.

Chromatography

Column method. Two Waters μBondapak C_{18} columns (30 cm × 3.9 mm), connected in series and maintained at 32°C, were used. Modified nucleosides were eluted by applying a linear gradient (1 ml/min) of 100% buffer A (0.01 *M* ammonium dihydrogen phosphate (pH 5.1, 1% methanol) to 100% buffer B (buffer A containing 15% methanol) in 90 min. Isocratic separation with buffer B was used for quantitation of m_2^2G and t^6A [10].

Radial-Pak method. A C_{18} Waters Radial-Pak cartridge (5 μm particle size) housed in an RCM-100 compression module at room temperature was used. Modified nucleosides were separated by applying a linear gradient flow-rate (2 ml/min) of 100% buffer C [0.01 *M* ammonium dihydrogen phosphate (pH 3.0), $2.0 \cdot 10^{-5}$ *M* dibutylamine, and 5% methanol] to 100% buffer D (buffer C containing 30% methanol) in 30 min.

RESULTS AND DISCUSSION

The procedures used for the separation of modified nucleosides from urine and sera are essentially based on the methods developed by Gehrke and co-workers [8–10]. The three-buffer system of Gehrke et al. [10] resolves modified nucleosides in 2–5 h. In an attempt to improve their system, we tested different columns and buffers. μBondapak C_{18} columns were found to be superior to Supelco C_{18} DB (5 μm), Nova-Pak C_{18} (5 μm) column or cartridge. Modified nucleosides with the exception of m_2^2G and t^6A were clearly resolved on μBondapak columns in 100 min by applying a linear gradient of 1–15% methanol in 0.01 *M* ammonium phosphate (pH 5.1). However, a second run using isocratic separation with 0.01 *M* ammonium phosphate (pH 5.1) containing 15% methanol was required to resolve m_2^2G and t^6A (data not shown). More rapid and complete separation of nucleosides in a single run including m_2^2G, m^6A and t^6A was accomplished in 30 min using a Radial-Pak cartridge (Fig. 1). In order to compare the reliability of Radial-Pak method with the separation accomplished on μBondapak C_{18} columns (column method), the nucleoside content of twelve specimens of normal adult male urine was determined. There was an excellent correlation between the two methods (correlation coefficient, $r > 0.995$) and furthermore, sensitivity of detection of nucleosides was increased to 5 pmol. Modified nucleoside content of urine

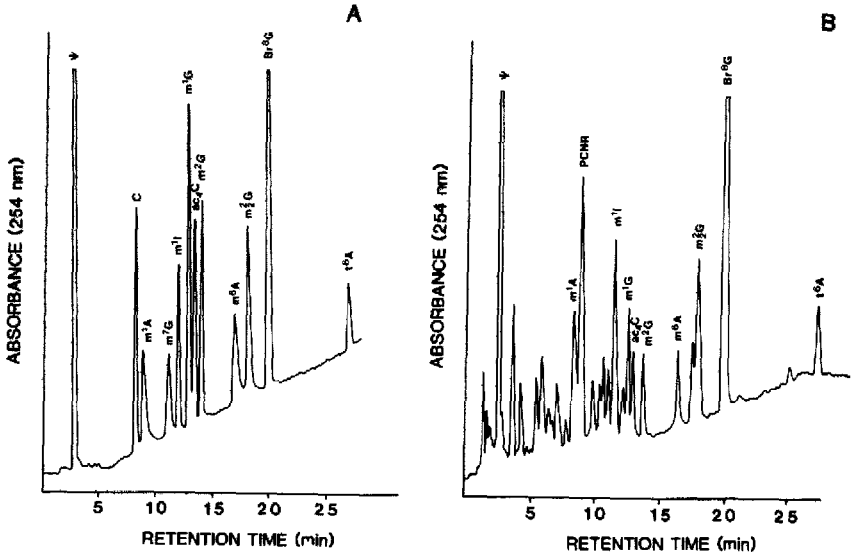

Fig. 1. Reversed-phase HPLC separation of modified nucleosides on a Radial-Pak cartridge. (A) Standard nucleoside mixture consisting of 100 pmol of each nucleoside, except Ψ which was 400 pmol; (B) urine from a cancer patient; injection corresponds to 4 μl of urine. Increase in baseline with time is due to a substantial increase in the sensitivity of detection. However, this does not present a problem in the quantitation of various peaks by the integrator. PCNR = 2-Pyridone-5-carboxamide-N'-ribofuranoside.

from healthy adult subjects is presented in Table I. These values are very similar to those reported by Speer et al. [13]. We have found higher levels of t^6A compared to those determined by Vold et al. [14] using radioimmunoassay. There was a significant positive correlation in excretion of modified nucleosides and age for Ψ ($r = 0.28$, $p = 0.078$) and m^1I ($r = 0.30$, $p = 0.067$). We have also noticed a small but significant difference in the average elution profiles for nine nucleosides and β-aminoisobutyric acid, represented as Z values, between smokers ($0.18Z$) and non-smokers ($-0.032Z$).

For the determination of modified nucleosides in serum, methods to separate nucleosides from serum proteins (i.e. precipitation with trichloracetic acid, perchloric acid, methanol, ethanol, ultrafiltration, gel filtration and affinity chromatography on boronate gel) were evaluated. Selective enrichment of nucleosides from serum by affinity chromatography on a phenylboronate (Aff-Gel) affinity column prior to HPLC analysis was found to be the most satisfactory. The nucleosides Ψ, m^1I, ac^4C, m^1G, m^2G, m^2_2G and t^6A were identified and quantitated in serum (Table II). These nucleosides in serum were identified by comparing their retention times with standard nucleosides, by a comparison of A_{254} and A_{280} and by co-chromatography with authentic nucleosides. The concentrations of these nucleosides in serum were much lower than in urine — Ψ was the major modified nuceloside (2.8 ± 0.5 nmol/ml for male and 2.6 ± 0.56 nmol/ml of serum for female) — and are nearly identical to those observed by Colonna et al. [6] and Gehrke [15]. In the present method Ψ could be detected with precision in sample injections equivalent to 2 μl of serum and this method is at least ten times more sensitive compared to the method of Colonna et al. [6] for Ψ. Since other modified nucleosides were

TABLE I

MODIFIED NUCLEOSIDE CONTENT IN URINE (nmol/μmol OF CREATININE)

Modified nucleosides were determined in duplicate aliquots of the urine and analyses of samples with more than 5% variation were repeated.

	Ψ	m^1A	PCNR*	m^1I	m^1G	ac^4C	m^2G	m_2^2G	t^6A
Males									
Non-smokers									
n	31	21	19	28	11	18	24	22	8
Mean	26.4	2.02	1.12	1.34	0.85	0.56	0.39	1.18	1.33
S.D.	5.01	0.39	0.37	0.39	0.26	0.13	0.14	0.39	0.23
Mean + 2S.D.	36.4	2.81	1.86	2.12	1.36	0.81	0.67	1.97	1.79
Smokers									
n	12	8	6	11	4	4	8	12	4
Mean	29.1	1.89	1.40	1.59	1.02	0.54	0.36	1.45	1.32
S.D.	5.23	0.29	0.61	0.38			0.16	0.34	
Mean + 2S.D.	39.6	2.48	2.63	2.35			0.69	2.13	
Smokers and non-smokers									
n	50	36	29	42	16	26	36	40	13
Mean	27.2	1.98	1.19	1.42	0.85	0.56	0.40	1.27	1.34
S.D.	5.37	0.40	0.46	0.39	0.28	0.15	0.15	0.40	0.19
Mean + 2S.D.	37.9	2.78	2.11	2.19	1.42	0.86	0.70	2.07	1.73
Females									
Non-smokers									
n	22	17	17	21	11	19	21	19	11
Mean	23.2	1.60	1.12	1.39	0.86	0.53	0.41	1.26	1.02
S.D.	3.2	0.29	0.23	0.26	0.11	0.07	0.08	0.21	0.18
Mean + 2S.D.	29.6	2.18	1.58	1.91	1.08	0.67	0.57	1.68	1.38
Smokers									
n	18	14	12	19	11	13	18	19	8
Mean	25.0	1.65	1.12	1.48	0.91	0.51	0.40	1.32	1.15
S.D.	2.99	0.33	0.25	0.26	0.21	0.10	0.09	0.20	0.26
Mean + 2S.D.	31.0	2.31	1.62	2.00	1.33	0.71	0.58	1.72	1.67
Smokers and non-smokers									
n	42	33	31	42	24	34	40	40	21
Mean	24.0	1.62	1.11	1.43	0.88	0.53	0.40	1.28	1.08
S.D.	3.15	0.29	0.23	0.26	0.16	0.08	0.08	0.19	0.20
Mean + 2S.D.	30.3	2.20	1.57	1.95	1.20	0.69	0.56	1.66	1.48

*PCNR = 2-Pyridone-5-carboxamide-N'-ribofuranoside.

TABLE II

MODIFIED NUCLEOSIDE CONTENT IN SERUM

Nucleoside	Content (mean ± S.D.) (pmol/ml)			
	Male (n = 48)		Female (n = 73)	
Ψ	2830	506	2640	560
m^1I	42.1	9.78	45.8	19.9
m^1G	39.7	16.1	45.4	20.4
ac^4C	71.6	18.4	63.5	18.8
m^2G	21.0	10.2	19.5	5.72
m_2^2G	46.5	19.2	47.0	21.5
t^6A	71.9	21.7	59.3	19.6

present in quantities 40—100 fold less than Ψ, large injection volumes corresponding to 80—200 μl of serum are required for their determination. Because of improved resolution, speed and sensitivity, the method using Radial-Pak cartridge is the method of choice for determining modified nucleosides from urine and sera and is routinely used in our laboratory. We have found that the frequency of elevation of modified nucleosides was lower in serum than in the urine of the same cancer patient [16]. A more recent study of twenty male subjects with diagnosed colon—rectal cancer and age-matched controls (provided by NCI Serum Bank) revealed that one or more modified nucleosides were elevated above mean plus two standard deviations only in 75% of the subjects and thus nucleoside determination in urine is a more reliable indicator of malignancy.

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