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DIRECT CLEAN-UP AND ANALYSIS OF RIBONUCLEOSIDES IN PHYSIOLOGICAL FLUIDS

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SUMMARY

We describe the group-selective separation and quantification of unmodified, modified and hypermodified ribonucleosides in physiological fluids (urine, serum) by on-line multidimensional high-performance affinity chromatography (HPAC)—reversed-phase liquid chromatography (RPLC). The excretion levels and patterns of ribonucleosides such as N^1-methyladenosine, N^1-methylinosine, N^2-methylguanosine, N^2-dimethylguanosine, N^6-carbamoylthreonyladenosine and 2-pyridone-5-carboxamido-N-ribofuranoside were determined in urines from a control group and from patients with different diseases. The HPAC—RPLC method applied represents a powerful tool, e.g. as a non-invasive screening test, a method to investigate disorders in ribonucleoside and/or RNA metabolism, a method for drug monitoring during nucleoside chemotherapy, and a method to study renal ribonucleoside reutilization.

INTRODUCTION

One of the central problems in biomedical chemistry is the detection of specific compounds in highly complex physiological matrices such as blood,

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serum, urine, lymphatic fluid or liquor. During the last few years, elaborated column-switching techniques have been developed in the field of liquid chromatography. These techniques involve, for example, a selective trace-enrichment, a peak front-, heart- or end-cut procedure or a back-flush method. In principle, these techniques always use two or more columns, which are connected in parallel or in series and thus allow the selective prefractionation and subsequent analysis of the target compounds.

We have introduced an on-line multi-dimensional high-performance affinity chromatography (HPAC)—reversed-phase liquid chromatography (RPLC) method for the group-selective separation and quantification of unmodified, modified and hypermodified ribonucleosides in physiological fluids [1–3]. This was realized using a boronic silica pre-column connected directly to a reversed-phase column by a switching technique [1, 2].

The *cis*-diol system of the ribonucleosides was chosen as a selectivity criterion as *cis*-diols can form cyclic esters with borate [4, 5]. In addition, it is known from the literature that boronic acid linked to polymer matrices such as agarose, cellulose or polyacrylamide was used as an affinity ligand for the separation of *cis*-diol compounds under low-pressure conditions [6—12]. In order to get an appropriate affinity material that is suitable for high-pressure conditions, LiChrosorb Si 100 was substituted with γ-chloropropyltrimethoxysilane and subsequently reacted with *m*-aminobenzeneboronic acid, as described recently [13].

In principle, the desired group-selective prefractionation and clean-up of *cis*-diols is carried out by a simple pH-step elution [1, 2]. After sample application, the target compounds form (under slightly alkaline conditions) a complex with the boronate and thus are selectively retarded, whereas the sample matrix is discharged. The target compounds are then eluted by lowering the pH of the mobile phase to < 4.

The high selectivity and capacity of the prepared affinity material, as well as reduction of the clean-up time and elution volume, formed the basis for the successful application of a column-switching technique, i.e. the direct transfer of the acidic ribonucleoside-containing HPAC fraction onto an analytical RPLC column [1, 2].

EXPERIMENTAL

Materials

Adenosine (Ado), cytidine (Cyd), inosine (Ino), uridine (Urd) and guanosine (Guo) were purchased from Boehringer (Mannheim, F.R.G.). The methylated ribonucleosides N^1-methyladenosine (m^1Ado), N^1-methylinosine (m^1Ino), N^2-methylguanosine (m^2Guo), N^6-dimethyladenosine (m^6_2Ado) and pseudouridine (Ψ) were from **Sigma** (Munich, F.R.G.). N^2-Dimethylguanosine (m^2_2Guo), N^6-(carbamoylthreonyl)adenosine (t^6Ado) and 2-pyridone-5-carboxamido-N-ribofuranoside (PCNR) were isolated from urine. For further characterization, t^6Ado and PCNR were also chemically synthesized [14, 15]. One tablet of Isoprinosine® (Röhm-Pharma, Darmstadt, F.R.G.) contains 120.25 mg of inosine and 379.75 mg of 1-(dimethylamino)-2-propanol-(4-acetamidobenzoate). Double-distilled water and salts from Merck (Darmstadt,

F.R.G.), of the purest grade available, were used in all buffer preparations.

Apparatus

A basic Altex microprocessor-controlled gradient system (Altex, Berkeley, CA, U.S.A.), consisting of two Altex Model 110A pumps, a Model 420 microprocessor, a Rheodyne Model 7125 loop injector for sample introduction and a Kontron Uvicon Model 725 spectrophotometer, was additionally equipped with a third Altex Model 110A pump, a second Rheodyne 7125 loop injector and a Rheodyne Model 7010 six-port valve, as described in detail in a preceding paper [1]. Integration was performed with a Hewlett-Packard Model 3390A integrator (Hewlett-Packard, Frankfurt, F.R.G.).

Sample preparation

Human urine (500 μl) was membrane-filtered (Millox 0.22 μm; Millipore, Buc, France) and an aliquot of 10–50 μl was applied to the HPAC column. Samples (1 ml) of human serum were adjusted to pH 4 with formic acid and deproteinized within 10 min by centrifugal ultrafiltration (Amicon, Micropartition System, Witten, F.R.G.). A 200-μl volume of the ultrafiltrate was applied to the HPAC column. The 24-h urines were collected, adjusted to pH 4 and stored at $-20°$C until investigation. Further details concerning sample collection, age and sex of the subjects are given in the legends to the tables.

Analytical procedure

The HPAC column (column 1; 30 × 4 mm I.D.) was filled by an upward slurry-packing technique with a laboratory-prepared boronic acid-substituted silica [13]. Column 2 (250 × 5 mm I.D.) was laboratory-packed with LiChrosorb RP-18, 7 μm (Merck).

Column 1 was equilibrated for 2 min with 0.1 *M* ammonium phosphate (pH 8.3). After sample injection (10–200 μl of urine, deproteinized serum or a synthetic mixture), column 1 was washed for 2 min with the same buffer. During that time, ribonucleosides were selectively retarded on the HPAC column and the sample matrix was discharged. After this clean-up step, column 1 was series-connected in front of column 2. The group-specifically bound ribonucleosides on column 1 were then eluted under acidic conditions (0.15 *M* ammonium formate, pH 3.5) in a small volume (ca. 700 μl) and concentrated on top of the reversed-phase C_{18} column over a period of 1.5 min. The acidic buffer used for the elution of ribonucleosides from column 1 was also used as mobile phase for the subsequent analytical separation on column 2. Separation of nucleosides on column 2 was then carried out by increasing the amount of methanol as organic modifier in the mobile phase.

The quantitative determinations were carried out according to the external standard method. Calibration mixtures of the appropriate nucleosides were prepared, based on the following UV data (H_2O): m^1Ado (λ_{max} 257 nm, ϵ = 13.7 cm^2/μmol, pH 1.5); m^1Ino (λ_{max} 248 nm, ϵ = 9.6 cm^2/μmol, pH 1.5); m^2Guo (λ_{max} 258 nm, ϵ = 11.4 cm^2/μmol, pH 1.5); m^2_2Guo (λ_{max} 264 nm, ϵ = 12.8 cm^2/μmol, pH 1.5); t^6Ado (λ_{max} 269 nm, ϵ = 24.9 cm^2/μmol, pH 5.0); PCNR (λ_{max} 259 nm, ϵ = 11.3 cm^2/μmol, pH 5.0).

Determination of creatinine

Creatinine was measured with the Beckman Creatinine Analyzer 2 (Beckman, Munich, F.R.G.). Reliability of the overall system performance was monitored with Precinorm® level 2 (Boehringer) and control serum Desicion® level 2 (Beckman).

RESULTS AND DISCUSSION

Fig. 1A shows the on-line HPAC—RPLC separation of a synthetic mixture of eighteen ribonucleosides (the formulae of the nucleobases are given in Fig.

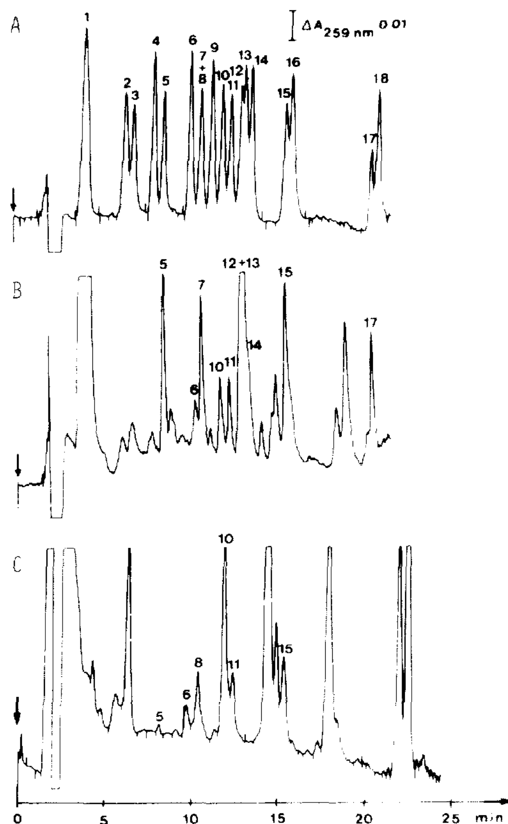

Fig. 1. On-line HPAC—RPLC analysis of ribonucleosides. (A) On-line analysis of a synthetic mixture of eighteen ribonucleosides which are presented in Fig. 2. Peaks: 1 = pseudouridine (Ψ); 2 = cytidine (Cyd); 3 = uridine (Urd); 4 = 5-aminoimidazole-4-carboxamido-N-ribofuranoside (AICAR); 5 = N^1-methyladenosine (m^1Ado); 6 = inosine (Ino); 7 = 2-pyridone-5-carboxamido-N-ribofuranoside (PCNR) [= 1,6-dihydro-6-oxo-1-(β-D-ribofuranosyl)-3-pyridinecarboxylic amide]; 8 = guanosine (Guo); 9 = N^3-methyluridine (m^3Urd); 10 = adenosine (Ado); 11 = N^1-methylinosine (m^1Ino); 12 = N^1-methylguanosine (m^1Guo); 13 = N^4-acetylcytidine (ac^4Cyd); 14 = N^2-methylguanosine (m^2Guo); 15 = N^2-dimethylguanosine (m^2_2Guo); 16 = N^6-methyladenosine (m^6Ado); 17 = N^6-carbamoylthreonyladenosine (t^6Ado); 18 = N^6-dimethyladenosine (m^6_2Ado). (B, C) On-line analysis of 10 μl of membrane-filtered human urine and 200 μl of serum ultrafiltrate, respectively. Chromatographic conditions: column 2 was operated with 0.15 mol/l ammonium formate (pH 3.5); after 2 min, linear gradient up to 11% methanol in 8 min, followed by a linear gradient up to 16% methanol in 4 min; after 2 min at 16% methanol, linear gradient up to 50% methanol in 9 min; flow-rate, 1.8 ml/min; detection, UV 259 nm. During the first 1.5 min of elution, column 1 was series-connected to column 2 (cf. *Analytical procedure*).

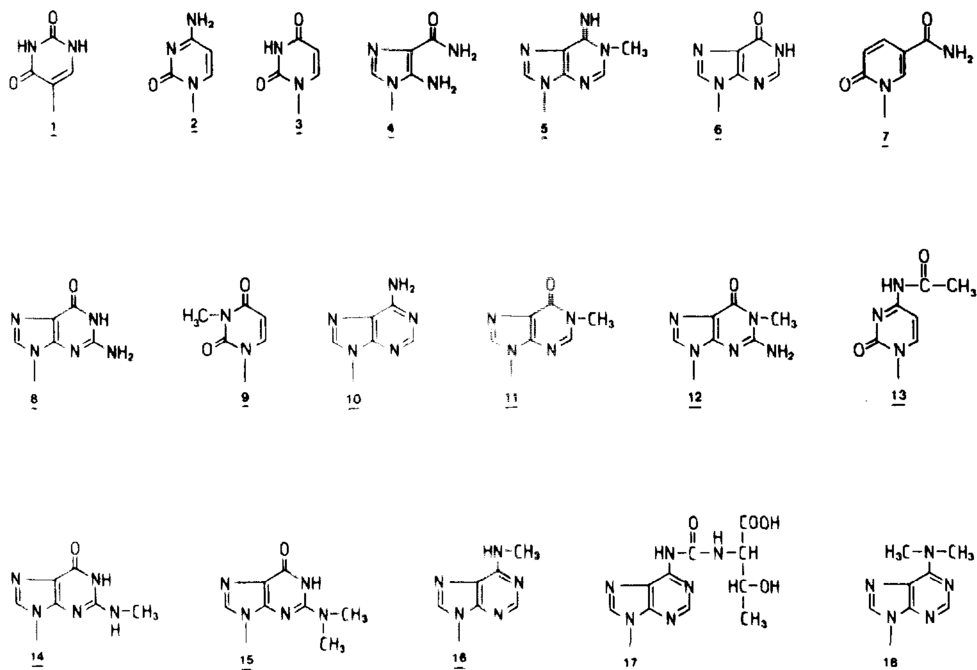

Fig. 2. Ribonucleosides separated by on-line HPAC–RPLC. Pseudouridine (Ψ), 1; cytidine (Cyd), 2; uridine (Urd), 3; 5-aminoimidazole-4-carboxamido-N-ribofuranoside (AICAR), 4; N^1-methyladenosine (m^1Ado), 5, inosine (Ino), 6; 2-pyridone-5-carboxamido-N-ribofuranoside (PCNR) [= 1,6-dihydro-6-oxo-1-(β-D-ribofuranosyl)-3-pyridinecarboxylic amide], 7; guanosine (Guo), 8; N^3-methyluridine (m^3Urd), 9; adenosine (Ado), 10; N^1-methylinosine (m^1Ino), 11; N^1-methylguanosine (m^1Guo), 12; N^4-acetylcytidine (ac^4Cyd), 13; N^2-methylguanosine (m^2Guo), 14; N^2-dimethylguanosine (m^2_2Guo), 15; N^6-methyladenosine (m^6Ado), 16; N^6-carbamoylthreonyladenosine (t^6Ado), 17; N^6-dimethyladenosine (m^6_2Ado), 18.

2), Fig. 1B that of 10 μl of membrane-filtered human urine and Fig. 1C that of 200 μl of ultrafiltrated human serum. By use of this on-line technique, the following ribonucleosides could be separated and characterized (Fig. 2): pseudouridine (Ψ), 1; cytidine (Cyd), 2; uridine (Urd), 3; 5-aminoimidazole-4-carboxamido-N-ribofuranoside (AICAR), 4; N^1-methyladenosine (m^1Ado), 5; inosine (Ino), 6; 2-pyridone-5-carboxamido-N-ribofuranoside (PCNR) [= 1,6-dihydro-6-oxo-1-(β-D-ribofuranosyl)-3-pyridinecarboxylic amide], 7; guanosine (Guo), 8; N^3-methyluridine (m^3Urd), 9; adenosine (Ado), 10; N^1-methylinosine (m^1Ino), 11; N^1-methylguanosine (m^1Guo), 12; N^4-acetylcytidine (ac^4Cyd), 13; N^2-methylguanosine (m^2Guo), 14; N^2-dimethylguanosine (m^2_2Guo), 15; N^6-methyladenosine (m^6Ado), 16; N^6-carbamoylthreonyladenosine (t^6Ado), 17; N^6-dimethyladenosine (m^6_2Ado), 18.

Linearity of concentration versus signal plots was verified over the concentration range of interest for the following compounds: m^1Ado, m^1Ino, m^2_2Guo, PCNR, t^6Ado, Ado, Guo and Ino. All correlation coefficients were between 0.998 and 0.999.

To monitor the accuracy of the overall chromatographic system, the matrix-dependent and matrix-independent recovery of ribonucleosides was determined and measured between 98 and 100%. Results are summarized in Tables I and II.

TABLE I

MATRIX-DEPENDENT RECOVERY OF m^1Ado, PCNR, m^1Ino, m_2^2Guo AND t^6Ado ADDED TO POOLED HUMAN URINE

Nucleoside	Concentration* (μmol/ml)			Recovery (%)
	Urine	Spike	Urine + Spike (mean ± S.D.)	
m^1Ado	11.49	5.17	16.41 ± 0.3	99
PCNR	8.57	4.82	13.25 ± 0.2	99
m^1Ino	7.46	9.91	17.03 ± 0.2	98
m_2^2Guo	9.91	4.04	13.65 ± 0.3	98
t^6Ado	4.39	2.60	6.97 ± 0.1	100

*Each value is an average of three assays.

TABLE II

MATRIX-INDEPENDENT RECOVERY OF m^1Ado, PCNR, m^1Ino, m_2^2Guo, t^6Ado, Ino, Guo AND Ado

Nucleoside	Recovery (mean ± S.D., n = 3) (%)
m^1Ado	99.4 ± 1.3
PCNR	100.2 ± 1.6
m^1Ino	99.7 ± 1.6
m_2^2Guo	99.8 ± 4.3
t^6Ado	99.4 ± 0.6
Ino	100.0 ± 2.9
Guo	100.5 ± 0.1
Ado	100.6 ± 0.7

TABLE III

URINARY EXCRETION OF RIBONUCLEOSIDES

Number: 24 (females). Age: 16—42 years. Creatinine: 9.32 ± 3.5 mmol per 24 h.

Nucleoside	Urinary excretion	
	μmol per 24 h	nmol/μmol of creatinine
m^1Ado	20.4 ± 5.8	2.19 ± 0.63
m^1Ino	15.4 ± 5.0	1.65 ± 0.55
Ino	13.1 ± 5.1	1.41 ± 0.50
m^2Guo	3.5 ± 1.2	0.38 ± 0.10
m_2^2Guo	21.0 ± 7.0	2.26 ± 0.29
t^6Ado	8.8 ± 2.9	0.95 ± 0.25
PCNR	25.1 ± 11.6	2.70 ± 1.02

Inter-individual means and standard deviations of urinary ribonucleoside excretion of a control group of subjects (females) are given in Table III. The values are expressed in μmol per 24 h and nmol nucleoside per μmol creatinine.

TABLE IV

URINARY NUCLEOSIDE EXCRETION PATTERNS OF CANCER PATIENTS INVESTIGATED WITH HPLC BY DIFFERENT LABORATORIES

Underlined values exceed control mean + 2S.D.

Patient	Age	Sex	Cancer	Elevated markers	Urinary excretion (nmol nucleoside per μmol creatinine)						Reference
					Ψ	m^1Ado	m^1Ino	m_2^2Guo	t^6Ado	PCNR	
1	18	Male	Myelotic leukemia	3	—*	1.51	9.29	11.22	1.70	1.02	This paper
2	59	Female	Myelotic leukemia	3	—	1.35	4.37	7.05	1.52	2.37	
3	45	Female	Praeleukemia (wrong diagnosis)	—	—	1.13	1.43	2.69	0.02	1.77	
1	5	Male	Lymphoblastic leukemia	2	—	—	4.26	3.85	—	2.87	22
2	6	Male		2	—	—	2.97	3.27	—	2.37	
3	16	Male		2	—	—	5.25	5.27	—	1.91	
4	11	Female		3	—	—	3.52	4.27	—	2.62	
1	Adult	Female	Lung carcinoma	4	—	3.30	2.50	2.50	—	2.40	19
2	Adult	Male		4	—	3.50	2.00	2.60	—	4.60	
3	Adult	Male		3	—	1.80	3.50	2.20	—	5.00	
4	Adult	Female	Breast cancer	1	—	2.50	1.50	1.50	—	1.90	
5	Adult	Female		2	—	2.90	2.20	1.80	—	1.20	
1	Adult		Hodgkin disease	3	40.62	9.37	2.02	1.40	—	—	26
2	Adult			3	36.66	7.17	2.94	2.25	—	—	
3	Adult			2	39.28	9.60	0.69	1.26	—	—	
4	Adult			3	61.10	—	2.20	3.80	—	—	
5	Adult			1	32.00	—	1.16	1.19	—	—	
6	Adult			3	36.00	12.00	0.45	1.55	—	—	
7	Adult			4	29.57	7.19	1.70	2.60	—	—	
8	Adult		Lymphocytic lymphoma	1	19.55	7.59	0.27	0.84	—	—	
9	Adult			2	44.88	8.96	1.20	1.48	—	—	
10	Adult			1	28.87	—	0.64	0.83	—	—	
11	Adult			3	41.45	12.63	2.04	2.18	—	—	
12	Adult			3	82.64	19.00	1.65	1.35	—	—	

*Not determined.

Creatinine proved to be a reliable basis for such comparisons [16], and its levels are a function of body mass [17].

Numerous results have been accumulated in several laboratories [7, 8, 12, 16, 18—26] during the last decade, showing that the analysis of urinary ribonucleosides might be useful as a non-invasive screening test. These groups could demonstrate that the excretion of modified ribonucleosides is altered in individuals suffering from cancer diseases. Table IV summarizes part of the studies carried out in several laboratories [19, 22, 26] on groups of patients suffering from different carcinoma. Urinary levels of some modified ribonucleosides were determined, whereby the underlined values exceed control means + 2S.D. The results indicate that Ψ, m^1Ado and m^1Ino represent marker molecules among the nucleosides investigated. m_2^2Guo and PCNR, on the other hand, were increased in fewer cases. As far as myelotic leukemia patients are concerned, the results indicate that among the nucleosides measured, m^1Ino, m_2^2Guo and t^6Ado represent marker molecules, whereas the excretion level of m^1Ado and PCNR remains unaltered in comparison to the control group (Table IV). These results do not answer but raise the question of whether there are specific excretion patterns of ribonucleosides for specific cancer malignancies, and, furthermore, whether increased excretion levels of distinct nucleoside markers may be observed in other diseases, such as metabolic disorders (diabetes) or during an activated metabolic phase such as pregnancy.

Table V shows the excretion levels and patterns of five ribonucleosides determined in the urine of diabetics. The inter-individual means for the

TABLE V

INTER-INDIVIDUAL MEAN AND STANDARD DEVIATION OF RIBONUCLEOSIDE EXCRETION AND RIBONUCLEOSIDE/CREATININE RATIO

Nucleoside	Collective	Excretion (μmol per 24 h)		Nucleoside/creatinine ratio *** (nmol/μmol)
		Mean	S.D.	
m^1Ado	Control group*	21.3	5.8	3.45
	Diabetics**	17.8	7.8	1.64
m^1Ino	Control group	14.7	5.0	2.75
	Diabetics	21.6	9.3	1.99
m_2^2Guo	Control group	41.9	13.8	2.84
	Diabetics	16.0	6.4	1.47
t^6Ado	Control group	8.8	2.9	1.45
	Diabetics	7.8	2.0	0.72
PCNR	Control group	25.1	11.6	4.74
	Diabetics	19.2	9.2	1.77

*Number: six (four females, two males); age: 20—57 years; creatinine: 10.85 ± 3.27 mmol per 24 h.

**Number: six (five females, one male); age: 42—70 years; creatinine: 12.90 ± 7.64 mmol per 24 h.

***Control mean + 2S.D.

TABLE VI

INTER-INDIVIDUAL MEAN AND STANDARD DEVIATION OF RIBONUCLEOSIDE EXCRETION AND RIBONUCLEOSIDE/CREATININE RATIO

Nucleoside	Collective	Excretion (μmol per 24 h)		Nucleoside/creatinine ratio *** (nmol/μmol)
		Mean	S.D.	
m^1Ado	Control group*	19.4	6.3	2.7
	Pregnant group**	19.8	7.9	2.6
m^1Ino	Control group	16.0	5.7	2.2
	Pregnant group	15.9	6.8	2.1
m_2^2Guo	Control group	21.0	7.0	2.8
	Pregnant group	22.1	8.5	2.9
m^2Guo	Control group	3.5	1.2	0.6
	Pregnant group	4.4	1.8	0.6

*Number: twenty females; age: 16—33 years; creatinine: 9.4 ± 2.9 mmol per 24 h.
**Number: seven; age: 19—27 years; creatinine: 7.8 ± 3.2 mmol per 24 h (each value expressed as a mean of longitudinal and transversal data of the pregnant group; the urine was collected as part of routine ambulatory control in four week intervals between the fifth month of pregnancy and one week post-partum).
***Control mean + 2S.D.

TABLE VII

INTER-INDIVIDUAL MEAN AND STANDARD DEVIATION OF URINARY RIBONUCLEOSIDE EXCRETION AND RIBONUCLEOSIDE/CREATININE RATIO

Nucleoside	Collective	Excretion (μmol per 24 h)		Nucleoside/creatinine ratio *** (nmol/μmol)
		Mean	S.D.	
m^1Ado	Control group*	45.2	10.8	3.91
	Inosiplex group**	23.2	5.9	2.19
Ino	Control group	13.1	5.1	1.66
	Inosiplex group	21.1	12.8	1.27
m^1Ino	Control group	40.6	9.0	4.05
	Inosiplex group	213.9	54.3	13.37
m_2^2Guo	Control group	64.8	33.2	4.83
	Inosiplex group	44.8	26.3	3.02
t^6Ado	Control group	16.8	1.0	1.37
	Inosiplex group	29.3	2.4	1.89
PCNR	Control group	20.3	6.7	2.41
	Inosiplex group	25.9	13.3	1.89

*Number: five (one female, four males); age: 25—41 years; creatinine: 16.55 ± 3.36 mmol per 24 h.
**Number: five (one female, four males); age: 25—41 years; creatinine: 15.99 ± 3.54 mmol per 24 h (after an oral Inosiplex application of 3.6 mmol of inosine = a daily dose of eight tablets of Isoprinosine).
***Control mean + 2S.D.

excretion of the ribonucleosides remain within the 2S.D.-range of the control mean. The ribonucleoside excretion of the diabetics does not show any pathological difference compared to the control group.

Table VI presents the excretion levels and patterns of four ribonucleosides determined in the urine of healthy non-pregnant and pregnant females. The inter-individual means for the excretion of the ribonucleosides examined remain within the 2S.D.-range of the control mean. This observation is surprising in sofar as an elevated level of ribonucleosides should be expected owing to increased protein biosynthesis and tRNA turnover during foetal development, especially during the third trimester of pregnancy.

Table VII gives an example of a direct course control during the treatment with the immunomodulator Inosiplex (Inoprinosine) and presents the excretion values of six ribonucleosides measured in 24-h urines without and after oral application of 3.6 mmol of inosine (corresponding to a daily dose of eight tablets of Isoprinosine) to a group of healthy subjects. t^6Ado and, in particular, m^1Ino show a markedly increased excretion after application of the immuno-modulator, whereas the other ribonucleosides and inosine itself remain

TABLE VIII

INTER-INDIVIDUAL MEAN AND STANDARD DEVIATION OF SERUM RIBONUCLEOSIDE CONCENTRATION AND RIBONUCLEOSIDE/CREATININE RATIO

Nucleoside	Collective	Concentration (μmol/l)		Nucleoside/creatinine ratio § (nmol/μmol)
		Mean	S.D.	
m^1Ado	Control group*	0.10	0.03	2.9
	1. Inosiplex group**	0.10	0.01	0.9
	2. Inosiplex group***	0.14	0.04	1.5
m^1Ino	Control group	<0.05		0
	1. Inosiplex group	0.95	0.63	12.4
	2. Inosiplex group	<0.05		0
Ino	Control group	1.22	0.72	43.9
	1. Inosiplex group	1.29	0.33	21.9
	2. Inosiplex group	1.12	0.52	25.0
Ado	Control group	0.23	0.07	5.6
	1. Inosiplex group	0.20	0.12	3.2
	2. Inosiplex group	0.17	0.05	3.7
Guo	Control group	0.22	0.08	4.9
	1. Inosiplex group	0.21	0.06	3.7
	2. Inosiplex group	0.19	0.06	3.8

*Number: five (one female, four males); age: 25—41 years; creatinine: 58.7 ± 13.9 μmol/l.
**Number: five (one female, four males); age: 25—41 years; creatinine: 64.6 ± 27.4 μmol/l (during Inosiplex application, five (= 448 μmol inosine per l blood) out of eight (= 717 μmol inosine per l blood) tablets of Isoprinosine).
***Number: five (one female, four males); age 25—41 years; creatinine: 51.3 ± 13.5 μmol/l (12 h after Inosiplex application, 717 μmol Inosine per l blood; blood volume: 5 l).
§Control mean + 2S.D.

unaltered. The amount of m^1Ino excreted (underlined value) represents about 5% of the orally applied inosine-dosis.

Table VIII presents the serum concentration of the major ribonucleosides adenosine, guanosine and inosine, as well as the minor modified ribonucleosides m^1Ado and m^1Ino, in the Inosiplex group and the control group. m^1Ino, but not the drug inosine, is the only nucleoside among the measured ones which exceeds control mean + 2S.D. These results suggest a direct methylation of the applied immunomodulator inosine. The question, however, of whether there is a relationship between the formation of m^1Ino and the therapeutic effect of Inosiplex remains open.

CONCLUSIONS AND OUTLOOK

Numerous results have been accumulated in several laboratories in the last decade, which indicate specific ribonucleoside excretion patterns for specific malignancies. Most of the data available seem to suggest a possible correlation between the excretion level as well as a pattern of specific modified ribonucleosides such as Ψ, m^1Ado, m^1Ino, m_2^2Guo and t^6Ado and neoplastic diseases. For example, most of the ribonucleosides given in Table III as m^1Ado, m^1Ino, m_2^2Guo, Ψ and t^6Ado have been exploited as marker molecules in the urine from patients with different malignancies.

The recent work of Nass and co-workers [27, 28] and Clark et al. [29] with rodents bearing experimentally induced tumors, demonstrate that the time course of nucleoside excretion shows increased urinary levels of modified ribonucleosides such as Ψ, m^1Ado, m^1Ino and m_2^2Guo several weeks before malignant lymphomas can be diagnosed clinically. To date, the question remains unanswered of whether the ribonucleosides present biochemical markers that indicate a developing neoplastic disease or whether these ribonucleosides are prerequisites for the development of tumour cells.

The described on-line analysis of ribonucleosides therefore represents a powerful tool, for example, as: (i) an early non-invasive screening test for cancer diseases in humans; (ii) a method to investigate disorders in ribonucleoside, ribonucleotide and/or RNA metabolism; (iii) a method for therapeutic drug monitoring during nucleoside chemotherapy; and (iv) a method to study renal reutilization processes.

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