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Chromatographic, capillary electrophoretic and matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of urinary modified nucleosides as tumor markers

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Abstract

Modified nucleosides are formed posttranscriptionally in RNA. During RNA turnover free modified nucleosides are formed which circulate in the blood stream and are excreted in the urine. Their levels are increased in a number of malignant diseases, and they can be used in clinical chemistry as tumor markers. The analysis includes the isolation of the nucleosides from urine with phenylboronate gel and their separation and quantitation by HPLC on C_{18} columns or by capillary electrophoresis on uncoated columns applying a sodium dodecyl sulfate–borate–phosphate buffer. Identification of the nucleosides is performed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry including post source decay spectra. In two clinical studies the diagnostic value of urinary modified nucleosides is investigated, in a study on children with leukemia and other malignant diseases and a study on women with breast cancer. Candidate markers are pseudouridine, 1-methylguanosine, *N*2-methylguanosine, 3-methyluridine and 1-methyl-inosine.

Keywords: Nucleosides; Tumor markers; Leukemia

1. Introduction

RNA, particularly t-RNA, contains a number of modified nucleosides, in addition to the normal ribonucleosides adenosine, guanosine, cytidine and uridine. The modifications are formed posttranscriptionally within the polynucleotide molecule by various modification enzymes, such as methyltransferases and ligases. During RNA turnover, free normal and modified nucleosides are created by the hydrolytic action of ribonucleases and phosphatases. In contrast to normal nucleosides, many of the modified nucleosides are biochemical end products and cannot be reutilized for de novo synthesis of RNA. They circulate in the blood stream and, together with small amounts of normal nucleosides, they are excreted in the urine. Their levels in serum and urine reflect the t-RNA turnover [1–3]. In several malignant diseases the levels of modified nucleosides are increased [4–7]. In addition to the absolute levels, the relative ratios of the nucleosides are changed in such pathological situations. This is due to aberrant modifying enzymes in tumor tissue, resulting in altered enzyme pattern and enzyme activity. Of diagnostic importance as tumor markers are mainly those nucleosides which are biochemical end products, such as 1-methylguanosine, *N*2-methylguanosine, 3-methyluridine, 1-methylinosine and pseudouridine.

The techniques usually applied for the analysis of the nucleosides include their isolation from the urine by phenylboronate affinity gel chromatography taking advantage of the reaction of phenylboronate with the vicinal OH groups of the ribonucleosides [8]. Separation of the nucleosides is achieved equally well by RP-HPLC [8,9] and by capillary electrophoresis in the micellar electrokinetic chromatog-

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raphy (MEKC) mode [10]. Identification of the modified nucleosides is based for the first time on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

2. Experimental

2.1. Urine samples

Random urine samples were collected from healthy children and adults, from children with leukemia and other malignant diseases and women with breast cancer. Criteria for inclusion of healthy control persons into the study were that at the time of sample collection no medication was taken or any form of medical treatment was performed, and that the collected urine samples had normal test results for glucose, ketone bodies, bilirubin, urobilinogen, protein, nitrite, leukocytes, erythrocytes and hemoglobin. After collection, the urine samples were immediately frozen without any preservative and stored at −20 °C. Directly before the analysis the samples were thawed at room temperature.

2.2. Chemicals

Reference substances for 14 ribonucleosides, i.e. uridine (U), cytidine (C), adenosine (A), guanosine (G), inosine (I), xanthosine (X), 3-methyluridine (M3U), 1-methyladenosine (M1A), *N*6-methyladenosine (M6A), 1-methylguanosine (M1G), *N*2-methylguanosine (M2G), 1-methylinosine (M1I), pseudouridine (Pseu) and 5′-deoxy-5′-methylthioadenosine (MTA) were purchased from Sigma–Aldrich (Seelze, Germany). The internal standard isoguanosine (isoG), was synthesized from guanosine according to the method of Divakar et al. [11]. Formic acid was obtained from Riedel-de Haen (Seelze, Germany) and Affi-gel 601 from Bio-Rad (Munich, Germany). Ammonia, ammonium acetate, methanol and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany), 2,5-dihydroxybenzoic acid (DHB) from Fluka (Taufkirchen, Germany) and α-cyano-4-hydroxycinnamic acid (CHCA) from Sigma–Aldrich.

2.3. Sample preparation

The nucleosides were extracted from urine applying a modification of the method described by Gehrke et al. [8]. In brief, a 10 ml volume of centrifuged urine with a pH value adjusted to 6.5 or higher by addition of an aqueous solution of ammonia, was mixed with 0.5 ml of a 0.25 mM aqueous solution of the internal standard isoguanosine. The extraction was performed on glass columns packed with 500 mg of phenylboronate gel (Affi-gel 601, Bio-Rad). After loading, the gel was washed with 20 ml of 0.25 M ammonium acetate and methanol–water (1:1, v/v) and the nucleosides were eluted with 25 ml of a 0.1 M solution of formic acid in methanol–water (1:1, v/v). After evaporation to dryness under vacuum at 35 °C, the residue was dissolved in 1 ml of a 25 mM solution of potassium dihydrogenphosphate at pH 5.0.

2.4. HPLC separation of the nucleosides

The separation of the nucleosides was performed on an HPLC system (Merck-Hitachi, Darmstadt, Germany) composed of an L-6200 pump, a 655A-40 column oven, an L-3000 photo diode array detector and an L-7200 autosampler, using a 250 mm × 4 mm, 5 μm LiChrospher 100 C18e column (Merck, Darmstadt, Germany) at 30 °C and an injection volume of 20 μl. The elution was carried out using a concentration gradient with 25 mM potassium dihydrogenphosphate at pH 5.0 and methanol–water (3:2, v/v). UV detection was performed at 260 nm.

2.5. Capillary electrophoretic separation of the nucleosides

For CE separation on a Dionex CES I capillary electrophoresis system (Idstein, Germany) an uncoated column (500 mm × 50 μm), a 300 mM sodium dodecyl sulfate (SDS)–25 mM borate–50 mM phosphate buffer (pH 6.7) and a voltage of 7.5 kV were used. The nucleosides were monitored by UV detection at a wavelength of 260 nm.

2.6. MALDI-TOF-MS analysis of the nucleosides

The analyses were performed on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer, model Autoflex (Bruker, Germany), with a 337 nm nitrogen laser. An AnchorChip target with 0.4 μm Anchors (Bruker, Germany) was used, applying DHB and CHCA as matrix substances.

For the analysis of the reference nucleosides, each nucleoside was dissolved in 0.1% trifluoroacetic acid (TFA) at a concentration of 1 mg/ml. One microlitre of these solutions was placed on target together with 0.5 μl of a DHB-solution (5 mg/ml 0.1% TFA–acetonitrile, 2:1, v/v). For high mass accuracy measurements, a thin layer of CHCA was prepared on the AnchorChip target and the analyte solution added afterwards. Urinary nucleosides were isolated by semipreparative HPLC. Fractions were collected in 1.5 ml tubes, lyophilised, redissolved in 10 μl of 0.1% TFA and 1 μl of this solution applied to the target.

In addition to the MALDI-TOF-MS spectra of the nucleosides, post source decay (PSD) spectra of the molecular ions were recorded.

2.7. Quantitation of the nucleosides

Quantitation was based on five calibration standards with different concentrations of the reference nucleosides, and

the concentrations of the urinary nucleosides, expressed in nmol/ml urine, were determined. These concentration values were transformed into nmol nucleoside/μmol creatinine. The urinary creatinine levels were determined by a modified Jaffé method whose principle is the reaction between creatinine and picric acid using colorimetric detection [12].

2.8. Statistical evaluation

Data processing and graphic presentations were performed with SPSS 8.0. The nucleoside levels were presented by boxplots which show the median value (50% percentile), the interquartile range with the 25% percentile on the bottom of the box and the 75% percentile on the top of the box, and extreme values outside the box.

3. Results and discussion

3.1. Methodical aspects

It has been shown that RP-HPLC and CE are equally suited for separation and quantitation of the nucleosides [9,10]. For the modified nucleosides the reproducibilities as expressed by the coefficients of variation are 11% or lower, except for M3U (17.7%). For the clinical studies on children with leukemia and other malignant diseases and on women with breast cancer, the nucleosides are measured by RP-HPLC. Fig. 1 shows a typical example of urinary nucleosides separated by HPLC.

With the MALDI-TOF-MS and PSD spectra the identities of urinary normal and modified nucleosides are proven. Examples are given in Figs. 2 and 3. To the authors' best knowledge this is the first time that nucleosides from urine were analyzed by MALDI-TOF-MS and PSD-MALDI. The PSD spectra of the nucleosides show a characteristic cleavage between the nucleic base and the sugar moiety. The resulting nucleic base peak is most informative for the identification of the nucleoside. Other fragments enhance the selectivity as they are characteristic for the different nucleosides, except those with isomeric nucleic bases which produce similar PSD spectra.

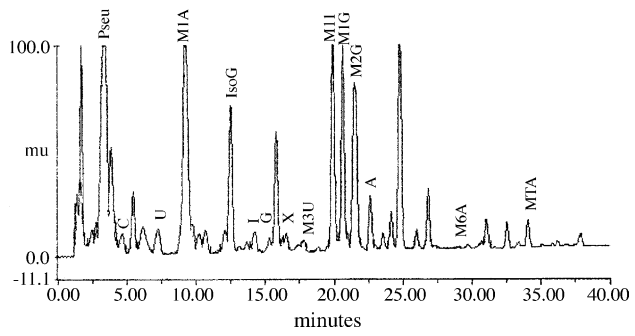

Fig. 1. HPLC separation of urinary nucleosides. UV detection at 260 nm.

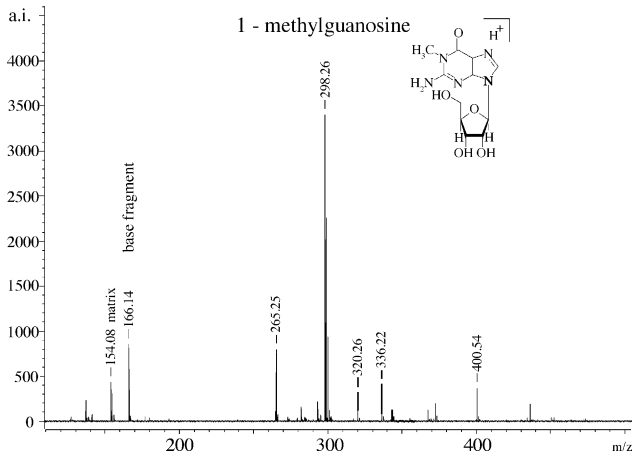

Fig. 2. MALDI-TOF-MS spectrum of 1-methylguanosine.

The TOF mass analyzer ensures a high mass accuracy of below 10 ppm when using internal calibration with a standard mixture of five calibrants in the mass range from 200 to 400. Nucleosides contain a minimum of nine carbon atoms, three oxygen atoms and two nitrogen atoms. With a mass accuracy of 10 ppm and looking for compounds fulfilling these restrictions, it is possible to narrow the number of totals formulas down to a few. The suggested total formulas are then used to search for nucleoside structures systematically. For this search, SciFinder 2003 was used.

With an accurate mass in connection with the fragmentation it is possible to obtain hints on the structure of yet unknown nucleosides. Our optimized protocol allows measurements with a limit of detection between 100 fmol and 10 pmol using CHCA as a matrix, depending on the basicity of nucleoside. These detection limits are satisfactory and are superior to frit fast atom bombardment (FAB) MS measurements [13]. However, in contrast to frit-FAB-MS, the MALDI method allows no coupling to separation systems such as

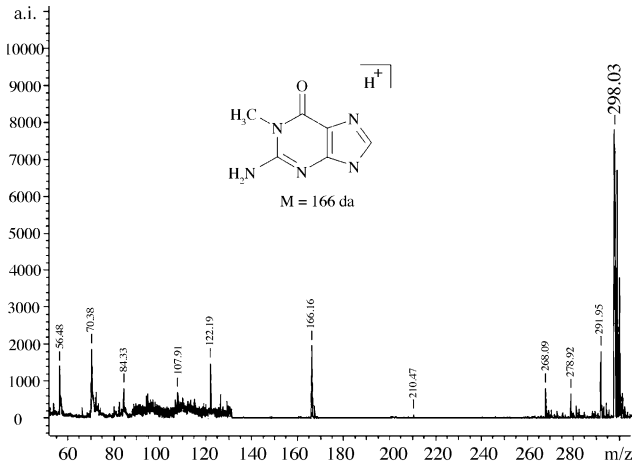

Fig. 3. PSD spectrum of mass *m/z* 298 of 1-methylguanosine.

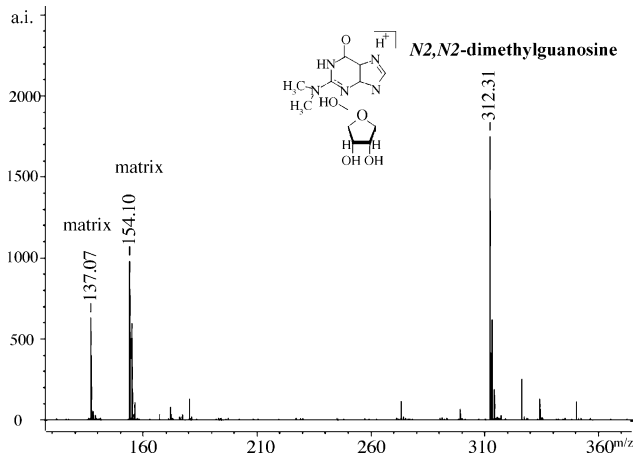

Fig. 4. MALDI-TOF-MS spectrum of *N*2,*N*2-dimethylguanosine.

HPLC or CE. The MALDI method is solely an off-line technique with increased sample preparation requirements.

In the two clinical studies presented as examples for applications of nucleoside analysis, the following 12 nucleosides are quantitated: Pseu, C, U, M1A, I, G, X, M3U, M1I, M1G, M2G and A (Fig. 1). Additional modified nucleosides identified by MALDI-TOF-MS, especially on the basis of the nucleic base fragments in the PSD spectra, are *N*4-acetyl-cytidine, *N*6-threonylcarbamoyladenosine, *N*2,*N*2-dimethylguanosine (Figs. 4 and 5) and *N*2,*N*2,7-trimethylguanosine. These nucleosides are not yet included in the clinical studies.

3.2. Clinical studies

The urinary excretion levels of the nucleosides are related to the urinary creatinine concentration, which is a standard manner in clinical chemistry to express urinary levels. This is justified because the excretion of creatinine is rather constant over a longer time interval, and allows that randomly collected urine specimens can be used instead of 24 h urine specimens.

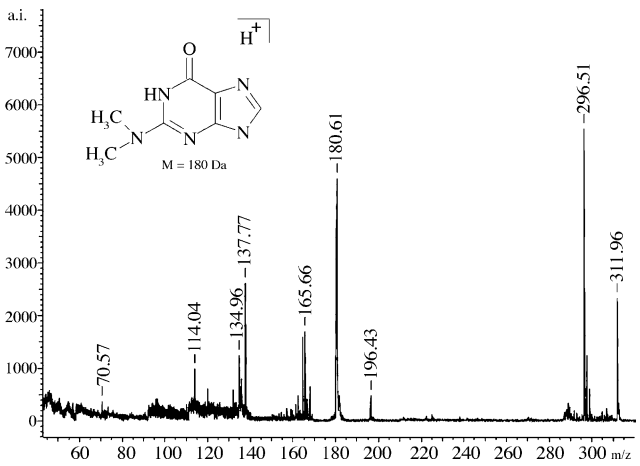

Fig. 5. PSD spectrum of mass *m*/*z* 312 of *N*2,*N*2-dimethylguanosine.

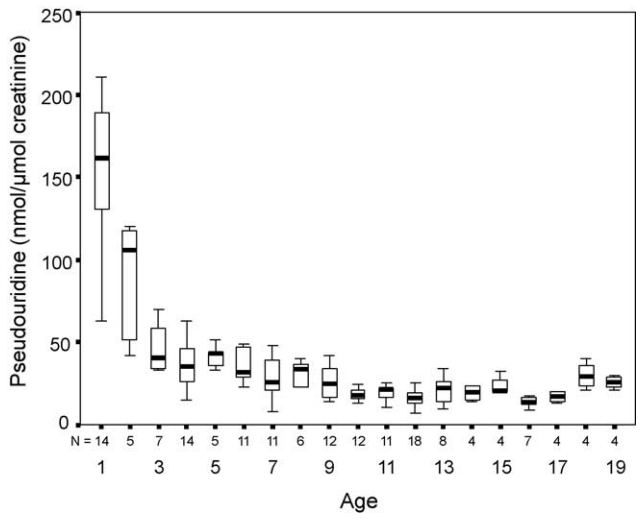

Fig. 6. Age-dependence of urinary pseudouridine.

The urinary excretion of the nucleosides in healthy persons is found not to vary with gender. No statistical difference is observed between males and females (Mann–Whitney–Wilcoxon Test, $\alpha = 0.05$), which is in parallel to the results of Itoh et al. [14]. Whereas in adults no age-dependence of the nucleoside levels is observed, our study with 166 healthy children shows a very pronounced age-dependence and higher levels in children, especially during the first 4 years of life. This is demonstrated in Fig. 6 for pseudouridine. Tumor marker studies with children require that this age-dependence is taken into account.

In the children with leukaemia and other malignant diseases ($N = 75$, age range 1–19 years), the age-related evaluation of the urinary modified nucleosides reveals for most age groups statistically significant elevations of pseudouridine, *N*2-methylguanosine and 1-methylinosine. At a diagnostic specificity of 95% the diagnostic sensitivities are 66%, 73%

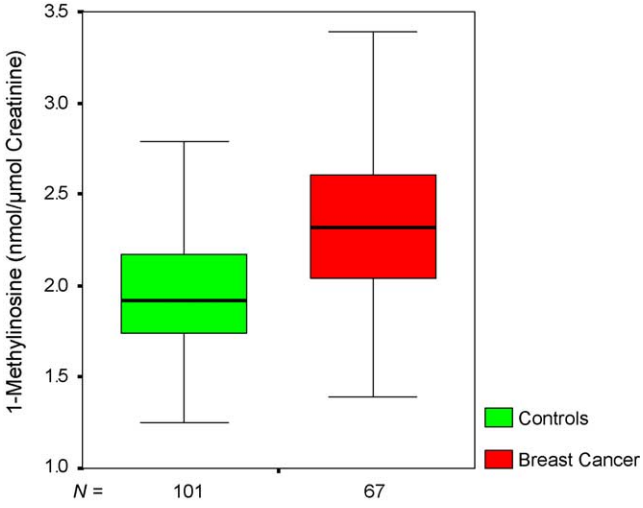

Fig. 7. Levels of urinary 1-methylinosine in healthy women and women with breast cancer.

and 59%, respectively. During remission of the disease the levels drop often to normal, in cases of a relapse they rise.

In the women with breast cancer ($N = 67$, age range 34–83 years) inosine, 1-methylinosine and 3-methyluridine are the nucleosides which are most characteristically increased. The diagnostic sensitivities are 50%, 57% and 67%, respectively. Fig. 7 describes the levels of 3-methylinosine in healthy controls and women with breast cancer.

Statistical evaluation of the data with artificial neural networks (ANNs) [15] demonstrates that the patterns of the modified nucleosides are even more useful as tumor markers for breast cancer than the separate nucleosides. At a diagnostic specificity of 83% the diagnostic sensitivity of the nucleoside pattern is 74%.

4. Conclusion

Quantitation of modified nucleosides in urine by HPLC opens the possibility to use these metabolites as tumor markers for malignancies in childhood and for breast cancer. MALDI-TOF-MS and the use of PSD spectra is a reliable method to identify the nucleosides and is newly applied to this class of substances.

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