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Age-dependence of urinary normal and modified nucleosides in childhood as determined by reversed-phase high-performance liquid chromatography

H.M. Liebich[a,*], S. Müller-Hagedorn[a], M. Bacher[b], H.-G. Scheel-Walter[c], X. Lu[d], A. Frickenschmidt[e], B. Kammerer[e], K.-R. Kim[f], H. Gérard[g]

[a] *Medizinische Universitätsklinik, Abteilung IV, Zentrallaboratorium, D-72076 Tübingen, Germany*
[b] *Zentrum für Zahn-, Mund- und Kieferheilkunde, Poliklinik für Kieferorthopädie, D-72076 Tübingen, Germany*
[c] *Universitäts-Kinderklinik, D-72076 Tübingen, Germany*
[d] *National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, PR China*
[e] *Universität Tübingen, Abteilung für Klinische Pharmakologie, D-72076 Tübingen, Germany*
[f] *College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea*
[g] *Laboratoire de Biologie de la Reproduction et du Développement, CHRU de Nancy, F-54042 Nancy, France*

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Abstract

Modified nucleosides have been characterized as tumor markers for a number of malignant diseases. In order to use these markers in children, the age-dependence of the nucleoside levels in healthy children has to be established and taken into account in diagnostic decisions. In this study, the levels of 12 normal and modified nucleosides in urine of 166 healthy children and adolescents with an age between 1 day and 19 years are determined by reversed-phase HPLC, and age-dependent reference ranges are defined. The urinary nucleoside concentrations are related to the creatinine concentrations, which allows the use of randomly collected urine samples. All nucleoside levels in urine of children decrease with age, most pronounced during the first 4 years of life, and the age-dependence of the reference values of the individual nucleosides can be approximated by a mathematical function $y = b_0 + b_1\ (1/x)$ with the regression coefficients b_0 and b_1, the nucleoside levels y and the age x between 1 year and 19 years. In the very young children, the shifts in the nucleoside concentrations are more differentiated. Starting with low levels on the first day of life, the concentrations of all studied nucleosides rise up to an age of 1–2 months, when they reach their absolute maximum for all age periods, and then decrease.

Keywords: Nucleosides; Age dependence

1. Introduction

RNA, in particular t-RNA, contains a large number of modified nucleosides, in addition to the normal ribonucleosides adenosine, guanosine, cytidine and uridine. They are formed post-transcriptionally by various modification enzymes, especially methyltransferases and ligases. The levels of the nucleosides in serum and urine reflect the turnover of RNA, in particular t-RNA [1–4]. Of clinical importance are mainly those modified nucleosides which are biochemical end products. Their concentrations in blood serum and urine can be considered as direct markers for the modification processes in the t-RNA molecule and for the t-RNA turnover. Such end products are for example 1-methylguanosine, 2-methylguanosine, 1-methyladenosine and pseudouridine. The end products cannot be rephosphorylated and are gener-

* Corresponding author. Tel.: +49 7071 2980602; fax: +49 7071 293188.
E-mail address: hartmut.liebich@med.uni-tuebingen.de (H.M. Liebich).

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ally not reincorporated during de novo synthesis of RNA [5]. In addition, many of the modified nucleosides are not further metabolized and are excreted in urine, e.g. pseudouridine.

In all tissues with increased t-RNA turnover, the formation of modified nucleosides can be expected to be increased. In addition, in cells with an altered enzyme pattern the modification products may also vary in their relative distribution. Consequently, in many pathological states the concentrations of all nucleosides as well as their relative patterns can be changed, especially in malignant diseases, in which increased t-RNA turnover and enzyme alterations can occur in the malignant cells. Therefore, for many years modified nucleosides have been suggested as tumor markers [6–12].

The physiological turnover of t-RNA may be age-dependent, in particular in childhood. Therefore, when modified nucleosides are to be used as tumor markers in children, decisions have to be based on age-dependent reference values. In the present study, the age-dependence of 12 urinary nucleosides is investigated in detail in children and adolescents between 1 day and 19 years of age.

Generally, the analytical procedures for 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. Separation of the nucleosides is achieved equally well by RP-HPLC [13] and by capillary electrophoresis in the mode of MEKC [14,15]. In this work RP-HPLC is used.

2. Experimental

2.1. Urine samples

The study included 166 healthy children and adolescents aged between 1 day and 19 years (84 males and 82 females). Random urine samples of newborns and 1-year old children were collected at the Department of Reproductive Medicine, University of Nancy. All other random urine samples were from children treated at the department of orthodontics of the dental clinic of the University of Tübingen. The urine samples from the older children were collected in sterile plastic 100 ml containers. Special collection bags (Urinocol® Pediatrie, B. Braun Biotrol SA, Paris, France) were used for the collection of the urine samples of the children up to 1 year of age.

Criteria for inclusion of children into the study were that at the time of sample collection the children were healthy, did not take any medication and did not undergo any form of medical treatment except teeth corrections. In particular, children with any infectious or malignant diseases were excluded. Likewise, all children suffering from an inherited disease or syndrome, such as trisomia 21, were excluded from the study. The collected urine samples were tested for glucose, ketone bodies, bilirubin, urobilinogen, protein, nitrite, leukocytes, erythrocytes and hemoglobin (Rapignost Total-Screen, Dade Behring, Marburg, Germany). When any abnormality was observed in these tests, the child was excluded from the study.

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 15 ribonucleosides, i.e. uridine (U), cytidine (C), adenosine (A), guanosine (G), inosine (I), xanthosine (X), 3-methyluridine (M3U), 5-methyluridine (M5U), 1-methyladenosine (M1A), N6-methyladenosine (M6A), 1-methylguanosine (M1G), 2-methylguanosine (M2G), 1-methylinosine (M1I), pseudouridine (Pseu) and 5′-deoxy-5′-methylthioadenosine (MTA) were purchased from Sigma (Deisenhofen, Germany). The internal standard isoguanosine (isoG) was synthesized from guanosine according to the method of Divakar et al. [16]. 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).

2.3. Extraction of the nucleosides from urine

The nucleosides were extracted from urine applying a modification of the method described by Gehrke et al. [17] and Kuo et.al. [18]. The extraction was performed on glass columns packed with 500 mg of phenylboronate gel (Affi-gel 601, Bio-Rad Laboratories) which possesses a specific affinity for the cis-diol structure characteristic for ribonucleosides.

A 10 ml volume of centrifuged urine, adjusted to pH 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 gel in the column was equilibrated with 45 ml of 0.25 M ammonium acetate at pH 8.5, and the sample was carefully applied to the column without disturbing the column's bed. After loading, the gel was washed once with 20 ml of 0.25 M ammonium acetate and then twice with 3 ml of methanol–water (1:1, v/v). In the following step, 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. By this procedure, an extract was obtained, which was concentrated by a factor of ten as compared to the original urine, and which was suitable for the quantification of the major modified nucleosides considered in this age-dependence study. For minor nucleoside components reconstitution in less than 1 ml of buffer is possible. Before reuse, the gel was regenerated with 25 ml of formic acid in methanol–water (1:1, v/v) and 25 ml methanol–water (1:1, v/v) and then reequilibrated with 45 ml of 0.25 M ammonium acetate at pH 8.5. Under these conditions, the gel could be used for up to 15 extractions. The same treatment was applied to the standard solutions containing the 15 reference nucleosides and the internal standard used for the calibration.

2.4. HPLC separation of the nucleosides

The HPLC system (Merck-Hitachi, Darmstadt, Germany) was composed of an L-6200 pump, a 655A-40 column oven, an L-3000 photo diode array detector, an L-7200 autosampler and a D-6000 interface, which connected the HPLC system with the HPLC manager software used (D-6000 chromatography data station software, version 2/revision 3).

The separation of the nucleosides was performed on a 250 mm × 4 mm, 5 μm LiChrospher 100 C18e column (Merck, Darmstadt, Germany) at 30 °C, with an injection volume of 20 μl. The elution was carried out using a concentration gradient with 25 mM KH_2PO_4 at pH 5.0 and methanol–water (3:2, v/v), beginning with 100% of the KH_2PO_4—solution and reaching 40% of the KH_2PO_4—and 60% of the methanol–water solution after 40 min. In addition, a flow gradient between 1.5 and 1.3 ml/min was used. The nucleosides were detected by measuring their UV absorbance at 260 nm.

2.5. Identification and quantification of the nucleosides

The peaks in the chromatograms were identified by comparing their retention times with those of the reference nucleosides in the standard solutions eluted under the same conditions, and by spiking the urine sample with standard solution. For calibration, a standard stock solution was prepared by dissolving the reference substances in 500 ml of a 25 mM solution of KH_2PO_4 at pH 5.0 to reach end concentrations of 16 μM for U, 8 μM for C, 34 μM for A, 8 μM for G, 32 μM for I, 32 μM for X, 16 μM for M3U, 32 μM for M5U, 167 μM for M1A, 32 μM for M6A, 32 μM for M1G, 8 μM for M2G, 8 μM for M1I, 1278 μM for Pseu and 18 μM for MTA. Five different volumes of this standard stock solution (0.625, 1.25, 1.875, 2.5 and 5.0 ml) were diluted with water to a volume of 10 ml to prepare five standard working solutions. To each of the five standard working solutions 0.5 ml of the aqueous internal standard solution (25 mM isoG) were added. The five standards were treated separately on the affinity chromatography gel, as described above, and for the 12 nucleosides included in this age-dependence study (Pseu, C, U, M1A, I, G, X, M3U, M1I, M1G, M2G, A) calibration curves were established. Based on the calibration curves, the concentrations of the 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 [19].

2.6. Validation of the method

The recoveries of the nucleosides were determined by measuring in triplicate their concentrations in a native urine sample and a urine sample spiked with standard working solution. The reproducibility of the analytical method was established by six-fold analysis of a urine sample. To determine the accuracy, the nucleosides in a standard working solution (2.5 ml of standard stock solution plus 7.5 ml of water) were analysed and the measured concentrations were compared with the known concentrations. The linearity of the method was derived from the analysis of the five standard working solutions. For the determination of the detection limit, a water blank sample containing only the internal standard was analysed, and the detection limit was calculated on the basis of an area three times as large as the mean area of the background noise. The peak purities of the nucleosides separated by HPLC were examined on the basis of their UV spectra. By multiple recording of spectra along each peak (23–72 UV spectra per peak), peak purities were calculated using the HP ChemStation for LC 3D Rev. A.09.01 software.

2.7. Statistical evaluation

Data processing and graphic presentations were performed with SPSS 8.0. To test the type of distribution of the nucleoside values in the population studied, P-P and Q-Q probability plots and the Lilliefors test were used. Gender-dependence was investigated by applying the Mann–Whitney–Wilcoxon test, suitable for concentration data, such as the nucleoside levels, which do not show a completely normal distribution. The nucleoside levels measured for the different age ranges 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. For presentation of the age-dependent reference values curve fitting was performed with the median and the 95% percentile values.

3. Results and discussion

3.1. Characteristics of the method

For the 12 nucleosides included in the age-dependence study the recoveries are shown in Table 1. They range between

Table 1
Recoveries of the nucleosides

Nucleoside	Recovery (%)
Pseu	89.2
C	83.6
U	90.5
M1A	108.6
I	106.1
G	95.3
X	94.5
M3U	98.4
M1I	102.2
M1G	114.2
M2G	98.2
A	90.4

Table 2
Precision and accuracy of the method

Nucleoside	Precision		Accuracy	
	Mean value (nmol/ml)	Coefficient of variation (%)	Theoretical concentration (nmol/ml)	Deviation (%)
Pseu	133.7	3.5	319.4	23.4
C	0.3	15.8	2.0	6.9
U	1.8	4.6	4.1	10.5
M1A	15.1	4.8	41.9	11.9
I	2.2	1.2	8.2	21.6
G	0.1	21.3	2.0	8.9
X	3.9	10.5	8.1	42.3
M3U	0.6	18.3	4.2	6.4
M1I	9.6	1.4	2.0	14.3
M1G	5.3	1.7	8.0	6.6
M2G	7.0	1.7	2.2	16.5
A	1.8	4.4	8.6	8.2

83.6% for C and 108.6% for M1A. Precision and accuracy of the analytical method are summarized in Table 2. The precisions as expressed by the coefficients of variation range between 1.2 and 21.3%, with most of the values below 5%. Values above 10% are observed mainly for the low-concentrated nucleosides C, G and M3U. The accuracies determined as the mean difference between measured concentration and known concentration ($n = 9$) is between 6.4 and 23.4%, except for X. The squared coefficients of variation (R^2) as the measure for the linearity range from 0.9314 to 0.9997, and the detection limits are between 0.1 and 0.5 nmol/ml. Isoguanosine is used as the internal standard because at the concentration levels of the urinary nucleosides determined in this study no isoguanosine peak is observed in the chromatogram. The peak purities (Table 3) as determined on the basis of the UV spectra are above 98% for the internal standard and for most of the nucleosides.

3.2. Analysis of the urine samples of healthy children

Under the conditions described above, the 15 normal and modified ribonucleosides of the standard solutions are separated and detected. Each of the nucleosides has a relative absorption maximum at a wavelength between 250 and 270 nm.

Table 3
Peak purities as determined from the UV spectra

Nucleoside	Peak purity (%)
Pseu	99.7
C	99.8
U	83.4
M1A	99.8
I	84.6
G	99.8
X	99.8
M3U	99.8
M1I	99.9
M1G	99.9
M2G	98,7
A	86.6
isoG	99.0

Therefore, 260 nm is used for UV-detection. In urine samples of healthy children, the levels of M5U and M6A are low and the two components are not measured in this study. Likewise MTA is not considered because of low methodical precision (coefficient of variation 41.7%). Therefore, 12 urinary nucleosides are evaluated with respect to their age-dependence in childhood. Figs. 1 and 2 demonstrate the chromatograms of a standard sample and a typical urine sample. In order to avoid cumbersome 24 h urine collections, randomly collected urine specimens are used. However, because the concentrations of urinary analytes are influenced by the variations in the physiological urinary excretion, quantitative analyte levels in randomly collected urine specimens should be expressed in relation to an endogenous reference parameter which in independent of diuresis. In clinical chemistry, urinary creatinine is used for this purpose. Therefore, in this study, the urinary excretion levels of the nucleosides are expressed relative to the urinary creatinine concentration, which is in accordance with the findings of Gehrke et al. [20] who demonstrated that randomly collected urine specimens can be used for the determination of urinary nucleosides instead of 24 h urine specimens when the levels are expressed relative to the creatinine values. The great advantage of urine samples as compared to serum samples is that their collection is completely non-invasive and therefore very suitable for studies in pediatrics. A further argument for using urine is that nucleosides occur in higher concentrations in urine than in serum [21], leading to higher analytical reproducibility and accuracy.

3.3. Gender-dependence of the nucleoside levels

The urinary excretion of the nucleosides in children is found not to vary with gender. No statistical difference is observed between males and females when the whole group of children and adolescents in the age range of 1 day and 19 years is tested (Mann–Whitney–Wilcoxon test, $\alpha = 0.05$). Likewise, when the population is split into two groups, one with children up to an age of 15 years and the other with adolescents up to an age of 19 years, no influence of gender

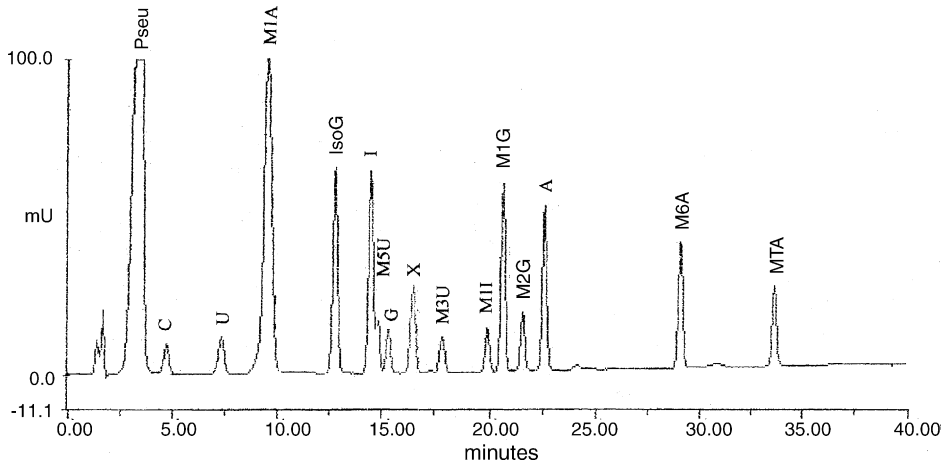

Fig. 1. Chromatogram of the RP-HPLC separation of nucleosides in a standard sample. UV detection at 260 nm, HPLC conditions as described under Section 2. Pseu, pseudouridine; C, cytidine; U, uridine; M1A, 1-methyladenosine; IsoG, isoguanosine (internal standard); I, inosine; M5U, 5-methyluridine; G, guanosine; X, xanthosine; M3U, 3-methyluridine; M1I, 1-methylinosine; M1G, 1-methylguanosine; M2G, 2-methylguanosine; A, adenosine; M6A, N^6-methyladenosine; MTA, 5′-deoxy-5′-methylthioadenosine.

on the nucleoside concentrations is noticed. This is in parallel to the results of Itoh et al. [22], who did not find any influence of gender on the urinary nucleoside levels in older children and in adults. On the other hand, Fischbein et al. [23] observed higher nucleoside concentrations in adult females than in males.

3.4. Age-dependence of the nucleoside levels

The age-dependence of the urinary nucleoside levels is studied under two aspects, firstly for all children between 1 day and 19 years of age choosing intervals of 1 year, secondly for children in the first year of life. The separate study of the age-dependence of the nucleoside concentrations in the very young children is advisable because of pronounced growth and development processes during the first year of life.

3.4.1. Age-dependence of the nucleoside levels in all children

The levels of all urinary nucleosides in children decrease with age as demonstrated for Pseu and M2G in Figs. 3 and 4. The decrease is most pronounced during the first 4 years of life. Later, the levels remain more constant. During the age of 11 years to 16 years, a slight increase of the concentrations is observed. It appears that the levels of the urinary nucleosides in children run parallel to the growth rate in the different age periods. Therefore, it seems possible to correlate the tRNA catabolism with the growth rate, and the nucleoside levels could therefore be considered as indicators of cell differentiation and of growth. During periods of high growth rates and important developmental processes higher levels of nucleosides are determined. In adults, no differences are found between different ages [24]. Itoh et al. [22] do not describe any influence of age on the urinary excretion of Pseu and

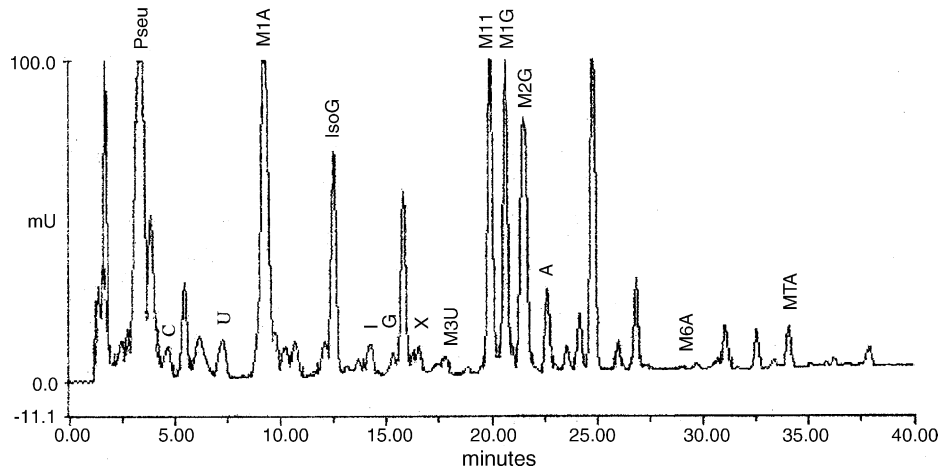

Fig. 2. Chromatogram of the RP-HPLC separation of nucleosides in a typical urine sample. Conditions and abbreviations as in Fig. 1.

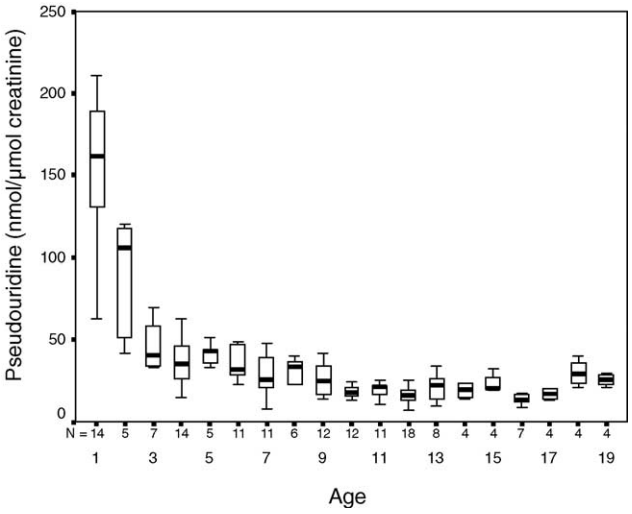

Fig. 3. Boxplot presentation of the age-dependence of the urinary pseudouridine levels in children and adolescents between 1 and 19 years of age.

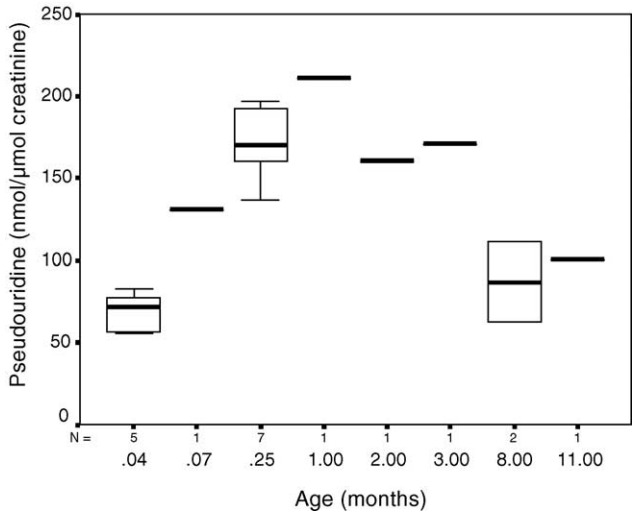

Fig. 5. Boxplot presentation of the age-dependence of the urinary pseudouridine levels in children between 1 day and 11 months of age.

M1A in a population aged between 6 and 70 years, not considering younger children, in which according to our study the age-dependence is very pronounced.

3.4.2. Age-dependence of the nucleoside levels in children in the first year of life

Characteristic shifts in the nucleoside concentrations occur during the first year of life, as shown for the examples Pseu and M2G (Figs. 5 and 6). In the first days of life, relatively low levels are observed. Then, the concentrations of all studied nucleosides rise up to an age of 1–2 months, reaching their absolute maximum for all age periods. After that, the nucleoside concentrations decrease with age as described above. Perhaps, the changes in the nucleoside concentrations shortly after birth (first week) could be explained by the change from intrauterine to extrauterine life. Even a loss of weight is observed in the first days of life. Once the adaptation to extrauterine life is attained, a period of very intensive processes of growth and development starts. Since many of these processes take place in the first year of life, pronounced changes in the nucleoside levels occur during that early period of life. Similar findings are published about amino acids whose urinary excretion decreases rapidly during the first year of life with a further slower decline thereafter [25]. This can be understood in parallel to the results of this study, because RNA and amino acids are linked by the genetic flux. Furthermore, the whole-body RNA turnover correlates quite well with the whole-body protein turnover [26].

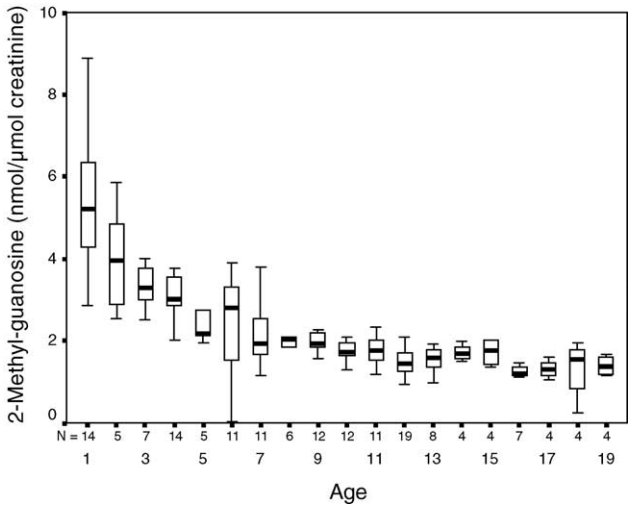

Fig. 4. Boxplot presentation of the age-dependence of the urinary 2-methylguanosine levels in children and adolescents between 1 and 19 years of age.

3.4.3. Curve-fitting and reference ranges

Because the excretion of the RNA metabolites, the nucleosides, is a function of age, the age-dependence of the nucleo-

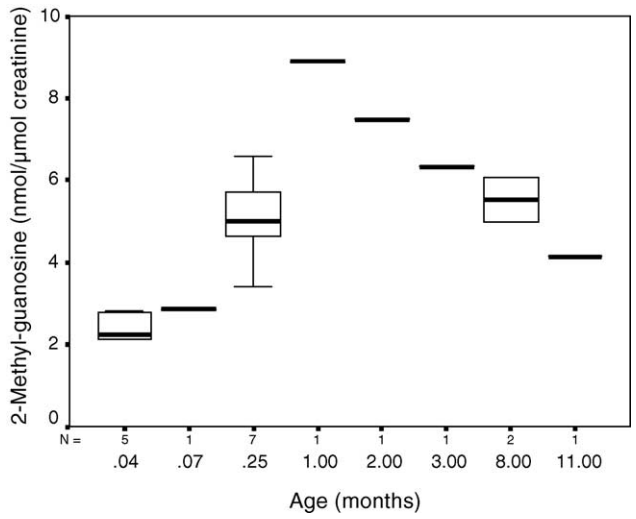

Fig. 6. Boxplot presentation of the age-dependence of the urinary 2-methylguanosine levels in children between 1 day and 11 months of age.

Table 4
Regression coefficients a_0, a_1, b_0 and b_1 and coefficients of determination R^2 of curve fitting by using a hyperbolic function

Nucleoside	a_0	a_1	R^2 (a)	b_0	b_1	R^2 (b)
Pseu	10.91	133.33	0.92	6.40	271.00	0.96
C	0.10	0.38	0.87	0.12	1.50	0.90
U	0.36	1.06	0.87	0.61	2.76	0.81
M1A	3.95	4.71	0.57	4.99	11.52	0.72
I	0.17	2.77	0.98	−0.79	13.95	0.84
G	0.05	0.77	0.95	−0.19	4.18	0.82
X	0.44	7.35	0.93	1.22	18.85	0.87
M3U	0.11	0.55	0.72	−0.02	4.28	0.90
M1I	1.39	8.52	0.98	1.41	17.25	0.94
M1G	1.00	2.82	0.81	1.14	7.58	0.89
M2G	1.42	4.10	0.88	1.78	7.72	0.87
A	0.004	4.45	0.94	−0.12	8.06	0.93

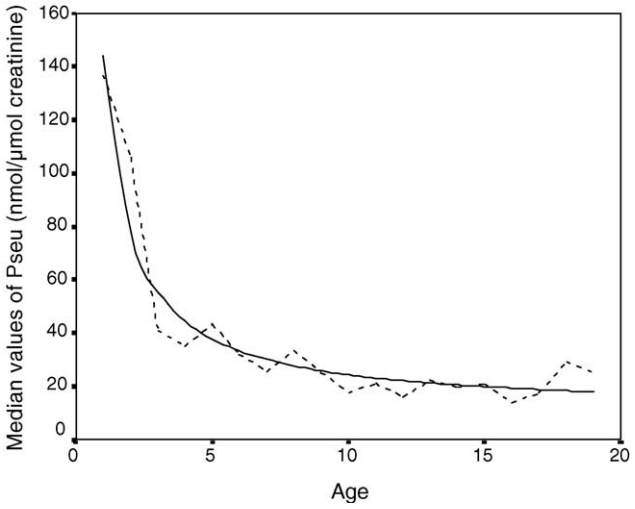

Fig. 7. Curve fitting, $y_{50} = 10.91 + 133.33\ (1/x)$ (—), and observed figures (- - -) for the median values of pseudouridine.

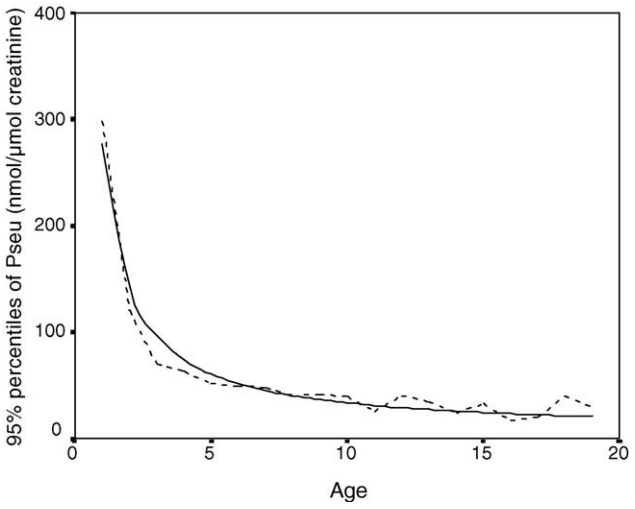

Fig. 8. Curve fitting, $y_{95} = 6.40 + 271.00\ (1/x)$ (—), and observed figures (- - -) for the 95 % percentile values of pseudouridine.

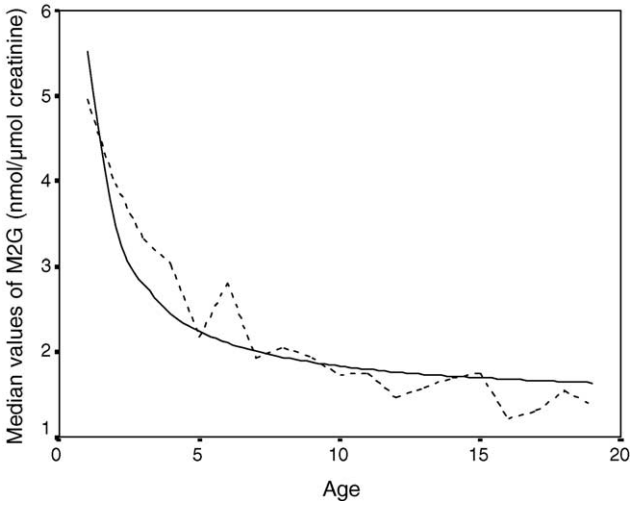

Fig. 9. Curve fitting, $y_{50} = 1.42 + 4.10\ (1/x)$ (—), and observed figures (- - -) for the median values of 2-methylguanosine.

sides can be described by a mathematical function, obtained by curve fitting of the median values (50% percentiles) of each age class of all children. In the age range 1–19 years, the best fitting curve for most of the nucleosides is obtained by the function:

$$y_{50} = a_0 + a_1 \cdot \frac{1}{x}$$

In the same way, the 95% percentiles, on the basis of which reference values are usually expressed in clinical chemistry, are calculated for each age class and used for curve fitting:

$$y_{95} = b_0 + b_1 \cdot \frac{1}{x}$$

The variable x is the age of the child in years, y_{50} and y_{95} are the median and the 95% percentile values, respectively,

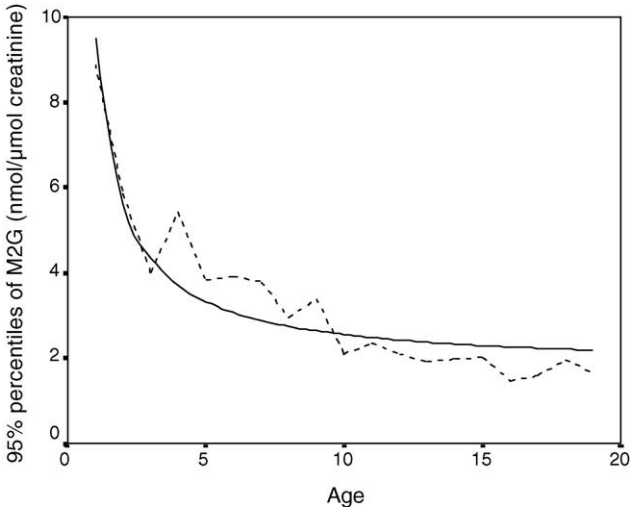

Fig. 10. Curve fitting, $y_{95} = 1.78 + 7.72\ (1/x)$ (—), and observed figures (- - -) for the 95% percentile values of 2-methylguanosine.

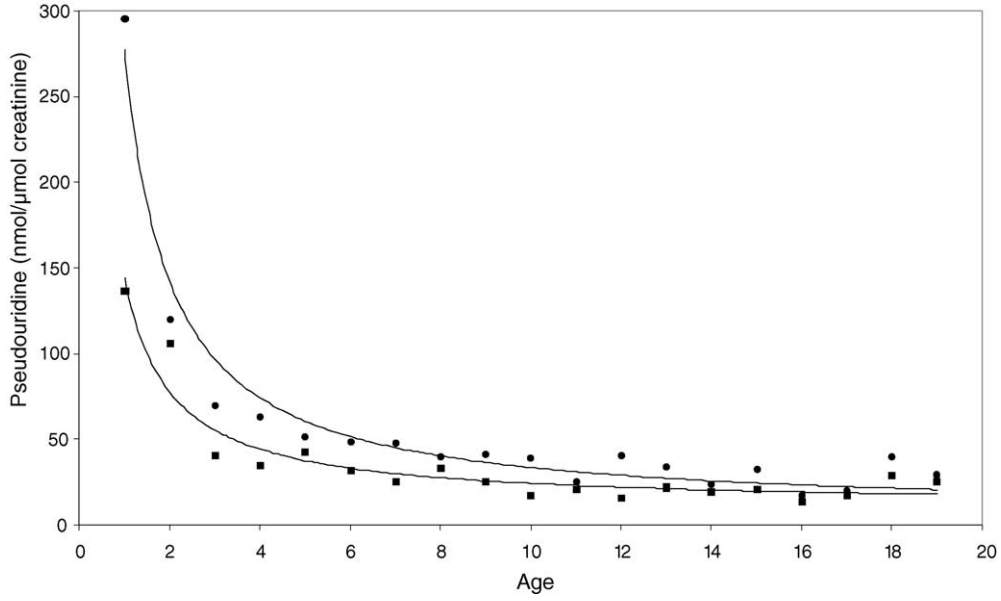

Fig. 11. Curve fittings of the median values (lower curve) and 95% percentile values (upper curve) and observed figures (■: median, ●: 95%) of pseudouridine.

of the nucleoside level in nmol/µmol creatinine and a_0, a_1, b_0 and b_1 are the regression coefficients.

Other correlations between urinary nucleoside levels and age have been described. Prankel et al. postulated a nearly logarithmic decrease of the nucleoside levels with age [27]. Heldman et al. [12] demonstrated for 15 children a linear decrease of the level of Pseu, M1G and M1I. However, they did not include children younger than 4 years. Graf et al. [28] described for a group of 74 healthy children between 1 and 18 years of age a decrease of the levels of Pseu using

Table 5
Median values of the urinary nucleoside levels in children in the first year of life

Nucleosides	Age			
	1 day	4 days	1 week	1 month
Pseu	69.03	130.92	187.48	210.97
C	0.58	0.50	0.86	1.25
U	1.25	0.66	2.14	2.66
M1A	5.83	5.21	9.01	14.92
I	6.42	2.26	3.29	2.61
G	2.08	0.74	0.89	1.46
X	11.62	3.24	11.33	7.96
M3U	0.40	1.37	1.63	2.61
M1I	9.23	10.30	12.92	13.72
M1G	3.33	2.35	4.56	5.44
M2G	2.43	2.87	5.09	8.89
A	0.63	0.62	1.04	2.49

	2 months	3 months	8 months	11 months
Pseu	160.45	171.04	87.06	100.52
C	0.42	0.97	0.25	0.36
U	1.97	2.05	1.08	1.12
M1A	13.25	10.73	6.39	6.49
I	2.93	5.02	1.49	1.70
G	0.42	0.95	0.45	0.41
X	4.52	13.56	2.62	6.83
M3U	0.48	1.65	0.62	1.35
M1I	8.95	9.52	7.42	5.32
M1G	5.76	4.65	3.13	2.69
M2G	7.48	6.34	5.53	4.13
A	1.17	1.67	1.76	1.49

curve fitting with a polynomial of fourth degree. In all these studies, relatively small numbers of children and only few of the nucleosides were included.

In this study, among several types of curve fittings tested, the described fitting has been shown to give the best characterization of the measured values. The quality of the hyperbolic curve fitting for the different nucleosides considered in this study is reflected in the values of the coefficients of determination R^2 (Table 4). Most of the nucleosides are well fitted by a hyperbola. The exception is M1A which would be better fitted by a linear function. Figs. 7–10 demonstrate the median value and 95% percentile value curve fittings for Pseu and M2G. In early childhood, the interindividual variance of the nucleoside levels is greater than in older children and adolescents. This is demonstrated for Pseu in Fig. 11. For young children, the difference between the median values and the 95% percentile values is larger than for older children, resulting in converging curves. The reference ranges for the different nucleosides can be described to be $<y_{95}$ nmol/μmol creatinine. For each age of the children and the adolescents the corresponding values can be calculated using the regression coefficients a_0, a_1, b_0 and b_1 for each nucleoside (Table 4).

For children in the first year of life, the nucleoside levels cannot by fitted by a hyperbolic function. In addition, because of the small number of individuals in each cluster of these children, the age-dependent reference ranges cannot be presented by any form of curve fitting. Instead, the values shown in Table 5 demonstrate numerically the course of the urinary nucleoside levels in the first year. The absolute values have to be considered as preliminary because of the small number of children in this age range.

4. Conclusions

With the described RP-HPLC method, suitable for routine applications, the levels of normal and modified nucleosides in urine can be determined in random urine samples of children including newborns. A distinct age-dependence is characteristic for children, particularly for children up to an age of 4 years. When the modified nucleosides are used as tumor markers in malignant diseases in childhood, their age-dependence has to be taken into account. The age-dependent reference values established in this study, can be used as decision limits for interpreting elevated modified nucleoside levels in children of all ages suffering from a tumor disease.

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