Journal of Chromatography, 434 (1988) 202–208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4429

Note

Determination of the age dependency of the creatinine-related pseudouridine excretion in children's urine by high-performance liquid chromatography

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(First received February 19th, 1988; revised manuscript received August 5th, 1988)

Some years ago pseudouridine excretion was postulated to be a possible tumour marker [1]. Many investigations have since been carried out (for a review see ref. 2) in order to evaluate normal and pathological pseudouridine excretion rates in adults.

Reports concerning pseudouridine excretion in children are scarce and non-uniform. Earlier publications [3–5] gave mean values and standard deviations derived from six to nine urine samples, without consideration of the age of the children. Borek et al. [6] reported individual values for eight children aged from 1 to 12 years but without statistical analysis. Heldman et al. [7] found a linear correlation between pseudouridine excretion and age based on the analysis of fifteen urine samples; the donors' ages ranged from 4 to 13 years.

Thus our aim was to get more information about pseudouridine excretion in relation to age by analysing urine samples of a larger group of children.

EXPERIMENTAL

Materials

High-performance liquid chromatographic (HPLC) determinations were carried out using an apparatus from Waters-Millipore (Milford, MA, U.S.A.) consisting of two pumps (Model 510), an injector (U6K) and a programmer (Model 680). Detection at 260 nm and 262 nm was carried out by UV detectors, Models ERC-7210 (ERMA Optical works, Tokyo, Japan) and LKB 2151 (LKB, Bromma, Sweden), respectively. The plotting and evaluation of the chromatograms were carried out by an electronic integrator (Model C-R3A, Shimadzu, Kyoto, Japan). Prepacked steel columns (250 mm × 4.6 mm I.D.) filled with Spherisorb ODS-II (5 μg) or Hypersil ODS-II (5 μm) were purchased from Bischoff (Leonberg,

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F.R.G.) and Grom (Ammerbuch, F.R.G.), respectively. Guard columns (20 mm × 4.6 mm I.D.) were packed with Spherisorb ODS-II (5 μm).

Creatinine determinations (Jaffé's method) were carried out using a photometer (Model 150-20 from Hitachi, Tokyo, Japan) and a special kit (No. 124192 from Boehringer, Mannheim, F.R.G.) according to the specifications of the manufacturer. In two series of fifteen urine samples each the creatinine concentrations were evaluated by HPLC and by Jaffé's method. The HPLC-related ratios obtained were 79.0 ± 24.8 and 92.8 ± 11.7%, respectively (for details see ref. 8). Pseudouridine was purchased from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

About 1 ml of the fresh urine samples was filtered (0.2 μm or 0.45 μm, Schleicher & Schüll, Dassel, F.R.G.) and frozen immediately (−18 to −24°C). Before the

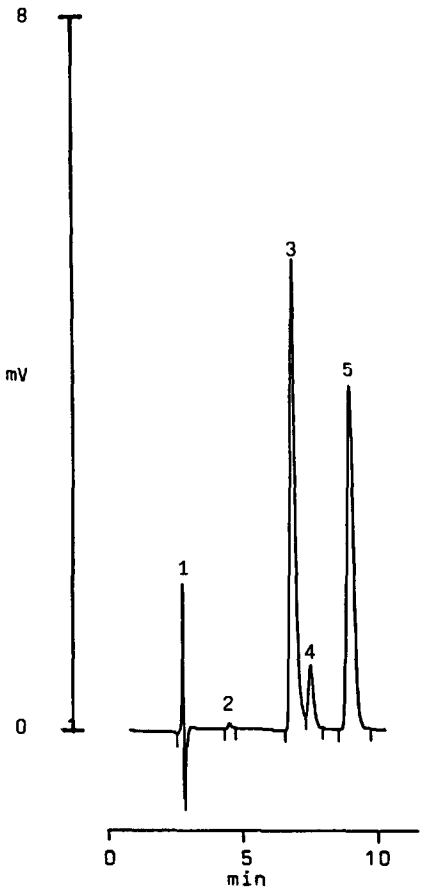

Fig. 1. Chromatogram of an aqueous stock solution (injection volume 15 μl) representing 200 ng of creatinine (peak 3, 6.7 min), 0.93 ng of uracil (peak 4, 7.4 min) and 38.5 ng of pseudouridine (peak 5, 8.8 min). The chromatographic conditions are described in Experimental; column, Hypersil ODS-II; mobile phase pH, 7.0; full scale, 8 mV.

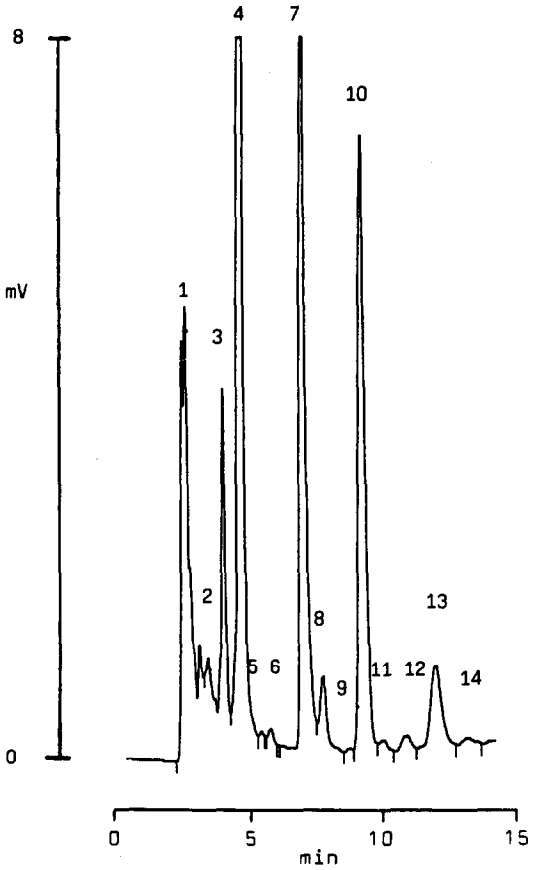

Fig. 2. Chromatogram of a urine sample (injection volume 10 μl, filtered and diluted). Peaks: 7 = creatinine; 8 = uracil; 10 = pseudouridine. The chromatographic conditions are described in Experimental; column, Hypersil ODS-II; mobile phase pH, 7.0; full scale, 8 mV.

HPLC analyses, the samples were thawed at room temperature and diluted with deionized and filtered water (Milli-Q water purification system, Waters-Millipore) to a standard dilution of 1:10, which was varied up to 1:30 if necessary. The standard injection volume was 10 μl (10-μl syringe from Hamilton, Darmstadt, F.R.G.).

Chromatographic conditions

Elution was performed isocratically with a mobile phase of 0.02 *M* potassium dihydrogenphosphate pH 5.4 (262 nm) or pH 7.0 (260 nm), the latter resulting in better peaks for creatinine and pseudouridine. The flow-rate was 1.0 ml/min. The main column was cooled to 10°C. The pressure, which was 131–145 bar when using Hypersil ODS-II and 145–166 bar when using Spherisorb ODS-II, was constant during analyses but tended to rise in the course of the day and with increasing column age. The retention time of pseudouridine was ca. 9.0 min (pH 7.0, Hypersil ODS-II) or 10.0 min (pH 5.4, Spherisorb ODS-II). The column was washed by adding up to 80% methanol to the mobile phase after each run (con-

stant flow 80:20, v/v for 10 min). At the end of the day, the column was washed for 90 min beginning with water (20 min), then by adding methanol in a gradient programme (ca. 15 min depending on the pressure), then 100% methanol for 45 min, then by adding 10% water (10 min). Each sample was at least analysed twice. The chromatograms of an aqueous stock solution and of a urine sample are shown in Figs. 1 and 2, respectively.

Quantitation

For calibration the external standard method (two-point calibration) was used. When Spherisorb ODS-II was used at pH 5.4 or 7.0, peak areas were calculated. With Hypersil ODS-II, at pH 7.0 peak heights were calculated. Calibration was controlled every morning and several times a day. Peaks were identified by comparison of retention times and by adding aqueous pseudouridine stock solution to the sample in daily routine.

The response curve was linear over the range 46.4–639 ng pseudouridine (r = 0.9995). The minimum detection limit was not determined because the pseudouridine peaks always exceeded 47% of the full-scale deflection.

The assay-to-assay coefficient of variation (n = 23, Spherisorb ODS-II, pH 5.4, calculation of peak areas) was ± 1.97%.

RESULTS

All creatinine-related pseudouridine values were plotted as a function of the age of the donors. The scatter diagram showed ten values lying far above the others. Comparing them with the excretion values of children with the same diagnoses as these ten children it became obvious that all the other four children suffering from enteritis had the highest pseudouridine excretions of their age group. The index patient with enteritis showed the most extreme elevation found (686.3 mg pseudouridine per g creatinine; 0.42 years; male).

After these fourteen excretion values had been omitted the mean values and standard deviations of the defined age groups were calculated (Table I) and plotted as a function of the age (Fig. 3). The resulting curve can be described by the

TABLE I

PSEUDOURIDINE EXCRETION IN DIFFERENT AGE GROUPS

Age group (years)	Total number of children	Total number of girls	Mean age of the group	Excretion (mean ± S.D.) (mg pseudouridine per g creatinine)
0.00– 0.49	11	5	0.12	283.5 ± 69.0
0.50– 0.99	5	1	0.83	227.8 ± (89.7)
1.00– 5.99	10	4	3.00	166.8 ± 55.0
6.00– 8.99	9	5	7.25	103.8 ± 20.8
9.00–12.99	10	2	10.50	77.5 ± 13.6
13.00–16.99	12	3	14.16	64.6 ± 17.6

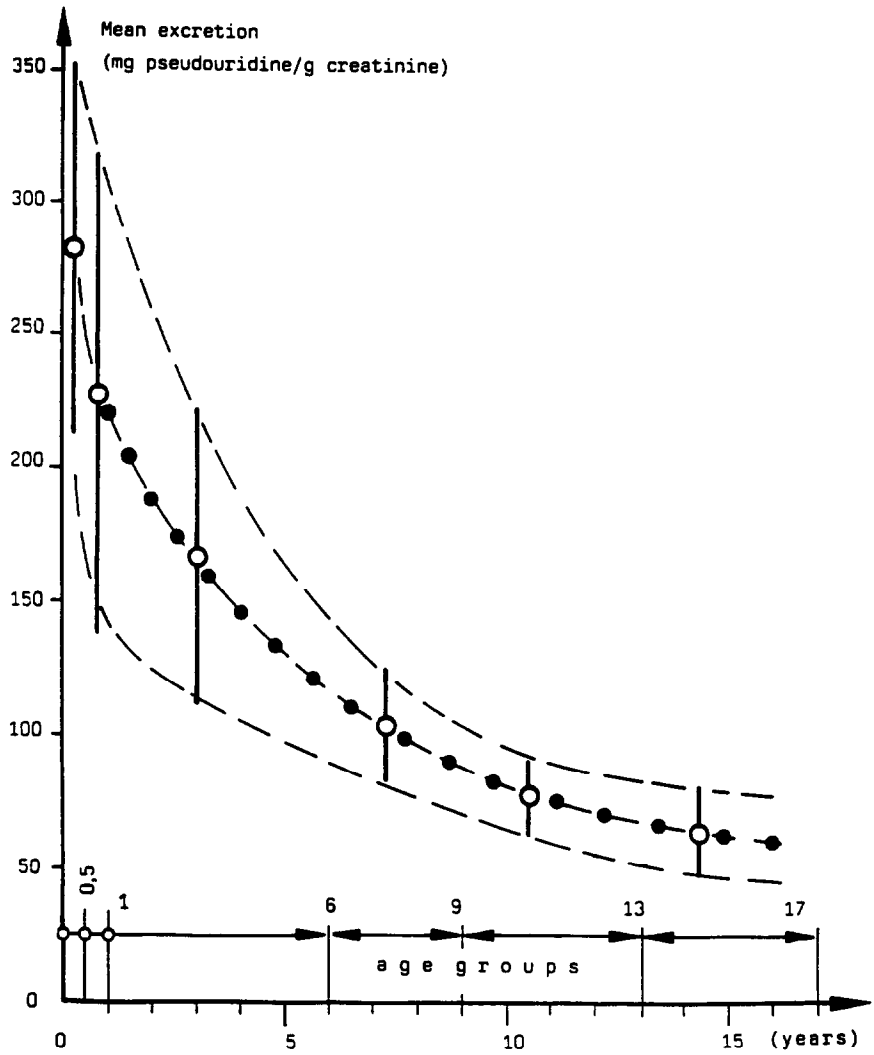

Fig. 3. Pseudouridine excretion as a function of age: (o) received mean values and S.D.; (•) values calculated from the function $y = 49.64 + 202.4\ e^{-0.1844x}$ where x is the age in years and y is the concentration in mg pseudouridine per g creatinine).

function $y = 49.64 + 202.4\ e^{-0.1844x}$ where x is the mean age in years and y is the mean concentration in mg pseudouridine per g creatinine. From the age of 0.83 years on, the deviation of the calculated values from the mean values observed is less than 2%.

Taking the curve of Fig. 3 as reference, ten of the omitted values exceed y + 2S.D. They are derived from the following diagnoses: enteritis, encopresis, mental debility with epilepsy, muscular dystrophy, hydrocephalus with probable infection of the valve, asthma, Crohn's disease, primary chronic polyarthritis and mucoviscidosis.

If the four additionally omitted values (enteritis) had been included, the curve

of the mean values would have had a "step" in the age group 1.0–5.99 years, which appears to be improbable.

DISCUSSION

As pointed out earlier, information about pseudouridine excretion in children is scarce. The only common feature of the results published by authors who considered age dependency is that the excretion rates tend to decrease with increasing age.

The results of Müller-Wickop et al. [9], which were published after we had finished our investigation, are similar to ours. They were the first to include a sufficient number of very young children (29 male babies). In total, they analysed urine samples from 349 probands whose age ranged from 0 to 40 years. They likewise showed that creatinine excretion increases more than pseudouridine excretion in the first twenty years of life. However, their mean values for babies are smaller than ours, approaching our mean values up to the age of 10 years and continuing at a higher level. The authors described their curve with a fifth-grade polynomial, but excluding children younger than 1 year. We also had to exclude children younger than 0.83 year. However, we think that mathematical description of the age dependency of pseudouridine excretion should be done with an exponential function rather than a fifth-grade polynomial for two reasons. Firstly, it seems unlikely to us that the excretion rates should have maxima and even negative minima in the course of adult life, since pseudouridine excretion is related to growth rate or cell turnover more generally. Secondly, contrary to the fifth-grade polynomial, exponential functions do appear quite often in natural events, such as growth of bacteria, explosions, heat transfer, etc. Thus we tried to describe the results of Müller-Wickop et al. [9] with another exponential function, which fits their results with a deviation always smaller than 1.5%. The equation is $y = 61.71 + 134.3\, e^{-0.1965x}$, where x and y are the same as before.

One maximum, however, would appear to be evident since pseudouridine excretion seems to be related to growth rate: the age just before puberty. In accordance with Müller-Wickop et al. [9] we did not find such a maximum. In discussing this phenomenon it has to be considered that creatinine excretion is a function of the actual muscle mass, whereas pseudouridine excretion is apparently a function of growth dynamics. So we find during the first year of life a rapidly falling curve of pseudouridine excretion even when related to the moderately rising creatinine excretion rates. In the period of puberty or prepuberty the two opposed tendencies might compensate each other due to the relatively high body weight combined with a growth rate lower than that in the first year of life. Schöch et al. [10], who did not investigate pseudouridine excretion but several methylated purines, obtained a maximum for the excretion of N^2,N^2-dimethylguanine in the period of puberty, which was not found by Müller-Wickop et al. [9]. In comparing these findings one must consider that Schöch et al. [10] determined the nucleosides after hydrolysis, together with the native bases, which perhaps have excretion patterns different from those of nucleosides; uracil excretion, for example, is not dependent on age at all [8,11].

Most of the children whose excretion values were omitted for the fitting of our curve suffered from chronic diseases in which cell turnover is assumed to be higher than normal. This has to be considered when using pseudouridine as a marker of malignancy. In the meantime, the urines of 45 other healthy children (1–17 years) have been investigated in our laboratory. Their pseudouridine excretion rates follow the curve described above without exception (unpublished observations).

ACKNOWLEDGEMENTS

We thank Mr. B. Assmann for mathematical advice, Mr. K. Bach for technical assistance and Dr. C.M. Klein for helpful discussions.

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