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# DETERMINATION OF CREATININE-RELATED URINARY URACIL EXCRETION IN CHILDREN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Excretion rates of uracil and thymine in children ($n=140$) and circadian rhythms of urinary uracil excretion ($n=9$) were investigated by reversed-phase high-performance liquid chromatography with ultraviolet detection. Excretion values were related to urinary creatinine determined by Jaffé's method. Creatinine-related uracil excretion was not dependent on age or sex. The values seemed to be distributed according to a Gaussian graph in both school children and those in hospital. The intra-individual range was 1.32–23.70 mg uracil per g creatinine over a four-day period in one subject. Uracil excretion seems to be somewhat lower during the night.

## INTRODUCTION

In 1984, four children with cerebral dysfunction (epileptic manifestations, behavioural problems and other) associated with extremely high urinary excretions of uracil and thymine were described [1,2]. The authors detected an almost complete deficiency of dihydropyrimidine dehydrogenase (DHPDH, EC 1.3.1.2) in the patients' leucocytes [1] and fibroblasts [2], respectively. The enzyme activities in their parents' cells were ca. 50% of the controls ($n=4$) [1]. These findings are suggestive of an inborn error of pyrimidine catabolism with a modus of autosomal inheritance. In 1985 another child with DHPDH deficiency was described [3]. The boy, whose parents were first cousins, probably suffered from several metabolic diseases and died at the age of two months. However, the analytical methods used were time-consuming, requiring pre-

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fractionation with Dowex 1-X8 followed by two different high-performance liquid chromatographic (HPLC) separations for the analysis of uracil and thymine, respectively [1]. Interference by uric acid and cytosine was also a problem in the analysis of physiological amounts of uracil and thymine [2]. Consequently, we have developed a more convenient method for the evaluation of normal and pathologic excretions of uracil and thymine.

## EXPERIMENTAL

### *Materials*

HPLC determinations were carried out with a Waters-Millipore liquid chromatograph (Milford, MA, U.S.A.) consisting of two Model 510 pumps, a U6K injector and a Model 680 programmer. Detection at 260 and 262 nm was performed by UV detector Models ERC-720 (ERMA Optical Works, Tokyo, Japan) and LKB 2151 (LKB, Bromma, Sweden), respectively. The plotting and evaluation of the chromatograms were carried out with 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 μm particle size) or Hypersil ODS-II (5 μm particle size) were purchased from Bischoff (Leonberg, 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 particle size).

The creatinine determinations (Jaffé's method) were carried out using a Model 150-20 photometer from Hitachi (Tokyo, Japan) using a kit according to its specifications (Boehringer, Mannheim, F.R.G.). Uracil and thymine were purchased from Sigma (St. Louis, MO, U.S.A.).

### *Sample preparation*

About 1 ml of the fresh urine samples was filtered (0.2 or 0.45 μm, Schleicher & Schuell, Dassel, F.R.G.) and frozen immediately (−18 to −24°C). Before HPLC analysis the samples were thawed to 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 done isocratically under the following conditions: 1.0 ml/min $KH_2PO_4$ (20 m*M*) with pH 5.4 and 262 nm during the run-in phase (of ca. four months) or pH 7.0 and 260 nm; the latter resulted in better creatinine and pseudouridine peaks (Figs. 1–3). The main column was cooled to 10°C. The pressure was ca. 131–145 bar when using Hypersil ODS-II and 145–166 bar when using Spherisorb ODS-II. The pressure was constant during analyses but tended to rise in the course of a day and with increasing column age. The

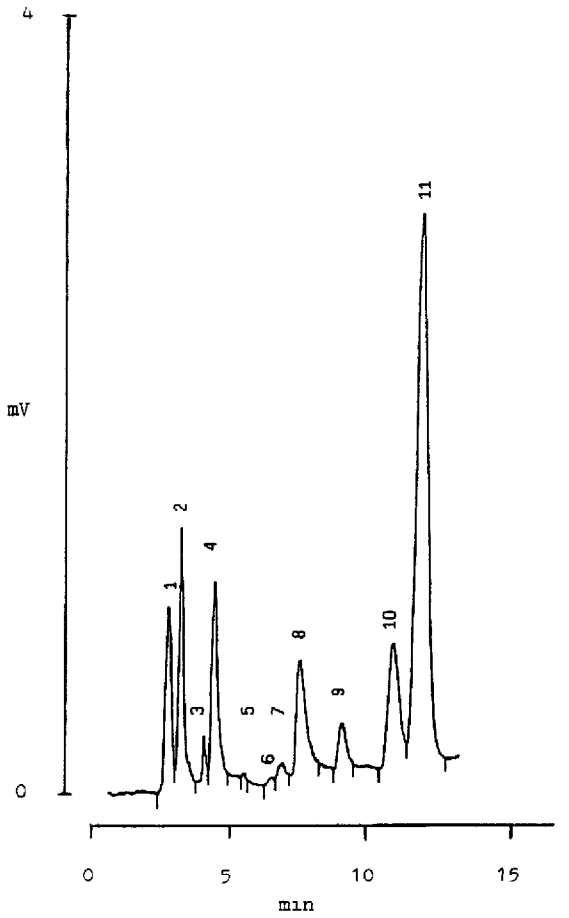


Fig. 1. Chromatogram of a urine sample (Spherisorb ODS-II, eluent pH 5.4, 262 nm). Peaks: 8 = creatinine; 9 = uracil; 10 = pseudouridine; 11 = uric acid.

retention time of uracil was ca. 7.5 min at pH 7.0 (Hypersil ODS-II) and ca. 8.5 min at pH 5.4 (Spherisorb ODS-II).

The column was washed by adding methanol to the mobile phase up to 80% after each run (constant flow of 80:20, v/v, for 10 min). At the end of the day the column was washed for ca. 90 min beginning with water (20 min), then adding methanol by a programmed gradient (ca. 15 min), 100% methanol for 45 min, then adding 10% of water (10 min). Each sample was analysed at least in duplicate.

*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

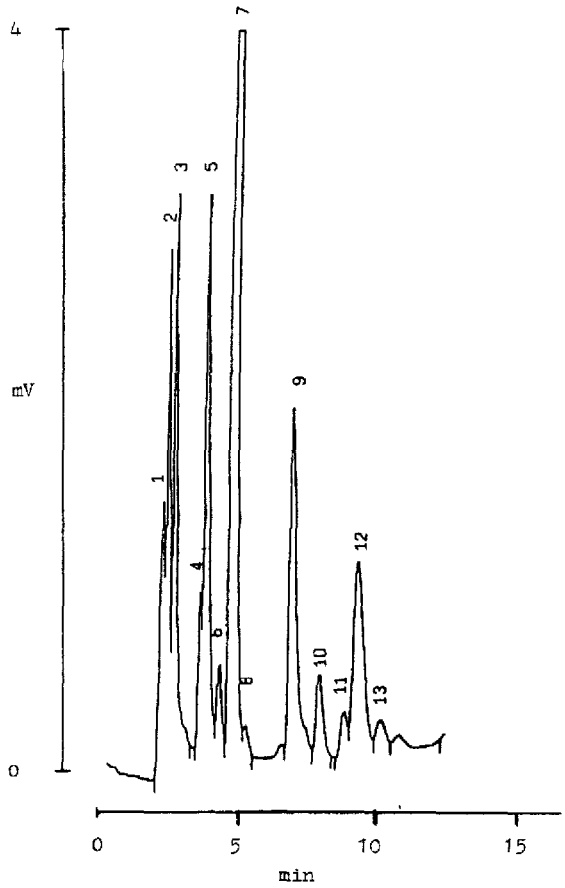


Fig. 2. Chromatogram of a urine sample (Spherisorb ODS-II, eluent pH 7.0, 262 nm). Peaks. 9 = creatinine; 10 = uracil; 12 = pseudouridine.

calculated, whereas peak heights were calculated when Hypersil ODS-II was used at pH 7.0 (Figs. 1–3). Calibration was controlled every morning and several times a day. Peaks were identified by a comparison of retention times and by adding uracil stock solution to the sample. The method afforded linearity of absorption over an uracil concentration range from 0.875 ng per 10 $\mu$l (0.781 $\mu M$) to 19.800 ng per 10 $\mu$l (17.664 $\mu M$) ($r = 0.9977$).

Accuracy and precision were tested by analysing 10 $\mu$l of an aqueous stock solution containing 2.000 ng (1.784 $\mu M$) of uracil twenty times in sequence. The mean was $2.182 \pm 0.067$ ng, which corresponds to 2.182 ng $\pm$ 3.07% (calculation of the peak heights). The average between-assay variation in urine samples was $\pm 3.90\%$ ($n = 20$) based on peak-area calculation (Spherisorb ODS-II, pH 5.4) and $\pm 3.24\%$ ($n = 20$) based on peak-height calculation (Hy-

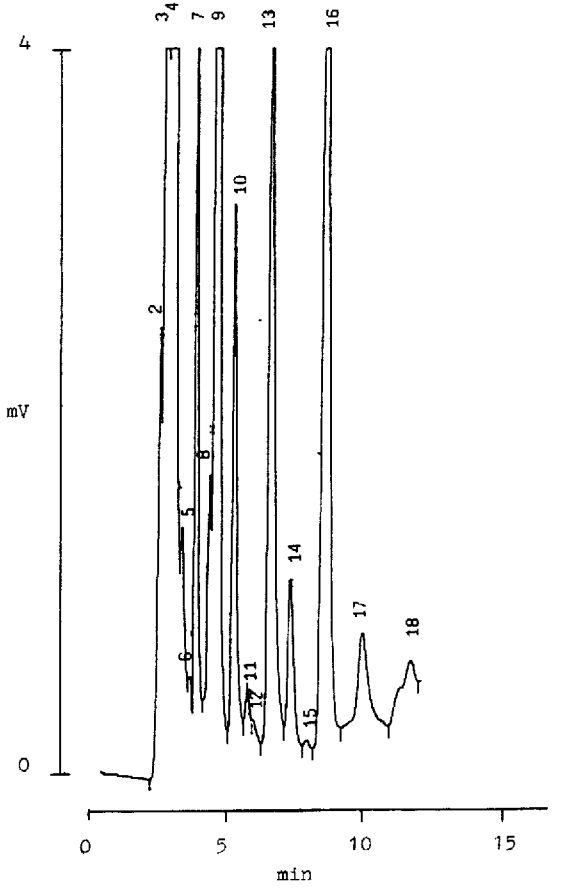


Fig. 3. Chromatogram of a urine sample (Hypersil ODS-II, eluent pH 7.0, 260 nm). Peaks: 13 = creatinine; 14 = uracil, 16 = pseudouridine.

persil ODS-II, pH 7.0). Additionally, accuracy and precision were assessed by recovery experiments against weighed amounts of uracil added to urine. Stock solutions added contained 4.98 mg/l (44.46 $\mu M$), 10.00 mg/l (89.25 $\mu M$), 14.80 mg/l (132.04 $\mu M$), 20.01 mg/l (178.52 $\mu M$) and 24.95 mg/l (222.59 $\mu M$) uracil, respectively. The urine sample contained 1.67 mg/l (14.93 $\mu M$) uracil. Mixtures of equal amounts of urine and stock solutions were diluted with deionized and filtered water (1:10). The injection volume was 20 $\mu$l. With five analyses each we found an average precision of $\pm 2.91\%$ and an average recovery of 99.43% (see Table I) by calculating the peak heights (Hypersil ODS-II, pH 7.0).

We collected urine spot samples from 120 patients (Department of Pediatrics, University of Saarland, Homburg/Saar, F.R.G., none of the children was suffering from a known malignant disease) aged from 4 days to 24 years, from

TABLE I

ACCURACY, PRECISION AND RECOVERIES OF URACIL ADDED TO URINE

| Uracil (mg/l) | Precision (±S.D., %) | Recovery (%) |
|---|---|---|
| 1.67[a] | 6 57 | – |
| 7.20 | 2 33 | 108.13 |
| 10.44 | 2.68 | 89.36 |
| 17.03 | 2.84 | 103.40 |
| 20.81 | 1.29 | 95.97 |
| 26.70 | 1.74 | 100.27 |
| Average | 2.91 | 99.43 |

[a]Urine without addition of uracil.

20 healthy children aged from 3 to 12 years and from the laboratory staff aged from 18 to 35 years, in order to investigate their urinary uracil excretion.

In order to elucidate possible circadian rhythms we determined 24-h urinary profiles of uracil and creatinine excretion. During nine periods of 24 h, urines were collected from five healthy subjects. One of the individuals (23 years, female) collected her urine every 2 h during four 24-h periods. Two of her daily profiles represent one 48-h excretion profile because they were received in direct sequence. The other four individuals were three boys and a girl aged from 3 to 9 years. The urine of the three-year-old boy was collected during two 24-h periods with an interval of six months. No special orders were given to the four children, except to avoid physical effort. In the case of the adult individual, urine was collected every 2 h including the nights and physical effort was avoided.

## RESULTS

The uracil excretion, expressed in mg uracil per g creatinine or in $\mu$mol per mol creatinine, seemed to be distributed according to a Gaussian graph (David's quotient). Mean values and ranges for the control group and the patients' group are given in Table II and seem to be independent of age and sex ($U$-test and $t$-test). In the subgroups of patients listed in Table II, approximately the same ranges of uracil excretion were seen. Their mean values were significantly different according to the Kruskel Wallis test, but it is most improbable that this reflects clinical significance because the mean values of the daily profiles of one healthy person do have nearly the same range and were significantly different as well (Table III). Thus we think that this is the consequence of inter-individual variation in addition to the rather large intra-individual vari-

TABLE II

MEAN VALUES AND STANDARD DEVIATIONS OF URINARY CREATININE-RELATED URACIL IN SPOT SAMPLES

| Group | *n* | Concentration (Mean ± S.D.) (mg/g of creatinine) |
|---|---|---|
| Control group (total) | 20 | 9.57 ± 4.51 |
| Control group (girls) | 10 | 9.15 ± 4.50 |
| Control group (boys) | 10 | 9.99 ± 4.72 |
| Patients (total) | 118[a] | 8.97 ± 5.88 |
| Patients with epilepsy | 25[b] | 7.41 ± 4.90 |
| Patients with hydrocephalus | 14[b] | 11.05 ± 7.02 |
| Patients with craniocerebral trauma | 10 | 13.18 ± 8.26 |
| Patients with acute infections | 21 | 8.67 ± 4.08 |

[a]In two samples from the neonatologic ward, uracil excretion could not be quantified reliably owing to interference with much higher peaks.
[b]Four of them in combination with hydrocephalus or epilepsy, respectively.

TABLE III

MINIMA, MAXIMA, MEAN VALUES, STANDARD DEVIATIONS AND RANGES OF CREATININE-RELATED URACIL IN URINE SAMPLES FOR EXCRETION PROFILES

| Subject No. | Sex | Age (years) | Concentration (mg/g of creatinine) | | | |
|---|---|---|---|---|---|---|
| | | | Minimum | Maximum | Mean ± S.D. | Range |
| 1 | M | 3.0 | 4.41 | 19.89 | 9.72 ± 4.86 | 15.48 |
| 1 | M | 3.6 | 8.09 | 26 36 | 19.47 ± 6.86 | 18.27 |
| 2 | M | 4.0 | 3.47 | 9.19 | 5.94 ± 1.76 | 5.72 |
| 3 | F | 7.0 | 4.86 | 24 02 | 11.63 ± 6.64 | 19.16 |
| 4 | M | 9.0 | 6.04 | 12.46 | 8.59 ± 2.43 | 6.42 |
| 5 | F | 23.0 | 3.10 | 23.70 | 12.73 ± 7.41 | 20.60 |
| 5 | F | 23.0 | 1.32 | 14.88 | 8.01 ± 4.25 | 13 66 |
| 5 | F | 23.0 | 2 45 | 20.08 | 8.90 ± 5.06 | 17.63 |
| 5 | F | 23.0 | 3.05 | 11.24 | 7.54 ± 3.03 | 8 18 |
| 1 (total) | | | 4.41 | 26.36 | 14.59 ± 7 63 | 21.95 |
| 5 (total) | | | 1.32 | 23.70 | 9.30 ± 4.94 | 22.38 |

TABLE IV

EXCRETED AMOUNTS OF URACIL AND CREATININE DURING 24 h

| Subject No. | Sex | Age (years) | Uracil (mg per 24 h) | Creatinine (mg per 24 h) |
|---|---|---|---|---|
| 1 | M | 3 0 | 2.72 | 254.44 |
| 1 | M | 3.6 | 3.75 | 218.24 |
| 2 | M | 4.0 | 1.73 | 304.06 |
| 3 | F | 7.0 | 3.34 | 504 46 |
| 4 | M | 9.0 | 4.46 | 457.41 |
| 5 | F | 23.0 | 11.96 | 899.07 |
| 5 | F | 23.0 | 9.05 | 1134.12 |
| 5 | F | 23.0 | 9.23 | 999.38 |
| 5 | F | 23.0 | 7.12 | 914.29 |

TABLE V

PUBLISHED EXCRETION RATES OF URACIL

| Sample | *n* | Method | Uracil excretion rate | Ref. |
|---|---|---|---|---|
| Adults | 23 | Photometry | 7 mg per 24 h | 4 |
| Normal urines | 6 | Ion-exchange + HPLC | 8.8–14.0 mg per g creatinine | 5 |
| Healthy infants | 20 | GC[a] after extraction | 9.91 mg per g creatinine | 6 |
| Control patients | 6 | Ion-exchange + HPLC | 3.36–10.09 mg per urine sample | 7 |
| Controls | 6 | Ion-exchange HPLC + GC–MS[b] | 7.85–33.63 mg per g creatinine | 1 |
| Controls | 20 | HPLC | 0.448 mg per urine sample | 2 |

[a]Gas chromatography.
[b]Gas chromatography–mass spectrometry.

ation. The individual uracil excretion profiles varied greatly, but there was a tendency to lower excretion values during the night. Table III shows the ranges of creatinine-related uracil excretion within one person as mentioned above. The relation of uracil excretion to body weight seems to be less close than that of creatinine excretion to body weight (Table IV).

## DISCUSSION

Physiological uracil excretion has not yet been investigated thoroughly. Some published excretion rates can be seen in Table V. In earlier reports normal uracil excretion was considered to be "0 mg per 24 h" [8] or "uracil was not found" [9]. In order to evaluate errors due to non-visible interferences with the uracil peak, the prefractionation using a mixed bed of ion-exchange resin according to Uziel [10] was found to be suitable (a photodiode array detector was not available). The recovery of uracil in water was $91.8 \pm 5.3\%$ ($n=9$) and

in urine $81.7 \pm 7.1\%$ ($n=9$). However, the prefractionation procedure required additional time and work, and in our system it was not always possible to avoid the column wash subsequent to analysis. Moreover the recovery was not stable enough (possibly owing to the manual rinsing of the shaken vessels) to compensate for the small errors due to the creatinine peak eluting just before the uracil peak. When Spherisorb ODS-II was used there was no interference with higher peaks at all. However, using Hypersil ODS-II, pH 7.0 was better for the analysis of creatinine and pseudouridine. Consequently we decided to omit this step from our procedure.

Thymine, which has a retention time of ca. 22 min, can be detected with the same method by simply prolonging the period of isocratic elution to 24 min. However, as in the case of DHPDH deficiency uracil excretion would be high as well, it is unnecessary to determine thymine excretion in every urine sample.

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