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Correlations of Pseudouridine in 8-Hour and 24-Hour Urinary Samples Determined by High-Performance Liquid Chromatography

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Summary

The correlations of Pseudouridine (ψ) values in 8 h and 24 h urinary samples from 23 healthy persons were determined. *Pseudouridine* in the 8 h urinary samples was measured by high-performance liquid chromatography and the 24 h excretion was calculated from the results of three 8 h determinations. Simultaneous determinations of urinary creatinine were performed by Jaffe's reaction. Based on the 8 h values of urinary *Pseudouridine* the results demonstrated a constant excretion of *Pseudouridine* in the 24 h periods, if the values were related to the urinary creatinine. The precision in using this ratio as an indicator for normal 24 h excretion of *Pseudouridine* was 90.5% for males and 92.5% for females.

Key words: Bladder tumours, Pseudouridine excretion, RNA, Markers.

Introduction

Pseudouridine (ψ) originates in RNA, predominantly transfer RNA (tRNA) [2, 4, 8]. Since ψ is neither metabolised nor recycled [2, 3], it is quantitatively excreted, in humans in the urine. Thus the urinary excretion of ψ reflects the whole body tRNA turnover.

Standard values for 24 h urinary ψ are constant in a normal human population [1, 2, 7, 16]. Increased values have been reported in different kinds of malignancies [7, 10, 14, 16] and in a few rare types of non-malignant diseases [17]. The results in patients with urothelial tumours have earlier been reported [6, 10].

The present study is a preliminary investigation related to a current bladder cancer study using urinary β-amino-isobutyric acid (β-AIB) and ψ as biochemical markers. The validity of 8 h urinary values of ψ in relation to the 24 h values is displayed among healthy controls in the same way as earlier reported on urinary β-AIB [15].

Method

The applied method of assay of pseudouridine has been described before [6, 9].

Apparatus

An ion-exchange column 150 x 5 mm was custom-fitted with a 50 ml reservoir (from Danish Laboratory Equipment, DK-2100 Ø, Denmark). Affi-Gel 601, a boronate affinity gel to separate ribonucleotides, ribonucleosides, sugars, catecholamines and coenzymes prior to HPLC was used (Bio-Rad Laboratories, Richmond, California 94804). A microcentrifuge Type 155 SL (Ole Dich, Avedøre Holme, DK-2650 Hvidovre), was used for protein precipitation.

The chromatographie studies were conducted with an Isocratic Liquid Chromatograph Model 330 (Altex Scientific Inc., Berkeley, California 94710 with Model 110 Solvent metering Pump, Model 153 Analytical UV Detector and W + W Recorder Series 600/601 Tarkan, W + W Electronic Inc., CH-4002 Basel). The column was a Waters Associates μ Bondapak C_{18}/porasil, 4 x 300 mm. The column was equipped with anlet filter 0.2 μ.

Chemicals

Pseudouridine (P-0509) was purchased from Sigma Chemical Co. (St. Louis, Missouri 63178 U.S.A.). Ammoniumacetate pro analysi (Art. 1116), Ammoniumdihydrogenphosphate pro analysi (Art. 1126), Ammoniumhydroxide pro analysi min. 25% (Art. 5432), Methanol pro analysi (Art. 6009) were supplied from E. Merck (Darmstadt, West Germany). Double distilled ion-exchanged, millipore (0.5 μ) filtrated water was produced by Dumex DK-2300 Copenhagen S, Denmark.

Buffers

A 2.0 M solution of $NH_4H_2PO_4$ was prepared as a stock solution. This solution was filtrated through a millipore SMWP 04700 filter (5 μ) and stored a 4 °C. Every second day 2 l buffer solution was prepared in the following way: to 20 ml aliquots of the buffer concentrate in a 2 l volumetric flask was added 200 ml ion-free water, then 20 ml methanol. This solution was diluted to 2 l with distilled ion-free water (Dumex) and then filtered through a Millipore

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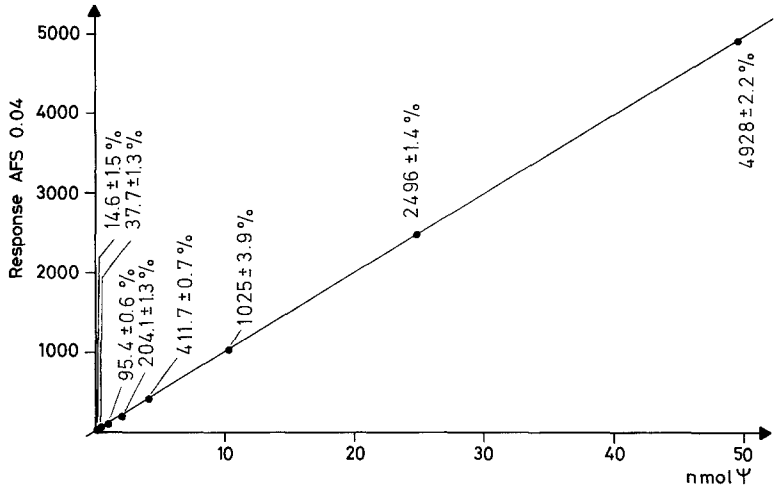

Fig. 1. Linearity of Urine Calibration for ψ. r = 0.9999, y = 95,566 + (– 1.87), n = 36 (9 conc.)

SMWP filter (5 μ). Diluting the buffer concentrate prior to adding methanol prevents the salt from precipitation out. The diluted buffer was stored at 4 °C and discarded after 2 days. Before the buffer was used it was degasified 15 min in an ultrasonic bath (Bronsonic Branson, Solst, Netherlands).

Samples, Collection, and Storage

The urine samples were collected from healthy males and females. The 24 h excretion of ψ and creatinine were calculated form the results of quantitative determinations of ψ and creatinine of three 8 h urinary samples (see material). From each urinary sample an aliquot of 5 ml was stored at –170 °C in polypropylen tubes 5 ml (Nunc, DK-4000 Roskilde, Denmark).

Preparation of ion-exchange column

1. 1 ml 0.25 M Ammonium Acetate buffer pH 8.8 was placed in the column.

2. A suspension of 2.5 g gel in 10 ml 0.1 M NaCl was transferred to the column with a plastic pipette.

3. The column was allowed to drain and more gel was added to the height of 40 mm (bed volume 0.8 ml).

4. The gel was rinsed with about 20 ml 0.25 M Ammonium Acetate pH 8.8. No pressure was used on the column.

5. The buffer was allowed to drain to the top of the gel. Then 50 ml of 0.1 M Formic Acid were added for rinsing. The Formic Acid caused the gel to contract visibly but the bed volume was based on the initial volume of the gel in 0.25 M Ammonium Acetate buffer pH 8.8

6. The column was percolated with 10 ml of 0.25 M Ammonium Acetate buffer pH 8.8 to equilibrate it with this buffer. The gel column was then ready for sample loading when the buffer had drained to the top of the gel bed.

Sample Cleanup

1. From the urine 0.50 ml aliquot was drawn and placed in a 1.5 ml Eppendorf microcentrifuge tube.

2. 200 μl of 2.5 M Ammonium Acetate buffer pH 9.5 was added to the sample and shaked and mixed for 5 min.

3. The sample was centrifuged for 5 min at 12,000 x g in a microcentrifuge.

4. The supernatant was transferred with a Pasteur pipette to the column.

5. 0.25 M Ammonium Acetate buffer pH 8.8 was added to the sample and mixed for 5 min.

6. The sample was centrifuged for 5 min at 12,000 x g.

7. The wash was transferred onto the column with the same Pasteur pipette.

8. The column was washed 6 ml 0.25 M Ammonium Acetate buffer pH 8.8. After this wash had drained to the top of the gel bed, the column was ready for elution.

Elution of Nucleosides

9. The nucleosides were eluted with 5 ml of 0.1 M Formic Acid, and collected in a 10 ml volumetric flask.

10. The volume was increased to 10 ml with distilled pure water (Dumex) and mixed.

11. The sample was millipore filtrated (0.5 μ, FHLP 01300 Millipore corporation, Bedford, Massachusetts 01730) and injected in the HPLC.

Reversed-Phase HPLC Chromatography of Pseudouridine

A 50 μl aliquot of each cleaned sample (equivalent to 2.5 μl urine) was injected and chromatographed at 1.2 ml/min with 0.01 M $NH_4H_2PO_4$ buffer containing 1% v/v methanol, pH 5.0, on a 4 x 300 mm Bondapak C_{18} column, temperature 25 °. Detector 254 nm, 0.8 AFS. Units nmol. Precision of HPLC determination see later.

Calibration Standard Solution

A stock solution of pseudouridine was prepared to give a concentration of 16.54 mM (20.2 mg pseudouridine in 5 ml redistilled water, Dumex). The following were applied: 0.2, 0.4, 1.0, 2.1, 4.1, 10.3, 24.8, 49.6 nmol (Fig. 1). r = 0.9999, y = 95,566 + (– 1.87).

Table 1. Values and ditribution of ψ and $\frac{\mu mol\ \psi}{mmol\ creatinine}$ in 8 h urinary collections from healthy males and females

Period	n	μmol ψ			μmol ψ / mmol creatinine		
		$\bar{x}$	σ	RSD%	$\bar{x}$	σ	RSD%
Males							
A	32	108.98	38.60	35.42	19.14	5.13	26.80
B	32	99.84	48.20	48.27	20.61	4.27	20.72
C	31	127.33	53.27	41.84	20.24	4.31	21.32
D	31	334.90	89.11	26.00	19.99	3.44	17.19
Females							
A	37	63.59	26.04	40.949	25.16	5.30	21.08
B	37	67.67	41.50	61.333	25.22	9.29	36.83
C	36	63.86	30.93	48.439	26.50	6.96	26.27
D	37	193.39	56.17	29.044	25.28	5.32	21.03

n is the number of assays in 12 males and 11 females, respectively

A, B, and C are consecutive 8 h urine samples in a 24 h period, from 7 a.m.

D is the calculated 24 h excretion $\bar{x} = \left(\frac{\Sigma(A+B+C)}{n} \right)$

Precision of HPLC Determination of Pseudouridine

Precision of the HPLC determination of pseudouridine was investigated using 11 aliquots of urine from the same person. To 0.5 ml urine with 0.2485 μmol ψ was added 2.7027 μmol ψ. Each solution was injected two times, so totally 22 analyses were performed. The standard deviation was 1.3%.

Determination of creatinine

Creatinine in urine was determined spectrophotometrically by Jaffe's reaction at E 515 nm. The results have been published earlier [5].

Material

In 11 healthy females aged 22 to 56 years and 12 healthy males aged 15 to 54 years the 24 h excretion of ψ (D) and creatinine (D) were calculated from the results of quantitative determinations ψ and creatinine in three 8 h urinary samples. The 8 h urinary samples were collected from (A) 7 a.m. to 3 p.m.; (B) 3 p.m. to 11 p.m.; (C) and 11 p.m. to 7 a.m. Each person was examined in 1-5 non-consecutive 24 h periods, on average 3.2 times, within 3 months. In total, 205 determinations of ψ and creatinine in 8 h urinary samples were made. The 8 h urine samples were collected without preservative and stored at 4 °C until collection was completed. Aliquots were stored at −170 °C.

Results

The 8 h values of urinary ψ and the calculated 24 h value in males and females respectively appear in Table 1. The 24 h value was about 1.7 times higher in males than in females. The 8 h values in both males and females demonstrated a rather constant excretion of ψ within the 24 h periods, except for the night excretion (C) in the males which was higher than in the other 8 h periods. The difference mentioned in the 24 h excretion of ψ in males and females was similarly reflected in the different 8 h values.

The uniformity in the 8 h values of ψ was more marked, if the values were related to urinary creatinine (Table 1), but in consequence of the significant difference in urinary creatinine excretion in males and females [15], the ratio ψ / creatinine was about 25% higher in females than in males.

The range of normal limits for urinary ψ in relation to urinary creatinine (ratio = μmol ψ/mmol cr.) is demonstrated in Table 1 for males and females.

These results indicated a rather narrow range for normal 24 h values of urinary ψ in healthy males and females, if these values were related to the creatinine excretion. The average values of this 8 h ratio in the three different 8 h periods (A + B + C) showed only minor variations from the corresponding calculated 24 h values. However the standard deviations on a 5% significance level were slightly higher for 8 h ratios than for 24 h ratios.

The precision in using this ratio from 8 h urinary samples as an indicator for low 24 h excretors of urinary ψ was tested on our 205 results. If the value for normal low excretion was fixed as less than 25 for males and less than 35 for females, the precision was 90.5% for males and 92.5% for females. The failures were equally distributed over the A, B and C 8 h periods both among males and females.

However, these precisions were increased to 99% and 96.5% respectively if the limits for normal values were elevated to 30 in males and 40 in females. Linear regression analysis for ratios in A, B, and C 8 h urinary samples showed

good correlation to the 24 h excretion of ψ (r: females 0.71–0.89, r: males 0.67–0.93).

Discussion

In the last 10 years, the search for new biochemical markers for cancer has included break-down products from the highly complex tRNA molecule [2, 7, 12–14, 16]. Most of these products are modified nucleosides, which occur in cancerous tissue in high amounts and are excreted in the urine in unchanged forms [1, 3, 10]. Among these are ψ, which is found in increased levels in the urine from patients with different types of cancer [2, 10, 13, 14, 16]).

In an extensive up-to-date survey Borek (1980) has given a detailed description of our present knowledge on the tRNA molecule, its normal degradation products and the modifications occurring in cancerous tissue [2].

Among the modified nucleosides we have chosen to use the urinary excretion of ψ as tumour marker in the control of patients with urothelial cancer. Besides, we have measured the β–AIB excretion [15]. Earlier we have demonstrated an increased urinary excretion of these two markers in urothelial cancer population [13, 14], but the explosive evolution in new technical methods has necessitated a change to new methods of which high pressure liquid chromatography is described in detail in this publication. This method is almost identical with that of Gehrke et al. [6, 9]. The significance of increased excretion of urinary ψ in cancer patients is at the present time obscure. However, Borek et al. [1] has indicated that tRNA in cancerous tissue contains a subpopulation with an increased turnover, which can be as high as ten-fold the normal turnover.

The present work is an initial part of a screeing programme for control of patients with urothelial cancer, to fix the range of urinary ψ among normal, healthy persons, and to test the validity of using 8 h urinary values in relation to the urinary creatinine.

The overall precision in using the creatinine related ψ values in 8 h urine collections as an expression for the 24 h excretion was in our results determined at 90.5% and 92.5% in normal males and females, respectively, if the upper limits for normal excretion were fixed at 25 for males and 35 for females. If these limits were increased to 30 and 40, respectively, the precisions were 99% and 96.5%. In accordance with the earlier demonstrated constant excretion of ψ within a 24 h period [9], our failures were equally distributed in the different 8 h periods. However, at present we are not able to determine the precision for high-excretors of urinary ψ. Preliminary results from cancer patients with increased excretions indicate to some extent a limited area of interference of creatinine-related ψ values in healthy persons and cancer patients. These critical areas are 25–30 for males and 35–40 for females. So the use of urine samples at random, as proposed by Kuo et al. [9] will probably be the most practical solution, since such samples are easy to obtain and repeat, especially if the results are within the mentioned critical areas.

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