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Isolation and identification of urinary nucleosides

Applications of high-performance liquid chromatographic methods to the synthesis of 5′-deoxyxanthosine and the simultaneous determination of 5,6-dihydrouridine and pseudouridine

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ABSTRACT

Modified nucleosides from pooled normal human urine were extracted using a boronate affinity gel column and fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). The major constituents in each of the 30 RP-HPLC fractions were determined by gas chromatography–mass spectrometry of the trimethylsilyl derivatives of the fractions. The same RP-HPLC method was used in the synthesis of 5′-deoxyxanthosine from authentic 5′-deoxyadenosine. In addition, the simultaneous determination of urinary 5,6-dihydrouridine (D) and pseudouridine (Ψ) was carried out by RP-HPLC using two ODS columns in series. The level of D in pooled normal urine was 4.87 nmol/μmol creatinine. The RP-HPLC method was applied to the measurement of D and Ψ levels in urines collected before and after surgery from four patients with gastrointestinal cancer. A large decline in both nucleoside levels in urines after surgery was observed in three of the four cancer patients.

INTRODUCTION

Modified nucleosides excreted in human urine have been studied to examine their biomedical significance as possible biomarkers of cancer[1–7] and certain immunodeficiency diseases[8], including AIDS[9,10]. In human urine, about 20 out of

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more than 40 urinary nucleosides are known to be derived from tRNAs. Among them, several modified nucleosides occurring at comparatively high levels such as pseudouridine (Ψ), 1-methyladenosine (m^1A), 1-methylinosine (m^1I), N^2-methylguanosine (m^2G) and N^2,N^2-dimethylguanosine (m^2_2G), have frequently been used to examine the differences between urinary nucleoside levels of cancer patients and normal subjects. Studies monitoring the correlation between urinary levels of these nucleosides and the degree of tumour involvement or response to therapy have also been performed[2–7,11].

In addition, more than ten urinary modified nucleosides derived from other biochemical processes not related to tRNA have been found. Recently, the presence of the unusual nucleosides 5′-deoxyinosine (5′-dI)[12], 5′-deoxy-5′-methylthioadenosine sulphoxide[12,13] and 7-β-D-ribofuranosylhypoxanthine[14] in human urine has been reported. The origin of these nucleosides is not known to occur from any biochemical metabolism. Chheda *et al.*[12] suggested that one possible source of 5′-dI in mammals appeared to be 5′-deoxyadenosine (5′-dA), which is liberated from coenzyme-vitamin B_{12}, adenosylcobalamin.

Through our collaborative studies on the isolation and identification of urinary nucleosides in human urines, 3-methyluridine (m^3U)[15] and the novel nucleoside 5′-deoxyxanthosine (5′-dX)[16] were detected by means of gas chromatography–mass spectrometry (GC–MS) analysis following partial purification by boronate gel affinity chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC). In addition, 5,6-dihydrouridine (D) was recently identified in pooled normal human urine by GC–MS[17]. Although D is present in the tRNAs of most organisms, and is second in abundance to Ψ among the modified nucleosides in tRNAs, there was little evidence of the presence of D in human urines; a thin-layer chromatographic method has been used for the determination of D in tRNAs[18,19] and LC–MS for the detection of D in human urine[20].

In this paper, we describe methods used for the isolation and identification of nucleosides in pooled normal human urines, the RP-HPLC analysis of 5′-dX synthesized from authentic 5′-dA and the simultaneous determination of urinary D and Ψ by another RP-HPLC method.

EXPERIMENTAL

Chemicals and standards

Ammonium acetate, formic acid, methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Wako (Osaka, Japan), A Wako Creatinine Test Kit using the Jaffe method was used for the measurement of urinary creatinine. Authentic samples of D, m^1A, Ψ, 5′-dA, purine nucleoside phosphorylase (PNP; N3003, from bovine spleen) and xanthine oxidase (XO; X1875) were purchased from Sigma (St. Louis, MO, U.S.A.). A milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.) was used for water purification.

Urine collection

For the isolation of urinary nucleosides, a 188-ml aliquot of pooled normal urine obtained from more than 300 apparently normal male and female subjects at the PL Osaka Health Control Centre was centrifuged to remove particulate matter and stored at $-20°C$ prior to analysis.

Urine samples (24-h) were collected 4 or 5 days before and 8 or 9 days after surgical operations on two gastric cancer patients and two sigmoid colon cancer patients at the PL Hospital, and used for the determination of urinary D and Ψ.

The case history of cancer patients is briefly summarized as follows. Case No. 1: sigmoid colon cancer (well differentiated adenocarcinoma); stage, Dukes A; resection, absolutely curative; carcinoembryonic antigen (CEA), 1.8 ng/ml (pre-surgery), 1.0 ng/ml (post-surgery). Case No. 2: gastric cancer (moderately differentiated tubular adenocarcinoma); stage, Ia (TNM classification of International Union Against Cancer); resection, absolutely curative; CEA, 2.3 ng/ml (pre-surgery), 2.9 ng/ml (post-surgery). Case No. 3: gastric cancer (signet-ring cell carcinoma, scirrhous type); stage, IV (TNM classification); resection, absolutely non-curative; CEA, not quantitated (pre-surgery), 13.6 ng/ml (post-surgery). Case No. 4: sigmoid colon cancer (mucinous carcinoma); stage, Dukes B; resection, absolutely curative; CEA, 2.9 ng/ml (pre-surgery), 0.8 ng/ml (post-surgery).

Boronate gel affinity chromatography

The boronate gel affinity chromatographic methods used to isolate the urinary nucleosides were a modification of a published procedure[11]. Affi-gel 601 (Bio-Rad Labs., Richmond, CA, U.S.A.) possessing a specific affinity for *cis*-hydroxyl groups was packed in a plastic column (60 × 9 mm I.D.; 1.66 ml bed volume) and equilibrated with 20 ml of 0.25 *M* ammonium acetate (pH 8.8). A 20-ml aliquot of urine adjusted to pH 8.8 with 4 ml of 2.5 *M* ammonium acetate (pH 9.5) was centrifuged to remove precipitable material and its supernatant was loaded onto the column. The sample tube and column were then washed with 3 and 7 ml of 0.25 *M* ammonium acetate (pH 8.8) and the nucleosides were eluted with 7 ml of 0.2 *M* formic acid. The eluate was evaporated under reduced pressure and the residue dissolved in 1 ml of water for HPLC purification. However, in the experiments for the determination of urinary D, the boronate column extract was lyophilized. The sample was passed through an ultrafiltration membrane filter (Nihon Millipore Kogyo, Yonezawa, Japan) just before the HPLC fractionation in order to remove macromolecules.

RP-HPLC fractionation of urinary nucleosides

The RP-HPLC conditions for fractionation of the urinary nucleosides were as follows: an LC-6A HPLC instrument (Shimadzu, Kyoto, Japan); two pumps; an SCL-6A system controller; an SPD-6AV UV–VIS detector; a C-R6A Chromatopac integrator; a Develosil ODS-5 (5 μm) column (250 × 4.6 mm I.D.) (Nomura Chemicals, Nagoya, Japan); a Develosil ODS (15–30 μm) precolumn (50 × 4.0 mm I.D.); linear gradient elution from water at 0 min to methanol–water 13:87 (v/v) at 25 min, methanol–water 45:55 (v/v) at 35 min and water at 40 min; flow-rate 1.1 ml/min; sample injection volume 300 or 350 μl; and UV detection at 260 nm (0.64 a.u.f.s.). A total of 30 HPLC fractions were collected and lyophilized for GC–MS analysis.

Preparation of reference sample 5′-dX from 5′-dA

A reference sample of 5′-dI was prepared by the deamination of 5′-dA with sodium nitrite[21]. A 10-mg amount of authentic 5′-dA was dissolved in 4 ml of 3.5 *M* acetic acid and treated with 4 ml of 4 *M* sodium nitrite at 25°C for 2.5 h. The product was purified and isolated using the RP-HPLC method as described above, and the

structure of the sample was confirmed by comparing the GC–MS analysis of the trimethylsilyl (TMS) derivative with data from the literature[12].

5-Deoxyribose-1-phosphate (5-dR-1-P) was obtained by the enzymatic cleavage of 5′-dI using a modification of a literature method[22]. The reaction mixture for the preparation of 5-dR-1-P contained 0.6 mmol 5′-dI, 2.5 units of PNP, p 0.125 units of XO and 50 m*M* potassium phosphate buffer (pH 7.5 adjusted using 5 *M* sodium hydroxide) in a total volume of 5 ml. Prior to the reaction, the two enzymes had been dialysed against 50 m*M* phosphate buffer (pH 7.5) to remove ammonium sulphate. The reaction was allowed to proceed for 12 h at 23°C on a rotary shaker. Following the reaction, the enzymes were removed by an ultrafiltration membrane. Inorganic phosphate was precipitated by strictly maintaining the pH between 8 and 9 through the addition of either saturated barium hydroxide or 1 *M* barium acetate. The mixture containing 5-dR-1-P was used without further purification in the synthesis of 5′-deoxyguanosine (5′-dG).

5′-dG was prepared[22] by the reaction of 140 μmol of guanine with 80 μmol of 5-dR-1-P in the presence of 4 units of PNP in 10 m*M* Tris–HCl (pH 7.5) in a total volume of 20 ml. The reaction was carried out at 23°C for 5 h on a rotary shaker. 5′-dG was isolated using RP-HPLC and the structure of the product was confirmed by GC–MS. 5′-dG was then deaminated using sodium nitrite as described earlier[21]. Attempts to couple xanthine directly to 5-dR-1-P using PNP were unsuccessful.

GC–MS analysis

The methods and results of the GC–MS analyses for the TMS derivatives of the RP-HPLC nucleoside fractions have been described elsewhere[15–17].

RP-HPLC analysis of urinary D and Ψ

For the determination of urinary D and Ψ, two Capcell Pak C_{18} (5 μm) columns (250 × 4.6 mm I.D.) (Shiseido, Tokyo, Japan) were used in series. The urinary nucleoside samples were analysed using isocratic elution with water alone at a flow-rate of 0.8 ml/min. After 20 min the columns were washed with a gradient of acetonitrile–water. UV detection was performed at 230 nm (0.04 a.u.f.s.). A precolumn (20 × 4.6 mm I.D.) packed with 15–30-μm Develosil ODS was fitted between the injector and the analytical column.

RESULTS AND DISCUSSION

HPLC analysis of urinary nucleosides

Urinary nucleosides extracted with the boronate gel column were fractionated by RP-HPLC. Water–methanol was adopted as mobile phase for RP-HPLC to simplify sample preparation for the subsequent GC–MS analysis. Fig. 1 shows a typical RP-HPLC trace for urinary nucleoside extract. A total of 30 peaks present in pooled normal urine were numbered as shown in Fig. 1.

GC–MS analyses of these HPLC fractions revealed that some of the fractions included well known nucleosides and bases as major constituents: HPLC fraction No. 1, m^1A; No. 2, Ψ; No. 2.5, hypoxanthine; No. 3, uric acid; No. 4, uridine (U); No. 9, inosine (I); No. 10, 1-ribosylpyridin-4-one-3-carboxamide (4,3-PCNR) + guanosine (G); No. 11, m^3U[15]; No. 12, m^1I; No. 14, m^1G; No. 15, adenosine (A) + xanthosine (X); No. 16, m_2^2G; and No. 18, 5′-dX[16].

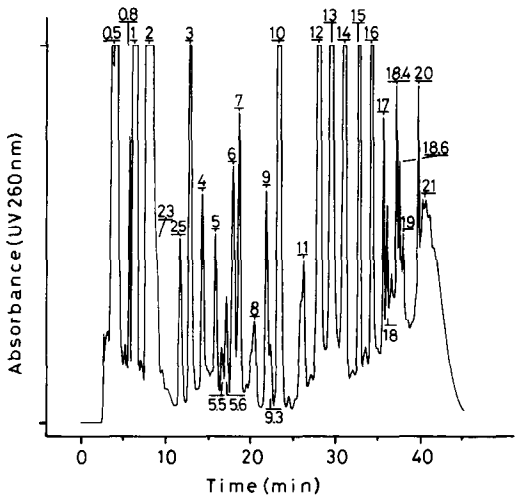

Fig. 1. Preparatory HPLC separation of a nucleoside sample in pooled normal human urine. Injection volume: 350 μl of nucleoside sample, corresponding to nucleosides present in 7 ml of original urine. UV detection at 260 nm (0.64 a.u.f.s.). For other chromatographic conditions, see Experimental.

Several unknown nucleosides were also revealed by the GC–MS analysis of the HPLC fractions. The structures of these unknown nucleosides are now under investigation.

The RP-HPLC fraction No. 1 (retention time 6.6 min) exhibited an absorption maximum at 257 nm, corresponding to the λ_{max} of m^1A. However, as reported previously[17], the GC–MS examination of HPLC fraction No. 1 indicated the presence of D in addition to m^1A. The presence of D was also confirmed in HPLC fraction No. 2, which contained Ψ as a major nucleoside. From this evidence, D was considered to be eluted between peaks 1 and 2 detected at 260 nm.

HPLC analysis for the preparation of 5′-dX

For the identification of urinary 5′-dX, the nucleoside was synthesized from an authentic sample of 5′-dA. Fig. 2 shows the RP-HPLC traces of an authentic 5′-dA and 5′-dI formed by sodium nitrite deamination of 5′-dA.

Fig. 3 shows the RP-HPLC separation of enzymatically synthesized 5′-dG and 5′-dX resulting from deamination of 5′-dG. 5′-dG and 5′-dX were purified by RP-HPLC and confirmed by GC–MS[16,23]. The UV absorption spectra of the 5′-dX thus obtained and the reference sample of X are shown in Fig. 4. Significant differences between the spectrum of 5′-dX and that of X were observed.

RP-HPLC analysis of urinary D and Ψ

Because D was not resolved from m^1A and Ψ under the HPLC conditions in Fig. 1, the HPLC determination of urinary D was further developed. As D does not exhibit UV absorption around 260 nm, 230 nm (corresponding to the λ_{min} of Ψ) was selected as the detection wavelength for urinary D. The following HPLC conditions for the separation of D and Ψ were examined using mainly a Develosil ODS column: cations, anions, pH, ionic strength of buffers, organic solvents used as mobile phase, column

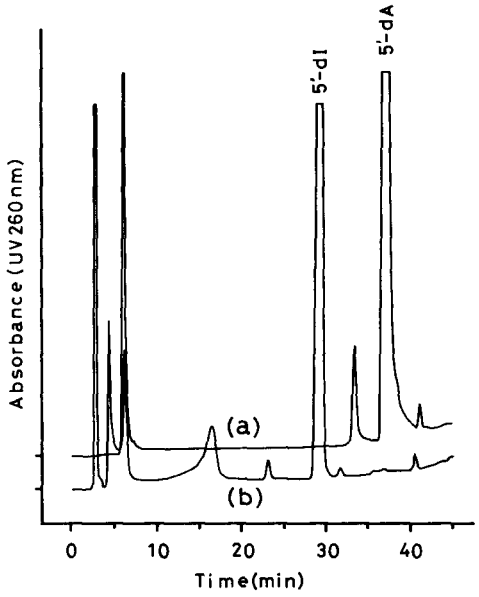

Fig. 2. HPLC separation of authentic sample of $0.995 \cdot 10^{-2}$ *M* 5'-deoxyadenosine in 3.5 *M* acetic acid (a) and 5'-deoxyinosine in the reaction mixture prepared by deamination of 5'-deoxyadenosine with sodium nitrite (b). UV detection at 260 nm (0.04 a.u.f.s.). Injection volume: 5 μl. For other chromatographic conditions, see Experimental.

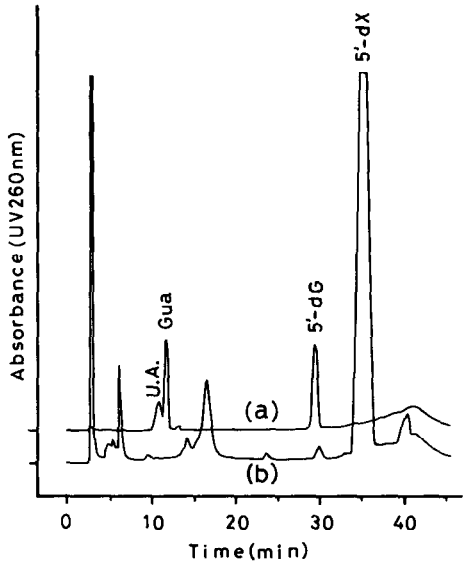

Fig. 3. HPLC separation of 5'-deoxyguanosine in the reaction mixture of 5-deoxyribose-1-phosphate and guanosine catalysed by purine nucleoside phosphorylase (a) and 5'-deoxyxanthosine in the reaction mixture prepared by deamination of 5'-deoxyguanosine with sodium nitrite (b). UV detection at 260 nm (0.04 a.u.f.s.). Injection volume: (a) 20 μl and (b) 5 μl. For other chromatographic conditions, see Experimental.

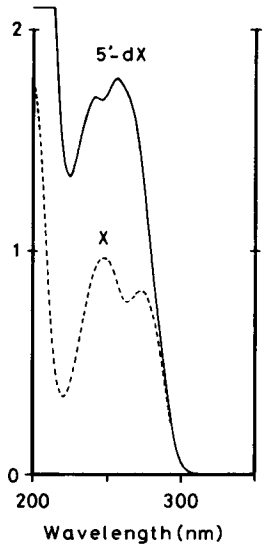

Fig. 4. Absorption spectra of 5'-deoxyxanthosine in water (solid line) and $1.0 \cdot 10^{-4}$ *M* authentic xanthosine in 0.01 *M* KH_2PO_4, pH 6.1 (broken line).

temperature, counter ions for ion-pair techniques; and stationary phases in columns from several suppliers.

When the reversed-phase column was used, elution with water alone as the mobile phase gave a fairly good separation; the use of buffers and organic solvents shortened the retention times of both D and Ψ compared with those obtained using water alone. However, the use of a single column did not provide a sufficient separation of the two nucleosides.

Finally, the use of two Capcell Pak C_{18} columns in series gave an adequate separation for the quantitative analysis of urinary D and Ψ. Fig. 5 shows a chromatographic separation of D (retention time 14.4 min) and Ψ (retention time 15.7 min) in a nucleoside sample from 40 μl of pooled normal human urine, and a chromatogram of a nucleoside sample co-injected with an authentic sample of D. HPLC detection at 230 nm showed equal peak heights (or areas) for authentic samples of D and Ψ prepared at the same concentrations.

The recovery of authentic D using boronate gel column extraction of an aqueous solution of D was found to be 100%. After repeated analyses of standard solutions of D and Ψ with several known concentrations, a calibration graph was obtained to determine the levels of urinary D and Ψ using an external standard method. Both plots resulted in straight lines passing through the origin for samples concentrations up to 10 nmol.

Urinary D and Ψ levels of cancer patients

Fig. 6 shows a chromatogram of D and Ψ in nucleoside samples from urines collected before and after a surgical operation on a patient with malignant gastric cancer. In the analysis, the creatinine level (Jaffe method) in 20 μl of pre-operative

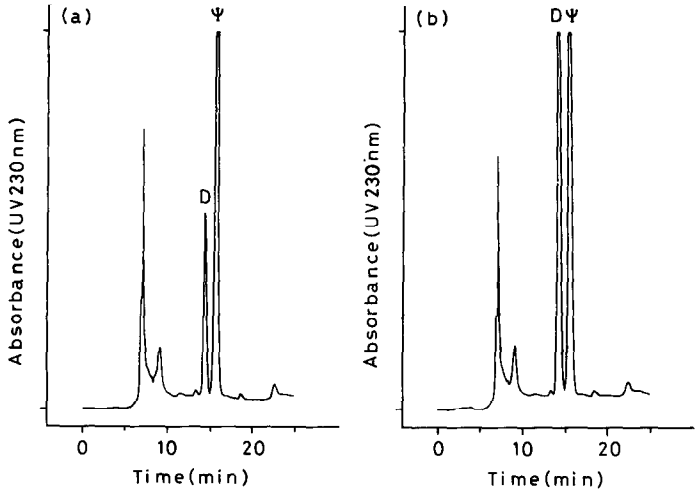

Fig. 5. HPLC separation of 5,6-dihydrouridine and pseudouridine in a nucleoside sample from pooled normal human urine (a) and a nucleoside sample co-injected with 4.06 nmol of authentic 5,6-dihydrouridine (b). Injection volume: 10 μl of nucleoside sample, corresponding to nucleoside present in 40 μl of original urine. UV detection at 230 nm (0.04 a.u.f.s.). Column conditions and elution gradients are given under Experimental.

sample injected (corresponding to 80 μl of original urine) was the same as that in the pooled normal human urine shown in Fig. 5, but that in the post-operative sample was about two-thirds of that level.

The levels of D and Ψ in urines collected before and after surgery from four

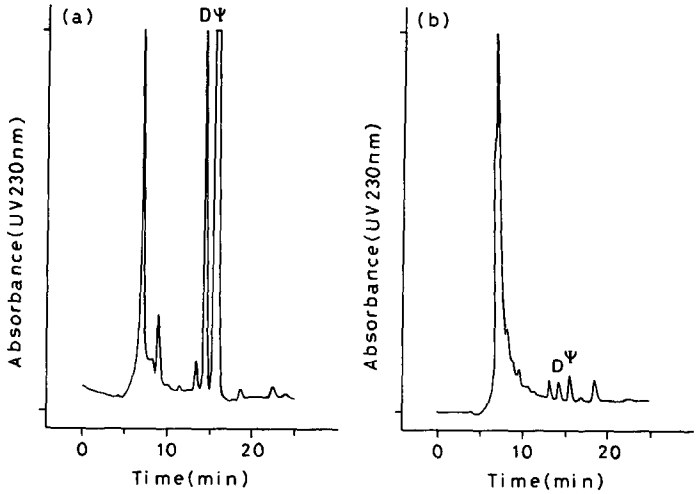

Fig. 6. HPLC separation of 5,6-dihydrouridine and pseudouridine in a nucleoside sample from urines collected before (a) and after (b) surgical procedures on a patient with malignant gastric cancer. Injection volume: 20 μl, corresponding to nucleoside present in 80 μl of original urine. UV detection at 230 nm (0.04 a.u.f.s.). Column conditions and elution gradients are given under Experimental.

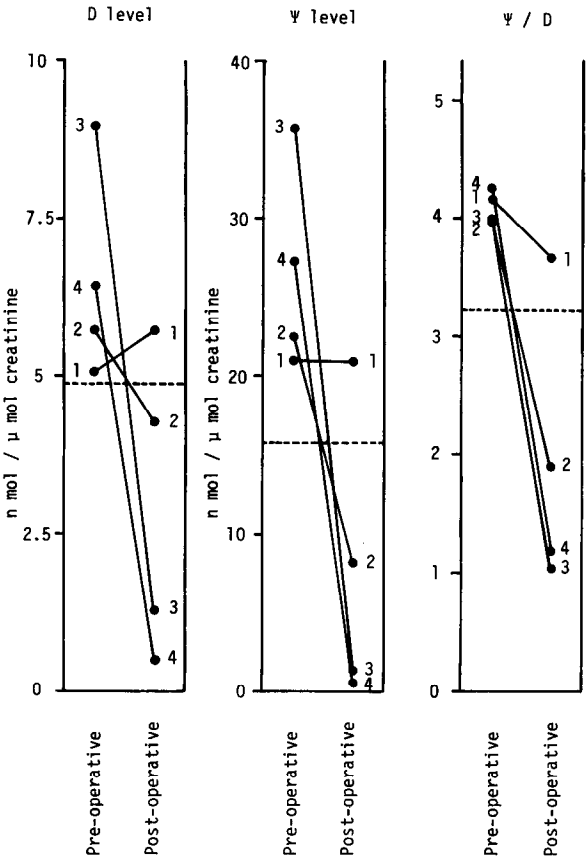

Fig. 7. Levels of urinary 5,6-dihydrouridine (D) and pseudouridine (Ψ) and Ψ/D molar ratio in four pre- and post-operative patients with gastrointestinal cancer. The broken lines indicate data from pooled normal human urine. The numbers 1–4 correspond to the case numbers given under Experimental.

patients with malignant gastrointestinal cancer were determined by the RP-HPLC method described above, and then normalized by the urinary creatinine level of each urine sample. Fig. 7 shows a comparison of normalized urinary D and Ψ levels and the Ψ/D ratio in the pre- and post-operative cancer patients. Compared with our previous report[11], a large decrease in both nucleoside levels in urines after surgery was observed in three of the cancer patients (Nos. 2–4 in Fig. 7) in a comparatively advanced stage. The other cancer case (No. 1 in Fig. 7), exhibiting no significant change in urinary D and Ψ levels, was diagnosed as a early stage of sigmoid colon cancer. These results seem to indicate a direct relationship between the change in urinary D and Ψ levels before and after surgical operation and the cancer burden or the turnover rate of cancer cells.

In this experiment, the rates of change of D levels before and after surgery appear to be almost the same as those of the Ψ levels. The decrease in the Ψ/D molar ratio after surgery compared with that before surgery, as seen in Fig. 7, suggests that tRNAs in cancer cells have a higher molar content of Ψ than D.

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