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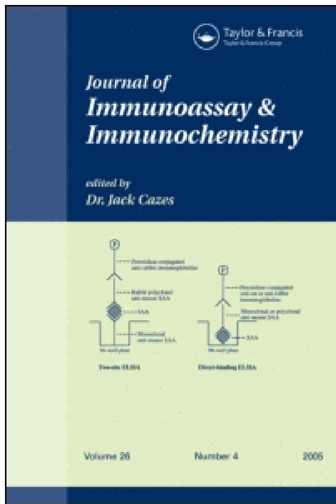

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MONOCLONAL ANTIBODY TO PSEUDOURIDINE USED TO DEVELOP A RADIOIMMUNOASSAY

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ABSTRACT

Pseudouridine, a component of tRNA, was modified to yield the derivatives: succinyl, palmitoyl pseudouridine, and protein conjugates. These derivatives were used in preparation of monoclonal antibodies specifically directed to pseudouridine. MAbs from three hybridomas (OAL 881,812 and 814) were established and shown to be directed to pseudouridine, uridine and uracil. OAL 881 was equally reactive with the three substrates, OAL 812 showed the same reactivity for pseudouridine and uracil, while OAL 814 showed a reactivity mainly for pseudouridine. MAb OAL 814 was used in a radioimmunoassay(RIA) system unique for pseudouridine. A good dose-response curve was observed in the range between 31.3 and 2000 nmole/ml. Intra- and inter-assay CV values were below 4.1 % and 7.4 % respectively, and good results were obtained from recovery of added material and dilution tests. The ratio of pseudouridine/creatinin in urine was sigificantly higher in patients with cancer than in normal subjects.

KEY WORDS: Pseudouridine, RIA, Monoclonal Antibody

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INTRODUCTION

Modified ribonucleosides, derived primarily from transfer ribonucleic acid (tRNA), are excreted in abnormal amounts in the urine of cancer patients(1-2). These excretion products include pseudouridine and methylated nucleosides(3). Interest in these substances as potential tumor markers(4) was stimulated by reports that tRNA produced by certain cancer tissues had increased activity when compared to that from the corresponding normal tissues. Borek et al(5) showed that tRNA from cancer tissue had a much higher turnover rate than that from the corresponding normal tissue, and that pseudouridine is not catabolized further but excreted in urine as the intact molecule.

Some of the present authors(6,7) have recently reported an analytical method for urinary nucleosides using reversed-phase high performance liquid chromatography following concentration by means of boronated gel(8). The level of modified nucleosides in urine is an important clinical marker for cancer diagnosis (9,10)

In the present study, we prepared derivatives of pseudouridine, established specific MAbs against these derivatives, and developed an RIA system for pseudouridine based on one(OAL 814) of these MAbs.

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MATERIALS AND METHODS

Materials used were: pseudouridine, and bovine lipoprotein (Sigma Chemical Co., St.Louis, Mo.USA), uridine, uracil, other nucleosides, anhydride succinate and ovalbumin (WAKO PURE Chemical Co., Osaka, Japan), peroxidase-goat anti mouse IgG + IgA + IgM (H+L), Iodogen (Pierce Chemical Co., Rockford, IL, USA) and water soluble carbodiimide (The Peptide Institute, Inc., Osaka, Japan). NMR was by Brucker WH-400 NMR Spectrometer (Rheinstettn W-Germary), and infrared spectroscopy by Nippon Bunko Industries (Tokyo, Japan). Urine samples were collected for 24 hours and stored at -20 ℃ until analysis.

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Modification of pseudouridine

Pseudouridine (1 mM) was dissolved in 5 ml pyridine, 1.5 mM anhydride succinate was added to this solution, and the reaction mixture kept overnight at room temperature. The reactant was concentrated under reduced pressure and purified on HPLC with DEAE-5PW (TOSOH Co., Osaka, Japan) on a linear gradient from 10 mM (pH 4.5) to 0.5 M ammonium acetate (pH 6.8). Pseudouridine (1 mM) was dissolved in 5 ml pyridine containing 2 mM palmitoyl chloride. After standing overnight, the reactant was successively precipitated with excess diethylether, filtered, washed with diethylether, dissolved in hot ethanol,

recrystallized at 4 ℃, filtered, again washed with diethylether, and dried under reduced pressure.

BSA (1 μM) and pseudouridine succinate (20 μM) were dissolved in 5 ml of 50 mM PBS and supplemented with 100 μM water soluble carbodiimide. After standing for several hours, the reaction mixture was dialyzed extensively against distilled water and lyophilized.

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Immunization of Balb/c mice and preparation of hybridomas

Three groups of 5 week old male Balb/c mice were immunized subcutaneously with 10 μg palmitoyl pseudouridine emulsified in complete Freund's adjuvant with 5 % methyl BSA(11). Second and third immunizations were given at 2 weeks intervals. Spleen cells were prepared 3 days after the third immunization, fused with P3U1 mouse myeloma cells(10 spleen cells per myeloma cell) in the presence of 35% polyethylene glycol-1500, and plated in 24 -well tissue culture plates($1-2 \times 10^6$ cells/well). Cell growth was followed by microscopic examination and culture supernatants were collected daily for screening of antibody production.

Screening of monoclonal antibody

Pseudouridinyl BSA was dissolved in 0.1M sodium bicarbonate at 5μg/ml, and 100 μl aliquots of solution were dispensed into

96 wells and stood overnight. Each well was washed twice with distilled water, blocked with 50 mM PBS with 0.2 % gelatin, and stored at 4 ℃.

The culture fluids were each tested against a well coated with pseudouridinyl BSA and a well containing 10 ug pseudouridine as an inhibitor. Each well was incubated for 2 hours at room temperature, washed twice with distilled water, supplemented with 100 μl of peroxidase-goat antimouse IgG + IgA + IgM (H+L), stood for 1 hour, and then each washed twice with distilled water. Peroxidase conjugate reacting with the well was colored by a solution of 0.25 % o-phenylenediamine / 0.05% hydrogen peroxide.

The reaction was stopped by 1 M sulfonic acid, and the absorbance of the reaction mixture was measured at 490 nm (Titertek Multiskan MCC, Abbott, Chicago Co. USA). MAbs from three hybridomas were established (OAL 881, 812, 814), and their subclass determined by Ouchterlony's method.

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Radiolabelling of monoclonal antibody (OAL 814)

Fifty μg MAb dissolved in 200 μl of 0.1 M sodium borate, pH 8.2, containing 1 mCi of $Na^{125}I$ was transferred to a glass tube coated with 40 μg of Iodogen and stood for 5 min at 0 ℃. The iodinated product was purified by gel chromatography on a column (1.0 x 30 cm) of Sephacryl S-300 developed with 50 mM PBS

containing 0.1 % gelatin and 0.02 % NaN_3. The specific radioactivity of iodinated MAb was about 5 μCi/μg protein.

Assay procedure for urinary pseudouridine

One thousand polystyrene beads (6.3 mm diameter) were soaked in 200 m of 0.1M sodium bicarbonate buffer (containing 10μg/ml of pseudouridinyl BSA) overnight at 4℃. The buffer was drained off and the beads were soaked in 50 mM PBS with 0.2 % gelatin to block the surface not covered, and stored at 4 ℃. 25 μl of sample and a bead were added to the test tube, supplemented with 200 μl of ^{125}I OAL 814 (ca 50000cpm), and stood overnight at 4 ℃. The bead was washed 3 times with distilled water and its radioactivity was measured in an auto γ-counter.

Procedure for urinary pseudouridine by HPLC

Determination of urinary pseudouridine was performed according to the method of Amuro et al(7). The analytical system was a combined HPLC system with an affinity and reversed-phase column and both columns were equilibrated with 0.1 mole/l sodium-potassium phosphate buffer(pH 8.7). The chromatography was initiated by injecting the sample into the affinity column of Boronate 5PW (7.5 mm x 7.5cm,TOSOH), and the elute containing pseudouridine from the affinity column was separated on the

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reversed-phase column of Octadecyl 4PW (4.6 mm x 15 cm,TOSOH) and was detected by UV spectrophotometry.

RESULTS AND DISCUSSION

The mixture of pseudouridine succinate were separated and purified with DEAE 5PW column (TOSOH Co., Tokyo, Japan) on a linear gradient from 10mM (pH 4.5) to 0.5 M ammonium acetate (pH 6.8). Four major fractions were obtained and identified by chemical analysis: (1) non-react-ed; (2) 5'-mono-succinyl derivatives; (3) 2'-and 3'-mono-succinyl derivatives; (4) 2'-, 3'-, and 5'-di-succinyl derivatives. The yields of the 4 fractions were 19.0 %, 21.2 % , 29.9 % , and 29.9% respectively. Infrared spectra of all succinate derivatives showed an absorbance peak at 1750 cm^{-1} due to the carboxyl ester group. NMR analysis showed chemical shifts attributable to methylene protons on succinyl groups: 5'-CH2OSuc at 4.21 and 4.31 (d,d 2H H-5'), 2'-CHOSuc at 5.23 (t 1H H-2'), and 3'-CHOSuc at 5.08 (t 1H H-2')ppm. Methylene on succinate caused shift at 2.38 and 2.56 (t 4H) ppm. These NMR and IR finding were attributed to succinylation of pseudouridine. Palmitoyl pseudouridine(yield 86 %) was identified chemically as follows. In infrared analysis, the absorbance peaks appearing at 1750 and 2900 cm^{-1} were due to carboxyl ester and alkyl groups; in NMR analysis, shifts due to

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TABLE 1

Cross reactivity of three MAbs with various inhibitors

	Inhibition (%)			
MAbs	Pseudouridine	Uracil	Uridine	other inhibitor
OAL 881	100	120	55	<5
OAL 812	100	50	25	<5
OAL 814	100	20-30	<5	<5

Inhibition assay was performed as described in "Materials and Methods". The inhibitors used were pseudouridine, uracil, uridine, adenosine, guanosine, cytosine, c-AMP, and ribose.

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methylene of the 5'-hydroxyl group appeared at 4.35 and 4.24 (d,d 2H H-5') ppm, and those due to alkyl of palmitoyl group at 0.88 (t 3H CH_3), 1.26 and 1.66 (m 24H CH_2), and 2.33 (t2H CH_2O-) ppm.

Conclusively, these data suggest that pseudouridine was palmitoylated and the palmitoyl group was attached only to the 5' -hydroxyl residue of ribose. Palmitoyl pseudouridine has a melting point of 169-171 ℃. Pseudouridinyl BSA was obtained with good yields (> 80 %), and identified from U.V. spectra (absorbance peak at 254nm) as belonging to the pyrimidine group. It was estimated that in pseudouridinyl BSA 15 μg pseudouridine residue was combined with 1 mg BSA.

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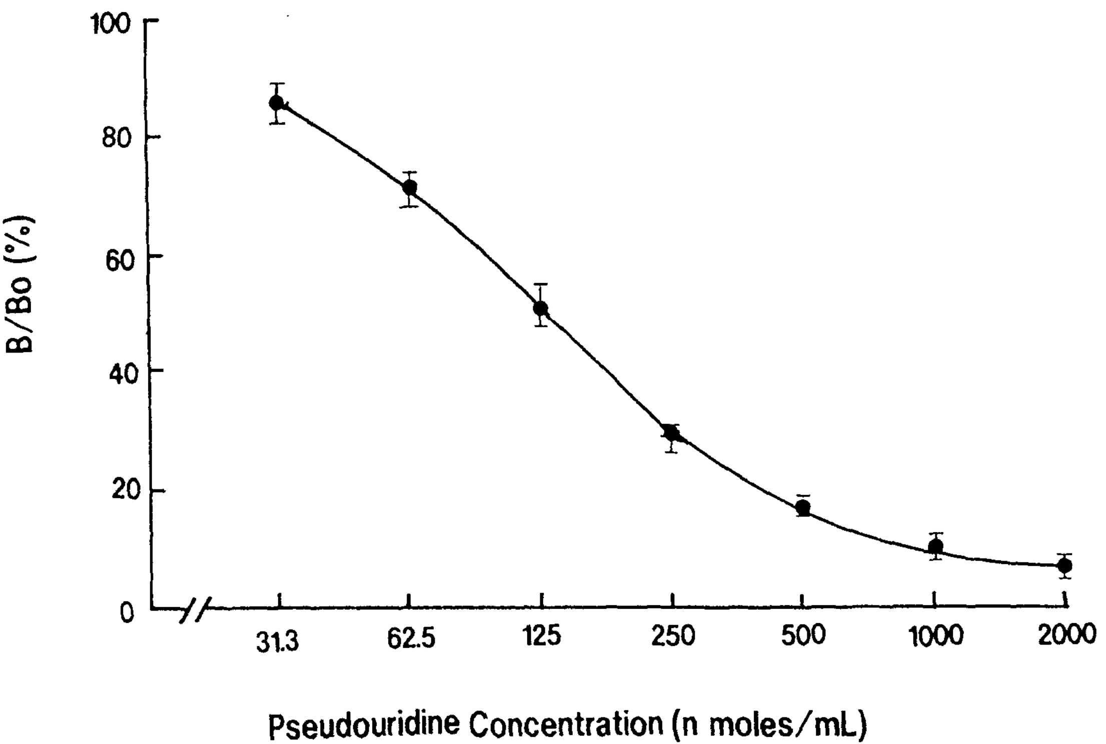

FIGURE 1 Standard curve of RIA for pseudouridine using OAL 814 Each point represents mean value of triplicate determination.

TABLE 2

Inner and inter assay of urinary pseudouridine by RIA

Inner	Sample	Mean	SD	CV %
n=6	A	190[a]	6.7	3.5
	B	289	7.7	2.6
	C	683	42	6.1
Inter n=5[b]	A	185	9.9	5.3
	B	256	25	9.8
	C	687	48	7.0

[a] nmole/mL
[b] Sample n=6, 5 times trial

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The three MAbs from hybridomas OAL 881, 812, and 814, were characterized as subclass IgG_1 by Ouchterlony's method. Specifity of the MAbs was determined by RIA inhibition assay as described in "Materials and Methods". Inhibitors used were pseudouridine, uracil, uridine, adenosine, guanosine, cytosine, c-AMP and ribose (Table 1). The results show that OAL 881 was reactive with the pyrimidine group, OAL 812 reacted slightly with pyrimidine and c-glycoside, and OAL 814 was specific for pseudouridine.

In previous studies(12,13), immunogens usually consisted of the modified proteins combined with the aldehyde group of ribose following treatment with $NaIO_4$. Because such immunogens include

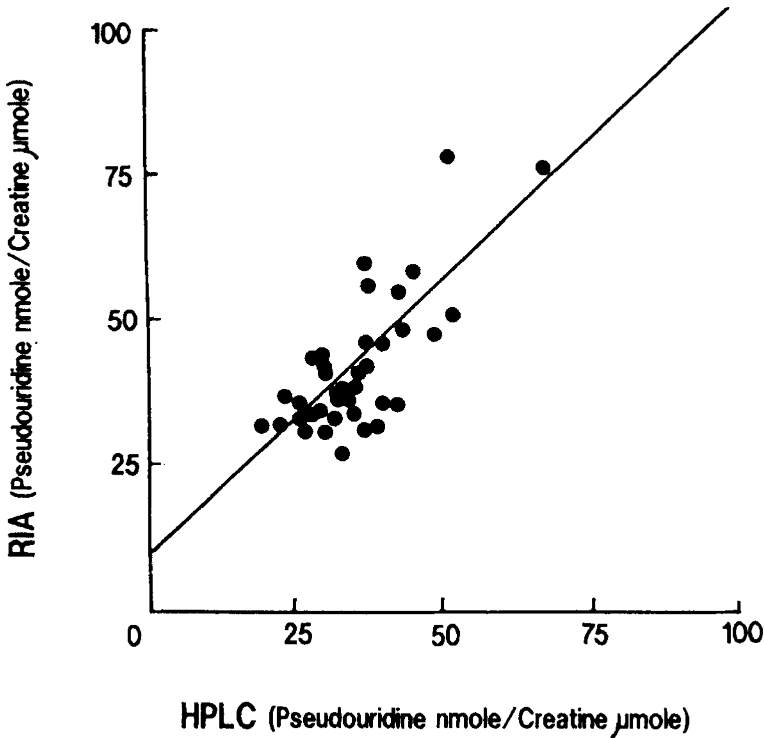

FIGURE 2 Correlation between urinary pseudouridine levels as detected by HPLC and RIA (r=0.764 n=40).

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both the pyrimidine group and the ribose residue, it was difficult to obtain MAbs specific for pseudouridine.

In our first experiment, pseudouridinyl BSA having the succinyl group as a spacer was used as immunogen in rabbits and mice. All antibodies obtained by this method were directed to the succinyl group spacer, but not to the pseudouridine residue.

TABLE 3

Analytical recovery of urinary pseudouridine by RIA

Sample	Inital conc. of PU[c] in urine, nmole/mL	Increase in PU concn.[a] nmole/mL			
		63	125	250	500
1	140	102.4[b]	87.2	101.6	97.4
2	183	91.2	92.0	112.8	102.8
3	289	91.2	86.4	106.4	106.2
4	391	107.2	84.8	107.6	107.2

[a] A known increment was made by addition of authentic PU
[b] Mean values, n=3 each, for percentage recoveries.

$$\text{Percentage recovery (\%)} = \frac{\text{(Found conc.)} - \text{(Inital conc.)}}{\text{(Added conc.)}} \times 100$$

[c] PU:Pseudouridine

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Next, pseudouridine which was palmitoylated instead of having a succinyl spacer was prepared, emulsified in complete Freund's adjuvant with 5% methyl BSA, and used as immunogen in mice. Using this method, three MAbs were obtained. The immunogenic reactivity of the succinyl spacer was thus eliminated and palmitoylated pseudouridine functioned as the immunogen.

MAb OAL 814 was purified with Affi-Gel Protein A (Bio-Rad Laboratories, Richmond, USA), and labelling by Iodogen Method. The specific activity of iodinated MAb was about 5 μCi/μg protein. ^{125}I MAb OAL 814 was used for development of a pseudouridine specific RIA. Fig. 1 shows the standard curve of

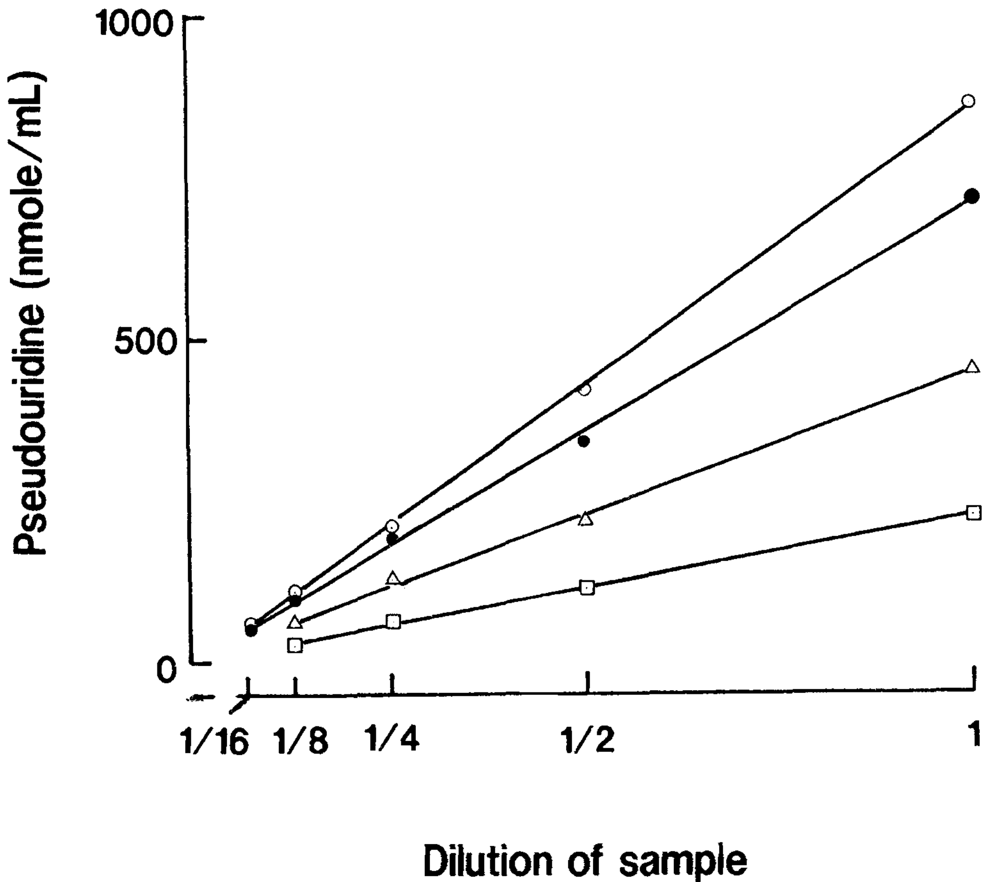

FIGURE 3 Dilution test of urinary pseudouridine of patients specimens. Each point represents mean value of triplicate determination.

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RIA for pseudouridine using MAb OAL 814. An acceptable dose-response curve was obtained in RIA system. A good dose-response curve was observed in the range between 31.3 and 2000 nmole/ml. Intra- and inter-assay coefficient of variation values were under 4.1 % and 7.4 % respectively (Table 2).

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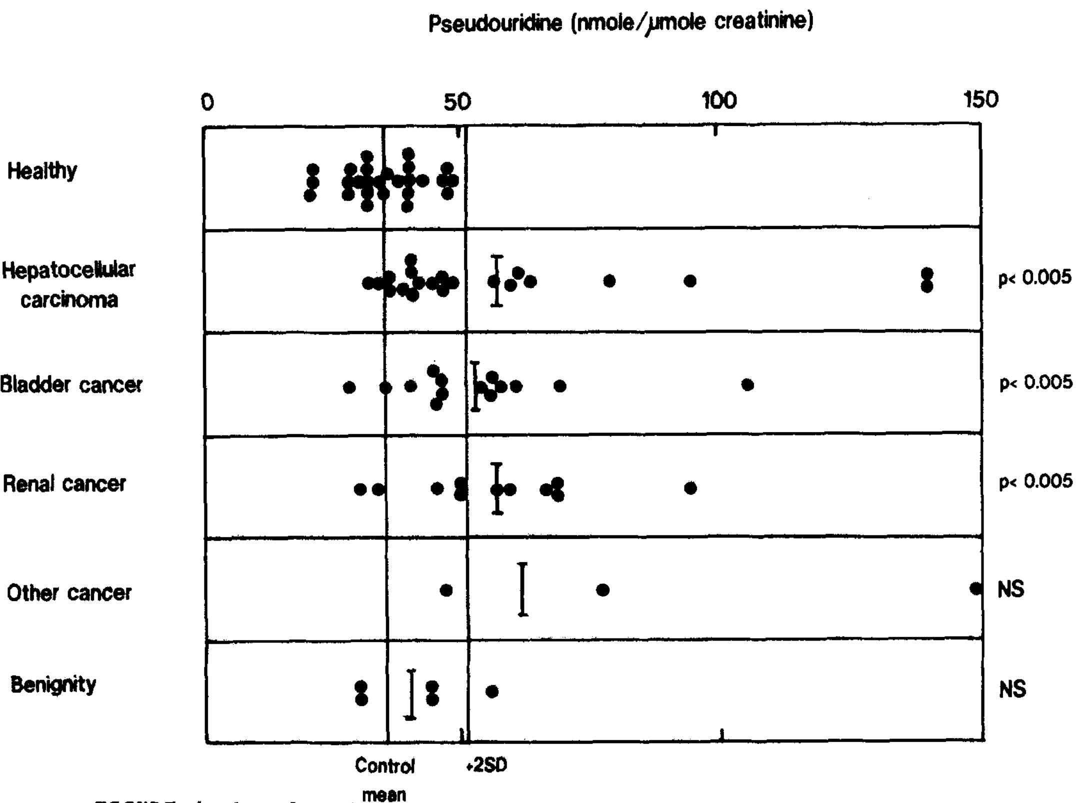

FIGURE 4 Levels of pseudouridine in urine from healthy donors and cancer patients. "Other cancer" denotesd cytoadenocarinoma and two prostatic adenoma. *p* values obtained from *t* tests are shown to the right of the plot. NS: not significant.

There was a good correlation in urinary pseudouridine levels measured by HPLC and RIA systems (r=0.764 n=40) (Fig.2). We performed analytical-recovery studies at four concentrations of pseudouridine in four urine samples. The results were good (mean 99.3 %) and acceptable for clinincal application(Table 3). Good results were also obtained in the dilution test (Fig. 3).

The amounts of urinary pseudouridine in 26 healthy donors was 34.8 ± 8.0 nmole/mol creatinine. The cut off value was set at the mean value for the healthy donors plus 2 standard deviation (50.8 nmole/mole creatinine).

Next, urinary levels of pseudouridine in cancer patients are shown in Fig. 4. Significantly elevated levels of pseudouridine were detected in patients with hepatocellularcarcinoma, bladder cancer, and renal cancer.

The direct RIA system for urinary pseudouridine developed in this study would have potential application in cancer diagnosis.

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FOOTNOTES

Abbreviations: BSA, bovine serum albumin; RIA, radioimmunoassay; NMR, nuclear magnetic resonance spectroscopy; MAb, monoclonal antibody; PBS, sodium phosphate buffered saline(pH 7.4).

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