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Journal of Chromatography B, 724 (1999) 221–230

JOURNAL OF CHROMATOGRAPHY B

Highly sensitive determination of *N*-terminal prolyl dipeptides, proline and hydroxyproline in urine by high-performance liquid chromatography using a new fluorescent labelling reagent, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride

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Received 14 September 1998; received in revised form 23 December 1998; accepted 24 December 1998

Abstract

A highly sensitive pre-column HPLC method for simultaneous determination of prolyl dipeptides, Pro and Hyp in urine was developed. The analytes were labelled with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride at 70°C for 20 min. The derivatives separated on tandem reversed-phase columns by a gradient elution and were monitored with fluorescence detection at 318 nm (excitation) and 392 nm (emission). The detection limits for prolyl dipeptides, Pro and Hyp were 1–5 fmol/injection ($S/N=3$). Urine samples were treated with *o*-phthalaldehyde, followed by purification on a Bond Elut C_{18} column before conducting the labelling reaction. Pro–Hyp, Pro–Gly and Pro–Pro were identified as prolyl dipeptides in urine. The within-day and between-day relative standard deviations were 1.5–4.8 and 1.7–5.8%, respectively. The concentrations of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp in normal human urine were 97.6±28.2, 2.74±1.48, 2.08±1.13, 6.71±3.34 and 2.30±1.59 nmol/mg creatinine, respectively.

Keywords: Prolyl dipeptides; Proline; Hydroxyproline; 4-(5,6-Dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride

1. Introduction

Proline (Pro) and hydroxyproline (Hyp) formed by hydroxylation of Pro during collagen biosynthesis are commonly found in connective tissue proteins such as collagen at high concentrations. These amino acids are released after degradation of collagen and excreted into urine as amino acids, small peptides and large peptides. According to previous reports [1–4], total Hyp excreted in urine is comprised of ~85% small peptides and 1–5% free Hyp. The main peptide containing Hyp was prolylhydroxyproline (Pro–Hyp) which accounted for about 60% of peptide Hyp or 44% of total urinary Hyp [2,4]. The concentration of total urinary Pro and Hyp varied in association with various diseases including bone diseases [1,5–7] and tumor [8–10] and excretion of dipeptides containing Pro or Hyp increases in patients with disorders involving collagen metabolism

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00009-2

[11–13]. Therefore, sensitive and reliable determinations of these metabolites in human urine are useful for understanding of various disorders.

Currently, a few methods for the determination of dipeptides that contain Pro or Hyp in urine by gas chromatograpgy–mass spectrometry (GC–MS) [11–14], liquid chromatography (LC)–MS [15–17] and high-performance liquid chromatography (HPLC) [18] have been reported. However, the GC–MS and LC–MS methods require tedious procedures including a two-step column separation for pre-treatment of urine and further, the GC–MS methods require a two-step derivatization procedure to convert dipeptides into volatile derivatives after pre-treatment. In the HPLC method employing a pre-column derivatization technique with 4-chloro-7-nitrobenzofurazan as a fluorescent labelling reagent, a relatively large sample volume (5 ml) is required because of low sensitivity to prolyl dipeptides.

Recently, we developed an extremely sensitive fluorescent labelling reagent, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl), which reacts quantitatively with amino acids to form fluorescent sulfonamides (maximum wavelength: 318 nm for excitation and 392 nm for emission), and applied the reagent for determination of urinary free Hyp by a pre-column HPLC method [19].

In the present study, we found DMS-Cl reacted with prolyl dipeptides to give fluorescent derivatives. In addition, a highly sensitive HPLC method for simultaneous determination of prolyl dipeptides, Pro and Hyp in urine by fluorescence detection after pre-column derivatization with DMS-Cl was established.

2. Experimental

2.1. Chemicals and solvents

All chemicals were of analytical reagent grade, unless stated otherwise. DMS-Cl was prepared as described previously [19]. *o*-Phthalaldehyde (OPA), acetonitrile (HPLC-grade) and Creatinine Test Wako were obtained from Wako Pure Chemicals (Osaka, Japan). Pro–Asp, Pro–Gln, Pro–Glu, Pro–Leu, Pro–Pro and Pro–Ser were purchased from Kokusan Chemical Works (Tokyo, Japan) and Pro–Ala, Pro–Asn, Pro–Gly, Pro–Hyp, Pro–Ile, Pro–Met, Pro–Phe, Pro–Trp, Pro–Tyr, Pro–Val and 3,4-dehydro-DL-proline (internal standard, I.S.) were from Sigma (St. Louis, MO, USA). L-Amino acids except Asn, Hyp and Ser (Nacalai Tesque, Kyoto, Japan) were purchased from Kyowa Hakko (Tokyo, Japan). Organic solvents except acetonitrile were of highest purity available and used as received. Bond Elut C_{18} (100 mg/1 ml, Varian, CA, USA) was conditioned with 2 ml of methanol followed by 2 ml of water and 1 ml of Na_2HPO_4 (50 m*M*) prior to use.

2.2. Instrumental conditions

The HPLC system consisted of two LC-10AD HPLC pumps (Shimadzu, Kyoto, Japan), a CTO-10AC column oven (Shimadzu), a DGU-14A on-line degasser (Shimadzu), an SIL-10AXL auto injector (Shimadzu), an L-7480 fluorescence detector (Hitachi, Tokyo, Japan) and a CLASS-LC10 LC work-station (Shimadzu) with a CBM-10A communications bus module (Shimadzu). A TSK-guard gel ODS-80T_s (15×3.2 mm I.D., Tosoh, Tokyo, Japan) and tandem Nova Pak C_{18} columns (150×3.9 mm I.D., 4 μm, Waters, Milford, MA, USA) were used at 55°C with a gradient system of (A) aqueous acetic acid (10 m*M*)–(B) acetonitrile–aqueous acetic acid (50 m*M*) (8:2, v/v). The elution program consisted of an isocratic elution of 25% B for 20 min, followed by a linear gradient elution from 25 to 55% of B for 30 min, and finally a stepwise decrease to 25% of B to re-equilibrate the column for 10 min. The flow-rate was 1 ml/min. The fluorescence intensities were monitored at excitation and emission wavelengths of 318 nm and 392 nm, respectively.

2.3. Derivatization procedure

To a test solution (20 μl) of prolyl dipeptides, Pro, Hyp and the I.S. were placed in a screw-capped glass vial, Na_2HPO_4 (50 m*M*, 280 μl) and DMS-Cl (2 m*M* in acetone, 500 μl) were successively added and mixed. The mixture was allowed to stand for 20 min at 70°C. After cooling, the reaction mixture was mixed with dichloromethane (0.8 ml) and Na_2CO_3 (0.1 *M*, 30 μl) and then centrifuged (500 *g*) for 10

min. An aliquot (10 μl) of the aqueous layer was subjected to HPLC.

2.4. Procedure for determination of urinary prolyl dipeptides, Pro and Hyp

2.4.1. Method A (recommended method): solid-phase extraction method

To human urine (25 μl) were added I.S. (10 μ*M*, 25 μl), Na_2HPO_4 (50 m*M*, 150 μl) and OPA [4%, w/v, in acetonitrile–Na_2HPO_4 (50 m*M*) (1:1, v/v), 50 μl]. After standing for 3 min, the OPA-treated mixture (200 μl) was passed through a Bond Elut C_{18} column and then the column was washed with acetonitrile–Na_2HPO_4 (50 m*M*) (1:9, v/v, 800 μl). The effluent with the OPA-treated mixture and the washings were mixed. The mixture (300 μl) was reacted with DMS-Cl (2 m*M* in acetone, 500 μl) at 70°C for 20 min. The reaction mixture was mixed with dichloromethane (0.8 ml) and Na_2CO_3 (0.1 *M*, 30 μl) and then centrifuged (500 *g*) for 10 min. The aqueous layer (10 μl) was subjected to HPLC. The Bond Elut C_{18} column was used at least 20 times by washing twice sequentially with methanol and water (2 ml each) after each use.

2.4.2. Method B: solvent extraction-stripping method

To human urine (20 μl) were added I.S. (10 μ*M*, 20 μl), Na_2HPO_4 (50 m*M*, 260 μl) and OPA (4%, w/v, in acetone, 50 μl). After standing for 3 min, DMS-Cl (2 m*M* in acetone, 450 μl) was successively added and mixed. The mixture was allowed to stand for 20 min at 70°C. After cooling, the reaction mixture was acidified with HCl (5 *M*, 50 μl), saturated with NaCl (ca. 150 mg) and then extracted with dichloromethane (1 ml). The organic layer (1 ml) was mixed with Na_2CO_3 (10 m*M*, 300 μl) and then centrifuged (500 *g*) for 10 min. The aqueous layer (10 μl) was subjected to HPLC.

3. Results and discussion

3.1. Chromatographic separation

DMS-Cl reacted with prolyl dipeptides in a basic medium to give the corresponding fluorescent derivatives (Fig. 1). The separation of the derivatives of prolyl dipeptides (16 species), Pro, Hyp and I.S. labelled with DMS-Cl were examined by use of tandem reversed-phase columns and gradient elution. A typical chromatogram of the reaction mixture of a standard solution is shown in Fig. 2. The peaks due to 12 species of prolyl dipeptides (retention time: Pro–Asn, 14.8; –Gln, 16.2; –Hyp, 17.1; –Glu, 23.9; –Gly, 27.0; –Ala, 33.8; –Pro, 35.0; –Tyr, 37.7; –Met, 42.1; –Val, 43.7; –Trp, 47.3; –Phe, 49.1 min),

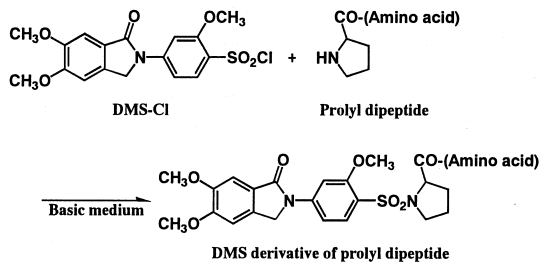

Fig. 1. Reaction of prolyl dipeptide with DMS-Cl.

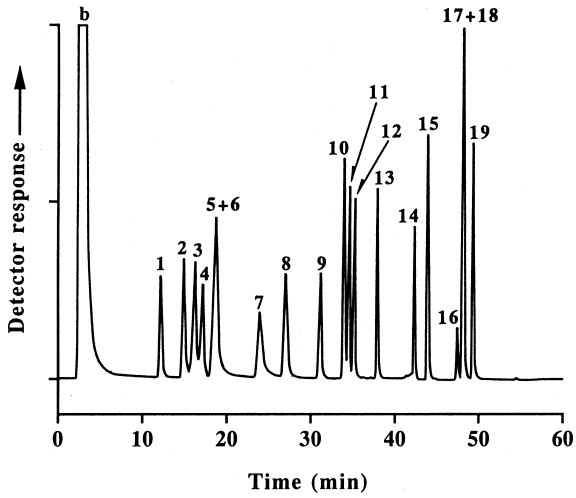

Fig. 2. Chromatogram obtained from a standard solution of prolyl dipeptides, Pro, Hyp and I.S. (10 μ*M*, each) labelled with DMS-Cl according to the derivatization procedure described in Section 2.3. Peaks: 1=Hyp; 2=Pro–Asn; 3=Pro–Gln; 4=Pro–Hyp; 5=Pro–Asp; 6=Pro–Ser; 7=Pro–Glu; 8=Pro–Gly; 9=I.S.; 10=Pro–Ala; 11=Pro; 12=Pro–Pro; 13=Pro–Tyr; 14=Pro–Met; 15=Pro–Val; 16=Pro–Trp; 17=Pro–Ile; 18=Pro–Leu; 19=Pro–Phe; b=Reagent blank. HPLC conditions as in Section 2.2.

Pro (34.4 min), Hyp (12.2 min) and I.S. (31.0 min) were separate from each other and the reagent blank components, although the peaks due to Pro–Asp (18.6 min) and Pro–Ser (18.7 min) and those due to Pro–Ile (47.9 min) and Pro–Leu (48.0 min) overlapped.

As the chromatographic separation of DMS derivatives of prolyl dipeptides, Pro, Hyp and I.S. was affected by the pH in the mobile phase, the effect of the pH was examined using acetate buffer [10 m*M* for mobile phase (A) and 50 m*M* for mobile phase (B), pH 4.0–6.0] instead of acetic acid. As shown in Fig. 3, the retention times of all peaks became shorter with increased pH and the prolyl dipeptide peaks overlapped at pH greater than pH 4.5. The pH of acetic acid (10 m*M*) in mobile phase was about 3.4.

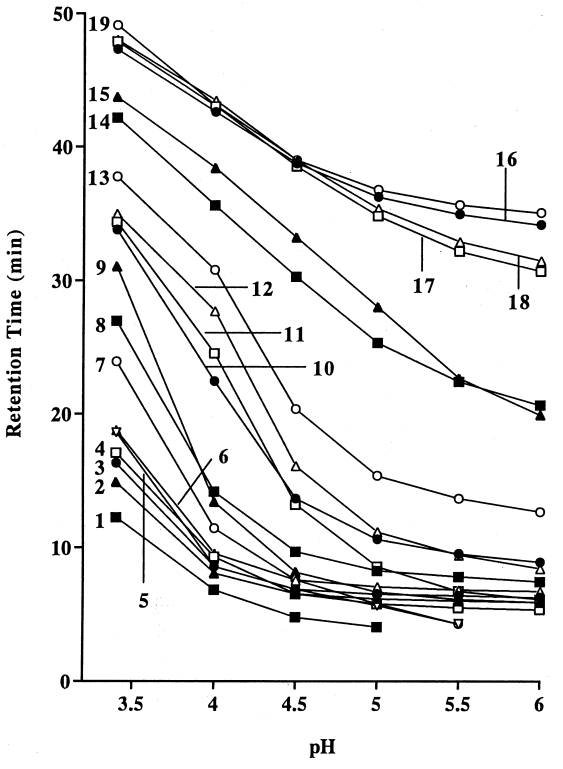

Fig. 3. Effect of pH of acetate buffer in the mobile phase on the retention times of peaks due to prolyl dipeptides, Pro, Hyp and I.S. Legend numbers are as in Fig. 2.

3.2. Labelling reaction conditions

A standard solution of prolyl dipeptides, Pro and Hyp (10 μ*M* each) was used to determine the optimum labelling conditions.

The reaction of Hyp with DMS-Cl was complete within 5 min regardless of the temperature (labelling yield: 99.7%) [19]. However, the labelling reactions of prolyl dipeptides with DMS-Cl were incomplete under the above conditions, while the reactions of Pro and I.S. with DMS-Cl were similar to that of Hyp. Therefore, the effect of the reaction time on the labelling reaction was examined at various temperatures. The results obtained for Pro–Hyp are shown in Fig. 4. The maximum peak area was obtained from the reactions performed at above 70°C, for 10 min. Similar results were obtained from other prolyl dipeptides. Consequently, subsequent labelling reactions for prolyl dipeptides, Pro, Hyp and I.S. with DMS-Cl were carried out at 70°C for 20 min. The reaction mixture was stable for at least 48 h at room temperature.

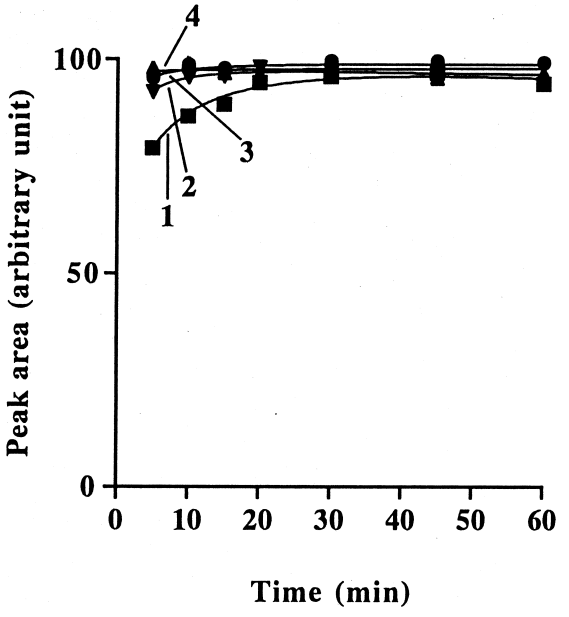

Fig. 4. Effect of reaction time and temperature on labelling reaction of Pro–Hyp with DMS-Cl. Curves: 1=25°C; 2=50°C; 3=70°C; 4=100°C.

As the labelling reaction of prolyl dipeptides, Pro, Hyp and I.S. with DMS-Cl proceeded in a basic medium, the effect of the pH of phosphate buffer (50 m*M*) on the labelling reaction was examined. The results for Pro–Hyp, Pro–Gly, Pro–Pro and I.S. are shown in Fig. 5. Each peak exhibited a maximum and constant area in the range of pH 8–10. Similar results were obtained from other prolyl dipeptides, Pro and Hyp. The effect of the anionic component of the buffer solution was also examined using acetate, borate and phosphate buffer (50 m*M*, pH 8). The maximum peak areas of prolyl dipeptides were obtained in phosphate buffer (peak area relative to phosphate buffer: acetate buffer, 75–98%; borate buffer, 91–100% except 61% for Pro–Tyr). Therefore, Na_2HPO_4 (50 m*M*, pH ca. 9.3) was adopted. When the effect of the concentration of Na_2HPO_4 (10–100 m*M*) was examined, the maximum and constant peak areas were obtained at a concentration of more than 25 m*M*.

The concentration of DMS-Cl was determined by the peak areas due to prolyl dipeptides, Pro and Hyp (100 μ*M* each). The maximum peak areas were obtained at more than 1.5 m*M* DMS-Cl.

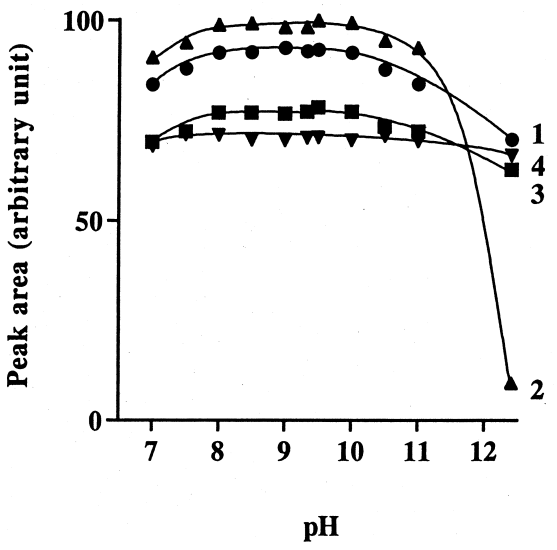

Fig. 5. Effect of pH of phosphate buffer (0.1 *M*) on labelling reaction of Pro–Hyp, Pro–Gly, Pro–Pro and I.S. with DMS-Cl. Curves: 1=Pro–Hyp; 2=Pro–Gly; 3=Pro–Pro; 4=I.S.

Dichloromethane extraction was carried out to remove excess DMS-Cl, which was suspected of causing the guard column to degrade. The extraction was achieved after addition of Na_2CO_3 (0.1 *M*, 30 μl) because parts of the derivatives of prolyl dipeptides that eluted after the derivatives of Pro–Ala on the HPLC column were lost with dichloromethane extraction without Na_2CO_3.

3.3. Linearity and detection limit

Linearity was studied over wide ranges of concentration of prolyl dipeptides, Pro and Hyp (between 0.1 and 100 μ*M* each). The peak-area ratios of analytes to I.S. were linear in the concentration ranges investigated ($r>0.999$ each). When the precision was tested using standard solutions (1, 10 and 100 μ*M* each), the relative standard deviations (RSDs, $n=10$) were less than 4.1%. The detection limits (S/N=3) for prolyl dipeptides were 1–5 fmol/injection.

3.4. Pretreatment of urine and HPLC chromatograms

For determination of urinary prolyl dipeptides, Pro and Hyp by pre-column HPLC, OPA treatment [20] was employed to eliminate primary amino compounds coexisting with analytes in urine. However, the reaction mixture of urine with OPA was colored darkly. Therefore, two methods, method A (solid-phase extraction) and method B (solvent extraction-stripping) described in Section 2.4 were tested to remove the colored products. The colored products were retained on the Bond Elut C_{18} column in method A and were not extracted with dichloromethane at acidic pH in method B.

The typical chromatograms of a standard solution and urine obtained according to the procedures of methods A and B are shown in Fig. 6A and B and Fig. 7A and B, respectively. As shown in Fig. 6A, some prolyldipeptides (Pro–Tyr, –Val, –Met, –Trp, –Ile, –Leu and –Phe) were unable to be measured by method A, because these peptides were retained on the Bond Elut C_{18} column under the conditions used. When urine was analyzed by method A, peaks due to

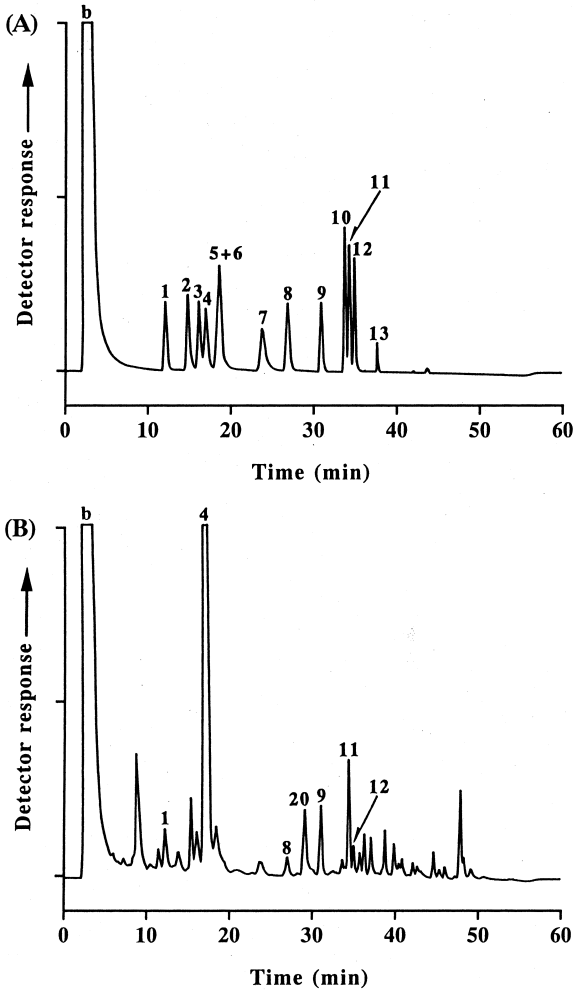

Fig. 6. Chromatograms obtained from (A) a standard solution and (B) a human urine according to method A described in Section 2.4.1. Legend numbers except 20 (sarcosine) as in Fig. 2. Concentration: (A) prolyl dipeptides, Pro and Hyp=10 μ*M* each; (B) Pro–Hyp=179.4 μ*M*; Pro–Gly=2.62 μ*M*; Pro–Pro=1.93 μ*M*; Pro=9.42 μ*M*; Hyp=5.47 μ*M*. HPLC conditions as in Section 2.2.

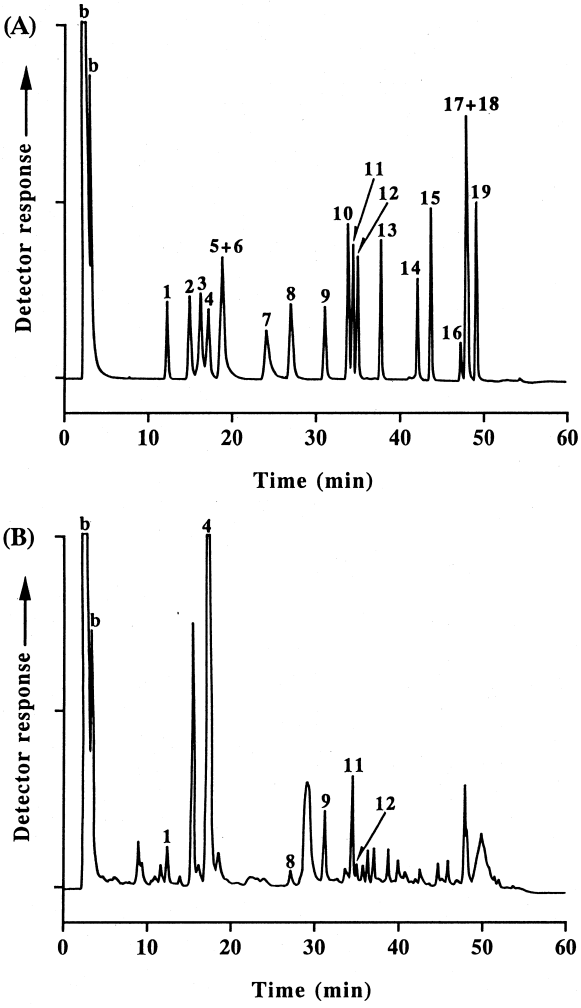

Fig. 7. Chromatograms obtained from (A) a standard solution and (B) a human urine according to method B described in Section 2.4.2. Legend numbers as in Fig. 2. Concentrations of analytes as in Fig. 6. HPLC conditions as in Section 2.2.

Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp at retention times of 17.1, 27.0, 35.0, 34.3 and 12.2 min, respectively, were observed (Fig. 6B). In contrast, for method B, all prolyl dipeptides in a standard solution were detected (Fig. 7A). However, the seven prolyl dipeptides that could not be measured by method A were not recognized in urine (Fig. 7B), although the three prolyl dipeptides mentioned above were recognized in urine. Despite these issues, urinary Pro–Hyp, Pro–Gly, Pro–Pro, Pro, Hyp and I.S. were separated from the reagent blank and other urinary components (Figs. 6B and 7B). These urinary analytes were identified by comparing the retention times with those of standard solutions and also by co-chromatography of a standard solution and urine.

3.5. Recovery and precision

Recovery tests were examined by means of both methods using three urine samples spiked with various amounts of standard Pro–Hyp (urinary concentration: 25, 50, 100, 250 and 500 μ*M*), Pro–Gly, Pro–Pro, Hyp (0.5, 1, 2, 5 and 10 μ*M* each) and Pro (1, 2, 4, 10 and 20 μ*M*). The relationships between the peak-area ratios of analytes to I.S. and the concentrations of analytes were linear. According to method A, the regression equations for standard solutions without urine were $y=0.1300x+0.1183$ ($r=0.9999$) for Pro–Hyp, $y=0.1384x-0.0001$ ($r=0.9999$) for Pro–Gly, $y=0.1069x+0.0020$ ($r=0.9998$) for Pro–Pro, $y=0.1135x+0.0735$ ($r=0.9994$) for Pro and $y=0.1140x+0.0066$ ($r=0.9996$) for Hyp, where y is the peak-area ratio of analyte to I.S. and x is the concentration of analyte (μ*M*). The recoveries were obtained from the slope ratios of regression equations of analytes with/without urine. The slopes of regression equations and the recoveries by methods A and B are shown in Table 1. The recoveries of the analytes except Pro–Gly by method A were almost 100%, while those by method B were very low. In method B, some organic solvents such as chloroform, benzene, ethyl acetate and diethyl ether instead of dichloromethane were also used. However, the maximum peak areas of DMS-derivatives were obtained with use of dichloromethane. In addition, when the concentration of Na_2CO_3 (1, 5, 10 and 20 m*M*) for stripping of the derivatives of prolyl dipeptides was examined, maximum peak areas were obtained by use of more than 5 m*M* Na_2CO_3. These results suggest that method A is appropriate for the determination of urinary prolyl dipeptides. Incidentally, when the recoveries of standard prolyl dipeptides, Pro, Hyp (0.02, 0.2, 2 and 10 nmol each) and I.S. from Bond Elut C_{18} were examined, the recoveries were 96.1–101.1% (except the seven species prolyl dipeptides mentioned above). Therefore, for determination of urinary prolyl dipeptides, Pro and Hyp, method A was employed and the regression equations in water described above were used. However, the estimated values of urinary Pro–Gly were corrected using the mean value (0.866, $n=6$) of recovery derived from additional experiments using another three urine samples (the recoveries: 85.8, 85.9 and 86.8%).

The within-day and between-day precisions of method A were evaluated using three urine samples. The within-day precision was examined with ten replicate assays in each one day and the between-day precision by assays on five different days. As shown in Table 2, RSDs of the within-day and between-day precisions were 1.5–4.8 and 1.7–5.8%, respectively.

3.6. Selection of I.S.

Some secondary amino acids (10 μ*M* each) were examined to select I.S. Although the peaks due to L-azetidine-2-calboxylic acid, L-thioproline, isonipecotic acid, nipecotic acid and DL-pipecolic acid were eluted at 23.0, 34.7, 36.9, 39.9 and 42.0 min, respectively, those peaks overlapped with peaks due to urinary components. The peak due to 3,4-dehydro-DL-proline (31.0 min) was successfully separated from those of urinary components.

3.7. Influence of amino acids

The influence of amino acids on determination of prolyl dipeptides, Pro and Hyp by method A was examined with urine spiked with 25 species of primary amino acids (Ala, Arg, Asp, Asn, Cit, Cys, Cys–Cys, Glu, Gln, Gly, His, Hse, Ile, Leu, Lys, Met, Orn, Phe, Ser, Thr, Trp, Tyr, Val, γ-aminobutyric acid and ε-aminocaproic acid, 5 nmol each). These amino acids did not interfere with the determination of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp.

3.8. Determination of urinary prolyl dipeptides, Pro and Hyp

The concentrations of prolyl dipeptides, Pro and Hyp in urine from ten healthy volunteers (Japanese staff and students in our laboratory) who were eating self-selected diets were measured by method A. Overnight urine was collected in the early morning and stored at −20°C until use. The concentrations of urinary prolyl dipeptides, Pro and Hyp are given in Table 3. The mean values (mean±SD) of urinary

Table 1
The slope of regression equation of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp with and without urine and the recovery

	Urinary concentration[a] (μ*M*)	Method A		Method B	
		Slope[b]	Recovery[c] (mean±SD) (%)	Slope[b]	Recovery[c] (mean±SD) (%)
Pro–Hyp					
–[d]		0.1300		0.1386	
Urine 1	81.8	0.1317	101.3	0.1234	89.0
Urine 2	157.6	0.1325	101.9	0.1153	83.2
Urine 3	430.0	0.1352	104.0	0.1018	73.4
			(102.4±1.4)		(81.9±7.9)
Pro–Gly					
–[d]		0.1384		0.1452	
Urine 1	3.83	0.1288	93.1	0.1265	87.1
Urine 2	3.63	0.1162	84.0	0.0983	67.7
Urine 3	3.00	0.1166	84.2	0.0759	52.3
			(87.1±5.2)		(69.0±17.4)
Pro–Pro					
–[d]		0.1069		0.1115	
Urine 1	1.29	0.1042	97.5	0.0990	88.8
Urine 2	2.17	0.1021	95.5	0.0894	80.2
Urine 3	6.59	0.1085	101.5	0.0785	70.4
			(98.2±3.1)		(79.8±9.2)
Pro					
–[d]		0.1135		0.1191	
Urine 1	6.08	0.1121	98.8	0.1420	119.2
Urine 2	10.70	0.1164	102.6	0.1427	119.8
Urine 3	17.10	0.1192	105.0	0.1332	111.8
			(102.1±3.1)		(116.9±4.5)
Hyp					
–[d]		0.1140		0.1208	
Urine 1	1.14	0.1135	99.6	0.1148	95.0
Urine 2	6.28	0.1110	97.4	0.1123	93.0
Urine 3	3.18	0.1145	100.4	0.0994	82.3
			(99.1±1.6)		(90.1±6.8)

[a] Urinary concentration was determined by method A.
[b] Concentration of standards: Pro–Hyp=25, 50, 100, 250 and 500 μ*M*; Pro–Gly, Pro–Pro and Hyp=0.5, 1, 2, 5 and 10 μ*M*; Pro=1, 2, 4, 10 and 20 μ*M*.
[c] The recovery was obtained from the ratio of a regression equation with urine to that without urine.
[d] Distilled water was used instead of urine.

Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp were 97.6±28.2, 2.74±1.48, 2.08±1.13, 6.71±3.34 and 2.30±1.59 nmol/mg creatinine, respectively. When the concentration of Hyp in hydrolysed urine (total Hyp) was measured according to the method reported previously [20], the concentration ratios (%, mean±SD) of Pro–Hyp and free Hyp to total Hyp were 45.7–64.5% (53.1±6.7%) and 0.4–2.2% (1.3±0.7%), respectively. These values were similar to the values reported previously [2,3,19].

The proposed method for the determination of prolyl dipeptides, Pro and Hyp in urine is highly

Table 2
Precision of determination of urinary Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp

	Mean±SD (μ*M*), RSD[a] (%)				
	Pro–Hyp	Pro–Gly	Pro–Pro	Pro	Hyp
Within-day[b] (*n*=10)					
Urine 1	84.3±1.7	4.38±0.21	1.29±0.06	6.21±0.21	1.08±0.04
	2.0	4.8	4.7	3.4	3.7
Urine 2	164.3±3.6	3.68±0.17	2.38±0.08	10.10±0.20	6.26±0.17
	2.2	4.6	3.4	2.0	2.7
Urine 3	440.9±6.4	2.98±0.12	6.79±0.12	16.50±0.43	3.08±0.06
	1.5	4.0	1.8	2.6	2.0
Between-day[c] (*n*=5)					
Urine 1	83.5±1.7	4.14±0.24	1.29±0.04	6.07±0.12	1.07±0.06
	2.0	5.8	3.1	2.0	5.6
Urine 2	163.9±3.6	3.73±0.16	2.33±0.09	10.35±0.34	6.18±0.15
	2.2	4.3	3.9	3.3	2.4
Urine 3	433.5±7.2	2.85±0.13	6.60±0.14	16.84±0.40	3.14±0.08
	1.7	4.6	2.1	2.4	2.6

[a] RSD=Relative standard deviation.
[b] Within-day precision was obtained from ten replicate assays on one day.
[c] Between-day precision was obtained from five different days.

sensitive and reliable. As the sensitive and reliable determination of these components can provide useful information for diagnosis and prognosis of diseases, the proposed method may be useful for biochemical and clinical research. Further clinical research including a determination of bone metas-

Table 3
Urinary excretion of Pro–Hyp, Pro–Gly, Pro–Pro, Pro, Hyp and total Hyp in normal subjects

Age	Sex[a]	Concentration (nmol/mg Cre[b])					
		Pro–Hyp	Pro–Gly	Pro–Pro	Pro	Hyp	Total Hyp[c]
23	M	46.2	1.54	0.77	4.35	2.02	95.8
24	M	64.2	3.26	0.99	4.70	0.83	123.5
25	M	117.4	0.92	1.80	4.47	0.83	195.3
25	M	111.0	0.42	1.43	2.58	0.94	191.3
25	M	121.2	4.10	3.34	5.14	1.11	204.7
23	F	84.5	2.72	1.46	11.21	3.41	184.7
23	F	136.9	2.91	1.14	5.39	5.91	285.0
24	F	86.0	2.34	3.95	6.91	2.77	181.4
24	F	91.9	4.95	3.19	9.82	3.11	142.4
27	F	116.8	4.25	2.77	12.57	2.09	244.5
Mean		97.6	2.74	2.08	6.71	2.30	184.9
SD		28.2	1.48	1.13	3.34	1.59	55.4

[a] M=Male; F=female.
[b] Cre=Urinary creatinine. Cre was measured by Creatinine Test Wako.
[c] Total Hyp=Hyp in hydrolysed urine. Total Hyp was measured according to the method reported previously [20].

tasis stages in cancer patients is now progressing in our laboratories.

References

[1] R. Smith, Clin. Sci. 59 (1980) 215.
[2] J.D. Smiley, M. Ziff, Physiol. Rev. 44 (1964) 30.
[3] J. Dubovskÿ, E.V.A. Dubovská, E. Pacovskÿ, J. Haba, Clin Chim. Acta 19 (1967) 387.
[4] A. Szymanowicz, A. Malgras, A. Randoux, J.P. Borel, Biochim. Biophys. Acta 576 (1979) 253.
[5] L. Hyldstrup, P. McNair, G.F. Jensen, H.R. Nielsen, I. Transbøl, Calcif. Tissue Int. 36 (1984) 639.
[6] H. Nishino, T. Tanaka, K. Shiroishi, S. Sato, Y. Naruse, S. Kagamimori, Bull. Environ. Contam. Toxicol. 47 (1991) 609.
[7] S.M. Mohammed, S.A. Suleiman, S.K. Addae, S.H. Annobil, F.K. Adzaku, O.F. Kadoummi, J.T. Richards, Clin. Chim. Acta 203 (1991) 285.
[8] P.C. Kelleher, C.J.P. Smith, Clin. Lab. Med. 2 (1982) 519.
[9] S.C. Hopkins, G.M. Palmieri, H.B. Niell, M. Moinuddin, M.S. Soloway, Cancer 53 (1984) 117.
[10] W.M. O'Brien, J.H. Lynch, J. Urol. 139 (1988) 66.
[11] R.A.W. Johnstone, T.J. Povall, J.D. Baty, J.-L. Pousset, C. Charpentier, A. Lemonnier, Clin. Chim. Acta 52 (1974) 137.
[12] K.F. Faull, G.M. Schier, P. Schlesinger, B. Halpern, Clin. Chim. Acta 70 (1976) 313.
[13] C. Charpentier, R.A.W. Johnstone, A. Lemonnier, I. Myara, M.E. Rose, D. Tuli, Clin. Chim. Acta 138 (1984) 299.
[14] J. Jandke, G. Spiteller, J. Chromatogr. 382 (1986) 39.
[15] H. Kodama, H. Nakamura, K. Sugahara, J. Chromatogr. 527 (1990) 279.
[16] K. Sugahara, H. Kodama, J. Chromatogr. 565 (1991) 408.
[17] H. Kodama, K. Sugahara, Anal. Chim. Acta 352 (1997) 141.
[18] M. Codini, C.A. Palmerini, C. Fini, C. Lucarelli, A. Floridi, J. Chromatogr. 536 (1991) 337.
[19] Y. Tsuruta, H. Inoue, Anal. Biochem. 265 (1998) 15.
[20] H. Inoue, Y. Date, K. Kohashi, H. Yoshitomi, Y. Tsuruta, Biol. Pharm. Bull. 19 (1996) 163.