ELSEVIER

Journal of Chromatography B, 757 (2001) 369–373

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Fluorometric determination of *N*-terminal prolyl dipeptides, proline and hydroxyproline in human serum by pre-column high-performance liquid chromatography using 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride

Hirofumi Inoue[a], Haruo Iguch[b], Akira Kouno[b], Yasuto Tsuruta[a,*]

[a]*Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan*
[b]*National Kyusyu Cancer Center Research Institute, Notame, Minami-ku, Fukuoka 811-1347, Japan*

Received 13 October 2000; received in revised form 2 February 2001; accepted 7 March 2001

Abstract

A highly sensitive HPLC method for the determination of prolyl dipeptides, Pro and Hyp in serum was developed. After deproteinization of serum and pretreatment with *o*-phthalaldehyde, the analytes were derivatized with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride at 70°C for 10 min. The fluorescent derivatives of prolyl dipeptides, Pro and Hyp, were separated on tandem reversed-phase columns by a gradient elution at 55°C and detected by fluorescence measured at 318 nm (excitation) and 392 nm (emission). The detection limits for prolyl dipeptides were 2–5 fmol/injection ($S/N=3$). Pro–Hyp, Pro–Gly and Pro–Pro were identified as serum prolyl dipeptides. The within-day and between-day relative standard deviations were 1.5–7.9 and 2.4–10.8%, respectively. The recoveries were in the range of 90.8–97.3%. The concentrations of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp in normal human serum ($n=10$) were 0.64 ± 0.35, 0.078 ± 0.047, 0.022 ± 0.016, 177.0 ± 43.0 and 11.1 ± 3.5 μM, respectively. The concentrations of Pro–Hyp and Pro–Pro in serum of a patient with bone metastases of prostatic cancer were about three times and 50 times, respectively, higher than those in normal human serum.

Keywords: Derivatization, LC; Prolyl dipeptides; Proline; Hydroxyproline

1. Introduction

Proline (Pro) and hydroxyproline (Hyp) contained in collagen at high concentrations are released into serum as amino acids and peptides. The concentrations of Pro and Hyp in serum vary in association with various diseases involving collagen metabolism such as bone diseases [1–4] and tumors [5] and the concentration of prolyl dipeptide, Pro–Hyp, is increased in the serum of patients with prolidase deficiency [6]. Therefore, sensitive and reliable determinations of these amino acid and prolyl dipeptides in human serum are useful for the understanding of various disorders.

As a method for the determination of prolyl

*Corresponding author. Tel.: +81-849-362-111; fax: +81-849-362-024.
E-mail address: tsuruta@fupharm.fukuyama-u.ac.jp (Y. Tsuruta).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00162-1

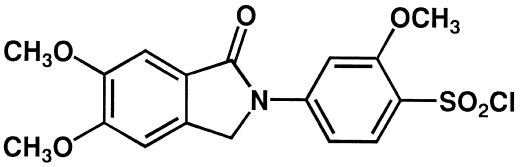

Fig. 1. Molecular structure of DMS-Cl.

dipeptides in serum, only liquid chromatography–mass spectrometry (LC–MS) [6] has been reported. However, the determination of prolyl dipeptides in normal human serum could not be achieved by this method because of low sensitivity, although the concentration of Pro–Hyp elevated in the serum of patients with prolidase deficiency has been measured. Furthermore, the LC–MS method requires a relatively large sample volume (2 ml) and is tedious and time consuming.

Recently, we established a highly sensitive and reliable high-performance liquid chromatography (HPLC) method for the determination of prolyl dipeptides (Pro–Hyp, Pro–Gly and Pro–Pro) in urine [7]. With this method, prolyl dipeptides were derivatized with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl, shown in Fig. 1) [8], to give corresponding highly fluorescent derivatives. However, the method employed solvent extraction with dichloromethane, and there was a problem of environmental pollution.

In the present study, we modified the method to exclude dichloromethane extraction and developed a highly sensitive HPLC for determination of prolyl dipeptides, Pro and Hyp in serum by pre-column derivatization with DMS-Cl.

2. Experimental

2.1. Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. DMS-Cl was prepared as described previously [8]. 3,4-Dehydro-DL-proline and prolyl dipeptides except Pro–Asp, –Gln, –Glu, –Leu, –Pro and –Ser (Kokusan, Tokyo, Japan) were purchased from Sigma (St. Louis, MO, USA). Organic solvents except acetonitrile (HPLC grade; Wako, Osaka, Japan) were of the highest purity available and used as received.

2.2. Instrumental conditions

The HPLC conditions were as previously described [7] except for the elution program to separate the peak due to Pro–Pro from that due to Pro. That is to say, tandem Nova Pak C_{18} columns (150×3.9 mm I.D., 4 μm; Waters, Milford, MA, USA) connected with a TSK-guard gel ODS-80Ts (15×3.2 mm I.D.; Tosoh, Tokyo, Japan) 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 26% of B for 25 min, a stepwise increase to 100% of B to wash the column for 10 min, and finally a stepwise decrease to 25% of B to re-equilibrate the column for 15 min. The flow-rate was 1 ml/min. The fluorescence intensities were monitored at excitation and emission wavelengths of 318 and 392 nm, respectively.

2.3. Procedure for determination of prolyl dipeptides, Pro and Hyp in serum

To human serum (200 μl) were added 3,4-dehydro-DL-proline (10 μ*M*, 20 μl) as an internal standard (I.S.) and methanol (800 μl). The mixture was vortex-mixed and then centrifuged at 2500 *g* for 10 min. An aliquot (500 μl) of the supernatant was evaporated to dryness in stream of nitrogen gas at 50°C. The residue was dissolved in *o*-phthalaldehyde (OPA) solution [2%, w/v, in acetonitrile–Na_2HPO_4 (50 m*M*) (1:9, v/v), 100 μl] and then allowed to stand for 3 min at room temperature. The mixture was reacted with DMS-Cl (7.5 m*M* in acetonitrile, 100 μl) at 70°C for 20 min and then *N*-methyltaurin (0.1 *M*, 50 μl) was added. After standing for 3 min at room temperature, the mixture was acidified with acetic acid (1 *M*, 50 μl) and an aliquot of the resulting mixture (10 μl) was subjected to HPLC.

3. Results and discussion

3.1. Labeling reaction and chromatographic separation

The labeling of prolyl dipeptides, Pro, Hyp and I.S. with DMS-Cl was completed within 10 min at 70°C in the basic medium (pH 8–10) [7].

When the reaction mixtures from serum samples were measured under the previous HPLC conditions [7] by which the separation of 12 species of prolyl dipeptides was achieved, Pro–Hyp, Pro–Gly and Pro–Pro were recognized as serum prolyl dipeptides. However, the separation conditions were modified to measure the prolyl dipeptides, Pro and Hyp in serum, as a large peak due to Pro in serum interfered with the measurement of Pro–Pro under the previous conditions. To obtain the sufficient separation of serum prolyl dipeptides, the elution program described in the Experimental section was employed, since the peaks due to Pro and Pro–Pro did not elute within 60 min with isocratic elution of 25% B and the peak due to Pro–Hyp overlapped with that of other serum component with isocratic elution of 26% B.

Typical chromatograms obtained from a standard solution and a human serum are shown in Fig. 2. The peaks due to prolyl dipeptides (seven species), Pro, Hyp and I.S. were separated from each other and the reagent blank. When serum was analyzed, the peaks due to Pro–Hyp, Pro–Gly, Pro–Pro, Pro, Hyp and I.S. eluted at 16.4, 26.1, 46.7, 42.7, 11.9 and 32.9 min, respectively, and were separated from the peaks due to the reagent blank components and other serum components. The peaks due to Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp in serum were identified by comparing the retention time with those of standard solutions and co-chromatography of a standard solution and serum.

3.2. Concentration of DMS-Cl

The concentration of DMS-Cl in acetonitrile was determined by use of serum (200 μl) spiked with Pro–Hyp, Pro–Gly, Pro–Pro (0.2 nmol each), Pro (40 nmol) and Hyp (2 nmol). As the most intense and constant peak areas were obtained at more than 5 m*M*, 7.5 m*M* of DMS-Cl was employed.

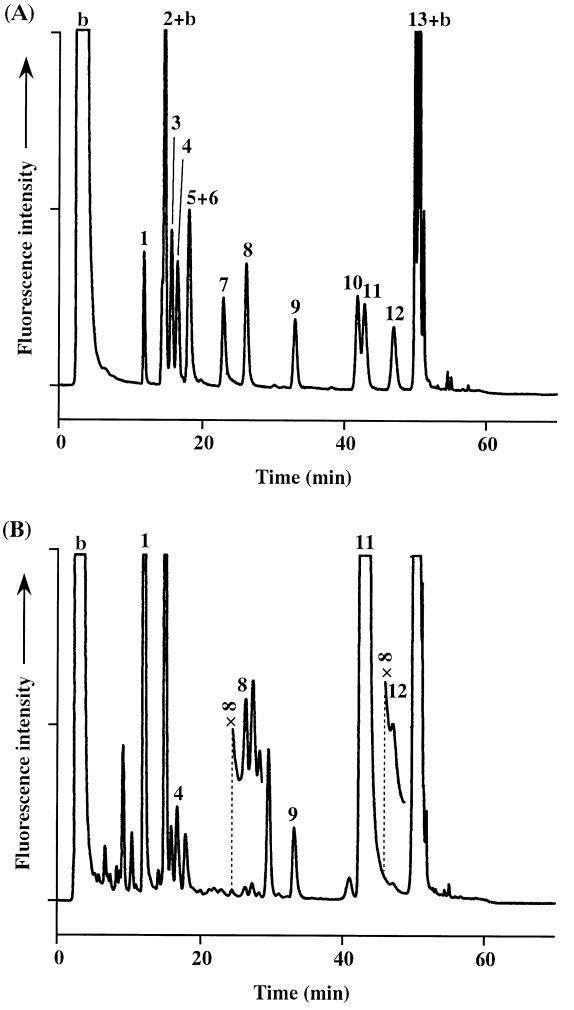

Fig. 2. Chromatograms obtained from (A) a standard solution of prolyl dipeptide, Pro, Hyp and I.S. and (B) a normal human serum according to the procedure described in Section 2.3. Peaks: 1, Hyp; 2, Pro–Asn; 3, Pro–Gln; 4, Pro–Hyp; 5, Pro–Asp; 6, Pro–Ser; 7, Pro–Glu; 8, Pro–Gly; 9, I.S.; 10, Pro–Ala; 11, Pro; 12, Pro–Pro; 13, Pro–Tyr, Pro–Met, Pro–Val, Pro–Trp, Pro–Ile, Pro–Leu and Pro–Phe; b, reagent blank. Concentration: (A) prolyl dipeptide, Pro and Hyp=1 μ*M* each; (B) Pro–Hyp=0.74 μ*M*; Pro–Gly=0.086 μ*M*; Pro–Pro=0.030 μ*M*; Pro=179.9 μ*M*; Hyp=15.4 μ*M*. HPLC conditions as in Section 2.2.

3.3. Addition of N-methyltaurin and acetic acid

After the labeling reaction, *N*-methyltaurin was added to remove the excess of DMS-Cl, which was

suspected of causing the guard column to degrade. In a previous study on the determination of urinary prolyl dipeptides [7], the excess of DMS-Cl in the reaction mixture was removed by dichloromethane extraction. However, it is preferable to avoid the use of halogenated organic solvents such as dichloromethane which cause of environmental pollution. Therefore, the excess of DMS-Cl was converted to the *N*-methyltaurin derivative, which was formed by the reaction with *N*-methyltaurin at room temperature within 2 min. Incidentally, the derivative of *N*-methyltaurin eluted at 3.2 min.

When the reaction mixture without acidification was subjected to HPLC, the peaks due to some DMS derivatives (Hyp, Pro–Asn, –Gln, –Hyp, –Asp, –Ser, –Glu, and –Gly) eluted as broad peaks. However, this problem was solved by acidification of the reaction mixture with acetic acid, although the reason was unknown.

3.4. Pretreatment of serum

The effect of the organic solvents such as methanol, ethanol, acetonitrile and acetone on the deproteinization was studied using serum (200 μl) spiked with Pro–Hyp, Pro–Gly, Pro–Pro (0.2 nmol each), Pro (40 nmol) and Hyp (2 nmol). The maximum peak areas of analytes and I.S. were obtained when methanol was used (peak areas relative to methanol; ethanol: 87.5–93.5%; acetonitrile: 54.5–79.6%; acetone: 59.1–81.0%).

The supernatant of deproteinized serum was treated with OPA prior to the labeling reaction with DMS-Cl to eliminate primary amino compounds coexisting with analytes in serum [9]. The influence of amino acids on the determination of prolyl dipeptides, Pro and Hyp was examined using serum (200 μl) spiked with 25 species of primary amino acids (Ala, Arg, Asn, Asp, Cit, Cys–Cys, Cys, Glu, Gln, Gly, His, Hse, Ile, Leu, Lys, Met, Orn, Phe, Ser, Thr, Try, Tyr, Val, γ-aminobutyric acid and ε-aminocaproic acid, 20 nmol each). These amino acids did not interfere with the determination of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp.

3.5. Precision and detection limit

The within-day and between-day precisions were evaluated using three sera. The within-day precision was examined with 10 replicate assays in 1 day and the between-day precision by assays on 5 different days. The within-day relative standard deviations (RSDs) of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp were 2.7–5.4, 2.3–4.0, 6.1–7.9, 1.7–2.9 and 1.5–3.1%, respectively, and the between-day RSDs were 5.2–6.0, 3.9–8.9, 6.6–10.8, 2.4–4.5 and 2.8–4.5%, respectively. The detection limits (signal-to-noise ratio=3) for prolyl dipeptides, Pro and Hyp, were 2–5 fmol/injection.

3.6. Linearity and recovery

Linearity was studied using standard solutions with and without pooled serum (concentration: Pro–Hyp=0.52 μ*M*; Pro–Gly=0.025 μ*M*; Pro–Pro=0.018 μ*M*: Pro=162.5 μ*M*; Hyp=14.7 μ*M*). The concentration ranges of standard solutions investigated were 0.1–2 μ*M* for Pro–Hyp, 0.01–0.2 μ*M* for Pro–Gly, 0.005–0.1 μ*M* for Pro–Pro, 20–400 μ*M* for Pro and 2–40 μ*M* for Hyp. The relationships between the peak-area ratios of analytes to I.S. and the concentrations of analytes were linear. The recoveries obtained from the slope ratios of regression equations of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp with/without serum, were 97.3, 92.1, 90.8, 96.5 and 96.6%, respectively.

3.7. Determination of prolyl dipeptides, Pro and Hyp in serum

The concentrations of prolyl dipeptides, Pro and Hyp in serum from 10 healthy volunteers (staff and students in our laboratory) who were eating self-selected diets were measured by the present method. Serum samples were collected and stored at −20°C until use. The concentrations of prolyl dipeptides, Pro and Hyp in normal human serum are given in Table 1. The mean values (mean±SD) of Pro–Hyp, Pro–Gly, Pro–Pro and Pro and Hyp were 0.64±0.35, 0.078±0.032, 0.022±0.016, 177.0±43.0 and 11.1±3.5 μ*M*, respectively. The concentrations of Pro–Hyp, Pro–Gly and Pro–Pro in normal human serum were determined for the first time by the present method. The mean values of the concentration of Pro and Hyp were similar to values reported previously [1,2,4,9].

The serum of a patient with bone metastases of prostatic cancer was also analyzed. The chromato-

Table 1
Concentration of Pro–Hyp, Pro–Gly, Pro–Pro, Pro and Hyp in normal human serum

Age (years)	Sex[a]	Concentration (μ*M*)				
		Pro–Hyp	Pro–Gly	Pro–Pro	Pro	Hyp
21	M	1.43	0.082	0.059	251	17.9
23	M	0.88	0.041	0.010	157	13.0
23	M	1.00	0.061	<0.005[b]	141	11.2
25	M	0.80	0.055	0.016	138	11.7
30	M	0.69	0.067	0.032	173	15.1
21	F	0.75	0.181	0.021	219	14.0
21	F	0.34	0.025	0.009	133	9.9
21	F	0.48	0.074	0.005	140	6.6
21	F	0.35	0.058	0.023	196	9.8
23	F	0.36	0.120	0.035	233	8.0
24	F	0.23	0.138	0.025	211	6.4
26	F	0.36	0.029	0.005	134	9.2
Mean		0.64	0.078	0.022	177	11.1
SD		0.35	0.047	0.016	43	3.5

[a] M=Male; F=female.
[b] Below the determination limit.

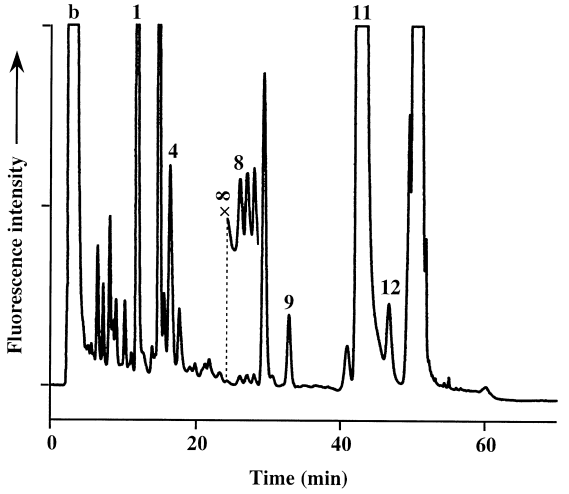

Fig. 3. Chromatogram obtained from serum of a patient with bone metastases of prostatic cancer according to the procedure described in Section 2.3. Peaks: 1, Hyp; 4, Pro–Hyp; 8, Pro–Gly; 9, I.S.; 11, Pro; 12, Pro–Pro; b, reagent blank. Concentration: Pro–Hyp=1.78 μ*M*; Pro–Gly=0.071 μ*M*; Pro–Pro=1.059 μ*M*; Pro=248.9 μ*M*; Hyp=10.0 μ*M*. HPLC conditions as in Section 2.2.

gram obtained from the serum is shown in Fig. 3. The concentrations of Pro–Hyp and Pro–Pro were about three times and 50 times, respectively, higher than those in serum from normal subjects, while there was no significant difference in the concentration of Pro–Gly, Pro and Hyp in sera between normal subjects and the patient.

In conclusion, a pre-column HPLC method for the simultaneous determination of prolyl dipeptides, Pro and Hyp in serum using DMS-Cl as a fluorescent labeling reagent has been established. As the proposed method is highly sensitive and reliable, it may be useful for clinical and biochemical research.

References

[1] M.C. Bishop, R. Smith, Clin. Chim. Acta 33 (1971) 403.
[2] Z. Varghese, J.F. Moorhead, G.L.V. Tatler, R.A. Baillod, M.R. Wills, Proc. Eur. Dial. Transplant Assoc. 10 (1973) 187.
[3] Z. Varghese, J.F. Moorhead, M.R. Wills, Clin. Chim. Acta 110 (1981) 105.
[4] H. Nishino, T. Tanaka, K. Shiroishi, S. Sato, Y. Naruse, S. Kagamimori, Bull. Environ. Contam. Toxicol. 47 (1991) 609.
[5] M.J. Kontturi, E.A. Sotaniemi, T.K.I. Larmi, Scand. J. Urol. Nephrol. 8 (1974) 91.
[6] H. Kodama, K. Sugahara, Anal. Chim. Acta 352 (1997) 141.
[7] H. Inoue, H. Iguchi, A. Kono, Y. Tsuruta, J. Chromatogr. B 724 (1999) 221.
[8] H. Inoue, Y. Tsuruta, Anal. Biochem. 265 (1998) 15.
[9] H. Inoue, K. Moritani, Y. Date, K. Kohashi, Y. Tsuruta, Analyst 120 (1995) 1141.