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Sensitive determination of carnosine in urine by high-performance liquid chromatography using 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride as a fluorescent labeling reagent

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ABSTRACT

A simple and highly sensitive high-performance liquid chromatography procedure was developed for the determination of carnosine in urine. Carnosine was derivatized with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride at 70 °C for 15 min in borate buffer (20 mmol l^{-1}, pH 9.0) to produce fluorescent sulfonamides. After hydrolysis of the reaction mixture with formic acid at 100 °C for 15 min, the fluorescent derivative of carnosine was separated on a reversed-phase column with a linear gradient elution using solvents of (A) acetate buffer (0.1 mmol l^{-1}, pH 7.0) and (B) acetonitrile at a flow-rate of 1.0 ml/min and was detected at excitation and emission wavelengths of 318 and 400 nm, respectively. The detection limit of carnosine was 4 fmol at a signal-to-noise ratio of 3. The within-day and day-to-day relative standard deviations were 2.7–4.6% and 0.4–5.2%, respectively. The concentration of carnosine in normal human urine was found to be 4.6–125 nmol (mg creatinine)$^{-1}$ (mean ± SD: 21.6 ± 26.6 nmol (mg creatinine)$^{-1}$, $n = 20$).

1. Introduction

Carnosine (β-alanyl-L-histidine) is a dipeptide that is highly concentrated in the muscles, heart, and brain tissues in mammals [1,2]. Carnosine is known to have various physiological functions; for example, it acts as a regulator of intracellular pH, a modulator of immune system, a neurotransmitter, a metal-ion chelator, an antioxidant, and a free-radical scavenger [2–5] and also to associate with some disease such as serum carnosinase deficiency and carnosinuria [2]. However, it is necessary, for the clarification of physiological function and clinical significance of carnosine, to develop new methods with improved sensitivity enough for the determination of carnosine in biological fluids, because of insufficiency for sensitivity of methods reported previously [6–19].

To measure the carnosine level in animal tissues and fluids, the following methods have been developed: HPLC-UV detection [6–11], HPLC-fluorescence detection [12–15], HPLC-amperometric detection [16], LC–MS [17,18], and capillary electrophoresis [19]. Among these methods, an HPLC-fluorescence detection method [12] involving the reaction of primary amine with o-phthalaldehyde/2-mercaptoethanol and HPLC-UV detection methods involving an ODS column [7] and an ion-exchange column [10], respectively, were tried to measure urinary carnosine. However, urinary carnosine was not detected by the HPLC-fluorescence method [12] or the HPLC-UV method involving the ion-exchange column [10] because these methods were not sensitive enough. The HPLC-UV method using the ODS column [7] is the only method reported before for determining the concentration of urinary carnosine. However, the separation conditions and the precision for urinary carnosine analysis by this method are not shown.

We previously developed an extremely sensitive fluorescent labeling reagent, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl), for the determination of amino acids [20]. This reagent reacts quantitatively with amino acids to form stable and highly fluorescent sulfonamides with a high labeling yield (about 100%). In this paper, a highly sensitive, simple HPLC method for the determination of carnosine in urine using fluorescence detection after pre-column derivatization with DMS-Cl is described.

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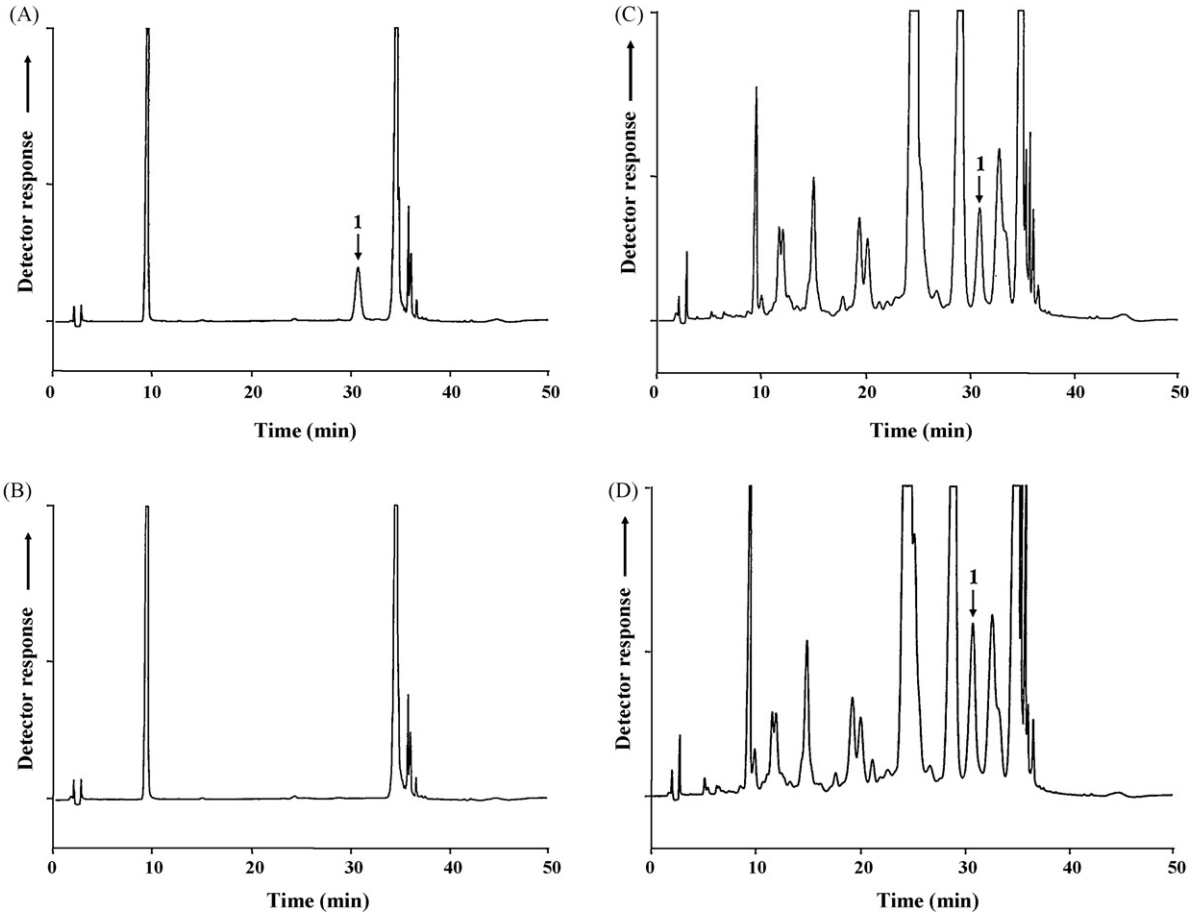

Fig. 1. Chromatograms obtained from (A) a standard solution, (B) a reagent blank, (C) human urine, and (D) human urine spiked with standard carnosine according to the procedure described in Section 2. HPLC conditions (A) described in Section 2 were used. Peak 1: derivative of carnosine. Concentration of carnosine: (A) 20 μmol l^{-1}; (C) 35.4 μmol l^{-1}; (D) 55.4 μmol l^{-1}.

2. Materials and methods

2.1. Chemicals

DMS-Cl was prepared as described in a previous paper [20]. Carnosine and HPLC grade acetonitrile were obtained from Wako Pure Chemicals (Osaka, Japan). The OASIS^{R}MCX column (Waters, MA, USA) was conditioned with methanol (1 ml) and water (1 ml) by aspiration at 10 in. Hg. All other chemicals were of an analytical grade. Deionized-distilled water was prepared by the Milli-QII system (Yamato, Tokyo, Japan).

2.2. Instrumentation

The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-10AD HPLC pumps, a CTO-10AC column oven, a DGU-14A online degasser, an SIL-10A_{XL} auto injector, an RF-10A_{XL} fluorescence detector, and a CLASS-LC10 LC workstation with a CBM-10A communications bus module. Fluorescence was monitored at excitation and emission wavelengths of 318 and 400 nm, respectively. The uncorrected fluorescence spectra of the derivative of carnosine produced by its reaction with DMS-Cl were measured with a Shimadzu RF-530 spectrofluorometer (Shimadzu, Kyoto, Japan) using a quartz cell (optical path length: 10 mm).

2.2.1. HPLC conditions (A) for urine analysis

A TSK-gel ODS-100Z column (150 mm × 4.6 mm, i.d., 5 μm, Tosoh, Tokyo, Japan) and a TSK-guard gel ODS-100Z as a guard column (15 mm × 3.2 mm, i.d.) were used at 40 °C with solvents of (A) acetate buffer (0.1 mol l^{-1}, pH 7.0) and (B) acetonitrile. The elution program consisted of linear gradient elution from 16% B to 17% B for 30 min, followed by a stepwise increase to 80% B for 10 min, and finally a stepwise decrease to 16% B to re-equilibrate the column for 10 min. The flow-rate was 1.0 ml/min.

2.2.2. HPLC conditions (B) for the study of the labeling reaction using standard solution

A TSK-gel ODS-120T column (150 mm × 4.6 mm, i.d., 5 μm, Tosoh, Tokyo, Japan) and a TSK-guard gel ODS-120T as a guard column (15 mm × 3.2 mm, i.d.) were used at 35 °C with solvents of (A) phosphoric acid (2 mM) and (B) acetonitrile. The elution program consisted of linear gradient elution from 22% B to 52% B for 30 min, followed by a stepwise increase to 80% of B for 10 min and finally a stepwise decrease to 22% B to re-equilibrate the column for 10 min. The flow-rate was 1.0 ml/min.

2.3. Sample collection and assay of creatinine

Urine was collected from healthy volunteers who were eating self-selected diets and used within 1 h or stored at −20 °C until use. Urinary creatinine was measured by the Jaffe' method using the Creatinine Test Wako (Wako, Osaka, Japan).

2.4. Analytical procedure

This analytical procedure consisted of two reaction steps, a labeling and a hydrolysis reaction. To the urine sample (30 μl), borate buffer (0.1 mol l^{-1}, pH 9.0, 30 μl) and DMS-Cl (10 mmol l^{-1}, in acetonitrile, 100 μl) were added. After performing the labeling reaction at 70 °C for 15 min, proline (0.1 mol l^{-1}, 50 μl) was added to deactivate the DMS-Cl. Following the second hydrolysis reaction with formic acid (40%, v/v, 50 μl) at 100 °C for 15 min, hydrochloric acid (0.1 mol l^{-1}, 250 μl) was added. An aliquot of the reaction mixture (400 μl) was applied to an OASIS^{R}MCX column and successively washed with hydrochloric acid (0.1 mol l^{-1}, 1 ml) and methanol (2 ml), before being eluted with 5% (v/v) triethylamine (in methanol, 1.5 ml). The eluate diluted with water (500 μl) was neutralized with formic acid (40%, v/v, 50 μl), and an aliquot of the mixture (20 μl) was subjected to HPLC.

3. Results and discussion

3.1. HPLC separation

Before HPLC analysis, urine samples were reacted with DMS-Cl to convert carnosine to fluorescent derivatives, and then hydrolysis with formic acid was selected to obtain a predominant fluorescent derivative, because fluorescence derivatization of carnosine with DMS-Cl produced mainly three derivatives (see Section 3.2.1). In addition, purification on the OASIS^{R}MCX column was also selected to eliminate the interfering fluorescence materials.

The derivative of carnosine produced by its reaction with DMS-Cl was successfully separated on a reversed-phase column. A TSK-gel ODS-100Z column was appropriate for the separation of the DMS derivative of carnosine in comparison with a TSK-gel ODS-120T column, as the peak assigned to the DMS derivative of carnosine was disturbed by peaks due to urine components. With HPLC condition (A) described in Section 2, the typical chromatograms obtained using this analytical procedure from a standard solution, a reagent blank, human urine, and human urine spiked with standard carnosine are shown in Fig. 1. A single peak due to carnosine was observed at 30.8 min and was completely separated from the peaks of the reagent blank and other urine components under the described conditions. The peak of the fluorescent derivative of carnosine in human urine was identified by comparing its retention time with that of the standard solution by co-chromatography of urine and the standard. The maximum fluorescence wavelengths of the eluate corresponding to the peak due to carnosine were 318 nm (excitation) and 400 nm (emission).

3.2. Hydrolysis conditions and reaction conditions

First of all, it was necessary to get DMS derivatives of carnosine to predominant one (see below). To determine the optimum labeling conditions, HPLC conditions (B) described in Section 2 were employed to shorten the analysis time, and a standard solution of carnosine (10 μmol l^{-1}, 20 μl each) was used instead of the urine sample in the analytical procedure without pretreatment using the OASIS^{R}MCX column.

3.2.1. Hydrolysis conditions

Hydrolysis is essential for this HPLC method. The HPLC chromatograms obtained from the reaction mixtures of carnosine with DMS-Cl with/without hydrolysis are shown in Fig. 2. When the hydrolyzed reaction mixture was analyzed, a single peak due to carnosine was observed at 8 min as shown in Fig. 2(A), while

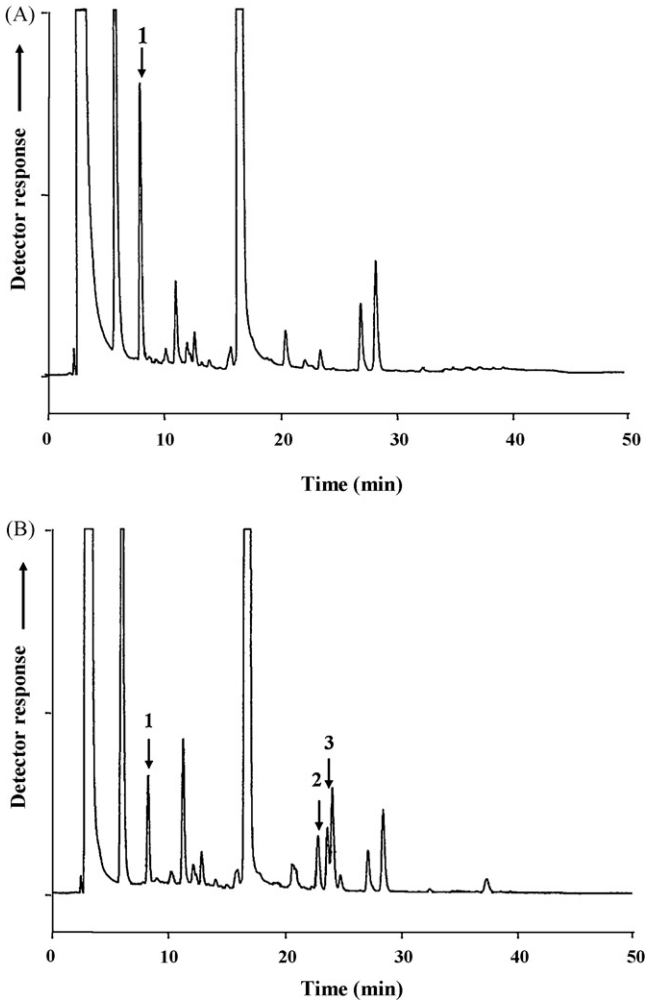

Fig. 2. Chromatograms obtained from a reaction mixture containing a standard solution (A) with hydrolysis and (B) without hydrolysis according to the procedure described in Section 2. HPLC conditions (B) were used. Peaks 1, 2 and 3: derivative of carnosine. Concentration of carnosine: 10 μmol l^{-1}.

three peaks (retention time: 8, 23, and 24 min) were observed on the chromatogram of the reaction mixture without hydrolysis as shown in Fig. 2(B). As DMS-Cl reacts with primary and secondary amino compounds in basic medium to give the corresponding fluorescent sulfonamides, these peaks were considered to be the products of the reaction of carnosine with DMS-Cl. Although the labeling reaction conditions were investigated in an attempt to integrate these three peaks into one by changing the reaction temperature and pH, the three peaks were always observed on the chromatogram with peak-1 (retention time: 8 min) being predominant. Therefore, the reaction mixture was treated with formic acid to hydrolyze the sulfonamides produced by the labeling reaction of the imidazole ring with DMS-Cl, as the sulfonamides that are obtained from the reaction of the imidazole ring of histidine with sulfonyl chlorides are readily hydrolyzed in the presence of formic acid [21]. The structure of the DMS derivative of carnosine after hydrolysis is shown in Fig. 3.

The conditions of the hydrolysis reaction involving formic acid were examined at 70 and 100 °C. As shown in Fig. 4, the most intense and constant peak areas of peak-1 were obtained, and peak-2 disappeared when hydrolysis was carried out at 100 °C for more than 15 min. The behavior of peak-3 was similar to that of peak-2.

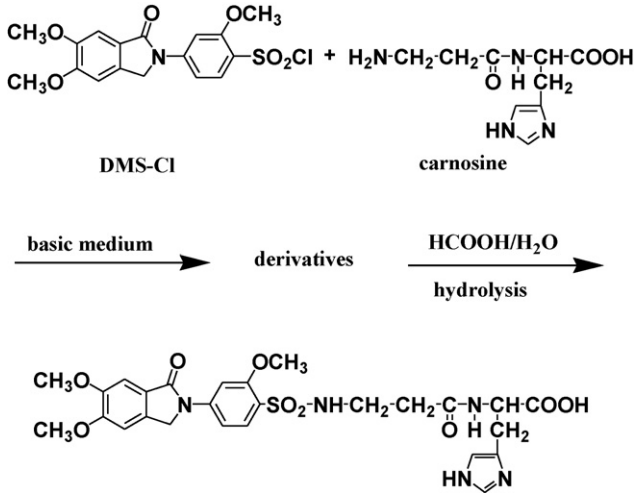

Fig. 3. Reaction scheme of carnosine labeled with DMS-Cl.

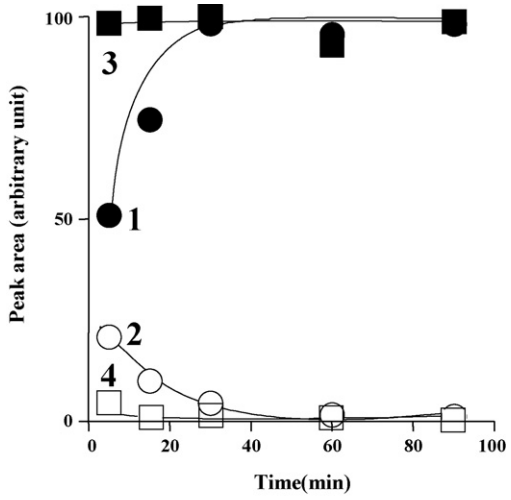

Fig. 4. Effect of reaction time at 70 and 100 °C on the hydrolysis of carnosine labeled with DMS-Cl by formic acid. Curves: 1: peak-1 at 70 °C; 2: peak-2 at 70 °C; 3: peak-1 at 100 °C; 4: peak-2 at 100 °C. The peak numbers are as shown in Fig. 2.

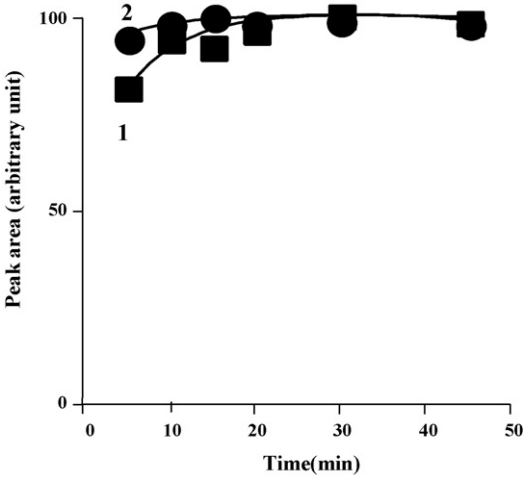

Fig. 5. Effect of reaction time and temperature on the labeling reaction of carnosine with DMS-Cl. Curves: 1: 50 °C; 2: 70 °C.

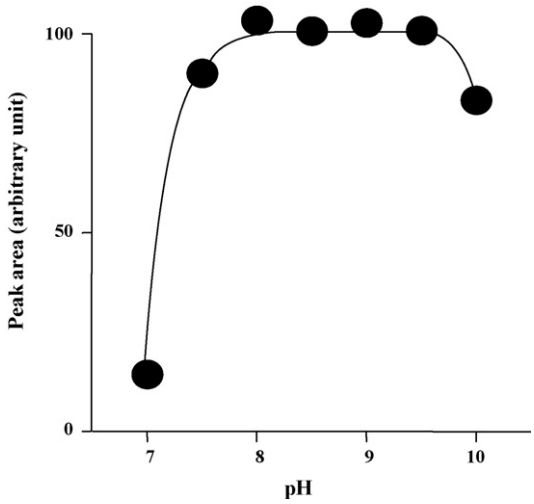

Fig. 6. Effect of pH of borate buffer (0.1 mol l^{-1}) on the labeling reaction of carnosine with DMS-Cl.

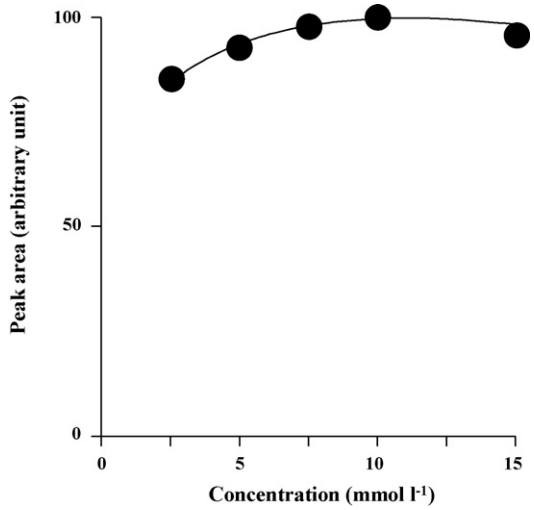

Fig. 7. Effect of DMS-Cl concentration on the labeling reaction of carnosine with DMS-Cl.

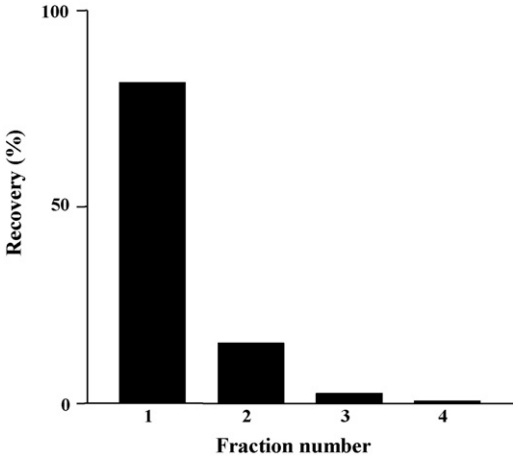

Fig. 8. Recovery of the DMS derivative of carnosine in the eluate fraction produced with triethylamine (5%, v/v, in methanol). Each fraction collected was a 500 μl sample of triethylamine (5%, v/v, in methanol) eluant.

3.2.2. Reaction conditions

The effect of reaction time on the labeling reaction was examined at various temperatures. As shown in Fig. 5, the maximum peak area of peak-1 was obtained by performing the reaction at 70 °C for 15 min.

As the labeling reaction of carnosine with DMS-Cl proceeded in basic medium, the effect of the pH of borate buffer (0.1 mol l^{-1}) was examined. The maximum peak area was obtained in the pH range 8.0–9.5, as shown in Fig. 6.

3.2.3. DMS-Cl concentration

The effect of the concentration of DMS-Cl in acetonitrile in the range of 2.5–10 mmol l^{-1} was examined. As shown in Fig. 7, the most intense and constant peak areas were obtained when the concentration of the reagent solution was more than 7.5 mmol l^{-1}.

3.3. Pretreatment

To determine the level of urinary carnosine, pretreatment with the OASIS^{R}MCX column was employed, since the fluorescence peak due to carnosine was disturbed by other urinary fluorescence materials without such pretreatment. The DMS derivative of carnosine was eluted from the OASIS^{R}MCX column with triethylamine (5%, v/v, in methanol). Most of all DMS derivative of carnosine was eluted out from the column in preceding three fractions (fractions 1–3) of consecutive four 500 μl triethylamine eluate fractions as shown in Fig. 8, while the interfering fluorescence materials were retained on the column. When the DMS derivative of carnosine was eluted with 1.5 ml of triethylamine (5%, v/v, in methanol), the recovery was 95.3 ± 3.5% (n = 3).

3.4. Precision, linearity, recovery, and detection limit

The within-day precision was examined with ten replicate assays in 1 day, and the day-to-day precision was examined on 3 different days. As shown in Table 1, the within-day and day-to-day relative standard deviations (RSD) were 2.7–4.6% and 0.37–5.2%, respectively.

Table 1
Precision of the determination of urinary carnosine.

	Within-day (n = 10)[a] Mean ± SD (μmol l^{-1}) RSD[c] (%)	Day-to-day (n = 3)[b] Mean ± SD (μmol l^{-1}) RSD[c] (%)
Urine 1	8.5 ± 0.32 3.8	8.5 ± 0.03 0.4
Urine 2	31.9 ± 0.87 2.7	31.6 ± 0.69 2.2
Urine 3	49.4 ± 2.27 4.6	47.7 ± 2.49 5.2

[a] Within-day precision was tested in ten replicates in 1 day.
[b] Between-day precision was tested on 3 different days.
[c] RSD = relative standard deviation.

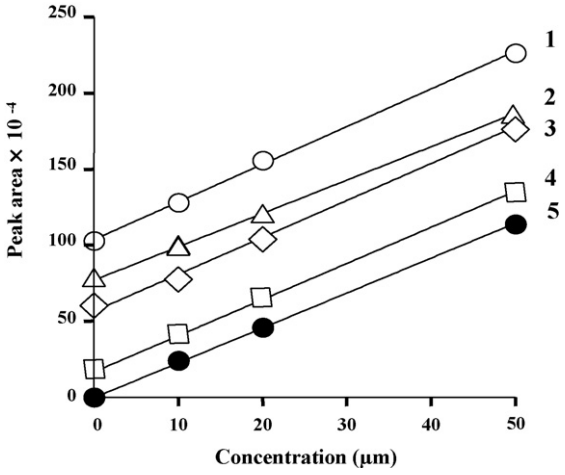

Fig. 9. Calibration curves of carnosine. Curves: 1: with urine-1 (carnosine concentration: 41.7 μmol l^{-1}; y = 2.48x + 103.5); 2: with urine-2 (carnosine concentration: 35.2 μmol l^{-1}; y = 2.19x + 77.0); 3: with urine-3 (carnosine concentration: 23.2 μmol l^{-1}; y = 2.43x + 56.5); 4: with urine-4 (carnosine concentration: 7.0 μmol l^{-1}; y = 2.38x + 16.6); 5: without urine (y = 2.30x − 0.44).

Table 2
Concentration of urinary carnosine in normal subjects.

Sample	Age	Sex[a]	Concentration		
			Carnosine (μmol l^{-1})	Cre[b] (mg/dl)	Carnosine/Cre (nmol/mg)
1	23	M	13.0	10.4	125
2	23	M	54.9	156.4	35.1
3	38	M	7.3	83.7	8.7
4	21	M	32.7	93.6	34.9
5	22	M	56.8	219.5	25.9
6	22	M	36.9	195.2	18.9
7	25	M	4.6	77.6	5.9
8	22	M	31.4	82.7	38.0
9	22	M	3.2	69.5	4.6
10	24	M	9.3	82.6	11.2
Mean ± SD (male)			25.0 ± 20.4	116.5 ± 53.9	30.8 ± 35.4
1	24	F	4.1	41.1	10.0
2	23	F	8.0	88.1	9.1
3	24	F	10.3	128.1	8.0
4	25	F	21.0	177.6	11.8
5	28	F	6.9	46.0	15.0
6	23	F	1.9	21.7	8.8
7	23	F	30.3	238.6	12.7
8	23	F	11.8	136.5	8.6
9	25	F	22.8	75.0	30.4
10	24	F	4.5	46.9	9.6
Mean ± SD (female)			12.2 ± 9.4	100 ± 69.5	12.4 ± 6.7
Mean ± SD (all)			18.6 ± 16.8	108 ± 61.1	21.6 ± 26.6

Cre was measured by Creatinine Test Wako.
[a] M = male; F = female.
[b] Cre = urinary creatinine.

The relationship between the peak area of carnosine and the concentration of carnosine was linear in the concentration range from 0.2 to 10 μmol l^{-1} ($r > 0.999$). The recovery tests were performed using urine (concentration of urinary carnosine: 7.0, 23.2, 35.2, and 41.7 μmol l^{-1}) spiked with various amounts of standard carnosine (concentration added to urine: 10, 20, and 50 μmol l^{-1} each). The mean recovery obtained from the slope ratio of regression equations with/without urine was 104.3 ± 0.81% ($n = 4$, Fig. 9.). The detection limit of carnosine was 4 fmol at a signal-to-noise ratio of 3 (S/N = 3). The detection limit of the present method is very low compared with that of a HPLC-UV method (10 pmol at S/N = 3) reported previously [10].

3.5. Determination of carnosine in human urine

The concentrations of carnosine in urine from 20 healthy volunteers (10 males: aged 21–38 years, 10 females: aged 23–28 years) who were eating self-selected diets were determined by the present method. As shown in Table 2, the mean concentration (mean ± SD) was 18.6 ± 16.8 μmol l^{-1} (21.6 ± 26.6 nmol (mg creatinine)$^{-1}$), and the mean value for males was about twofold larger than that for females. The mean value of the concentration for females (mean ± SD: 12.2 ± 9.4 μmol l^{-1}) was almost one third of that for young women (mean ± SD: 33.5 ± 40.5 μmol l^{-1}) reported previously [7]. It was likely that the concentrations of carnosine in urine were determined near the limit of quantification with the method utilizing the HPLC-UV, although the quantification limit was not shown. Therefore our method described here, as well as our quantification data of urinary carnosine, could be more reliable.

4. Conclusion

We have established a pre-column derivatization HPLC method for the determination of carnosine in urine using a fluorescent labeling reagent. As the proposed method is simple, highly sensitive, and reproducible in comparison with other methods previously reported, it could contribute to clarify physiological functions and clinical significance of carnosine.

References

[1] J.J. O'Dowd, D.J. Robins, D.J. Miller, Biochim. Biophys. Acta 967 (1988) 241.
[2] P.J. Boldyrev, QuinnAF A., V.E. Formazuyk, Mol. Aspects Med. 13 (1992) 379.
[3] R. Kohen, Y. Yamamoto, K.C. Cundy, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 3175.
[4] S.E. Gariballa, A. Sinclair, Age Ageing 29 (2000) 207.
[5] H.J. Stuerenburg, Biochemistry (Moscow) 65 (2000) 862.
[6] H. Abe, S. Ohmama, Comp. Biochem. Physiol. 88 (1987) 507.
[7] H. Abe, E. Okuma, H. Sekine, A. Maeda, S. Yoshiue, Int. J. Biochem. 25 (1993) 1245.
[8] J.J. O'Dowd, M.T. Cairns, M. Trainor, D.J. Robins, D.J. Miller, J. Neurochem. 55 (1990) 446.
[9] M. Dunnett, R.C. Harris, J. Chromatogr. 579 (1992) 45.
[10] Y. Yokoyama, S. Horikoshi, T. Takahashi, H. Sato, J. Chromatogr. A 886 (2000) 297.
[11] L. Mora, M.A. Sentandreu, F. Toldrá, J. Agric. Food Chem. 55 (2007) 4664.
[12] K. Teahon, J.M. Rideout, Biomed. Chromatogr. 6 (1992) 16.
[13] M. Dunnett, R.C. Harris, J. Chromatogr. B 688 (1997) 47.
[14] J. Schonherr, J. Agric. Food Chem. 50 (2002) 1945.
[15] M. Ducci, S. Pacchini, A. Niccolini, A. Gazzano, D. Cerri, J. Gadea, R. Bobowiec, C. Sighieri, F. Martelli, Pol. J. Vet. Sci. 9 (2006) 159.
[16] D. Nardiello, T.R.I. Cataldi, J. Chromatogr. A 1035 (2004) 285.
[17] G. Aldini, M. Orioli, M. Carini, R.M. Facino, J. Mass Spectrom. 39 (2004) 1417.
[18] M. Orioli, G. Aldini, G. Beretta, R.M. Facino, M. Carini, J. Chromatogr. B 827 (2005) 109.
[19] Y. Huang, J. Duan, H. Chen, M. Chen, G. Chen, Electrophoresis 26 (2005) 593.
[20] Y. Tsuruta, H. Inoue, Anal. Biochem. 265 (1998) 15.
[21] Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, S. Udenfriend, Anal. Biochem. 52 (1973) 595.