〈Original Article〉

Preliminary study of a high-sensitivity method to determine sarcosine in urine using high-performance liquid chromatography

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Summary In recent years, urinary sarcosine has emerged as an early detection marker for prostate cancer because it is found in increased levels in the urine of prostate cancer patients and exhibits better sensitivity and specificity in the receiver operating characteristic (ROC) curve than prostate-specific antigen (PSA). Sarcosine in urine can be analyzed using various methods that are complicated, time-consuming, and expensive.

Herein, we have developed a method for determining urinary sarcosine using high-performance liquid chromatography (HPLC) with NBD-F (4-fluoro-7-nitrobenzofurazan), a fluorescent derivatizing agent for amines.

Using this method with a C18 column and gradient elution, we obtained a single peak at a retention time of 8.37 min using an aqueous solution of derivatized sarcosine. We confirmed this peak assignment was accurate by observing disappearance of the peak after adding sarcosine oxidase (SOD), which decomposes sarcosine to a urine sample spiked with sarcosine solution.

Linearity was obtained in the sarcosine concentration range of 0–100 μmol/L. The average

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Received for Publication: April 12, 2017
Accepted for publication: May 28, 2017

ratio of sarcosine peak area to internal standard peak area was 12.95 using a 100 μmol/L sarcosine solution (n=14), with a coefficient of variation of 1.42%.

In summary, we have developed a highly sensitive and highly specific fluorescence method for determining sarcosine in urine by HPLC. Compared with gas chromatography/mass spectrometry methods, the HPLC method is simpler and allows rapid analyses.

Key words: Sarcosine, High-performance liquid chromatography, NBD-F (4-fluoro-7-nitrobenzofurazan), Sarcosine oxidase, Prostate cancer

1. Introduction

In recent years, owing to an increasingly ageing society and westernization of dietary habits, the number of people suffering from prostate cancer in Japan has increased, exceeding 70,000 in 2012. According to a nationwide estimate[1], the incidence rate for prostate cancer ranks fourth among cancers in male patients, and the prevalence rate (against a population of 100,000) has increased 26-fold since 1975. Furthermore, according to 2016 statistical cancer predictions, the number of prostate-related cancers is predicted to increase further[2]. Early-stage prostate cancer is hard to diagnose because symptoms develop only once the disease has progressed considerably. Therefore, by the time the patient experiences symptoms and seeks diagnosis, successful treatment becomes difficult. As the age of onset is expected to decrease in the future, early detection and treatment are becoming increasingly important. A highly sensitive method for the accurate quantification of markers specifically overexpressed in prostate cancer would be an effective tool for early detection[3].

Currently, prostate-specific antigen (PSA) is used as a diagnostic marker for prostate cancer. However, PSA is a protein specific to prostate tissue and can appear in other prostate diseases, such as benign prostatic hyperplasia and prostatitis. Moreover, PSA levels also increase with increasing prostate weight[4]. The specificity of PSA is only approximately 80%, making it difficult to detect in the early stages of prostate cancer. Furthermore, PSA levels may decrease during treatment with drugs, including: chlormadinone acetate (Prostal), used to treat benign prostatic hyperplasia; Finasteride (Propecia), used to treat male pattern alopecia; non-steroidal anti-inflammatory drugs; statins, used to treat dyslipidemia; and thiazide diuretics, used to treat hypertension[5,6,7].

Reference PSA levels are set at 0.0–4.0 ng/mL for all ages, making PSA difficult to detect in the early stages of prostate cancer. Furthermore, normal PSA levels have been found in some rare cases of prostate cancer[8,9].

As it has been suggested that accurately diagnose prostate cancer is not possible based on PSA levels alone, an additional rectal examination is recommended in medical checkups[10,11].

Furthermore, it is necessary to collect blood for serum PSA measurements, which involves invasive procedures. Additionally, as the method used to determine PSA levels involves immunological analyses, special reagents are required and the process is time-consuming. Moreover, to determine total PSA from free PSA and PSA bound to α_1-antichymotrypsin and α_2-macroglobulin, antibody specificity is required[12,13].

Pro PSA, a PSA precursor that can be determined with even higher sensitivity than PSA, has recently attracted wide attention and is expected to be useful for early stage detection[14,15].

A study published in *Nature* recently claimed that sarcosine levels in urine are significantly higher with prostate cancer[16]. For PSA levels of 0–20 ng/mL, no significant differences were reported. However, in the low concentration range of 0–10 ng/mL, sarcosine showed better sensitivity and specificity in the ROC curve. Furthermore, as urine was used as

the sample, the process was non-invasive.

Sarcosine (*N*-methylglycine), an amino acid with a molecular weight of 89 Da, is an intermediate in the synthesis of glycine and a degradation product in muscle and other internal tissues.

In healthy people, the glycine-*N*-methyltransferase (GNMT) gene is expressed in the nucleus. Sarcosine is synthesized from glycine by stimulating GNMT expression and is further converted to glycine by sarcosine dehydrogenase (SARDH). In prostate cancer patients, overexpression of the GNMT gene causes excessive production of sarcosine, resulting in higher sarcosine levels in urine[17]. Sarcosine is also known to induce upregulation of HER2 expression in androgen-dependent prostate cancer cells[17,18].

It has been suggested that sarcosine levels markedly increase as prostate cancer progresses and that its sensitivity and specificity are better than PSA in the receiver operating characteristic (ROC) curve. Therefore, urinary sarcosine may be a new early detection marker for prostate cancer[19].

Currently, sarcosine levels in human urine are determined using gas or liquid chromatography/mass spectrometry (GC/MS and LC/MS, respectively)[20,21] or fluorescent antibody methods[22]. However, these methods are time-consuming, cumbersome, and expensive.

Therefore, in this study, we aimed to establish a method to determine urinary sarcosine using high-performance liquid chromatography (HPLC) using a fluorescent derivatizing agent.

2. Materials and methods

2.1. Chemicals

HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid) was purchased from Dojindo Laboratories (Kumamoto, Japan). Boric acid (JIS special grade) and sodium tetraborate dehydrate (JIS special grade) were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Derivatizing reagent NBD-F (4-fluoro-7-nitrobenzofurazan) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). SOD (sarcosine oxidase, from microorganisms EC 1.5.3.1) was purchased from the Toyobo Biochemical Department (Osaka, Japan). Acetonitrile (HPLC grade) was obtained from Sigma-Aldrich Co. LLC (Tokyo, Japan) and methanol (HPLC grade) was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Trifluoroacetic acid (Wako Special Grade), sarcosine (*N*-methylglycine, Wako 1st Grade) and 6-aminohexanoic acid (Wako 1st Grade) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Hydrochloric acid (guaranteed reagent grade) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Principle

Sarcosine, a secondary amine, has no specific absorbance or fluorescence. Therefore, detecting sarcosine in chromatography requires prior column derivatization with NBD-F (4-fluoro-7-nitrobenzofurazan), a fluorescent derivatizing agent for amines. Derivatized sarcosine was detected using a fluorescence detector at 530 nm under 470 nm excitation (Fig. 1).

2.3. HPLC conditions and instrumentation

A Hitachi HPLC system (La Chrom Elite, HPLC, Tokyo, Japan) with an L-2130 pump, L-2200 autosampler, and L-2480 fluorescence detector, was used. A COSMOSIL $3C_{18}$-EB packed analytical column (4.6 × 250 mm, φ = 3 μm; Nacalai Tesque, Inc. Kyoto, Japan) was used. A D-51 glass electrode pH meter (Horiba Ltd., Kyoto, Japan), Taitec Thermo Minder Jr-100 (Taiyo Chemical Industry CO., LTD., Saitama, Japan) and Dry Thermo Unit DTU-1B (Ditech Corporation) thermostatic baths, an MX-307 micro high-speed refrigerated centrifuge (Tomy Digital Biology Co., Ltd.), and an 8910 inverter multi-frame cooling centrifuge (Kubota Seisakusho Co., Ltd.) were also used.

As shown in Fig. 2, gradient elution was performed using two eluent solutions, which were prepared as follows: (a) deionized water and methanol were mixed in a 90:10 (v/v) ratio and trifluoroacetic acid was added to a final

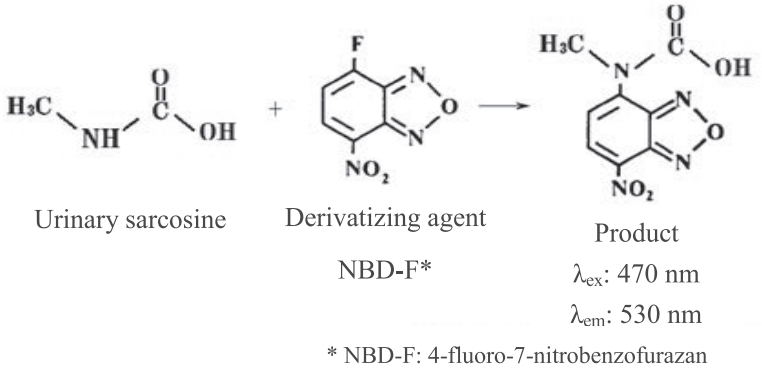

Fig. 1 Derivatization of sarcosine with a fluorescent agent.

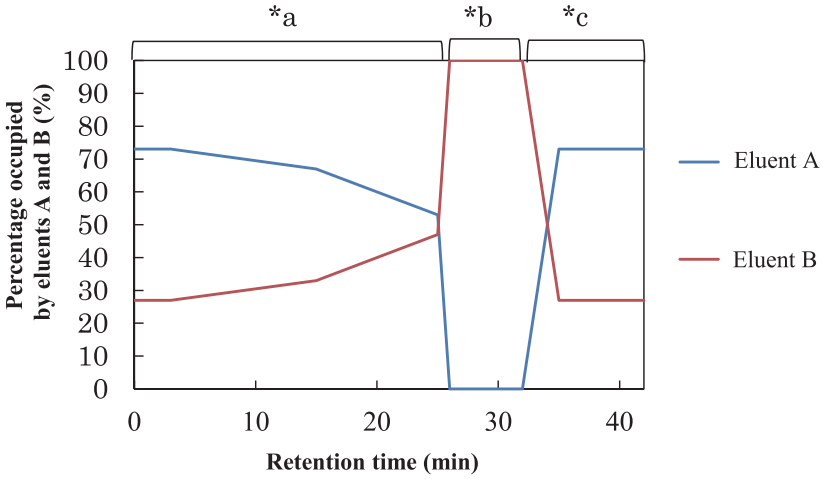

Fig. 2 Elution conditions.
*a. Elution of sarcosine and IS, b. column cleaning, c. column equilibration.

concentration of 0.12% (v/v); (b) methanol and deionized water were mixed in a 90:10 (v/v) ratio and trifluoroacetic acid was added to a final concentration of 0.12% (v/v). Fig. 2-a shows the elution of sarcosine and IS, Fig. 2-b shows the column cleaning stage, and Fig. 2-c shows column equilibration. The fluorescence of NBD-amino acid was detected at 530 nm under excitation at 470 nm. The flow rate was 1.0 mL/min, the column thermostat was set at 60 °C, the injection volume was 10 µL, and the interval between measurements was 10 min to allow the column to equilibrate.

2.4 Reagent preparation

The sarcosine stock solution was diluted to various concentrations to obtain standard solutions for the calibration curve. The 100 µmol/L reference solution was obtained by performing a 10-fold dilution of the stock solution. For the internal standard (IS) solution, certified 6-aminohexanoic acid was dissolved in purified water to a concentration of 10 mmol/L. This 10 mmol/L IS solution was then diluted 100-fold with purified water to a concentration of 100 µmol/L.

Urine samples spiked with sarcosine at various concentrations were prepared by mixing the urine of healthy volunteers with sarcosine standard solutions in a ratio of 9:1 (v/v).

2.5 Sample preparation for HPLC analysis

Deproteinization. After urine samples were centrifuged at 3000 × g for 10 min at 4 °C, 90 µL of the upper layer was separated and IS solution (40 µL), acetonitrile (160 µL), and methanol (160 µL)

were added. This mixed solution was vortexed and centrifuged at 3000 × g for 10 min to obtain the deproteinized sample in the upper layer.

Derivatization of sarcosine. A 90-μL aliquot of deproteinized sample was transferred to a screw-capped test tube and evaporated to dryness at 60 °C for 90 min. To this sample was added borate buffer (162 μL, 0.2 mol/L, pH 8.5) and NBD-F solution (36 μL, 2.5 mmol/L), followed by mixing and incubation at 50 °C for 30 s. After cooling in ice, hydrochloric acid (252 μL, 50 mmol/L) was added to stop the reaction. This final solution was the sample used for analysis.

2.6 Analytical procedures

Identification of sarcosine and IS peaks using standard solutions. To determine the retention time of sarcosine and the IS in the chromatogram, we analyzed a sample prepared according to the procedure described in the "Reagent preparation" section.

Confirmation of the sarcosine peak using a urine sample spiked with sarcosine. To confirm whether the peak ascribed to sarcosine was correct, sarcosine oxidase (SOD), which decomposes sarcosine, was added as follows: To a urine sample spiked with sarcosine (50 μL) was added 14.4 kU/L SOD solution (50 μL) and the mixture was incubated at 37 °C for 10 min.

2.7 Method validation

Calibration curve. We analyzed six sarcosine solutions with different concentrations (0, 20, 40, 60, 80, and 100 μmol/L) and constructed a calibration curve by plotting the ratio of sarcosine peak area to IS peak area against sarcosine concentration.

Recovery. Three pooled urines of known sarcosine concentration were supplemented with sarcosine, and performed the assay.

Imprecision. We analyzed the 100 μmol/L sarcosine solution for the within-day (n=14) and between-day imprecision (n=5) and calculated the average value and coefficient of variation for the ratio of sarcosine peak area to IS peak area.

Limits of detection and quantitation. The limits of detection (LOD) and quantitation (LOQ) were calculated by determining the mean ratio of the base line area at the retention time of sarcosine and IS for three measurements using purified water as the sample. The LOD and LOQ were expressed as three times the signal/noise ratio and ten times the signal/noise ratio, respectively.

3. Results

Identification of the sarcosine peak. The chromatogram of the 100 μmol/L sarcosine solution is shown in Fig. 3. The retention times (RT) of sarcosine and IS were found to be 8.37 and 21.85 min, respectively. Although the retention time shown in Fig. 3 appears to be 8.37 min, we can report that the retention time changed from 8.37 to 8.39 min depending on the analytical conditions.

Confirmation of the sarcosine peak. Fig. 4 shows chromatograms for before and after SOD treatment. In the chromatogram of non-treated urine, a peak was present at 8.39 min, but no peak was detected for the SOD-treated urine sample. Therefore, the peak at 8.39 min was confirmed to be attributed to sarcosine.

Calculation of results. Quantitative results for the internal standard method were obtained from the ratio of sarcosine peak area to IS peak area.

Calibration curve. The calibration curve showed good linearity in the sarcosine concentration range 0–100 μmol/L, with a correlation coefficient of 0.998 (Fig. 5).

Recovery. Three urines of known sarcosine concentration (4.44, 2.08, and 22.15 μmol/L) were supplemented with sarcosine (10.0, 25.0, and 50.0 μmol/L), and assayed on our method. The average analytical recovery of the three concentrations of sarcosine added to the three urines was 93.5%.

Imprecision. Within-day (n=14) and between-day imprecision (n=5) were determined by performing measurements on the 100 μmol/L sarcosine solution, which gave an average ratio of sarcosine peak area to IS peak area of 12.95 and 12.97, with a coefficient of variation of 1.42% and 3.49%, respectively.

Limits of detection and quantitation. Following

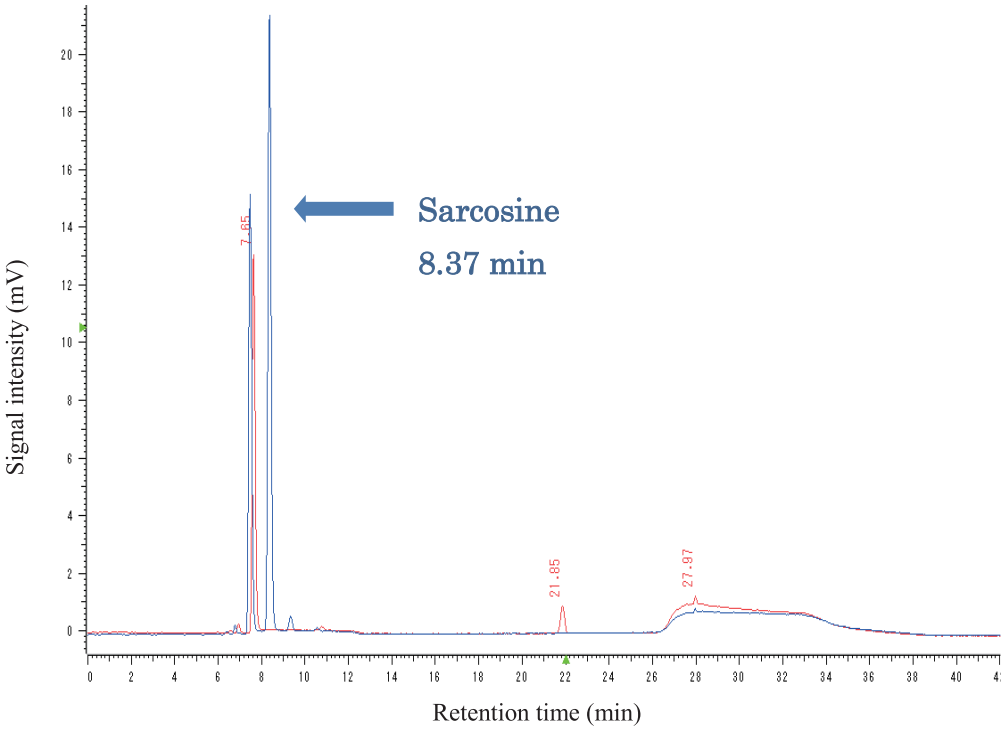

Fig. 3 Chromatogram of a 100 μmol/L sarcosine solution.
Red chromatogram: purified water (with IS). Blue chromatogram: 100 μmol/L sarcosine aqueous solution (without IS).

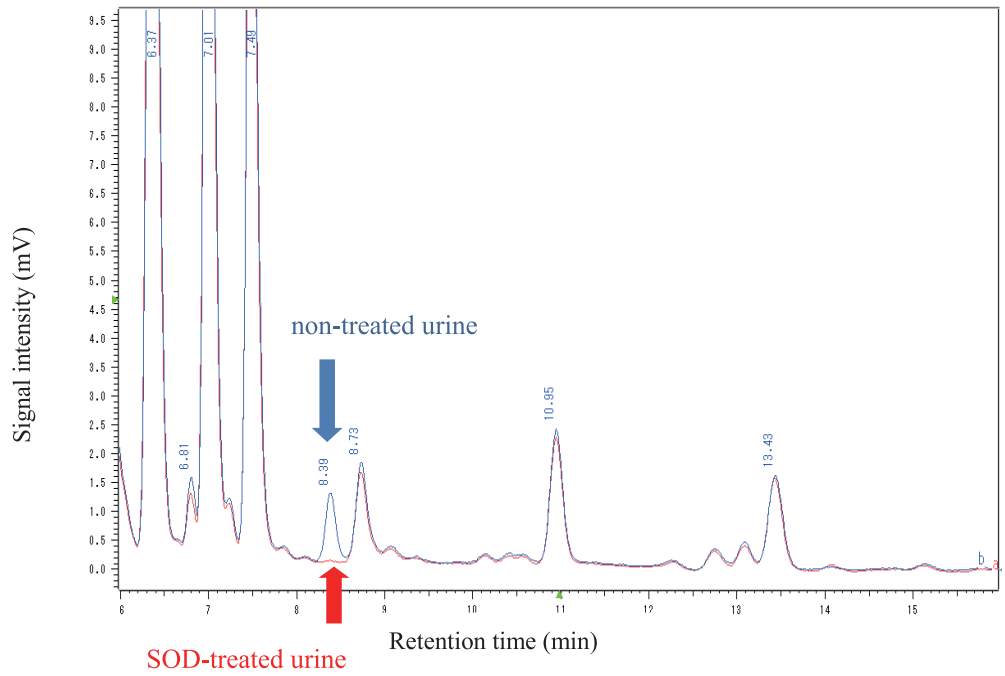

Fig. 4 Comparison of chromatograms of SOD-treated and non-treated urine samples.
The urine sample was spiked with a 100 μmol/L sarcosine solution.
Red chromatogram corresponds to urine sample treated with sarcosine oxidase (SOD); blue chromatogram corresponds to a non-treated urine sample.

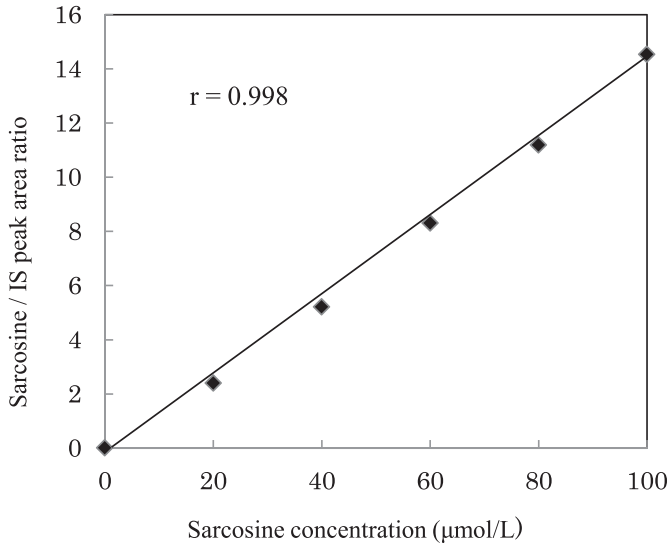

Fig. 5 Calibration curve for the sarcosine determination. Y-axis represents the ratio of sarcosine peak area to IS peak area; x-axis represents the sarcosine concentration.

the method described above, the LOD and LOQ were determined as 0.10 μmol/L and 0.33 μmol/L, respectively.

4. Discussion

According to metabolomics research, urinary sarcosine is expected to be a marker for prostate cancer, as it increases considerably during prostate cancer progression. Furthermore, using urine as the sample presents a non-invasive option to the patient. From a clinical examination perspective, urinary sarcosine is better than serum PSA, which is widely used as a screening marker for prostate cancer, both in terms of sensitivity and specificity. Therefore, in future, urinary sarcosine determination could potentially find clinical application for the diagnosis of early-stage prostate cancer.

To develop a method for measuring sarcosine levels in urine, we focused on sarcosine as a secondary amine and used NBD-F as the fluorescent derivatizing agent[23]. We established experimental conditions for determining urinary sarcosine levels and performed a method validation of the developed method.

In prostate cancer patients, the sarcosine concentration in urine is as low as approximately 5 μmol/L[16]. Therefore, the analytical method must be able to determine trace sarcosine with high sensitivity. To achieve this, conditions were set for derivatization and HPLC measurements accordingly, with NBD-F concentration, reaction time, and reaction temperature for derivatization optimized to minimize urine contaminant peaks.

The NBD-F concentration was set at 2.5 mmol/L. NBD-F reacts under weakly alkaline conditions at 60 °C within 1 min[23], but we set reaction conditions to 50 °C for 30 s to reduce the analysis temperature and time, assuming that sarcosine could react with NBD-F at a lower temperature and in a shorter time than other substances (data not shown).

Acetonitrile, widely used in HPLC analysis, was initially chosen as the solvent. Acetonitrile exhibits low absorption, providing decreased noise and higher sensitivity. Furthermore, many organic compounds are highly soluble in acetonitrile.

However, after treating the sarcosine-spiked urine sample with SOD, using acetonitrile in the analysis resulted in a peak at a retention time identical to that of the sarcosine peak. Therefore, we concluded that sarcosine was not adequately separated in acetonitrile, and methanol was selected as

the solvent instead.

Using methanol, the sarcosine peak was adequately separated. As methanol has higher viscosity, the pressure on the column increased. We examined the flow rate and minimized the pressure on the column by setting the flow rate to 1.0 mL/min, which afforded optimal peak separation for sarcosine and other substances.

Regarding column temperature, it was possible to reduce the effect of methanol viscosity by adopting an optimal upper limit of 60 °C.

We selected a gradient elution method and gradually changed the concentration gradient of the eluents until IS was eluted. Sarcosine separation was improved by reducing the slope of the concentration gradient near the eluent composition at which sarcosine was eluted.

We increased the slope after sarcosine elution to shorten analysis time. The analytical conditions are shown in Fig. 3.

The individual peaks for sarcosine and IS were first identified in the aqueous samples. Then, the chromatograms of SOD-treated and non-treated samples were compared to confirm the peak attributed to sarcosine in urine (Fig. 4).

As shown in Fig. 4, when urine sample spiked with sarcosine was treated with SOD, the peak at 8.39 min mostly disappeared, giving and almost the same area as the reference obtained with purified water. Therefore, this peak was confirmed as the only sarcosine peak.

The analysis of biological samples is complicated by the sample composition with content of both structure analog of sarcosine and inorganic components. Therefore the influence of urea (16.7 mmol/L), Uric acid (0.59 mmol/L), and creatinine (381 μmol/L) was studied. None of these components had significant on the retention time of sarcosine (data not shown). Additionally the influence of glycine, L-proline, and *N*-ethylglycine was tested due to their similarity in chemical structure. We found that it was confirmed to be different from retention time of sarcosine (data not shown).

Furthermore, the calibration curve showed good linearity from 0 to 100 μmol/L with a correlation coefficient of 0.998, as determined by method validation.

The within-day and between-day coefficient of variation for the ratio of sarcosine peak area to IS peak area were 1.42% and 3.49%, respectively. These were a good result.

Using this method, sarcosine, which is reportedly found in higher levels in the urine of prostate cancer patients, was detected as a single peak, allowing its traces to be determined. In conclusion, a highly sensitive analytical method for determining urinary sarcosine by HPLC using NBD-F as a fluorescent derivatizing agent was developed and evaluated.

Conflicts of interest

The authors declare no conflict of interests.

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