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Analysis of Urinary Amino Acids by High-Performance Liquid Chromatography with Fluorescence Detection Using 2,3-Naphthalenedicarboxaldehyde as Fluorescence Derivatization Reagent

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Abstract: Amino acids are involved in various chemical reactions in vivo, and changes in several amino acids in urine are related to certain disease states. Therefore, developing an efficient method to analyze the amino acids in urine is useful in the timely diagnosis of diseases. In this study, we developed a high-performance liquid chromatography (HPLC) fluorescence method for the quantitative analysis of urinary amino acids using the fluorescence derivatization reagent 2,3-naphthalenedicarboxaldehyde (NDA). NDA was selected because it does not require heating for the reaction and can react within a short time, rendering its use in clinical settings feasible. The reaction temperature, reaction time, and other derivatization conditions were optimized, and the reaction was found to be completed in 5 min at 25 °C. The separation of NDA–amino acids was investigated on an octadecylsilyl (ODS) column under gradient conditions. The mobile phase was a mixture of water–acetonitrile–trifluoroacetic acid. Eighteen NDA–amino acids (histidine (His), arginine (Arg), asparagine (Asn), glutamine (Gln), citrulline (Cit), serine (Ser), aspartic acid (Asp), threonine (Thr), glutamic acid (Glu), glycine (Gly), tyrosine (Tyr), alanine (Ala), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and 5-aminovaleric acid (internal standard)) were separated within 100 min under optimal conditions. The calibration curves showed good linearity in the range of 0.25–25 pmol per injection with correlation coefficients of >0.998. The limits of quantification for NDA–amino acids were 16.7–74.7 fmol. The developed analytical method was applied to a human urine sample and 16 amino acids (His, Arg, Asn, Gln, Cit, Ser, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, and Leu) were quantified. The urinary amino acid concentrations were 5–960 μM. Urinary amino acid analysis using this method is expected to be clinically applicable as a novel biomarker for diseases affecting the bladder, urinary tract, and kidneys.

Keywords: derivatization; fluorescence; urine; biomarker

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1. Introduction

"Amino acid" is a general term for organic compounds containing both amino and carboxyl functional groups. An amino acid is a component of the proteins that make up living organisms. Twenty amino acids (histidine (His), arginine (Arg), asparagine (Asn), glutamine (Gln), serine (Ser), aspartic acid (Asp), threonine (Thr), glutamic acid (Glu), glycine (Gly), tyrosine (Tyr), alanine (Ala), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), proline (Pro), and cysteine (Cys)) are the building blocks of proteins in the human body. They play various roles in protein synthesis, neurotransmission, and many other biological reactions [1–3]. For example, branched-chain amino acids (BCAAs) (Leu, Ile, and Val) are involved in the modulation of protein synthesis and the reduction of protein catabolism. Phe and Tyr are important compounds in the biosynthesis of trace amines and catecholamines such as

dopamine and adrenaline [4]. Glu and γ-aminobutyric acid are the major excitatory and inhibitory neurotransmitters, respectively.

The concentrations of amino acids in urine fluctuate within a certain range under healthy conditions. However, in various disease conditions, the metabolic pathways of some amino acids have been shown to be disrupted, resulting in changes in the amino acid concentrations [5–7]. For example, in phenylketonuria, in which the metabolic pathway of Phe is disrupted, the concentration of Phe increases in the urine and the urinary excretion of phenylalanine-derived metabolites, such as phenylpyruvate, increases [8]. Additionally, many amino acids are closely related to the biosynthesis and functioning of metabolic pathways [9]. Therefore, the concentration of a specific amino acid alone and the abnormal fluctuation of multiple amino acids have been reported to be associated with disease states. For example, urine from patients with acute renal failure, who require dialysis, has been shown to contain less BCAA metabolites and more Cys, Trp, and Glu than that from healthy subjects [10]. Thus, the multi-component analysis of urinary amino acids may be useful for detecting a high risk of disease and understanding health conditions. The comprehensive analysis of urinary amino acids might be clinically applicable to screening for diseases such as urinary tract infection and vesicoureteral reflux disease, in which the bladder, urinary tract, and kidneys are affected.

Biological samples contain a wide variety of inorganic and organic substances, and fluorescence detection is one of the most useful methods for analyzing trace amounts of amino acids owing to its high selectivity and sensitivity [11,12]. However, amino acids other than aromatic amino acids (Trp, Tyr, and Phe) do not emit fluorescence; therefore, the amino acids must undergo derivatization for fluorescence detection, which is also useful for chromatographic separation on an octadecylsilyl (ODS) column [12]. Derivatization often involves the modification of amino groups. As described in a review article [12], many derivatization reagents, such as 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), o-phthaldialdehyde (OPA), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and 1,3,5,7-tetramethyl-8-(N-hydroxysuccinimidyl butyric ester)difluoroboradiaza-s-indacene (TMBB-Su), have been applied for fluorescence detection of amino acids. AQC and NBD-F do not react well at room temperature and the reaction requires the addition of heat. However, OPA can be derivatized within a few minutes at room temperature with a reaction in the presence of a thiol, and no heating is required [13]. Nevertheless, OPA derivatives are unstable, and their combination with pre-column fluorescence derivatization is inaccurate. 2,3-Naphthalene-dicarboxyaldehyde (NDA), an analog reagent of OPA, can react at room temperature in the presence of cyanide ions [14,15]. In addition, NDA derivatives have been shown to exhibit a higher sensitivity and stability than OPA derivatives [16].

Therefore, in this study, NDA was chosen as a highly sensitive fluorescence derivatization reagent that does not require heating during the reaction and can be useful for on-site analysis in clinical chemistry (Figure 1). To the best of our knowledge, there have been no reports of the multi-component analysis of amino acids in urine using the high-performance liquid chromatography (HPLC)-fluorescence method with NDA. Roach and Harmony performed the pioneer work concerning LC separation of NDA–amino acids, and 16 NDA–amino acids (His, Arg, Asn, Gln, Ser, Asp, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, and Met) were separated on an ODS column [14]. However, Gly and Thr were not well-separated. Moreover, it was not applied to biological samples such as urine. We optimized the conditions for the fluorescence derivatization of NDA–amino acids and the mobile phase and validated the developed method. Furthermore, the developed method was applied to human urine samples.

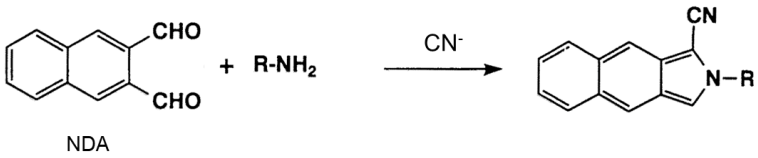

Figure 1. Reaction scheme of NDA and amine in the presence of cyanide ions.

2. Materials and Methods

2.1. Reagents

Amino acid mixture standard solution Type H, 5-aminovaleric acid (5-AVA), and boric acid were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Asparagine monohydrate, glutamine, tryptophan, citrulline, histidine, arginine, asparagine, serine, aspartic acid, threonine, glutamic acid, glycine, tyrosine, alanine, valine, phenylalanine, isoleucine, leucine, methionine, lysine, cystine, 6-aminocaproic acid, potassium cyanide, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,3-Naphtlenedicarboxaldehyde (NDA) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was from Dojindo Laboratories (Kumamoto, Japan). Water was purified with a Milli-Q system (Merck Millipore). Other reagents were of analytical grade.

2.2. Sample Preparation

The urine of four healthy male (21–23 years of age) volunteers was obtained with informed consent. The urine samples were stored at −30 °C until use. Before derivatization, the urine was diluted one hundred times with 10 mM 5-AVA solution. To a solution containing amino acids (30 µL of a standard aqueous solution or 30 µL of diluted human urine), 24 µL of 400 mM borate buffer (pH 8.5), 3 µL of 100 mM potassium cyanide solution, and 3 µL of 100 mM NDA in acetonitrile were added. The mixture was reacted at 25 °C under light-shielded conditions for 5 min; thereafter, 60 µL of water was added. Aliquots (3 µL) were injected into the HPLC system.

For NBD-F derivatization, the following procedure was used based on our previous studies [17–19]. To the amino acid solution (10 µL of standard or 10 µL of diluted human urine), 70 µL of 400 mM borate buffer (pH 8.5), 10 µL of 10 mM 6-aminocaproic acid solution as an internal standard, and 10 µL of 20 mM NBD-F in acetonitrile were added. The mixture was reacted at 60 °C under light-shielded conditions for 5 min; subsequently, 100 µL of 0.1 N HCl was added. Aliquots (5 µL) were injected into the HPLC system.

2.3. High-Performance Liquid Chromatography Conditions

The HPLC system was composed of a pump (PU-980, JASCO, Tokyo, Japan), an auto sampler (AS-2057 Plus, JASCO, Tokyo, Japan), a column oven (CO-1560, JASCO, Tokyo, Japan), and a fluorescence detector (FP-920S, JASCO, Tokyo, Japan); Chromato-Pro (Run Time Corporation, Kanagawa, Japan) software was used to analyze the chromatograms. The column temperature was 40 °C. Inertsil ODS-4V (250 mm × 3 mm i.d., 5 µm; GL Sciences, Tokyo, Japan) was used as the analytical column.

Amino acids derivatized with NDA were detected using fluorescence with excitation and emission wavelengths of 420 and 480 nm, respectively, and separated using a binary solvent system running under gradient conditions with the composition of the mobile phase. Solvent A was a mixture of water–acetonitrile–TFA (90/10/0.1, *v*/*v*/*v*), and solvent B was a mixture of water–acetonitrile–TFA (10/90/0.1, *v*/*v*/*v*). The flow rate was 0.5 mL/min. The gradient elution was carried out as follows: 0 min, 25% B; 20 min, 25% B; 95 min, 50% B; 95.1 min, 100% B; 105 min, 100% B; 105.1 min, 25% B; 120 min, 25% B.

Amino acids derivatized with NBD-F were detected using fluorescence with excitation and emission wavelengths of 470 and 530 nm, respectively, and separated using a binary solvent system running under gradient conditions with the composition of the mobile phase. Solvent A was an aqueous solution containing 10 mM citrate buffer (pH 6.2), 75 mM $NaClO_4$, and solvent B was a mixture of water and acetonitrile (50/50, *v*/*v*). The flow rate was 0.6 mL/min. The gradient elution was carried out as follows: 0–20 min, 10% B to 50% B; 20–30 min, 50% B to 100% B; 30–34 min, 100% B.

The resolutions (Rs) were calculated using the following equation:

$$Rs = 2\,(t_{R1} - t_{R2})/(W_1 + W_2) \quad (1)$$

where t_{R1} = retention time of compound 1, t_{R2} = retention time of compound 2, and W is the baseline peak width. Triplicate determinations were performed for the measurements to obtain mean values.

2.4. Validation

The analytical method was validated according to the guidelines of the International Conference on Harmonization (ICH) [20]. The limit of detection (LOD) was determined as the sample concentration that produced a peak with a height three times the level of the baseline noise, and the limit of quantification (LOQ) was calculated that produced a peak with 10 times the signal-to-noise ratio. Linearity was studied by measuring six concentrations of standard amino acid solutions in the range 0.25–25 pmol (0.25, 0.5, 2.5, 5, 10, and 25 pmol). Concerning the intra-day precision (n = 4) and inter-day precision (4 days, n = 4/day), the injection amounts of amino acids for the lower limit of quantification (LLOQ), low, middle, and high were 0.25, 2.5, 10, and 25 pmol, respectively. The precision of the urine samples was verified by injecting the samples four times on the same day. The accuracy was confirmed by adding a standard solution of three concentrations to the urine sample in the linearity range. The spiked quantities of amino acids were 0.25 pmol (0.625 pmol for His, Ser, Gly, and Ala) (low), 1.56 pmol (middle), and 12.5 pmol (high). For the accuracy determination, a mixture of one-hundred-times diluted urine (25 μL) and amino acid standard aqueous solution (5 μL) was used.

3. Results and Discussion

3.1. Investigation for NDA–Amino Acid Derivatization Conditions

Optimizing the derivatization conditions is crucial, as there are many factors that influence the reaction, such as the reaction pH, temperature, time, and derivatization reagent concentrations.

First, the effects of the concentrations of NDA (25–200 mM) and KCN (25–300 mM) were investigated on the fluorescence intensities of the NDA–amino acids. The peak intensities of all the analytes increased with increasing NDA and KCN concentrations up to 100 mM (Figure 2). Higher KCN concentrations reduced the reaction yield. CN^- is known to condense two aromatic aldehydes to form α-hydroxyketones, and because there are two adjacent aldehyde groups in NDA, many condensates are possible in principle [14]. Therefore, if the molar ratio of KCN to NDA is greater than 1:1, it would favor the antagonistic mechanism of benzoin condensation and reduce the effective NDA concentration available for the derivatization of amino acids. The optimum concentration for NDA and KCN was determined to be 100 mM.

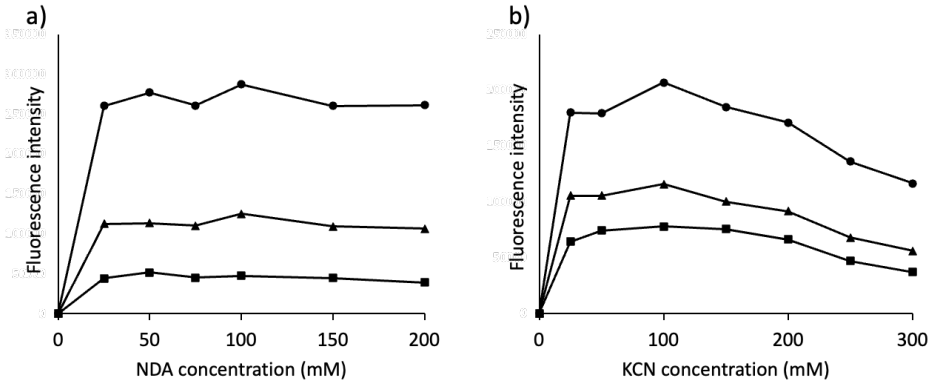

Figure 2. Effects of (**a**) NDA and (**b**) KCN concentrations on fluorescence intensities of NDA–amino acids. ●, His; ■, Phe; ▲, Trp.

Subsequently, the reaction temperature and time were examined. The derivatization of amino acids with NDA was performed at 25, 30, 40, 50, 60, and 70 °C for 5 min. No increase in the fluorescence intensity was observed with increasing temperature. Next, the

reaction times were investigated at 0.5, 1, 5, 15, 30, and 60 min at 25 °C. While the fluorescence intensity increased significantly by 5 min, no significant changes in the fluorescence intensity were observed after 5 min. Accordingly, the fluorescence derivatization of amino acids with NDA was performed at 25 °C for 5 min.

3.2. Optimization of Separation Conditions of NDA–Amino Acids

The mobile phase conditions were examined for the efficient separation of 17 NDA–amino acids (His, Arg, Asn, Gln, Cit, Ser, Asp, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, and Leu) and an internal standard (5-aminovaleric acid). NDA does not react with secondary amino acids such as Pro. Moreover, it has been reported that multi-derivatized amino acids such as Lys and Cys cannot be detected because of fluorescence quenching. Under the initial conditions (solvent A was a mixture of water–acetonitrile–TFA (90/10/0.05, $v/v/v$) and solvent B was a mixture of water–acetonitrile–TFA (10/90/0.05, $v/v/v$). The gradient elution was carried out as follows: 0 min, 25% B; 20 min, 25% B; 60 min, 50% B; 75 min, 60% B; 75.1 min, 100% B; 90 min, 100% B). The separation of NDA–Ile and NDA–Leu, which were eluted last, was low (Rs = 0.61). Hence, TFA concentrations (0.025%, 0.05%, 0.75%, and 0.1%) were examined, and with 0.1% TFA, the best resolution was obtained (Rs = 0.81). Further, by modifying and optimizing the gradient conditions, the separation of NDA–Ile and –Leu was increased to 0.86. Finally, 18 NDA–amino acids (His, Arg, Asn, Gln, Cit, Ser, Asp, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, Leu, and 5-AVA (internal standard)) were separated within 100 min, as shown in Figure 3a. Notably, the first paper on NDA–amino acid separation using LC did not separate the two amino acids, as Ile and Leu have the same molecular weight [14].

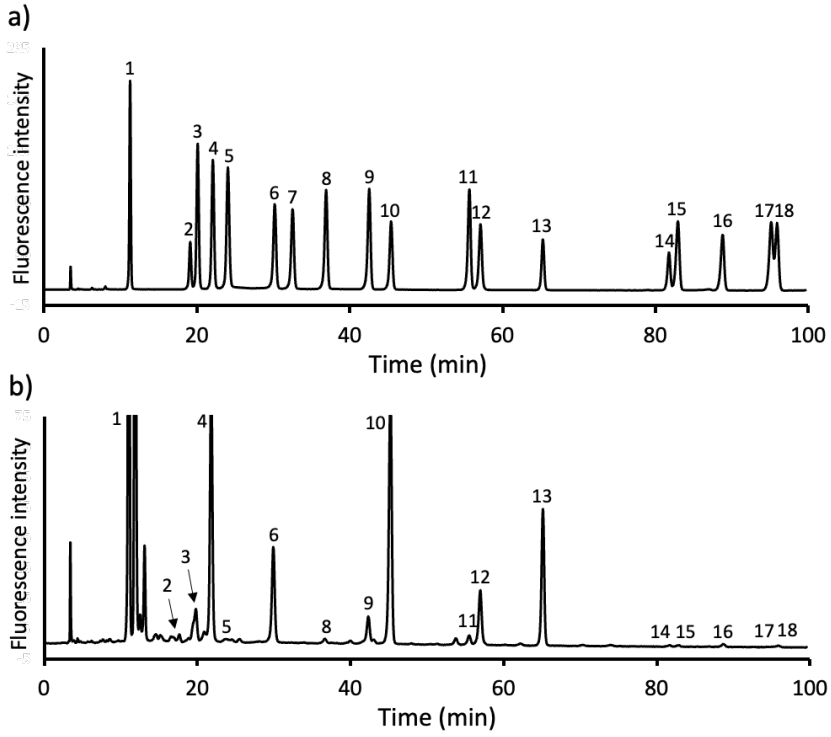

Figure 3. Chromatograms of (**a**) NDA–amino acid standards and (**b**) human urine sample. Inertsil ODS-4V (250 mm × 3 mm i.d., 5 µm) was used for separation at 40 °C. Solvent A was a mixture of water–acetonitrile–TFA (90/10/0.1, $v/v/v$), and solvent B was a mixture of water–acetonitrile–TFA (10/90/0.1, $v/v/v$). The flow rate was 0.5 mL/min. The gradient elution was carried out as follows: 0 min, 25% B; 20 min, 25% B; 95 min, 50% B; 95.1 min, 100% B; 105 min, 100% B; 105.1 min, 25% B; 120 min, 25% B. NDA–amino acids were detected using fluorescence with excitation at 420 nm and emission at 480 nm. Peaks: 1, His; 2, Arg; 3, Asn; 4, Gln; 5, Cit; 6, Ser; 7, Asp; 8, Thr; 9, Glu; 10, Gly; 11, Tyr; 12, Ala; 13, 5-AVA (IS); 14, Trp; 15, Val; 16, Phe; 17, Ile; 18, Leu.

3.3. Method Validation

The assay was validated with calibration curves, precision, and accuracy using amino acid standards and urine samples. The validation data for these standards are listed in Table 1. The LODs of 17 NDA–amino acids (His, Arg, Asn, Gln, Cit, Ser, Asp, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, and Leu) were in the range of 5.0 to 22.4 fmol per injection, and the LOQs were in the range of 16.7 to 74.7 fmol per injection. The values are similar to those in the previous study [14]. For each NDA–amino acid, the calibration curves were linear and exhibited high correlation coefficients ($r^2 > 0.998$). As shown in Table 2, the RSD values for intra-day ($n = 4$) and inter-day ($n = 4$) precision with amino acid standards were ~0.6–13.2% and ~1.0–15.5%, respectively.

Table 1. Linearity and sensitivity for the determination of NDA–amino acids.

Amino Acids	Limit of Detection (LOD) (fmol)	Limit of Quantification LOQ (fmol)	Calibration Curve		
			Range (pmol/inj)	Equation	r^2
His	5.0	16.7	0.25–25	y = 0.076x + 0.0151	0.999
Arg	22.4	74.7	0.25–25	y = 0.017x + 0.0039	0.999
Asn	6.2	20.7	0.25–25	y = 0.054x + 0.0039	1.000
Gln	6.2	20.7	0.25–25	y = 0.067x + 0.0089	0.999
Cit	7.2	24.0	0.25–25	y = 0.044x + 0.0053	0.999
Ser	9.8	32.7	0.25–25	y = 0.038x − 0.0026	1.000
Asp	9.0	30.0	0.25–25	y = 0.044x + 0.0068	0.999
Thr	7.1	23.7	0.25–25	y = 0.050x + 0.0164	0.999
Glu	6.2	20.7	0.25–25	y = 0.055x − 0.0011	1.000
Gly	8.2	27.3	0.25–25	y = 0.052x − 0.0001	0.998
Tyr	6.3	21.0	0.25–25	y = 0.063x − 0.0081	1.000
Ala	8.2	27.3	0.25–25	y = 0.057x − 0.0173	0.999
Trp	13.4	44.7	0.25–25	y = 0.038x − 0.0044	0.999
Val	6.1	20.3	0.25–25	y = 0.064x + 0.007	0.999
Phe	6.9	23.0	0.25–25	y = 0.059x + 0.0023	0.999
Ile	5.9	19.7	0.25–25	y = 0.029x + 0.0015	0.999
Leu	6.1	20.3	0.25–25	y = 0.035x − 0.0061	0.998

Calibration curves were constructed by plotting the amounts of NDA–amino acids (x, injection amount, pmol) and the ratios of peak area of NDA–amino acid to that of internal standard.

Table 2. Intra- and inter-day precisions of the developed method for determination of NDA–amino acids.

Amino Acids	Precision (RSD, %)							
	Intra-Day ($n = 4$)				Inter-Day ($n = 4$)			
	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
His	4.0	4.6	2.7	1.5	12.6	3.6	4.6	8.4
Arg	3.0	3.6	2.7	2.6	6.8	9.1	9.9	9.1
Asn	3.8	2.4	3.0	1.3	12.2	7.4	7.2	6.8
Gln	4.3	2.1	3.2	1.6	10.5	6.3	5.6	8.5
Cit	9.0	1.6	2.9	1.0	12.5	7.3	6.8	6.6
Ser	7.8	2.3	1.9	1.1	10.1	4.2	2.5	6.2
Asp	7.9	4.1	3.5	2.8	3.8	3.4	1.0	7.6
Thr	10.1	3.8	4.0	2.3	5.2	3.8	2.1	6.5
Glu	2.8	2.0	2.7	2.1	9.0	4.0	2.0	7.4
Gly	1.4	2.5	2.1	1.3	15.5	5.9	7.0	4.5
Tyr	3.8	0.6	3.3	1.4	6.1	5.9	3.9	6.0
Ala	13.2	2.5	1.1	1.0	15.3	2.0	2.1	5.3
Trp	12.6	9.6	1.8	2.6	13.5	4.6	1.7	7.0
Val	4.7	3.0	3.9	1.0	9.1	4.0	3.0	5.9

Table 2. *Cont.*

Amino Acids	Precision (RSD, %)							
	Intra-Day (n = 4)				Inter-Day (n = 4)			
	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
Phe	6.0	3.5	3.4	1.5	10.4	4.7	4.2	7.7
Ile	5.7	1.3	8.9	4.9	3.5	4.3	4.3	9.3
Leu	7.3	4.3	3.9	3.8	10.6	4.5	3.6	7.7

LLOQ: Lower limits of quantification.

Table 3 shows the accuracy and precision of the urine samples. With respect to precision, the RSD values are in the range ~0.8–8.2% (n = 4). The accuracy was confirmed by adding three concentrations of standard amino acids to the urine sample and calculating the recovery rate. The accuracies were in the range ~82.8–119.2%. The stability of the NDA–amino acids was investigated at 4 °C, and all NDA–amino acids were found to be stable for 12 h. However, after 24 h, the peak intensities of NDA–amino acids were reduced to less than 80%. These results confirm the applicability of the proposed method to determine amino acids in urine samples.

Table 3. Amino acid concentrations in urine samples and precision and accuracy of amino acids in urine samples.

Amino Acids	Concentration in Urine (μM) (n = 4, Mean ± SD)	Precision (RSD, %)	Accuracy (%)		
			Low	Middle	High
His	922 ± 70	1.4	86.3	117	100
Arg	13.7 ± 1.4	5.2	95.4	101	97.1
Asn	59.3 ± 1.5	4.0	99.1	96.8	101
Gln	956 ± 63	2.1	85.1	104	101
Cit	16.2 ± 1.0	3.6	119	100	107
Ser	315 ± 10	1.7	119	101	101
Thr	4.7 ± 0.2	4.8	88.8	102	101
Glu	106.3 ± 4.7	2.6	82.8	99.4	97.0
Gly	801 ± 27	0.8	91.9	104	102
Tyr	30.6 ± 1.9	8.2	100	96.9	99.5
Ala	306 ± 4	2.1	110	101	97.2
Trp	10.6 ± 1.0	3.5	89.7	98.5	101
Val	12.0 ± 1.1	3.7	105	101	101
Phe	15.2 ± 1.2	5.8	96.3	104	94.8
Ile	4.7 ± 0.2	7.7	84.8	103	98.8
Leu	9.2 ± 0.5	3.9	102	105	95.3

3.4. Application to Urine Samples

This optimized analytical method was applied to urine samples. First, the pretreatment of urine samples was examined: urine samples diluted 10, 20, 50, and 100 times with water were derivatized and analyzed; the changes in fluorescence intensities when converted to the pre-dilution concentration were examined. The results for Asn and Phe are shown in Figure 3.

Although only the data for the two amino acids (Asn and Phe) are shown in Figure 4, similar trends were observed for the other amino acids. The fluorescence intensity of the amino acids tended to increase as the dilution factor increased. A dilution factor of 100 times was chosen because there was no significant difference between the 50- and 100-times dilutions, and the concentrations of the target analytes in the urine samples

were too low with the >100-times dilution. At low urine-dilution factors, amino acid quantification is affected by the compounds present in the urine.

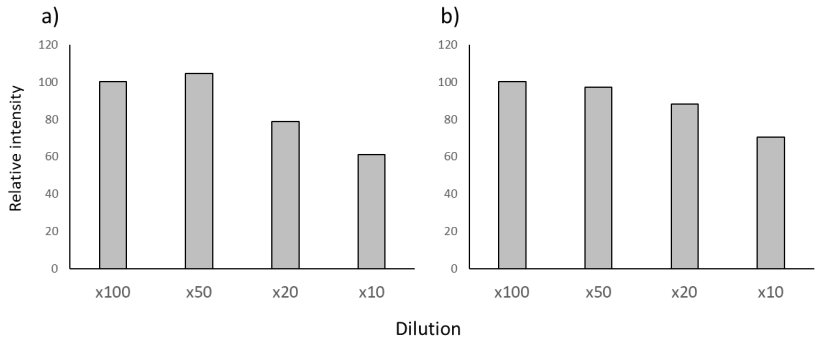

Figure 4. Effect of urine dilution on fluorescence intensity of (**a**) NDA–Asn and (**b**) NDA–Phe. The relative intensities are shown when the 100-times dilution is set as 100.

After the dilution of the urine sample, the sample was derivatized with NDA under optimum conditions, followed by HPLC–fluorescence detection. The chromatograms obtained from the urine samples are shown in Figure 3b. Sixteen NDA–amino acids other than Asp (His, Arg, Asn, Gln, Cit, Ser, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, and Leu) could be detected without interference from endogenous urinary compounds. NDA–Asp was not detected as the peak was too small. The amino acid concentrations in each urine sample are summarized in Table 3. These values are consistent with those reported previously [21].

We previously developed analytical methods for amino acids using NBD-F as a fluorescence derivatization reagent [17]. Therefore, we analyzed the same urine samples using this method. The derivatization conditions for NBD-F with amino acids and the LC conditions are described in the Materials and Methods section. The urinary amino acid concentrations were not significantly different between NDA and NBD-F. This suggests that urinary amino acid analysis using NDA is suitable for routine analysis.

4. Conclusions

In this study, an HPLC–fluorescence detection system was developed and validated for the determination of amino acids in human urine samples. The method was successfully applied to the analysis of 16 amino acids (His, Arg, Asn, Gln, Cit, Ser, Thr, Glu, Gly, Tyr, Ala, Trp, Val, Phe, Ile, and Leu) in urine. The present analytical method has sufficient sensitivity and selectivity for the investigation of amino acids in urine samples. Urinary amino acid analysis using this method is expected to be clinically applicable as a novel biomarker for diseases affecting the bladder, urinary tract, and kidneys, and further studies using samples from diseased individuals are ongoing.

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