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Measurement of ^{15}N enrichment of glutamine and urea cycle amino acids derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate using liquid chromatography–tandem quadrupole mass spectrometry

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ABSTRACT

6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) is an amino acid-specific derivatizing reagent that has been used for sensitive amino acid quantification by liquid chromatography–tandem quadrupole mass spectrometry (LC–MS/MS). In this study, we aimed to evaluate the ability of this method to measure the isotopic enrichment of amino acids and to determine the positional ^{15}N enrichment of urea cycle amino acids (i.e., arginine, ornithine, and citrulline) and glutamine. The distribution of the *M* and *M* + 1 isotopomers of each natural AQC–amino acid was nearly identical to the theoretical distribution. The standard deviation of the (*M* + 1)/*M* ratio for each amino acid in repeated measurements was approximately 0.1%, and the ratios were stable regardless of the injected amounts. Linearity in the measurements of ^{15}N enrichment was confirmed by measuring a series of ^{15}N-labeled arginine standards. The positional ^{15}N enrichment of urea cycle amino acids and glutamine was estimated from the isotopic distribution of unique fragment ions generated at different collision energies. This method was able to identify their positional ^{15}N enrichment in the plasma of rats fed ^{15}N-labeled glutamine. These results suggest the utility of LC–MS/MS detection of AQC–amino acids for the measurement of isotopic enrichment in ^{15}N-labeled amino acids and indicate that this method is useful for the study of nitrogen metabolism in living organisms.

Among the precolumn derivatization procedures for amino acid analysis, the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)[1] method is widely accepted. AQC reacts with primary and secondary amines to produce highly stable fluorescent derivatives. This derivatization reaction is straightforward and can be completed within 10 min. The AQC derivatization method has been used in conjunction with high-performance liquid chromatography (HPLC) [1,2] or ultra-performance liquid chromatography (UPLC) [3,4] for the measurement of amino acid concentrations.

Armenta and coworkers applied the AQC method together with UPLC coupled with tandem quadrupole mass spectrometry (MS/MS) for fast, selective, and sensitive amino acid quantification in biological samples [5]. Because amino acids derivatized with AQC generate the *m*/*z* 171 ion in MS/MS, this specific ion is selected as the daughter ion in all selected reaction monitoring (SRM) transitions. Although it has also been used to measure the isotopic enrichment of amino acids [6,7], insufficient data are available regarding the accuracy and precision of the AQC method in measuring the isotopic enrichment of amino acids.

Urea cycle amino acids such as arginine, ornithine, and citrulline have various biological functions. They play important roles in not only synthesizing urea in the liver but also generating bioactive substances such as nitric oxide, creatinine, polyamines, and proline [8,9]. For the investigation of their metabolism in vivo, tracer techniques using stable isotopes are essential, and these techniques require sensitive analytical methods for determining isotopic enrichment. Although various methods to measure

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[1] *Abbreviations used:* AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; MS/MS, tandem quadrupole mass spectrometry; SRM, selected reaction monitoring; GC–MS, gas chromatography–mass spectrometry; N, nitrogen; LC, liquid chromatography; ESI, electrospray ionization; SD, standard deviation; CV, coefficient of variation.

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isotopic enrichment with gas chromatography–mass spectrometry (GC–MS) have been reported [10–12], they are not entirely satisfactory because of complicated precolumn derivatization steps or contaminant peaks, as reported previously [13]. Furthermore, the measurement of the positional ^{15}N enrichment of each nitrogen (N) in urea cycle amino acids is important for more precise investigation of their N metabolism because one amino acid contains multiple N atoms of distinct origins. Marini reported a method to measure the positional ^{15}N enrichment of glutamine and citrulline derivatized with dansyl chloride [14]. However, there has been no report of positional ^{15}N enrichment using AQC as a derivatization reagent despite the advantages of this derivatization method compared with others [15].

In the current study, we evaluated the accuracy of the AQC method for measuring the ^{15}N enrichment of amino acids and developed new methods for determining the positional ^{15}N enrichment of urea cycle amino acids and glutamine. Furthermore, we investigated the efficacy of these methods for analyzing biological samples.

Materials and methods

Materials

A solution of 41 authentic amino acids was prepared from commercially available L-amino acid solutions (type B and AN-II, Wako Pure Chemical Industries, Osaka, Japan) [16] and crystallized L-glutamine, L-tryptophan, and L-asparagine (Wako Pure Chemical Industries). ^{15}N-labeled L-arginine (U-$^{15}N_4$ and guanidino-$^{15}N_2$) and ^{15}N-labeled L-glutamine (2-^{15}N and 5-^{15}N) (>98% ^{15}N for all) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The AccQ-Fluor Reagent Kit was purchased from Waters (Milford, MA, USA). Acetic acid and acetonitrile were purchased from Junsei Chemical (Tokyo, Japan).

Derivatization of amino acids with AQC

AQC was dissolved in acetonitrile to a concentration of 10 mg/ml. A 10 µl aliquot of the authentic amino acid solution was mixed with 30 µl of 0.2 M sodium borate buffer at pH 8.8 in a sealed 1.5 ml polypropylene microtube. A 10 µl aliquot of the AQC reagent was added to the above solutions, and the mixture was heated at 55 °C for 10 min. A 200 µl aliquot of 0.2% aqueous formic acid was then added to the reaction mixture. The samples were stored in a tightly sealed container at 4 °C until liquid chromatography (LC)–MS/MS analysis.

Preparation of blood samples

All animal studies were reviewed and approved by the animal care committee of Ajinomoto. Male 6-week-old Fischer (F344) rats (Charles River Japan, Atsugi, Japan) were maintained under controlled conditions with 12 h cycles of dark (10:00–22:00) and light (22:00–10:00) at 23 ± 1 °C and 55 ± 10% humidity. The rats were fed an amino acid-defined purified diet based on the AIN-93G composition [17]. The amino acid composition followed the recommendations of the National Research Council (values in g/kg: arginine, 4.3; histidine, 2.8; isoleucine, 6.2; leucine, 10.7; lysine hydrochloride, 11.5; methionine, 6.5; cysteine, 3.3; phenylalanine, 6.8; tyrosine, 3.4; threonine, 6.2; tryptophan, 2.0; valine, 7.4; alanine, 4.0; aspartic acid, 4.0; glycine, 6.0; proline, 4.0; serine, 4.0; asparagine monohydrate, 4.6; glutamic acid, 20.0; glutamine, 20.0; Ajinomoto, Tokyo, Japan). The rats were adapted to these conditions for 2 weeks and were then fed hourly with 1.0 g (~80% of the ad libitum intake) of an experimental diet in which all glutamine was replaced with ^{15}N-labeled glutamine (2-^{15}N or 5-^{15}N). Thus, hourly consumption rates of [2-^{15}N]glutamine and [5-^{15}N]glutamine were 20 mg/h. Six hours after the initiation of feeding, the rats were anesthetized with ether to collect blood from the descending aorta. Blood containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant was centrifuged at 2010*g* for 20 min at 4 °C to separate the plasma. The plasma samples were stored at −80 °C until analysis. A 20 µl aliquot of the pooled plasma was deproteinized by adding 80 µl of methanol. A 10 µl aliquot of the deproteinized supernatant was derivatized with AQC as described above.

Instrumentation

An Agilent 1200 series LC system (Agilent Technologies, Waldbrunn, Germany) with a binary pump, degasser, autosampler, column compartment, and ultraviolet (UV) detector was used. The system was coupled to a triple-quadrupole Agilent 6400 series mass spectrometer (Agilent Technologies) equipped with a JetStream interface.

LC–MS/MS selected reaction monitoring for quantitative analysis of amino acid derivatives

Amino acid derivatives were injected into an Ascentis Express C18 HPLC column (2.7 µm particle size, 15 cm × 2.1 mm length × inner diameter, Supelco, Bellefonte, PA, USA). The column temperature was maintained at 40 °C. Mobile phase A was 25 mM formic acid (pH 6.0 adjusted with aqueous ammonium), and mobile phase B consisted of 60% acetonitrile in MilliQ water (v/v). The gradient conditions (B%) were 0.00 min = 10%, 0.00 to 21.00 min = 10 to 20%, 21.00 to 21.01 min = 20 to 40%, 21.01 to 23.00 min = 40 to 80%, 23.00 to 23.01 min = 80 to 100%, 23.01 to 25.00 min = 100%, 25.00 to 25.01 = 100 to 10%. Reequilibration was performed for 10 min at 10% of mobile phase B. The flow rate was 0.2 ml/min. The autosampler was maintained at 4 °C. The electrospray ionization (ESI) was operated in the positive mode at 4000 V. Other MS parameters were as follows: nebulizer pressure, 15 psi; nozzle voltage, 1500 V; drying gas temperature, 300 °C; drying gas flow, 10 L/min; capillary voltage, 4000 V; sheath gas temperature, 400 °C; sheath gas flow, 12 L/min; fragmentor voltage, 120 V. Quantitative analysis of the amino acids was performed by SRM. MassHunter software (Agilent) was used for HPLC system control, data acquisition, and data processing.

Fragmentation

A collision energy of 25 eV generated the common product ion of *m*/*z* 171 originating from the AQC molecule for all amino acids. The ^{15}N enrichment of amino acids was estimated from the mass distribution of their precursor ions using the common fragment *m*/*z* 171 as a reporter ion, as shown in Table 1. In cases where amino acids contained multiple N atoms in a single molecule, the enrichments were the sum of those in all Ns because the precursor ions contained all of the N atoms. For the measurement of positional ^{15}N enrichments in urea cycle amino acids (arginine, citrulline, and ornithine) and glutamine, other fragment ions generated from the same precursor ions were analyzed. The ^{15}N enrichment of the alpha-amino Ns of these amino acids was estimated using smaller fragment ions (*m*/*z* 70 for urea cycle amino acids and *m*/*z* 84 for glutamine) generated at collision energies of 25 and 38 eV. For the measurement of the guanidino N of arginine, *m*/*z* 158 generated at a collision energy of 25 eV, which lacks one of the two

Table 1
Mass spectral settings for each ^{15}N measurement.

Test amino acid (positional N)	Collision energy (eV)	Monitoring ions (precursor → reporter, *m/z*)	
		M	*M* + 1
M + 1 enrichment for whole molecule			
Alanine	25	260 → 171	261 → 171
Arginine	25	345 → 171	346 → 171
Asparagine	25	303→ 171	304→ 171
Aspartic acid	25	304 → 171	305 → 171
Citrulline	25	346 → 171	347 → 171
Glutamic acid	25	318 → 171	319 → 171
Glutamine	25	317 → 171	318 → 171
Glycine	25	246 → 171	247 → 171
Histidine	25	326 → 171	327 → 171
Lysine[a]	25	487 → 171	488 → 171
Methionine	25	320 → 171	321 → 171
NH_3	25	188 → 171	189 → 171
Ornithine[a]	30	473 → 171	474 → 171
Phenylalanine	25	336 → 171	337 → 171
Proline	25	286 → 171	287 → 171
Serine	25	276 → 171	277 → 171
Threonine	25	290 → 171	291 → 171
Tryptophan	25	375 → 171	376 → 171
Tyrosine	25	352 → 171	353 → 171
Valine	25	288 → 171	289 → 171
M + 1 enrichment for positional N			
Ornithine (amino N)	38	473 → 70	474 → 71
Citrulline (amino N)	38	346 → 70	347 → 71
Citrulline (ureido N)	8	346 → 329	347 → 329
Arginine (amino N)	38	345 → 70	346 → 71
Arginine (guanidino N)[b]	25	345 → 158	346 → 158
Arginine (guanidino N_2 and delta N)[c]	38	345 → 60	346 → 61
Glutamine (amino N)	38	317 → 84	318 → 85

Note. Sets of precursor and product ions for the measurement of ^{15}N enrichment of each amino acid are shown with MS/MS settings (collision energy). Targeted positional Ns are shown in parentheses.
[a] Two moles of AQC are bound to lysine and ornithine.
[b] The ^{15}N enrichment of one of the two guanidino Ns was measured.
[c] The sum of ^{15}N enrichments in the two guanidino and one delta Ns was measured.

guanidino Ns, was used. At a collision energy of 38 eV, a fragment ion of *m/z* 60 is generated from AQC–arginine derivatives, and this ion was used for the total ^{15}N enrichments of the delta and the two guanidino Ns of arginine. For the analysis of the ureido N of citrulline, the fragment ion *m/z* 329 generated at a collision energy of 8 eV, which is unique to the ureido N, was used. The collision energy and precursor and reporter ions for each measurement of positional ^{15}N enrichments in the current study are also shown in Table 1.

Calculation

The isotopic enrichment (*IE;* mole percentage excess, mol%) of each amino acid was calculated from the tracer–tracee ratio (*TTR*) as follows:

$$IE = \frac{TTR}{1 + TTR} \times 100$$

$$TTR = IR_{obs} - IR_{bkd},$$

where IR_{obs} represents the observed isotopic ratio and IR_{bkd} represents the isotopic ratio of the background.

The TTR of the *M* + 2 isotopomer for [guanidino-$^{15}N_2$]arginine was corrected according to a previous report [18]. Theoretical values of the isotopic ratios for each amino acid were calculated using MS-Isotope in Protein Prospector (available at http://prospector.ucsf.edu/prospector/mshome.htm).

Data analysis

The data are presented as means ± standard deviations (SDs). Significant differences between two groups were analyzed using Student's *t*-test.

Results

Chromatograms and accuracy of isotopic ratio measurements

A mixture of 41 authentic amino acids was derivatized with AQC and analyzed by LC–MS/MS. Monitoring of *m/z* 171 as an AQC-specific product ion allowed the detection of AQC–amino acids with high sensitivity (Fig. 1). Urea cycle amino acids such as arginine, citrulline, and ornithine were detected as peaks with precursor ions of *m/z* 345, 346, and 473, respectively. A larger *m/z* of precursor ion for ornithine compared with arginine and citrulline indicated that two molecules of AQC bind to a single ornithine. SRM measurement of the mixture of authentic amino acids detected the peaks of *M* and *M* + 1 isotopomers for each amino acid without any interfering peaks (Fig. 2). The isotopic ratios of the *M* + 1 and *M* areas were nearly identical to the theoretical values (Table 2). The SDs of their (*M* + 1)/*M* ratios in repeated measurements ($n = 6$) of authentic amino acids were generally approximately 0.1 mol% (0.01–0.36 mol%), and the coefficients of variation (CVs) were less than 4%. This method was also applicable to the measurements of the *M* and *M* + 1 isotopic ratios of various amino acids in rat plasma samples with similar high accuracy. The SDs of the ratios in repeated measurements of plasma amino acids were also approximately 0.1 mol% (0.01–0.56 mol%), and the CVs were less than 4% (Table 2).

Dynamic range and linearity in measurement of ^{15}N enrichment

The influence of the injected dose of each urea cycle amino acid on the isotopic enrichment was investigated (Fig. 3). The signal intensity of *m/z* 171 increased nonproportionally with larger injected doses of each urea cycle amino acid from 0.1 to 12 pmol. However, the (*M* + 1)/*M* ratios for all amino acids tested remained nearly constant up to 12 pmol per injection.

Next, linearity in the measurement of ^{15}N enrichment was tested using a series of [guanidino-$^{15}N_2$]arginine standards containing 0, 5, 10, 15, 20, 25, 30, 50, or 100 mol% of [guanidino-$^{15}N_2$]arginine in place of natural arginine. The measured isotopic enrichment of the *M* + 2 isotopomer for arginine calculated from the (*M* + 2)/*M* ratio (i.e., the ratio of *m/z* 347 to 345 using *m/z* 171 as a reporter ion) increased linearly and was nearly identical to the actual enrichment levels ($r^2 = 0.9995$, $y = 1.0086x$) (Fig. 4).

Measurement of positional ^{15}N enrichment of amino acids

The applicability of the method to determine positional ^{15}N enrichment in glutamine was assessed using [2-^{15}N]glutamine and [5-^{15}N]glutamine. A signal of *m/z* 85, rather than *m/z* 84, appeared when fragment ions of [2-^{15}N]glutamine were analyzed (Fig. 5B). In contrast, no signal for *m/z* 85 was observed when [5-^{15}N]glutamine was analyzed, but the signal at *m/z* 84 was observed (Fig. 5C). Thus, the positional ^{15}N enrichment of the amino N in glutamine can be determined by monitoring this fragment ion. Besides, there was no detectable signal of ions containing only amide N of glutamine. The accuracy of this method was further tested by measuring standard mixtures of natural glutamine and [2-^{15}N]glutamine or [5-^{15}N]glutamine (the theoretical ^{15}N enrichment for both is 19.6 mol%). Monitoring of the transitions of *m/z* 317 to 84 and *m/z* 318 to 85 revealed that the ^{15}N

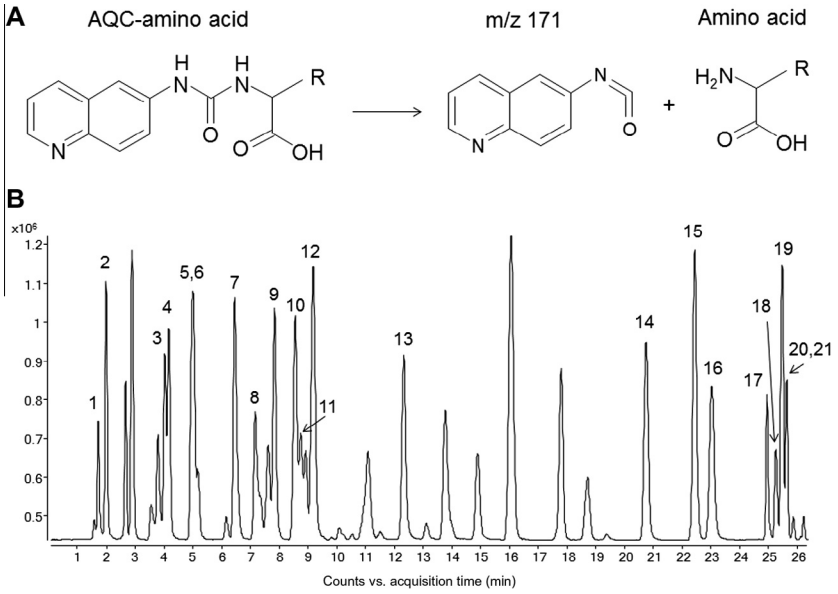

Fig.1. Chromatogram of the AQC–amino acids. (A) General cleavage pattern of AQC–amino acids in a tandem mass spectrometer. A common product ion *m/z* 171 is generated from all amino acids derivatized with AQC. (B) Chromatogram of authentic amino acid mixture derivatized with AQC. AQC–amino acids were separated by reverse phase HPLC, and *m/z* 171, a common product ion for AQC–amino acids, was detected in precursor ion scan mode. Peaks of representative amino acids are shown as follows: 1, aspartic acid; 2, glutamic acid; 3, asparagine; 4, serine; 5, glutamine; 6, glycine; 7, alanine; 8, histidine; 9, citrulline; 10, threonine; 11, arginine; 12, ammonia; 13, proline; 14, tyrosine; 15, cysteine; 16, valine; 17, methionine; 18, lysine; 19, ornithine; 20, phenylalanine; 21, tryptophan.

enrichment of the amino N of the [2-^{15}N]glutamine standard was 19.9 ± 0.1 mol%, whereas the ^{15}N enrichment of the [5-^{15}N]glutamine standard was 1.0 ± 0.2 mol%. In contrast, the ^{15}N enrichments of the amide N of [2-^{15}N]glutamine and [5-^{15}N]glutamine standards were 0.0 ± 0.2 and 18.1 ± 0.2 mol%, respectively (Table 3).

Next, the ability of the methods to determine positional ^{15}N enrichments in arginine was assessed using [U-$^{15}N_4$]arginine and [guanidino-$^{15}N_2$]arginine standards. In the analysis of natural arginine, the major fragment ions at 25 eV of collision energy were *m/z* 60, 158, and 175 in addition to the common fragment (*m/z* 171) for all amino acids. The structures of the fragments of *m/z* 60, 70, 158, and 175 were predicted as $[C(NH_2)_3]^+$, $[C_4NH_8]^+$, $[C(NH_2)NHC_3H_6CH(NH)COOH]^+$, and $[(NH_2)_2C(NH)C_3H_6CH(NH_2)COOH]^+$, respectively, from their mass and the molecular structure of the precursor ion as shown in Fig. 6 [19]. Based on these predictions, the *m/z* 175 fragment contains all of the Ns of arginine, the *m/z* 70 fragment contains only the amino N of arginine, and the *m/z* 60 and 158 fragments lack the amino N and one of the two guanidino Ns, respectively. Mass shifts from *m/z* 175 to 179, *m/z* 158 to 161, *m/z* 60 to 63, and *m/z* 70 to 71 in the analysis of [U-$^{15}N_4$]arginine indicated that these fragment ions contain four, three, three, and one N atoms, respectively. The lack of change in the *m/z* 70 fragment in the analysis of [guanidino-$^{15}N_2$]arginine indicated that this fragment does not contain any guanidino Ns. Mass shifts from *m/z* 158 to 159 and *m/z* 60 to 62 in this analysis indicated that these fragment ions contain one and two guanidino Ns, respectively. These results were consistent with the predicted structures of the fragment ions described above. An increase in the collision energy from 25 to 38 eV changed the fragmentation pattern of the same precursor ion in the analysis of arginine. The signal intensity at *m/z* 70 was tripled by the increase in collision energy, whereas some of the other fragments such as *m/z* 158 and 175 disappeared in this condition (Fig. 6D–F).

In the analysis of citrulline, the *m/z* 70 fragment ion was detected at 25 and 38 eV of collision energy as in the analysis of arginine (Fig. 7A and B). The *m/z* 70 fragment was also observed in the analysis of ornithine at 38 eV of collision energy. $[C_4NH_8]^+$ is the only plausible structure for this fragment ion common to urea cycle amino acids. An *m/z* 329 fragment was generated at 8 eV of collision energy in the analysis of citrulline (Fig. 7A). The mass difference of 17 (corresponding to NH_3) from the precursor ion indicates that this fragment ion lacks the ureido N and that its structure is likely $[AQC\text{-}(NH)CH(COOH)C_3H_6(NH)CO]^+$.

Animal experiments

The methods for the measurement of the ^{15}N enrichment of amino acids were applied to a rat tracer study using ^{15}N-labeled amino acids (Table 4). Rats were fed hourly on small diets containing [2-^{15}N]glutamine (experiment 1) or [5-^{15}N]glutamine (experiment 2) for 6 h, and their plasma samples were collected. The isotopic enrichment of [2-^{15}N]glutamine (amino-^{15}N; 16.9 ± 0.6 mol%) was much higher than that of [5-^{15}N]glutamine (amide-^{15}N; 2.6 ± 0.3 mol%) in rats fed [2-^{15}N]glutamine (experiment 1), whereas the amide N was the primary site labeled with ^{15}N (22.3 ± 2.0 mol%) in rats fed [5-^{15}N]glutamine (experiment 2). The ^{15}N enrichments of amino Ns in various amino acids such as glutamic acid (9.3 ± 0.3 mol%) and aspartic acid (7.6 ± 0.3 mol%) were greater in experiment 1 than in experiment 2 (4.7 ± 0.4 and 3.4 ± 0.3 mol%, respectively), whereas the ^{15}N enrichment of ammonia in experiment 1 (0.6 ± 0.1 mol%) was lower than that in experiment 2 (2.4 ± 0.1 mol%). The ^{15}N enrichment of the alpha-N of ornithine, originating from glutamic acid, was 4.8 ± 0.6 mol% in experiment 1 but was only 0.2 ± 0.2 mol% in experiment 2. The ^{15}N enrichment of the amino N of citrulline, which is derived from the alpha-N of ornithine, was also higher in experiment 1 (8.6 ± 0.1 mol%) than in experiment 2 (0.9 ± 0.3 mol%). The enrichment differences between experiments 1 and 2 were also evident in the amino N of arginine (2.2 ± 0.2 vs. 0.3 ± 0.1 mol%, respectively). In addition, the ^{15}N enrichment of the ureido N of citrulline, which is derived from ammonia, was as high as 22.6 ± 1.2 mol% in experiment 2, whereas it was only 3.0 ± 0.3 mol% in experiment 1. The ^{15}N enrichment of the guanidino N of arginine, which is derived from the ureido N of citrulline or the amino N of aspartic acid, was labeled to a greater extent in experiment 2 (2.5 ± 0.3 mol%) than in experiment 1 (1.8 ± 0.1 mol%).

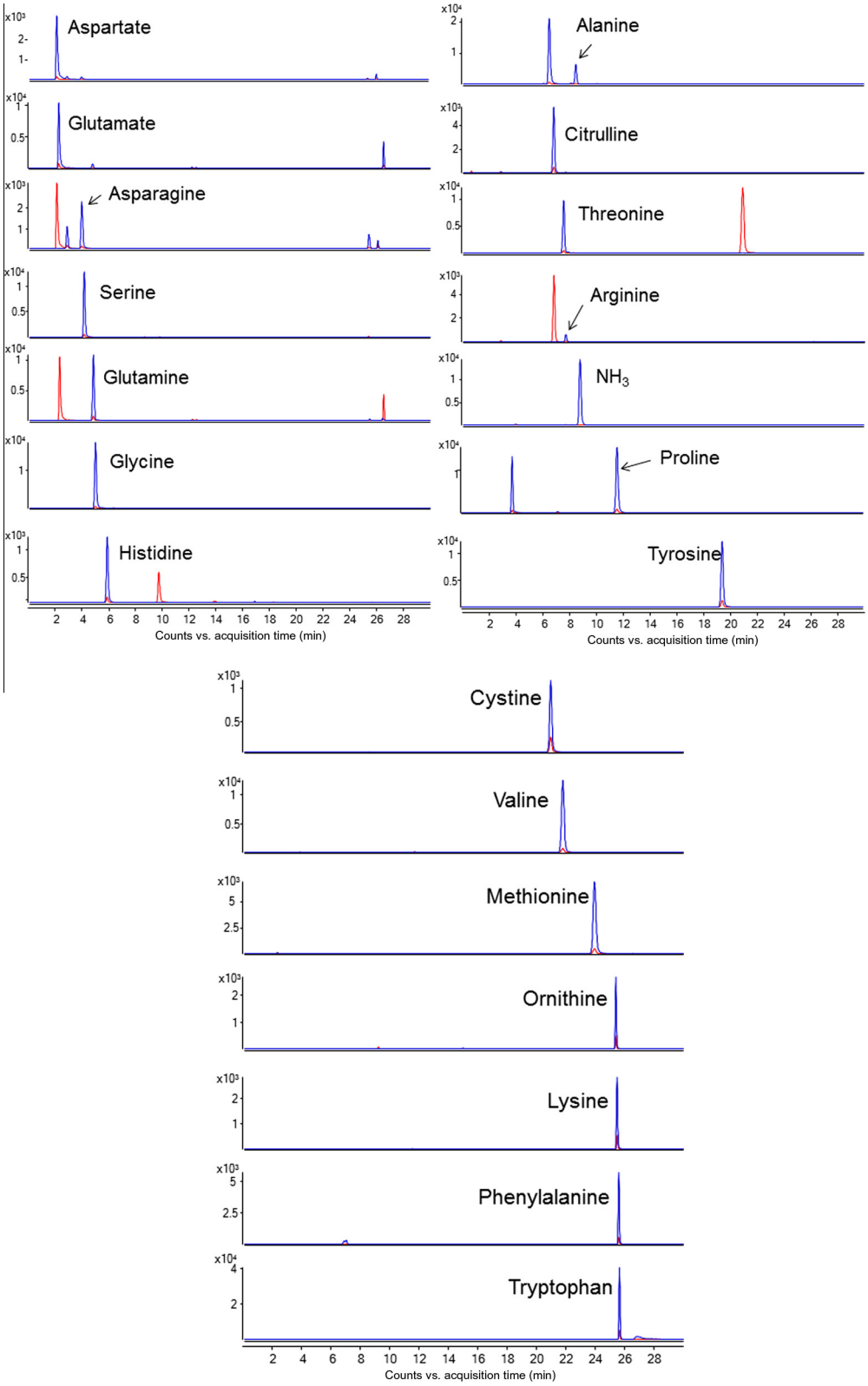

Fig.2. SRM measurement of *M* and *M* + 1 isotopomers of AQC–amino acids. An authentic amino acid mixture derivatized with AQC was analyzed in SRM mode, as shown in Table 1. Each amino acid was detected without any interfering peaks for both *M* (blue) and *M* + 1 (red) isotopomers.

Discussion

In the current study, we applied the conventional LC–MS/MS analytical method for amino acids to measure their isotopic enrichment and evaluated the accuracy of the measurements. The method was shown to determine the positional ^{15}N enrichment of glutamine and urea cycle amino acids such as ornithine, citrulline, and arginine.

AQC is a specific derivatization reagent for the amino moiety that enables selective measurement of amino acids through

Table 2
Accuracy of the measurement of $(M+1)/M$ isotope ratios.

	Theoretical $(M+1)/M$ ratio (%)	Measured $(M+1)/M$ ratio (%)					
		Authentic amino acids			Plasma amino acids		
		Average	SD	CV	Average	SD	CV
Arginine	8.47	8.08	0.30	3.68	8.09	0.22	1.91
Citrulline	8.13	8.26	0.08	1.03	8.36	0.11	1.28
Ornithine	18.64	18.48	0.16	0.88	18.08	0.56	3.09
Alanine	3.91	3.99	0.10	2.43	3.85	0.03	0.78
Asparagine	5.45	5.43	0.17	3.08	5.60	0.09	1.55
Aspartic acid	5.10	4.97	0.10	1.95	5.11	0.20	3.95
Glutamine	6.59	6.57	0.06	0.91	6.52	0.06	0.97
Glutamic acid	6.25	6.18	0.05	0.87	6.21	0.05	0.76
Glycine	2.76	2.74	0.02	0.90	2.77	0.04	1.60
Histidine	8.03	7.93	0.22	3.37	8.00	0.16	2.01
Lysine	19.78	19.25	0.36	1.89	18.97	0.25	1.30
Methionine	6.99	6.97	0.04	0.60	6.86	0.10	1.40
NH_3	0.41	0.48	0.01	1.35	0.48	0.01	2.81
Phenylalanine	10.68	10.72	0.15	1.4	10.37	0.06	0.58
Proline	6.17	6.15	0.06	0.91	6.16	0.07	1.19
Serine	3.94	3.94	0.06	1.42	3.98	0.02	0.55
Threonine	5.09	5.08	0.06	1.13	5.01	0.04	0.86
Tryptophan	13.3	13.45	0.14	1.04	13.13	0.17	1.32
Tyrosine	10.72	10.57	0.05	0.47	10.41	0.12	1.12
Valine	6.20	6.21	0.03	0.45	6.14	0.05	0.45

Note. Mixtures of authentic amino acid solutions and deproteinized rat plasma samples were mixed with AQC reagent and incubated as indicated in Materials and Methods to generate AQC–amino acids. The natural abundance of the $M+1$ isotopomer of each AQC–amino acid [$(M+1)/M$ ratio] was repeatedly measured by LC–MS/MS ($n=6$ for authentic amino acids and $n=8$ for plasma amino acids).

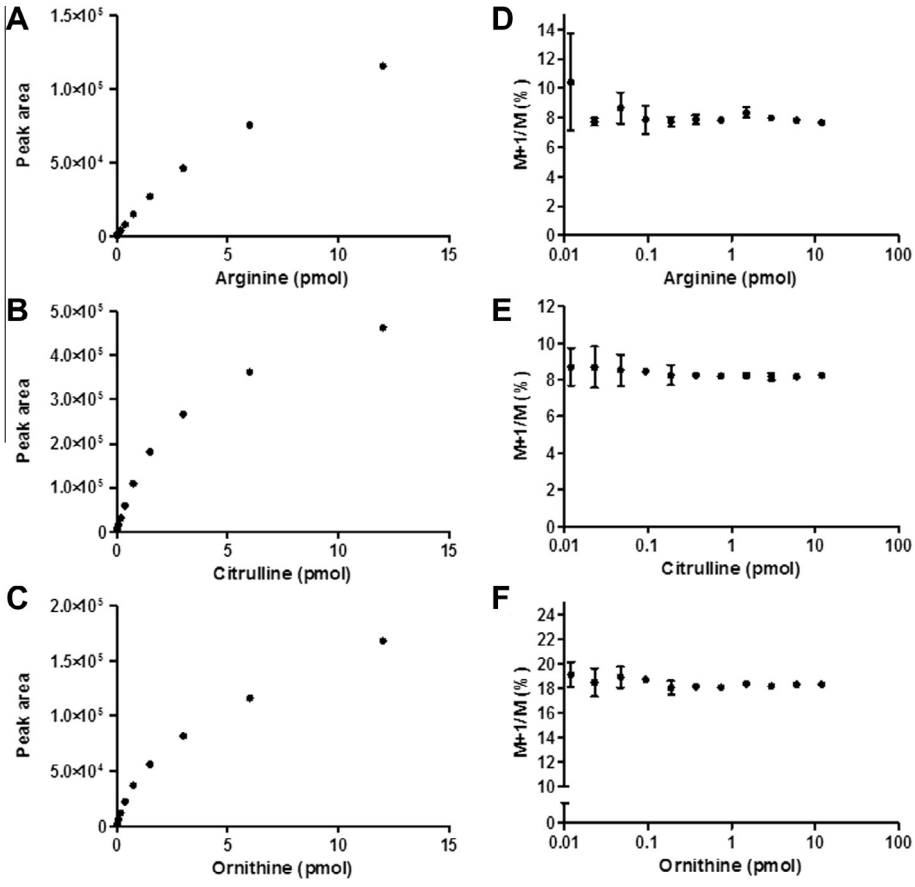

Fig.3. Effect of injected amounts of each AQC–amino acid on the isotopic ratios. The relationships of the injected amounts of AQC–amino acids (ranging from 0.01 to 12 pmol) with peak area responses for M isotopomers of arginine (A), citrulline (B), and ornithine (C), and with $(M+1)/M$ ratios (%) for arginine (D), citrulline (E), and ornithine (F), were investigated. The data are expressed as means ± SDs ($n=3$). Although the peak area of each amino acid (M) did not increase linearly in accordance with the increased quantities injected, the $(M+1)/M$ ratios remained nearly constant within the range of 0.1 to 12 pmol per injection.

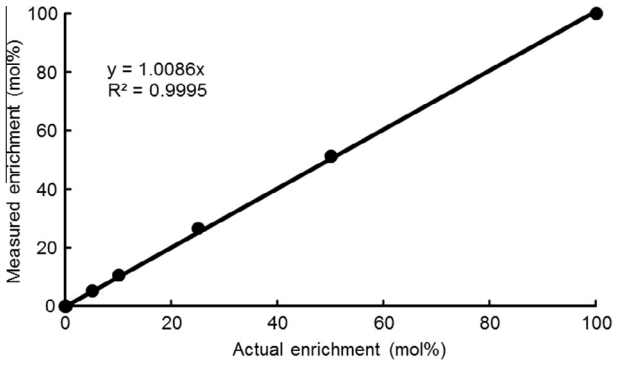

Fig.4. Linearity in the measurement of $M+2$ enrichment for [guanidino-$^{15}N_2$]arginine. Arginine standards containing 0, 5, 10, 15, 20, 25, 30, 50, and 100 mol% of [guanidino-$^{15}N_2$]arginine were prepared. After derivatization with AQC, the $M+2$ isotopic enrichments were calculated from the peak areas as described in Materials and Methods. The peak area of the $M+2$ isotopomer of arginine was determined by monitoring the transitions of m/z 347 to 171. Relationships between the actual and measured enrichments were plotted. The regression line is $y = 1.0086x$ ($r^2 = 0.9995$).

monitoring of the common m/z 171 ion that is derived from AQC. This analytical method also has a great advantage in evaluating the isotopic distribution of each amino acid because of its derivatization specificity. When $M+1$ isotopomers of major amino acids were analyzed, no interfering signals were detected, as shown in Fig. 2, and this was also confirmed in rat plasma samples (data not shown). The measured isotope ratios of $M+1$ to M for all of the amino acids were nearly identical to their theoretical ratios in the measurements of both the standard amino acid mixture and the rat plasma samples, as shown in Table 2. Furthermore, the accuracy of the measurements of the isotopic ratios was as high as that of the conventional methods using GC–MS; the SDs of the $(M+1)/M$ ratios of the natural amino acids were generally as low as 0.1% with minor exceptions such as arginine, ornithine, and lysine, which have multiple Ns in their molecules. The reproducibility of the measurements of isotope ratios was tested in different analytical conditions, and hundred-fold differences in the injection amount (0.1–10 pmol) gave nearly identical results in the measurement of $(M+1)/M$ ratios, although the peak areas of their ions did not show linearity, presumably due to ion suppression [20]. Finally, the linearity and accuracy of the measurements of isotopic enrichment were confirmed using a series of arginine standards containing 0 to 100 mol% of [guanidino-$^{15}N_2$]arginine. The measured values were nearly identical to the actual values (slope = 1.0086), and the correlation coefficient was as high as 0.9995 (Fig. 3). Iwatani and coworkers already applied this method to the measurement of the ^{13}C isotopic enrichment of amino acids to determine the metabolic flux of amino acids in *Escherichia coli* but did not show any data for the accuracy of the measurements [6]. Thus, this is the first study to evaluate the adequacy of this method for the measurement of the isotopic enrichment of amino acids derivatized with AQC.

Table 3
Measurements of isotopic enrichment of positional ^{15}N in glutamine.

	Measured ^{15}N enrichment (mol%)		
	Total ^{15}N	2-^{15}N	5-^{15}N
[2-^{15}N]glutamine	19.9 ± 0.1	19.9 ± 0.1	−0.0 ± 0.2
[5-^{15}N]glutamine	19.1 ± 0.1	1.0 ± 0.2	18.1 ± 0.2

Note. Glutamine standards containing 20 mol% of [2-^{15}N]glutamine or [5-^{15}N]glutamine were prepared to evaluate the method to measure the positional ^{15}N enrichment in glutamine. The $M+1$ isotopic enrichment of total [^{15}N]glutamine (including both [2-^{15}N]glutamine and [5-^{15}N]glutamine) and [2-^{15}N]glutamine was measured as described in Materials and Methods. Under the assumption that there are no $M+2$ isotopomers labeled with two ^{15}Ns, the enrichment of [5-^{15}N]glutamine was determined as the difference in the isotopic enrichment between total [^{15}N]glutamine and [2-^{15}N]glutamine. Values are reported as means ± SDs ($n = 3$).

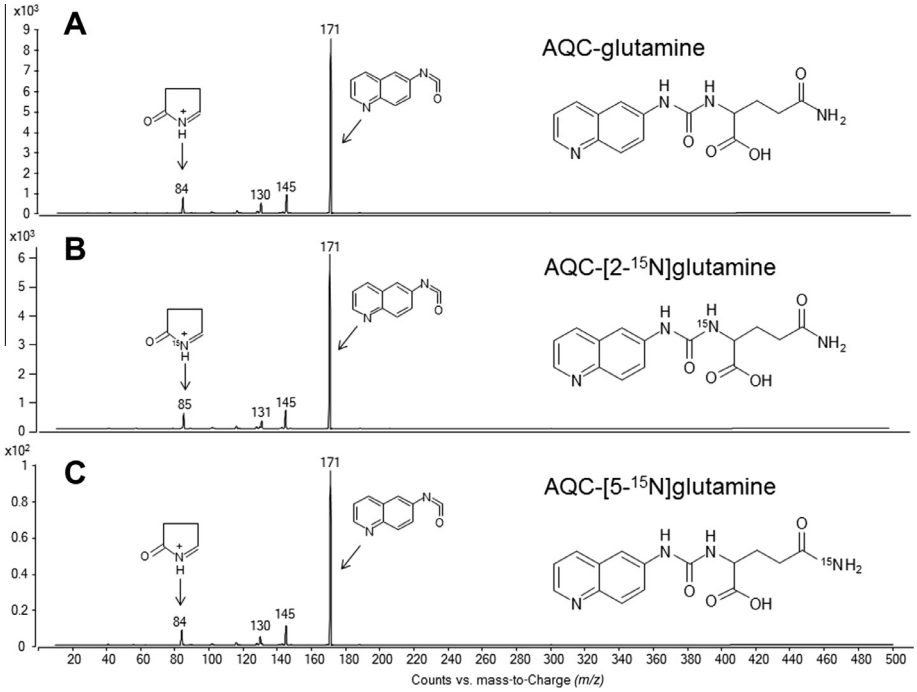

Fig.5. Fragmentation patterns of glutamine. Natural glutamine (A), [2-^{15}N]glutamine (B), and [5-^{15}N]glutamine (C) derivatized with AQC were analyzed in a product ion scan mode at 38 eV of collision energy. All three glutamine derivatives generated the m/z 171 ion, a fragment common to AQC–amino acids, whereas a fragment at m/z 84 was shifted to m/z 85 in the analysis of [2-^{15}N]glutamine, indicating that this fragment contains only the amino N of glutamine. Structures of fragments were predicted based on a previous report [19].

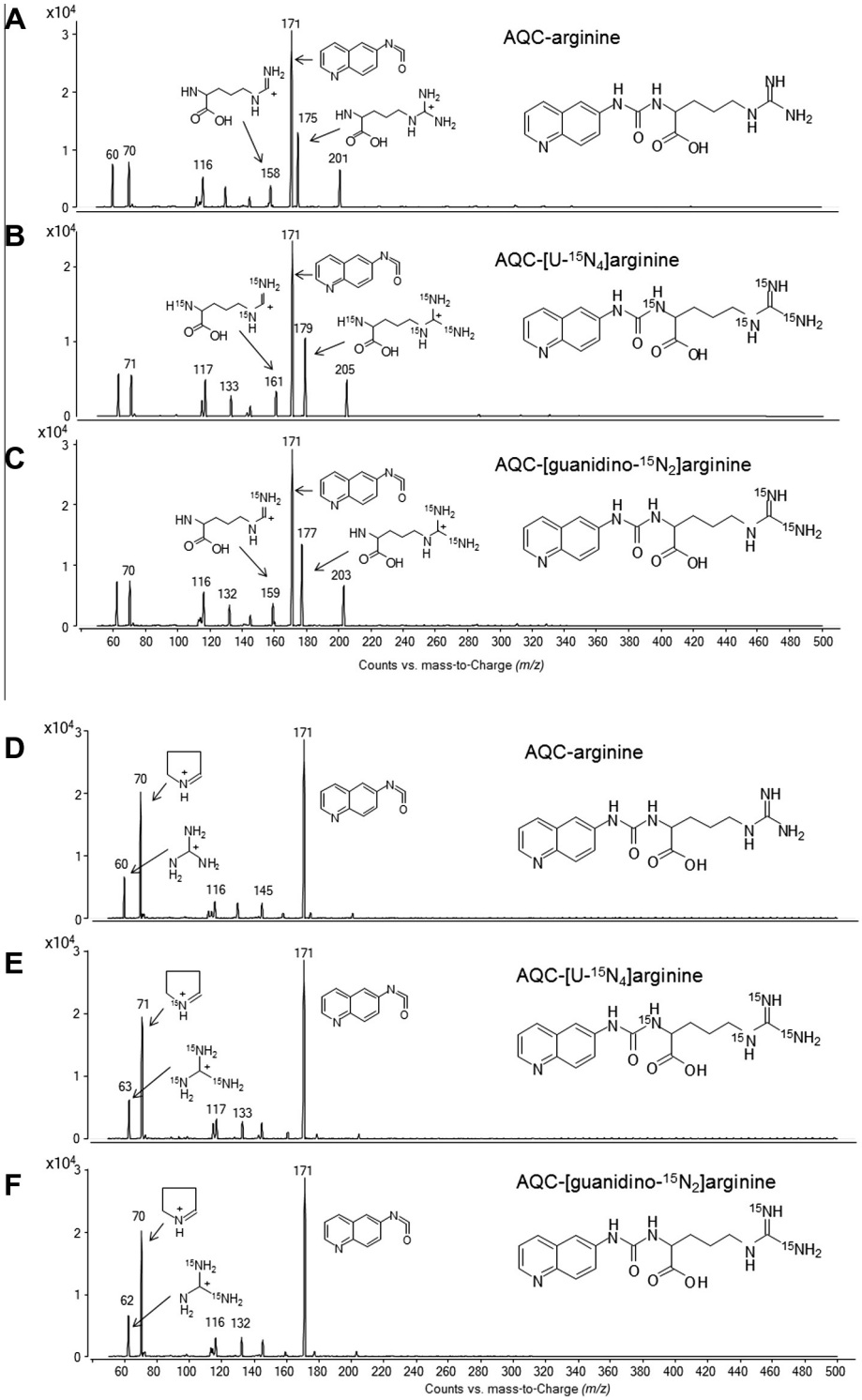

Fig.6. Fragmentation patterns of AQC–arginine under different collision energy settings. Natural arginine (A, D), [U-$^{15}N_4$]arginine (B, E), and [guanidino-$^{15}N_2$]arginine (C, F) derivatized with AQC were analyzed in a product ion scan mode at 25 eV (A–C) or 38 eV (D–F) of collision energy. Fragments of *m*/*z* 60, 70, 116, 158, 171, 175, and 201 were primarily observed at 25 eV of collision energy in the analysis of natural arginine (A). Among these ions, mass numbers of fragments with *m*/*z* 60, 70, 116, 158, 175, and 201 were increased by 3, 1, 1, 3, 4, and 4, respectively, in the analysis of [U-$^{15}N_4$]arginine (B). In the analysis of [guanidino-$^{15}N_2$]arginine (C), fragments with *m*/*z* 60, 158, 175, and 201 were increased by 2, 1, 2, and 2, respectively. Signals for *m*/*z* 60 and 70 increased in magnitude at high collision energy (38 eV) (D–F). Structures of fragments were predicted based on a previous report [19].

The current analytical method using LC–MS/MS and AQC derivatives has advantages compared with conventional methods using GC–MS. In general, GC–MS methods for amino acid detection require complex precolumn derivatization steps. For example, two reaction steps and drying steps are needed to form heptafluorobutyl propyl ester or trifluoroacetyl methyl ester derivatives of amino acids. Furthermore, in biological samples, partial purification of amino acids is necessary to remove interfering peaks. However, the current AQC method has more convenient sample preparation. The current method requires only one

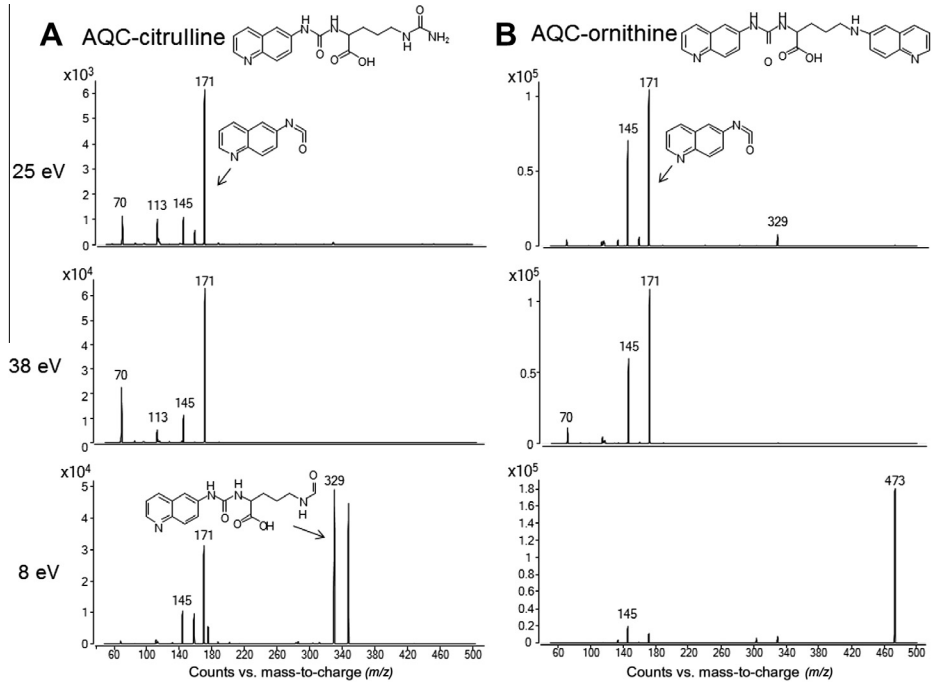

Fig.7. Fragmentation patterns of AQC–citrulline and AQC–ornithine under different collision energy settings. Citrulline (A) and ornithine (B) derivatized with AQC were analyzed in a product ion scan mode. The higher collision energy at 25 and 38 eV produced a common fragment (m/z 70) in both amino acids and in arginine (see Fig. 6). The predicted structure of the fragment ion with m/z 329 produced at lower collision energy (8 eV) in the analysis of citrulline lacks ureido N but contains the amino N and delta N of citrulline.

10 min reaction step to form stable AQC–amino acid derivatives without any drying steps and does not require any purification steps before derivatization even in the measurements of biological samples. Armenta and coworkers reported that AQC–amino acids are stable for more than 1 month when stored at 4 °C [5], which is another advantage of the current method.

Although the conventional methods using GC–MS have been applied to measurements of isotopic enrichment in various amino acids, GC–MS methods have not provided satisfactory results for measuring urea cycle amino acids [10–12]. For example, a previous report showed that the derivatization of arginine with *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) produced the same derivative as that of ornithine [21]. Smith and coworkers reported that citrulline showed the same peak as the ornithine derivative [13]. Furthermore, Amelung and Zhang reported that ensuring the complete derivatization of arginine (*N*-pentafluoropropionyl-arginine isopropyl esters) is difficult [22]. In contrast, the current AQC method produced unique derivatives of ornithine, citrulline, and arginine without any molecular cleavage, and these derivatives were distinguishable from others both in chromatography and in mass detection (Figs. 2, 6 and 7). Thus, this method was quite suitable for measuring the isotopic enrichment of these amino acids, similarly to the other amino acids mentioned above. The measured isotopic ratios agreed well with the actual ratios in the series of arginine standards labeled with ^{15}N (Fig. 2).

The current method was also applicable to the measurement of the positional ^{15}N enrichment of glutamine. Increasing the collision energy from 25 to 38 eV in the analysis of AQC–glutamine was effective for detecting an m/z 84 product ion that contains only the amino N and not the amide N. Using this fragment ion, the enrichment of the amino N of glutamine was determined and the enrichment of the amide N was calculated as the difference between the total ^{15}N and the amino ^{15}N enrichments. The relevance of this method was confirmed by measuring [2-^{15}N]glutamine and [5-^{15}N]glutamine standards (Table 3). It should be noted that the current method to measure positional ^{15}N enrichment of glutamine does not require any purification steps, whereas the conventional method using GC–MS requires separation of glutamine and glutamic acid before derivatization [23].

The positional ^{15}N enrichment of urea cycle amino acids (i.e., arginine, ornithine, and citrulline) was also determined using fragment ions containing Ns originating from these amino acids. In the analysis of AQC–arginine, multiple fragment ions were generated at

Table 4
Measurements of positional ^{15}N enrichment of plasma amino acids in rats fed with [2-^{15}N]glutamine or [5-^{15}N]glutamine.

Amino acid	Position of the target N	^{15}N enrichment (mol%)		
		[2-^{15}N]glutamine feeding (experiment 1)	[5-^{15}N]glutamine feeding (experiment 2)	*P* value
Glutamine	Total	19.4 ± 0.9	23.1 ± 2.0	<0.05
	Amino N	16.9 ± 0.6	0.7 ± 0.0	<0.001
Glutamate	Amino N	9.3 ± 0.3	4.7 ± 0.4	<0.001
Aspartate	Amino N	7.6 ± 0.3	3.4 ± 0.3	<0.001
NH_3	–	0.6 ± 0.1	2.4 ± 0.1	<0.001
Ornithine	Total	10.2 ± 1.0	0.8 ± 0.1	<0.001
	Amino N	4.8 ± 0.6	0.2 ± 0.2	<0.001
Citrulline	Total	18.0 ± 0.4	23.3 ± 1.3	<0.01
	Amino N	8.6 ± 0.1	0.9 ± 0.3	<0.001
	Ureido N	3.0 ± 0.3	22.6 ± 1.2	<0.001
Arginine	Total	6.9 ± 1.0	7.5 ± 0.9	NS
	Amino N	2.2 ± 0.2	0.3 ± 0.1	<0.001
	Guanidino N	1.8 ± 0.1	2.5 ± 0.3	<0.05
	Guanidino N_2+ delta N	5.4 ± 0.7	7.7 ± 1.0	<0.05
	Guanidino N, amino N and delta N (M + 1)	5.8 ± 0.9	5.7 ± 0.6	NS

Note. Diets containing [2-^{15}N]glutamine (experiment 1) or [5-^{15}N]glutamine (experiment 2) were fed to rats hourly, and blood was collected from the descending aorta after 6 h of feeding. Deproteinized plasma samples were derivatized with AQC and analyzed by LC–MS/MS. ^{15}N enrichment of the positional Ns in individual amino acids was measured as described in Materials and Methods. The data are expressed as means ± SDs (n = 3). NS, not significant.

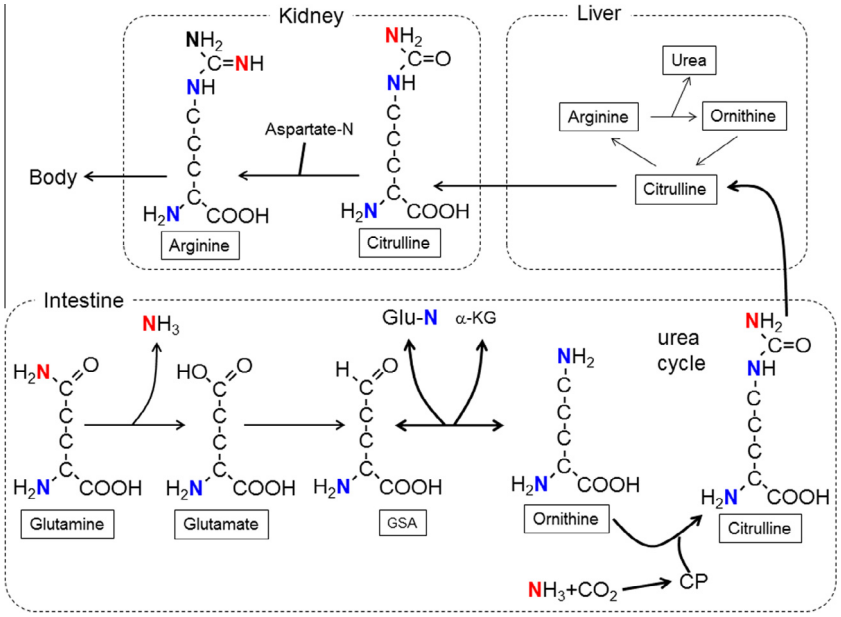

Fig.8. Schematic representation of N flow from glutamine to urea cycle amino acids. The amino N of glutamine is transferred to the amino N of ornithine via glutamic acid and glutamate–semialdehyde (GSA), as is their carbon skeleton in the gut. The amino N of glutamine also supplies the delta N of ornithine via glutamic acid through ornithine aminotransferase. Ammonia (NH_3) released from glutamine via glutaminase is used for the synthesis of carbamoyl phosphate (CP), which is incorporated into the ureido N of citrulline in the gut and liver and then into one of the guanidino Ns of arginine mainly in the kidney. Aspartic acid N, derived from glutamic acid N, is incorporated into the other guanidino N of arginine. N derived from the amino N and the amide N of glutamine are shown in blue and red, respectively.

25 or 38 eV of collision energy in addition to the common *m/z* 171 fragment for AQC derivatives. Among these fragments, those with *m/z* 60, 70, 116, 158, 175, and 201 contained one to three Ns originating from arginine. The predicted molecular structures indicated that *m/z* 70 only contains the amino N of arginine and that *m/z* 60 and 158 lack the amino N and one of the guanidino Ns, respectively. These predictions were confirmed by the mass shifts in the analysis of [U-$^{15}N_4$]arginine and [guanidino-$^{15}N_2$]arginine. The *m/z* 70 fragment ion was also observed in the analysis of AQC–citrulline and AQC–ornithine, and it was used for the measurement of their amino ^{15}N enrichment. The *m/z* 329 fragment ion generated in the analysis of AQC–citrulline at 8 eV of collision energy was used for the measurement of ureido ^{15}N enrichment based on the predicted molecular structure of the fragment. Although we did not validate these methods for the positional ^{15}N analysis of ornithine and citrulline using their ^{15}N-labeled standards, no other plausible molecular structures for fragments with *m/z* 70 and 329 were predicted.

An animal study using [2-^{15}N]glutamine and [5-^{15}N]glutamine suggested the adequacy of these methods (Table 4). The amino N of plasma ornithine was labeled with ^{15}N in rats treated with [2-^{15}N]glutamine, which agrees with the stoichiometry of ornithine synthesis from glutamine in the intestines as shown in Fig. 8. The greatly decreased enrichment of the total ^{15}N and the amino ^{15}N of plasma ornithine in rats given [5-^{15}N]glutamine also agrees with the general consensus that the amide N of glutamine is catabolized to ammonia in the first metabolic process in the gut and does not transfer to ornithine directly [24,25]. The amino N of plasma citrulline, presumably originating from the amino N of intestinal ornithine, was also labeled with ^{15}N in rats fed [2-^{15}N]glutamine, whereas the ^{15}N enrichment was much lower in rats fed [5-^{15}N]glutamine. In contrast, the enrichment of the ureido ^{15}N of citrulline, which is derived from ammonia via carbamoyl–phosphate, was much higher in rats fed [5-^{15}N]glutamine than in those fed [2-^{15}N]glutamine. These labeling patterns of citrulline also agree with the stoichiometry of glutamine N metabolism. Regarding arginine that is synthesized from citrulline in kidney and released into the circulation [26], feeding of [2-^{15}N]glutamine labeled the amino N but not the guanidino N to a greater extent than feeding of [5-^{15}N]glutamine, which was consistent with the labeling patterns of citrulline and ornithine. Thus, these results suggest the adequacy of the current methods to analyze the positional ^{15}N enrichment of urea cycle amino acids.

Conclusions

The current study evaluated the accuracy and sensitivity of the LC–MS/MS method for determining the isotopic enrichment of amino acids derivatized with AQC and demonstrated the relevance of the method. Furthermore, this study developed methods for estimating the positional ^{15}N enrichment in glutamine and urea cycle amino acids. These methods were all sufficiently applicable to biological samples in a tracer study using ^{15}N-labeled glutamine. Because AQC derivatization is known to be a highly sensitive method for MS detection, these methods will provide powerful tools to measure isotopic enrichment, thereby enabling the investigation of the N metabolism of amino acids in living organisms.[2]

[2] Cooper and coworkers used ^{13}N (positron-emitter, $t_{1/2}$ = 9.96 min) to study ammonia metabolism in the rat liver [27]. In this study, the authors showed that despite the need for five enzyme steps and two mitochondrial transport steps, the conversion of ammonia to urea in the rat liver is remarkably fast. The authors suggested that this finding is evidence that the urea cycle enzymes and transporters form a metabolon. The authors also showed that N exchange among components of the alanine and aspartate aminotransferase reactions is also very fast in rat liver [28]. In a separate study, Cooper and coworkers investigated the short-term metabolic fate of ^{13}N-labeled glutamate, alanine, and glutamine(amide) in rat liver [28]. The authors verified the rapid N exchange among components of the alanine and aspartate aminotransferase reactions. They also showed that glutamine amide N is a source of urea. However, although these studies were instrumental in showing the previously unappreciated rapidity of N exchange among certain metabolites in rat liver, the use of ^{13}N is not suitable for studies of N flux over periods of more than 30 min or so. Moreover, ^{13}N label in the amide group of glutamine can only be distinguished from ^{13}N label in the amine group of glutamine by enzymatic analysis with glutaminase. The current procedure has the advantage that long-term studies over many hours are feasible. Moreover, determination of isotopomer content of glutamine N does not require analysis with glutaminase and can be determined directly.

Acknowledgment

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References

[1] Q.Z. Li, Q.X. Huang, S.C. Li, M.Z. Yang, B. Rao, Simultaneous determination of glutamate, glycine, and alanine in human plasma using precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and high-performance liquid chromatography, Korean J. Physiol. Pharmacol. 16 (2012) 355–360.

[2] G. Sharma, S.V. Attri, B. Behra, S. Bhisikar, P. Kumar, M. Tageja, S. Sharda, P. Singhi, S. Singhi, Analysis of 26 amino acids in human plasma by HPLC using AQC as derivatizing agent and its application in metabolic laboratory, Amino Acids 46 (2014) 1253–1263.

[3] I. Boogers, W. Plugge, Y.Q. Stokkermans, A.L. Duchateau, Ultra-performance liquid chromatographic analysis of amino acids in protein hydrolysates using an automated pre-column derivatisation method, J. Chromatogr. A 1189 (2008) 406–409.

[4] G. Fiechter, H.K. Mayer, Characterization of amino acid profiles of culture media via pre-column 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivatization and ultra performance liquid chromatography, J. Chromatogr. B 879 (2011) 1353–1360.

[5] J.M. Armenta, D.F. Cortes, J.M. Pisciotta, J.L. Shuman, K. Blakeslee, D. Rasoloson, O. Ogunbiyi, D.J. Sullivan Jr., V. Shulaev, Sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ: tag ultra performance liquid chromatography–electrospray ionization–MS/MS with multiple reaction monitoring, Anal. Chem. 82 (2010) 548–558.

[6] S. Iwatani, S. Van Dien, K. Shimbo, K. Kubota, N. Kageyama, D. Iwahata, H. Miyano, K. Hirayama, Y. Usuda, K. Shimizu, K. Matsui, Determination of metabolic flux changes during fed-batch cultivation from measurements of intracellular amino acids by LC–MS/MS, J. Biotechnol. 128 (2007) 93–111.

[7] Z. Gaudin, D. Cerveau, N. Marnet, A. Bouchereau, P. Delavault, P. Simier, J.B. Pouvreau, Robust method for investigating nitrogen metabolism of ^{15}N labeled amino acids using AccQ*Tag ultra performance liquid chromatography–photodiode array–electrospray ionization–mass spectrometry: application to a parasitic plant–plant interaction, Anal. Chem. 86 (2014) 1138–1145.

[8] M.E. Jones, Conversion of glutamate to ornithine and proline: pyrroline-5-carboxylate, a possible modulator of arginine requirements, J. Nutr. 115 (1985) 509–515.

[9] A.A. Reyes, I.E. Karl, S. Klahr, Role of arginine in health and in renal disease, Am. J. Physiol. 267 (1994) F331–F346.

[10] C. Rouge, C. Des Robert, A. Robins, O. Le Bacquer, M.F. De La Cochetiere, D. Darmaun, Determination of citrulline in human plasma, red blood cells, and urine by electron impact (EI) ionization gas chromatography–mass spectrometry, J. Chromatogr. B 865 (2008) 40–47.

[11] I. Nissim, M. Yudkoff, T. Terwilliger, S. Segal, Rapid determination of [guanidino-^{15}N]arginine in plasma with gas chromatography–mass spectrometry: application to human metabolic studies, Anal. Biochem. 131 (1983) 75–82.

[12] S.J. Gaskell, A.W. Pike, Gas chromatography mass spectrometry of androstanolones as methyl oxime, *tert*-butyldimethylsilyl ether derivatives, Biomed. Mass Spectrom. 8 (1981) 125–127.

[13] P.A. Smith, V. Villa, G.L. King, Artifacts related to *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide derivatization of citrulline revealed by gas chromatography–mass spectrometry using both electron and chemical ionization, J. Chromatogr. A 1217 (2010) 5444–5448.

[14] J.C. Marini, Quantitative analysis of ^{15}N-labeled positional isomers of glutamine and citrulline via electrospray ionization tandem mass spectrometry of their dansyl derivatives, Rapid Commun. Mass Spectrom. 25 (2011) 1291–1296.

[15] R.M. Callejon, A.M. Troncoso, M.L. Morales, Determination of amino acids in grape-derived products: a review, Talanta 81 (2010) 1143–1152.

[16] K. Shimbo, T. Oonuki, A. Yahashi, K. Hirayama, H. Miyano, Precolumn derivatization reagents for high-speed analysis of amines and amino acids in biological fluid using liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 23 (2009) 1483–1492.

[17] H. Nakamura, Y. Kawamata, T. Kuwahara, K. Torii, R. Sakai, Nitrogen in dietary glutamate is utilized exclusively for the synthesis of amino acids in the rat intestine, Am. J. Physiol. Endocrinol. Metab. 304 (2013) E100–E108.

[18] J. Rosenblatt, D. Chinkes, M. Wolfe, R.R. Wolfe, Stable isotope tracer analysis by GC–MS, including quantification of isotopomer effects, Am. J. Physiol. 263 (1992) E584–E596.

[19] N.N. Dookeran, T. Yalcin, A.G. Harrison, Fragmentation reactions of protonated α-amino acids, J. Mass Spectrom. 31 (1996) 500–508.

[20] T.M. Annesley, Ion suppression in mass spectrometry, Clin. Chem. 49 (2003) 1041–1044.

[21] G. Corso, M. Esposito, M. Gallo, A.D. Russo, M. Antonio, Transformation of arginine into ornithine during the preparation of its *tert*-butyldimethylsilyl derivative for analysis by gas chromatography/mass spectrometry, Biol. Mass Spectrom. 22 (1993) 698–702.

[22] W. Amelung, X. Zhang, Determination of amino acid enantiomers in soils, Soil Biol. Biochem. 33 (2001) 553–562.

[23] D. Darmaun, D.E. Matthews, D.M. Bier, Glutamine and glutamate kinetics in humans, Am. J. Physiol. 251 (1986) E117–E126.

[24] N.P. Curthoys, M. Watford, Regulation of glutaminase activity and glutamine metabolism, Annu. Rev. Nutr. 15 (1995) 133–159.

[25] W.G. Bergen, G. Wu, Intestinal nitrogen recycling and utilization in health and disease, J. Nutr. 139 (2009) 821–825.

[26] R.F. Bertolo, D.G. Burrin, Comparative aspects of tissue glutamine and proline metabolism, J. Nutr. 138 (2008) 2032S–2039S.

[27] A.J. Cooper, E. Nieves, A.E. Coleman, S. Filc-DeRicco, A.S. Gelbard, Short-term metabolic fate of [^{13}N]ammonia in rat liver in vivo, J. Biol. Chem. 262 (1987) 1073–1080.

[28] A.J. Cooper, E. Nieves, K.C. Rosenspire, S. Filc-DeRicco, A.S. Gelbard, S.W. Brusilow, Short-term metabolic fate of ^{13}N-labeled glutamate, alanine, and glutamine(amide) in rat liver, J. Biol. Chem. 263 (1988) 12268–12273.