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Determination of amino acids by precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and high-performance liquid chromatography with ultraviolet detection

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Abstract

A precolumn derivatization method for the determination of amino acids using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by high-performance liquid chromatography is described. Ultraviolet detection was used for the assay of AQC derivatives of amino acids with the detection wavelength set at 248 nm. The reagent peak interference was minimized by optimizing the pH of the eluent and the gradient elution profile to improve the resolution between the reagent peak and amino acid derivatives. All nineteen amino acids were separated in 35 min with resolutions ≥ 1.6. The correlation coefficients of the calibration graphs for seventeen amino acids were fairly good ($r \geq 0.9999$) at concentrations of 25–500 μM. The detection limits for all common amino acids including cystine and tryptophan were at the range 0.07–0.3 pmol. Good reproducibility and accuracy of the method were demonstrated by the determination of amino acids in three typical kinds of samples (protein, peptide and feed). The average relative standard deviations for bovine serum albumin (BSA) and neuromedin were 0.86% and 1.36, respectively, and the average relative errors were 3.2% and 2.3%, respectively. The results of the analysis of feed hydrolysates agreed with those obtained by an ion-exchange method and the average recovery of the method for feed hydrolysates was 98%.

1. Introduction

Since it was found that amino acids are basic elements of proteins, the separation and determination of amino acids has become very important to those interested in protein studies. Liquid chromatography (LC) is now the most widely used technique for such determinations. The two types of LC systems used are the postcolumn and precolumn derivatization methods, respectively. The former is characterized by an ion-exchange separation mechanism and postcolumn derivatization with ninhydrin. Since it was developed by Spackman *et al.* [1] in 1958, this method has been used for amino acid determinations in a wide variety of samples and has become a classical method because of its accuracy, reproducibility and automation. However, some disadvantages such as long run times, low sensitivity and high instrument expense make this postcolumn derivatization method unsuitable in many instances.

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Since the 1970s, reversed-phase high-performance liquid chromatography has been used for the isolation of precolumn-derivatized amino acids and many reagents [2–5] have been used for derivatization. Speed, sensitivity and flexibility are the main advantages of the precolumn strategy over the postcolumn one approach. However, all the precolumn derivatization reagents have various weaknesses which make precolumn methods labour-intensive or the reproducibility and accuracy are not good enough for amino acid analyses.

Recently, Cohen and Michaud [6] developed a precolumn derivatization method in which a novel reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), was used for amino acid derivatization. AQC can react quantitively with all primary and secondary amino acids in a few seconds with little matrix interference and single and very stable derivatives are formed. Fluorescence detection was applied with excitation at 250 nm and emission at 395 nm, which allowed for direct injection of the reaction mixture because of the 60-nm blue shift of the wavelength of amino acid derivatives compared with 6-aminoquinoline (AMQ), the hydrolysate of AQC [6]. As the 6-aminoquinolyl derivatives of amino acids have very strong ultraviolet (UV) absorbance, UV detection can also be used for the determination of AQC-derivatized amino acids provided that the huge reagent peak does not interfere with the isolation of the amino acid derivatives. The purpose of this study was to develop a UV detection method for AQC-derivatized amino acids by modifying the chromatographic conditions described by Cohen and Michaud [6].

2. Experimental

2.1. Materials

Analytical-reagent grade chemicals were used unless indicated otherwise and ultrapure water was generated using a Milli-Q water purification system (Millipore, Milford, MA, USA).

The derivatization reagent kit (AQC, 0.2 *M* borate buffer and DNA-grade acetonitrile) was obtained from Millipore. Phosphoric acid, hydrochloric acid and phenol were purchased from Wako (Osaka, Japan). Amino acid standard was purchased from Pierce (Rockford, IL, USA). Bovine serum albumin (BSA) was purchased from Pharmacia (Uppsala, Sweden). Neuromedin was supplied by Millipore. Sodium acetate, Triethylamine, Acetonitrile (HPLC grade) and ethylenediaminetetraacetic acid disodium salt (EDTA) were all local products. Sodium azide was obtained from Farco Chemical Supplies (Hong Kong).

2.2. Apparatus

Two HPLC systems from Millipore were used. System 1 consists of two Model 510 pumps, a Model M717 autosampler, a Waters Model 486 tunable absorbance detector or a Waters Model 996 photodiode-array detector and a temperature control module. System 2 was a Waters LC Module 1 system with a column heater option. Millinnium 2010 Chromatography Manager was connected with the systems for data acquisition and management.

2.3. Sample hydrolysis and derivatization

Approximately 14.3 μg of BSA and 2 μg of neuromedin (a synthesized basic peptide containing eight amino acid residues) were hydrolysed using the following standard gas-phase hydrolysis procedure. An aliquot of sample was pipetted into the bottom of a 6×50 mm tube and dried under vacuum. A 200-μl volume of 6 *M* HCl containing 0.5% phenol was placed in the bottom of a vacuum vial and then the tube was inserted into the vial. The vial was sealed under vacuum after three alternate vacuum–nitrogen flushing steps. Hydrolysis was then carried out at 115°C for 24 or 42 h. Feed samples were hydrolysed using the liquid-phase hydrolysis protocol as follows. A 59-mg amount of feed powder was added to 10 ml of 6 *M* HCl and hydrolysed for 24 h. After filtration through a 0.45-μm Millex-HV filter (Millipore), the hydrolysate was diluted with ultrapure water to a

concentration of approximately 1.5 mg/ml of amino acids and 10 μl of the diluted solution were pipetted into a 6 × 50 mm tube. After drying under vacuum, all these protein, peptide and feed hydrolysates were reacted with AQC using the procedure optimized by Cohen and Michaud [6].

2.4. Chromatographic conditions

Both systems were operated under the same chromatographic conditions: eluent A concentrate was prepared as described by Cohen and Michaud [6] except that the pH was lowered to 4.95 from 5.05 and that 0.01% of sodium azide was present in the concentrate to protect the buffer from bacterial growth. The working eluent A was prepared by mixing 100 ml of the concentrate with 900 ml of water. Eluent B was acetonitrile–water (60:40, v/v) containing 0.01% acetone. A 4-μm AccQ-Tag C_{18} column (150 mm × 3.9 mm I.D.) was connected to the systems with the temperature controlled at 37°C. The gradient condition were as follows: initial, 100% A; 17 min, 93% A; 21 min, 90% A; 32 min, 66% A (all linear). After a 1-min hold, the column was washed with 100% eluent B for 3 min and then re-equilibrated with 100% A for 7 min. The run time (injection-to-injection) was 45 min. The flow-rate was maintained at 1 ml/min over the whole gradient.

3. Results and discussion

3.1. Improvement of the separation of amino acid derivatives and reagent peak

Many previous precolumn derivatization methods for amino acid determinations suffered from interference from excess of reagent because it has similar absorbance or fluorescence emission properties to those of the derivatives, so extra steps, *e.g.*, vacuum or extraction, had to be taken to remove the excess of reagent after derivatization. With AQC using fluorescence detection [6], the reagent interference was minimal because the resolution between the reagent peak (AMQ) and Asp, the fastest eluted amino acid, was sufficient for accurate determination of Asp as the AMQ peak was small and 1.8 min from that of Asp. A 1.8-min separation was not sufficient, however, if the fluorescence detector was replaced with a UV detector under the same chromatographic conditions because AMQ and its amino acid derivatives gave very similar UV absorbances so the reagent peak was huge one compared with those of the amino acid derivatives and the Asp peak would be eluted before the AMQ peak had returned to the baseline. This problem was especially serious when highly sensitive analysis was required: the small Asp peak was eluted on the tail of the reagent peak, which made accurate integration of the Asp peak difficult. Hence the resolution between these two peaks had to be increased before UV detection method could be applied. In this study, the separation between the AMQ and Asp peaks was improved in two ways: by lowering the pH of buffer A or by making the gradient shallower.

As AMQ is basic and Asp is acidic, AMQ would be eluted faster and Asp more slowly as the pH of mobile phase A decreased. Fig. 1 shows that when the pH of eluent A was lowered to 4.90 from 5.05, the distance between the two peaks increased sharply from 1.8 to 3.3 min. Considering that lowering the pH could worsen the resolution of the Asp–Ser and Glu–Gly pairs

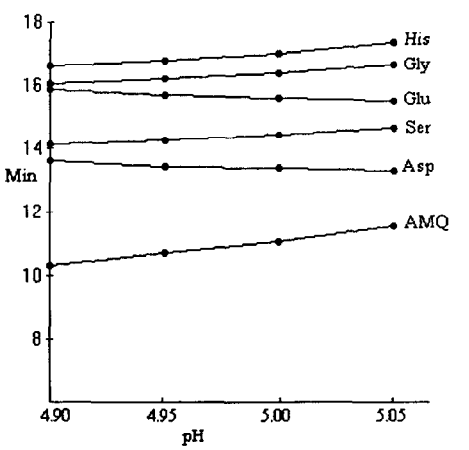

Fig. 1. Effect of pH of mobile phase A on retention time of AMQ and other early-eluted amino acids. Gradient conditions as described in ref. 6.

and that the separation between Glu and Gly would become unacceptable if the pH was lowered to 4.90, 4.95 was chosen as the final pH of mobile phase A. Another approach, namely making the elution gradient shallower, was also tried as the 2.7 min distance between the AMQ and Asp peaks was still unsatisfactory for the analysis of samples with low Asp concentrations. Under the final gradient conditions adopted, AMQ was isolated 3.4 min away from Asp on both systems and all other amino acids were separated from each other with a resolution larger than 1.6 (Fig. 2). It could be expected that as little as 10 pmol or less of Asp could still be determined accurately (Fig. 2b) because of the lack of reagent interference, which apparently was impossible when UV detection was applied under the fluorescence chromatographic conditions [6]. The purpose of adding acetone to eluent B was to eliminate the baseline drift of the chromatogram caused by eluent A having a stronger UV absorbance than eluent B.

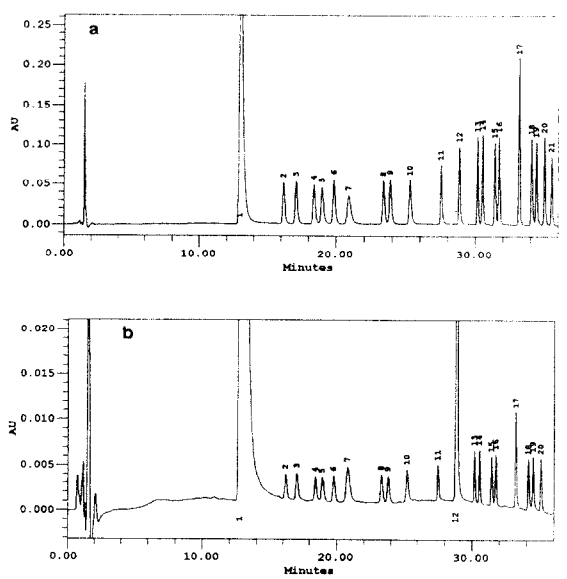

Fig. 2. Chromatogram of AQC derivatives with amino acid standard containing nineteen amino acids. Derivatization procedure: (1) 70 μl of 0.2 *M* borate buffer added to 10 μl of amino acid solution and vortex mixed; (2), 20 μl of 3 mg/ml AQC solution in acetonitrile added and vortex mixed immediately for 2 s; (3) derivatized sample transferred into an autosampler vial; (4) vial heated for 10 min at 55°C. Volumes of 5 μl of the derivatized samples were injected. Amount injected: (a) 250 and (b) 12.5 pmol. Peaks: 1 = AMQ; 2 = Asp; 3 = Ser; 4 = Glu; 5 = Gly; 6 = His; 7 = NH_3; 8 = Arg; 9 = Thr; 10 = Ala; 11 = Pro; 12 = α-aminobutyric acid (AABA); 13 = cystine (Cys2); 14 = Tyr; 15 = Val; 16 = Met; 17 = Lys; 18 = Ile; 19 = Leu; 20 = Phe; 21 = Trp.

The reproducibility of the responses of seven replicate analyses of derivatives of amino acid standards is shown in Fig. 3. Among all eighteen amino acids, methonine, one of the most unstable amino acids, displayed the highest relative standard derivation (R.S.D.) of 2.3%; for Asp, because of its distance from the reagent peak, the R.S.D. was 0.99%, below the average value for all the amino acids.

3.2. UV absorbance properties of AQC–amino acid derivatives

The maximum absorbance wavelengths (λ_{max}) of all the AQC-derivatized amino acids were 248 nm with the exception of cystine and Lys, the two disubstituted derivatives with AQC, with λ_{max} 243 nm. The λ_{max} of AMQ was 257 nm, so 248 nm was chosen as the detection wavelength to increase the detection sensitivity of the method and to decrease the response of the reagent peak.

3.3. Compositional analysis of BSA and neuromedin

Some data have been reported [6] to demonstrate the accuracy of the AQC derivatization method with fluorescence detection for the determination of amino acids. However, this is the

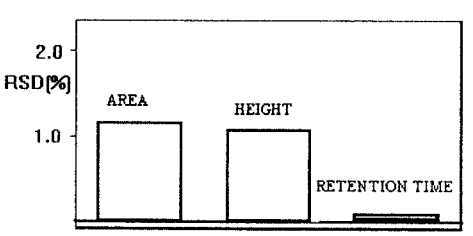

Fig. 3. Average R.S.D. bar plot for seven replicate analyses of derivatized amino acid standard. Seven aliquots of amino acid standards containing 5 nmol of each amino acid were derivatized and analysed under the same conditions. A 250 pmol amount of each sample was injected.

first report in which AQC-derivatized amino acids were determined with UV absorbance detection, so it was necessary to conduct more experiments to investigate the accuracy of the method with UV detection as it is less selective than fluorescence detection. Fig. 4 shows the chromatograms of BSA and neuromedin hydrolysates. It is interesting that the two minor reagent-related interference peaks mentioned in ref. 6 were not found in the chromatograms with UV detection even on a very sensitive scale (Fig. 4c), which suggests that negligible interference from these two peaks could be expected in the UV detection method.

Table 1 summarizes the composition of BSA samples and Table 2 gives the results for neuromedin. The average R.S.D. for five replicate analyses of BSA and neuromedin samples were 0.86% and 1.36%, respectively, which, resulting from the stability of the derivatized amino acids, are comparable to those for any techniques for amino acid determination including the post-column method. The average relative errors of the results were also good. The two largest errors in BSA sample analysis were for Met and Gly, a possible reason for which is background contamination. Another reason for the error for Met is its relative smaller residue number in BSA as compared with other amino acids. Ser and Thr are degraded during hydrolysis. Because of the easy dehydration, the recovery of Ser was 70–90% and that of Thr was 85–95%, Correction of the data for these two amino acids for the losses during hydrolysis were made by hydrolysing the protein for different times and extrapolating to zero time. Because the compositional results for BSA after hydrolysis for 42 h were 19.44 for Ser and 27.81 for Thr (normalized to Val), the corrected results were 27.35 for Ser and 34.58 for Thr, which were comparable to the sequence of BSA. After correction the average relative error of the calculated composition was 3.2%.

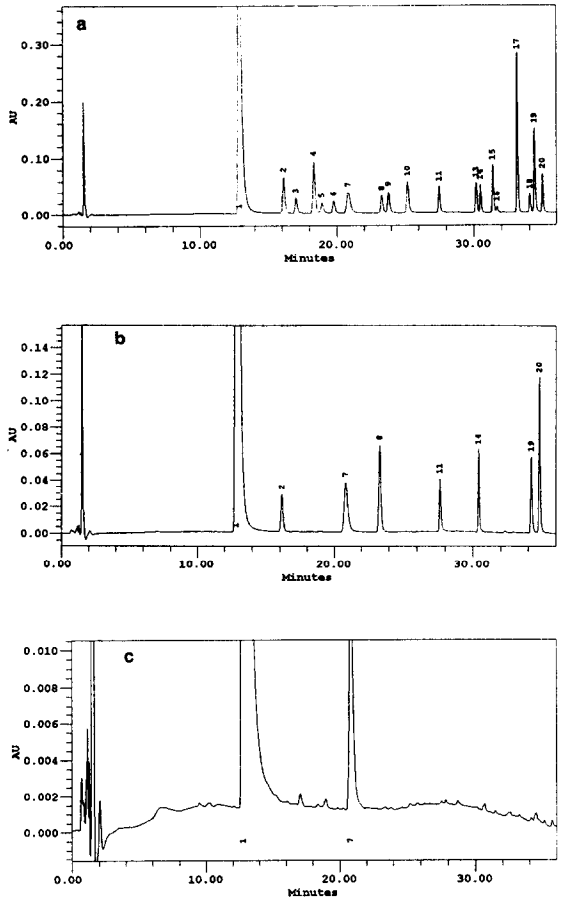

Fig. 4. Chromatograms of derivatives of amino acids in (a) BSA and (b) neuromedin hydrolysates and (c) hydrolysis blank. One twentieth of the derivatized amount was injected. Injection volume, 5 μl. Peaks as in Fig. 1.

3.4. Determination of amino acids in feed hydrolysates

The chromatographic profile of AQC-derivatized amino acids in feed hydrolysates is shown in Fig. 5. Table 3 summarizes the results obtained with the proposed method and also for the

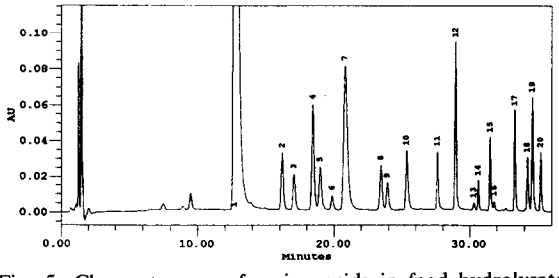

Fig. 5. Chromatogram of amino acids in feed hydrolysate. One twentieth of the derivatized amount was injected. Injection volume, 5 μl. Peaks as in Fig. 1.

Table 1
Determination of composition of five replicated BSA samples

Amino acid	Sequence of BSA	Calculated composition[a] (average, $n = 5$)	R.S.D. (%)	Error (%)
Ala	46	45.66	0.9	−0.74
Arg	23	22.95	0.53	−0.22
Asp	54	54.41	1.45	0.75
Glu	79	79.78	0.81	0.99
Gly	16	18.13	2.52	13.31
His	17	16.85	0.53	−0.86
Ile	14	14.00	0.46	0.00
Leu	61	59.64	0.29	−2.23
Lys	59	57.93	1.29	−1.81
Met	4	4.75	0.64	−1.81
Phe	27	27.23	0.56	0.84
Pro	28	28.47	0.53	1.53
Ser	28	22.83	1.6	−18.46
Thr	34	30.71	0.41	−9.68
Tyr	19	19.98	1.31	5.16
Val	36	36	0.00	0.00

Five aliquots of BSA samples were hydrolysed for 24 h and then analysed under the same conditions.
[a] All data were normalized to Val without correction for losses during hydrolysis.

Table 2
Determination of composition of five replicate neuromedin samples

Amino acid	Sequence	Calculated composition[a] (average, $n = 5$)	R.S.D. (%)	Error (%)
Asp	1	1.041	1.40	4
Arg	2	2.062	1.40	3.1
Pro	1	1.051	2.10	5.1
Tyr	1	0.998	2.20	−0.2
Leu	1	1	0.00	0
Phe	2	2.026	1.10	1.3

Five aliquots of neuromedin sample were hydrolysed for 24 h and then analysed under the same conditions.
[a] All data were normalized to Leu without correction for losses during hydrolysis.

analysis of the same vial of sample with an ion-exchange method performed on a Hitachi 835 amino acid analysis system. The two sets of data agree fairly well. The precolumn method data (Data 1) were calculated by internal calibration method with AABA as internal standard. External calibration was also tested for the analysis of the same sample and the two sets of data were almost identical (not shown), which suggested that the complex matrix of the feed hydrolysate had a minimal effect on the yield of the derivatization reaction. The reproducibility of the results with the internal calibration method, however, was generally better than that with external calibration (Table 4).

Another validation test for the accuracy of the method was done by measuring a known amount of amino acid standard added to the feed hydrolysate. The average recovery was about 98% (Table 5).

Table 3
Comparison of results for feed hydrolysate obtained using the proposed method (Data 1) and an ion-exchange method (Data 2)

Amino acid	Data 1 (mg per 100 mg feed)	Data 2 (mg per 100 mg feed)	Data 1/Data 2
Ala	1.85	1.76	1.05
Arg	2.65	2.25	1.17
Asp	2.92	2.74	1.07
Glu	6.09	5.9	1.03
Gly	1.38	1.27	1.09
His	0.746	0.75	0.99
Ile	1.28	1.38	0.93
Leu	2.63	2.69	0.98
Lys	1.36	1.37	0.99
Pro	1.74	1.58	1.1
Ser	1.41	1.36	1.04
Thr	0.95	1.12	0.85
Tyr	0.9	0.97	0.93
Val	1.61	1.73	0.93

Table 4
Comparison of reproducibilities of results for feed hydrolysates ($n = 5$)

Amino acid	R.S.D. (%)	
	Internal calibration	External calibration
Asp	0.66	2.3
Ser	1.7	2.8
Glu	0.68	1.8
Gly	1.6	2.6
His	1.4	2.5
Arg	0.4	2.1
Thr	0.29	1.5
Ala	0.64	1.6
Pro	0.34	1.8
Cys_2	0.51	1.5
Tyr	0.33	2.2
Val	0.32	1.8
Met	4.1	2.9
Lys	0.1	2
Ile	0.17	1.9
Leu	0.1	1.9
Phe	0.73	1.5

Five aliquots of the same feed hydrolysate were derivatized and analysed. The raw data were processed with the internal and external calibration methods.

3.5. Linearity of calibration graphs and detection limits of the method

The UV detection method was not as insensitive as the fluorescence method. The detection limits for UV detection of all amino acids including cystine and Trp were in the range 0.07–0.30 pmol, as shown in Table 6, whereas for the fluorescence method they were 0.04–0.32 pmol except for cystine (0.8 pmol) and Trp (unknown) [6]. Table 6 also shows the linearity of the calibration graphs for seventeen amino acids which were obtained by analysing a series of dilutions of the standard mixture ranging from 25 to 500 μM. Most of the correlation coefficients were greater than 0.9999, which demonstrates excellent linearity of the calibrations.

4. Conclusions

Although UV detection is not as sensitive and selective as fluorescence detection, the results for BSA, neuromedin and feed samples described in this paper demonstrate that the accuracy and reproducibility of the method with AQC pre-

Table 5
Recovery of standards added to feed hydrolysate

Amino acid	Amount added (nmol)	Amount found (nmol)	Recovery (%)
Ala	1.00	1.01	101
Arg	1.00	1.12	112
Asp	1.00	0.94	94
Glu	1.00	0.93	93
Gly	1.00	1.03	103
His	1.00	0.92	92
Ile	1.00	0.97	97
Leu	1.00	0.95	95
Lys	1.00	0.97	97
Pro	1.00	1.01	101
Ser	1.00	0.94	94
Thr	1.00	0.98	98
Tyr	1.00	0.99	99
Val	1.00	0.98	98
Average			98

Table 6
Linearity of calibration graphs and detection limits of the UV method with AQC derivatization

Amino acid	Correlation coefficient	Detection limit (pmol)[a]
Asp	0.99997	0.26
Ser	0.999926	0.24
Glu	0.999976	0.29
Gly	0.999875	0.29
His	0.999941	0.26
Arg	0.999997	0.24
Thr	0.999991	0.26
Ala	0.999981	0.21
Pro	0.99998	0.19
Cys_2	0.999965	0.06
Tyr	0.999974	0.13
Val	0.999995	0.15
Met	0.999907	0.14
Lys	0.999977	0.071
Ile	0.999986	0.13
Leu	0.999958	0.13
Phe	0.9990951	0.13
Trp	N.D.[b]	0.16

[a] The detection limits were calculated from a 12.5-pmol injection and based on signal-to-noise ratio of 3 (noise level: $\pm 2 \cdot 10^{-5}$ absorbance, estimated from the baseline at 26.2–27.2 min).
[b] Not determined.

column derivatization of amino acids and UV detection are similar to those with fluorescence detection and sensitive enough for the routine analysis of most kinds of amino acid samples.

5. Acknowledgements

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