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Direct extract derivatization for determination of amino acids in human urine by gas chromatography and mass spectrometry

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Abstract

The purpose of this study was to develop a simple and accurate analytical method to determine amino acids in urine samples. The developed method involves the employment of an extract derivatization technique together with gas chromatography–mass spectrometry (GC–MS). Urine samples (300 μl) and an internal standard (10 μl) were placed in a screw tube. Ethylchloroformate (50 μl), methanol–pyridine (500 μl, 4:1, v/v) and chloroform (1 ml) were added to the tube. The organic layer (1 μl) was injected to a GC–MS system. In this proposed method, the amino acids in urine were derivatized during an extraction, and the analytes were then injected to GC–MS without an evaporation of the organic solvent extracted. Sample preparation was only required for ca. 5 min. The 15 amino acids (alanine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, tryptophan, valine) quantitatively determined in this proposed method. However, threonine, serine, asparagine, glutamine, arginine were not derivatized using any tested derivatizing reagent. The calibration curves showed linearity in the range of 1.0–300 μg/ml for each amino acid in urine. The correlation coefficients of the calibration curves of the tested amino acids were from 0.966 to 0.998. The limit of detection in urine was 0.5 μg/ml except for aspartic acid. This proposed method demonstrated substantial accuracy for detection of normal levels. This proposed method was limited for the determination of 15 amino acids in urine. However, the sample preparation was simple and rapid, and this method is suitable for a routine analysis of amino acids in urine.

Keywords: Derivatization, GC; Amino acids

1. Introduction

When there is a lack of enzymes associated with an amino acid metabolic pathway, amino acids and their precursors abnormally accumulate in the body. As a result, amino acid concentrations rise in blood and urine. If this is a genetic defect, it causes major intellectual disturbance in humans. If a neonatal diagnosis is made, appropriate treatment can be done. Therefore, it is important to develop a simple and accurate method to determine amino acids in biological samples [1].

Numerous screening methods were published to determine amino acids in blood or urine [2–9]. However, it is difficult to analyze amino acids in biological samples due to their high polarity, twitterion, and the lack of the typical UV spectrum. High-performance liquid chromatography with fluorescence detection and gas chromatography with mass spectrometry (GC–MS) are major analytical methods. In these methods, amino acids need to be

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isolated from biological samples and then derivatized prior to chromatography. They require, however, time consuming sample preparations and relatively large samples.

The purpose of this study was to develop a simple and accurate analytical method to determine amino acids in urine samples. The developed method involves the employment of the extract derivatization technique together with GC–MS.

2. Experimental

2.1. Materials

The amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan and valine were obtained from Sigma (St. Louis, MO, USA). Stock standards (10 mg/ml) were prepared in hydrochloric acid (10 m*M*). The deuterated methionine (methionine-d_3, 98 atom% D) was obtained from Isotec (Mianisburg, OH, USA).

Methylchloroformate, ethylchloroformate, propylchloroformate and butylchloroformate were obtained from Sigma. All other chemicals were of analytical-reagent grade and obtained from Wako (Osaka, Japan).

2.2. Extraction and derivatization

The extract derivatization technique for amino acids with alkylchloroformates was a slightly modified version of the method described by Husek [10]. In his method, amino acids were derivatized in an aqueous solution and the derivatives were then extracted with an organic solvent. In order to shorten the sample preparation time, amino acids in urine were derivatized and extracted simultaneously. Urine samples (300 μl) and an internal standard (1.0 mg/ml, 10 μl) were placed in a screw tube. Alkylchloroformate (50 μl), alcohol–pyridine (500 μl, 4:1, v/v) and chloroform (1 ml) were added to the tube. The tube was gently shaken for 1 min. During shaking of the sample, amino acids were derivatized in the mixture and the derivatives were transferred to a chloroform layer. After 5 min, the organic layer was transferred to a new vial. The organic layer (1 μl) was injected to the GC–MS system.

Table 1
Mass fragment ions of *N*-ethoxycarbonyl amino acid methyl esters

Name	Fragment ions			
Glycine	102	88	161	
Alanine	116	88	175	
Valine	144	88	116	72
Proline	142	70	98	128
Leucine	158	102	88	
Isoleucine	158	101	129	88
Aspartic acid	174	70	102	
Glutamic acid	188	84	128	142
Methionine	161	129	61	235
Phenylalanine	162	88	192	120
Cysteine	88	220	190	118
Lysine	156	226	272	84
Histidine	224	81	154	254
Tyrosine	107	178	250	135
Tyrptophan	130	290	201	

Underlined ions were used for the quantitation.

2.3. Gas chromatography–mass spectrometry

The GC–MS system used was a Hewlett-Packard 5890 series II gas chromatograph and a 5971 A mass-selective detector, equipped with a 30 m×0.25 mm I.D. fused-silica capillary column (Hewlett-Packard, HP-5MS, film thickness 0.25 μm). The oven temperature was set at 50 °C for 1 min, and then programmed from 50 °C to 300 °C at 10 °C/min, and held at 300 °C for 6.5 min. The temperatures of the injection port and the detector were set at 250 and 280 °C, respectively. The splitless injection mode was used. The mass-selective detector was operated in the electron impact (EI) mode at 70 eV of electron energy. Helium with a flow-rate of 0.8 ml/min was used as a carrier gas. To confirm the mass fragment

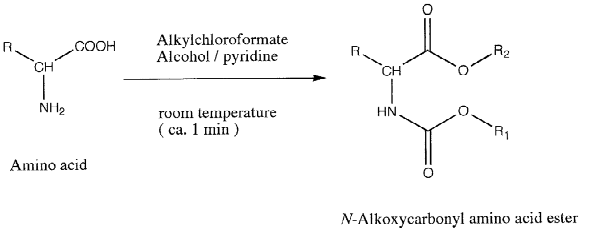

Fig. 1. Reaction scheme of the derivatizing reagents and amino acid.

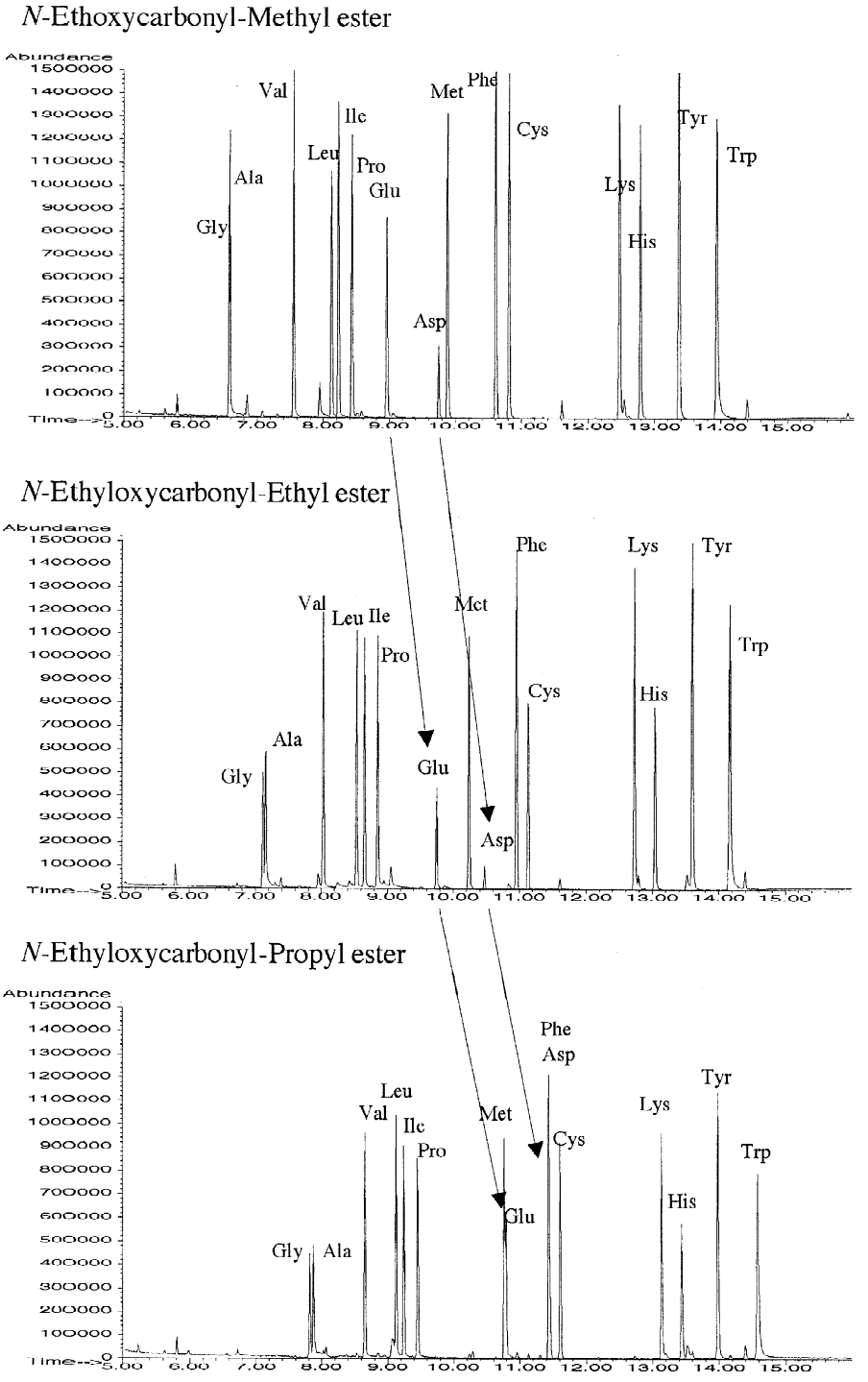

Fig. 2. The influence of the alkyl length of carboxyl group. These chromatograms were obtained *N*-ethoxycarbonyl derivatives of amino acids extracted from spiked waters.

of the derivatives, data were obtained in the full scan mode with a scan range from m/z 50 to 550. Ions used for quantitation are shown in Table 1. Data were collected and integrated with a personal computer using Chemistation®.

2.4. Method validation

To determine calibration curves, urine samples spiked with amino acids at concentrations ranging from 0.5 to 300 μg/ml were prepared and analyzed using the above procedure. The calibration curves were obtained by plotting the peak area ratio between the derivatives of amino acids and that of methionine-d_3 (I.S.). Reproducibility was evaluated by analyzing urine samples containing three different concentrations of amino acids (1, 10 and 100 μg/ml) on the same day in five replicates (intra-day reproducibility) and over 5 consecutive days in duplicates (inter-day reproducibility).

3. Results

3.1. Selection of alkylchloroformates and alcohols

Four kinds of alkylchloroformates (methyl-, ethyl-, propyl- and butylchloroformate) and four kinds of alcohols (methanol, ethanol, propanol and butanol) were examined for derivatization of amino acids in order to achieve a good chromatographic separation. The reaction scheme of these reagents and amino acid is shown in Fig. 1. The typical chromatograms are shown in Fig. 2. The length of the alkyl group was influenced by retention time. This influence was typically in the case of aspartic acid and glutamic acid. When ethylchloroformate and propanol were used as the derivatizing reagents, the derivatives of aspartic acid and glutamic acid were overlapped with the peaks of methionine and phenylalanine. Whereas using ethylchloroformate and methanol as the derivatizing reagents, better chromatographic separation and the highest peak area were obtained. Therefore ethylchloroformate and methanol were adopted as the derivatizing reagents.

3.2. Sample volume

To avoid the formation of an emulsion is the most important factor in a liquid–liquid extraction. The sample volume was changed from 0.1 to 1.0 ml in order to solve this problem. When using a 1.0 ml sample, the extraction mixture made an emulsion and there was a need to centrifuge. When using 0.1 or 0.3 ml samples, the extraction mixture made no emul-

Table 2
Quantitation limit and linearity of the method

Name	Limit of detection (μg/ml)	Range of linearity (μg/ml)	Correlation coefficient
Glycine	0.5	1–200	0.987
Alanine	0.5	1–200	0.992
Valine	0.5	1–200	0.993
Proline	0.5	1–200	0.994
Leucine	0.5	1–200	0.991
Isoleucine	0.5	1–200	0.990
Aspartic acid	1.0	2–300	0.992
Glutamic acid	0.5	1–200	0.984
Methionine	0.5	1–200	0.998
Phenylalanine	0.5	1–200	0.992
Cysteine	0.5	1–200	0.966
Lysine	0.5	1–200	0.990
Histidine	0.5	1–300	0.969
Tyrosine	0.5	1–300	0.991
Tyrptophan	0.5	1–300	0.987

sion and there was no need to centrifuge. However, the limit of detection was not acceptable in the 0.1 ml sample. Therefore, the sample volume adopted was 0.3 ml.

3.3. Calibration curves and reproducibility

The calibration curves showed linearity in the range of 1.0–300 μg/ml for each amino acid in

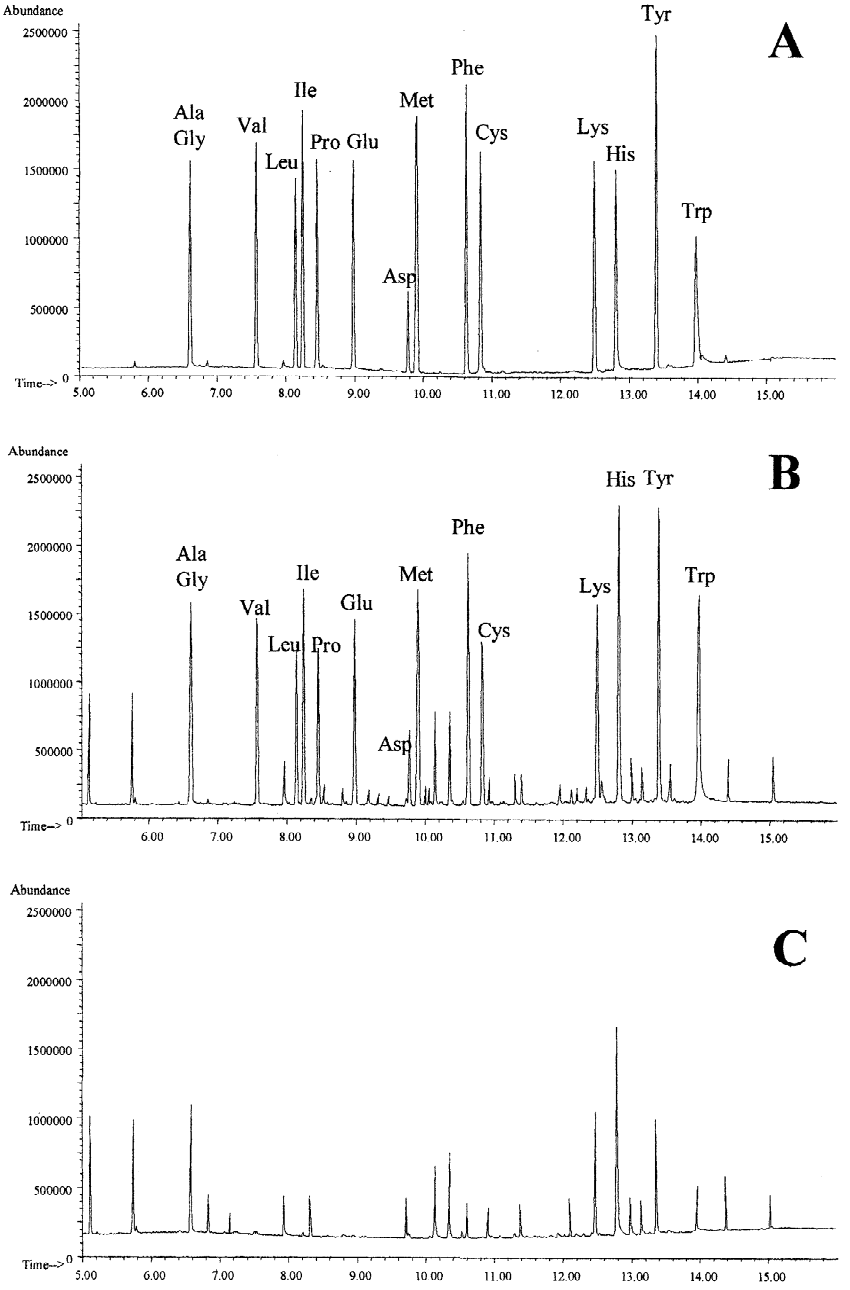

Fig. 3. The typical chromatograms of *N*-ethoxycarbonyl amino acid methyl esters extracted from spiked water, spiked urine and blank urine. (A) Spiked water (5.0 μg/ml, each amino acid), (B) spiked urine (5.0 μg/ml, each amino acid), (C) blank urine.

urine (Table 2). The correlation coefficients of the calibration curves of the tested amino acids were from 0.966 to 0.998. The limit of detection in urine was 0.5 μg/ml except for aspartic acid. This proposed method gave substantial accuracy for detection of normal levels. The typical chromatograms extracted from the spiked urine are shown in Fig. 3. The intra-day and inter-day relative standard deviations (RSDs) for three different concentrations (1.0, 10 and 100 μg/ml) in urine were 2.5 to 21.6% and 3.2 to 23.9%, respectively (Table 3). Considering the relative standard deviations with the other methods [2–9], the proposed method was found to be acceptable except for glutamic acid.

Table 3
Extraction recovery and relative standard deviation

Name (μg/ml)	Intra-day		Inter-day RSD (%)	Name (μg/ml)	Intra-day		Inter-day RSD (%)
	Recovery (%)	RSD (%)				RSD (%)	
Glycine				Methionine			
1	75.3	5.8	9.5	1	82.3	5.5	6.9
10	77.1	2.5	3.8	10	79.8	4.3	5.8
100	84.5	5.6	6.9	100	83.3	6.9	9.8
Alanine				Phenylalanine			
1	78.0	6.7	9.2	1	84.1	4.3	7.9
10	78.6	3.0	3.2	10	88.5	2.8	4.3
100	80.2	7.4	8.6	100	72.3	6.7	7.8
Valine				Cysteine			
1	89.6	7.2	8.4	1	78.3	5.1	7.2
10	87.2	4.2	5.7	10	75.9	5.2	6.4
100	80.5	6.7	7.5	100	73.1	10.9	11.9
Proline				Lysine			
1	84.7	7.2	7.7	1	79.6	7.2	10.2
10	87.9	3.7	3.4	10	77.1	7.9	8.7
100	81.9	7.0	8.0	100	82.6	6.2	7.6
Leucine				Histidine			
1	91.8	6.7	7.7	1	72.6	5.8	8.5
10	91.6	4.1	4.9	10	77.4	6.1	6.8
100	87.5	7.1	8.1	100	72.7	7.1	8.2
Isoleucine				Tyrosine			
1	84.4	4.6	8.5	1	85.3	5.7	8.9
10	91.6	4.5	5.9	10	83.1	3.6	5.1
100	87.5	6.1	7.1	100	85.4	6.2	7.3
Aspartic acid				Tryptophan			
1	83.0	6.9	10.8	1	77.8	8.7	11.8
10	87.6	5.1	6.2	10	81.3	2.7	3.9
100	78.6	6.7	8.3	100	84.4	9.6	9.8
Glutamic acid							
1	85.1	21.6	23.9				
10	85.1	11.9	13.3				
100	72.3	8.1	17.6				

Intra-day assay was performed on a single day with five replicates ($n=5$).
Inter-day assay was performed over 5 consecutive days in duplicate ($n=10$).

4. Discussion

Silylation is the most frequently used method for the GC assay of amino acids. The silyl derivative of amino acid has many advantages; good fragmentation, high intensity. However, the derivative procedure is time consuming (30~60 min). In order to shorten the procedure, alkylchloroformate was used for the derivatizing reagent of the amino group. The alkylchloroformate reacts quickly with a primary or secondary amine in an aqueous medium [10,11]. Therefore, it is a useful reagent for the derivatization of the amino group.

In order to achieve a good chromatographic separation of the amino acid derivatives, four alkylchloroformates (methyl-, ethyl-, propyl- and butylchloroformate) and four alcohols (methanol, ethanol, propanol and butanol) were examined as derivatizing reagents. In the proposed method, threonine, serine, asparagine, glutamine, arginine were not derivatized using any tested derivatizing reagent. A report stated that it was important to adjust the pH to make the double-derivative formation with amino acids [10]. In this study, the pH was not adjusted to the optimal condition of the reaction medium. As a result, the above five amino acids were not derivatized.

Both the alkyl length of the derivatives were influenced by retention time. The influence was typical in the case of aspartic acid and glutamic acid. When methanol was used as the derivatizing reagent of a carboxyl group, the derivatives of aspartic acid and glutamic acid had the highest peak intensity and were not overlapped with any peak. However, when propanol was used as the derivatizing reagent of a carboxyl group, the derivatives of aspartic acid and glutamic acid moved and were overlapped with phenylalanine and cysteine.

5. Conclusion

This extractive derivatization is a simple and rapid procedure for determination of amino acid in urine. No emulsion is made in this procedure. This method can be applied to a routine analysis using a robotic machine.

References

[1] I. Matsumoto, S. Sakamoto, T. Kuhara, M. Sudo, M. Yoshino (Eds.), GC–MS Practical Chemical Diagnosis—Inherited Metabolic Disorders, Soft Science Publications, Tokyo, 1995.
[2] Z. Deyl, J. Hyanek, M. Horakova, J. Chromatogr. 379 (1986) 177.
[3] T. Kuhara, I. Matsumoto, Proc. Jpn. Soc. Biomed. Mass Spectrom. 20 (1995) 45.
[4] T. Kuhara, J. Chromatogr. B 758 (2001) 3.
[5] M.S. Rashed, J. Chromatogr. B 758 (2001) 27.
[6] H. Bruckner, A. Schieber, Biomed. Chromatogr. 15 (2001) 166.
[7] T. Kuhara, T. Shinka, Y. Inoue, M. Ohse, X. Zhen-Wei, I. Yashida, T. Inokuchi, S. Yamaguchi, M. Takayanagi, I. Matsumoto, J. Chromatogr. B 731 (1999) 141.
[8] C.D. Marquez, S.T. Weintraub, P.C. Smith, J. Chromatogr. B 658 (1994) 213.
[9] S.P. Stabler, P.D. Marcell, E.R. Podell, R.H. Allen, Anal. Chem. 162 (1987) 185.
[10] P. Husek, J. Chromatogr. 552 (1991) 289.
[11] J. Vessman, K.E. Karlsson, O. Gyllenhaal, J. Pharm. Biomed. Anal. 4 (1986) 825.