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Liquid chromatographic–mass spectrometric analysis of N-acetylamino acids in human urine

Kazunori Sugahara*, Zhang Jianying, Hiroyuki Kodama

Department of Chemistry, Kochi Medical School, Kohasu, Oko-cho, Nankoku-shi, Kochi 783, Japan

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Abstract

Liquid chromatography–atmospheric pressure chemical-ionization mass spectrometry (LC–APCI-MS) was used for the analysis of N-acetylamino acids that could not be determined with an amino acid analyzer. LC–APCI-MS could directly detect the protonated molecular ions of various synthetic N-acetylamino acids, distinguishing N-acetylserine from O-acetylserine and N(α)-acetyllysine from N(ε)-acetyllysine. Furthermore, N-acetylasparagine, N-acetylaspartic acid, N-acetylglutamine and N-acetylglutamic acid were identified in normal human urine. The assay data for N-acetylaspartic acid agree with a previous report using gas chromatography–mass spectrometry. These results demonstrate the usefulness of the apparatus described above for the analysis of N-acetylamino acids in biological samples.

1. Introduction

Various amino acids that are excreted in normal urine can be easily analyzed with an amino acid analyzer. However, N-acetylamino acids fail to react with ninhydrin and can not be detected by that technique. Determination of N-acetylamino acids in biological samples has been performed by analysing free amino acids after hydrolysis [1], gas chromatography [2], gas chromatography–mass spectrometry [3], or high-performance liquid chromatography with fluorimetric detection [4].

Liquid chromatography–atmospheric pressure chemical-ionization mass spectrometry (LC–APCI-MS) [5,6] has recently shown to be promising for the analysis of non-volatile compounds in biological samples. We have applied LC–APCI-MS to the study of urinary amino acids in a cystathioninuric patient [7] and cystinuric patients [8], and of urinary iminodipeptides in patients with prolidase deficiency [9,10]. In this study this technique was used to analyse synthetic N-acetylamino acids and identify N-acetlyamino acids in normal human urine.

2. Experimental

2.1. Materials

Amino acids and several acetylamino acids were purchased from Sigma (St. Louis, MO,

* Corresponding author.

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USA). All other chemicals used were of analytical grade.

2.2. Syntheses of N-acetylamino acids

N-acetylamino acids as standards were prepared as follows. A 10-mg amount of amino acid dissolved in 1 ml of 2 *M* NaOH was reacted with 0.5 ml of acetic anhydride at room temperature for 30 min. The solution was transferred to a column (230 × 10 mm I.D.) containing a cation-exchange resin (Diaion SK-1, H-form, 100–200 mesh, Mitsubishi Kasei Kogyo, Tokyo, Japan). The column was washed with water, and the eluent and washings were combined and evaporated to dryness under reduced pressure.

2.3. Instrumentation

The apparatus used was a high-performance liquid chromatograph (Hitachi L-6200) equipped with an Inertsil ODS packed column (250 × 4.6 mm I.D., particle size 5 μm) (Gasukuro Kogyo, Tokyo, Japan), connected to an atmospheric pressure chemical-ionization double-focussing mass spectrometer (Hitachi M-80B). LC–MS data were obtained with a Hitachi M-0101 data acquisition system. The nebulizer temperature was 270°C, desolvator temperature 390°C, drift voltage 275 V and flow-rate 0.9 ml/min. The mobile phase was 0.05 *M* ammonium acetate–acetonitrile (9:1, v/v).

2.4. Isolation of N-acetylamino acids from urine

Samples of normal human urine were obtained from laboratory personnel. The urine samples were weakly acidified with 2 *M* acetic acid and filtered through filter paper. The filtrate was transferred to a column containing Diaion SK-1. The column was washed with water and the eluent and washings were combined and made weakly basic with 2 *M* ammonia. The solution transferred to a column (230 × 10 mm I.D.) containing an anion-exchange resin (Diaion SA-100, HCOOH-form, 100–200 mesh, Mitsubishi Kasei Kogyo). After washing with water, the N-acetylamino acids were eluted with 10% formic acid. The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in a small amount of water and then subjected to LC–APCI-MS.

2.5. Assay of creatinine in urine

A creatinine assay kit from Wako Pure Chemical Industries (Osaka, Japan) was used.

3. Results and discussion

The mass chromatogram and mass spectrum of synthesized N-acetylalanine are shown in Fig. 1. A clear peak of the protonated molecular ion $[M + H]^+$ (m/z 132) of N-acetylalanine was observed on the mass chromatogram. In addition to the protonated molecular ion as base peak, the fragment ion with an acetyl group (m/z 90) and the adduct ion with ammonia (m/z 149) were detected in the mass spectrum. All N-acetylamino acids used were easily detected by scanning each protonated molecular ion (Fig. 2). N-Acetylleucine and N-acetylisoleucine, which have the same molecular mass, could be distinguished according to this difference in retention time (t_R) (Fig. 2). LC–APCI-MS could also distinguish between N-acetylserine and O-acetylserine and between N(α)-acetyllysine and N(ε)-acetyllysine on the basis of their retention times (Fig. 3).

A normal human urine sample was prepared as described in Experimental. The sample was finally fractionated with 10% formic acid (0–5 ml, 5–10 ml, and 10–30 ml) and each fraction was analyzed by LC–APCI-MS (scan mode). In the 5–10 ml fraction, only weak peaks were observed on the mass chromatogram (Fig. 4), thus this fraction was further fractionated at 30-s intervals and each 30-s fraction was concentrated.

The mass chromatogram and mass spectrum of the t_R 3.5–4.0 min fraction are shown in Fig. 5. The retention time of a clear peak of m/z 175 ([M + H]) coincided with that of authentic N-acetylasparagine, and in the mass spectrum the

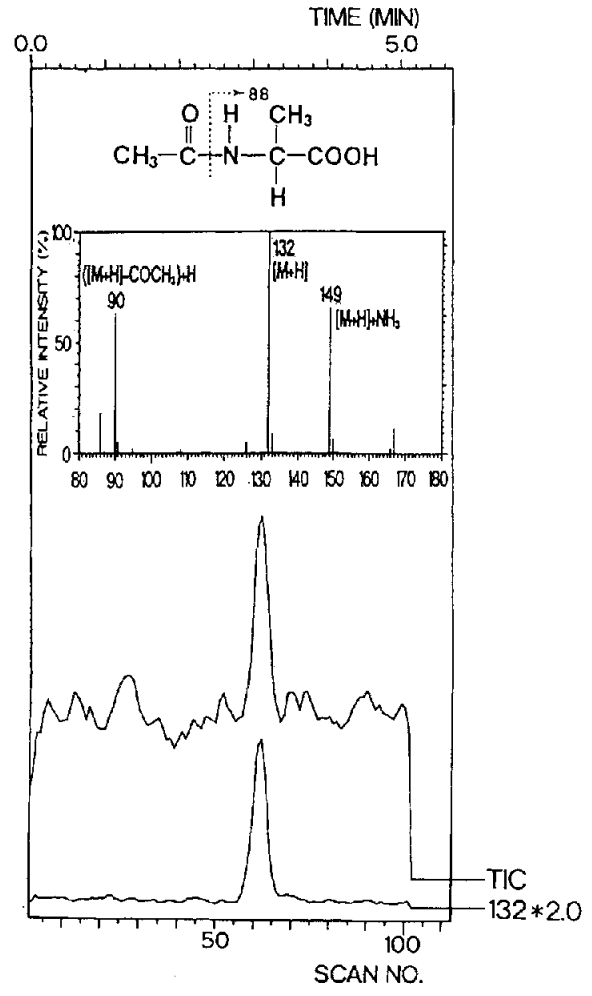

Fig. 1. Mass chromatogram and mass spectrum of authentic N-acetylalanine m/z 132 [M + H]: TIC means total-ion chromatogram of mass range from 80 to 180; This analysis was carried out on the calibration table of which m/z range was from 0 to 500, and with a scan rate of 4 s.

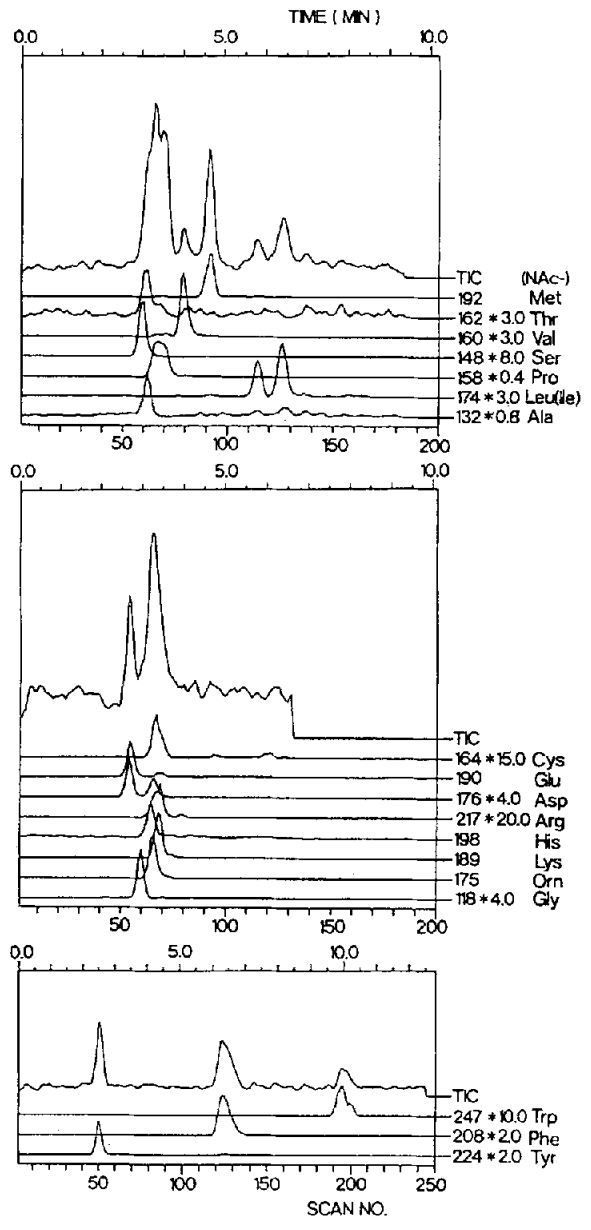

Fig. 2. Mass chromatograms of various N-acetylamino acids. The calibration table used was the same as in Fig. 1.

fragment ion (m/z 133) and the adduct ion (m/z 192) characterizing N-acetylasparagine were detected (Fig. 5-1,2). After hydrolysis of this fraction the peak m/z 175 disappeared and the peak m/z 134 appeared, indicating aspartic acid (Fig. 5-3). These results convinced us that the peak m/z 175 in the urine sample was N-acetylasparagine.

The mass chromatogram and mass spectrum of the t_R 4.0–4.5 min fraction are shown in Fig. 6. The retention time of a peak of m/z 189 ([M + H] corresponded to that of authentic N-acetylglutamine, and in the mass spectrum the fragment ion m/z 147 and the adduct ion m/z 206 were observed (Fig. 6-1,2). However, the protonated molecular ion of N-acetyllysine is also m/z 189, and its retention time was almost

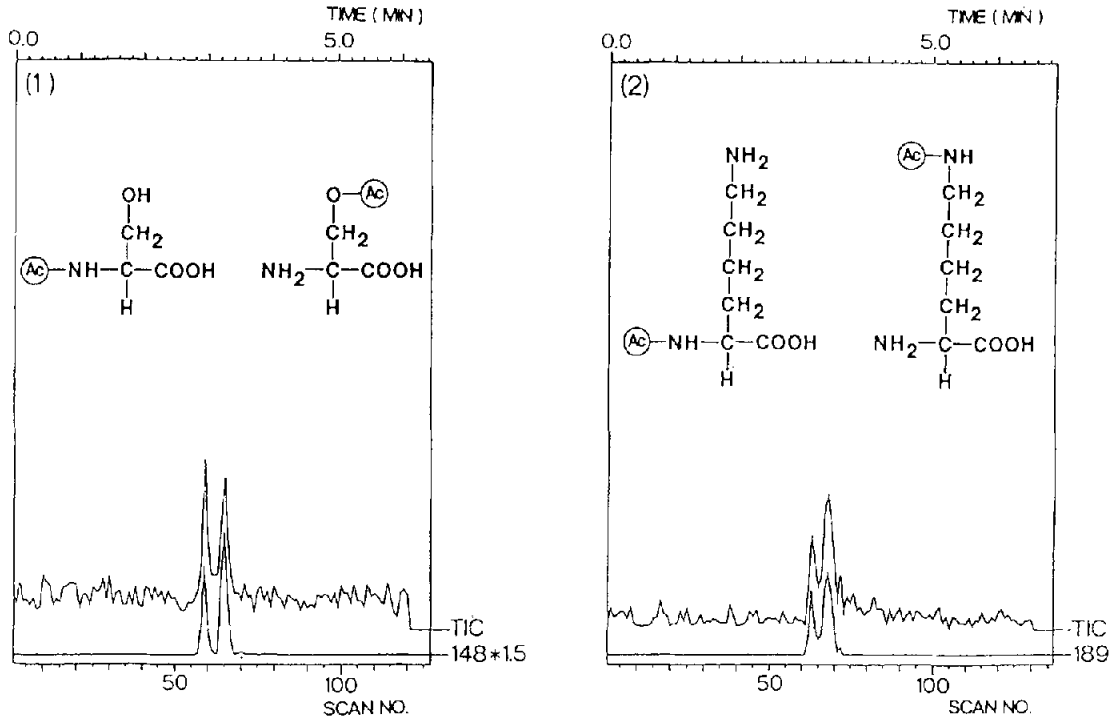

Fig. 3. Mass chromatograms of a mixture of N-acetylserine and O-acetylserine (1), and a mixture of N(α)-acetyllysine and N(ε)-acetyllysine (2).

the same as that of N-acetylglutamine. The mass spectrum could not distinguish between the two. After hydrolysis of the fraction the peak m/z 189 was replaced with a peak m/z 148 corresponding to glutamic acid, showing no peak of lysine (Fig. 6-3). This means that the peak m/z 189 in the urine sample is not N-acetyllysine but N-acetylglutamine.

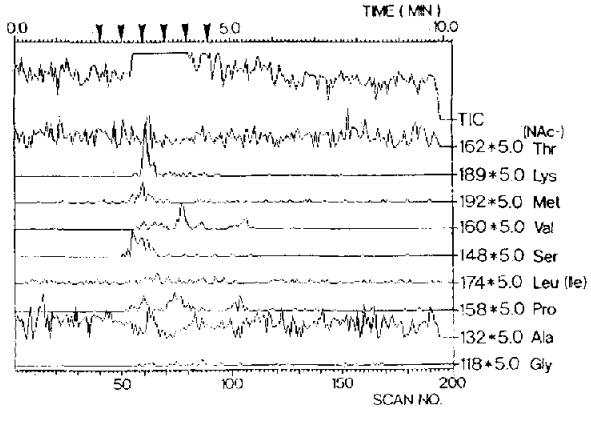

Fig. 4. Mass chromatogram of the 10% formic acid fraction (5–10 ml) of a urine sample scanned at various [M + H].

Quantitative analysis of urinary N-acetylamino acids was carried out in the selected-ion monitoring (SIM) mode. Calibration curves (concentration *versus* peak area) for the N-acetylamino acids studied were linear over the concentration range 2.5–60 μg/ml. The linear regression equation, for example, of N-acetylasparagine was $y = (1300 \pm 9.007)x - (24\,380 \pm 11\,710)$, and the correlation coefficient was 0.9989.

Prior to the assay of urine samples, the recovery of the treatment with cation- and anion-exchange resins described in Experimental was tested. Known concentrations of standard N-acetylamino acids were treated with resins just as urine samples and determined by external calibration (Table 1). The high recoveries obtained show that our procedure for the measurement of N-acetylamino acids is acceptable. In addition to N-acetylasparagine and N-acetylglutamine, the

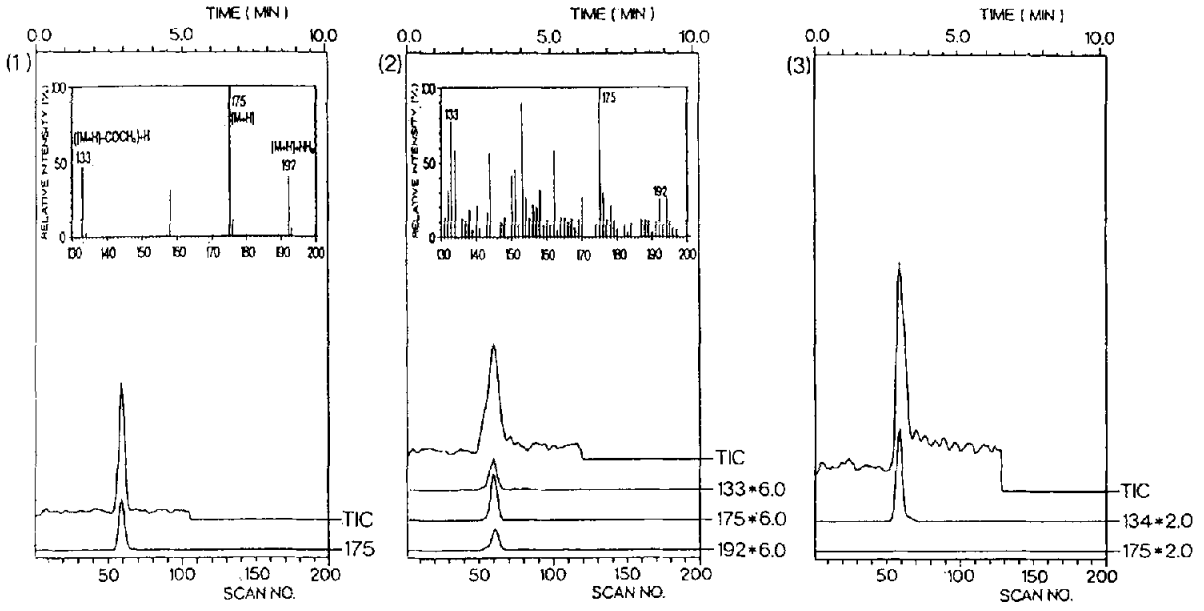

Fig. 5. Mass chromatograms and mass spectra of authentic N-acetylasparagine (1), and urinary N-acetylasparagine (t_R 3.5 min fraction) (2). (3) Mass chromatogram of aspartic acid in the hydrolysed urine sample.

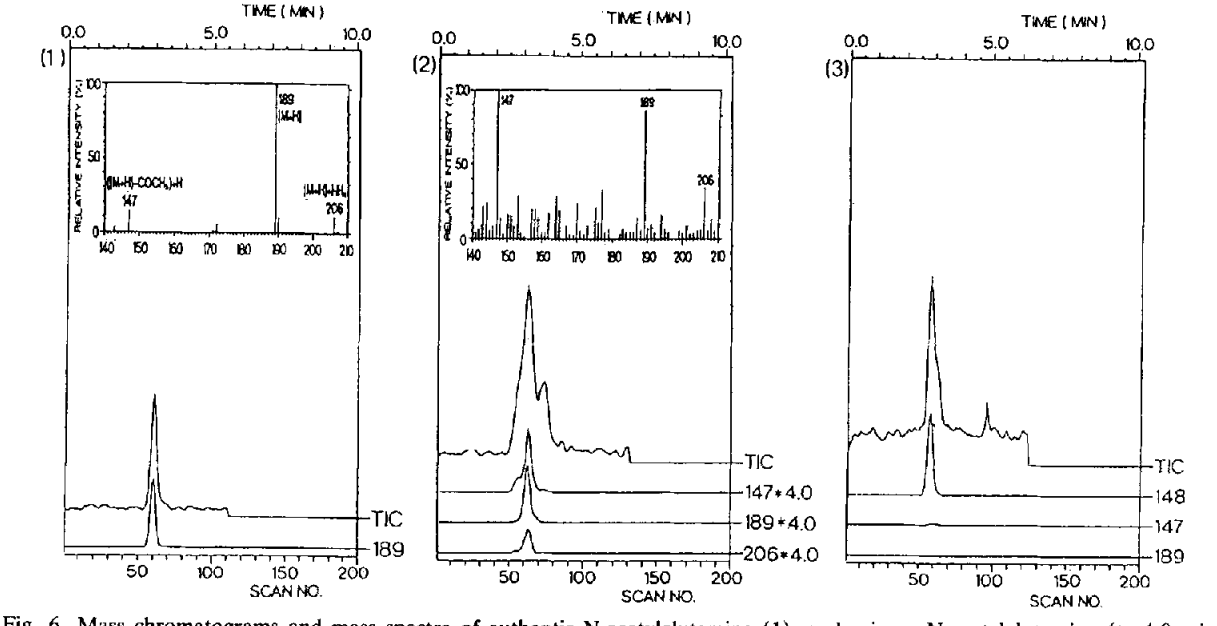

Fig. 6. Mass chromatograms and mass spectra of authentic N-acetylglutamine (1), and urinary N-acetylglutamine (t_R 4.0 min fraction) (2). (3) Mass chromatogram of glutamic acid in the hydrolysed urine sample.

Table 1
Recovery of standard N-acetylamino acids treated with cation-exchange resin (Diaion SK-1, H-form) and anion-exchange resin (Diaion SA-100, HCOOH-form)

N-Acetylamino acid	Added (ng)	Detected[a] (ng)	Recovery[a] (%)
NAc-glutamine	500	402.97 ± 12.30	80.59 ± 2.46
NAc-glutamic acid	500	410.36 ± 16.49	82.07 ± 3.30
NAc-asparagine	500	437.99 ± 21.15	87.60 ± 4.23
NAc-aspartic acid	500	401.52 ± 28.71	80.30 ± 5.74

[a] (mean ± S.D., $n = 3$)

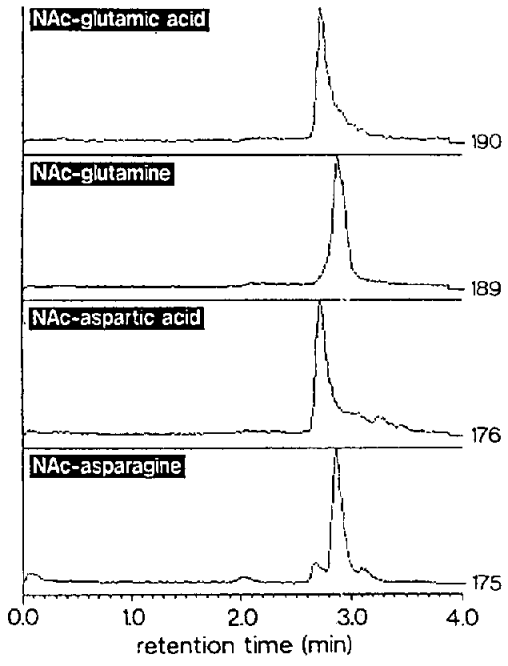

Fig. 7. SIM chromatograms of N-acetylamino acids in a urine sample from a normal volunteer. This analysis was carried out on the calibration table of which m/z was from 0 to 1500, and with a scan rate of 128 s.

SIM mode of LC–APCI-MS revealed urinary N-acetylaspartic acid and N-acetylglutamic acid which were difficult to identify in the scan mode (Fig. 7). The assay data for N-acetylaspartic acid, N-acetylasparagine, N-acetylglutamic acid and N-acetylglutamine in normal urine are presented in Table 2. The value for N-acetylaspartic acid agrees with a previous report using gas chromatography–mass spectrometry [3].

The results described above demonstrate that LC–APCI-MS without derivatization of samples is useful for the study of those N-acetylamino acids in biological samples that can not be analyzed with an amino acid analyzer.

4. References

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Table 2
Concentrations of N-acetylamino acids detected in urine samples from four normal volunteers

N-Acetylamino acid	Concentration (μg/mg creatinine)				Mean ± S.D.
	1	2	3	4	
NAc-glutamine	2.97	2.82	3.78	5.66	3.81 ± 1.13
NAc-glutamic acid	2.27	1.81	2.02	3.19	2.32 ± 0.53
NAc-asparagine	2.45	2.67	3.35	4.55	3.26 ± 0.82
NAc-aspartic acid	4.34	4.17	4.05	6.05	4.65 ± 0.81

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