Journal of Chromatography, 622 (1993) 263–268
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7144

Short Communication

Analysis of urinary organic acids by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

Mitsuharu Kajita*

Department of Pediatrics, Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466 (Japan)

Toshimitsu Niwa

Department of Internal Medicine, Nagoya University Branch Hospital, Higashi-ku, Nagoya 461 (Japan)

Kazuyoshi Watanabe

Department of Pediatrics, Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466 (Japan)

(First received March 22nd, 1993; revised manuscript received September 17th, 1993)

ABSTRACT

We developed a new method for the rapid determination of urinary organic acids using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. Mass spectra of authentic organic acids obtained in the negative-ion mode showed intense $[M-H]^-$ ions with some fragment ions. Urine samples of patients with methylmalonic aciduria, ornithine transcarbamylase deficiency, and phenylketonuria were extracted using anion-exchange columns. The mass chromatograms of the extracts showed some dominant peaks of abnormal metabolites characteristic of each disorder. This is a useful method for the analysis of urinary organic acids for the diagnosis of organic aciduria, because the sample preparation is simple.

INTRODUCTION

In the diagnosis of inborn errors of organic acid metabolism, organic acids in serum and urine have been analysed by gas chromatography–mass spectrometry (GC–MS) [1,2] and high-performance liquid chromatography (HPLC) [3,4]. However, the GC–MS method requires time-consuming sample preparation, such as extraction and derivatization. HPLC analysis does not require derivatization, but the identification of the compounds detected by HPLC is unconvincing.

Liquid chromatography–mass spectrometry (LC–MS) can offer better separation and identification of the various compounds because it yields information about the molecular mass and the structure. Because there are technological problems associated with the interface between

* Corresponding author.

0378-4347/93/$06.00

two disparate systems, the operating pressures of which are incompatible, various interfaces, including thermospray, plasmaspray, atmospheric pressure ionization (API) and continuous-flow fast-atom bombardment, have been developed, but the ideal one does not yet exist [5].

Recently a new HPLC technique combined with atmospheric pressure chemical ionization MS (LC–APCI-MS) has been developed, which has wide applicability and high sensitivity [6,7]. There are a few reports on the application of LC–APCI-MS to the diagnosis of inherited metabolic disorders, such as cystinuria and prolidase deficiency [8] and cystinuria [9]. In this study, we developed a new simple method for the determination of organic acids abnormally excreted into the urine of patients with inherited metabolic disorders by LC–APCI-MS.

EXPERIMENTAL

Materials

p-Fluorobenzoic acid was purchased from Tokyo Kasei (Tokyo, Japan), phenyllactic acid (PLA) and phenylpyruvic acids (PPA) from Sigma (St. Louis, MO, USA), orotic acid from Wako (Osaka, Japan), methylmalonic acid and 2-hydroxyphenylacetic acid (2-HPAA) from Aldrich (Milwaukee, WI, USA), and HPLC-grade methanol and distilled water from Kanto (Tokyo, Japan). All other chemicals used were of analytical grade.

Urine samples

Urine samples were obtained from the following three patients and from three normal children and stored at −20°C until analysis. The urine samples from the patients were collected in metabolically stable periods.

Case 1 is a 14-year-old girl with vitamin B_{12} responsive methylmalonic aciduria (MMA). At 11 months old she vomited and had metabolic acidosis, and was diagnosed as having MMA. She has been given vitamin B_{12} and 1-carnitine orally and has no symptoms.

Case 2 is a 9-year-old boy with ornithine transcarbamylase deficiency (OTCD). He was found to be lethargic at two years old, and thereafter had recurrent hyperammonemia attacks. Liver OTC activity was found to be 22% of normal control. His protein intake has been limited within 2 g/day and he has been given l-arginine and l-carnitine. In a stable period he has no symptoms and has a normal blood level of ammonia.

Case 3 is a 19-year-old male with phenylketonuria (PKU). He was found to have a high blood level of phenylalanine by neonatal screening with the Guthrie test. He was diagnosed as having PKU and had been treated dietically. He showed no symptoms except for an abnormality in the electroencephalogram and has been given phenobarbital.

Sample preparation

p-Fluorobenzoic acid was used as the internal standard (I.S.), and 100 μg were added to each urine sample containing 2.5 mg creatinine. The sample was transferred to a SAX (strong anion-exchange) column (100 mg/ml, Analytichem International, Harbor City, CA, USA), washed with 2 ml of distilled water and 2 ml of methanol, and eluted with 0.5 ml of 0.5 *M* HCl. A 50-μl volume of the eluate was directly subjected to LC–APCI-MS.

Each stock standard solution of organic acids was prepared by dissolving 1 mg of the authentic sample in 1 ml of ethanol or water–ethanol (1:1, v/v). The calibration mixtures were prepared from the stock solution (0, 12.5, 25 and 50 μl) by adding 100 μg of the I.S. and 1 ml of water. Thereafter these were prepared by the same method as used for the urine sample.

Liquid chromatography

HPLC was performed using an Hitachi (Tokyo, Japan) Model L-6200 and an Hitachi L-6000 chromatographic pump equipped with a TSKgel ODS-120T packed column (150 mm × 4.6 mm I.D., particle size 5 μm, Tosoh, Tokyo, Japan). Sample solutions were injected with a syringe through a Rheodyne Model 7125 loop injector (Cotati, CA, USA). The loop volume was 100 μl. The starting mobile phase was 50 m*M* ammonium acetate (pH 6.8). After 10 min, methanol was added in a linear gradient to 66.7% in 20 min. The flow-rate was 1 ml/min.

APCI-MS

The APCI-MS system used was a Hitachi Model M-1000S (Hitachi, Tokyo, Japan) equipped with a quadrupole mass spectrometer. The temperature of the vaporizer was 300°C and that of the desolvator was 399°C. The mass spectrometer was operated in a negative-ion detection mode and the drift voltage of the APCI system was −40 V. The multiplier voltage was 2 kV. The scan range was *m/z* 60–300, and the scan time was 4 s.

Quantification

The standard curves were obtained by plotting the concentration ratios *vs.* the relative peak areas of the base peak ions of the organic acid to those of the I.S. from the measurement of the calibration mixtures. The amounts of the organic acids in the urine sample were calculated from the peak-area ratios determined by measuring peak areas on the mass chromatograms.

RESULTS

The authentic organic acids were analysed by negative-ion mode LC–APCI-MS, and their mass chromatograms and mass spectra were obtained. The lower detection limit of each organic acid was *ca.* 1 μg. These acids could not be detected in the extracts from urine samples of three normal children. The mass spectrum of *p*-fluorobenzoic acid showed characteristic ions at *m/z* 139 ($[M-H]^-$) as the base peak and 95 ($[M-COOH]^-$), and so these ions were monitored. Under these conditions, the column life was *ca.* 500 sample injections.

Fig. 1 shows the mass chromatogram of authentic methylmalonic acid (A) and the extract

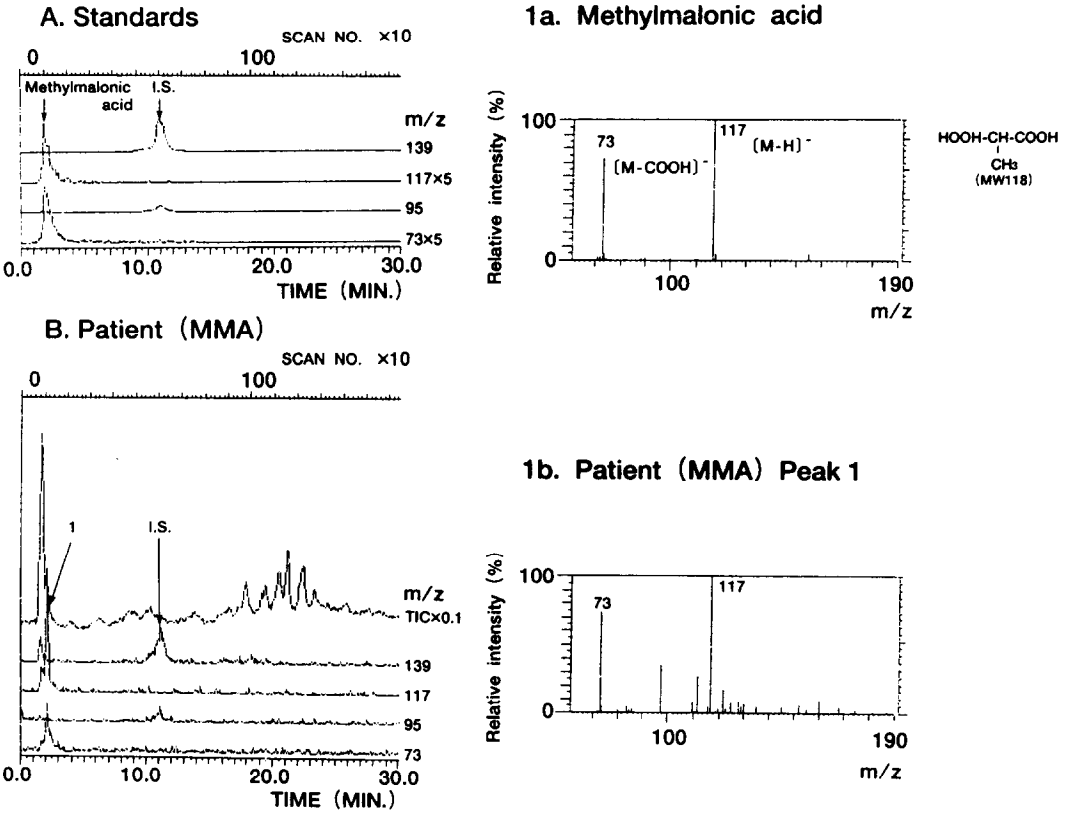

Fig. 1. Mass chromatograms of authentic methylmalonic acid (A) and the extract from the urine of a patient with methylmalonic aciduria (B). Mass spectra of authentic methylmalonic acid and peak 1 are shown in 1a and 1b, respectively.

from the urine of the case 1 (MMA) (B). Peak 1 in Case 1 showed a retention time of 2.0 min, identical to that of authentic methylmalonic acid. As shown in Fig. 1-1a, the mass spectrum of authentic methylmalonic acid showed intense ions at m/z 117 ($[M-H]^-$) and 73 ($[M-COOH]^-$). Fig. 1-1b shows the mass spectrum of peak 1 in Fig. 1B, and this included these peaks with an almost identical peak-height ratio (m/z 117/73) to that of the authentic acid. The obtained standard showed that the concentration of methylmalonic acid (Y) was well correlated with the peak-area ratio to the I.S. (X), ($Y = 0.0096 + 0.0113X$, $r = 0.99777$). It was found that the concentration of methylmalonic acid in the urine of Case 1 was 38.6 μg/mg creatinine.

Fig. 2 shows the mass chromatograms of authentic orotic acid (A) and the extract from the urine of Case 2 (OTCD) (B). Peak 1 in Case 2 showed an identical retention time (2.3 min) to that of authentic orotic acid. As shown in Fig. 2-1a, the mass spectrum of authentic orotic acid showed intense ions at m/z 111 ($[M-COOH]^-$) and 155 $([M-H])^-$. Fig. 2-1b shows the mass spectrum of peak 1 in Fig. 2B, and this included these peaks with an almost identical peak-height ratio (m/z 155/111) to that of the authentic one. The obtained standard curve showed that the concentration of orotic acid (Y) was well correlated with the peak intensity ratio to the I.S. (X), ($Y = -0.0086 + 0.0106X$, $r = 0.99763$). It was found that the concentration of orotic acid in the urine of Case 2 was 14.7 μg/mg creatinine.

Moreover, in another study using extraction with ethyl acetate, uracil could be detected in the urine sample of this patient by LC–APCI-MS under the same conditions (data not shown).

The mass chromatogram of the mixture of authentic PLA, 2-HPAA and PPA is shown in Fig. 3A and the extract from the urine of Case 3

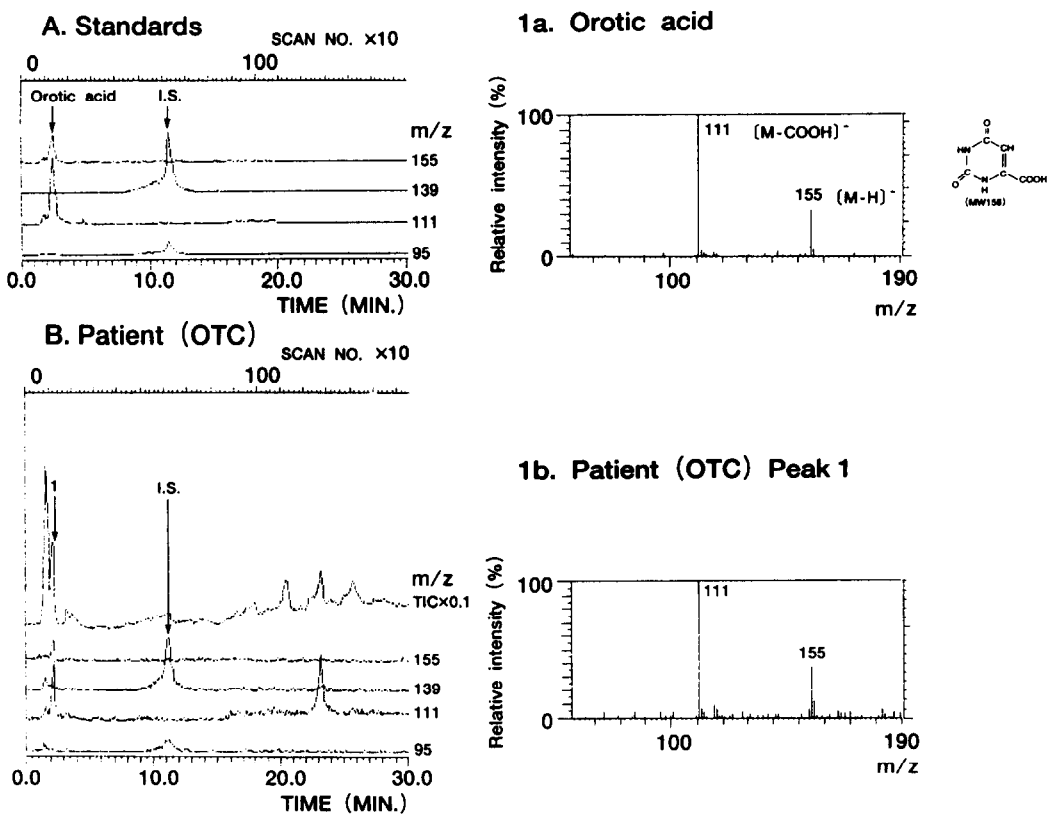

Fig. 2. Mass chromatograms of authentic orotic acid (A) and the extract from the urine of a patient with ornithine transcarbamylase deficiency (B). Mass spectra of authentic orotic acid and peak 1 are shown in 1a and 1b, respectively.

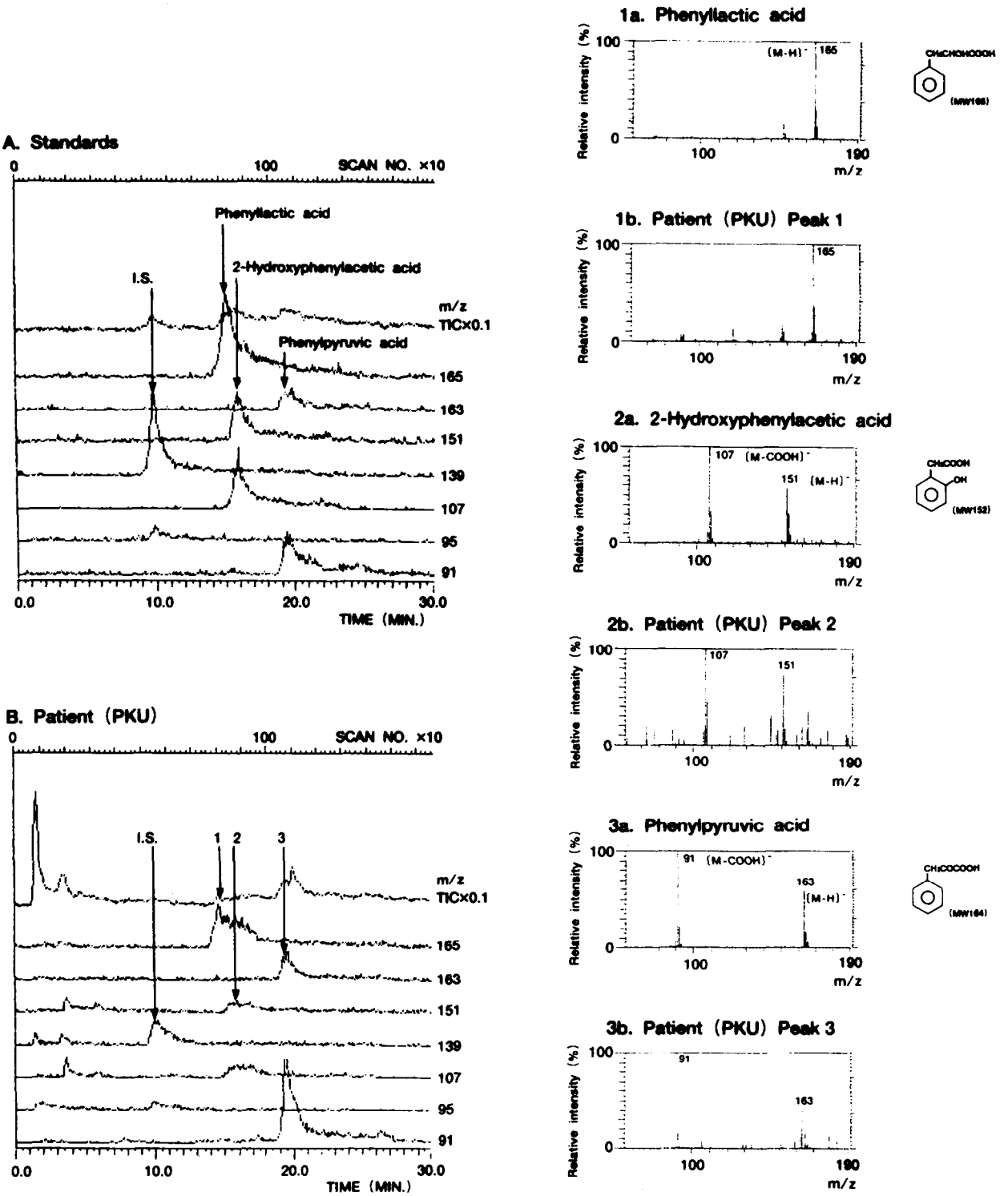

Fig. 3. Mass chromatograms of the mixture of authentic phenyllactic acid (PLA), 2-hydroxyphenylacetic acid (2-HPAA) and phenylpyruvic acid (PHA) (A) and the extract from the urine of a patient with phenylketonuria (B). Mass spectra of authentic PLA (1a) and peak 1 (1b), 2-HPAA (2a) and peak (2b), PPA (3a) and peak 3 (3b) are also shown.

(PKU) in Fig. 3B. The mass spectrum of authentic PLA showed a characteristic ion at m/z 165 ($[M-H]^-$) (Fig. 3-1a), 2-HPAA at m/z 107 ($[M-COOH]^-$) and 151 ($[M-H]^-$) (Fig. 3-2a), and PPA at m/z 91 ($[M-COOH]^-$) and 163 ($[M-H]^-$) (Fig. 3-3a). The peaks 1, 2 and 3 in Fig. 3B were confirmed to be PLA, 2-HPAA acid and PPA, because the peaks showed identical retention times at 14.5, 15.8 and 19.4 min, respectively, and included mass spectra almost identical with those of the authentic compounds (Figs. 3-1b, 2b and 3b). The concentration of each one (Y) was well correlated with the peak-area ratio to the I.S. (X), (PLA: $Y = -0.0633 + 0.0732X$, $r = 0.99821$; 2-HPAA: $Y = -0.5833 + 0.0334X$, $r = 0.99276$; PPA: $Y = -0.1640 + 0.0509X$, $r = 0.99696$). The concentrations of PLA, 2-HPAA and PPA in the urine of Case 3 were 11.6, 8.4 and 18.1 μg/mg creatinine, respectively.

DISCUSSION

Analytical methods for urinary organic acids using LC–MS were reported by Mills *et al.* [10] using a negative-ion plasmaspray interface, and by Buchanan *et al.* [11] using positive-ion thermospray. These methods require little or no sample preparation and can detect some organic aciduria, such as MMA.

The API method was first reported by Horning *et al.* [12] in 1973. In 1988, Sakairi and Kambara [6] developed a new LC–APCI-MS method to determine concentrations of drugs in human serum. Because ionization occurs at atmospheric pressure in the APCI instrument, the system can be easily manipulated. This method was applied to the diagnosis of some inherited metabolic diseases [8,9], but the analysis of urinary organic acids by LC–APCI-MS has not yet been reported.

In this study, we could detect excessively excreted organic acids in urine samples from patients with organic aciduria (MMA, OTCD and PKU) by LC–APCI-MS with very simple preparation using a SAX column and without derivatization.

The analytical method using LC–APCI-MS does not require laborious and time-consuming sample preparation, it is very useful for the clinical examination of organic aciduria, which is accompanied with excessive urinary excretion of organic acids. Organic acids could not be identified in the urine samples from healthy children because of the relatively poor sensitivity of the LC–APCI-MS system. When the sensitivity is improved using selected-ion monitoring, smaller amounts of organic acids can be detected. Under the HPLC conditions used in this study, the organic acids with aromatic groups were well separated, but the separation of the other organic acids was insufficient because of their early retention times. We intend to establish better HPLC conditions for the analysis of these organic acids.

In comparison with GC–MS, this LC–APCI-MS method has the disadvantages of poor sensitivity and identification of unknown organic acids, but it has the advantage of simpler sample preparation. It would be useful for quantitative analyses of a given organic acid to monitor the efficiency of treatment.

ACKNOWLEDGMENTS

This study was partially supported by the Mother and Child Health Foundation, Japan.

REFERENCES

1 D.C. Lehotay, *Biomed. Chromatogr.*, 5 (1991) 113.
2 P.T. Ozand and G.G. Gascon, *J. Child Neurol.*, 6 (1991) 288.
3 M.J. Bennett and C.E. Brandy, *Clin. Chem.*, 30 (1984) 542.
4 G. Rumsby, J. Belloque, R.S. Ersser and J.W.T. Seakins, *Clin. Chim. Acta*, 163 (1987) 171.
5 K.B. Tomer and C.E. Parker, *J. Chromatogr.*, 492 (1989) 189.
6 M. Sakairi and H. Kambara, *Anal. Sci.*, 4 (1988) 199.
7 Y. Kato and Y. Numajiri, *J. Chromatogr.*, 562 (1991) 81.
8 H. Komada, H. Nakamura and K. Sugahara, *J. Chromatogr.*, 527 (1990) 279.
9 H. Watanabe, K. Sugahara, K. Inoue, Y. Fujita and H. Kodama, *J. Chromatogr.*, 568 (1991) 445.
10 G.A. Mills, V. Walker, M.R. Clench and V.C. Parr, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 259.
11 D.N. Buchanan, J. Muenzer and J.G. Thoene, *J. Chromatogr.*, 534 (1990) 1.
12 E.C. Horning, M.G. Horning, D.I. Caroll, I. Dzidie and R.N. Stillwell, *Anal. Chem.*, 45 (1973) 936.