Journal of Chromatography, 626 (1992) 239–243
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 545

Selective determination of secondary amino acids as their N-dimethylthiophosphoryl methyl ester derivatives by gas chromatography with flame photometric detection

Hiroyuki Kataoka, Katsuyuki Nagao, Noriko Nabeshima, Mayumi Kiyama and Masami Makita

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)

(First received May 7th, 1992; revised manuscript received August 3rd, 1992)

ABSTRACT

A selective and sensitive method for the determination of secondary amino acids by gas chromatography (GC) was developed. After removal of primary amino acids by reaction with *o*-phthaldialdehyde and subsequent extraction with diethyl ether, secondary amino acids were converted into their N-dimethylthiophosphoryl methyl ester derivatives and determined by GC with flame photometric detection using a DB-5 capillary column. The derivatives were volatile enough and stable, giving single and symmetrical peaks. The detection limits for proline, pipecolic acid, thioproline, hydroxyproline and hydroxypipecolic acid were 0.1–0.7 pmol injected. By using this method, low-nanomole amounts of secondary amino acids could be accurately and precisely determined without any influence from primary amino acids and other amines.

INTRODUCTION

The determination of secondary amino acids by gas chromatography (GC) has been carried out after conversion into their N-acyl alkyl ester, trimethylsilyl, thiohydantoin, nitrophenyl methyl ester, oxazolinone and other ester derivatives [1–5]. However, these methods are not specific for secondary amino acids. Therefore, when small amounts of secondary amino acids are measured in matrices containing large amounts of primary amino acids, the secondary amino acid peaks may be buried in the primary amino acid peaks. Moreover, sensitivity can be adversely affected, as the sample may have to be dilluted to such an extent to avoid overloading of the primary amino acids that the lower abundance secondary amino acids may now be below the detection limits.

It is well known that *o*-phthaldialdehyde (OPA) reacts only with primary amino groups. Therefore, it is considered that secondary amino acids might be selectively detected as suitable derivatives if a sample was previously treated with OPA. On the basis of this idea, we investigated a selective and sensitive method for the determination of secondary amino acids. In this work, after primary amino acids had been eliminated through reaction with OPA, secondary amino acids were analysed as their N-dimethylthiophosphoryl (DMTP) methyl ester derivatives by GC with flame photometric detection (FPD).

EXPERIMENTAL

Reagents

L-Proline (Pro), L-thioproline (Tpr) and 4-hydroxy-L-proline (Hyp) were purchased from Naca-

Correspondence to: Dr. Hiroyuki Kataoka, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan.

0021-9673/92/$05.00

lai Tesque (Kyoto, Japan). L-Pipecolic acid (Pip), 5-hydroxy-L-pipecolic acid (Hpi) and 3,4-dehydro-DL-proline as an internal standard (I.S.) were purchased from Sigma (St. Louis, MO, USA). All of the primary amino acids used were purchased from Ajinomoto (Tokyo, Japan). Each amino acid was dissolved in water to make a stock solution at a concentration of 2 m*M*. OPA (Nacalai Tesque) was used as a 2 *M* solution in acetonitrile. Dimethyl chlorothiophosphate (DMCTP) (Tokyo Kasei Kogyo, Tokyo, Japan) was used as a 1% solution in acetonitrile after distillation. N-Methyl-N-nitroso-*p*-toluenesulphonamide (MNTSA) to generate diazomethane was obtained from Nacalai Tesque. All other chemicals were of analytical-reagent grade.

Derivatization procedure

An aliquot of the sample solution containing 0.4–25 nmol of secondary amino acids was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. To this solution were added 0.1 ml of 20 μM I.S. solution and 0.2 ml of 0.5 *M* phosphate buffer (pH 8) and the total reaction volume was made up to 0.8 ml with distilled water. After addition of 0.2 ml of 0.2 *M* OPA, the reaction mixture was allowed to stand for 2 min at room temperature. The reaction mixture was acidified to pH 1–2 with 2 *M* hydrochloric acid and then extracted twice with 3 ml of diethyl ether in order to remove the OPA derivatives of primary amino compounds. After the aqueous layer was basified to pH >10 with 10% sodium carbonate, 0.2 ml of 1% DMCTP was added and the mixture was incubated at 60°C for 5 min. The reaction mixture was extracted twice with 3 ml of diethyl ether in order to remove the excess of reagent and DMTP derivatives of secondary amines. The aqueous layer was acidified to pH 1–2 with 2 *M* hydrochloric acid, saturated with sodium chloride and then extracted twice with 3 ml of diethyl ether. The pooled ethereal extracts were methylated by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman [6] as follows: a stream of nitrogen was saturated with diethyl ether in the first side-arm tube (130 mm × 16 mm I.D.) and then passed through a diazomethane-generating solution containing 2 ml of diethyl ether, 1 ml of calbitol, 1 ml of 60% potassium hydroxide and an adequate amount of MNTSA in the second side-arm tube. The diazomethane generated was carried into the sample tube via the nitrogen stream. The sample was treated until a yellow tinge became visible. After standing at room temperature for 5 min, the solvents were evaporated to dryness at 60°C until also the oily residue had disappeared. The residue was dissolved in 0.1 ml of ethyl acetate and 1 μl of this solution was injected into the GC–FPD system.

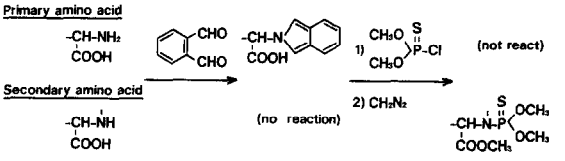

Fig. 1. Selective derivatization of primary and secondary amino acids.

The derivatization process is summarized in Fig. 1. Primary amino acids are previously derivatized with OPA, and therefore these amino acids cannot react with DMCTP any more. On the other hand, secondary amino acids are derivatized with DMCTP and then methylated with diazomethane, because these amino acids do not react at all with OPA.

Gas chromatography

The GC analysis was carried out with a Shimadzu 14A gas chromatograph equipped with a flame photometric detector (P-filter). A fused-silica capillary column (15 m × 0.53 mm I.D., 1.5 μm film thickness) of cross-linked DB-5 (J & W, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed from 150 to 280°C at 5°C/min; injection and detector temperature, 290°C; and nitrogen flow-rate, 12 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard Model 2890A gas chromatograph was operated in conjunction with a VG Analytical 70-SE mass spectrometer and a VG 11-250J mass data system. The GC column was of the same type as used for GC analysis, with an ionizing voltage of 40 eV, an ion-source temperature of 240°C and a helium flow-rate of 8 ml/min.

RESULTS AND DISCUSSION

Phosphorus-containing derivatives of amines and

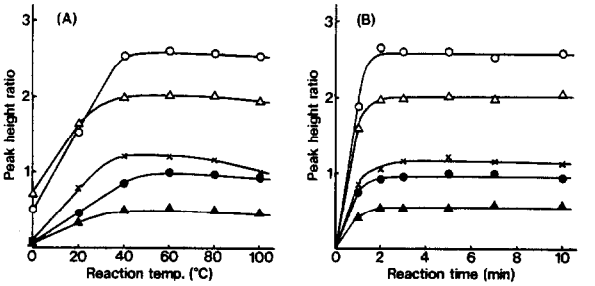

Fig. 2. Effects of (A) reaction temperature and (B) time on the N-dimethylthiophosphorylation of secondary amino acids. ○ = Proline; ● = pipecolic acid; × = thioproline; △ = hydroxyproline; ▲ = hydroxypipecolic acid.

amino acids have been prepared with diethylchlorophosphate [7] and dimethylthiophosphinic chloride [8], and analysed by GC with alkali flame ionization detection. Although these methods are highly sensitive, the preparation of the derivatives requires a lengthy procedure and anhydrous conditions. We investigated a simple and rapid derivatization method using DMCTP as phosphorus-containing reagent. The N-dimethylthiophosphorylation of secondary amino acids with DMCTP proceeded rapidly and quantitatively in aqueous alkaline media. As shown in Fig. 2, this reaction was accomplished within 2 min at 60°C. Excess of reagent and DMTP derivatives of amines were removed by extraction with diethyl ether in alkaline media. Subsequently, the DMTP derivatives of secondary amino acids were quantitatively extracted into diethyl ether in acidic media and gave the final derivatives, DMTP amino acid methyl esters, by methylation with diazomethane.

The structures of the derivatives were confirmed by GC–MS analysis. As shown in Fig. 3, a molecular ion peak (M^+) was observed for each of the derivatives and other common ion peaks which were useful for structure elucidation were $[M - 59]^+$ ($COOCH_3$), $[M - 125]^+$ $[PS(OCH_3)_2]$, m/z 125 and m/z 93 $[P(OCH_3)_2]$. The derivatives were very stable and no decomposition was observed even after standing in ethyl acetate for 2 weeks at room temperature.

In the above procedure, primary amino acids were also derivatized and detected by GC–FPD (Fig. 4A). In order to remove the primary amino acids, the reaction with OPA was tested prior to the

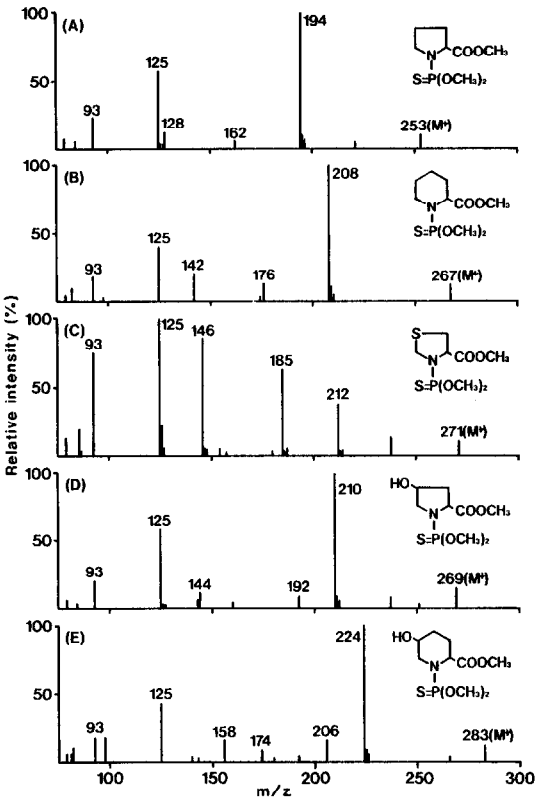

Fig. 3. Mass spectra of the N-dimethylthiophosphoryl methyl ester derivatives of (A) proline, (B) pipecolic acid, (C) thioproline, (D) hydroxyproline and (E) hydroxypipecolic acid.

N-dimethylthiophosphorylation. The reaction of primary amino acids with OPA was accomplished within 30 s in phosphate buffer (pH 8), and the OPA derivatives no longer reacted with DMCTP. As shown in Fig. 4B, primary amino acids were not detected at all when the sample was previously treated with OPA. On the other hand, as shown in Fig. 4C and D, secondary amino acids were detected as single and symmetrical peaks irrespective of OPA treatment, because these amino acids did not react with OPA. These results indicate that secondary amino acids could be selectively analysed in the sample containing primary and secondary amino acids. In fact, the determination of secondary amino acids were not affected even when the amount of total primary amino acids was 500 times greater than that of total secondary amino acids.

In order to test the linearity of the calibration

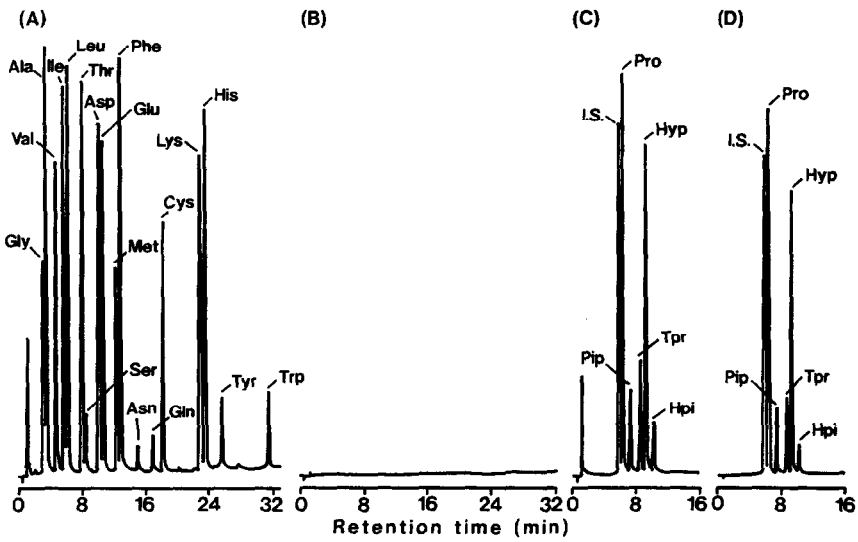

Fig. 4. Chromatograms of the N-dimethylthiophosphoryl methyl ester derivatives of primary and secondary amino acids. (A) Primary amino acids; (B) primary amino acids pretreated with OPA; (C) secondary amino acids; (D) secondary amino acids pretreated with OPA. GC conditions are given under Experimental. Each peak represents 20 pmol of amino acid.

TABLE I

LINEAR REGRESSION DATA FOR SECONDARY AMINO ACIDS

Amino acid	Range (nmol)	No. of data	Regression line[a]	Correlation coefficient (r)
Proline	0.4–10	20	$y = 0.5396x + 0.0721$	0.9997
Pipecolic acid	1–25	20	$y = 0.0849x + 0.0233$	0.9949
Thioproline	1–25	20	$y = 0.0991x + 0.0281$	0.9959
Hydroxyproline	0.4–10	20	$y = 0.4366x - 0.0063$	0.9959
Hydroxypipecolic acid	1–25	20	$y = 0.0384x + 0.0246$	0.9956

[a] y = Peak height-ratio; x = amount of each amino acid.

graph, various amounts of secondary amino acids ranging from 0.4 to 25 nmol were derivatized in a mixture according to the OPA treatment and subsequent reaction with DMCTP, and aliquots representing 4–250 pmol of each amino acid were injected into the GC–FPD system. In each instance, a linear relationship was obtained and the reproducibility was found to be satisfactory (Table I). The minimum detectable amounts of Pro, Pip, Tpr, Hyp and Hpi to give a signal three times the noise under our instrumental conditions were 0.1, 0.3, 0.3, 0.2 and 0.7 pmol injected, respectively.

These experiments have conclusively demonstrated that the method proposed in selective and sensitive for the determination of secondary amino acids. Further investigations on the application of this method to biological samples are in progress.

REFERENCES

1 D. R. Knapp, *Handbook of Analytical Derivatization Reactions*, Wiley, Toronto, 1979, p. 242.
2 R. W. Zumwalt, K. C. T. Kuo and C. W. Gehrke (Editors), *Amino Acid Analysis by Gas Chromatography*, CRC Press, Boca Raton, FL, 1987.

3 I. M. Moodie, B. J. Hough and D. Labadarios, *J. High Resolut. Chromatogr.*, 12 (1989) 437.
4 M. F. Grubb and P. S. Callery, *J. Chromatogr.*, 469 (1989) 191.
5 P. Husek, *J. Chromatogr.*, 552 (1991) 289.
6 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
7 G. Ertingshausen, C. W. Gehrke and W. Aue, *Sep. Sci.*, 2 (1967) 681.
8 K. Jacob, C. Falkner and W. Vogt, *J. Chromatogr.*, 167 (1978) 67.