Journal of Chromatography, 348 (1985) 213–220
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 054

SELECTIVE DETERMINATION OF SECONDARY AMINO ACIDS USING PRECOLUMN DERIVATIZATION WITH 9-FLUORENYLMETHYLCHLOROFORMATE AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received July 2nd, 1985)

SUMMARY

A new method for the determination of secondary amino acids is reported. Primary amino acids are removed with *o*-phthaldialdehyde–mercaptoethanol, followed by fluorescence labelling of secondary amino acids with 9-fluorenylmethylchloroformate (FMOC-Cl). The reactions are run in sequence and are completed in less than 2 min at room temperature. The stable FMOC derivatives are easily separated on short reversed-phase liquid chromatography columns. The detection limits for hydroxyproline, sarcosine and proline are in the low femtomole range. The method is shown to be free of interferences from primary amino acids and has been applied to physiological fluids and seawater samples.

INTRODUCTION

Derivatization is necessary for sensitive detection of amino acids. Although they work well for primary amino acids, the most commonly used reagents suffer from low or non-existent response to secondary amino acids.

The molar extinction coefficients of the products formed by the reaction of ninhydrin with proline and hydroxyproline are 1/5 to 1/10 of that of the derivative formed with primary amino acids[1].

The fluorescent reagents fluorescamine and *o*-phthaldialdehyde (OPA) react only with primary amines. Post-column derivatization methods have been described based on oxidation of secondary amino acids with hypochlorite (ClO^-) or chloramine T followed by derivatization with OPA[2–6]. A detection limit reported for proline is 20 times higher than that for primary amino acids[6]. The problems encountered are hypochlorite destruction of primary amino acids, which necessitates that hypochlorite is added only during the elution of secondary amino acids[4], and different optimal conditions for ClO^- conversion of cyclic and aliphatic secondary amino acids[5].

Reagents are available, suitable for precolumn derivatization of amino acids,

0021-9673/85/$03.30

that give similar responses for primary and secondary amino acids, *e.g.* Dns-Cl[7] and FMOC-Cl[8]. However, when small amounts of secondary amino acids are being studied in matrices containing large concentrations of primary amino acids, high resolution is required in the separation, which may result in a long analysis time. To simplify the analysis, more specific methods are desirable.

NBD chloride (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) has been observed to yield higher fluorescence intensities with secondary than primary amino acids[9] owing to higher reaction rates[10]. This has been utilized for specific determinations of imino acids[11]. The reaction is complete in 3 min at 60°C, and the derivatives have to be protected from light[10].

The efficiency of the OPA reaction with primary amino acids has been used to reduce background due to these acids in Edman degradation of peptides[12] and in secondary amino acid assay using NBD-Cl and thin-layer chromatography[13]. FMOC-Cl has recently been shown to be a suitable reagent for the determination of primary and secondary amino acids, giving products that are stable and highly fluorescent[8]. The present paper describes a new method for selective determination of secondary amino acids. Primary amino acids are eliminated through reaction with OPA, and secondary amino acids are then determined with FMOC-Cl. Both reactions proceed in seconds in a basic solution at room temperature. The derivatives are separated by reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Varian 5000 gradient delivery system and a Valco injection valve, fitted with either a 8-μl or a 250-μl loop. For detection a Shoeffel Model FS 970 fluorescence detector was used, equipped with liquid filters as described previously[8]. The emitted light was monitored between 300 and 350 nm, with an excitation wavelength of 260 nm. The output was recorded on a Perkin-Elmer Model 56 recorder or a Spectra Physics SP 4270 integrator. Columns 33 × 4.6 mm I.D. and 50 × 4.6 I.D. packed with 3 μm Spherisorb ODS-2 material and 150 × 4.6 mm I.D. packed with 5 μm Spherisorb C-8 material were used for the separations. Emission spectra were recorded with an Aminco-Bowman Spectrophotofluorometer.

Reagents and chemicals

The amino acid standards and iodoacetamide were obtained from Sigma (St. Louis, MO, U.S.A.); OPA and FMOC-Cl from Fluka (Buchs, Switzerland) and 2-mercaptoethanol (p.a.) from Roth (Karlsruhe, F.R.G.). Acetonitrile (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, U.K.).

The elution buffers were made of $3^0/_{00}$ glacial acetic acid in double distilled water. The pH was adjusted with sodium hydroxide. The OPA–mercaptoethanol reagent was dissolved in acetonitrile (50 mg of OPA and 26 μl of mercaptoethanol per millilitre). A fresh solution was prepared every day. The iodoacetamide reagent (140 mg/ml) was dissolved in acetonitrile. The reaction buffer was a 0.8 *M* borate buffer, pH 9.5, made by mixing boric acid (Merck, p.a.) and sodium hydroxide. The FMOC-Cl reagent was dissolved in acetone and had a concentration of 5 mmol/l.

Derivatization procedure

The sample (900 μl) and borate buffer (100 μl) are mixed in a 3-ml or 5-ml reaction vial (Supelco, U.S.A.). OPA-mercaptoethanol reagent (100 μl) is added and allowed to react for 30 s. Then 100 μl of the iodoamide reagent are added and after another 30 s 300 μl of the FMOC-Cl reagent. Immediately afterwards the vial is almost filled with diethyl ether and the mixture is extracted to remove excess reagent. The extraction is repeated once, and the ether extracts are discarded. The sample is then ready for injection.

Preparation of samples

In the analysis of serum samples, proteins have to be removed to protect the analytical column. Equal volumes of serum and acetonitrile are mixed and then centrifuged. The procedure is reported to precipitate 97% of the proteins in plasma samples[14]. The supernatant is diluted with distilled water (1:9, v/v) before derivatization.

Urine samples are hydrolysed by mixing equal volumes of urine and 37% hydrochloric acid, followed by heating at 110°C for 18 h in a sealed tube.

RESULTS AND DISCUSSION

Derivatization

The basis of the method presented here is the selectivity obtainable with fluorescence detection. Fig. 1 shows uncorrected emission spectra of OPA and FMOC amino acid derivatives. The spectra are well separated and the FMOC derivatives are easily measured in the presence of the OPA derivatives with the use of a monochromator or suitable filters[8].

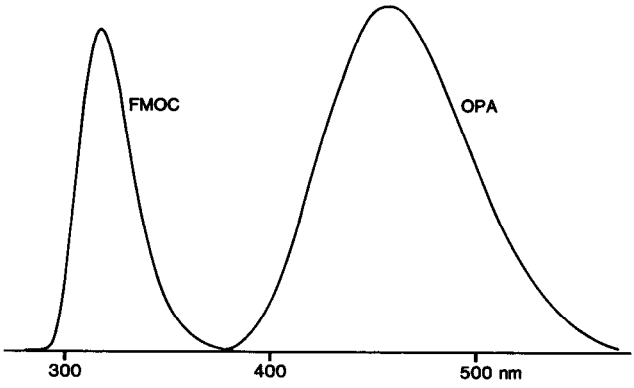

Fig. 1. Uncorrected emission spectra of an OPA derivative of alanine and a FMOC derivative of proline.

High chemical yields in the OPA–mercaptoethanol reaction with primary amines are crucial for the subsequent derivatization of the secondary amino acids with FMOC-Cl. To investigate this, 33 primary amino acids were mixed together and treated in the usual way. The results are depicted in Fig. 2a. Fig. 2b shows a chromatogram of the same mixture with hydroxyproline, sarcosine and proline added.

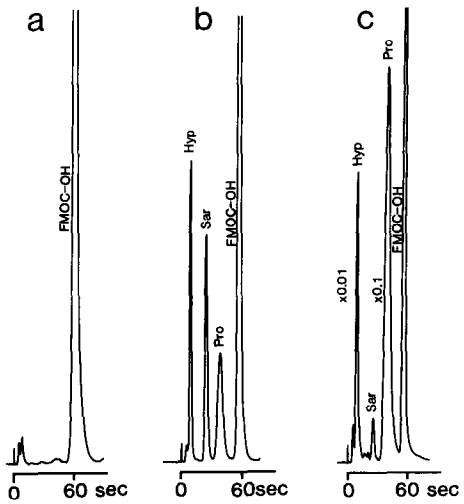

Fig. 2. (a) Chromatogram of a mixture of the following compounds (100 pmol each): Ala, Arg, Asp, Asn, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Orn, Phe, Ser, Thr, Trp, Tyr, Val, γ-amino-*n*-butyric acid, creatinine, δ-hydroxylysine, 1-methylhistidine, 3-methylhistidine, β-alanine, α-aminoadipic acid, β-aminoisobutyric acid, citrulline, cystationine, homocystine, *o*-phosphoethanolamine, *o*-phosphoserine, taurine. (b) Chromatogram of the same mixture as in (a) with hydroxyproline, sarcosine and proline added (1 pmol each). Detector response is the same in (a) and (b). (c) Chromatogram of a serum sample. Chromatographic conditions: 3 μm particle size Spherisorb ODS-2 column (33 × 4.6 mm I.D.); isocratic elution, 35% acetonitrile, 65% acetic acid buffer ($3^0/_{00}$, pH 4.3), 5 ml/min.

The injected amount of secondary amino acids is 1 pmol each, and the amount of each primary amino acid is 100-fold higher (100 pmol). The detector response is the same in Fig. 2a and b. The results show that the yields of the OPA reaction are better than 99.9%, which is sufficient for most applications.

Since the derivatization of the secondary amino acids is performed directly after the OPA reaction, it is essential that reagents and products of the first reaction do not interfere with the second reaction. Mercaptoethanol reacts with FMOC-Cl to give a late eluting peak that interfered in subsequent runs. Mercaptoethanol also consumed reagent, which means that the concentration of FMOC-Cl had to be kept high. To avoid this, iodoacetamide was added before the reaction with FMOC-Cl to block the excess of mercaptoethanol.

Limits of detection, precision and linearity

The limits of detection for the method were evaluated from a calibration curve in the concentration range 25–125 nmol/l. The separation conditions were the same as in Fig. 2, with an 8-μl injection volume and a mobile phase flow-rate of 2 ml/min. Good linearity was obtained in this range with a correlation coefficient $r > 0.998$. The detection limits presented in Table I were calculated[15] using a signal-to-noise ratio of 3.

An examination of the linearity at high concentrations showed good linearity ($r = 0.999$) up to a total concentration of 1350 μmol/l, which corresponds to 81% of the FMOC-Cl reagent concentration. The precision was determined at the 1 pmol

TABLE I

DETECTION LIMITS AND PRECISION

Chromatographic conditions as in Fig. 2, except flow-rate (2 ml/min). Precision was determined at the 1 pmol level. Detection limits were calculated with a signal-to-noise ratio of 3.

Substance	*k'*	*Detection limit (fmol)*	*Relative standard deviation of peak areas (%)*
Hydroxyproline	1.2	26	2.1
Sarcosine	5.4	48	3.5
Proline	9.2	61	3.6

level by measurements of ten replicate samples. The coefficients of variation (C.V.) for the peak areas are presented in Table I. Somewhat lower variations (C.V. = 2.2% for Pro) were obtained for peak heights.

Applications

The method is currently used to measure hydroxyproline concentrations in serum samples. A separation is shown in Fig. 2c. A study of the recovery of hydroxyproline, sarcosine and proline in serum gave 98%, 101% and 103%, respectively. These amino acids were measured in nine individuals after 12 h of fasting, and the following serum concentrations were obtained (mean ± S.D.): hydroxyproline 7.5 ± 1.5 μmol/l, range 5.5–9.6 μmol/l; sarcosine 1.5 ± 0.5 μmol-l, range 1.1–2.4 μmol/l; proline 207 ± 60 μmol/l, range 139–309 μmol/l. These results are comparable with results obtained by other investigators[11,16].

The low natural concentrations of amino acids in seawater make their determination difficult. In order to be able to make these measurements directly, without a preconcentration step, high sensitivity in the detection is required, coupled with the possibility of injecting large sample volumes. The proportion of organic solvent in the sample and the ionization state of the FMOC-amino acid derivatives are critical in the use of the peak compression effect in the injection. Organic solvents (acetone and acetonitrile) added in the derivatization step are essentially removed in the subsequent ether extractions. Fig. 3 shows a chromatogram of a seawater sample spiked with hydroxyproline and sarcosine to a concentration of 10 nmol/l. The injection volume was 250 μl. The samples were acidified with 10 μl of glacial acetic acid prior to the injection to protonate the amino acid derivatives.

Hydrolysed urine contains 4-hydroxyproline, sarcosine and proline as major secondary amino acids and traces of 3-hydroxyproline. Because it is found at relatively high levels in basement membrane collagen, 3-hydroxyproline has interested a number of workers, but commercial unavailability of analytical standards together with a lack of convenient analytical methods have limited its studies[17]. The seeds of *Delonix regia* contain 3-hydroxyproline as a major secondary amino acid[18]. The core of a *D. regia* seed was extracted with an alcohol–water mixture, and analysis of the extract gave one dominant compound. This compound concluded to be 3-hydroxyproline, separated from 4-hydroxyproline only at low pH of the mobile phase. Fig.

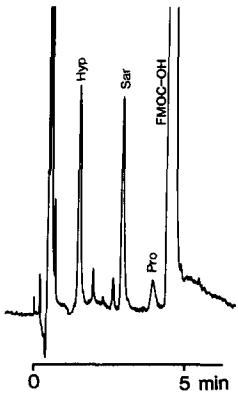

Fig. 3. Separation of a seawater sample on a 3 μm particle size Spherisorb ODS-2 column (50 × 4.6 mm I.D.). Elution: 0–0.2 min, 25–35% acetonitrile; 0.2–3 min, 35% acetonitrile; 3–4 min, 35–50% acetonitrile; 4–6 min, 50–25% acetonitrile. Elution buffer, as in Fig. 2 (pH 4.2); flow-rate, 2 ml/min.

4a illustrates a separation of a *D. regia* extract spiked with 4-hydroxyproline, sarcosine, proline and pipecolic acid. The peak shape of 3-hydroxyproline is the same as characterizes the FMOC derivatives of 4-hydroxyproline and proline, which elute as significantly broader peaks than the FMOC derivatives of primary amino acids and sarcosine. Hydrolysed urine (Fig. 4b) was run under the same conditions as in Fig. 4a. Proline, 4-hydroxyproline and sarcosine dominate, but at higher sensitivity

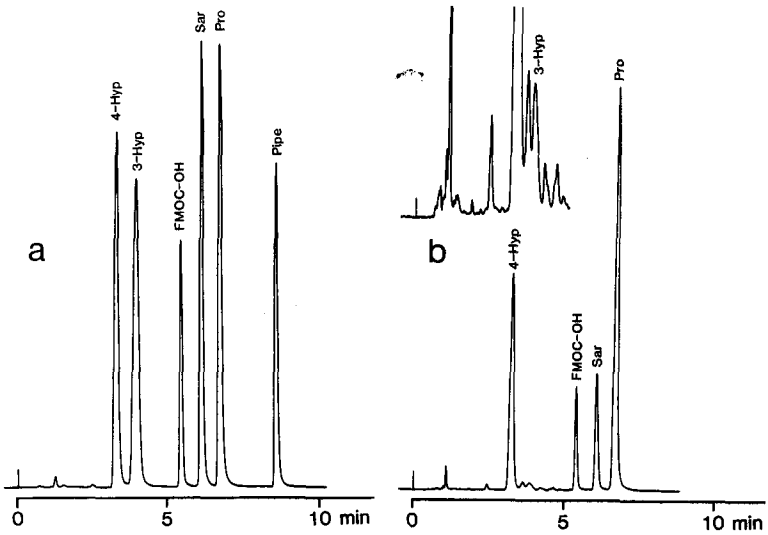

Fig. 4. (a) Chromatogram of an extract of a *Delonix regia* seed, spiked with 4-hydroxyproline, sarcosine, proline and pipecolic acid. Column: 5 μm particle size Spherisorb C_8 column (150 × 4.6 mm I.D.). Elution: 0–2 min, 37% acetonitrile; 2–6 min, 37–50% acetonitrile. Elution buffer, phosphoric acid (100 mmol/l); flow-rate, 2 ml/min. (b) Separation of a hydrolysed urine sample. The chromatographic conditions were the same as in (a). The sample was neutralized with sodium hydroxide and diluted (1:19, v/v) with distilled water prior to derivatization. The upper chromatogram is the same sample run at higher detector sensivity.

(upper part of Fig. 4b) a compound nearly identical with 3-hydroxyproline in retention time and peak shape was observed.

The identification of 3-hydroxyproline was based on chromatographic behaviour, but the stability of the FMOC derivatives makes collection and further identification after separation possible. Furthermore, the FMOC group is easily removed under mildly basic conditions[19] leaving the native compound accessible for examination.

CONCLUSIONS

A rapid and highly selective method for the determination of secondary amino acids was obtained by a selective removal of primary amino acids with OPA, followed by a derivatization of secondary amino acids with FMOC-Cl. The high chemical yields obtained in the OPA reaction make the method suitable for direct determination of low concentrations of secondary amino acids in samples such as urine and serum, which contain high concentrations of primary amino acids. High sensitivity in the detection, and the possibility of injecting large sample volumes, make it possible to analyse secondary amino acids at the very low concentrations present in seawater.

The simple derivatization procedure and the ease with which the derivatives can be separated should make this method feasible for routine applications. It is possible to extend the method to include small peptides with a secondary amine end-group, but when dealing with relatively hydrophobic compounds a change of extraction solvent in the derivatization procedure from diethyl ether to a less polar solvent such as pentane should be considered, to reduce risks of losses in the extractions.

ACKNOWLEDGEMENTS

The author thanks Björn Josefsson for useful comments on the manuscript, Sören Lagerkvist for fruitful discussions, William Landing for revising the English text and Magnus Neuendorf for the *D. regia* seeds.

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