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Note

Pre-column *o*-phthalaldehyde derivatization of amino acids and their separation using reversed-phase high-performance liquid chromatography

I. Detection of the imino acids hyroxyproline and proline

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The analysis of amino acids using pre-column derivatization with *o*-phthalaldehyde–2-mercaptoethanol (OPA–MCE) and reversed-phase high-performance liquid chromatography (HPLC) is a rapid and sensitive technique[1]. However, the inability of OPA–MCE to react with the imino acids hydroxyproline and proline[2,3] is a major reason that has precluded its general acceptance for amino acid analysis. Attempts to overcome this problem have involved oxidation of the imino acid ring[4,5] using chlorinated oxidizing agents. Although this approach has been used for post-column derivatization, reduced derivative formation occurs with a very poor fluorescence response for the imino acid derivatives. One advantage of pre-column derivatization is that complex reaction procedures may be performed without an apparent decrease in chromatographic efficiency.

This paper reports a pre-column derivatization technique using OPA–MCE and reversed-phase HPLC for the detection of the imino acids. The procedure employs a chloramine T oxidation step followed by sodium borohydride treatment. The technique is rapid and produces imino acid derivatives with fluorescence intensities similar in magnitude to OPA–MCE amino acid derivatives.

EXPERIMENTAL

Apparatus

The gradient HPLC apparatus used consisted of an Altex 420 system, a 150 × 4.6 mm I.D. column pre-packed with 5-μm Ultrasphere ODS (Anachem, Luton, U.K.) and a Schoeffel FS970 fluorescence detector (Kratos, Manchester, U.K.) using an excitation wavelength of 240 nm and an emission cut-off filter of 417 nm. The analytical column was fitted with a pre-column of 50 × 2.0 mm I.D. packed with 25–37 μm diameter Co:PELL ODS (Whatman, Maidstone, U.K.). Injections were made using a Rheodyne valve fitted with a 20-μl loop. The chromatographic data were processed with an SP4100 Integrator (Spectra-Physics, St. Albans, U.K.).

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Reagents

Unless otherwise stated, all chemicals including L-serine, L-proline and L-hydroxyproline were of analytical-reagent grade obtained from BDH (Poole, U.K.). Distilled, deionized water was used for all reagent preparations. 2-Mercaptoethanol, chloramine T and sodium borohydride were obtained from Sigma (Poole, U.K.).

OPA–MCE reagent was prepared by dissolving 500 mg of OPA (Sepramar grade; BDH) in 10 ml of methanol, then diluting to 100 ml with 400 m*M* sodium borate buffer solution (pH 9.5) and adding 400 μl of MCE. This reagent was stable indefinitely at room temperature.

Standard solutions of L-proline and L-hydroxyproline with concentrations ranging from 0 to 1.0 m*M* were prepared in water. L-Serine, 500 μ*M* in water, was used as an internal standard. Chloramine T solutions (0–20 m*M*) were prepared in 2.0 ml of dimethyl sulphoxide and 8.0 ml of 200 m*M* sodium borate buffer (pH range 8.0–10.0). Sodium borohydride solutions (0–1500 m*M*) were prepared in 600 m*M* lithium hydroxide solution. Both the chloramine T and borohydride solutions were stable for 24 h.

Chromatographic conditions

The HPLC solvents were filtered through a 0.45-μm filter (Anachem) and degassed with helium before use. The gradient solvent compositions were based on those described previously[1]. Stock aqueous solutions of 250 m*M* propionic acid and 350 m*M* anhydrous disodium hydrogen phosphate were prepared. A 500-ml volume of the stock phosphate solution was added to 500 ml of the stock propionic acid solution and the pH of the mixture was adjusted to 6.5 with 4 *M* sodium hydroxide solution. Solvent A was phosphate/propionate–acetonitrile–water (40:8:52) and solvent B was acetonitrile–methanol–water (30:30:40). The gradient programme used was as follows: at time 0, 20% B; at time 0.5 min, 100% B in 5 min; at time 8 min, 0% B in 0.5 min; solvent flow-rate, 2.5 ml/min.

Derivatization

The following procedure was used to obtain optimal conditions for the OPA–MCE fluorescence detection of the imino acids. Volumes of 50 μl of L-serine, L-proline and L-hydroxyproline solutions were added to 200 μl of chloramine T solution pre-heated to 60°C in a water-bath. After incubating for exactly 1.0 min, 200 μl of sodium borohydride solution were added and the mixture was incubated for a further 10 min at 60°C, after which 1.0 ml of OPA–MCE reagent was added and immediately 20 μl were injected on to the column using the filled loop technique.

RESULTS

The oxidation of the proline ring to pyrroline and the reaction of the pyrroline with borohydride was optimized by observing the yield of the final OPA–MCE derivative.

Optimization of the chloramine T reaction

When using 250 m*M* sodium borohydride in lithium hydroxide solution, temperatures greater than 50°C were required to obtain detectable OPA–MCE imino

acid derivatives. A temperature of 60°C was adopted in order to achieve a maximum fluorescence response after an incubation period of 1 min. Incubation times greater than 1 min at this temperature resulted in a decreased fluorescence response of OPA–MCE serine, proline and hydroxyproline derivatives. It was found necessary to include dimethyl sulphoxide in the reaction mixture to effect maximum oxidation of the proline and hydroxyproline rings.

Fig. 1a shows the fluorescence responses obtained for the OPA–MCE derivatives of serine, proline and hydroxyproline with varying concentrations of chloramine T. Maximum fluorescence of the OPA–MCE imino acid derivatives was obtained between pH 8.0 and 9.0 for the chloramine T reaction.

A chloramine T concentration of 12.5 m*M* in the dimethyl sulphoxide–borate buffer (pH 8.5) with an incubation time of 1 min at a temperature of 60°C was used in subsequent experiments.

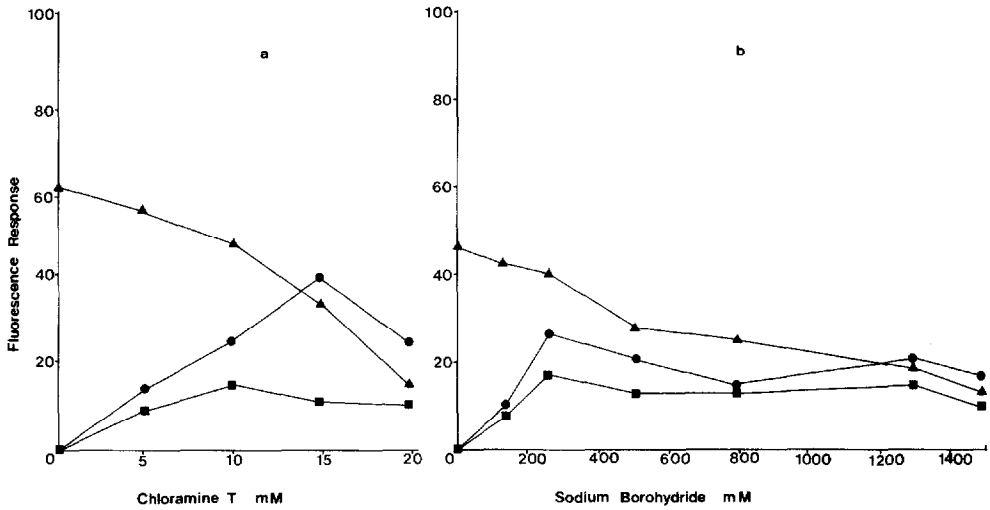


Fig. 1. Fluorescence responses obtained for the final OPA-MCE derivatives at various (a) chloramine T and (b) sodium borohydride concentrations. In both instances the concentration scale refers to the concentration in the reagent solutions. ▲, Serine; ●, hydroxyproline; ■, proline.

Optimization of the borohydride reaction

Increases in the incubation temperature were found to increase the yield of the OPA–MCE imino acid derivatives. To simplify the procedure, a reaction temperature of 60°C was chosen. At this temperature maximum yields OPA–MCE derivatives occurred after a 10-min incubation. When the analytes were allowed to stand at room temperature for 24 h after borohydride treatment, and then reacted with the OPA–MCE reagent, gradual decreases in the absolute fluorescences were observed for both serine and the imino acids. However, the relative fluorescence response of the OPA–MCE imino acid derivatives to the OPA–MCE serine derivative remained constant. To promote maximum reaction of the oxidized products of proline and hydroxyproline, it was found necessary to dissolve the borohydride in 600 m*M* lithium hydroxide solution.

Fig. 1b shows the results obtained with varying concentrations of borohydride on the fluorescence responses of the OPA–MCE proline, hydroxyproline and serine derivatives. A borohydride concentration of 250 m*M* in the lithium hydroxide solution was used in subsequent experiments.

Fig. 2a and b show the chromatograms obtained using the procedure described, and Fig. 2a and c indicate that the incorporation of the borohydride reaction was essential to obtain detectable yields of the OPA–MCE imino acid derivatives. Serine was used as the internal standard because it was well resolved from the OPA–MCE imino acid derivatives. Evidence that the derivative of proline produced by the chloramine T–borohydride treatment was 4-amino-1-butanol was confirmed by adding 4-amino-1-butanol (Aldrich, Gillingham, U.K.) to the hydroxyproline–proline standard whereby co-elution with proline was observed. It was also reacted directly with the OPA–MCE reagent and a single peak was obtained having the same elution time as the proline derivative.

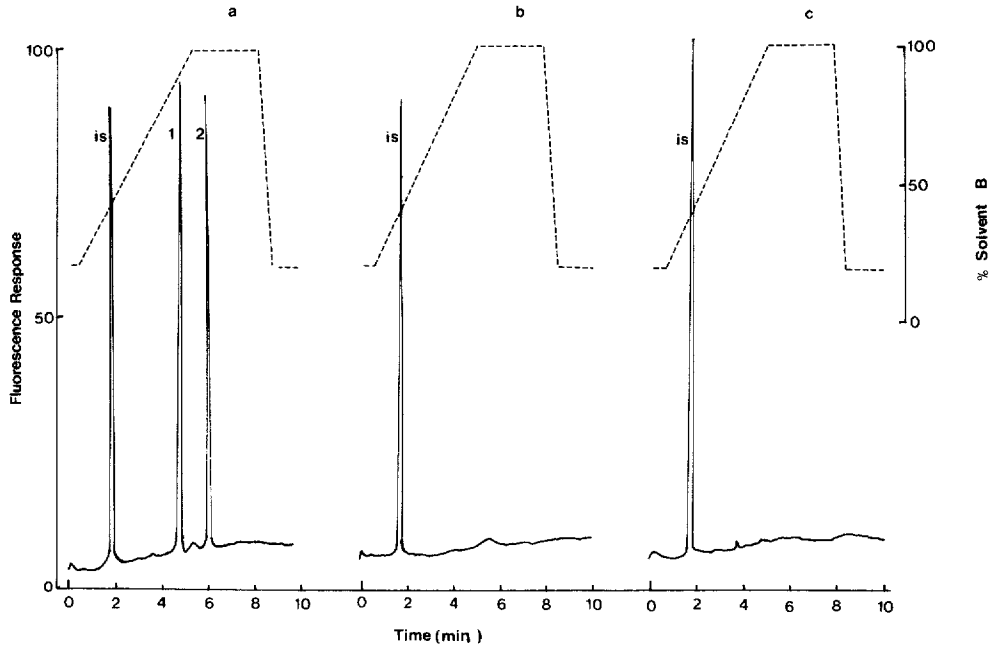


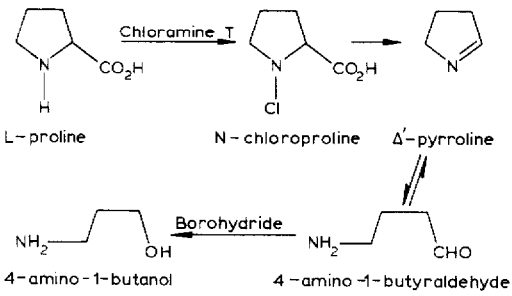
Fig. 2. Chromatograms of (a) a mixture of 500 μM of serine (internal standard) and 1.0 m*M* each of hydroxyproline (1) and proline (2); (b) 500 μM of the internal standard (serine); (c) a mixture identical with (a) but with borohydride omitted from the reaction sequence. In all instances the concentrations referred to are those of the original standard solutions. The broken lines indicate the gradient programme.

Competitive interference with the reaction

As chlorinated oxidizing agents can react with amino acids, imino acids and urea, the proline, hydroxyproline and serine concentrations were varied independently whilst at the same time the reaction was also carried out with and without the presence of 500 m*M* of urea. The fluorescence responses were found to be constant, regardless of the concentrations of analytes present. The quantitation of both imino acids was found to be linear up to 1500 μM using serine as an internal standard.

DISCUSSION

Weigele *et al.*[6] showed that the reaction of the oxidizing agent chlorosuccinimide with proline formed a pyrroline product that is in equilibrium with 4-amino-1-butyraldehyde. The primary amine group on the 4-amino-1-butyraldehyde then reacts with the OPA–MCE reagent to form the fluorophore. It was surmized that the oxidation of proline with other chlorinated oxidizing agents follows the same reaction pathway.



In order to increase the yield of the primary amine, it was found essential to reduce the 4-amino-1-butyraldehyde to 4-amino-1-butanol using borohydride. The formation of this compound was confirmed by the chromatographic co-elution of pure 4-amino-1-butanol and proline after treatment with chloramine T and borohydride. Hydroxyproline will produce 4-aminobutane-1,3-diol in this reaction sequence. Chloramine T was chosen as the oxidant because it undergoes negligible reaction with urea and thus permits the detection of the imino acids in biological samples such as urine, where wide variations of urea concentration occur. The use of hypochlorite as the oxidant in this procedure was found to be unsatisfactory as it reacts strongly with urea and amino acids. Even though chloramine T is a mild oxidant compared with hypochlorite, excessive concentrations can oxidize primary amines to mono- and disubstituted chloro compounds. This can be seen with respect to serine in Fig. 1a. Chloramine T reagent concentrations between 10 and 15 m*M* should be used. Using this concentration of chloramine T the linearity of the procedure is not affected by variations of the imino acid concentrations, indicating that the oxidation by chloramine T is not competetive. The incubation period for the reaction should not exceed 1 min at a temperature of 60°C to obtain maximum fluorescence of the imino acid derivatives and to minimize any loss of sensitivity of the amino acids.

High concentrations of borohydride can also cause a reduced yield of the OPA–MCE serine derivative. The mechanism of this reaction is unclear and the concentration of borohydride in the reagent should range between 125 and 250 m*M* to minimize losses but still give good yields of 4-amino-1-butanol and 4-aminobutane-1,3-diol. After treatment with borohydride, these products are sufficiently stable for up to 24 h, provided that quantitation is performed with reference to an internal standard. It is therefore practicable to batch process samples if a manual procedure is used.

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