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Note

Determination of free methylhistidine and amino acids by high-performance liquid chromatography

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o-Phthalaldehyde (OPA) plus a reducing agent [α-mercaptoethanol or ethanethiol (ET)] have been employed for the fluorogenic detection of amino acids[1,2]. The OPA reacts specifically with the primary amino group of the amino acid to form a stable and strongly fluorescent derivative that is readily separated and detected by high-performance liquid chromatography (HPLC)[3–5].

N^{τ}-Methylhistidine (3-MeHis) is a quantitative index of myofibrillar breakdown in some animal species[6,7]. Its experimental importance has led to a number of methods of detection, including colorimetry[8], ion-exchange paper and column chromatography[9–11], gas–liquid chromatography[12,13] and HPLC[14,15]. These methods generally require rather lengthy preparation and subsequent separation and detection, or else allow only the determination of 3-MeHis and in some cases a limited number of other amino acids. This report describes a modification of an existing HPLC method to allow baseline separation of the OPA derivatives of 3-MeHis, N^{π}-methylhistidine (1-MeHis), and fourteen other amino acids in a standard amino acid mixture.

MATERIALS AND METHODS

Chemicals

An amino acid solution was purchased from Sigma (St. Louis, MO, U.S.A.). It contained, in 1 ml of 0.1 *N* hydrochloric acid, 1.25 μmole L-cystine (CyS–SCy) and 2.50 μmoles of the following L-amino acids; Ala, Arg, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr and Val. These amino acids and α-aminoisobutyric acid (α-AIB), β-alanine (β-Ala), N-methylalanine (MeAla), anserine (Ans), Asn, cyteine (CySH), Gln, 1-MeHis, 3-MeHis, norleucine (Nor) and taurine (Tau) were obtained in crystalline form (Sigma or Fisher Scientific, Pittsburgh, PA, U.S.A.) and 5-mg samples were dissolved individually in 10 ml of distilled water. All chemicals were analytical-reagent grade unless specified otherwise, and organic solvents were HPLC grade.

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Preparation of solvents and reagents

Potassium borate buffer (0.5 mmole/ml). Boric acid (31 g) was dissolved in distilled water (1 l) and titrated to pH 10.5 with potassium hydroxide pellets.

o-Phthalaldehyde solution (74.5 μmole/ml). OPA (100 mg) (Sigma) was dissolved in methanol (10 ml).

Ethanethiol solution (135 μmole/ml). Ethanethiol (ET) (100 μl) (Fisher) was mixed with methanol (10 ml).

Sodium phosphate stock solution (300 μmole/ml). Anhydrous Na_2HPO_4 (30.67 g) and NaH_2PO_4 monohydrate (11.6 g) were dissolved in distilled water (1 l).

HPLC Solvent A. The above sodium phosphate stock solution (50 ml) was diluted to 1 l with distilled water and filtered through a 0.45-μm type HA filter (Millipore, Bedford, MA, U.S.A.) under vacuum with constant stirring.

HPLC Solvent B. Tetrahydrofuran (THF)–acetonitrile–Solvent A (5:50:45) was mixed and filtered.

HPLC

The chromatograph (Waters Assoc.) consisted of a 6000A and M45 solvent-delivery system, a U6K injector, a 660 solvent programmer, a 420 fluorescence detector equipped with an excitation monochromator (338 nm) and an emission cut-off filter (440 nm), and a 30 cm × 3.9 mm I.D. μBondapak C_{18} reversed-phase stainless steel analytical column.

Preparation of amino acid standards

The amino acid standard was prepared by adding to a screw-capped test tube, 25 μl of the Sigma amino acid solution, 25 μl of Asn (3.78 μmole/ml), 25 μl of Gln (3.42 μmole/ml), 50 μl of 1-MeHis (2.96 μmole/ml) and 50 μl of 3-MeHis (2.96 μmole/ml). This was followed by the addition of 50 μl of each of potassium borate buffer, methanol, OPA and ET. This amino acid standard was derivatized at room temperature for 4 min followed by the addition of 25 μl of ethylenediamine (98–100%, Anachemia, Toronto, Ontario, Canada), and the reaction was allowed to continue for a further 2 min. Methanol (5 ml) was then added to the tube and, after mixing, 20 μl of the sample was injected into the chromatograph. Samples, each 25 μl, of the individually prepared amino acids were derivatized in an identical manner and used for peak identification of the amino acid standard.

Standard curves

The linearity of the chromatographic response was established by derivatizing graded quantities of the amino acid standard (5–25 μl) followed by HPLC analysis. Standard curves for each amino acid were obtained by plotting instrument response (recorded as the integrated peak area) against the amount of added amino acid (in pmoles). The molar response (MR, area × 10^{-3} per pmole injected) and the correlation coefficient (r)[16] were estimated from these plots over the concentration range 50–500 pmoles per 20-μl injection.

RESULTS AND DISCUSSION

A typical chromatogram of the amino acid standard is shown in Fig. 1. The

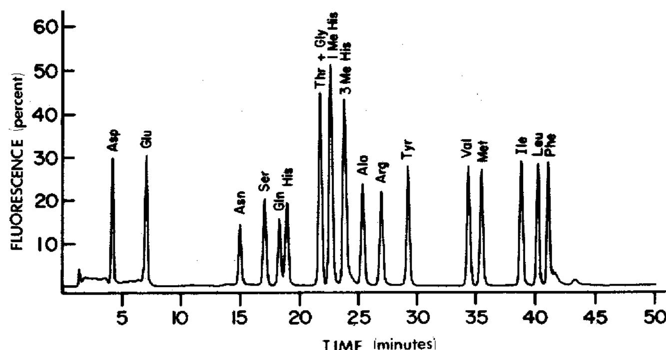

Fig. 1. Chromatogram of OPA–ET derivatives of amino acid standard mixture. Sample injection, 20 μl; guard column, 22 × 3 mm packed with μBondapak C_{18}/Corasil; column, μBondapak C_{18}, 300 × 3.9 mm stainless steel analytical column (Waters Assoc.); flow-rate, 2 ml/min; solvent A, 15 m*M* sodium phosphate buffer; solvent B, THF–acetonitrile–solvent A (5:50:45, v/v/v); mobile phase, 15–60% solvent B in 45 min with a linear program (curve 6); detector, fluorescence detector with excitation monochromator at 338 nm and emission filter at 440 nm, coarse sensitivity (gain) at 64; recorder, Spectra-Physics 4100 with chart-speed at 0.5 cm/min, attenuation at 128.

complete analysis of the eighteen amino acids required slightly over 1 h, including 15 min for the column to return to its initial conditions. Peak identification was possible for twenty crystalline amino acids and for the dipeptide Ans, but no detectable response was given for Pro, CySH, CyS–SCy, Lys or MeAla.

The following amino acid groups were eluted either too close together or at the same time, thus precluding accurate identification and quantitation: (a) Thr, Gly; (b) α-AIB, 1-MeHis; (c) Arg, β-Ala; (d) Leu, Nor, Phe; and (e) Ile, Try, Leu. Since Thr and Gly were eluted together (Fig. 1) and their relative molar responses were similar (6.05 ± 0.43 and 6.72 ± 0.62 per pmole, n = 3), they are quantitated as a pair. The non-naturally occurring amino acids α-AIB, Nor, and MeAla cannot be used as internal standards for this method as they either gave no response or coeluted with a naturally occurring amino acid. The sulphonated amino acid Tau coeluted with the dipeptide Ans at *ca.* 31 min, and so neither of these were included in the standard.

A similar elution order of the OPA-amino acid derivatives was reported by Hill *et al.*[3], but Ala and 3-MeHis co-elute under the chromatographic conditions of Hill *et al.*[3]. The addition of THF to solvent B delayed the elution of Ala, but not 3-MeHis. Solvent B, containing 5% THF, 50% acetonitrile and 45% solvent A, optimized the separation of Ala, 3-MeHis and the other amino acids.

The unreacted OPA–ET in the derivatizing mixture lowered the retention times of the amino acid standard after each chromatographic analysis. Decreasing the volume of OPA–ET from 200 to 100 μl and adding 25 μl of ethylenediamine 4 min after OPA–ET did not change the relative retention time or area response of the amino acid standard but reduced the loss in retention time by approximately 50% (from 0.1–0.2 to 0.05–0.10 min per injection).

Furthermore, the unreacted OPA–ET reduced the resolving power of certain amino acids, possibly by interacting with the column packing material (μBondapak C_{18}). After less than 100 injections, the resolution of Arg and Tyr using the present

HPLC conditions was completely lost. By increasing the sodium phosphate buffer concentration to 1.5–2.0 times that described in Materials and Methods, the column life can be doubled or tripled. This enhancement of Arg and Try resolution has been attributed to the effects of an increased sodium ion concentration[3].

The molar response MR was similar for the majority of amino acids (5.33–7.70 per pmole), except for Asp and Glu where the values were 2.26 and 2.76 per pmole, respectively. This similarity between the values implies that the reaction of the OPA–ET with the amino acids is stoichiometric and that the fluorescent characteristics of each derivative are similar and due primarily to the OPA moiety. Once formed, the OPA derivatives were stable; no change was detected in the standard chromatographic response after 8 h at room temperature or 16 h at 0–5°C.

The correlation coefficient (r) between area response and molar concentration in the range 50–500 pmole per 20-μl injection of each amino acid approached unity (0.976–0.994), except for Asp and Glu (0.963 and 0.943, respectively). Except for Asp and Glu, similar r values have been reported previously for OPA derivatives[5].

The coefficient of variation (C.V.) of the area response between different injections of the same preparation was small (1.3–5.9%). The C.V. between preparations and days was comparable (1.0–9.1%). The combined C.V. between preparations × days was higher (4.6–11.8%) than either those for preparations or days alone. Asp and Glu had generally higher values than the other amino acids. The C.V. of retention times between injections, preparations, days, and preparations × days for these amino acids was small (0.1–1.3%), except for Asp and Glu (1.3–6.5%).

Asp and Glu had lower MR and r values and a higher C.V. value than the other amino acids. The short retention time of these amino acid derivatives indicates their relatively low affinity for the column packing material. It is also possible that these derivatives are less stable than those formed by the other amino acids. Both explanations could lead to the observed low chromatographic response and high variation for these amino acids. The reliability of this method for quantitating Asp and Glu must be questioned; however, the concentrations of these two amino acids are generally low in biological samples.

Trichloroacetic acid (TCA) is commonly used to precipitate proteins from biological samples. A preliminary study found that its strong acidic nature inhibited the formation of the OPA-amino acid derivatives. Neutralizing the TCA with sodium hydroxide alleviated this problem. This procedure led to higher Asp and Glu values, but lower Asn and Gln values, possibly due to the breakdown of the amides to the parent amino acids in an alkaline medium. These factors can be corrected by calibrating the area response using those of the amino acid standard prepared and determined under identical conditions.

The limit of amino acid detection by the present HPLC method is in the pmole range. This limit can change a further four- to eight-fold by increasing the coarse sensitivity (gain) of the fluorescence detector (from 64 to 128), and by doubling the amount of biological sample derivatized and/or doubling the amount injected into the chromatograph.

There are several advantages of the present technique over the existing HPLC method[3]. It allows baseline separation and accurate determination of 1-MeHis, 3-MeHis, and fourteen other amino acids. Addition of ethylenediamine after amino acid derivatization reduces the amount of unreacted OPA–ET, thus reducing the loss

in retention time and increasing column life. Increases in the sodium phosphate concentration by 1.5–2.0 times will further prolong the column life by two to three times. This paper also identifies a possible problem in the analysis of the OPA derivatives of Asp and Glu.

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