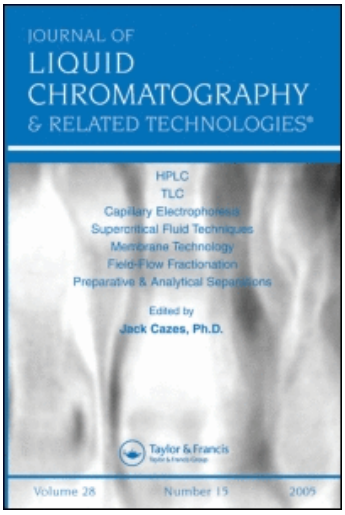

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THE SEPARATION OF PRE-COLUMN *o*-PHTHALALDEHYDE DERIVATIZED AMINO ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

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ABSTRACT

A method is presented in which a pre-column fluorescent derivative of primary amino acids is formed using *o*-phthalaldehyde (OPA). After a one minute derivatization period, the OPA amino acids are separated by high performance liquid chromatography using a ternary gradient in the reversed phase mode and fluorometric detection. The complete analysis time, including the derivatization step is less than 40 minutes with an average minimum detectable quantity of 40 picograms.

INTRODUCTION

The major difficulty of amino acid analysis by liquid chromatography is high sensitivity detection. Various derivatives have been employed to enhance amino acid detection by using either post-column (1, 2) or pre-column techniques (3, 4, 5, 6), the former being the most commonly used system. Some of the more

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popular post-column derivatives are ninhydrin, fluorescamine and *o*-phthalaldehyde and two of the more popular pre-column derivatives are dansyl chloride and phenylthiohydantoins (PTH). Neither of the pre-column derivatives mentioned has very high fluorescence quantum yields, especially in aqueous systems such as used in reversed phase liquid chromatography (6). Therefore, a need exists for a pre-column derivative for amino acids which is easily prepared and which has a high fluorescence quantum yield.

o-Phthalaldehyde (OPA) has been used successfully for the fluorometric detection of amino acids in the post-column derivatization technique (7, 8, 9). In the presence of a reducing agent such as 2-mercaptoethanol, OPA reacts with all primary amines to form thio-substituted isoindoles. It does not react with secondary amines (7). Recently, three reports have appeared in which OPA has been successfully used as a pre-column derivative for biogenic primary amines (10, 11, 12).

For this reason, it seemed plausible that a similar pre-column technique could be applied to the analysis of amino acids. Therefore, the purpose of this study was to explore the feasibility of using OPA as a pre-column derivative for all amino acids with subsequent separation by reversed phase liquid chromatography. Since proline and hydroxyproline are secondary amines, they were not examined.

MATERIALS AND METHODS

Apparatus

The instrument used throughout the study was a component HPLC system consisting of, 1) a Model 750 Solvent Delivery System, 2) a Model 753 Ternary Solvent Mixer, 3) a Model 740 Control Module, and 4) a Model 735 Push-Button Injector (Micromeritics Instrument Corp., Norcross, GA 30093). The detector was a Model A-4 Fluorometer (Farrand Optical Co., Inc., Valhalla, NY 10595) equipped with a 10 µl flow cell and optical filters to allow a wavelength of excitation at 340 nm and a wavelength of emission at 450 nm.

The column, 150 mm x 5.0 mm ID, was packed with MicroSil C_8. This material is a spherical silica with an average particle diameter of 7.5 μm and a monolayer coverage of octyl groups via a Si-C bond. The residual hydroxyl groups are capped with CH_3- groups and the total carbon content is approximately 10% (w/w).

Chemicals

All solvents were distilled-in-glass grade (Burdick and Jackson, Muskegon, MI 49442) and used without further treatment. Water was processed through a Continental Water Conditioning System (Continental Water Conditioning Co. of Atlanta, Atlanta, GA 30359). The amino acid standards and *o*-phthalaldehyde were obtained through Sigma Chemical Co. (St. Louis, MO 63178). The 2-mercaptoethanol was obtained from K&K Labs Division, ICN Pharmaceuticals (Plainview, NY 11803) and the boric acid, acetic acid, potassium acetate and potassium hydroxide from Fisher Scientific (Norcross, GA 30091).

Procedure

The derivatization reagent was prepared by dissolving 400 mg of anhydrous *o*-phthalaldehyde into 400 ml of 0.4 M boric acid buffer adjusted to a pH of 10.40 with potassium hydroxide. To this solution were added 2.00 ml of 2-mercaptoethanol. The solution was purged of oxygen with nitrogen gas and stored in the dark. Prepared in this manner, the solution was useful for two weeks.

Stock solutions of each amino acid and of a mixture of all amino acids were prepared to contain 100 μg/ml of each amino acid. These solutions were used to prepare all sample solutions and were refrigerated when not in use. The mixture was used for separation development while the individual amino acids were used for peak identification and the kinetics experiments.

The aqueous buffer used as mobile phase was 0.02 M potassium acetate adjusted to a pH of 5.50 with glacial acetic acid. This

solution was prepared fresh daily. All mobile phase solvents, tetrahydrofuran, methanol, and the buffer were degassed by continuous helium purging.

The general operation procedure was as follows. A two volume excess of derivatizing reagent was added to a solution containing 100 ng/µl of amino acid or less. This mixture was then agitated for one minute and a 5 µl aliquot withdrawn with a microliter syringe and injected into the liquid chromatograph. The average amount of amino acid injected was 20 ng. All runs were done in duplicate.

RESULTS AND DISCUSSION

Separation Development

Simple isocratic solvent systems were used to determine which solvents and buffer pH provided the best resolution. Organic solvents investigated were acetonitrile, methanol and tetrahydrofuran (THF) and combinations of these. Potassium acetate buffer was used over phosphate buffer because of the latter's reputed quinching effects on the fluorescence of OPA amino acids (7). The best combination of solvents was determined to be methanol, THF, and 0.02 M potassium acetate buffer adjusted to a pH of 5.50 with acetic acid (Figure 1). Tetrahydrofuran concentration was important. Absence of THF increased retention time by approximately 20% while loss of resolution of the polar OPA amino acids occurred with concentrations greater than 7% (v/v).

Figure 2 shows a linear methanol-buffer gradient holding the THF concentration at constant 1% (v/v). Although the separation was an improvement over the isocratic separations, there was still room for improving resolution. Since changing gradient curvature or range gave no apparent improvement in resolution, step gradients were employed. The step gradient used to generate Figure 3 showed enhanced resolution over the isocratic and linear gradient separations. All components with the exception of four were separated. The peak labeled J in Figure 3 contained these four

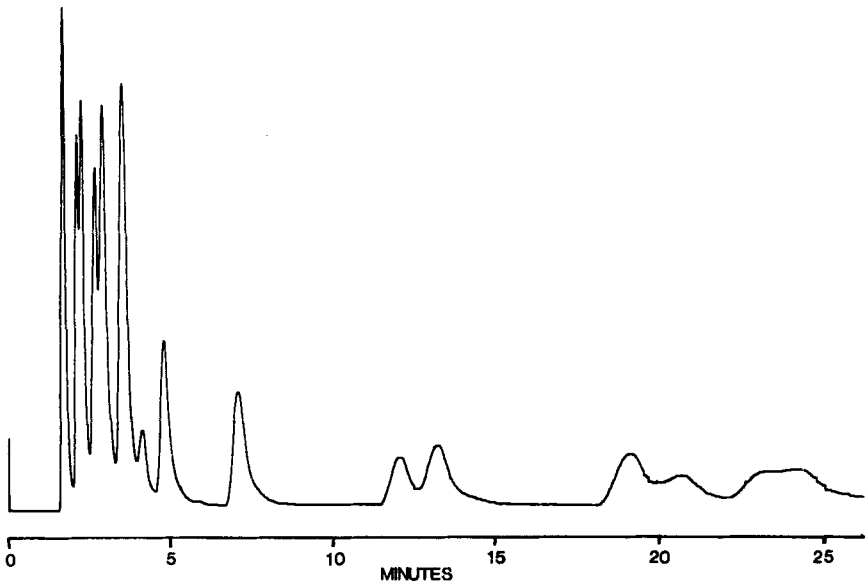

FIGURE 1. Isocratic separation of OPA amino acids. Column, MicroSil C_8 150 mm x 5 mm ID; Solvents, 6% THF/24% Methanol/70% 0.02 M potassium acetate pH 5.50; Flow, 1.5 ml/min.

components. By increasing buffer concentration at this point in the chromatogram, these four OPA amino acids, as well as the others, were separated (Figure 4). The gradient profile for this separation is shown in Figure 5. It is interesting to point out the selectivity change which occurred due to the increase in buffer concentration. The retention time of peak I (cysteine) in Figures 3 and 4 shifted from seven minutes in Figure 3 to fourteen minutes in Figure 4, while the components under peak J which eluted after peak I in Figure 3 were less retained but separated in Figure 4. Since these conditions (Figure 5) provided the best separation of the OPA amino acids, they were used to determine

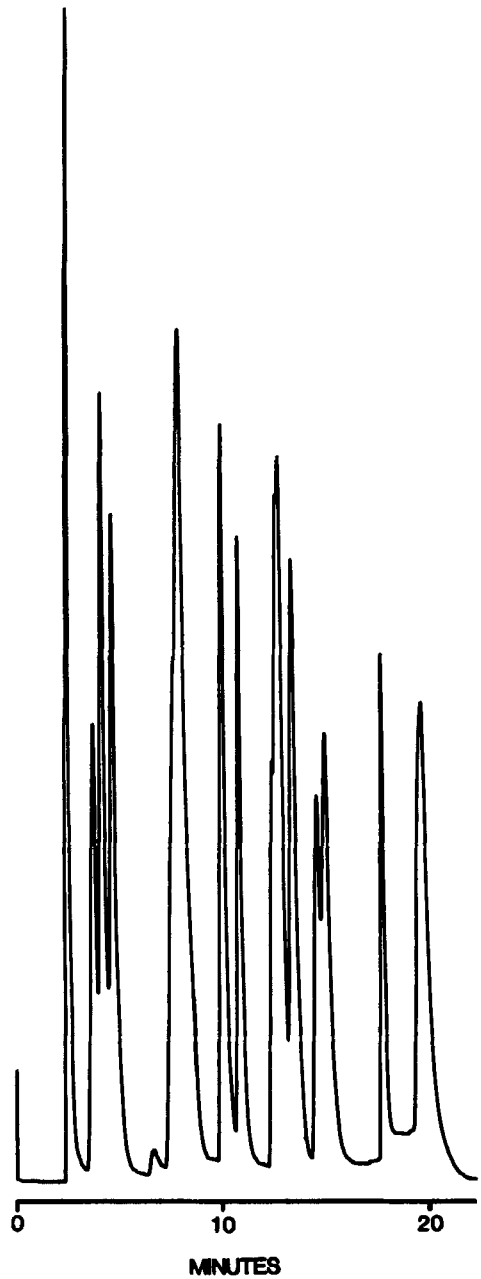

FIGURE 2. Linear gradient separation. Column, MicroSil C_8 150 mm x 5 mm ID; Gradient, 19% to 79% methanol in 0.02 M potassium acetate pH 5.50, 1% THF throughout; Flow, 2.00 ml/min.

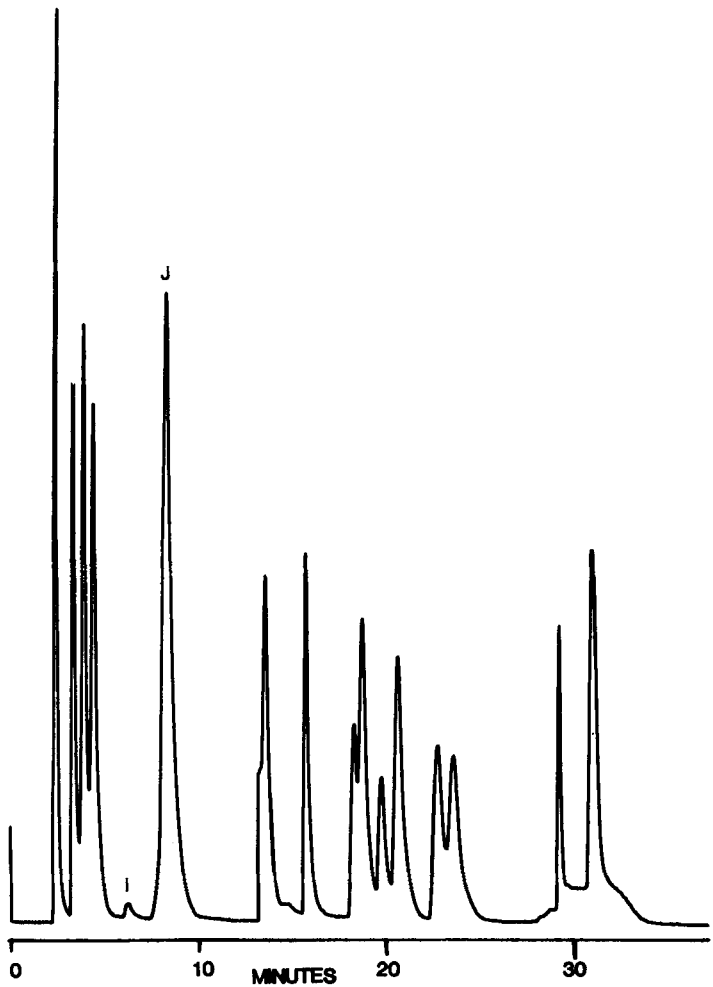

FIGURE 3. Step gradient separation. Column, MicroSil C_8 150 mm x 5 mm ID; Solvent Steps, 1) 1% THF/19% Methanol/80% 0.02 M potassium acetate pH 5.50 for 5 minutes, 2) 1% THF/39% Methanol/60% 0.02 M potassium acetate pH 5.50 for 15 minutes, and 3) 10% THF/60% Methanol/ 30% 0.02 M potassium acetate pH 5.50 for 10 minutes; Flow, 2.00 ml/min.

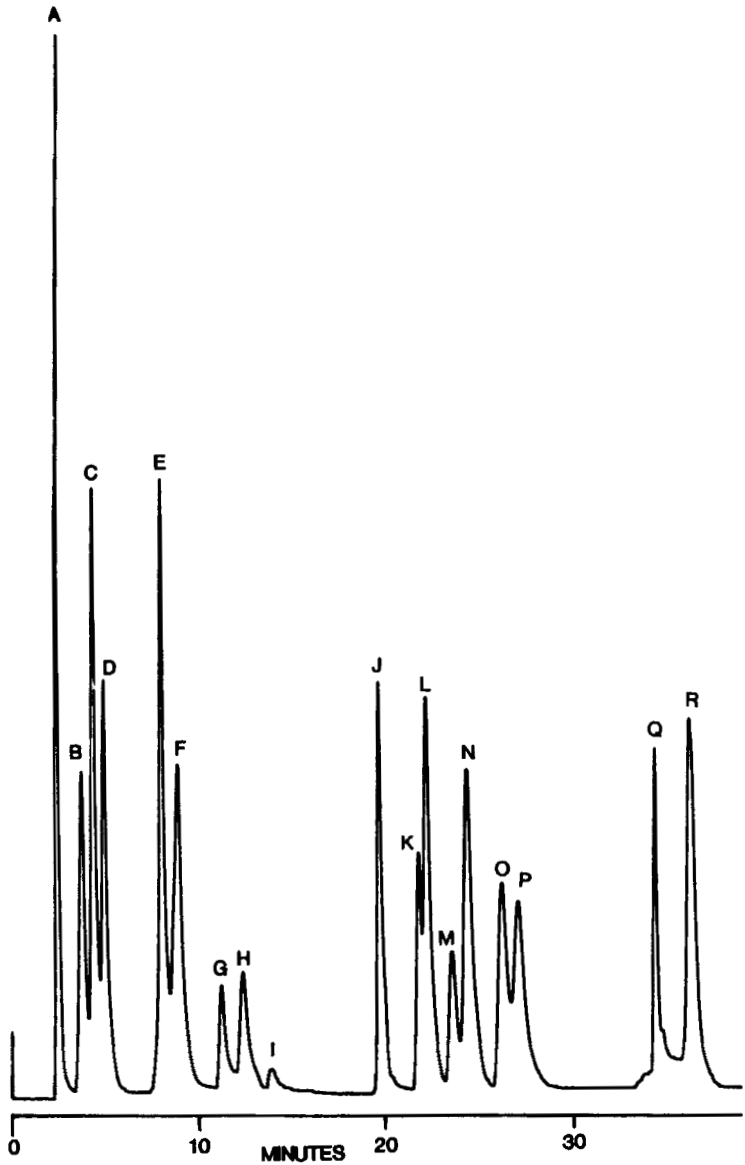

FIGURE 4. Final step gradient separation. Conditions, see Figure 5. Peaks, A) Asp, B) Thr, C) Ser, D) Glu, E) Gly, F) Ala, G) Tyr, H) Val, I) Cys, J) His, K) Met, L) Glu (NH_2), M) Ile, N) Leu, O) Phe, P) Arg, Q) Try, and R) NH_3 (?).

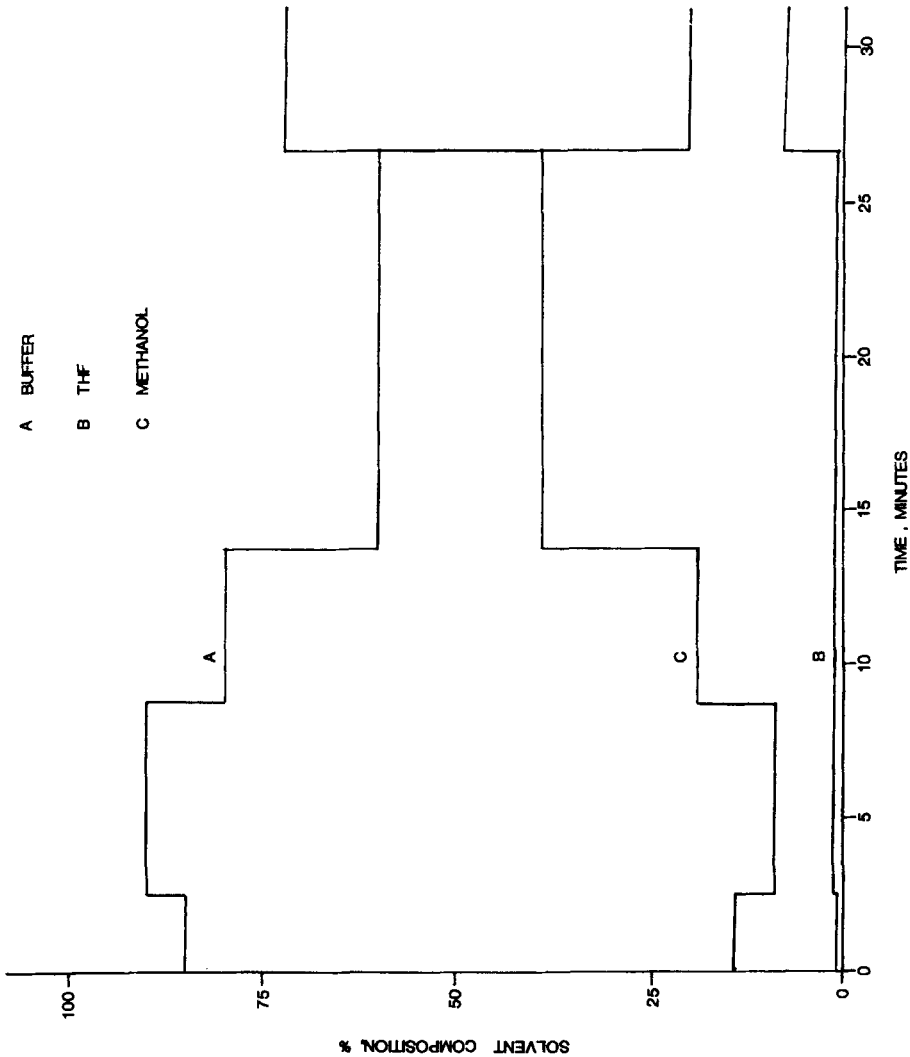

FIGURE 5. Solvent profile for separation in Figure 4. Column, MicroSil C_8 150 mm x 5 mm ID; Flow, 2.00 ml/min.

OPA amino acid elution order and to perform a kinetic study to determine optimum mixing time for maximum fluorescence.

Kinetic Study

According to Roth (7), the mixing time required to achieve maximum fluorescence intensity was five minutes. Benson and Hare (8), from their observation with post-column derivatization, disagreed with this and stated that maximum fluorescence was obtained upon mixing reagent and sample. In order to determine the optimum mixing time for pre-column derivatization, a simple kinetics experiment in the fashion of Mell *et al* (10) was conducted. Excess OPA reagent was mixed with the amino acid standards and the resulting mixture agitated for a measured amount of time. An aliquot of this mixture containing 20 ng was then chromatographed and the peak heights measured. The results for selected amino acids are shown in Figure 6. It was concluded from this experiment that the optimum mixing time for pre-column derivatized amino acids was one minute.

All amino acids except cysteine and lysine had good response and had an average minimum detectable quantity of 40 picograms. The linear response for four amino acids in the general working range is shown in Figure 7. The low response for cysteine and lysine has been reported (7, 8). This problem has been rectified in post-column derivatization by adding the surfactant, Brij 35, to the OPA reagent (8); however, this was not employed for this study.

CONCLUSION

The pre-column derivatization of amino acids with *o*-phthalaldehyde has been demonstrated to be a feasible method for amino acid analysis. The derivatization period is rapid and yields a highly detectable fluorescent derivative. The total analysis time, including derivatization, is less than 40 minutes, faster than all current amino acid analyzers which require approximately 60 to 70 minutes on the average.

The major disadvantages are the low response of cysteine and lysine, and OPA's inability to directly derivatize proline and

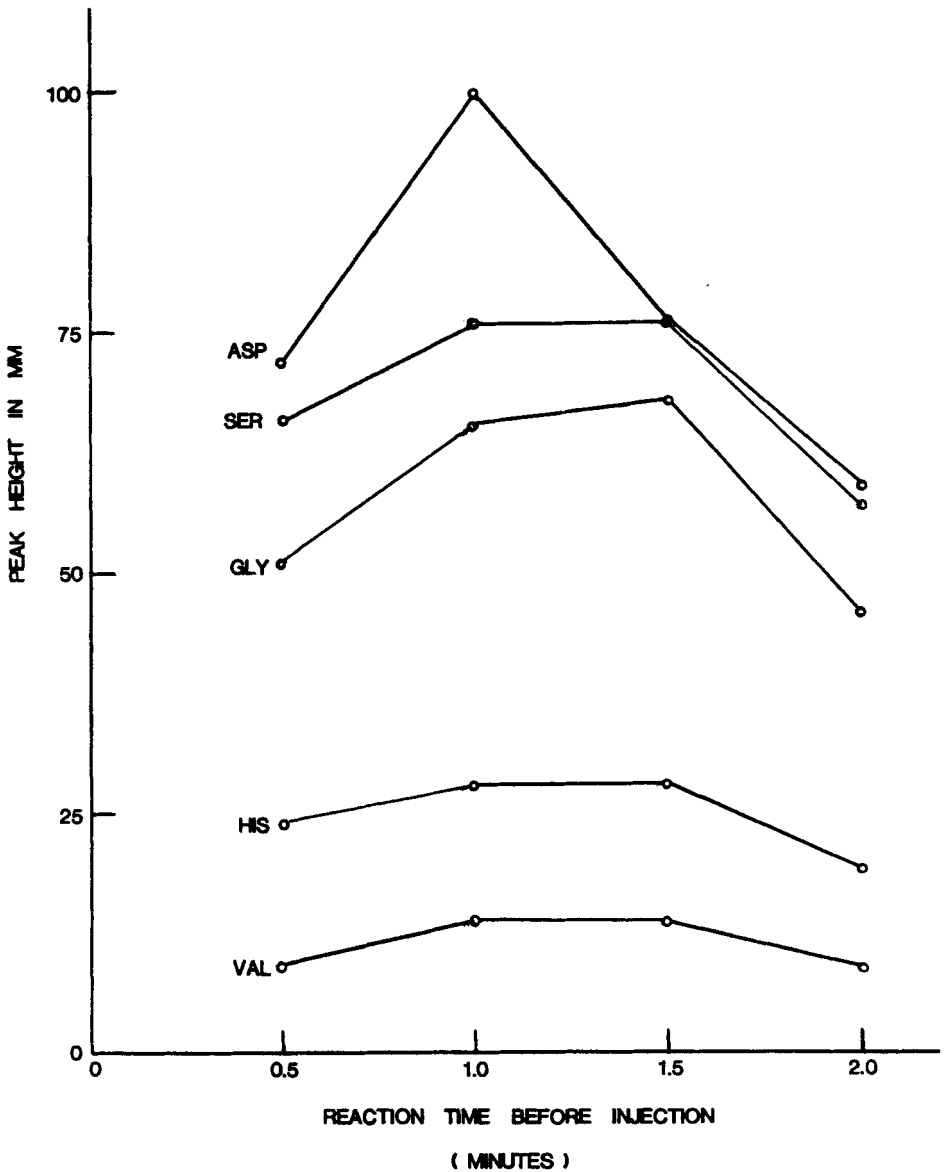

FIGURE 6. Optimum reaction time for five OPA amino acids. Chromatographic conditions as in Figure 5.

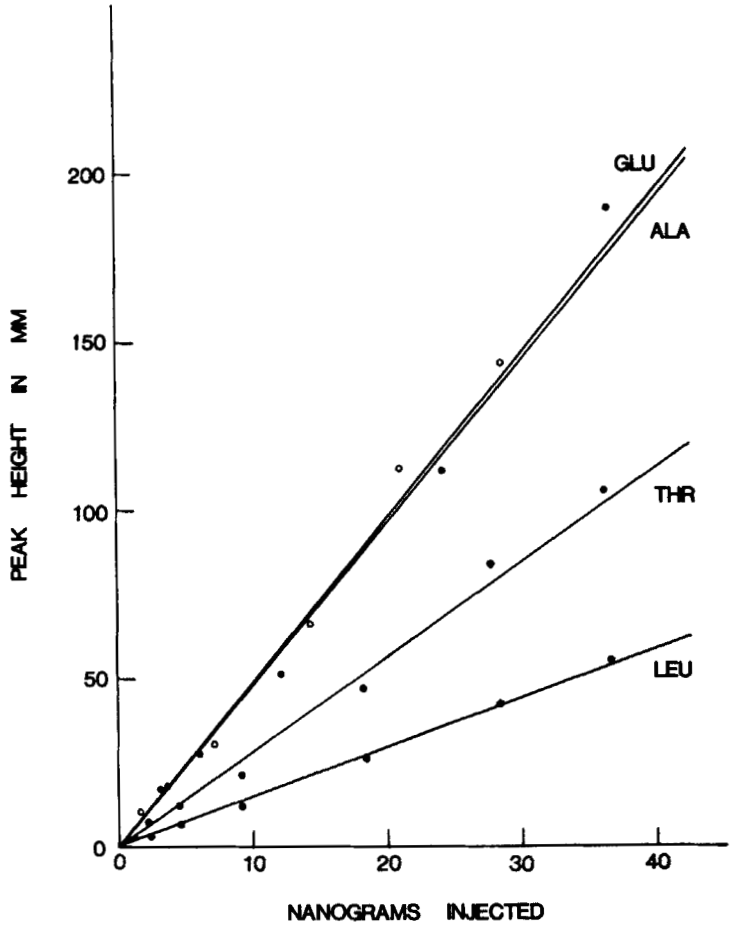

FIGURE 7. Linearity of response for four OPA amino acids. Conditions as shown in Figure 5.

hydroxyproline. Future work will concern the sensitivity enhancement of lysine and cysteine.

ACKNOWLEDGMENTS

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