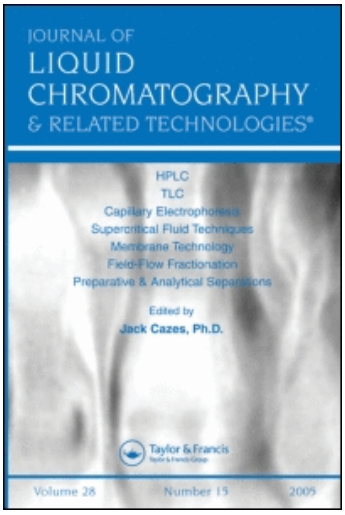

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Smith, Robert J. and Panico, Karen A.(1985) 'Automated Analysis of O-Phthalal-Dehyde Derivatives of Amino Acids in Physiological Fluids by Reverse Phase High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 8: 10, 1783 — 1795
To link to this Article: DOI: 10.1080/01483918508074095
URL: http://dx.doi.org/10.1080/01483918508074095

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(10), 1783-1795 (1985)

AUTOMATED ANALYSIS OF O-PHTHALALDEHYDE DERIVATIVES OF AMINO ACIDS IN PHYSIOLOGICAL FLUIDS BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An automated method is described for the determination of free amino acids in biological fluids using precolumn derivatization with o-phthalaldehyde and reverse phase high performance liquid chromatography. Chromatographic separation of amino acids is accomplished in 24 minutes (cycle time 44 minutes). As little as 1.5 pmol of most commonly occurring amino acids can be accurately quantified. Accuracy and reproducibility are optimized by automating the derivatization-injection sequence and by correcting for variations in the fluorescence response of each amino acid in each run. A total of 31 analyses can be completed in 24 hours on a single column (7 standards and 24 unknowns). The method can be used in the general determination of free amino acids in biological fluids, or can be further accelerated and used for the quantitation of specific amino acids simply by altering the elution conditions.

 0148-3919/85/0810-1783$3.50/0

INTRODUCTION

The concentrations of free amino acids in blood, urine, tissues, and other biological fluids have traditionally been determined by ion-exchange chromatography coupled with post-column detection with ninhydrin or fluorescent reagents (1,2). These methods assure reproducible separation and quantitation of most amino acids, but they require relatively long elution times, provide unreliable results for acid-labile amino acids such as glutamine and asparagine, and may not be sensitive enough when limited material is available (for example from needle biopsies of tissues or cell cultures). An alternative methodology has recently been developed, which utilizes the pre-column derivatization of amino acids with compounds which generate fluorescent products that can be separated by reverse phase high performance liquid chromatography (HPLC) (3-6). Utilizing pre-column derivatization and HPLC, we have assembled an automated chromatographic system for amino acid analysis. Both precision and convenience are maximized by automating the derivatization and the analysis of chromatographic data. The system runs unattended for up to 24 hours, generating and analyzing 31 runs per day on a single column. In this report, we describe the method and demonstrate its usefulness for the rapid analysis of picomolar quantities of free amino acids in biological samples.

MATERIALS AND METHODS

Apparatus and Materials

The chromatographic system consisted of a Model 721 System Controller, Wisp 710B injector, two Model 6000A pumps (all from

Waters), an FS970 fluorescence detector (Schoeffel Instrument Corp.), and a model 7000A chromatogram processor with two floppy disk drives (System Instruments Corporation). Amino acid derivatives were separated on an Altex 3 micron Ultrasphere ODS 4.75 x 75 mm column (Beckman Instruments) after passage through a column (3.9 x 22.5 mm) containing 0.5 mm glass beads (Thomas Scientific) and a precolumn (3.9 x 22.5 mm) containing C18/Corasil (Waters).

Preparation of O-Phthalaldehyde and Solvents

O-Phthalaldehyde (OPA) (Pierce) was dissolved in methanol (50 mg/ml) and then diluted with 9 volumes of 0.4 M sodium borate buffer (pH 9.5). 2-Mercaptoethanol (4 µl/ml of diluted OPA solution) was added, followed by an additional 0.4 µl/ml of OPA solution daily thereafter. The resulting OPA reagent was stable for at least one week when stored in the dark at $4^{o}C$.

Solvents were similar to those described by Jones et al. (5). Solvent A consisted of 1 part tetrahydrofuran, 19 parts methanol and 80 parts 0.05 M sodium acetate buffer (pH 5.9). Solvent B was 80 parts methanol and 20 parts 0.05 M sodium acetate buffer (pH 5.9). Both were passed through a 0.5 micron Type FH filter (Millipore) and degassed prior to use.

Preparation of Amino Acid Standards and Biological Samples

A neutralized solution of perchloric acid (PCA) was prepared by adding 7.5 ml of 0.2 M sodium acetate buffer (pH 4.9) to 50 ml of 5% (w/v) PCA. The pH was titrated to 4.75-4.90 with 5N KOH, the volume brought up to 100 ml with H_2O, the precipitated potassium perchlorate salt removed by centrifugation, and the final volume then brought to 400 ml. Amino acid standards

and p-fluoro-DL-phenylalanine (F-phe) (Sigma) internal standard were dissolved in this neutralized PCA solution at 2.5 uM concentrations and stored in aliquots at -25°C. Under these conditions, amino acids were stable for at least 4 months. Just prior to use, equal volumes of the amino acid standard mixture and F-phe were combined and centrifuged for 1 minute at 12,500 g to remove any additional precipitate.

Plasma or whole blood samples were deproteinized by combining with equal volumes of ice cold 10% PCA, vortexing, and centrifuging for 10 min at 2000 g. A 2 ml aliquot of the resulting supernatant was combined with 0.3 ml of 0.2 M sodium acetate buffer (pH 4.9), titrated to pH 4.75-4.90 with 5 N KOH, and adjusted to a final volume of 4 ml. After removal of precipitated potassium perchlorate by centrifugation, the neutralized samples were stored at -25°C. Samples of skeletal muscle obtained by needle biopsy were homogenized in 10% PCA and processed in a similar manner. Neutralization of PCA supernatants to pH 4.75-4.90 was necessary to prevent hydrolysis of glutamine and asparagine during storage.

Prior to chromatography, biological samples were appropriately diluted with neutralized PCA solution and then combined with an equal volume of F-phe internal standard solution. Preparation and dilution of samples and standards in water rather than the neutralized PCA solution resulted in the gradual loss of several amino acids during 24 hours at room temperature, possibly as a result of their adsorption to the injector vials.

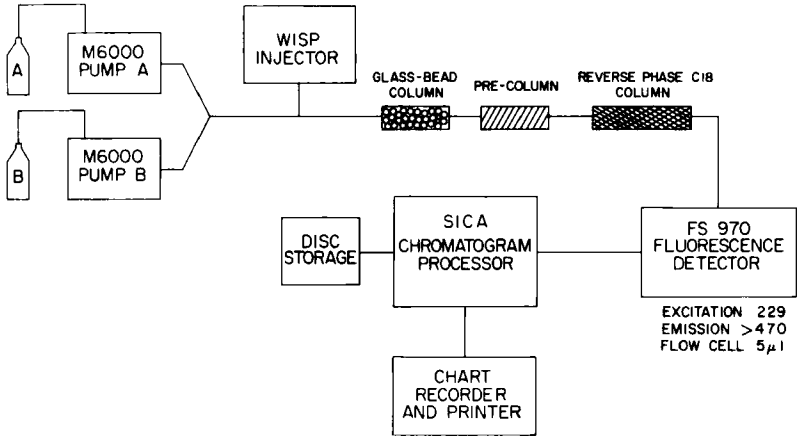

Figure 1. Diagram of pre-column OPA chromatographic system.

Automated Amino Acid Analysis Method

The instrumentation for amino acid analysis is depicted schematically in Figure 1. A vial containing the OPA solution is placed in position 1 in the 48-position rack of the WISP autosampler. An appropriate number of amino acid standards and unknown samples then are placed in the rack in sequence such that a standard will be analyzed before and after each 4 samples.

The OPA derivatization is carried out according to the Auto.TAG OPA method described by Waters (7). After equilibration with Solvent A, flow is stopped for 10 minutes to allow pressure in the injection system to decrease. A 5 μl aliquot of OPA solution is then injected, followed immediately by 5 μl of standard or unknown sample. Flow is increased to 0.1 ml/min for 2 min, during which time the OPA and sample are carried through a glass

bead column where mixing and the derivatization reaction occur at room temperature. Flow is then increased to 1.0 ml/min and gradient elution of amino acids is carried out over the next 24 minutes. Chromatographic runs are automatically sequenced after re-equilibration with solvent A and a return to 0 flow (total cycle time of 44 minutes).

The digitalized output from the fluorescence detector is stored on disk and processed during the equilibration phase between runs. Individual amino acid peaks are identified by analyzing the chromatogram in two parts (0 to 15 min and 15 to 25 min). Reference peaks are identified in each segment of the chromatogram (glycine and valine, respectively) and preset retention times for the other amino acids are adjusted in accordance with the shift in reference peak retention times. Following integration, the area of each peak is stored in computer memory until a set of 4 unknowns and 2 standards (preceding and following) have been similarly analyzed. After correction for changes in internal standard peak area, the areas for each standard peak are then compared. It is assumed that any changes in corrected standard peak areas occur linearly with time between the two standard runs, and corrected concentration factors (CF_1 to CF_4) are thus calculated individually for each peak in each unknown chromatogram as follows:

$$CF_{Initial} = CF_1 = \frac{(\text{Standard Concentration})}{(\text{Area of Initial Standard})}$$

$$CF_{Final} = CF_F = \frac{(\text{Std. Conc.})}{(\text{Std. Area}_F)} \times \frac{(\text{Int. Std. Area}_F)}{(\text{Int. Std. Area}_I)}$$

$$CF_1 = \left(\frac{CF_1 - CF_I}{5}\right) + CF_I$$

$$CF_2 = (2) \times \left(\frac{CF_F - CF_I}{5}\right) + CF_I$$

$$CF_3 = (3) \times \left(\frac{CF_F - CF_I}{5}\right) + CF_I$$

$$CF_4 = (4) \times \left(\frac{CF_F - CF_I}{5}\right) + CF_I$$

The individual CF values for each amino acid in each chromatogram and the internal standard area in each chromtogram are then used to calculate amino acid concentrations according to the following formula:

Concentration on Unknown in Run #1

$$= (CF_I)\ (\text{Unknown Area}) \times \frac{(\text{Int. Std. Area I})}{(\text{Int. Std. Area in Run 1})}$$

Unknown sample data are finally corrected for dilution during preparation for chromatography. All of the computations are completed automatically and reported in tabular form.

RESULTS

The separation of a standard mixture of amino acids is depicted in Figure 2. A 5 µl aliquot containing a total of 6.25 pmol of each amino acid was injected. All amino acids that commonly occur in proteins were quantified except lysine, cysteine, proline, and hydroxyproline. Tryptophan and methionine co-eluted, but can be separated with a minor change in the gradient as shown in Figure 3. In most determinations on biological samples, the method in Figure 2 has been used because it separates the large alanine peak from the small tyrosine peak more effectively, and tryptophan and methionine are of less interest in this laboratory.

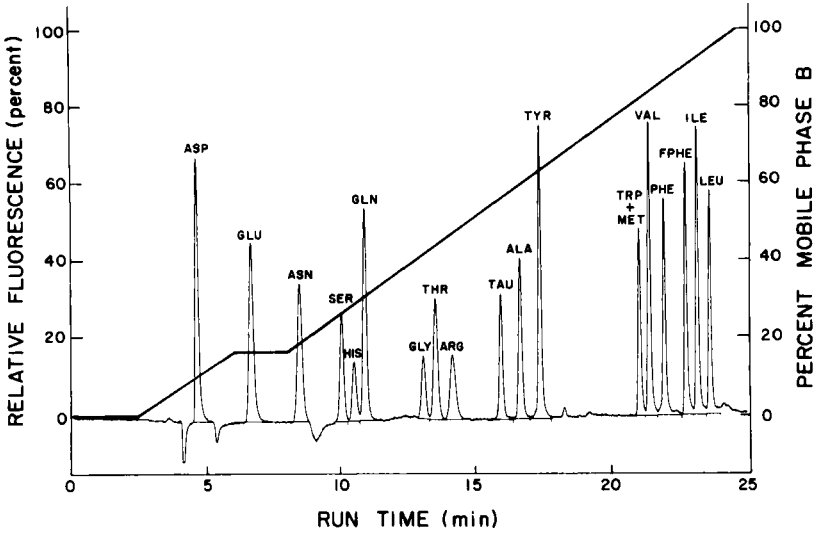

FIGURE 2. Elution profile of amino acid standards (6.25 pmol).

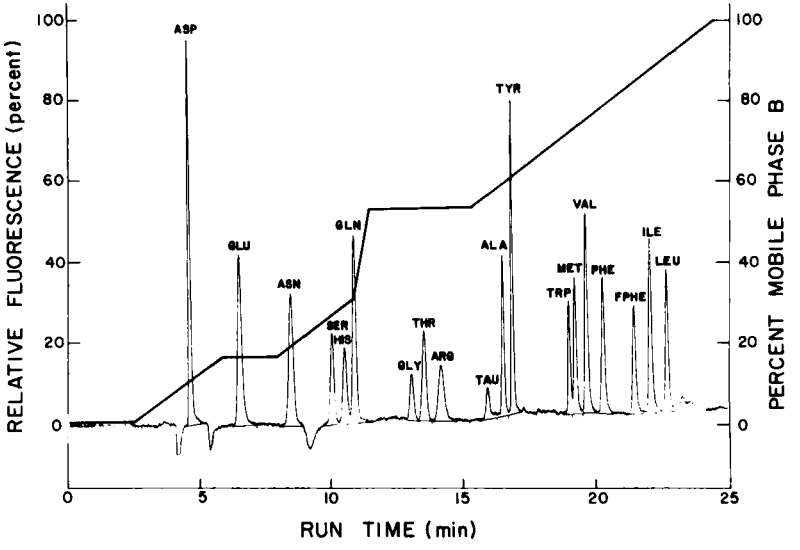

FIGURE 3. Elution profile of amino acid standards with gradient program optimized to separate TRY and MET (6.25 pmol).

TABLE 1

Coefficients of Variation for Individual Amino Acids

(n=5)

Amino Acid	CV (%)	Amino Acid	CV (%)
Aspartic Acid	5.20	Taurine	4.97
Glutamic Acid	5.40	Alanine	4.39
Asparagine	4.44	Tyrosine	5.32
Serine	4.44	Methionine	5.08
Histidine	4.72	Valine	3.85
Glutamine	3.57	Phenylalanine	4.93
Glycine	5.01	Isoleucine	2.64
Threonine	3.37	Leucine	2.53
Arginine	3.74		

The fluorescence response of all measured amino acids correlated linearly with amount injected over a range of at least 1.5-6.3 pmol/5 µl injection (0.3 - 1.3 µM). Coefficients of variation are depicted in Table 1.

The chromatographic separation of free amino acids from several biological fluids is illustrated in Figures 4-7. Skeletal muscle was analyzed at two different dilutions because of the wide range of free amino acid concentrations.

DISCUSSION

A chromatographic system has been assembled from commercially available components that is suitable for the analysis of most free amino acids in biological fluids by reverse phase HPLC. The system has been fully automated so that pre-column derivatization

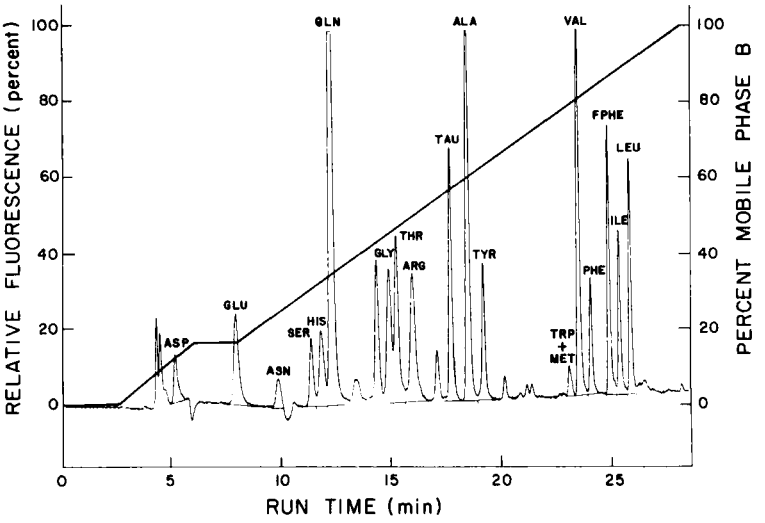

FIGURE 4. Free amino acids in dog arterial blood (1/80 final dilution, 5 µl injection).

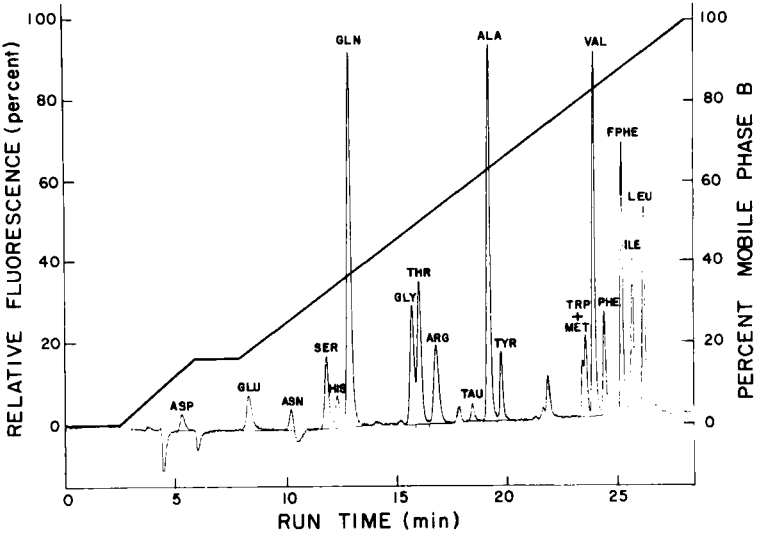

FIGURE 5. Free amino acids in 5 µl of dog plasma (final dilution 1/120).

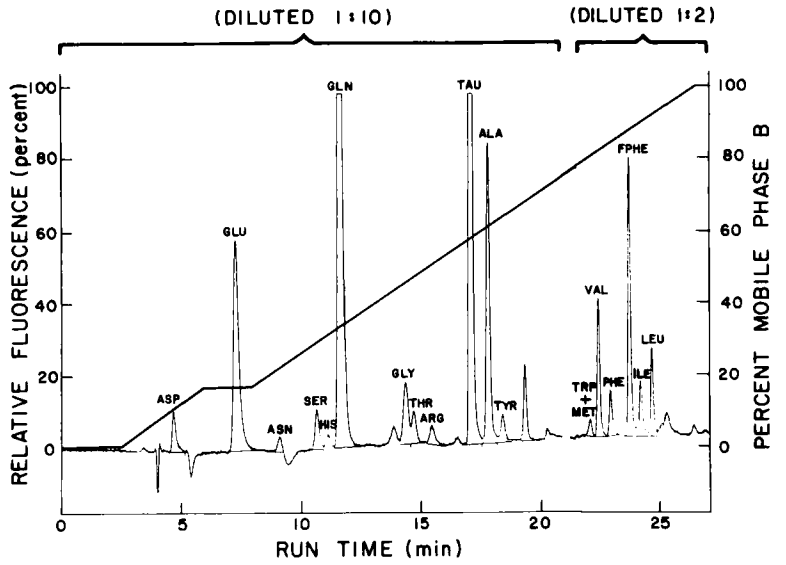

FIGURE 6. Free amino acids in dog skeletal muscle (100 mg in 0.5 ml of 10% PCA). Combined results from runs at 1/10 and 1/2 dilution.

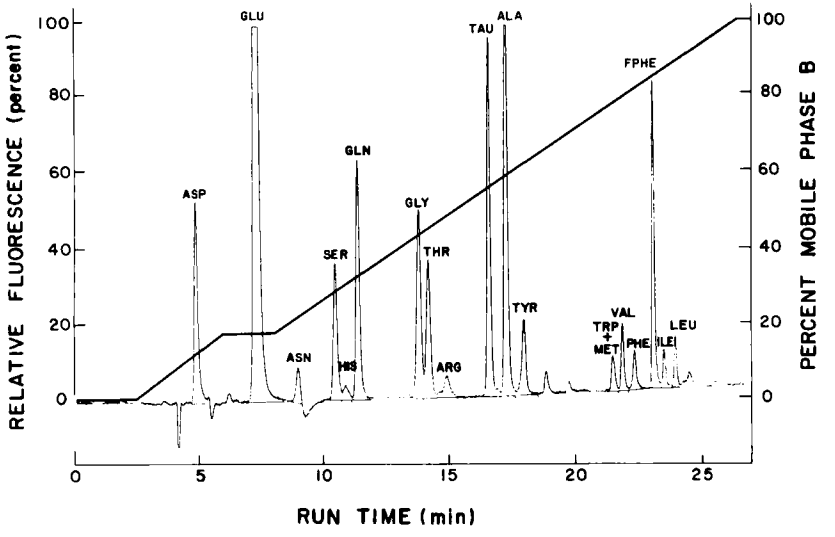

FIGURE 7. Free amino acids in cultured skeletal muscle (one 60 mm petri dish of L6 cells in 0.5 ml of 10% PCA, 1/4 dilution).

of amino acids, chromatography, and data analysis are completed sequentially on multiple samples without the requirement for operator intervention for periods up to 24 hours. Reproducibility of amino acid determinations has been increased both by automating and thus standardizing the derivatization reaction and by analyzing standards at frequent intervals and calculating an individualized response factor for each amino acid in each run.

In comparison with ion exchange methods (7) the major disadvantage of the pre-column OPA method is the failure to quantify secondary amines and several other quantitatively important amino acids (e.g. hydroxyproline, proline, cysteine, and lysine). There are numerous advantages of the OPA method, however. Each chromatographic elution requires only 24 minutes (cycle time 44 minutes), allowing a total of 31 runs per 24 hours on a single column (7 standards and 24 unknowns). The sensitivity is extremely high (<1.5 pmol), making it possible to determine intracellular amino acids in mg quantities of tissue obtained by needle biopsy or in small quantities of cultured cells. For studies of protein degradation (8), this method is far more sensitive for determinations of phenylalanine and tyrosine than other published fluorometric methods (9). Finally, the chromatography can easily be altered to allow even more rapid determination of specific amino acids.

ACKNOWLEDGMENTS

The authors wish to thank Rick Carberry and his associates at Waters for assistance with the pre-column derivatization method, and Arthur Leung of Systems Instruments Corporation

for designing the data processing programs. This work was supported by the Howard Hughes Medical Institute and a grant from the National Institutes of Health (NIGM P50 GM 29327).

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