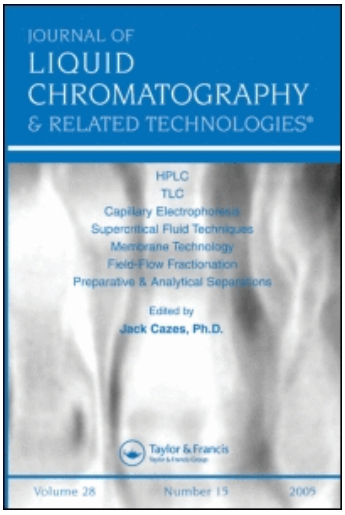

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Determination of Amino Acids by Micellar High-Performance Liquid Chromatography and Pre-column Derivatization with *O*-Phthalaldehyde and N-Acetyl-L-cysteine

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DETERMINATION OF AMINO ACIDS BY MICELLAR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRE-COLUMN DERIVATIZATION WITH *O*-PHTHALALDEHYDE AND N-ACETYL-L-CYSTEINE

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ABSTRACT

Micellar liquid chromatography of proteic primary amino acids with pre-column derivatization with *o*-phthalaldehyde (OPA) and N-acetyl-L-cysteine was studied, using mobile phases containing a short-chain alcohol. The modification of pH gave a large variation of the retention as a result of the protonation of the carboxylate group of amino acids. Maximum resolution and adequate retentions were achieved with a 0.05 M sodium dodecyl sulphate/3% propanol mobile phase at pH 3. The reproducibility was lower than 1.0% at a $1x10^{-4}$ M concentration level and between 0.6 and 2.2% for $1x10^{-6}$ M. The determination of glycine, lysine, methionine and threonine in pharmaceutical formulations gave recoveries, with respect to the values declared by the manufacturers, in the 90-105% range.

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INTRODUCTION

High-performance liquid chromatography of aminoacids with spectrophotometric detection usually requires the formation of derivatives, because of little absorption of ultraviolet light above 210 nm. Precolumn derivatization is usually preferable, because of sensitivity increases. *o*-Pthalaldehyde (OPA) is the derivatization reagent that probably has the best characteristics (1,2). It reacts with primary amino groups, in the presence of a thiol at pH 9.5 and room temperature, to form 1-alkylthio-2-alkyl substituted isoindoles. The derivatives show maximum absorption at 335 nm and are highly fluorescent, with excitation wavelength at 340 nm and emission at 445 nm. Mercaptoethanol has been more extensively used than other thiols for the derivatization of amino acids, however the OPA-mercaptoethanol isoindoles are unstable. It has been demonstrated that N-acetyl-L-cysteine (NAC) improves the stability of isoindoles when used instead of mercaptoethanol (3).

An extensive investigation is being performed in our laboratory on the applicability of Micellar Liquid Chromatography in routine analysis. Procedures for the evaluation of diuretics (4,5), anabolic steroids (6), sulphonamides (7), and beta-blockers (8) in pharmaceuticals, and diuretics (9) and catecholamines (10) in urine, have been developed. The reasons which may impel the analyst to use a micellar solution, instead of a conventional hydroorganic mobile phase, in reversed-phase liquid chromatography, are the lower cost, toxicity and biodegradability of the solvent, the increased sensitivity in the spectrophotometric, fluorimetric and phosphorimetric detection achieved for some compounds (11), the possibility of describing the chromatographic behaviour with simple equations (12), the change in selectivity (13), the performance of elution gradients of surfactant without the need of reequilibration of the column

(14), and finally, the easy solubilization of analytical samples, that avoids the long previous separation steps which are often necessary.

Proteic primary amino acids are found in diverse pharmaceutical formulations, such as vitaminic preparations or appetite stimulants. Cystine, cysteine and methionine are used in formulations indicated in the treatment of alopecia. Finally, some preparations contain an amino acid in the excipient. Thus, glycine is used to increase the rate of dissolution of acetylsalicylic acid, improving its action and stomachic tolerance (15).

In this work, the first investigations on the chromatographic behaviour of the OPA-amino acid derivatives with micellar eluents, are reported. An analytical procedure for the determination of amino acids in pharmaceutical formulations is developed.

MATERIALS AND METHODS

The micellar mobile phases were prepared by mixing aqueous sodium dodecyl sulphate (99%, Merck, Darmstadt, Germany) solutions with an alcohol to obtain the working concentration. The alcohols studied were methanol (HPLC, Panreac, Barcelona, Spain), 1-propanol (analytical reagent, Panreac) and 1-pentanol (analytical reagent, Merck). The pH of the micellar eluent was adjusted with 0.01 M phosphate buffer, prepared with disodium hydrogen phosphate and phosphoric acid (analytical reagent, Panreac).

A 2×10^{-4} M stock solution of the following amino acids were prepared: DL-alanine, L-arginine, L-asparagine, L-glutamic acid, L-glutamine, L-histidine, L-leucine, L-serine, DL-tyrosine, DL-valine (very pure, Scharlau, Barcelona), DL-isoleucine, L-lysine hydrochloride, L-threonine, and DL-tryptophan (very pure, Fluka, Buchs, Switzerland), L-

cysteine, L-methionine and L-phenylalanine (biochemical use, Merck), L-aspartic acid (analytical reagent, Merck), and glycine (pure, Carlo Erba, Rodano, Milano, Italy). Some drops of 6 M HCl was added to dissolve those amino acids which are sparingly soluble at pH 7. The solutions were stored at 4 °C.

The isoindole derivatives were obtained with solutions of $2x10^{-3}$ M *o*-phthalaldehyde (biochemical use, Fluka) and $2x10^{-3}$ M N-acetyl-L-cysteine (very pure, Fluka). The 0.1 M boric-borate buffer of pH 9.5 was prepared with boric acid and NaOH (analytical reagent, Probus). The OPA-NAC reagent was stored at 4° C.

Barnstead nanopure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph were vacuum-filtered through 0.45 μm and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA).

A Hewlett-Packard (Palo Alto, CA, USA) 8452A diode-array spectrophotometer and a Perkin-Elmer LS50 fluorimeter (Beaconsfield, Buckinghamshire, England) were used.

The chromatographic system included a modular HPLC unit consisting of isocratic pump, automatic injector, UV-visible detector (Hewlett-Packard, Palo Alto, CA, USA, Model 1050), and integrator (Hewlett-Packard, Model 3396A). Data acquisition was performed through the PEAK-96 software (Hewlett Packard, Avondale, PA, USA). The solutions were injected into the chromatograph through a Rheodyne valve (Cotati, CA, USA) with a 20 μl loop. The detection was performed at 336 nm. A Spherisorb ODS-2 analytical C_{18} column (5 μm particle size, 120 x 4.6 mm I.D.) was used, together with a C_{18} precolumn (35 x 4.6 mm I.D.) of similar characteristics (Scharlau, Barcelona, Spain) to saturate the mobile phase with silica. The dead volume was determined by injection of water. The mobile phase flow-rate was 1 ml/min.

The derivatization was performed by mixing an aliquot of the amino acid solution with the OPA-NAC reagent in a 10 ml vial, being the OPA-NAC:amino acid molar ratio ≥ 10. Complete formation of the isoindoles and good reproducibility were achieved by injecting the solutions into the chromatograph one minute after mixing the reagents. The concentration of OPA should not be larger than 10^{-3} M. A more concentrated reagent produced a variation in the retention times for the derivatives of alanine, arginine, cysteine, lysine, methionine and valine. The larger instability of the amino acid isoindoles at an increasing OPA concentration is known.

Pills, capsules and powder were dissolved in 0.05 M SDS, by immersion in an ultrasonic bath. An adequate volume of the drops was taken and diluted with 0.05 M SDS. Other dilutions were made with 0.05 M SDS.

RESULTS

Optimization of experimental conditions

The OPA-NAC derivatives of amino acids showed maximum absorption at 336 nm in a micellar medium at pH 9.5, the molar absorptivities being similar to those obtained in a solution in the absence of surfactant ($\epsilon \approx 6800$) (3). Maximum absorption shifted to ≈ 330 nm in acidic medium. At low pH the emission intensity of the derivatives also shifted to lower wavelengths, from 440 nm to 390 nm, and the emission intensity decreased appreciably. A basic medium is required to form the isoindoles, thus these spectral changes may suggest the formation of other compounds in acidic medium.

The pH of the mobile phase is an important parameter to be controlled when ionizable solutes are eluted in a reversed-phase system with a micellar mobile phase of an ionic surfactant. Therefore, the influence of the pH of the eluent on retention was first studied. It was observed that at pH > 4 the amino acid derivatives were eluted at the beginning of the chromatogram. The variation of the capacity factors with pH is plotted in Figure 1 for some amino acid derivatives. Sigmoidal curves were obtained for all amino acids in the 3-4.5 range. The signal increase corresponds to the protonation of the free carboxylate group of the derivatives. At a higher pH the retention is reduced, due to the increase in the concentration of the anionic form, that is repelled by the negatively charged heads of the surfactant monomers adsorbed on the stationary phase.

The presence of micelles originates a modification in the acid-base properties of solutes, giving rise to shifts in the protonation constants (6,16,17). The extent of the modification depends on the properties of solutes and micelles. The mathematical treatment of the capacity factors (k') vs pH data, at a constant concentration of SDS, allows the determination of the protonation constants (log K) in the micellar medium. Equation [1] was used:

$$\log K = pH + \log \frac{k'_0 - k'}{k' - k'_1} \qquad [1]$$

where k'_1 and k'_0 are the capacity factors of the protonated and unprotonated species, obtained at pH 3 and >4, respectively. Table 1 indicates the protonation constants for a 0.05 M SDS solution. The values of log K were found in the 2.8-4.0 range, whereas the log K for the carboxylate group of free amino acids in a non-micellar medium is in the 1.7-2.7 range, that is, one logarithmic unit lower. In a previous study on the formation and spectrophotometric behaviour of a phthalimidine of

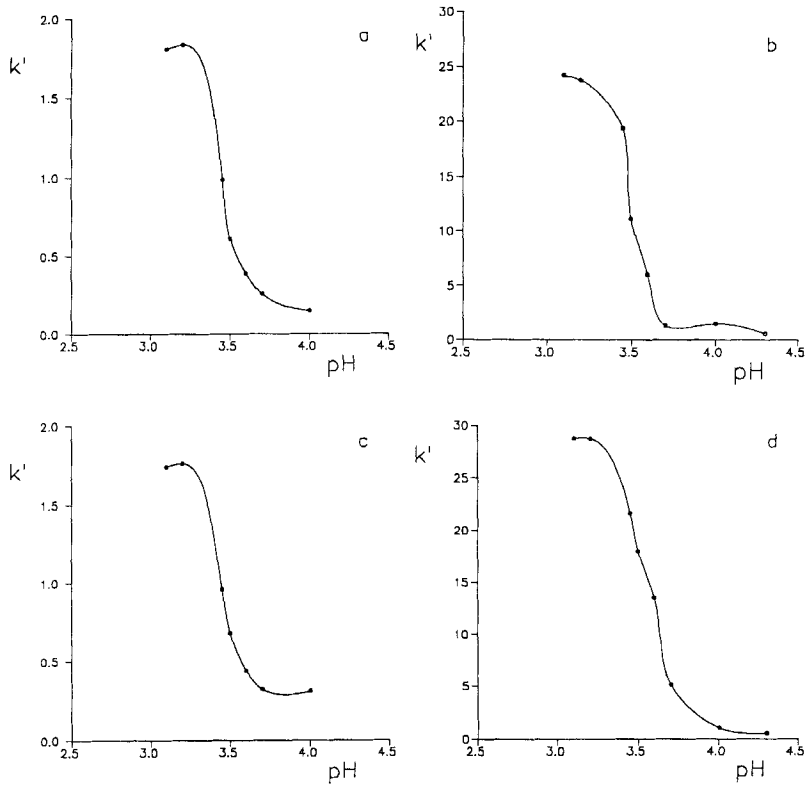

Figure 1. Dependence of the capacity factors on pH with a 0.05 M SDS mobile phase for the OPA-NAC derivatives of: a) aspartic acid; b) phenylalanine; c) glutamine; d) isoleucine.

cystine, obtained by reaction of this amino acid with OPA in a non-micellar basic medium, log K = 3.7 was found for the protonation of the carboxylate group (18).

It was observed that maximum resolution and adequate retention times of the chromatographic peaks of the amino acids was achieved at pH 3. Therefore, the composition of the mobile phase was optimized at this pH. The influence of the concentration of SDS on the retention of the

Table 1. Protonation constants (log K) of the carboxylate group of amino acids

Amino acid	log K	
	Free amino acid[a]	OPA-NAC derivatives[b]
Aspartic acid	1.88	3.1 ± 0.3
Alanine	2.34	3.3 ± 0.2
Arginine	2.17	4.02 ± 0.15
Asparagine	2.02	3.2 ± 0.8
Cysteine	1.71	3.39 ± 0.15
Phenylalanine	1.83	3.5 ± 0.5
Glycine	2.34	3.1 ± 0.2
Glutamine	2.17	3.2 ± 0.2
Histidine	1.82	2.9 ± 0.3
Isoleucine	2.36	3.7 ± 0.2
Leucine	2.36	3.7 ± 0.4
Lysine	2.18	3.29 ± 0.16
Methionine	2.28	2.8 ± 0.5
Serine	2.21	3.1 ± 0.7
Threonine	2.71	3.2 ± 0.4
Tryptophan	2.38	3.2 ± 0.4
Valine	2.32	3.4 ± 0.2

[a] Protonation constants for free amino acids in non-micellar aqueous solution (1)

[b] Protonation constants for OPA-NAC-amino acid derivatives in a 0.05 M SDS solution, obtained in this work.

OPA-NAC derivatives was studied in the 0.05-0.15 M range. The k' values were between 1.9-2.0 (serine and asparagine), and 30.4 (isoleucine) for 0.05 M SDS, and between 1.7 (asparagine) and 13.5 (lysine) for 0.15 M SDS. The amino acid derivatives behaved as binding solutes in a purely micellar mobile phase (without alcohol), decreasing their retention at increasing concentrations of SDS, except the aspartic acid and glutamine derivatives, which presented a non-binding behaviour. The retention of these solutes was almost constant in the 0.05-0.15 M SDS concentration range.

A short-chain alcohol (methanol, propanol and pentanol) was added to the micellar eluent to improve the efficiency of the chromatographic peaks and to achieve adequate retentions. Propanol was selected as the most appropriate. The k' values in a 0.05 M SDS/3% (v/v percentage) propanol mobile phase were between 0.7 (serine and asparagine), and 16.0 (isoleucine).

Analysis of pharmaceutical formulations

A 0.05 M SDS/3% propanol eluent at pH 3 was used to analyze five pharmaceutical formulations found in the spanish market, which contain glycine, lysine, methionine and threonine, together with a number of other components (Table 2 and Figure 2). The formulations were Polivitaendil minerales (vitaminic preparation), Pantobamín and Tres-orix Forte (appetite stimulant), Vincogelatín soluble (dermatological preparation), and Okal, where glycine is present to improve the assimilation and tolerance of acetylsalicylic acid.

The calibration plots, obtained by performing the derivatization of the amino acids by triplicate at five concentrations of each amino acid, were linear in the $4x10^{-5}$ M to $5x10^{-4}$ M range, with correlation coefficients $r > 0.999$. The reproducibility was evaluated from series of

Table 2. Determination of amino acids in pharmaceutical formulations. Contents declared by the manufacturers, found values and reproducibility

Formulation	Composition	Found (mg)	CV (%)
Okal (pills, Puerto Galiano, Madrid)	Glycine (100 mg), acetylsalicylic acid (500 mg), caffeine (30 mg), excipient	Glycine (102.9)	0.8
Pantobamín (drops, Medix, Madrid)	per ml: L-Lysine[a] (62.5 mg), L-threonine (25 mg), cyproheptadine clorhydrate (0.5 mg), L-carnosine (75 mg), thiamine nitrate (5 mg), pyridoxine clorhydrate (2.5 mg), hydroxocobalamine acetate (2.5 μg), dexpanthenol (1.25 mg), riboflavine sodium phosphate (1.25 mg), excipient	L-Lysine (56) L-Threonine (26.3)	0.9 0.8

Polivitaendil minerales (pills, Wasserman, Barcelona)	Glycine (50 mg), retinol (5000 U.I.), ergocalciferol (1000 U.I.), thiamine (10 mg), riboflavine (5 mg), pyridoxine (2.5 mg), cyanocobalamine (5 μg), ascorbic acid (125 mg), vitamin H (50 μg), nicotinamide (10 mg), calcium pantothenate (8 mg), phosphorus (17 mg), calcium (23 mg), iron (10 mg), magnesium (5 mg), manganese (2 mg), copper (1 mg), zinc (2 mg), molybdenum (0.5 mg), fluorine (0.05 mg), iodine (0.1 mg), adenosine triphosphoric acid (3 mg), saccharose (365 mg), excipient	Glycine (47.5)	0.2
Tres-orix Forte (capsules, Prodes, Barcelona)	Lysine[b] (150 mg), cyproheptadine orotate (1.5 mg), carnitine (150 mg), cobamamide (1000 μg), excipient	Lysine (147.5)	0.4
Vincogelatin soluble (powder, Reig Jofré, Barcelona)	Methionine (500 mg), gelatine (6 g), L-cystine (10 mg), calcium pantothenate (100 mg), vitamin B_6 (40 mg), zinc sulphate (60 mg)	Methionine (515)	0.5

[a] The height of the peak at 8 min was measured.

[b] The sum of the heights of the peaks at 3 and 8 min was measured.

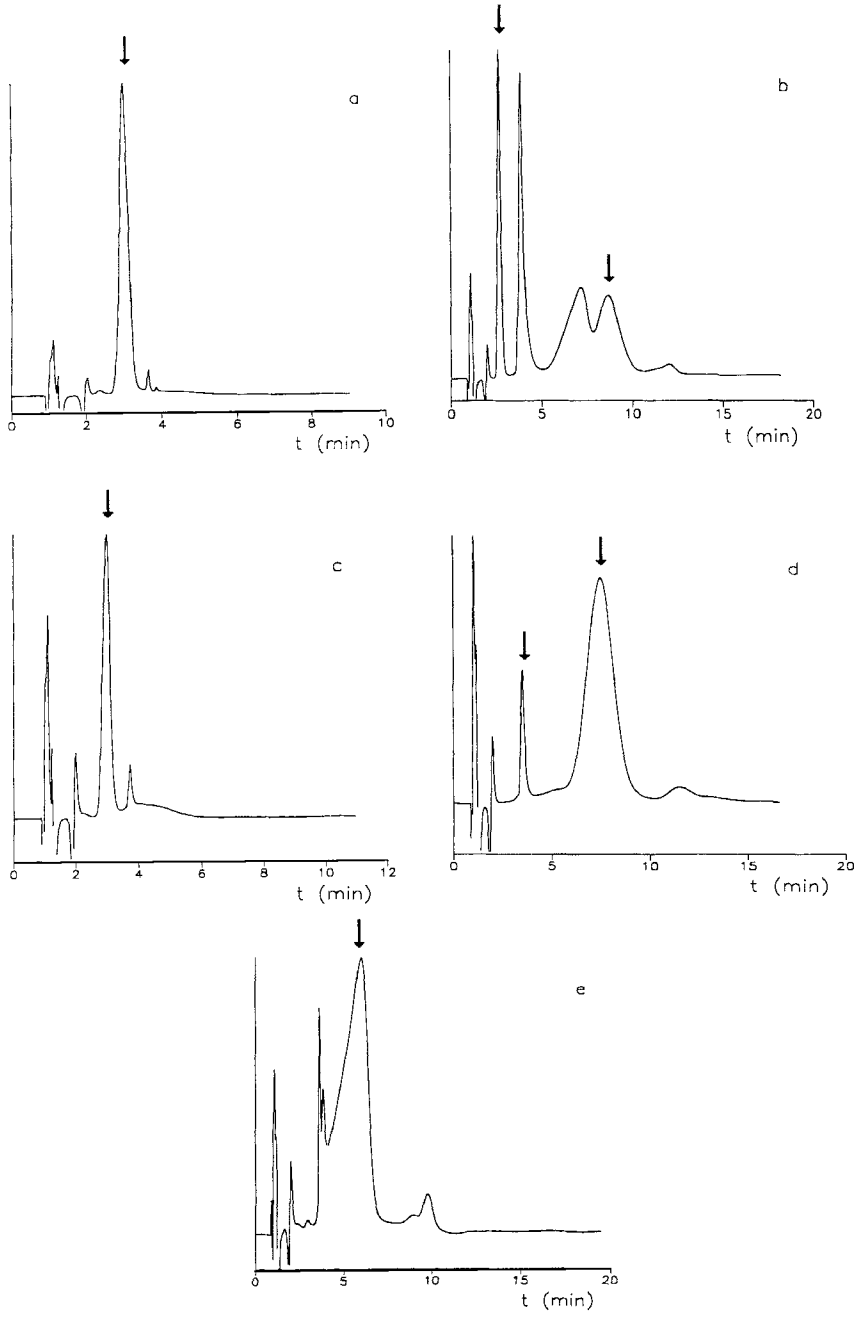

Figure 2. Chromatograms of the formulations: a) Okal (glycine); b) Pantobamín (threonine, 2.5 min, and lysine, 8 min); c) Polivitaendil minerales (glycine); d) Tres-orix Forte (lysine, 3 min and 8 min); e) Vincogelatín soluble (methionine).

five aliquots of the amino acids, which were independently derivatized. The coefficients of variation were lower than 1.0% at a $1x10^{-4}$ M concentration level, and between 0.6 and 2.2% for $1x10^{-6}$ M. The limits of detection were between $1.3x10^{-7}$ M and $6.5x10^{-7}$ M (3s criterium, n = 6).

The amino acid contents were obtained by taking five aliquots of the dissolved formulations, which were derivatized and one minute later injected into the chromatograph. The results were reproducible and the recoveries with respect to the values declared by the manufacturers were in the 90-105% range.

Lysine gave two peaks in the chromatograms at 3 min and 8 min, which indicated the formation of two different derivatives. The determination of lysine in the formulations where this amino acid was found, required the construction of two different calibration curves. In the analysis of Pantobamin, the height of the peak at a higher retention, close to 8 min was measured, due to the presence of other compounds that eluated at retention times close to the peak at 3 min. For Tres-orix Forte, the sum of the heights of the two peaks of lysine gave better results.

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