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HPLC-fluorescence determination of amino acids in pharmaceuticals after pre-column derivatization with phanquinone☆

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Abstract

4,7-Phenanthroline-5,6-dione (phanquinone) was used as a fluorogenic labeling reagent in pre-column derivatization for the quality control of amino acids in pharmaceuticals. The amino acid adducts were efficiently separated by C12 RP high-performance liquid chromatography (HPLC) using a ternary mixture of triethylamine (TEA) phosphate buffer (pH 2.5, 0.05 M)–methanol–tetrahydrofuran (THF) as mobile phase by varying composition gradient elution and detected fluorometrically. The results obtained by the proposed method were compared statistically, by means of the Student's *t*-test and the variance ratio F-test, with those obtained by a rapid reference method, which involved *o*-phthaldialdehyde (OPA) as pre-column reagent; no significant difference was found. The stronger derivatization conditions (60 °C, pH 8, 60 min) required for the method with phanquinone are compensated by the major stability of derivatives and by the absence of fluorescent degradation products.
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1. Introduction

Several diseases are associated with defects in amino acid metabolism. The analysis of these compounds is reckoned to be one of the most important applications in the biomedical and pharmaceutical fields, but the determination is difficult owing to their poor detectability. High-performance liquid chromatography (HPLC) in conjunction with a pre- or post-column chemical derivatization constitutes an effective approach to overcome the problem.

The more common derivatizing fluorescent reagents reported for the purpose are fluorescein isothiocyanate [1,2], 4-(2′-phthalimidyl) benzoyl chloride [2,3], fluorescamine [1–3], nynhydrin [1–3], dansyl chloride (Dns-Cl) [1–4], *o*-phthaldialdehyde (OPA) [1–8], 9-fluorenylmethyl chloroformate (FMOC) [1–4,9,10], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (6-AQC) [2–4,11], 4-fluoro-7-nitro-2,1,3-benzoxadiazole

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(NBD-F) [1,2,4,12,13]. OPA is the most reported post-column reagent, but it is also used for pre-column derivatization procedure despite the instability of the reaction products [1,4]. OPA, in the presence of an alkylthiol, has to a considerable extent replaced ninhydrin and it is selective reagent reacting only with primary amino groups [1]. Fully automated instruments for the post-column derivatization are available from different sources, which reduces manual labour and variability in the results [3]. Ion-exchange or ion-pair reversed-phase chromatography have been used with adequate sensitivity. However, to improve the sensitivity further and achieve short analysis time, pre-column derivatization procedures are preferred. The pre-column derivatization also offers the advantage of increasing the hydrophobicity of the analytes sufficiently to retain on the reversed-phase stationary phase.

Previous studies showed 4,7-phenanthroline-5,6-dione (phanquinone) [14,15], molecule devoid of a significant native fluorescence, to be a suitable fluorogenic reagent for pre-chromatographic derivatization of amino acids and was found to react selectively with the primary amino function. The present study was therefore aimed at verifying the applicability of phanquinone to the analysis of amino acids, such as D,L-*p*-Ser, in dosage forms. The LC separations were performed on a Phenomenex Synergi^{TM} MAX-RP column. The performance of the method was evaluated with respect to linearity, accuracy, precision and detection limits. In addition, results obtained by pre-column analysis using OPA reagent were included, as a reference method and evaluated critically.

2. Experimental

2.1. Materials

Individual crystalline samples of amino acids: D,L-phosphoserine (D,L-*p*-Ser) glycine (Gly), L-alanine (L-Ala), L-valine (L-Val), L-isoleucine (L-Ile), L-leucine (L-Leu), L-phenylalanine (L-Phe), L-aspartic acid (L-Asp), L-glutamic acid (L-Glu) were purchased from Fluka (Buchs, Switzerland). L-Serine (L-Ser) and quinidine (used as internal standard, IS) were obtained from Sigma (St. Louis, MO). L-Glutamine (L-Gln) and TLC plates RP-18 F_{254s} (20 cm × 20 cm) were purchased from Merck (Darmstadt, Germany). Methanol and tetrahydrofuran (THF) for chromatography were of HPLC grade from Romil (Delchimica Scientific Glassware, Naples, Italy). Triethylamine (TEA) was purchased from Carlo Erba (Milano, Italy). Deionized, double distilled water was used for the mobile phase preparation. Potassium dihydrogen phosphate and all the other chemicals were of analytical reagent grade. *o*-Phthaldialdehyde was purchased from Fluka (Buchs, Switzerland). 4,7-Phenanthroline-5,6-dione (phanquinone) was prepared and purified as previously described [15] and compared with those kindly obtained from Novartis Pharma AG (Basel, Switzerland). Galenicals were purchased from Toschi Chemist Shop (Bologna, Italy).

2.2. Solutions

The reagent phanquinone solution (about 2.4 mg/ml) was prepared in a mixture of water–methanol (50:50, v/v) and was found to be stable for at least 2 weeks at ambient temperature. The reagent OPA solution (about 7.8 mg/ml) was prepared by dissolving 100 mg of compound in 1.5 ml of methanol; to this solution 100 μl of 2-mercaptoethanol and 11.2 ml of sodium borate (pH 9.5; 0.4 M) were then added. The mixture was stored in the dark at 4 °C and was allowed to stand for 24 h before use. An aliquot of 10 μl of 2-mercaptoethanol was added every 2 days to help to maintain the reagent strength. It remains stable for about 2 weeks [16,17]. Standard solutions of the described amino acids were prepared in water (concentration under calibration graphs). TEA phosphate buffer (pH 2.5; 0.05 M) was prepared adding orthophosphoric acid to an aqueous TEA solution up to the desired pH value. The internal standard (IS) solution (0.4 mg/ml), used for the method with phanquinone, and the IS solution (7.8 μg/ml), used for the procedure with OPA, were obtained by dissolving the quinidine in TEA phosphate buffer (pH 2.5, 0.05 M) in presence of about 40 and 18% methanol, respectively. Borate buffer (pH 9.5, 0.4 M) solution was prepared by adjusting the pH of a boric acid solution to 9.5 with 10 M sodium hydroxide [2]. Borate buffer (pH 9, 0.1 M), acetate buffer (pH 5, 0.1 M) and phosphate buffer (pH 8, 0.1 M) solutions were prepared according to the standard methods [18].

2.3. Equipment

The liquid chromatograph consisted of a Jasco model LG-980-02S ternary gradient unit, a Jasco PU-1580 pump and a Jasco FP-920 fluorescence detector (Tokyo, Japan) connected to a personal computer AcerView 34TL. The integration program Borwin (Jasco, Tokyo, Japan) was used. The solvents were degassed on line with a degasser model Gastorr 153 S.A.S Corporation (Tokyo, Japan). Manual injections were carried out using a Rheodyne model 7125 injector with 20 μl sample loop. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication. IR spectra were recorded on a Perkin-Elmer FT-IR spectrophotometer spectrum 2000 (Boston, MA, USA) by using KBr pellets. UV spectra were recorded on a Hewlett-Packard 8453 spectrophotometer (Waldbronn, Germany). ^{1}H NMR spectra were recorded on a Varian Gemini spectrometer (Palo Alto, CA, USA) at 300 MHz in DMSO.

2.4. Derivatization procedure

The derivatization reaction was carried out using phanquinone reagent (method A) and by a reference method (method B) which requires OPA reagent [16,17].

2.4.1. Method A

A 25 μl aliquot of the amino acid solution was treated with 100 μl of phosphate buffer (pH 8, 0.1 M) and 0.5 ml of the reagent solution were added. The reaction was carried out at 60 °C for 60 min under magnetic stirring in a micro reaction vessel (3.0 ml); then, to 200 μl of the reaction mixture the IS solution (0.4 mg/ml), equivalent to 150 μl for the method development and 50 μl for the pharmaceutical analysis, was added. After dilution to 10 ml with a mixture of TEA phosphate buffer (pH 2.5, 0.05 M)–methanol, 82:18 (v/v), 20 μl aliquots of the resulting clear solution were injected into the chromatograph.

When the reaction was performed on a preparative scale, a previous method [14,15] was used and the residue was purified by reversed-phase TLC on silica RP-18 F_{254s} using acetonitrile–water (75:25, v/v, for L-Ser iminoquinol or 60:40 (v/v), for D,L-*p*-Ser iminoquinol). To monitor the course of the reaction, thin-layer chromatography (TLC) on silica gel F_{254} was used; the solvent mixture was ethyl acetate–methanol–TEA 6:3:0.7 (v/v/v), for L-Ser iminoquinol and 2:8:0.2 (v/v/v), for D,L-*p*-Ser iminoquinol. The new adducts were characterized as follows.

2.4.1.1. 3-Hydroxy-2-[(6-hydroxy[4,7]phenanthrolin-5-yl)imino]propanoic acid.

mp 139–141 °C.

IR (cm^{-1}): 3410 (OH), 1735 (C = O), 1635 (C = N).

UV (methanol) λ_{max} = 403 nm (ε = 7300).

^{1}H NMR (DMSO, 300 MHz): δ (ppm) 4.85 (s, 2H, CH_2), 7.44–7.50 (m, 1H, ArH), 7.81–7.86 (dd, 1H, ArH), 8.61–8.65 (d, 1H, ArH), 8.88–8.91 (dd, 1H, ArH), 8.95–8.99 (dd, 1H, ArH), 9.04–9.08 (dd, 1H, ArH).

2.4.1.2. 2-[(6-Hydroxy[4,7]phenanthrolin-5-yl)-imino]-3-(phosphonooxy)propanoic acid.

mp > 290 °C (dec).

IR (cm^{-1}): 3417 (OH), 1723 (C = O), 1635 (C = N), 1196 (P = O), 1041 (P–O–C), 1000 (P–OH).

UV (methanol) λ_{max} = 402 nm (ε = 6100).

^{1}H NMR (DMSO, 300 MHz): δ (ppm) 5.40 (d, 2H, CH_2), 7.62–7.68 (m, 1H, ArH), 8.74–8.78 (m, 1H, ArH), 8.96–9.01 (dd, 2H, ArH), 9.19–9.26 (dd, 2H, ArH).

2.4.2. Method B

A 50 μl aliquot of the amino acid solution was treated with 50 μl of sodium borate buffer (pH 9.5, 0.4 M) and 40 μl of the reagent OPA solution were added. The reaction was carried out at ambient temperature in a tube (1.0 ml) for 1 min covering 15 s ultrasonication, then 200 μl of the IS solution (7.8 μg/ml) was added. Twenty microliters aliquot of the resulting clear solution was injected into the chromatograph.

2.5. Chromatographic conditions

The routine LC separations were performed at 33 ± 2 °C on a Phenomenex Synergi^{TM} 4μ MAX-RP 80A (250 mm × 4.6 mm i.d.) stainless steel column, under gradient elution using a mobile phase consisting of a

Table 1
Data for calibration graphs ($n = 5$)

Compounds	Method[a]	Slope[b]	y-intercept[b]	Correlation coefficient	Concentration range (μg/ml)
D,L-*p*-Ser	A	0.015729	0.002249	0.9996	10.55–105.5
	B	0.010122	0.001466	0.9997	10.56–105.6
D,L-*p*-Ser[c]	A	0.015012	0.003329	0.9992	10.55–105.5
	B	0.009626	0.000951	0.9985	10.56–105.6
L-Gln	A	0.005463	0.001014	0.9999	9.99–149.8
	B	0.008081	0.00122	0.9998	6.284–150.8
L-Ser	A	0.013021	−0.000988	0.9996	6.225–99.60
	B	0.010001	−0.000896	0.9992	6.225–99.60
Gly	A	0.028868	0.002725	0.9997	6.225–99.60
	B	0.020631	0.001175	0.9999	6.225–99.60
L-Ala	A	0.012004	0.001488	0.9997	6.375–102.0
	B	0.019266	0.00185	1.0000	6.375–102.0
L-Val	A	0.036362	0.003692	1.0000	6.500–104.0
	B	0.031990	0.023896	0.9998	6.500–104.0
L-Leu	A	0.024136	0.001955	0.9997	7.060–105.9
	B	0.024574	0.002577	0.9999	6.344–101.5

[a] A: pre-column derivatization with phanquinone; B: pre-column derivatization with OPA.
[b] According to $y = ax + b$, where x = analyte concentration expressed as μg/ml and y = ratio of analyte peak-area to IS peak-area.
[c] D,L-*p*-Ser spiked in placebo (effervescent tablets).

mixture A:B, where A is TEA phosphate buffer (pH 2.5, 0.05 M) and B is methanol–THF 98:2 (v/v) at a flow-rate of 0.6 ml/min. The multi-linear line gradient profile was $t = 0$ min, 18% B; $t = 12$ min, 18% B; $t = 28$ min, 80% B; $t = 32$ min, 18% B (method A) and $t = 0$ min, 16% B; $t = 18$ min, 72% B; $t = 20$ min, 80% B; $t = 28$ min, 80% B; $t = 32$ min, 16% B (method B), respectively.

Fluorescence detection at $\lambda_{em} = 460$ nm with $\lambda_{ex} = 400$ nm (method A) and $\lambda_{em} = 455$ nm with $\lambda_{ex} = 340$ nm (method B) was used.

2.6. Chromatographic conditions

Individual standard solutions of amino acids were prepared in water (concentration ranges in Table 1). A 25 μl (method A) or 50 μl (method B) volume of amino acid standard solution was subjected to the appropriate described derivatization procedure. Triplicate injections for each standard solution were made and the peak-area ratio of analyte to IS was plotted against the corresponding amino acid concentration to obtain the calibration graphs.

2.7. Analysis of pharmaceutical formulations

2.7.1. Sample preparation

2.7.1.1. Effervescent tablets. Twenty tablets were powered and an amount equivalent to about 1.05 mg of D,L-*p*-Ser was treated with 19 ml of water by ultrasonication for 3 min at ambient temperature and then diluted to 20 ml with water. Finally, the sample was filtered with 0.45 μm nylon filters to obtain a clarified solution.

2.7.1.2. Extemporaneous solution. The reservoir-plug power, equivalent to about 40 mg of D,L-*p*-Ser and 70 mg of L-Gln was mixed, stirring till to homogeneity, with the appropriate solution of the bottle and was treated with 500 ml of water by ultrasonication for 3 min at ambient temperature; then, an aliquot of 4 ml was diluted to 10 ml with water.

2.7.1.3. Capsules. The contents of five capsules were powdered and an amount, equivalent to about 2.5 mg of Gly, L-Gln, L-Ser, L-Ala, L-Val, L-Ile, L-Leu, respectively, was treated with 100 ml of water by ultrasonication for 5 min at ambient temperature.

2.7.2. Assay procedure

A 25 μl (method A) or 50 μl (method B) aliquot of the sample solution was subjected to the suitable derivatization procedure and the drug content in each sample was determined by comparison with an appropriate standard solution.

3. Results and discussion

3.1. Derivatization reaction

The first objective of this study was to apply 4,7-phenanthroline-5,6-dione (phanquinone) to the analysis of D,L-*p*-Ser, a phosphoamino acid, and to optimize the derivatization system. At the same time, an additional aim was the comparison of the developed method with a reference procedure using the well-known OPA reagent. A much more general application of OPA showed that nearly all amino acids can form fluorescent condensation products in the presence of 2-mercaptoethanol [1]. The derivatization reaction with OPA, used as pre-column reagent, was carried out according to described methods [16,17].

To achieve optimum derivatization conditions of D,L-*p*-Ser with phanquinone, the effect of temperature, pH, reagent concentration was investigated.

At first, as continuation of previous studies developed at 68 °C in water–methanol 60:40 (v/v) [14,15], the course of the reaction under different pH values was evaluated (Fig. 1). At pH 8, the derivatization reaction was found to be complete after 30 min. Different pH values did not offer advantages and reduced responses were observed. Subsequently, in order to obtain mild reaction conditions, the effect of the temperature on the reaction course at pH 8 was also considered (Fig. 2). At 60 °C, the reaction was found to be complete after 60 min and only in stronger conditions (68°C) the observed derivatization profile reached a maximum more rapidly (30 min). Above 68 °C temperatures were not considered in order to avoid the methanol evaporation from the reaction mixture. Under the both described conditions (60 °C within 60 min or 68 °C within 30 min), the yield of the iminoquinol increased to reach a plateau at a molar ratio of reagent to amino acid of about 25 and a further reagent excess did not interfere. However, in general, the analysis was carried out at 60 °C for a better reproducibility.

The derivatization reaction for D,L-*p*-Ser and L-Ser was found to be essentially quantitative by comparison with an authentic specimen of synthetized iminoquinol. The derivatization reaction with phanquinone for these amino acids was performed on preparative scale and the analytical data (IR, ^{1}H NMR) were consistent with the expected structure according to other iminoquinols [14,15].

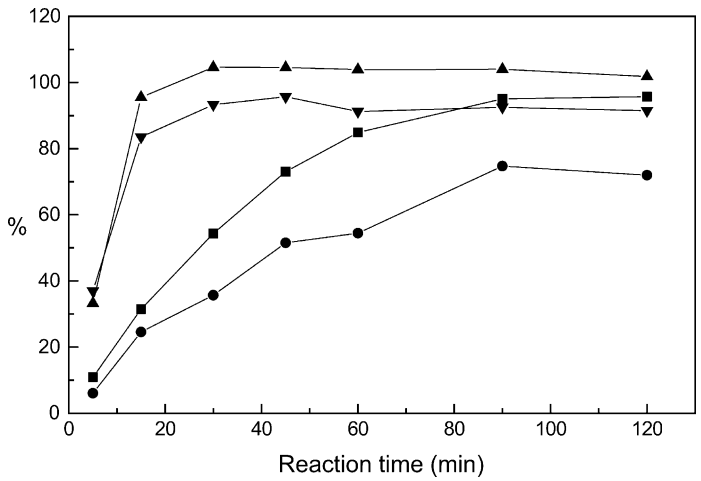

Fig. 1. Influence of the pH on the derivatization reaction of D,L-*p*-Ser with phanquinone at 68 °C: water without catalysis (●), acetate buffer (pH 5, 0.1 M) (■), phosphate buffer (pH 8, 0.1 M) (▲), borate buffer (pH 9, 0.1 M) (▼); (%) percent yield of the reaction.

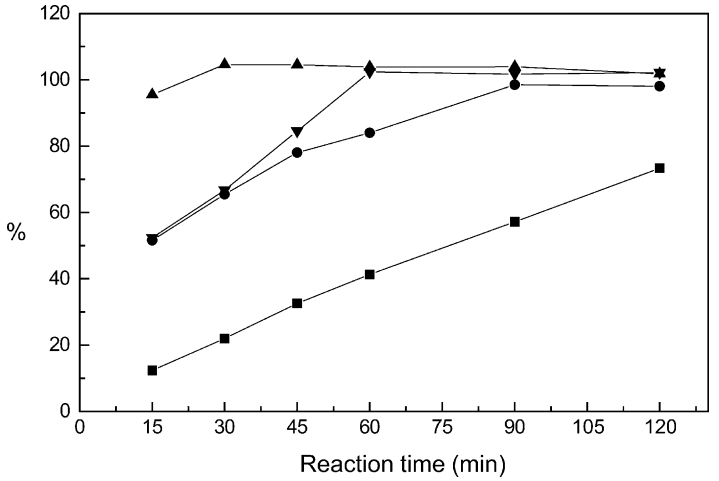

Fig. 2. Influence of the temperature on the derivatization reaction of D,L-*p*-Ser with phanquinone at pH 8: 35 °C (■), 50 °C (●), 60 °C (▼), 68 °C (▲); (%) percent yield of the reaction.

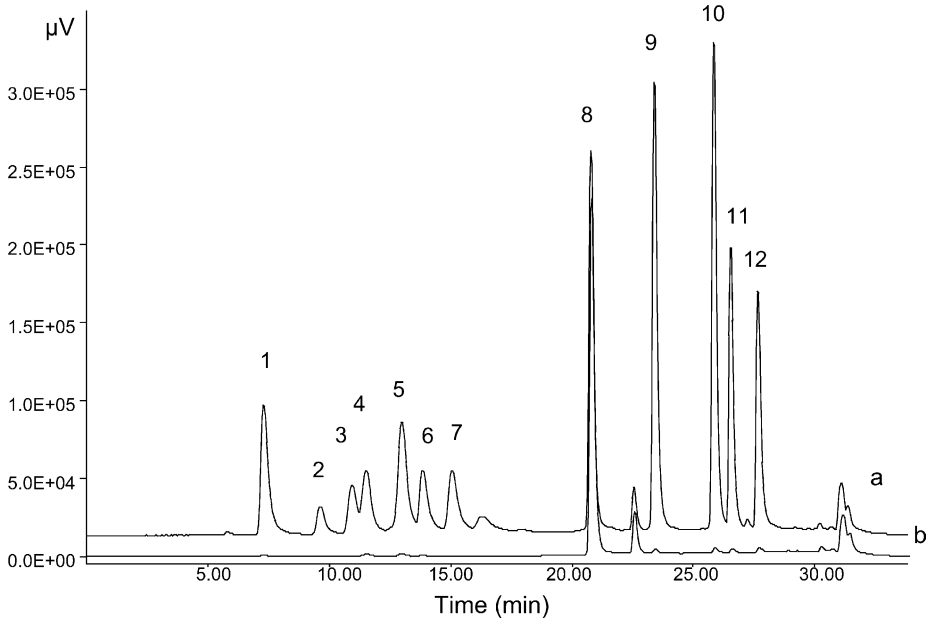

Fig. 3. Representative LC chromatogram at 33 ± 2 °C of: (a) amino acids derivatized with phanquinone; (b) phanquinone under reaction conditions. Peaks: 1 = D,L-*p*-Ser; 2 = L-Asp; 3 = L-Gln; 4 = L-Ser; 5 = Gly; 6 = L-Ala; 7 = L-Glu; 8 = quinidine (IS); 9 = L-Val; 10 = L-Ile; 11 = L-Leu; 12 = L-Phe. LC conditions: Phenomenex Synergi^{TM} 4μ MAX-RP 80A (250 mm × 4.6 mm i.d.) column with a mixture of A:B where A was TEA phosphate buffer (pH 2.5, 0.05 M) and B is methanol–THF, 98:2 (v/v), under the following gradient elution conditions as mobile phase: $t = 0$ min, 18% B; $t = 12$ min, 18% B; $t = 28$ min, 80% B; $t = 32$ min, 18% B. Flow-rate: 0.6 ml/min. Fluorescence detection: $\lambda_{ex} = 400$ nm; $\lambda_{em} = 460$ nm.

3.2. Chromatography

As a result of previous experience [14,15] RP chromatography was chosen and ternary mixtures of TEA phosphate buffer–methanol–THF were used to achieve adequate separation of derivatized amino acids. Columns (250 mm × 3.2 mm i.d. and 250 mm × 4.6 mm i.d.) packed with two different RP materials (Prodigy 5μ and Synergi 4μ, respectively) were used. Prodigy™ columns, made with high purity HPLC silica phases, represent a major advance in the analysis of basic, acidic and amphoteric compounds, at a cost savings. The ultra-low metal content silica (99.999% pure) and an inert bonded surface reduce the need for expensive and labor-intensive mobile phase modifiers or ion-pairing reagents. Synergi™ MAX-RP is a C12 bonded phase with TMS endcapping for method development of moderately polar or non-polar analytes, acids and bases, over a broad pH range. One of its benefits is that the 4μ, 80 Å silica gel gives higher efficiencies than 5μ columns and lower backpressures compared to 3μ high-performance C8 and C18 columns. The Synergi column proved to enable good peak separation in shorter time than the Prodigy column, but THF in the mobile phase was required for a better resolution of the amino acids. The separations were carried out under gradient conditions; isocratic conditions were unable to provide adequate resolution of complex samples in a reasonable analysis time. A representative separation of amino acids derivatized with phanquinone is reported in Fig. 3. As it can be seen, the non-fluorescent excess reagent is not detected and an easy chromatogram is obtained. On the other hand, the chromatogram obtained using method B (Fig. 4) shows that the peaks of the amino acids derivatized with OPA are fully resolved and separated from the reagent degradation products and from IS. The detection limit (signal-to-noise ratio = 3) was about 0.01 pmol for D,L-*p*-Ser and 0.05 pmol for the other amino acids (method A), and about 2.5 pmol for D,L-*p*-Ser and 0.1 pmol for the other amino acids (method B).

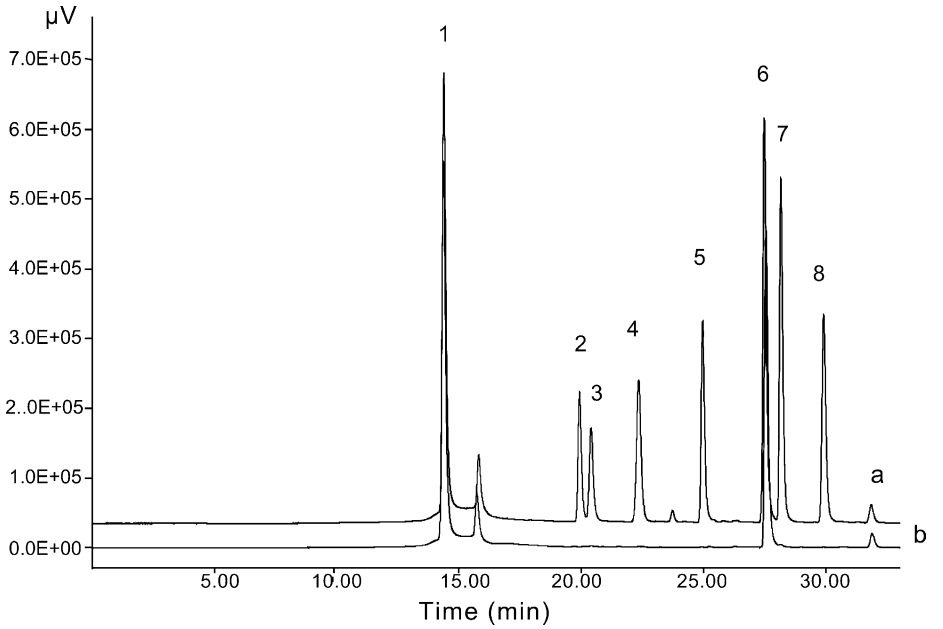

Fig. 4. LC chromatogram of: (a) a amino acid sample (capsules) after derivatization with OPA; (b) OPA under reaction conditions. Peaks: 1 = quinidine (IS); 2 = L-Gln; 3 = L-Ser; 4 = Gly; 5 = L-Ala; 6 = reagent; 7 = L-Val; 8 = L-Leu. Column and mobile phase as described in Fig. 3 with the following gradient profile: $t = 0$ min, 16% B; $t = 18$ min, 72% B; $t = 20$ min, 80% B; $t = 28$ min, 80% B; $t = 32$ min, 16% B. Fluorescence detection: $\lambda_{ex} = 340$ nm; $\lambda_{em} = 455$ nm.

Table 2
Results for the LC determination of amino acids in commercial pharmaceutical formulations

Formulation	Amino acid	% Found[a] (% R.S.D.)		t[b]	F[c]
		Method A[d]	Method B[e]		
Effervescent tablets[f]	D,L-*p*-Ser	96.57 (1.69)	97.05 (1.21)	0.53	1.94
Extemporaneous solution[g]	D,L-*p*-Ser	100.07 (2.64)	97.54 (3.09)	1.41	1.30
	L-Gln	96.44 (1.98)	97.49 (1.42)	0.99	1.90
Capsules[h]	L-Gln	101.65 (2.34)	98.65 (2.76)	1.86	1.31
	L-Ser	99.04 (2.00)	98.32 (2.32)	0.53	1.32
	Gly	96.73 (1.94)	99.41 (2.48)	1.92	1.73
	L-Ala	104.05 (2.52)	101.59 (2.22)	1.59	1.35
	L-Val	97.64 (2.06)	98.91 (1.73)	1.08	1.38
	L-Leu	96.28 (1.68)	97.11 (1.94)	0.75	1.36

[a] Mean of five determinations and expressed as a percentage of the claimed content.
[b] Calculated t-value; tabulated t-value = 2.306.
[c] Calculated F-value; tabulated F-value = 6.39.
[d] Pre-column derivatization with phanquinone.
[e] Pre-column derivatization with OPA.
[f] Inactive ingredients: citric acid anhydrous, magnesium glycerophosphate, sodium bicarbonate, sorbitol, sodium carbonate, sodium phosphate monobasic, vitamin C, 50% vitamin E, flavor, saccharin sodium, zinc sulphate heptahydrate, vitamin PP, calcium pantothenate, manganese sulphate monohydrate, vitamin B_6, riboflavin 5′-(dihydrogen phosphate) monosodium salt, 0.1% vitamin B_{12}, vitamin B_1, and folic acid.
[g] Inactive ingredients: cyanocobalamin, mannitol, methyl *p*-hydroxybenzoate sodium, propyl *p*-hydroxybenzoate sodium, sorbitol, citric acid, sour black cherry flavor, lemon flavor, and purified water.
[h] Inactive ingredients (galenicals): starch, magnesium stearate, talc, and silica.

3.3. Analysis of amino acids pharmaceutical formulations

Both described methods were applied to the determination of amino acids in commercial dosage forms (effervescent tablets, extemporaneous solution and capsules). Under the described conditions, linear calibration graphs were obtained by plotting the peak area ratio of derivatized amino acid to IS against the analyte concentration (μg/ml) and the calibration parameters are shown in Table 1. Calibration graphs, with slopes and intercepts not significantly different, were obtained for D,L-*p*-Ser spiked in a placebo, corresponding to the effervescent tablet components (Table 1); these data confirmed that the very complex matrix did not interfere with the analyses. The within-run precision (repeatability) of the methods was satisfactory as indicated by R.S.D. range (2.00–3.2 for the method A and 1.98–2.90 for the method B) obtained from replicated ($n = 8$) analyses (derivatization and LC separation) of a standard solution of amino acids (concentration for each amino acid: 0.28 μmol/ml). The obtained results (Table 2) were found in agreement with the claimed content for each analyte. The other ingredients of the formulations did not interfere with the analysis. As it can be seen, for each sample the results obtained using the method A were comparable with those of the method B. The Student's t-test values at a 95% confidence level for 8 degrees of freedom did not exceed the tabulated value of $t = 2.306$, indicating no significant difference between the described methods. The variance ratio F-test values calculated for $P = 0.05$ and $f_1 = f_2 = 4$ did not exceed the tabulated value of $F = 6.39$, again indicating that was no significant difference between the precision of two analytical procedure applied. Chromatograms obtained from commercial dosage forms are illustrated in Figs. 4 and 5. The accuracy of the methods was verified by analysing commercial samples fortified with known amounts (20 and 50% of the claimed drug content; quantitative recoveries were obtained in each instance (recovery = 98–101%; R.S.D. = 2.5–4.3%). Both proposed methods were also applied to the evaluation of the stability of

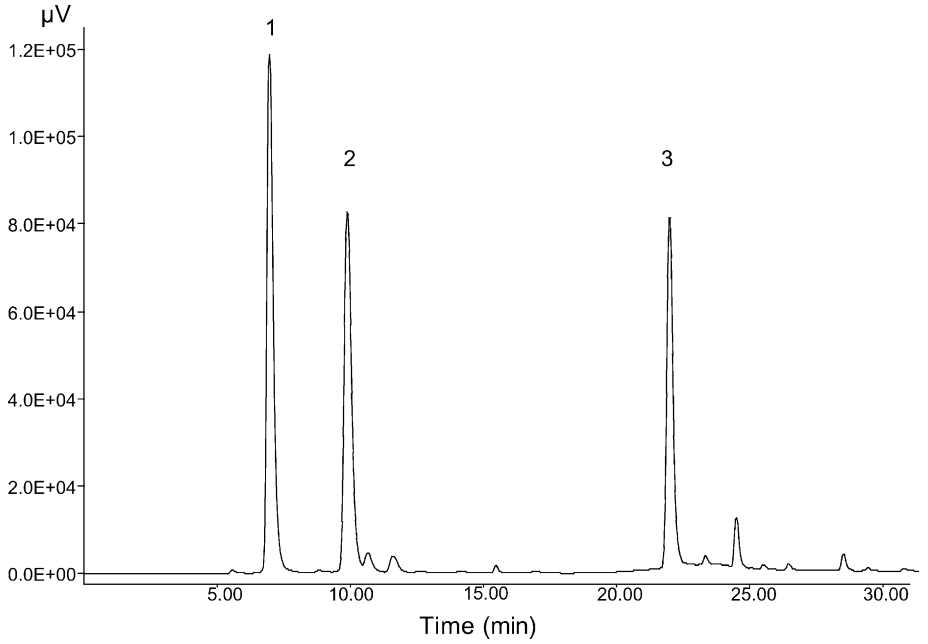

Fig. 5. LC chromatogram of a D,L-*p*-Ser and L-Gln sample (extemporaneous solution) after derivatization with phanquinone; Peaks: 1 = D,L-*p*-Ser; 2 = L-Gln; 3 = quinidine (IS); Chromatographic conditions and detection as in Fig. 3.

D,L-*p*-Ser from commercial preparations. The samples ($n = 8$) were stored under different conditions: 25 °C with 60% of relative humidity and 40 °C with 75% of relative humidity. The samples were analyzed after 6 months and triplicate analyses were performed for each sample. The results, expressed as a percentage of the claimed content, were found in the range of 93.50–99.30% (R.S.D. = 1.10–4.20%); no significant difference was found between the method A and the method B.

4. Conclusions

4,7-Phenanthroline has been confirmed to be a pre-column fluorogenic labeling reagent suitable for the quality control of amino acid dosage forms. The stronger derivatization conditions of the method which requires phanquinone are compensated by absence of degradation products, major stability and major fluorescence intensity for D,L-*p*-Ser adduct than the procedure which employs OPA. The proposed methods gave comparable and accurate results.

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