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Short communication

LC determination of leuprolide component amino acids in injectable solution by phanquinone pre-column derivatization labelling procedure

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Abstract

A sensitive LC method for the determination of leuprolide acetate component amino acids in injectable solution with fluorogenic pre-column derivatization has been developed. The derivatization reaction with phanquinone was optimised by a series of experiments. Histidine, arginine, serine, tryptophan, glutamic acid, tyrosine, methionine, isoleucine, leucine and phenylalanine were separated on a reversed-phase ODS column using as eluent a binary mixture of triethylammonium phosphate buffer–methanol, under gradient elution conditions. The derivatives were eluted in 30 min with good reproducibility. The hydrolysis reaction of the peptide was carried out at reflux with 12N hydrochloric acid for 2 h 30 min. The intra-day accuracy of the entire procedure (hydrolysis, derivatization, LC separation) ranged from 80.5 to 109.5% of the nominal concentration of leuprolide acetate and the precision (%R.S.D.) was less than 5.8%; the inter-day accuracy was in the range 81.5–107.2% and corresponding R.S.D. values were less than 4.6%. The detection limits (signal-to-noise ratio = 3) for the adducts are 30–800 fmol.

Keywords: Reversed-phase liquid chromatography; Phanquinone; Leuprolide acetate; Peptide hydrolysis; Fluorogenic pre-column derivatization

1. Introduction

Luteinizing hormone-releasing hormone (LH-RH, gonadorelin) is the blood-borne messenger between the hypothalamus and the anterior pituitary, which controls reproductive function. The therapeutic usefulness of LH-RH would be limited because of its short biological half-life and also because of rapid inactivation in vivo. The binding of 20 LH-RH analogues, including leuprolide, to the pituitary receptor has been investigated [1]. Results revealed high-affinity binding for agonist with strong in vivo activity. Subcutaneous injection of leuprolide, chemically defined as: *p*-Glu-L-His-L-Trp-L-Ser-L-Tyr-D-Leu-L-Leu-L-Arg-L-Pro-ethylamide, into immature male rats released 53 times more luteinizing hormone (LH) and 15 times more follicle-stimulating hormone (FSH) over a 6-h period than similar dose of LH-RH. Rat ovulation studies revealed leuprolide to be 50–80 times more active than LH-RH. Leuprolide presents also a higher resistance to enzymatic degradation than the natural hormone. It can thus be used for the treatment of a large number of diseases related with the regulation of sexual hormones, like masculine and feminine infertility, uterine myomas and prostatic and breast cancer.

There is a considerable literature on high-performance liquid chromatography (HPLC) of gonadorelin and its analogues including leuprolide, most of that concerning with the assay, identification or purification of a single peptide [1–4] and some LC methods for the analysis of leuprolide acetate in pharmaceuticals [5,6].

On the other hand, the functional components of leuprolide acetate and structural elucidation of this peptide were confirmed using the following methods [1]: hydrolysis of the peptide followed by quantitative analysis of amino acids residues by ion exchange chromatography; ionization by fast atom bombardment (FAB) and mass spectrometry; high-performance liquid chromatography (HPLC) following acid/alkaline exposure of the peptide and enzymatic hydroly-

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sis. In particular, an HPLC method was developed to analyze component amino acids in leuprolide acetate (except Pro) using *o*-phthaldialdehyde (OPA). OPA reacts selectively with primary amino groups in presence of a thiol compound as co-reagent to form fluorescent derivatives. No other papers appear to have dealt with this subject.

Amino acids and peptide analysis has long attracted considerable interest in biomedical research and pharmaceutical sciences. In recent years, as a result of its sensitivity, selectivity and high speed, HPLC has contributed substantially to the efficiency of amino acids and peptide analysis. Most amino acids have originally no intrinsic chromophores and fluorophores in their structures, while, because of their hydrophilicity, they afford a low affinity to a reversed-phase column. Pre- or post-column derivatization with fluorogenic reagents is commonly used to improve the detection limit of amino acids and proteins/peptides. Therefore, most studies have employed a pre-column derivatization with fluorogenic reagents followed by simultaneous determinations of derivatized amino acids by reversed-phase HPLC. The pre-column derivatization offers the advantage of increasing the hydrophobicity of the analytes sufficiently to retain on the reversed-phase stationary phase. The fluorogenic reagents frequently used, such as ninhydrin, fluorescamine, *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC), 1-dimethylamino-naphthalene-5-sulfonyl chloride (Dns-Cl), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), fluorescein isothiocyanate, acridone *N*-acetyl chloride (ARC-Cl), carbamazole-9-acetyl chloride (CRA-Cl) and carbazole-9-proprionyl chloride (CRP-Cl) are known to react mainly with primary and secondary amino groups [7–11].

Recent studies [12–14] have demonstrated that 4,7-phenanthroline-5,6-dione (phanquinone), compound devoid of a significant intrinsic fluorescence, can be used successfully as a pre-column derivatizing reagent. Phanquinone reacts with primary amino function forming highly fluorescent iminoquinols which can be analyzed with good selectively and sensitivity by reversed-phase HPLC.

In this report, the use of phanquinone as a pre-column derivatizing reagent for amino acids was further evaluated. The derivatization reaction was improved by a series of experiments. In addition, the applicability of the reagent to the component amino acid identification of leuprolide acetate, as well as to their determination, following acid hydrolysis, in commercially available pharmaceutical solution was investigated. To approach the hydrolysis procedure, the reaction conditions using hydrochloric acid on the tripeptide Met-Phe-Leu acetate was developed.

2. Experimental

2.1. Materials

Individual crystalline samples of amino acids: L-leucine (L-Leu), L-phenylalanine (L-Phe), L-*p*-glutamic acid (*p*-Glu), L-glutamic acid (L-Glu), L-serine (L-Ser), L-tryptophan (L-Trp), L-tyrosine (L-Tyr), L-histidine (L-His), L-arginine (L-Arg) and L-methionine (L-Met) were purchased from Fluka (Buchs, Switzerland). The pH indicator bromothymol blue and methanol for chromatography were from Carlo Erba (Milan, Italy). Met-Phe-Leu acetate, leuprolide acetate and triethylamine (TEA) were obtained from Sigma-Aldrich (St. Louis, MO). Deionized, double distilled water was used for the mobile phase preparation. All the other chemicals were of analytical reagent grade. 4,7-Phenanthroline-5,6-dione (phanquinone) and the L-Ile iminoquinol (used as internal standard, IS) were prepared and purified as previously described [12–14]. 2-Methoxy-4-nitroaniline, which was used for phanquinone synthesis [15,16], and TLC plates RP-18 F_{254s} (20 cm × 20 cm) for the purification of IS, were purchased from Merck (Darmstadt, Germany).

2.2. Solutions

The reagent phanquinone solution (about 11.4 mM) 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. Standard solutions of the described amino acids were prepared in water (concentration under calibration graphs). Triethylammonium phosphate buffer (pH 3; 0.05 M) was prepared adding orthophosphoric acid to an aqueous TEA solution up to the desired pH value. The internal standard (IS) solutions (13 μg/ml) were prepared in triethylammonium phosphate buffer (pH 3; 0.05 M)–methanol in the ratio 76:24 (v/v). Borate buffer (pH 9, 0.1 M), acetate buffer (pH 5; 0.1 M) and phosphate buffer (pH 8; 0.1 M and pH 7; 0.1 M) solutions were prepared according to the standard methods [17]. The pH indicator bromothymol blue solution was prepared as previously described [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. Manual injections were carried out using a Rheodyne model 7125 injector with 20 μl sample loop. A second liquid chromatograph consisted of a Jasco Model LG-1580-02 ternary gradient unit, a Jasco PU-1580 pump with a 500 μl sample loop and a Jasco MD-910 diode array detector connected to a personal computer Acer 54eL was used for the purification of the His iminoquinol. The solvents were degassed on line with a degasser model Gastorr 153 SAS Corporation (Tokyo, Japan). IR spectra were recorded on a Nicolet Avatar 320 FT-IR apparatus with software EZ Omnic. 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 CD_3OD.

2.4. Synthesis of 2-[(6-hydroxy[4,7]phenanthrolin-5-yl)imino]-3-(1H-5-imidazolyl) propanoic acid

Phanquinone of 0.30 mmol was dissolved in about 10 ml of a mixture water/methanol (60:40, v/v); then 2 ml of aqueous solution of His, equivalent to 0.30 mmol of amino acid, and 2 ml of phosphate buffer (pH 7; 0.1 M) were added. The reaction mixture was maintained at ambient temperature under magnetic stirring for 1 day and then was evaporated under reduced pressure. 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). The resulting residue was purified by reversed-phase UV-DAD HPLC, monitoring at the wavelength of MAX-ABS, on a semi-preparative Phenomenex Luna 10μ ODS (250 mm × 10 mm i.d.) column, under gradient elution using a mobile phase consisting of a mixture A:B, where A is water (adjusted to pH 4 by formic acid) and B is methanol at a flow-rate of 1.8 ml/min. The gradient profile was $t = 0$ min, 22% B; $t = 32$ min, 66% B; $t = 35$ min, 85% B; $t = 38$ min, 22% B. The obtained orange pitchy adduct was characterized as follows.

IR (KBr, cm^{-1}): 3250 (OH), 1736 (C=O), 1654 (C=N). UV (methanol) λ_{max}, nm: 239 ($\varepsilon = 1.43 \times 10^{-4}$), 276, 316, 408 ($\varepsilon = 0.49 \times 10^{-4}$). ^{1}H NMR ($CD_3OD$, 300 MHz), δ (ppm): 4.70 (s, 2H, CH_2), 7.30–7.50 (m, 1H, ArH), 7.77–7.87 (s + m, 2H, ArH), 8.10–8.20 (t, 1H, ArH), 8.45–8.53 (d, 1H, ArH), 8.68–8.75 (d, 1H, ArH), 8.90–9.00 (s + m, 2H, ArH).

2.5. Peptide hydrolysis

An amount of tripeptide, Met-Leu-Phe acetate, equivalent to about 2.6 μmol, and an amount of leuprolide acetate, equivalent to about 0.98 μmol, respectively, were hydrolyzed with 12N hydrochloric acid (5 ml) at reflux for 2 h 30 min. After cooling, the resulting solution was neutralized with 8N sodium hydroxide using bromothymol blue solution as pH indicator and then diluted to 20 ml with water. A 50 μl aliquot of the solution was subjected to the derivatization procedure.

2.6. Derivatization procedure

A 50 μl aliquot of the amino acid solution was treated with 100 μl of phosphate buffer (pH 7; 0.1 M) and 0.25 ml of the reagent solution were added. The reaction was carried out at 50 °C for 45 min under magnetic stirring in a micro-reaction vessel (3.0 ml); then, after cooling, to the reaction mixture 100 μl of the IS solution and 2 ml of a mixture of triethylammonium phosphate buffer (pH 3; 0.05 M)–methanol in the ratio 78:22 (v/v) were added. 20 μl aliquots of the resulting clear solution were injected into the chromatograph. The derivatization of the peptide hydrolysate followed a similar procedure using 50 μl of IS solution.

2.7. Chromatographic conditions

The routine LC separations were performed at 33 ± 2 °C on a Phenomenex Prodigy 5μ ODS (250 mm × 3.2 mm i.d.) stainless steel column, under gradient elution using a mobile phase consisting of a mixture A:B, where A is triethylammonium phosphate buffer (pH 3; 0.05 M) and B is methanol at a flow-rate of 0.4 ml/min. The gradient profile was $t = 0$ min, 22% B; $t = 32$ min, 68% B; $t = 38$ min, 22% B. Fluorescence detection at $\lambda_{em} = 460$ nm with $\lambda_{ex} = 400$ nm was used. The gain was modified as follows: $t = 0$ min, gain 10; $t = 18$ min, gain 1000; $t = 20$ min, gain 10.

2.8. Calibration graphs

Individual standard solutions of amino acids were prepared in water (concentration ranges in Table 1). A 50 μl volume of amino acid standard solution was subjected to the 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.

2.9. Accuracy and precision

Accuracy and precision studies were carried out on a placebo solution spiked of leuprolide acetate, which was prepared in laboratory as described [1].

A 62.5 μl aliquot of the solution was hydrolyzed using 1.25 ml of 12N hydrochloric acid. After hydrolysis and neutralization, the solution was diluted to 5 ml with water and derivatized with phanquinone.

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

Compound	Slope[a]	y-Intercept[a]	Correlation coefficient	Concentration range (μmol/ml)
His	6.99416	−0.00444	0.9988	0.01980–0.1488
Arg	0.70408	0.000352	0.9990	0.02017–0.1513
Ser	1.80689	0.000235	0.9995	0.01984–0.1488
Trp	0.57858	0.000303	0.9999	0.01981–0.1981
Glu	0.70556	−0.000155	0.9992	0.02134–0.2134
Tyr	2.4640	0.00160	0.9995	0.01965–0.1473
Leu	3.6275	0.00278	0.9988	0.03941–0.2955

[a] According to $y = ax + b$, where x is the analyte concentration expressed as μmol/ml and y the ratio of analyte peak-area to IS peak-area.

2.10. Analysis of injectable solution

2.10.1. Sample preparation

A 0.25 ml aliquot of injectable solution, equivalent to about 0.98 μmol of leuprolide acetate was hydrolyzed as described for the peptide hydrolysis (Section 2.5).

2.10.2. Assay procedure

A 50 μl aliquot of the sample solution was subjected to the derivatization procedure and the amino acid content in each sample was determined by comparison with an appropriate standard solution.

3. Results and discussion

3.1. Derivatization reaction

In the last years, the derivatization reaction of phanquinone with a variety of amino acids was studied at different temperature and pH conditions [12–14] and applied to the analysis of free amino acids in pharmaceutical formulations. In this work, in order to assay low quantities of peptide and protein samples, the optimum derivatization conditions using His, as amino acid, was further investigated.

In mild conditions (50 °C, pH 7), the derivatization reaction of His was found to be complete after 40 min. At lower temperature (40 °C) the reaction proceeds more slowly and at 60 °C not steady response was observed, while at different pHs (5, 8) and without buffer reduced responses were obtained. Under these conditions the yield of the derivative increases to reach a plateau at a reagent to amino acid molar ratio of about 55 and further reagent excess does not interfere. The reaction was found to be essentially quantitative by comparison with an authentic specimen of synthesized amino acid adduct, which was first performed on preparative scale. The analytical data (IR, ^{1}H NMR) were consistent with the expected structure. The phanquinone reagent is not useful for the amino acid analysis with a secondary amino group as Pro. According to the literature [1], *p*-Glu was determined as Glu, after verification that the pyrrolidone ring opens up during the acid hydrolysis.

3.2. Peptide hydrolysis

In accordance with the classical acid hydrolysis methods [19–25], leuprolide acetate was hydrolyzed [1] with 6N hydrochloric acid for 24 h at 110 °C, discarding alternative hydrolysis media such as alkali or alkyl sulphonic acids because these can react with amino acids like Arg and Ser. On the basis of that, to choice the adequate conditions for acid hydrolysis, before to handle the expensive leuprolide acetate sample, the course of the hydrolysis reaction was studied on the simple tripeptide Met-Leu-Phe acetate, under reflux with hydrochloric acid at different concentrations. The reaction was found to be complete after 2 h 30 min, in presence of 12N hydrochloric acid. All examined amino acids (Leu, Met and Phe) showed

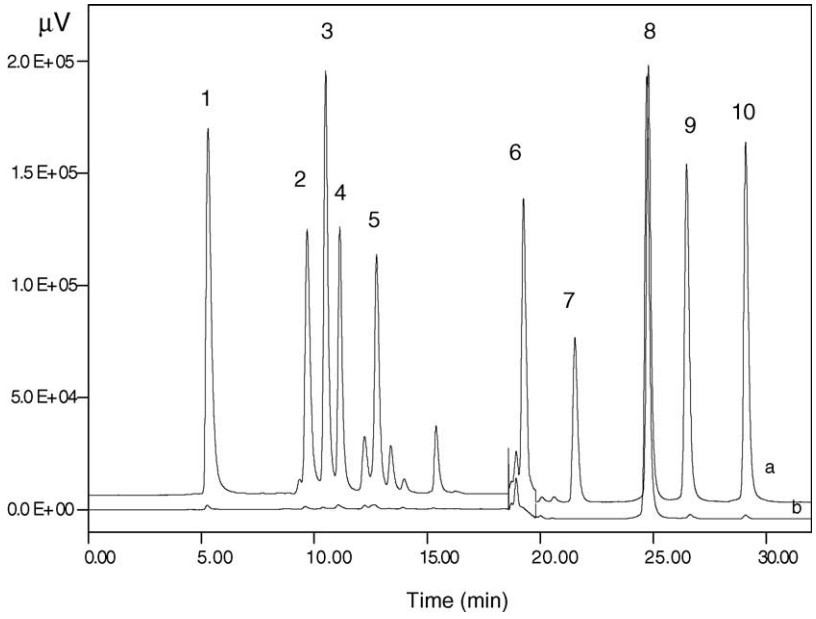

Fig. 1. Representative LC chromatogram at 33 ± 2 °C of: (a) amino acids derivatized with phanquinone; (b) phanquinone under reaction conditions. Peaks—1: His (0.09 μmol/ml); 2: Arg (0.20 μmol/ml); 3: Ser (0.08 μmol/ml); 4: Trp (0.10 μmol/ml); 5: Glu (0.20 μmol/ml); 6: Tyr (0.24 μmol/ml); 7: Met (0.51 μmol/ml); 8: Ile (IS); 9: Leu (0.15 μmol/ml); 10: Phe (0.10 μmol/ml). LC conditions: Phenomenex Prodigy 5μ ODS (250 mm × 3.2 mm i.d.) column with a mixture of A:B, where A was triethylammonium phosphate buffer (pH 3; 0.05 M) and B is methanol, under the following gradient elution conditions as mobile phase: t = 0 min, 22% B; t = 32 min, 68% B; t = 38 min, 22% B. Flow-rate: 0.4 ml/min. Fluorescence detection: λ_{em} = 460 nm with λ_{ex} = 400 nm. Gain: t = 0 min, 10; t = 18 min, 1000; t = 20 min, 10.

the same behaviour. At lower concentrations of hydrochloric acid (6 and 8N) less satisfactory results were obtained. In addition, at higher concentrations of peptide (about 10.6 μmol) an hydrolysis time at least of 6 h or 7–8 ml of 12N hydrochloric acid were necessary. Not significant degradation products in the described reaction conditions (12N hydrochloric acid for 2 h 30 min at reflux) were observed. Owing to these encouraging data, the described hydrolysis conditions were then applied to the analysis of leuprolide acetate.

3.3. Chromatography

Chromatographic separations of the derivatized amino acids were performed on a reversed-phase Phenomenex Prodigy 5μ (250 mm × 3.2 mm i.d.) column with a mobile phase consisting of a binary mixture of triethylammonium phosphate buffer–methanol, under gradient elution conditions, with fluorescence detection (λ_{ex} = 400 nm; λ_{em} = 460 nm). Typical chromatogram is illustrated in Fig. 1. As it can be seen the non-fluorescent excess reagent is not detected and the resolution of all derivatized amino acids in a quite short time was obtained with good reproducibility of the retention times (%R.S.D. = 0.18–0.62). No significant degradation products interfering with the analysis were observed. The gain was increased from t = 18 to 20 min for a better identification of the Tyr adduct peak, because of its lower detectability respect to other iminoquinols. Probably, the low sensitivity of Tyr, Arg, Trp and Glu derivatives can derive from the structural difference of the starting amino acids, which could have a significance on the fluorescence efficiency; in particular, the Trp and Tyr intrinsic fluorescence at wavelengths different from those of the adducts could quench further the fluorescence intensity. A decrease of fluorescence also by using other reagents was described [9]: with NBD-F, tryptophan does not yield a fluorescent product; with OPA, the derivatives of the individual amino acids show quite great variation in their fluorescence and the isoindoles from aspartate and glutamate show a low response. On the other hand, the different, even if reproducible, derivatization reaction yields of the iminoquinols have significant influence on the sensitivity.

Table 2
Intra- and inter-day accuracy and precision of amino acids in placebo solution spiked of leuprolide acetate

Amino acid	Accuracy (%)[a]			Precision (%R.S.D.)		
	Low[b]	Average[b]	High[b]	Low[b]	Average[b]	High[b]
Intra-day[c] (n = 3)						
Day 1						
His	90.3	94.7	95.2	4.0	3.0	3.1
Arg	96.8	101.8	100.6	4.5	3.9	4.6
Ser	83.5	82.0	81.6	4.4	4.0	4.5
Trp	82.0	84.4	80.8	4.1	5.3	4.3
Glu	102.4	105.4	107.1	5.8	3.4	2.7
Tyr	85.0	82.0	81.7	5.3	4.9	3.7
Leu	97.0	96.3	98.8	2.3	2.0	2.5
Day 2						
His	93.3	91.0	96.5	4.1	3.9	3.1
Arg	101.5	97.7	100.7	4.6	4.5	3.0
Ser	80.5	81.7	83.6	2.8	4.6	4.1
Trp	80.8	87.5	85.3	4.9	4.4	4.5
Glu	104.9	103.5	107.6	3.3	3.2	4.7
Tyr	84.4	83.3	80.6	4.5	4.1	5.5
Leu	97.9	96.6	97.7	2.5	2.5	2.4
Day 3						
His	94.9	93.9	96.3	2.6	2.0	3.8
Arg	99.8	101.0	103.0	4.1	3.3	5.4
Ser	84.6	81.1	80.6	5.7	4.3	4.4
Trp	87.1	83.5	84.3	4.7	4.0	3.8
Glu	109.5	104.0	107.0	4.7	5.2	2.9
Tyr	86.1	81.1	82.2	5.1	4.0	5.5
Leu	96.1	97.9	99.7	2.6	3.3	2.6
Inter-day (n = 9)						
His	92.8	93.2	96.0	3.8	3.2	3.0
Arg	99.4	100.2	101.4	4.4	3.9	4.1
Ser	82.8	81.6	81.9	4.5	3.8	4.1
Trp	83.3	85.0	83.5	5.3	3.9	4.4
Glu	105.6	104.3	107.2	4.6	2.7	3.1
Tyr	85.2	82.1	81.5	4.4	3.9	4.4
Leu	97.0	96.4	98.7	2.3	1.9	2.4

[a] (Mean found concentration of amino acid/nominal concentration of leuprolide) × 100/f, where f is mol amino acid/mol leuprolide.
[b] Nominal concentration of leuprolide subjected to hydrolysis: low = 3.1 μmol/ml; average = 3.9 μmol/ml; high = 4.7 μmol/ml.
[c] Three replicates of each leuprolide concentration (μmol/ml) level (n = 3).

3.4. Analysis of injectable solution

A commercial injectable solution of leuprolide acetate was subjected to the described HPLC method involving acid hydrolysis and pre-chromatographic derivatization with phanquinone.

Under the described conditions a linear relationship between peak-area ratio (analyte to IS) and analyte concentration (μmol/ml) was observed for each amino acid and the calibration parameters are shown in Table 1. The detection limit (signal-to-noise ratio = 3) was in the range 30–800 fmol.

Table 3
Results for the amino acid analysis of leuprolide acetate hydrolysate in injectable solution

Amino acid[a]	Molar ratio		% Recovery[b] (%R.S.D.)
	Found[c] (%R.S.D.)	Theoretic	
His	1.01 (3.3)	1.00	95.0 (2.9)
Arg	0.98 (2.6)	1.00	101.4 (4.7)
Ser	1.07 (5.2)	1.00	81.0 (4.0)
Trp	1.05 (4.9)	1.00	83.5 (3.3)
Glu	1.00 (4.8)	1.00	106.7 (2.5)
Tyr	1.05 (1.8)	1.00	81.3 (5.6)
Leu	1.96 (2.8)	2.00	98.1 (1.8)
Pro	–	1.00	–

(–) Not detected.
[a] Other ingredients: benzilic alcohol, sodium chloride, water. The solution includes sodium hydroxide and/or acetic acid to adjust the pH.
[b] Percentage of the added quantity (20%).
[c] Mean of five determinations.

The intra- and inter-assay accuracy and precision of the method were determined on simulated injectable solution by analysing three replicates at three concentration levels of leuprolide acetate, and the relative data are reported in Table 2. The intra-day accuracy ranged from 80.5 to 109.5% of the nominal concentration and the precision (%R.S.D.) was less than 5.8%; the inter-day accuracy was in the range 81.5–107.2% and corresponding R.S.D. values were less than 4.6%. The data were found to be quantitative for most amino acids; the lower recovery values of Trp, Ser and Tyr probably are due to the degradation of these amino acids which could arise during hydrolysis by hydrochloric acid. The degradation process seems to happen a part from the acid concentration. Using 6 N HCl at 110 °C for 18–24 h Trp is destroyed, while Ser and Tyr are partially lost [21,23–25]. Tyrosine, reported to be susceptible to oxidation, was degraded with the use of 9 and 12N HCl. However, similar concentrations of tyrosine were obtained with 6N HCl relative to those obtained with 3N HCl, although some of the values were slightly lower at 6N compared to 3N HCl [25].

On the basis of the accuracy and the precision of the method, the molar ratio of amino acid to leuprolide in the pharmaceutical formulation was evaluated. The results of the analysis (Table 3) agreed with the expected value respect the claimed content of peptide according to the literature [1]. The other components of the formulation did not interfere with the analysis. The accuracy of the method was further verified by analysing commercial samples spiked with a known amount of leuprolide acetate (20% of the claimed peptide content). The recoveries (Table 3), calculated by comparison with a standard amino acid solution, were in agreement with the intra- and inter-day accuracy data (Table 2). To illustrate the application of the procedure, the chromatographic profile of the amino acids derivatized with phanquinone, following leuprolide acetate hydrolysis, in injectable solution is shown in Fig. 2. The described procedure could be useful for the characterization of amino acids in protein hydrolysates when LC–MS instrumentation is not available and can be suitable to confirm the quality of the formulation.

Phanquinone, despite of the stronger derivatization conditions, compared with other known reagents can be favourable [7–11,14]. Phanquinone is more selective and sensitive than ninhydrin and presents also the advantage with respects to Dns-Cl, FMOC and fluorescein isothiocyanate of not exhibiting significant native fluorescence; the emission appears only after reaction. In addition, Dns-Cl, FMOC, NBD-F and fluorescein isothiocyanate have poor selectivity reacting not only with amino group, but also with phenols and thiols. In particular, ninhydrin can be used only as post-column reagent giving the same adduct with all amino acids. The phanquinone high stability, the high sensitivity, the absence of significant degradation products, the simplicity of the derivatization reaction without additional reagents, allowing a easy manipulation, made it competitive also to OPA and fluorescamine. These compounds are usually employed for the detection of primary amines as post-chromatographic derivatization reagents. Owing to the formation of fluorescent degradation products, the derivatization technique with fluorescamine or OPA is unfavourable for pre-chromatographic labelling. As regard the fluorescence property, the excitation and emission wavelengths range (λ_{ex} = 400 nm; λ_{em} = 460 nm), in any case wider than those of other known reagents such as CRA-Cl, CRP-Cl, and ARC-Cl, did not produce interference. The reduced bandwidth (18 nm) of the used spectrofluorimetric detector also allowed to avoid this problem.

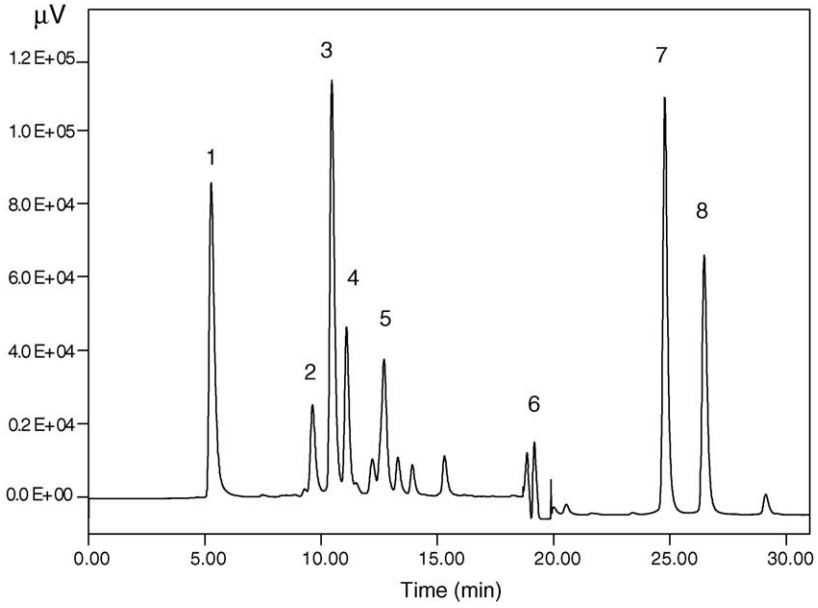

Fig. 2. LC chromatogram of leuprolide acetate (injectable solution) after hydrolysis and derivatization with phanquinone. Peaks—1: His; 2: Arg; 3: Ser; 4: Trp; 5: Glu; 6: Tyr; 7: Ile (IS); 8: Leu. Chromatographic conditions and detection as described in Fig. 1.

4. Conclusions

Phanquinone was found to be suitable for the pre-column fluorescence determination of amino acids, except Pro, in leuprolide acetate, following hydrolysis, without the extraction of excess derivatizing reagent. The classic hydrolysis step has been shortened from the usual time to 2 h 30 min using 12N hydrochloric acid, without formation of fluorescent degradation products for the examined amino acids. The method described here is simple and reproducible and can offer the opportunity to achieve further information useful for a reliable quality control of leuprolide in raw materials and dosage forms.

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