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Determination of glycine and threonine in topical dermatological preparations

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ABSTRACT

In the present study, a single HPLC method was developed for the determination of glycine and threonine in cicatrizants. Two different preparations of a cream and an ointment, and the corresponding bandages, onto which the formulations were applied, were studied.

The method involved matrix solubilisation with dichloromethane, liquid–liquid isolation of gly and thr with aqueous 1N NaOH, and derivatization with phenylisothiocyanate.

Reversed-phase HPLC separation was carried out by gradient elution with 20 mM aqueous $NaClO_4$ and acetonitrile (from 90% to 30% aqueous $NaClO_4$ in 10 min) on a LiChrospher® 100 RP-18 cartridge (125 mm × 4.6 mm). Analytes were determined with a UV detector set at 245 nm.

Quantitation was accomplished by internal standardization with methionine. Linearity was studied in the range 60–120% of the concentrations expected for gly and thr (*viz.* for gly from 200 to 400 μg ml^{-1}, and for thr from 100 to 200 μg ml^{-1}). In reference aqueous samples, linear correlation (r) was better than 0.99 for gly and thr, while in spiked matrix samples r ranged from 0.97 to 0.98. Recoveries were in the 95–105% interval, and precision (CV%, $N = 6$) was better than 5% for both analytes either in cream, ointment or bandages.

The method was successfully used for the quality control of topical dermatological preparations.

1. Introduction

Free glycine (gly), threonine (thr) and cysteine are used traditionally in pharmaceutical dermatologic preparations as active principles for cicatrization. It is believed that their external application is beneficial to the skin reparation process and may accelerate the overall wound-healing rate. Cicatrizant preparations, as well as the majority of dermatological pharmaceuticals, are custom-made and industrial products formulated specifically to soften and smooth the skin while releasing the aminoacids upon application [1,2]. They include topical creams and ointments as well as bandages, patches, sheets, and plasters onto which creams or ointments are applied. The matrixes in which the free aminoacids are incorporated can be totally fat-based ointments, or water-in-oil solid, gel, or liquid emulsions made of structural agents such as hydrocarbons (e.g. white mineral oil, petrolatum, and wax), silicones, alcohols (e.g. cholesterol), long-chain fatty acid esters (e.g. isopropyl palmitate), emulsifying agents (e.g. lanolin) and auxiliary or antimicrobic agents such as, for instance, benzalkonium chloride [1,2]. As expectable therefore, quality control (QC) of such pharmaceuticals may pose analytical problems due to the complexity of the matrix. However, assessing precisely the dose of excipients and active principles incorporated into the product is necessary for the manufacturer in order to establish if the production is appropriate, and is important for QC, stability assessment, and clinical practice.

Aminoacid analysis is an important field of research which find many applications in pharmaceutical, biochemical, and clinical research. Virtually all separation techniques have been used for the determination of aminoacids. The more popular involved reversed-phase high performance liquid chromatography [3], ion-exchange chromatography [4], gas chromatography [5], and capillary electrophoresis [6]. Among these, the technique most employed by bioanalysts is reversed-phase high performance liquid chromatography and ultraviolet light detection (RP-HPLC-UV). However, free gly and thr are not detectable by UV or visible light detectors and have to be derivatized.

Analysis of aminoacids including gly and thr is generally carried out by HPLC using C8 or C18 silica-based columns and sodium acetate or phosphate buffers together with pre-column or post-column derivatization. Ortho-phtalaldehyde (OPA) [7–9], phenylisothiocyanate (PITC) [3,5,9–18], butylisothiocyanate [17], benzylisothiocyanate [18], 5-dimethylamino-1-naphtalene sulfonylchloride (dansyl chloride or dansyl-Cl) [9,11], 9-fluorenylmethyl chloroformate (FMOC-Cl) [9,19], 5,5′-dithio-bis-nitrobenzoic acid [20] are among the derivatizing agents that

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have been reported for derivatization of aminoacids in biological matrixes including foods [5,8,17,18], wines [21], biological fluids [9,11,13,20,22,23], biological tissues [10,12], marine sediments [7], fodders [24], and even coniferous needles [15].

Since the last 10 years, however, only precolumn derivatizations with OPA and PITC have been widely used for the determination of aminoacids in biological specimens [3]. These methods are well established and are reported as simpler, faster, and using less expensive and complex instrumentation compared to dedicated analyzers based on MS or other sensitive detectors (e.g. LIF or electrochemical). OPA and PITC, however, are generally not suitable for derivatizing cysteine, sulphydryl peptides (e.g. glutathione), and disulphide aminoacids (e.g. cysteine). Thus most methods report on sample preparations in which cysteine is either determined separately from other aminoacids not containing sulfur groups using specific derivatizing agents [20], or determined with PITC after blocking the terminal thiol group with iodoacetic acid [3,5,13] or, exceptionally, determined with PITC directly together with cysteine [25].

Therefore, the primary aim of this study was the development of an analytical method for the determination of glycine and threonine in topical dermatological preparations (bandages, patches, sheets, and plasters onto which ointments or creams are applied) in order to demonstrate the feasibility of the proposed approach.

The method requirements were that it ought to be simple, inexpensive and based on a well-established pre-column derivatization-HPLC-UV approach. The method developed and presented here is thus based on pre-column derivatization of the free aminoacids with phenylisothiocyanate (PITC) and formation of the corresponding phenylthiocarbamyl (PTC) derivatives, followed by reversed-phase HPLC separation and UV detection.

The active principles selected as target analytes, namely gly and thr, were determined in two different lots of creams and in two types of bandages onto which the creams were applied. Although much has been published on HPLC analysis of PTC-aminoacid derivatives, to the authors' knowledge this is the first report describing the determination of gly and thr in a water-in-oil emulsion and an ointment or dermatologic pharmaceuticals.

2. Experimental

2.1. Chemicals

Reference standards of glycine and threonine (pharmaceutical-grade purity) were kindly supplied by a mid-size Italian pharmaceutical company together with all excipients and products reported in Table 1. All chemicals used for preparing pharmaceuticals were at least of pharmaceutical grade purity and met all European Pharmacopoeia requirements. L-Methionine (purity 98%) was purchased from Sigma–Aldrich (Milan, Italy). All solvents and general-purpose reagents were obtained from Carlo Erba Reagenti (Milan, Italy) and were of the highest purity commercially available or HPLC-grade.

Eastman Kodak (Rochester, NY, USA) sequentiation-grade pure phenylisothiocyanate (PITC) was a kind gift from the Department of Biochemistry of the University of Pavia.

2.2. Samples

This study was carried out on two lots of 1 kg each of aminoacid-containing bulk products (named cream and ointment in the following, see Table 1 for composition). Two lots of 1 kg each of the corresponding "placebo" bulk products consisting of cream and ointment prepared exactly as the pharmaceutical formulations but without the aminoacids were also used. Two lots of 7 cm × 9 cm canvas bandages onto which either products were applied were also supplied in order to test the method on samples similar to those designed for marketing. Cream and ointment bulk products, and the bandages covered with the formulations, were produced by a mid-size Italian pharmaceutical company for research purposes. Bulk creams and ointments were stored immediately after production in white polyethylene screw-capped air-tight vessels. The bandages were supplied in carton boxes containing 10 pieces each. Every bandage was closed in an aluminium envelope packing sealed and air-tight. All samples upon arrival in laboratory were stored at room temperature in ambient protected from diffuse sunlight until analysis.

2.3. Apparatus and chromatographic conditions

The apparatus consisted of Agilent Technologies series 1100 bench-top equipment (Agilent Technologies, Palo Alto, CA, USA) including a model G1311A quaternary gradient pump (GP), a model G1367A WPALS autosampler, set at 5 μl injection volume, a model G1322A degasser, a model G1316A COLCOM thermostated column oven set at 40.0 ± 0.5 °C, and a model G1314A VWD variable wavelength UV detector set at 245 nm. On purpose, for peak purity assessment and UV spectra aquisition a model G1315A DAD diode-array detector was included in the system.

The HPLC-UV system was controlled by a personal computer equipped with ChemStation Software Version A.10.01 operating in a Windows XP Professional operating system environment.

All separations were carried out using a Lichrospher® 100 RP-18 of 5 μm particle size stationary phase prepacked in a 125 mm × 4 mm cartridge assembled in a LiChroCART® 125–4 stainless steel holder (Merck, Darmstadt, Germany).

Gradient elution was carried out at constant flow of 1 ml/min, from 90%A to 30%A (corresponding to 10%B to 70%B) for 10 min, followed by an isocratic elution at 100%B for 10 min. Eluents compositions were (A) 20 mM aqueous $NaClO_4$ and (B) 20 mM $NaClO_4$ acetonitrile–water (60:40, v/v). At the end of each analysis, the cartridge was rinsed at 1.5 ml/min flow rate for 5 min with methanol–water (90:10,v/v), and then re-equilibrated at 1.5 ml/min flow rate for 5 min with the initial mobile phase (A:B 90:10, %).

2.4. Preparation of standard reference solutions and samples

2.4.1. Stock solutions

Stock solutions of gly, thr and internal standard (I.S.) methionine (met) were prepared in 1N aqueous NaOH at the concentration of 10, 5, and 5 mg ml^{-1}, respectively, and kept at 4 °C in the dark for 1 week. Working solutions were prepared by diluting the corresponding stock solutions with 1N NaOH before every working session at the concentrations of 200, 250, 300, 350, 400 μg ml^{-1}, for gly, and of 100, 125, 150, 175, 200 μg ml^{-1} for thr. These concentrations were chosen in order to have standard reference solutions at levels roughly corresponding to 60–120% of the concentrations expected for gly and thr in the extraction solvent, when processing about 2 g of either products, namely in the ointment about 280 μg ml^{-1} for gly, and 140 μg ml^{-1} for thr, and in the cream about 332 μg ml^{-1} for gly, and 166 μg ml^{-1} for thr. The I.S. was prepared in 1N NaOH at the level of 100 μg ml^{-1} at the beginning of every working session.

2.4.2. Reference and blank solutions

Standard reference solutions and blanks for HPLC analysis were obtained by adding 85 μl of HCl 37% (w/v) to 1 ml of the corresponding working standard 1N NaOH solution prior to derivatization;

Table 1
Glycine and threonine content of bulk products and 7 cm × 9 cm bandages

Components	Cream (mg/g)	Ointment (mg/g)	Bandages (μg/cm^2)
Active principles			
Threonine	0.83	0.7	15.8
Glycine	1.66	1.40	31.6
Other	L-Cysteine	L-Cysteine	L-Cysteine
Excipients	Benzalkonium chloride, benzyl benzoate, methylparaben, propylparaben, lavender essence, cholesterol, dimethicone, isopropyl palmitate, white mineral oil, anhydrous lanolin, paraffin, white wax, water (accounting for 98.7% in weight)	Benzalkonium chloride, benzyl benzoate, methylparaben, propylparaben, lavender essence, white mineral oil, paraffin, aerosil, isopropanol, sodium EDTA, chlorobutanol, butylated hydroxyanisole (accounting for 98.8% in weight)	According with the product applied on the bandage

0.4 ml of this solution were put into an Eppendorf tube, and derivatized with PITC as described in Section 2.5.

2.4.3. Cream and ointment samples

In a 20 ml glass vial, 2 g of each type of product (corresponding, respectively to 3.32 mg gly and 1.66 mg thr for the cream, and to 2.80 mg gly and 1.40 mg thr for ointment, see Table 1) were solubilised in 5 ml dichloromethane at about 40 °C under gentle mechanical stirring in a water bath. The solution was then extracted with 10 ml of 1N NaOH containing the internal standard (100 μg ml^{-1}). The vial was closed and left under mechanical (magnetic) stirring for about 40 min at 40–50 °C. The resulting emulsion was treated with 0.85 ml HCl (37%), heated further at about 60 °C in water bath until the emulsion was broken, and centrifuged at about 3000 rpm at 40 °C for 10 min with a thermostated centrifuge. An aliquot of 0.4 ml of the supernatant was sampled and put in an Eppendorff tube for PITC derivatization.

Analytes additions to placebo products were made using 1N NaOH containing the internal standard (100 μg ml^{-1}), and known amounts of gly, and thr were added to the samples as illustrated in Table 2 for a model standard additions calibration curve.

2.4.4. Bandage samples

One bandage of 7 cm × 9 cm was cut with scissors into about 1 cm × 2 cm strips and collected in a 100 ml glass vial together with 50 ml dichloromethane. The vial was screw-capped and put in a water bath at 40 °C under magnetic stirring for about 30 min in order to let the cream or ointment adsorbed on the bandage be solubilised by the organic solvent. To the resulting mixture, 10 ml of 1N NaOH containing the internal standard (100 μg ml^{-1}) were added, and the mixture was left under magnetic stirring at 40 °C for 30 min.

The mixture was brought to an acidic pH by adding 0.85 ml of concentrated HCl and the vial was put again in the water bath for a few minutes in order to let the emulsion separate completely. After disposing of the solid residues (paper and canvas) 1.5 ml of the supernatant were transferred into Eppendorff tubes and centrifuged at 6000 rpm for 10 min. One aliquot of 0.4 ml of the clear supernatant aqueous solution was transferred into another Eppendorff tube and evaporated to dryness under low pressure with a Büchi Rotavapor equipped with a membrane vacuum pump prior to PITC derivatization.

Table 2
Linearity data on reference standard 1N NaOH solutions and on placebo products spiked with known levels of glycine and threonine

Active principles levels (μg ml^{-1})	Curve equation	Correlation (r)
Reference standard NaOH 1N solution		
Gly: 200, 250, 300, 350, 400	$y = 0.0155x + 1.0903$	0.995
Thr: 100, 125, 150, 175, 200	$y = 0.0124x - 0.1047$	0.999
Standard additions to placebo cream		
Gly: 200, 250, 300, 350	$y = 0.0182x - 0.360$	0.98
Thr: 75, 100, 125, 150, 175	$y = 0.0166x - 1.526$	0.98
Standard additions to placebo ointment		
Gly: 200, 250, 300, 350, 400	$y = 0.0156x + 1.3856$	0.98
Thr: 100, 125, 150, 175, 200	$y = 0.0124x + 0.093$	0.97

The curve equations correlate y, the ratio between area of analyte peak and area of I.S. peak, and x the analyte concentration level. The I.S. was added to all calibration points at the concentration of 100 μg ml^{-1}. The concentrations levels were chosen in order to study the interval of roughly 60–120% of the expected concentrations for gly and thr in 2 g of sample (see also Table 1 for composition) extracted with 10 ml of 1N NaOH. The cream was expected to contain 1.66 mg g^{-1} gly and 0.83 mg g^{-1} thr, therefore target concentrations in 10 ml 1N NaOH were 332 μg ml^{-1} for gly and 166 μg ml^{-1} for thr. Analogously, the ointment was expected to contain 1.40 mg g^{-1} gly and 0.70 mg g^{-1} thr, thus target concentrations in 1N NaOH were 280 μg ml^{-1} for gly and 140 μg ml^{-1} for thr.

2.5. Phenylisothiocyanate (PITC) derivatization

The aqueous solution prepared for calibration or that recovered from the cream and bandage samples were brought to dryness with a Rotavapor.

To the dry residue, 100 μl were added of the derivatizing mixture composed of methanol–triethylamine–water–PITC (7:1:1:1, v/v). The reaction was soaked after 10 min by adding 1 ml of absolute ethanol, and the solution was directly evaporated to dryness again by using a rotary evaporator.

The dry residue was reconstituted in 100 μl of 100 mM sodium phosphate buffer at pH 7 and centrifuged at 6000 rpm for 10 min. Of the resulting solution, 5 μl were introduced into the HPLC system.

3. Results and discussion

3.1. Method development

The assay of gly, thr, and cysteine in pharmaceuticals used as cicatrizant for skin wounds is important. In particular, cysteine can be suitable marker for the stability assessment of such preparations because this aminoacid is easily oxidized to cystine under mild conditions, thus during manufacturing and in any process which may involve heating. The cysteine to cystine ratio determination actually involves a two-step approach in which the thiol function has to be blocked first, and then PITC can be used as derivatizing agent, as documented in previous works regarding biological samples [3,13,20], and foods [5]. The present study was designed to demonstrate the practicality of PITC derivatization for aminoacids in fat-based pharmaceutical preparations, and only gly and thr were considered.

A HPLC method was developed with the purpose of a know-how transfer to quality control (QC) laboratory units in industrial settings. It was therefore evaluated for QC of pharmaceutical preparations and studied accordingly in terms of selectivity, linearity, precision, accuracy, and robustness.

Particular attention was paid to set up a procedure as simple and inexpensive as possible which used commonly available instrumentation.

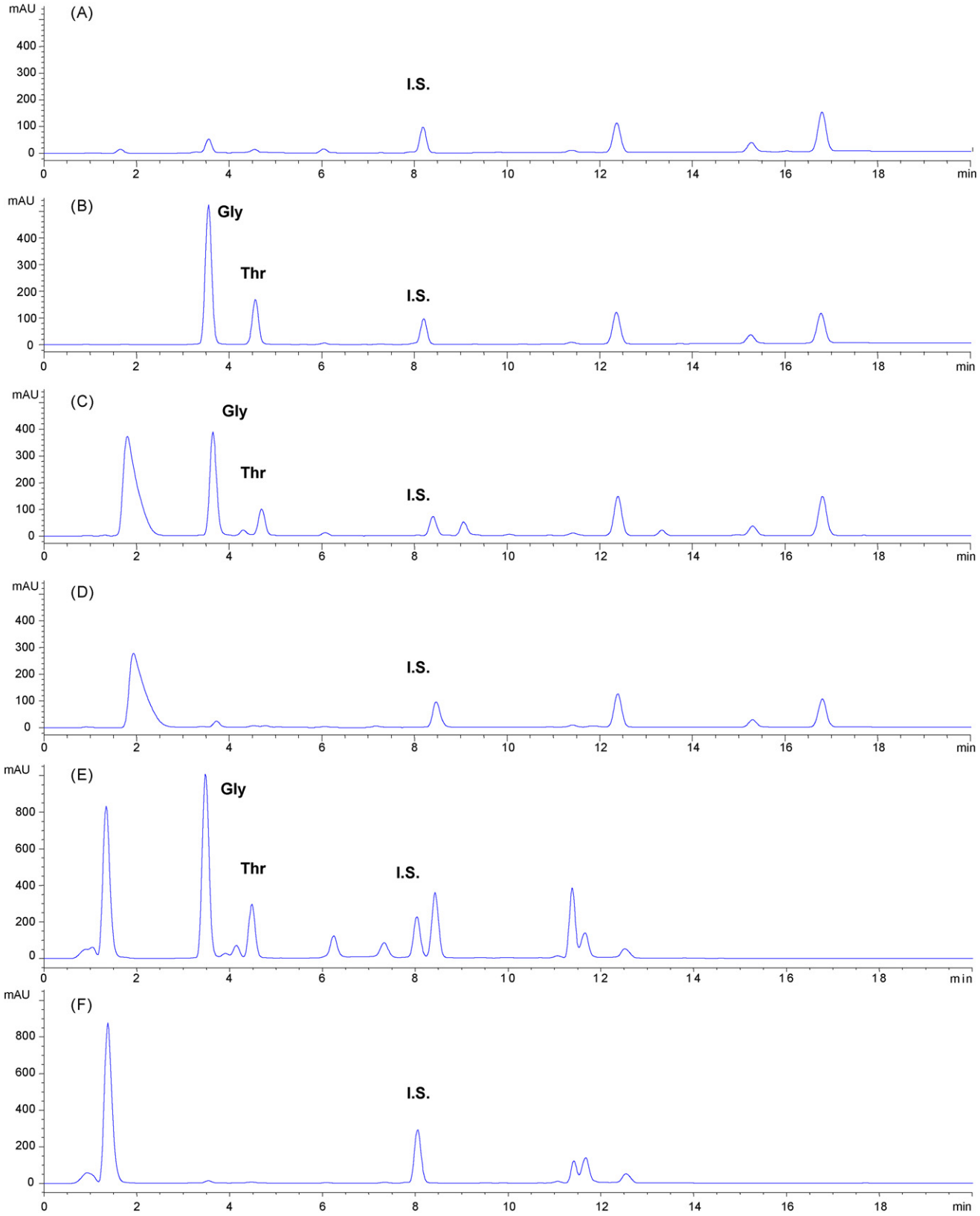

Fig. 1. Chromatographic selectivity: (A) blank NaOH 1N added with internal standard (0.1 mg ml^{-1}); (B) reference standard NaOH 1N solution, gly 0.3 mg ml^{-1}, thr 0.15 mg ml^{-1}, internal standard (I.S.) 0.1 mg ml^{-1}; (C) cream, gly 0.32 mg ml^{-1}, thr 0.17 mg ml^{-1}, I.S. 0.1 mg ml^{-1}; (D) Placebo cream spiked with 0.1 mg ml^{-1} I.S.; (E) ointment, gly 0.28 mg ml^{-1}, thr 0.14 mg ml^{-1}; (F) Placebo ointment spiked with 0.1 mg ml^{-1} I.S. Comparing chromatograms A–D with chromatograms E and F, it appears a slight contamination with gly of the blanks due to the evaporation step.

Table 3
Precision (expressed by CV%) and recoveries of six replicate determinations of gly and thr in bulk cream

Glycine (mg g^{-1})	Threonine (mg g^{-1})
1.80	0.83
1.63	0.82
1.77	0.83
1.70	0.82
1.60	0.85
1.61	0.82
Mean ± S.D.	
1.69 ± 0.09 (n = 6)	0.83 ± 0.01 (n = 6)
CV (%)	
5%	1.5%
Recovery ± S.D. (%)	
102 ± 5	100 ± 1

Expected concentrations were glycine 1.62 mg g^{-1} and threonine 0.83 mg g^{-1}; S.D., standard deviation; CV (%), coefficient of variation percent; recovery is calculated by the ratio percent between expected and measured concentration.

Analysis of creams and ointments required internal standardization because sample procedures like liquid–liquid extraction and derivatization may lead to errors on recoveries difficult to control otherwise. For this reason, methionine was chosen as suitable internal standard on the basis of structure similarity to gly and thr, and that its PTC-derivative is well separated from the corresponding PTC-derivatives peaks of gly and thr occurring in the chromatograms (Fig. 1).

3.1.1. Liquid–liquid extraction

Solubilisation of creams and ointments was achieved with *n*-hexane, chloroform, or dichloromethane. The latter solvent was chosen for safety reasons, based onto its lower toxicity on acute exposure and reduced flammability [26].

Extraction was initially attempted with plain water, with aqueous 2% (w/v) NaCl, and with aqueous 1N HCl, but recoveries were never better than 80% for either gly or thr (data not presented). Aqueous 1N NaOH produced thick emulsions with dichloromethane-solubilised creams. Acidification and further heating of the emulsions at 60 °C for few minutes was necessary to separate the aqueous from the organic layer and obtain satisfactory precision and recoveries (see Table 3). Our results with 1N NaOH agree with those reported by Nunn and Keil [7]. The emulsions formed by cream samples, however, obliged to operate with at least 10 ml volumes of solvents for practicality.

3.1.2. Derivatization and clean-up

Derivatization with PITC is well documented in its pros and cons [3,5,9–18,22,23], and here it is worth discussing briefly only the two main problems related with the use of the Edman's reagent. First, to obtain rapidly high yields in the derivatization, it is necessary to operate on dry residues of the samples. Unfortunately, aminoacids are generally isolated from aqueous solutions, as in this case, and this forces to evaporate to dryness aqueous solutions which is never a rapid step by any means. By using an efficient Rotary evaporator, 0.4 ml of sample were evaporated to dryness in about 20 min.

Second, the excess PITC that remains after derivatization is reported to damage the column, to disturb the chromatograhic partition of the analytes and cause retention time shifts [9]. Therefore, excess PITC must be eliminated before injecting the sample into the HPLC. The most common and easier way to eliminate PITC is to evaporate it under reduced pressure [5], but this is again not a practical approach because PITC has a boiling point of about 120 °C at the lower pressure achievable with conventional membrane pumps serving Rotavapors (*viz.* around 30 mmHg). It was observed, however, that the addition of about 1.0 ml absolute ethanol to the sample at the end of derivatization was beneficial because it apparently facilitated the distillation to dryness of the reaction mixture which was complete in about 10 min. This is thought to happen because ethanol under reduced pressure, at about 80 °C, rapidly distils together with the traces of water, triethylamine, and PITC.

The dry residue is then solubilised again in mobile phase and 5 μl of such solution are injected into the HPLC.

The chromatograms presented in Fig. 1 are typical of the experiments with the creams and ointments extracts after derivatization and evaporation. It was common to see blanks and samples affected by cross-contamination due to the high concentrations of analytes and excipients which are dispersed inside the evaporating system. All samples have been processed in disposable tubes and plastic vials in order to avoid glassware which was difficult to clean from the greasy film remaining inside. Nonetheless, contamination occurred albeit it was generally minimal when the evaporating system was rinsed periodically in routine work. Despite this unsolved problem, selectivity, precision, linearity, and accuracy were demonstrated, witnessing that the quantitative determinations actually did not suffer from this potential source of error.

The cream composition affected the derivatization reaction yields which resulted lower than those observed for standard solutions and ointment. In Fig. 1, peak heights of all analytes are lowered by roughly 50% in chromatograms C and D (cream sample and placebo) as compared with those in chromatograms E and F (ointment sample and placebo), and chromatograms A and B (blank NaOH and reference solution).

3.1.3. HPLC separation

In the present method, the mobile phase used was selected according to recent reports published by Shibue and coworkers [27–29]. This choice was adopted in order to avoid preparation of acetate- [10,12–16,23] or phosphate-based [17,18] mobile phases buffered at pH 6–7.4 reported in the separation of PTC aminoacids derivatives. Although most researchers found necessary to buffer binary and even ternary gradient mobile phases, as discussed thoroughly [3], no buffering was necessary in the present study applying a binary sodium perchlorate gradient mobile phase onto the C18 cartridge chosen. Under the conditions described, gradient elution was necessary to achieve a satisfactory separation between all peaks in the chromatograms originating from the sample preparation in run times shorter than 20 min.

Sample processing took about 45 min per sample owing to the slow rate of the evaporation steps, and every chromatographic run lasted 30 min. The total turnaround time therefore can be estimated in 75 min which is still acceptable for routine analysis. PTC-derivatives are stable over time for a few days and therefore overnight sessions of analysis can be planned if an autosampler is available.

3.2. Method evaluation

In the following, some method validation parameters are discussed. Definitions and criteria were adopted according to the recent analysis reported by Rozet et al. [30]. Full validation of the present method was beyond the scope of the study and was not taken into consideration.

3.2.1. Selectivity

Selectivity is illustrated in Fig. 1, where it is evidenced that neither creams excipients and preservatives, nor the byproducts of the PITC derivatization reaction interfered with the separation of gly, thr, and I.S. in the chromatographic region where the peaks eluted.

Table 4
Accuracy evaluated as recovery in standard reference 1N NaOH solutions and standard additions to placebos

Expected	1N NaOH solutions		Standard additions placebo cream		Standard additions placebo ointment	
	Measured	Recovery (%)	Measured	Recovery (%)	Measured	Recovery (%)
Glycine						
200	205	103	202	101	199	99
250	247	99	250	100	249	100
300	293	98	297	99	308	103
350	355	101	345	99	344	98
Threonine						
100	101	101	98	98	104	104
125	123	98	125	100	120	96
150	151	101	148	99	152	101
175	177	101	180	103	181	104
200	198	99	203	102	n.a.	n.a.

Concentrations expressed in $\mu g\,ml^{-1}$. Target concentrations were in cream 332 $\mu g\,ml^{-1}$ for gly and 166 $\mu g\,ml^{-1}$ for thr, and in ointment 280 $\mu g\,ml^{-1}$ for gly and 140 $\mu g\,ml^{-1}$ for thr; n.a., not assayed. Recovery is calculated as the ratio percent between expected and measured concentration.

3.2.2. Linearity

Linearity was studied in the interval roughly comprised between 60% and 120% of the expected analytes (gly and thr) concentrations based on the manufacturer preparation. The results are summarised in Table 2 and demonstrate that linearity was satisfactory in the interval studied in both bulk products, allowing not only the fresh product QC but, if necessary, also active principles stability assessment over time. The method sensitivity within the linearity range was not an issue for the goal of controlling the products examined and no limits of detection or quantitation were assessed [30].

3.2.3. Precision

Precision was evaluated measuring the active principles concentration in real samples of bulk creams, ointments, and bandages. Table 3 illustrates the results obtained for one sample of bulk cream as an example of the overall precision attained. The determinations were carried out during non-consecutive sessions for 6 weeks. Therefore, the data presented in Table 3 should be regarded as an inter-session precision set of data, representing the variability expectable by the method.

3.2.4. Accuracy

Accuracy was evaluated by comparing the results of the calibration curve of standard aqueous solutions and by adding known amounts of gly and thr to samples of placebos and authentic products of creams and ointments of the available lots. The results illustrated in Tables 4 and 5 show that accuracy was satisfactory and met the requirements of recoveries in the range of 95–105% for both gly and thr. Moreover, the analytes concentrations were determined with satisfactory accuracy on creams and ointments (Table 5) as well as on bandages covered with cream (Table 6, top data).

Table 5
Accuracy evaluated as recovery on standard additions to authentic cream and ointment

Expected	Glycine			Threonine	
	Measured	Recovery (%)	Expected	Measured	Recovery (%)
Cream					
332	341	103	166	163	98
492	480	98	246	251	102
972	975	100	486	485	100
Ointment					
274	276	101	138	135	98
414	410	99	208	216	104
554	556	100	278	273	98

Data are expressed in $\mu g\,ml^{-1}$; recovery is calculated as the ratio percent between expected and measured concentration.

Table 6
Glycine and threonine concentrations in 7 cm × 9 cm bandages covered with cicatrizant cream (top, determinations on four different samples), and ointment (bottom, determinations on eight different samples)

Cream bandage	Mean ± S.D. ($\mu g\,cm^{-2}$)	Expected ($\mu g\,cm^{-2}$)	Recovery (%)	CV (%)
Glycine	31.8 ± 0.3	31.6	100 ± 1	1
Threonine	16.1 ± 0.4	15.8	100 ± 1	2
Ointment bandage				
Glycine	24 ± 3	31.6	76 ± 10	12
Threonine	12 ± 1	15.8	76 ± 7	8

Inadequate distribution of the ointment onto the bandage surface is evidenced; S.D., standard deviation; recovery is calculated as the ratio percent between expected and measured concentration; CV, coefficient of variation.

3.3. Application on authentic samples

The method was applied on authentic samples of bandages prepared with a layer of either bulk products (*viz.* cream or ointment). In Table 6 some results are summarised and show that accurate measurement of gly and thr concentration was achieved also on canvas bandages onto which the cicatrizant cream or ointment was applied. These samples can create some practical difficulty, because the entire bandage must be processed in order to determine with sufficient accuracy the total content of aminoacids. The analysis of the entire bandage actually reduces the variability in the data that may result if only parts of a bandage are processed. Heterogenous and inadequate distribution of the cream or ointment onto the bandage surface is possible due to manufacturing, storage, and handling of the pharmaceutical (see results for bandage covered with ointment, Table 6). Therefore, if bandages are processed, one must choose either to prepare several samples of the same bandage in order to evidence the distribution of the aminoacids concentrations, or to process the entire bandage in one single vessel.

4. Conclusions

The presented method allowed determining gly and thr in cicatrizant preparations of creams and canvas bandages treated with cicatrizant products. The method proved to be simple and robust enough to be applied for routine analysis in QC laboratories in industrial settings. Sample turnaround time is severely reduced by the derivatization step with PITC which requires two steps of evaporation of solutions made of non-volatile aqueous reagents. Selectivity, linearity, precision and accuracy were assessed and resulted satisfactory for the purpose of QC.

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