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"For export only" medicines come back to Europe: A RP-LC method for the screening of six glucocorticoids in illegal and counterfeit anti-inflammatory and lightening creams

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ABSTRACT

"For export only" anti-inflammatory and lightening creams are medicinal products sold in African countries for their skin whitening action. In the last years, Rapid Alerts from European Medicinal Regulatory Agencies evidenced the presence of a large number of illegal and counterfeit anti-inflammatory products advertised for their whitening action on black skin in the European market.

These drugs, containing glucocorticoids, are illegally sold in Europe in unauthorized ethno-cosmetics-shops and mainly bought by immigrants.

This paper reports a new RP-LC method for the rapid simultaneous screening of six different active ingredients in anti-inflammatory and whitening products (creams, ointment and suspension): betamethasone dipropionate, dexamethasone, fluocinonide, fluocinolone acetonide, clobetasol propionate, methyl-prednisolone acetate. The method was developed and validated in view of its possible application in quality control laboratories, mainly those appointed to the control of illegal/counterfeit medicinal products. The associated measurement uncertainty was calculated from validation data. The method was then applied to the analysis of whitening products obtained from the Italian illegal market.

1. Introduction

Voluntary depigmentation is a skin bleaching practice called "khessal" largely diffused in sub-Saharan Africa. A list of products containing substances with skin bleaching action has been banned by some African countries such as Kenia, Gambia and Burkina Faso. However, in spite of their prohibition, products such as creams containing hydroquinone, glucocorticoids and mercury soaps are advertised and sold. Their use rises from anthropological and identitary questions and from racist advertising campaign, but it is mainly a public health problem. Several studies evidenced that voluntary depigmentation induces dermatological infections such as mycosis, skin lesions, burning, dyschromie. All-body depigmentation induces systemic diseases such as diabetes and high blood pressure [1–3]. In Europe the use of hydroquinone and mercury iodine based-on cosmetics is banned, while glucocorticoids creams are considered pharmaceutical products and, thus, can be sold in authorized shops only [4]. The production and sale of "for export only" anti-inflammatory and bleaching creams is a large profit business for many European pharmaceutical companies. These products are sold in African countries where they are sought for their skin whitening action. In the last years, the EU RAPEX (Rapid Alert System for non-food consumer products) [5] evidenced the presence in the European market of a large number of illegal and counterfeit creams claimed as cosmetics with anti-inflammatory and skin lightening action containing glucocorticoids. Thereafter numerous seizures were made by competent authorities. Two possible sources for these products in the European countries can be hypothesized: (i) "for export only" anti-inflammatory creams are legally exported from Europe to Africa and then illegally re-imported to Europe, where the selling price can be higher and a new interesting market is developing because of the large presence of immigrates from Africa and (ii) counterfeit creams mimicking the European "for export only" products are sold in the same African countries where the original products are commercialized and then illegally imported from there or from other extra-European countries to Europe. These products look like cosmetics, with brilliant coloured packaging and attracting images. They are advertised for their whitening action on black skin. Only an expert purchaser can realize that these products are medicines and thus potentially dan-

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Table 1
Details on extraction procedure for each medicinal sample.

Active substance (dosage form; label claim)	Extraction solvent	Dilution of medicinal sample; final mass concentration of active substance sample solutions (C_{fin})	Extraction procedure from medicinal samples
Betamethasone dipropionate (cream; 0.64 mg/g)	0.1% acetic acid methanolic solution	3 g in 50 mL; 0.038 mg/mL	Vortex, 5′ ultrasounds, 15′ water bath (60 °C), vortex, 5′ water bath
Clobetasol propionate (cream; 0.5 mg/g)	Methanol	3 g in 50 mL; C_{fin} = 0.03 mg/mL	Vortex, 15′ ultrasounds
Fluocinonide (cream; 0.5 mg/g)	Methanol	3 g in 50 mL; C_{fin} = 0.03 mg/mL	Vortex, 15′ ultrasounds
Methyl-prednisolone acetate (suspension; 40 mg/mL)	Methanol	0.5 mL of a mix of 10 vials dissolved in 50 mL then diluted 1:10; 0.04 mg/mL	Vortex, 15′ ultrasounds
Fluocinolone acetonide (cream; 0.25 mg/g)	Methanol	3 g in 50 mL; 0.015 mg/mL	Vortex, 15′ ultrasounds
Dexamethasone (ointment; 2 mg/g)	Absolute ethanol	300 mg of ointment in 50 mL, 0.012 mg/mL	Vortex, 15′ water bath (54 °C) under magnetic stirring, vortex, 5′ water bath

gerous for health if not appropriately used. These drugs are illegally sold in Europe in unauthorized shops such as "ethno-cosmetics-shops" and they are mainly bought by the immigrants looking for the same products used in their countries. Glucocorticoids such as betamethasone dipropionate, fluocinonide, clobetasol propionate are generally contained in these products.

The European Official Medicines Control Laboratories (OMCL) have been often asked by legal authorities to analyze these products to evaluate their quality, but, unfortunately, compendial methods (mainly BP [6] and USP [7]) are often obsolete and laborious and require different analytical conditions for each active ingredient. Scientific literature reports a number of different LC methods for each glucocorticoid in pharmaceutical formulations [8–11] or methods in biological matrices [12–15] but simple validated LC methods for the simultaneous assay of various glucocorticoids in pharmaceutical formulations are not usually reported [16,17].

In this study a new RP-LC method for the rapid simultaneous screening of six different active ingredients (betamethasone dipropionate, dexamethasone, fluocinonide, fluocinolone acetonide, clobetasol propionate, methyl-prednisolone acetate) in anti-inflammatory and skin lightening creams was developed and validated. These compounds were chosen because they were found to be the most commonly used in whitening products. Validation was designed with a view to a possible application of the method in different quality control laboratories. The associated measurement uncertainty was calculated from validation data. The method was applied to the analysis of whitening creams seized from illegal market by Carabinieri NAS (Nuclei Antisofisticazioni e Sanità), the Italian police force specialized on health matters.

2. Experimental

2.1. Chemicals

Betamethasone 17,21-dipropionate (98.7%), clobetasol propionate (98.5%) and fluocinonide (fluocinolone acetonide 21-acetate) (99.0%) were purchased from Sigma–Aldrich® (St. Louis, MO, USA); fluocinolone acetonide (100%), dexamethasone (100%) and methyl-prednisolone acetate (100%) were purchased from European Pharmacopoeia/EDQM (Strasbourg, France). HPLC-grade methanol and absolute ethanol were purchased from Panreac Quimica (Barcelona, Spain), while acetonitrile from Carlo Erba (Milano, Italy). All other reagents were of analytical grade.

Validation was performed on commercial glucocorticoids medicinal products from Italian market: (1) betamethasone 17,21-dipropionate 0.064% cream corresponding to 0.05% betamethasone alcohol (Diprosone, Schering-Plough S.p.A., Milano, Italy); (2) clobetasol propionate 0.05% cream (Clobesol, GlaxoSmithKline S.p.A., Verona, Italy); (3) fluocinonide 0.05% cream (Topsyn, Teofarma s.r.l., Pavia, Italy); (4) fluocinolone acetonide 0.025% cream (Localyn, Recordati, Milano, Italy); (5) dexamethasone 0.2% ointment (Luxazone, Allergan S.p.A., Roma, Italy) and (6) methyl-prednisolone acetate 40 mg/mL suspension (Depo-Medrol, Pfizer, Italia S.r.l., Latina, Italy). Illegal medicinal products containing fluocinonide, betamethasone dipropionate and clobetasol propionate were obtained from seizures made by police forces in the national territory.

2.2. Standard and sample solution preparation

Stock standard solutions were freshly prepared in methanol. Solutions were vortexed for 20 s and sonicated for 10 min and then diluted 5:20 in methanol to the final concentration (for each active substance the final mass concentration is reported in Table 1). Only dexamethasone stock solution was prepared in absolute ethanol at a concentration 10-fold the final one and then diluted with the same solvent.

Calibration curves were obtained by preparing standard solutions of each active substance in triplicate, while for chromatographic separation development and robustness studies, solutions containing a mix of the six analytes were prepared.

Sample solutions were freshly prepared following the extraction procedures reported in Table 1. Samples containing clobetasol propionate, fluocinonide and fluocinolone acetonide in creams were dissolved in methanol, while for betamethasone 17,21-dipropionate cream 0.1% acetic acid methanolic solution was used and samples were heated at 60 °C on a water bath to obtain the complete extraction of the active substance. Methyl-prednisolone acetate suspension was simply diluted in methanol taking care to mix well the suspension before dilution. Dexamethasone based product was dissolved in absolute ethanol, heated at 54 °C on a water bath under constant magnetic stirring. All sample solutions were pre-filtered on 1 μm glass filters (Acrodisc, Pall, Gelman Laboratory, USA) and then on 0.45 μm nylon filters (Superchrom S.r.l., Milano, Italy) prior to the analysis.

2.3. Chromatographic analysis

The chromatographic apparatus was a HPLC 1100 Series equipped with an automatic injector and a photo-diode array detector (Agilent Technologies, Waldbronn, Germany). For data collection and calculation Chemstation software from Agilent was used.

Chromatographic separation was obtained by isocratic elution with a Symmetry C18, 75 mm × 4.6 mm I.D., 3.5 μm particle size column (Waters Corporation, MA, USA), at a flow rate of 1.5 mL/min. Mobile phase was water/acetonitrile (50:50, v/v). Column temperature was 25 °C and the injection volume was 20 μL. Detection wavelength was 240 nm.

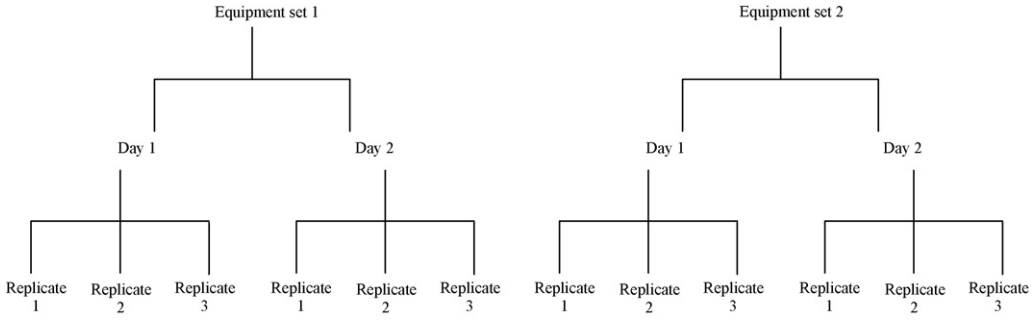

Fig. 1. The nested ANOVA design used to study the precision of the method is depicted. It permitted to trace sources of variability.

2.4. Validation of analytical procedure

Validation was performed considering the application field of the method (analysis of suspicious samples with possible dosage issues) and its exportability to other quality control laboratories.

For each analyte the method was validated in terms of specificity, linearity, precision, trueness (recovery studies) and robustness. Measurement uncertainty was investigated to understand how the analytical results obtained for each sample could change from one laboratory to another.

Recent studies [18–27] demonstrated that when uncertainty is evaluated from validation data, its estimate is comparable with the one obtained from an inter-laboratory study.

Hence in this work validation studies were designed specifically for this purpose. In particular indications from Barwick and Ellison [28–30] and Maroto et al. [31,32] were followed on how to properly plan validation studies to obtain information about uncertainty. In particular uncertainty assessment was performed combining precision, trueness and robustness uncertainty components. According to International Conference of Harmonization Guideline Q2(R1) [33], quantitation and detection limits were not evaluated because these parameters are not required for potency analysis of medicinal products. The test concentrations (C_{fin}) of analytes were chosen largely higher than the expected quantitation and detection limits.

2.4.1. System suitability testing

System suitability was performed on a solution containing standards of the six analytes at the test concentration. The acceptance criteria were: areas and retention times RSD% $\leq$1.0% for six replicate injections for each analyte and baseline separation for all peaks.

2.4.2. Specificity

Specificity was evaluated verifying the absence of chromatographic interferences with excipients, preservatives and related substances. Peak purity was checked on both standards and samples by the function "peak purity" of the chromatography management software, which was based on the analysis of the UV-spectra in various regions of the peak.

2.4.3. Linearity

Linearity was verified in the range 10–150% of the test concentration, considering that in counterfeit medicines the active substance content can greatly deviate from label claim. Stock standard solutions were prepared and then diluted to obtain a final concentration in the range 10–150% of the test concentration (C_{fin} are reported in Table 1).

2.4.4. Uncertainty assessment

Most of the information needed for uncertainty determination was collected from precision and trueness studies. The remaining sources of uncertainty were investigated through robustness evaluation.

Precision, trueness and robustness studies provided uncertainty components in form of relative standard deviations, indicated as $U_{precision}$, $U_{trueness}$ and $U_{robustness}$. All these components were then combined to obtain a relative *combined standard uncertainty* U_c:

$$U_c = \sqrt{U^2_{precision} + U^2_{trueness} + U^2_{robustness}}$$

A detailed description of the steps followed for uncertainty estimation, together with formulas and calculations were not reported in this paper since detailed procedures can be found elsewhere [18–34]. Only a schematic description will be given in the following paragraphs. Calculations were performed using a Microsoft Excel spreadsheet and the Statgraphics Centurion XV software [35].

2.4.5. Precision studies and evaluation of $U_{precision}$

Precision component of uncertainty (i.e. the uncertainty due to method imprecision) was determined as follows. A 3-factor different intermediate precision was determined, the 3 factors being equipment, time and random error. In particular a 3-factor nested experimental design was used. The nested array is recommended by ISO [36,37] and has been extensively described and tested by various authors [19–21,38]. The schematic layout of the design is given in Fig. 1. Samples were analyzed on two sets of equipment (comprising two HPLCs, two balances, two laboratory glasses sets, two reagents batches etc) in three replicate determinations in each of 2 different days. So a total of 12 data were collected for each studied substance. As suggested in Ref. [19], the two HPLC instruments were not operated on the same day to avoid an underestimation of the day effect. In each analysis the operator prepared a new standard solution in triplicate. Results were expressed as a percentage of the labelled amount. Outliers were determined by Grubb's test made on various combinations of data as indicated in Ref. [39], and deleted, while stragglers were retained. Cochran's tests were also performed. Analysis of variance (ANOVA) was used to obtain variance components from the 3 factors considered. In Refs. [20,36] guidance can be found on how to apply ANOVA for a nested experimental design. Variances were eventually combined (see Refs. [21,22] for further details) to obtain the precision component of uncertainty $U_{precision}$.

2.4.6. Trueness studies and evaluation of $U_{trueness}$

Trueness was investigated performing recovery studies for each substance by spiking experiments, following the experimental design and the scheme proposed in Ref. [26]. Three sample solutions were prepared and quantified as usual to obtain a mean dosage value C_{native}. Then other three sample solutions were prepared from the same cream tube and spiked with 1 mL of a standard solution so that a final concentration of approximately 150% of active substance could be recovered at the end of the analysis.

Table 2
Parameter variation in robustness studies.

Parameters	Determination number							
	1	2	3	4	5	6	7	8
Column temperature (°C)	20	20	20	20	30	30	30	30
Flow rate (mL/min)	1.3	1.3	1.7	1.7	1.3	1.3	1.7	1.7
Water/acetonitrile ratio (v/v)	47.5/52.5	52.5/47.5	47.5/52.5	52.5/47.5	47.5/52.5	52.5/47.5	47.5/52.5	52.5/47.5
Detection wavelength (nm)	235	235	245	245	245	245	235	235
Autosampler temperature (°C)	10	20	10	20	20	10	20	10
Column type[a]	A	B	B	A	A	B	B	A
Injection volume (μL)	15	25	25	15	25	15	15	25

[a] A and B were two different batches of the same column.

Table 3
Validation results and uncertainty estimation.

	Methyl- prednisolone acetate	Fluocinonide	Dexamethasone	Clobetasol propionate	Betamethasone dipropionate	Fluocinolone acetonide
Linearity (range 10–150%)	$Y = 25{,}123X - 3.1$	$Y = 23{,}980X + 0.1$	$Y = 28{,}765X + 7.2$	$Y = 24{,}338X + 1.1$	$Y = 25{,}489X - 9.9$	$Y = 26{,}195X - 1.2$
R^2	0.9999	0.9999	0.999	0.9999	0.9999	0.999
Mean value (%)	99.5	98.8	98.7	98.7	97.0	97.3
$RSD_{equipment}$	1.8	2.1	1.1	0.5	0	0.7
RSD_{day}	1.3	1.5	2.4	0.7	0.9	2.3
$RSD_{repeatability}$	0.5	1.3	1.4	1.0	1.1	1.0
Precision component of uncertainty ($U_{precision}$)	2.3	2.8	3.0	1.3	1.4	2.6
Mean recovery (%)	1.006	0.944	1.028	0.986	1.078	1.006
Trueness component of uncertainty ($U_{trueness}$)	1.7	1.9	2.1	1.7	1.8	1.7
Robustness component of uncertainty ($U_{robustness}$)	1.6	1.7	2.3	1.1	1.3	2
Combined uncertainty (U_c)	3.3	3.8	4.3	2.4	2.7	3.7

The mean recovery was determined as the change in observation divided by the change in concentration:

$$\bar{R} = \frac{1}{n}\sum_{i=1}^{n}\frac{C_{obs(i)} - C_{native}}{C_{spike(i)}}$$

where $C_{obs(i)}$ is the observed content of each spiked sample, $C_{spike(i)}$ is the amount of substance added by spiking with standard solution and n is the number of determinations for each recovery experiment ($n = 3$). See Refs. [26,30] for further details. A mean recovery was calculated from the three recovery values obtained.

The uncertainty associated with the estimate of recovery $U_{trueness}$ was calculated as reported in Ref. [26].

2.4.7. Robustness studies and evaluation of $U_{robustness}$

For each of the investigated substances the robustness testing procedure consisted of a two-level screening design as described in Ref. [40]. Seven parameters were investigated by eight determinations. The parameters considered were column temperature, flow rate, water/acetonitrile ratio, detector wavelength, autosampler temperature, column type and injection volume. Each parameter was examined at two levels. In Table 2 the experimental design is depicted together with the chosen investigated levels. To determine whether a variation in a parameter could affect significantly the result, a specific test was used as indicated in Ref. [25]. Although in Ref. [30] the use of a precision estimate assessed "over a short period of time" is suggested, here the time-different intermediate precision (RSD_{day} of Table 3) was used, following the recommendation by Hund et al. [18]. Uncertainty contributions from robustness studies were calculated as reported in Ref. [29]. Each parameter contributes with its own uncertainty. Each contribution, expressed as a relative standard deviation, was combined to obtain the global robustness uncertainty component:

$$U_{robustness} = \sqrt{U_{col.temp}^2 + U_{flow}^2 + U_{wat/ACN}^2 + U_{\lambda}^2 + U_{autos.temp.}^2 + U_{column}^2 + U_{inj.vol.}^2}$$

3. Results and discussion

The method developed provides a rapid separation (less than 7 min) of six glucocorticoids typically contained in skin whitening products. Fig. 2 reports the chromatographic separation of the six analytes. Even if these six components are not usually present in the same legal medicinal product, in the case of illegal/counterfeit products, replacement of an active substance with another or a co-presence of non-declared active substances can be observed. For this reason a method for counterfeit products should be able to resolve different active substances.

3.1. Precision studies

In Table 3 validation results and uncertainty estimates for each analyte are reported. The nested ANOVA statistical treatment of data provided information on the intra-day and inter-day variability and on the effects due to changing the laboratory equipment. In Table 3 these contributions to the total precision are reported as relative standard deviations and indicated as $RSD_{repeatability}$, RSD_{day}, and $RSD_{equipment}$ respectively.

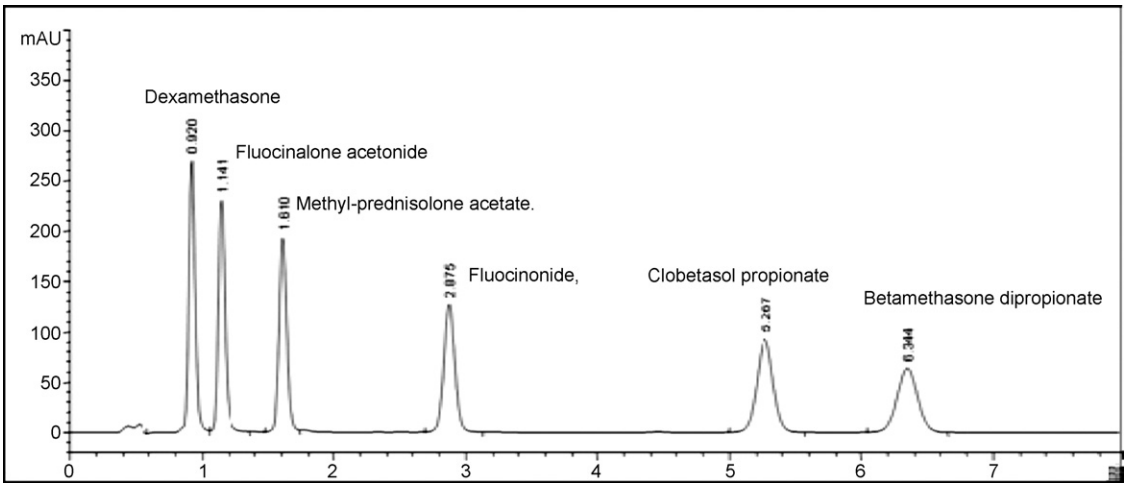

Fig. 2. Chromatographic separations of dexamethasone (R.T. = 0.92 min), fluocinolone acetonide (R.T. = 1.14 min), methyl-prednisolone acetate (R.T. = 1.61 min), fluocinonide (R.T. = 2.88 min), clobetasol propionate (R.T. = 5.27 min), betamethasone 17,21-dipropionate (R.T. = 6.34 min). Chromatographic conditions are reported in Section 2.

In general the largest contribution to method imprecision comes from equipment variation, followed by time effect, while intra-day random effect gives the smallest contribution. In this study, instead, inter-day and intra-day precision were quite similar. This possibly suggests that a variability source common to both intra-day and inter-day analyses is preponderant compared to other sources: the extraction process. Moreover the equipment component of precision is quite small and contributes to a less extent to the total method imprecision, especially for betamethasone dipropionate, clobetasol propionate, dexamethasone and fluocinolone acetonide. This data corroborates the hypothesis that the extraction alone explains the most part of method variability and thus the equipment effect is not very important compared to it.

Another possible explanation for the uncommon behaviour of the three precision components is related to the nature of the samples studied: in creams and ointments the active substance could not be homogeneously distributed inside the bulk and this alone may be a variation source preponderant over all other effects considered in the study.

The exact opposite was reported for methyl-prednisolone acetate: from Table 3 it can be noticed that in this case the trend of precision components is the one commonly expected. The intra-day precision was rather small compared to inter-day and equipment components. This data can be easily explained since methyl-prednisolone acetate analysis did not comprise an extraction process because it is a suspension and not a cream or ointment. Moreover the heterogeneity in active substance distribution inside the product is not an issue here because 10 vials were premixed in each experiment. Therefore these results indirectly confirm the hypothesis made to describe the precision behaviour reported for the other substances.

3.2. Trueness studies

For each substance no significant bias was detected as the mean recovery was not statistically different from 1. The recovery uncertainty values obtained showed that the method performed similarly in terms of trueness for all the investigated substances.

3.3. Robustness studies

For most of the active substances the method resulted totally robust. Only for fluocinolone acetonide injection volume and water/ACN ratio were borderline parameters, even if not statistically significant. This is reasonably explained by the fact that separation of fluocinolone acetonide from dexametasone is critical.

3.4. Application to illegal/counterfeit products

The method was applied to the analysis of illegal products seized by the Italian police force Carabinieri NAS. Products were first analyzed following the extraction procedure specific for the declared active substance which was then quantified if actually present in the extract. If a substance different from the declared one was present it would still be evidenced in the chromatogram even if the extraction procedure used was not the most suitable. So at this stage the substance found was only identified (comparing its retention time and UV spectrum with those of the six glucocorticoids) but not quantified because the extraction could be incomplete. Finally to carry out a correct quantitation a new extraction was performed by the procedure specific for the substance found.

In some cases the quality of the illegal/counterfeit products analyzed was found to be not compliant to European standards for medicinal products and often a high content of degradation products was noticed. In Table 4 some examples of results obtained are

Table 4
Results obtained on illegal/counterfeit medicinal products.

Illegal/counterfeit medicinal product sold as cosmetic	Active substance and label claim	Assay of label claim (CV%)
Sample A (gel)	Fluocinonide 0.5 mg/g	63.6% (0.5)
Sample B (gel)	Fluocinonide 0.25 mg/g	46.9% (1.0) + fluocinolone acetonide[a]
Sample C (gel)	Betamethasone dipropionate 0.65 mg/g	72.6% (0.3) + monoesters
Sample D (gel)	Betamethasone dipropionate 0.65 mg/g	92.9% (0.2)
Sample E (cream)	Clobetasol propionate 0.5 mg/g	99.8% (0.4)

[a] Equivalent to 47.0% (1.4) of fluocinonide.

reported. In sample B the presence of a high quantity of fluocinolone acetonide was evidenced in place of fluocinonide (fluocinolone acetonide 21-acetate) probably due to the ester hydrolysis and in sample C (betamethasone 17,21-dipropionate gel) the presence of monoesters was evidenced. In both cases the presence of hydrolysis products was evidenced by unknown peaks in the chromatograms, that were later identified by running a series of standards (commercially available or synthesized in house). The out-of-specification results highlighted the low quality of these products, probably due to bad storage conditions and bad manufacturing practices (non-stable formulations or partially hydrolysed active substance).

4. Conclusions

The presence of illegal/counterfeit medicinal products in illicit market is a serious public health problem that should be combated by reinforcing police controls, organizing information campaigns for citizens and immigrants and strengthening the analytical capabilities of control laboratories to rapidly analyze a large number of samples in a short time by simple methods. To this aim the present method was developed and validated allowing the simultaneous separation and determination of six different glucocorticoids (betamethasone dipropionate, dexamethasone, fluocinonide, fluocinolone acetonide, clobetasol propionate and methyl-prednisolone acetate) that are the most common active substances contained in illegal and counterfeit creams, gels and ointments for dermatological action. The discrimination among various glucocorticoids is of particular concern considering that a counterfeit medicine could contain non-declared substances or show a co-presence of different active pharmaceutical ingredients. Moreover a single chromatographic method for several active ingredients allows rapid and vast scale screening of large numbers of illegal and counterfeit products. Previous works have pointed out [41–43] that LC screening methods for counterfeit medicines should be easy to use and robust enough to be applied by other quality control laboratories even in the absence of last generation chromatographic apparatus.

The method presented in this paper resulted robust toward all the investigated parameters, linear in a wide concentration range and sufficiently accurate and precise. Moreover many different drug formulations were employed during validation: this allowed to prove that the method is suitable for a wide variety of matrixes and that excipients do not influence its performance noticeably.

Obviously, if a laboratory was equipped with a mass spectrometer detector, its capability in the identification of unknown substances would be much enhanced, but for all laboratories with poor analytical resources, this method can be a very useful tool in the fight against counterfeiting.

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