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ORIGINAL RESEARCH PAPER

# Development and validation of RP-HPLC method for quantification of trace levels of topical corticosteroids in ambiphilic cream

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**ABSTRACT**

Corticosteroids are anti-inflammatory and immunosuppressant drugs. Topical corticosteroids formulations (ointments, creams, gels) are used in the treatment of different types of dermatitis and urticaria. Considering their therapeutic and whitening effects, they are frequently used for counterfeiting of cosmetic products. Corticosteroids can cause different local and systemic side effects. HPLC method is often chosen for their analysis, because it is selective, sensitive, precise, simple and fast.

The aim of this study was optimization and validation of RP-HPLC method with UV detection for determination of trace levels of corticosteroids in ambiphilic creams. This method is used for qualitative and quantitative analysis of evaluated corticosteroids.

Mometasone furoate, hydrocortisone acetate, fluocinonide, fluocinolone acetonide, betamethasone, betamethasone dipropionate and triamcinolone acetonide were evaluated. Separation was performed on Inertsil® ODS-3V 250 × 4.6 mm, 5 µm chromatographic column. Mobile phase was mixture of acetonitrile and water 50:50 (v/v) with gradient elution and flow rate 1 mL $min^{-1}$. Column temperature was held on 40 °C and UV detection was performed at 240 nm.

Selectivity, linearity, accuracy, precision and limit of quantification (LOQ) were evaluated. Method is selective because ambiphilic cream base peaks and corticosteroids peaks were not overlapping. Linearity was confirmed since correlation coefficient was 1 for all compounds. Accuracy and precision were evaluated for hydrocortisone acetate and betamethasone dipropionate. Determined Recovery values were in range of 70–130%. Both RSD values (21.46% and 9.59%) were lower than 30%. Method is highly sensitive since LOQ concentrations were in ng $mL^{-1}$ range.

All evaluated parameters of validation were in accordance with regulatory requirements. Validated RP-HPLC method can be used for qualitative and quantitative analysis of selected corticosteroids in ambiphilic creams.

**KEYWORDS**

topical corticosteroids, RP-HPLC, validation, ambiphilic creams

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## INTRODUCTION

Corticosteroids are anti-inflammatory and immunosuppressive drugs, with effects of vasoconstriction, antiproliferative and antipruritic properties. They are derivatives of pregnane, which contains conjugated double bond (3-on-4-ene) in ring A and at the C21 position beta ketol with reductive properties. They are widely used for the treatment of systemic and local inflammatory diseases [1].

Topical corticosteroids are used for the treatment of different types of dermatitis, plaque psoriasis, *lichen simplex*, *lichen planus*, cutaneous *lupus erythematosus* and papular urticaria. The most common formulations for topical use containing corticosteroids are ointments, gels and creams. Their use is associated with different local adverse effects and risks, such as exacerbation of untreated infections, skin atrophy, irreversible stretch marks, red skin lesions, acne, hypertrichosis etc. When absorbed, topical corticosteroids can cause systematic side effects such are: hypertension, *diabetes mellitus*, bleeding, osteoporosis, infections, muscle weakness and delayed healing of wounds. Seriousness of adverse effects depends on corticosteroids' potency, skin area where the drug is applied and the duration of treatment. If the formulation is applied on areas of thin skin like armpit, face, scrotum, hair follicles or sweat glands or on damaged skin, the risk of systematic side effects is larger. The therapy should begin with low potency corticosteroids, if it is possible. In some severe conditions, the use of high potency corticosteroids is justified. The preparation should be applied only on affected skin area in thin layer, usually for 1 week. Topical corticosteroids should be used carefully with pediatric population. Higher ratio of child's skin area and body mass indicates higher risk for systematic absorption of corticosteroids and consequently Cushing disease, intracranial hypertension, retardation, slower growth and obesity in children. Regardless, corticosteroids have good risks/benefits ratio [2].

Corticosteroids are often used for counterfeiting of cosmetic products, claiming they have therapeutic effects. Skin whitening is one of the adverse effects which is used for counterfeiting of cosmetic products.

Figure 1 shows the structural formulas of the topical corticosteroids tested in this paper.

RP-HPLC (Reversed Phase High Performance Liquid Chromatography) with UV detection is often used for corticosteroids analysis, since it is selective, fast, sensitive and simple [3–12]. In order for the method to be used in quality control, its validation must be performed. If the purpose of the method is determination of impurities in finished product, following validation parameters should be tested: selectivity, linearity, accuracy, precision and limit of quantification. [13]. The aim of this paper was optimization and validation of RP-HPLC method for determination of trace levels of corticosteroids in ambiphilic creams. This method can be used for determination of seven different corticosteroids: mometasone furoate (MF) ([(8S,9R,10S,11S,13S,14S,16R,17R)-9-chloro-17-(2-chloroacetyl)-11-hydroxy-10,13,16-trimethyl-3-oxo-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] furan-2-carboxylate), hydrocortisone acetate (HA) ([2-[(8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,6,7,8,9,11,12,14,15,16-decahydro-1H-cyclopenta[a]phenanthren-17-yl]-2-oxoethyl] acetate), fluocinonide (F) ([2-[(1S,2S,4R,8S,9S,11S,12R,13S,19S)-12,19-difluoro-11-hydroxy-6,6,9,13-tetramethyl-16-oxo-5,7-dioxapentacyclo[10.8,0.02,9.04,8.013,18]icosa-14,17-dien-8-yl]-2-oxoethyl] acetate), fluocinolone acetonide (FA) ((1S,2S,4R,8S,9S,11S,12R,13S,19S)-12,19-difluoro-11-hydroxy-8-(2-hydroxyacetyl)-6,6,9,13-tetramethyl-5,7-dioxapentacyclo[10.8.0.02,9.04,8.013,18]icosa-14,17-dien-16-one), betamethasone (B) ((8S,9R,10S,11S,13S,14S,16S,17R)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-3-one), betamethasone dipropionate (BDP) ([2-[(8S,9R,10S,11S,13S,14S,16S,17R)-9-fluoro-11-hydroxy-10,13,16-trimethyl-3-oxo-17-propanoyloxy-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl]-2-oxoethyl] propanoate) and triamcinolone acetonide (TA) ((1S,2S,4R,8S,9S,11S,12R,13S)-12-fluoro-11-hydroxy-8-(2-hydroxyacetyl)-6,6,9,13-tetramethyl-5,7-dioxapentacyclo[10.8.0.02,9.04,8.013,18]icosa-14,17-dien-16-one), which improves the investigation of counterfeited pharmaceuticals and cosmetic products.

## EXPERIMENTAL

### Chemicals and reagents

Hydrocortisone acetate, mometasone furoate, fluocinonide, fluocinolone acetonide, triamcinolone acetonide, betamethasone and betamethasone dipropionate were all purchased from Sigma-Aldrich Chemie GmbH (Germany) and were European Pharmacopoeia reference standards. Methanol and acetonitrile, HPLC grade were purchased from Sigma-Aldrich Chemie GmbH (Germany). Purified water was obtained by water purifying system (TKA GenPure, Thermo Electron LED GmbH, Germany). Ambiphilic cream *Cremor Basalis* (germ. *Deutsche Arzneimittel Codex, DAC*) was used as a matrix.

### Chromatographic conditions

Analysis was performed on Dionex Ultimate 3000 HPLC system (Thermo Scientific, USA) equiped with Chromeleon 7 software. Separation of analytes was conducted on *Inertsil*® *ODS-3V*, 250 × 4.6 mm, 5 µm chromatographic column. Mobile phase consisted of acetonitrile and water with gradient elution (Table 1), with flow rate 1 mL $min^{-1}$ and injection volume 100 µL. Column temperature was hold on 40 °C. UV detection of all investigated steroids was performed on 240 nm. UV detector conditions were set as following: bandwith 4, peak width 0.100 min, data collection rate 25.0 Hz, response time 1.000 s. Chromatogram that represents topical corticosteroids' peaks under selected chromatographic conditions is shown on Fig. 2. Achieved retention times were: 5.3, 7.3, 8.4, 9.7, 14.5, 17.1 and 17.7 for B, TA, FA, HA, F, MF and BDP respectively.

### Validation procedure

***Solution preparation.*** The extraction of 1g of ambiphilic cream by 10 ml of acetonitrile was performed in ultrasonic bath for 10 min on room temperature. The extract was filtered through a 0.45 µm cellulose acetate filter and then injected in HPLC system. This solution has been used for testing selectivity. LOQ has been calculated based on signal

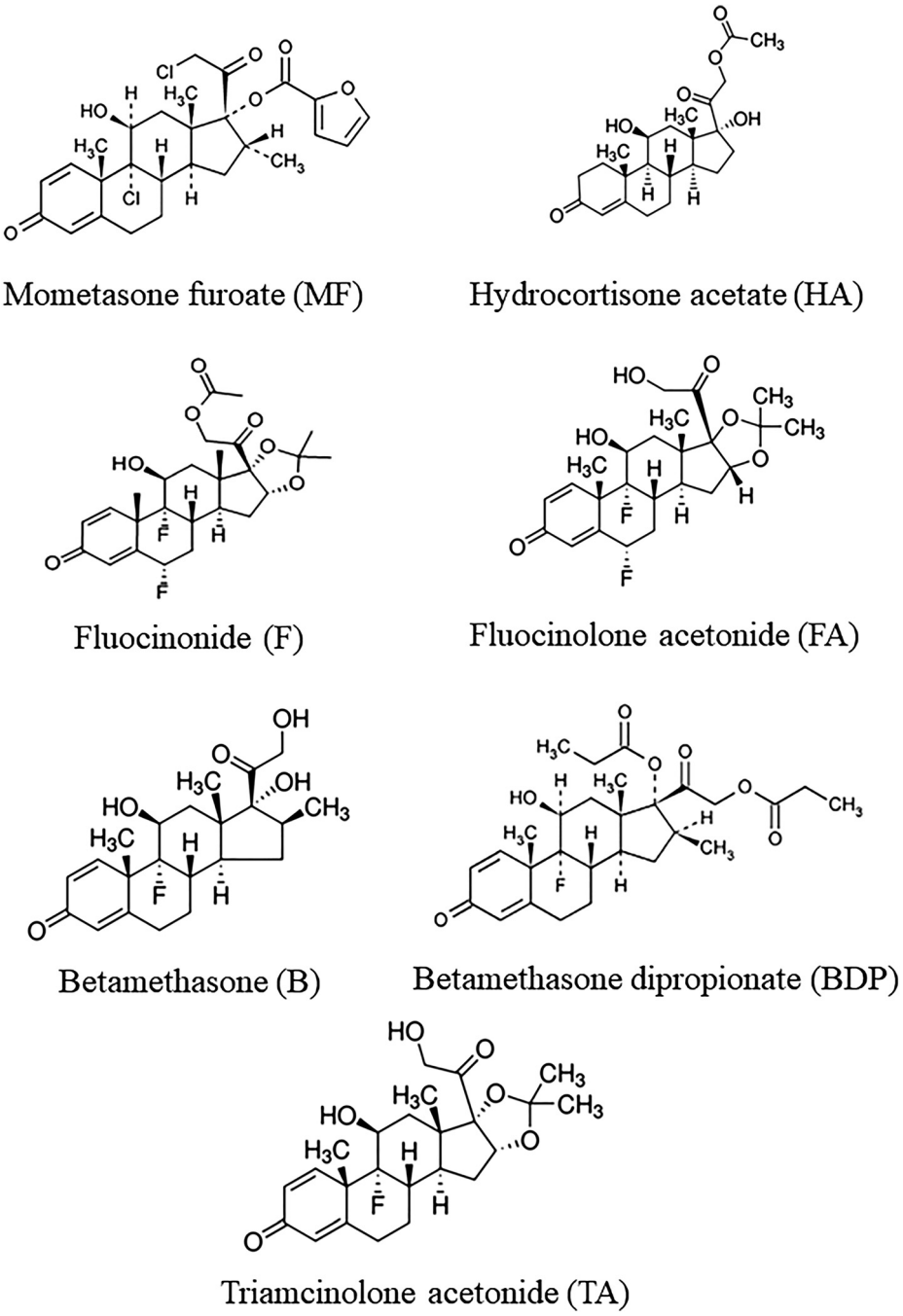


*Fig. 1.* Structures of investigated compounds

*Table 1.* Gradient elution

| Time (minutes) | Acetonitrile (%) |
|---|---|
| 0.00 | 35 |
| 10.00 | 35 |
| 11.00 | 60 |
| 16.00 | 60 |
| 19.00 | 35 |
| 20.00 | 35 |

to noise ratio (S/N). According to guidelines [13] determination of LOD is not necessary for analysis od trace levels of compounds. For stock solution, 5 mg of each standard substance separately was weighed and dissolved in 10 mL of methanol. Than, 100 µl of prepared solution was diluted to 10 mL using mixture of acetonitrile and water (50:50 v/v). In this way, a stock solution with a concentration of 5 µg $mL^{-1}$ was obtained. Working solutions for calibration curve determination were prepared in triplicates using acetonitrile/water mixture (50:50 v/v) in concentrations shown in Table 2.

For accuracy assessment, ambiphilic base was loaded with standard substances hydrocortisone acetate and betamethasone dipropionate, separately. Multiple extraction with 25 mL of acetonitrile on ultrasonic bath for 10 min on room temperature was performed. Concentration of obtained extract

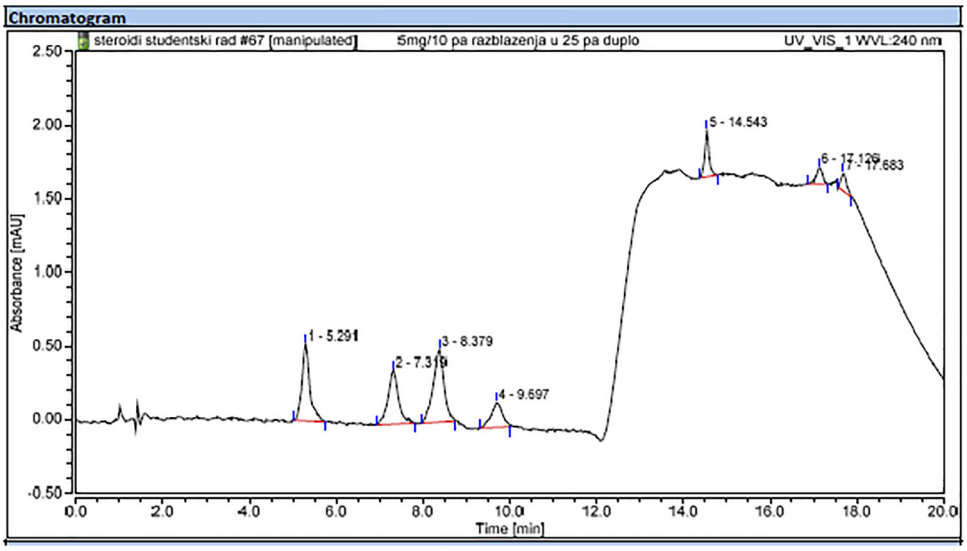


*Fig. 2.* Representative chromatogram of investigated corticosteroids (1-B, 2-TA, 3-FA, 4-HA, 5-F, 6-MF, 7-BDP)

*Table 2.* Concentration range for assessing linearity

| MF | HA | F | FA | B | BDP | TA |
|---|---|---|---|---|---|---|
| concentration (μg $mL^{-1}$) | | | | | | |
| 00.125 | 0.01563 | 0.009 | 0.03125 | 0.021 | 0.0105 | 0.0105 |
| 0.025 | 0.03125 | 0.018 | 0.125 | 0.084 | 0.021 | 0.042 |
| 0.05 | 0.125 | 0.036 | 0.25 | 0.168 | 0.042 | 0.084 |
| 0.125 | 0.3125 | 0.072 | 0.625 | 0.42 | 0.084 | 0.168 |
| 0.25 | 0.625 | 0.36 | 1.25 | 0.84 | 0.42 | 0.84 |
| 0.5 | 1.25 | 0.72 | 2.5 | 1.68 | 0.84 | 1.68 |
| 1 | 2.5 | 1.44 | 5 | 3.36 | 1.68 | 3.36 |
| 2 | 5 | 2.88 | 7.5 | 6.72 | 3.36 | 6.72 |

was approximately 0.01 mg $mL^{-1}$ for each compound. For analysis, extracts were diluted with mixture of acetonitrile and water (50/50 v/v). Accuracy was assessed in three concentration levels with three replicates each. Standard solutions were prepared in the same concentrations (0.025, 0.1 and 0.25 μg $mL^{-1}$ for HA and 0.0168, 0.0672 and 0.168 μg $mL^{-1}$ for BDP). For precision assessment, repeatability was tested in one concentration with six replicates (0.025 μg $mL^{-1}$ for HA and 0.0168 μg $mL^{-1}$ for BDP). Software used for data analysis was Microsoft Excell.

## RESULTS AND DISCUSSION

Selectivity was assessed by comparing ambiphilic base's chromatogram and chromatogram of mixture of corticosteroid standard substances. The presence of coelution of ambiphilic base's components with corticosteroids was tested (Figs 2 and 3). LOQ was calculated according to signal to noise ratio (S/N). Criteria was S/N = 10 (Table 3). Calibration curves were constructed to test linearity. The calibration curve represents the linear relationship of the corticosteroid solution concentration and the peak area and is obtained by the method of least squares. Linearity was evaluated using coefficient of correlation, *r*. Parameters of regression analysis are shown in Table 3.

Accuracy and precision were evaluated for hydrocortisone acetate and betamethasone dipropionate using Recovery value (R) (Table 3) for accuracy and standard deviation (SD) and relative standard deviation (RSD) for precision (Table 3).

The HPLC method with UV detection was chosen because it is selective, precise, sensitive, simple and not expensive. Based on the chemical structure and logP values that indicate the lipophilicity of the tested corticosteroids, the RP-HPLC system was selected. The stationary phase used is silica gel modified with octadecyl groups (C18), which is lipophilic column. The presence of a chromophore (3-on-4-ene) enables the detection of corticosteroids in the UV region, at a wavelength of 240 nm.

Optimization of chromatographic conditions was performed by changing the composition of the mobile phase, the appearance of the gradient and the wavelength of the detector. The mobile phases used in the optimization were: methanol and water in different ratios, acetonitrile and water in different ratios and methanol/acetonitrile/water in the ratio 60/30/10 (v/v). Chromatographic conditions were selected based on the following criteria: resolution factor

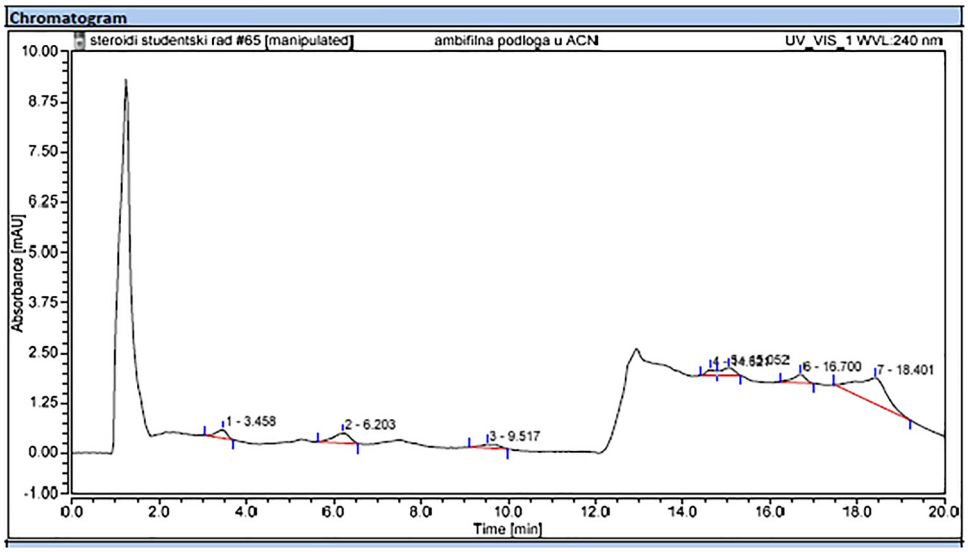


*Fig. 3.* Chromatogram of ambiphilic cream (*Cremor basalis*)

*Table 3.* Validation parameters

| Parameter | MF | HA | F | FA | B | BDP | TA |
|---|---|---|---|---|---|---|---|
| Linearity | | | | | | | |
| Range (μg mL$^{-1}$) | 0.0125–2 | 0.01563–5 | 0.009–2.88 | 0.03125–7.5 | 0.021–6.72 | 0.0105–3.36 | 0.0105–6.72 |
| Slope (a) | 2.072 | 2.260 | 2.459 | 2.858 | 3.049 | 1.696 | 2.523 |
| Coefficient of correlation (r) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **LOQ (ng mL$^{-1}$)** | 12.5 | 15.625 | 9 | 31.25 | 21 | 10.5 | 21 |
| **Precision (RSD, %)** | | 21.46 | | | | 9.59 | |
| **Accuracy (Recovery, %)** | | | | | | | |
| Level 1 | | 92.00 | | | | 80.11 | |
| Level 2 | | 88.70 | | | | 82.83 | |
| Level 3 | | 77.33 | | | | 87.50 | |

(Rs) greater than 1.2; number of theoretical plateaus (N) greater than 2000; peak symmetry factor (As) 0.8–1.2; retention factor ($k$) 1–10. Under selected chromatographic conditions, validation of the method for the determination of seven trace corticosteroids in ambiphilic creams was performed. As can be seen at Figs 2 and 3, at around 12 min the baseline of the chromatogram increase drastically which is due to selected gradient mobile phase. Selectivity, linearity, accuracy, precision and limit of quantification were examined.

Since the concentration of corticosteroids in the sample is very low (0.05%) and some excipients in the cream are lipophilic and can damage the chromatographic column, it is necessary to perform extraction [15]. The extraction of analytes from the samples is based on the QuEChERS (quick, easy, cheap, effective, rugged and safe) principle. After review of the literature [4, 7] we selected multiple extractions with acetonitrile with mixing on an ultrasonic bath for 10 min on room temperature. This way we achieved an adequate yield and prevented damage of the column by excipients, and the procedure is simple and fast.

By comparing the chromatograms of the ambiphilic base and the chromatograms of the mixture of corticosteroid standards, it was determined that there is no overlapping of the peaks originating from the ambiphilic base at the retention times of corticosteroids. It was confirmed that there is no coeulation of the components of the ambiphilic base with the tested corticosteroids and that the method is selective.

Calculated limits of quantification were low (ng mL$^{-1}$), which indicates that the method is highly sensitive (LOQ 9–31.25 ng mL$^{-1}$). Golubović et al. validated the LC-MS/MS method for the determination of illegal corticosteroids in cosmetic creams. The quantification limit for fluocinolone acetonide, fluocinonide, hydrocortisone acetate and betamethasone dipropionate in that paper was 3 ng mL$^{-1}$ [14]. This confirmed that the mass spectrometer (LOD 100 ag to 1 ng) has a higher sensitivity than the UV detector (LOD 1 pg to 1 ng) as expected. However, UV detector is most often used in quality control, due to the lower price and ease of use [15, 16].

Linearity was assessed over a wide range of concentrations. The requirement is that the value of the correlation coefficient $r$ should be ≥0.990, when the level of impurities in the finished product is <0.1% and the number of samples is ≥6. The correlation coefficient is 1 for all seven corticosteroids tested, so it was concluded that the method is linear.

The Recovery value is required to be in the range of 70–130%, when the impurity level is less than 0.2% in the finished product. All Recovery values belong to this range.

The requirement for the precision of the method is RSD≤30%, when the level of impurities in the finished product is less than 0.1% and the number of samples ≥6. RSD values for hydrocortisone acetate and betamethasone dipropionate meet the requirement.

## CONCLUSION

Validated HPLC method is fast, accurate and sensitive and as such, it can be used for quantitative and qualitative analysis of seven topical corticosteroids (mometasone furoate, hydrocortisone acetate, fluocinonide, fluocinolone acetonide, bethametasone, bethametasone dipropionate and triamcinolone acetonide) in the pharmaceuticals and cosmetic products, when cross contamination and counterfeiting of products are suspected.

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