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Liquid chromatography/tandem mass spectrometry for simultaneous determination of undeclared corticosteroids in cosmetic creams

Jelena B. Golubović*, Biljana M. Otašević, Ana D. Protić, Aleksandra M. Stanković and Mira L. Zečević

University of Belgrade-Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450, Belgrade, Serbia

RATIONALE: Undeclared corticosteroids in creams intended for frequent use might cause serious side-effects, especially in children. In order to prevent this or find the cause, it was essential to develop a method for quick detection and quantification of low levels of corticosteroids.

METHODS: Eleven corticosteroids were used in this study: prednisolone, methylprednisolone, prednisolone-21-acetate, fluocinolone acetonide, fluocinolone acetonide-21-acetate, hydrocortisone-21-acetate, dexamethasone, betamethasone, betamethasone dipropionate, clobetasol propionate and triamcinolone. Separation was achieved via liquid chromatography (LC), and mass spectrometric analysis was conducted by electrospray ionization triple-quadrupole mass spectrometry (MS/MS) in the multiple reaction monitoring mode using corticosterone as internal standard.

RESULTS: Good separation by using a gradient-elution LC/MS/MS method with run time of 25 min enabled the use of a segmented detection method and consecutive decrease in detection limits. The proposed method has been validated in the linearity range of 10–1000 ng/mL with coefficients of determination higher than 0.990. The method has shown to have very low limits of quantification (0.75–3 ng/mL) with satisfactory precision and accuracy for each of the corticosteroids.

CONCLUSIONS: An LC/MS/MS method for the rapid and simultaneous determination of low levels of eleven topical corticosteroids in creams was developed, optimized and validated. The proposed method can be used for testing of different products indicated for the treatment of atopic dermatitis, including "natural products", and "herbal creams" with "miraculous effects". Copyright © 2015 John Wiley & Sons, Ltd.

Corticosteroids are a class of steroid hormones naturally synthesized in the adrenal cortex from cholesterol. According to their biological activity and pharmacological effects, corticosteroids can be divided into two groups: glucocorticosteroids (i.e. cortisol and cortisone), which regulate many aspects of metabolism and immune functions, and mineralcorticosteroids, which regulate blood volume and electrolyte content.^[1] Chemical modifications of cortisol have led to synthetic derivatives with increased therapeutic potency but reduced mineralcorticoid effects. As drugs, synthetic corticosteroids are frequently used in human and veterinary medicine, often in combination with other compounds. Their use is related to their anti-inflammatory and immunosuppressant properties, which reduce the clinical manifestations of disease in a wide range of disorders. Treatment can be by oral administration, intramuscular, subcutaneous or intravenous injection, or as a topical application. Topical corticosteroids are known to be highly effective drugs widely used in dermatology for

the treatment of inflammatory diseases including atopic dermatitis. However, the improper use of potent or very potent topical steroids can cause serious side effects such as hypertension, diabetes mellitus, osteoporosis, rebound worsening of eczema on withdrawal, growth retardation, and suppression of the hypothalamic-pituitary-adrenal axis, particularly in children, and most common irreversible skin atrophy.^[2,3] Everyday care of inflamed skin requires very frequent use of emollients in order to maintain skin-barrier function. Patients with atopic dermatitis should liberally apply emollients to the entire body whether or not active symptoms are present.^[4,5] In addition, a number of "herbal creams" can be found on the market, especially the internet market. Worrying is the fact that a large percentage of "herbal creams" were found to contain corticosteroids.^[6,7] Frequent, long-term use of preparations that contain undeclared topical corticosteroids can cause serious side effects even in low doses, especially in children.

Liquid chromatography/mass spectrometry (LC/MS) and tandem mass spectrometry (LC/MS/MS) methods have been widely used to monitor corticosteroids in different matrices (urine and animal tissues).^[1,8–11] A couple of papers deal with non-allowed drugs in cosmetic creams.^[12,13] Also, simultaneous analysis of 2–6 corticosteroids at low level

* Correspondence to: J. B. Golubović, University of Belgrade-Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450, Belgrade, Serbia.
E-mail: golub@pharmacy.bg.ac.rs

concentrations in cosmetic preparations by means of LC/MS or LC/MS/MS has been reported.^[14–17]

The aim of this study was to propose an LC/MS/MS method fit for routine official control of low levels of unlabelled corticosteroids in creams. The method should be suitable for simultaneous determination of eleven topical corticosteroids, more than any published method.^[14–17] These corticosteroids are: prednisolone, methylprednisolone, prednisolone-21-acetate, flucinolone acetonide, flucinolone acetonide-21-acetate, hydrocortisone-21-acetate, dexamethasone, betamethasone, betamethasone dipropionate, clobetasol propionate and triamcinolone (Figs. 1(a)–1(k)). In addition, the proposed method should be selective enough to enable identification and determination of lower concentrations of corticosteroids than the published methods^[14–17] in a short run time.

This kind of method for the determination of unlabelled corticosteroids in creams is especially crucial in the prevention of side effects in children, since many of these creams are intended to be applied also to children's skin. Children are more prone to the development of systemic reactions to topically applied medication because of their higher ratio of total body surface area to body weight and skin structure. Cutaneous adverse effects occur regularly with prolonged treatment and are dependent on the chemical nature of the drug, the vehicle, and the location of its application.^[18]

EXPERIMENTAL

Chemicals, solvents and samples

LC/MS grade acetonitrile and 0.1% formic acid in water were obtained from Fisher Scientific (Loughborough, UK). Corticosteroid standards: prednisolone, methylprednisolone, prednisolone-21-acetate, flucinolone acetonide, flucinolone

acetonide-21-acetate, hydrocortisone-21-acetate, dexamethasone, betamethasone, betamethasone dipropionate and triamcinolone, were purchased from Tokyo Chemical Industry (Tokyo, Japan). The clobetasol propionate standard was purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). The corticosterone standard (Fig. 1(l)), used as internal standard (IS), was obtained from EMD Chemicals Inc. (San Diego, CA, USA). The purity of all standards was higher than 98%. Stock solutions of each standard substance (1 mg/mL) were prepared in acetonitrile. Seventeen different branded cosmetic products were obtained in local pharmacies. Two preparations prepared in a dermatologic clinic, and one "natural" product purchased on-line were also analyzed.

Instrumentation

An Accela high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific Inc.), consisting of autosampler, quaternary pump, degasser and hyphenated to a TSQ Quantum Access Max triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source, was used for this study. ChromQuest software (version 5.0.) was used for data acquisition and processing. Chromatographic separation was performed using an Atlantis T3 column (4.6 mm × 150 mm, 3 μm; Waters, Dublin, Ireland). Injection volume was 10 μL.

Mass spectrometric conditions

Firstly, the identification of a unique precursor and most abundant transitions was performed. Each analyte was dissolved in acetonitrile at concentration of 1 μg/mL and was directly infused into the ESI source at a flow rate of 20 μL/min via a syringe pump (Hamilton, Reno, USA). The two most abundant transitions were selected for further multiple reaction monitoring (MRM) confirmation. In order

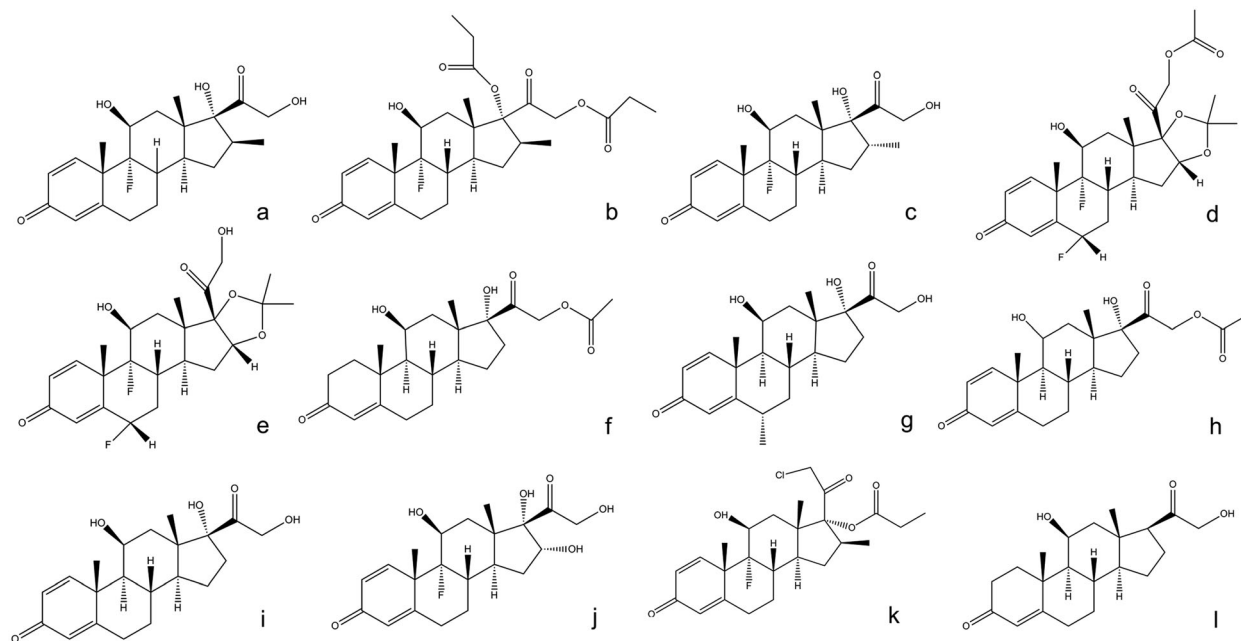


Figure 1. Chemical structures of corticosteroids: (a) betamethasone, (b) betamethasone dipropionate, (c) dexamethasone, (d) flucinolone acetonide-21-acetate, (e) flucinolone acetonide, (f) hydrocortisone-21-acetate, (g) methylprednisolone, (h) prednisolone-21-acetate (i) prednisolone, (j) triamcinolone, (k) clobetasol propionate, (l) corticosterone.

to enable simultaneous determination of eleven analytes, the method was divided into four segments. During each segment only the analytes which eluted in that period were scanned. In this way ionization and collision parameters were optimized separately for each segment for those analytes which were eluted during the segment time range. Each group of analytes which belonged to a specific segment was dissolved in acetonitrile at concentration of 1 µg/mL and infused into the ESI source and then MS parameters were optimized using the MRM optimization option. Optimization for the yield from collision energy was automatically performed by the instrument control software. ESI in positive ion mode was selected for the mass spectral analysis.

Table 1. Optimized ionization and collision parameters

ESI parameter	Segment 1	Segment 2	Segment 3	Segment 4
Spray voltage (V)	4000	4500	5000	5000
Vaporizer temperature (°C)	354	362	343	349
Sheath gas pressure (psi)	10	60	10	60
Capillary temperature (°C)	200	200	200	223
Auxiliary gas pressure (psi)	55	45	55	45
Collision gas pressure (mTorr)	2	1.8	1.6	2.1

Optimized parameters for each segment are presented in Table 1. These parameters were then included in the method. The scan time was 0.3 s, and scan width 0.2 u.

Validation procedures

Validation was carried out according to regulatory guidelines.^[19–22] Examined parameters were specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), recovery and matrix effect. Standard stock solutions (1 mg/mL) of analytes and IS were prepared in acetonitrile. Ten commercial products which suppose to contain all of the compounds that can be found in creams and can lead to certain interferences (excipients, preservatives, etc.) were previously subjected to screening to confirm absence of the analytes. These creams were mixed and used as blank. The validation has been performed using o/w creams as this is the major cosmetic formulation used for everyday care of atopic skin.

Quality control (QC) samples containing different amounts of corticosteroids (10, 100 and 1000 ng/mL) and calibration samples (LOQ, 10, 50, 100, 500, 1000 ng/mL) were prepared to be used for calculation of validation parameters in the following manner: 1 g of blank was spiked with a suitable amount of standard stock solutions of each analyte except for dexamethasone in a 10 mL volumetric flask. This was diluted with about 5 mL of acetonitrile and then ultrasonicated at 50 °C for 15 min. After cooling to room temperature, the flask was filled up to volume. A volume of 2.5 mL of this solution was transferred to a 5 mL volumetric flask filled up with 0.1% formic acid in water to volume. This solution was then filtered through a

Table 2. Parameters of the LC/MS/MS method

Segment	Duration of segment (min)	Drug	Retention time (min)	[M+H] ⁺	Collision energy (V)	Product ions (m/z)
1	(0–6.4)	Triamcinolone	4.19	395.0	28	147
		Prednisolone	5.55	361.0	13	357
2	(6.4–9.4)	Methylprednisolone	7.06	375.1	26	147
					26	171
		Bethametasone	7.66	393.0	17	161
		Dexamethasone	7.85	393.0	11	357
					27	147
					25	171
3	(9.4–13)	Corticosterone	8.76	347.0	25	147
		Fluocinolone acetonide	9.83	453.1	25	171
		Prednisolone-21-acetate	10.51	403.0	30	121
					20	279
		Hydrocortisone-21-acetate	11.11	405.0	27	147
					13	307
4	(13–25)	Fluocinolone acetonide-21-acetate	16.38	495.0	28	121
		Clobetasol propionate	18.36	467.0	17	309
					32	121
					17	319
		Bethametasone dipropionate	19.00	505.0	16	373
					16	355
					31	147
					17	279

*Internal standard was monitored in single ion monitoring (SIM) mode

0.45 μm filter paper into a 2 mL vial. The same procedure was conducted for dexamethasone separately because it cannot be quantified in the presence of betamethasone. IS was added to a 10 mL flask prior to extraction in order to obtain a final concentration of 100 ng/mL.

The method specificity was determined by checking the chromatogram obtained from the blank. Absence of peaks at the retention times of analytes was a criterion for confirmation of method specificity.

Linearity was studied by using a six-point calibration curve for corticosteroids (LOQ, 10, 50, 100, 500, 1000 ng/mL). Calibration curves were calculated by plotting the analyte and internal standard peak area ratios against the analyte concentrations in triplicate, and analyzed by linear regression.

Limits of detection (LODs, signal-to-noise ratio (S/N)=3) and limits of quantification (LOQs, S/N=10) were determined as concentrations of analytes that gave S/Ns of 3 and 10, respectively. To be accepted, the calculated LOQ had to show precision and accuracy within the 20% relative standard deviation (RSD) and relative error, respectively.

Precision of the method was evaluated by replicated analyses (intraday: $n=5$, interday: $n=15$ on three different days). Precision was expressed as the RSD of concentrations calculated for QC and LOQ samples.

Accuracy was calculated by comparing the concentrations of spiked samples to theoretical QC (10, 100 and 1000 ng/mL) and LOQ concentrations, and was expressed as the relative error of the calculated concentrations.

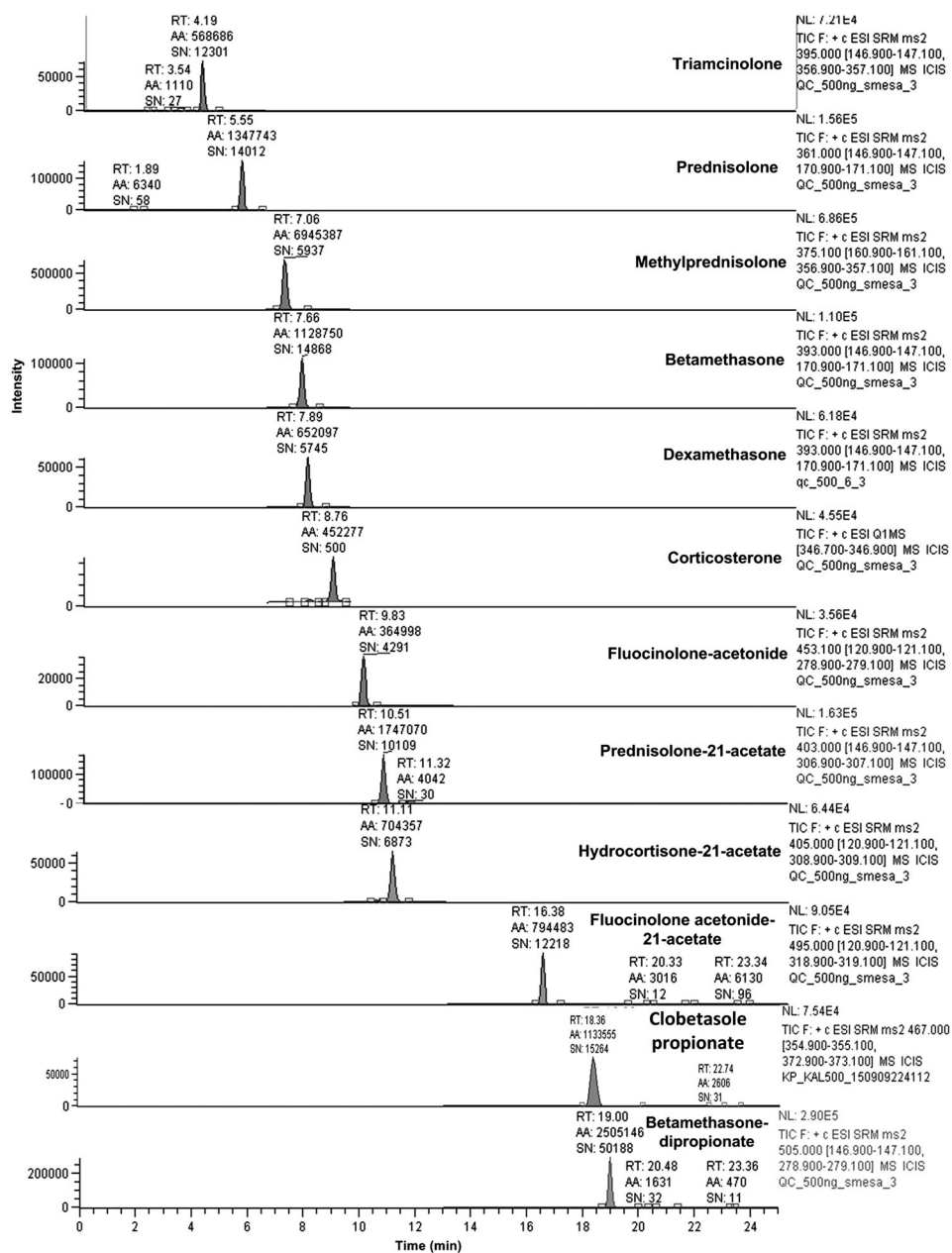


Figure 2. MRM chromatograms of standard substances spiked with matrix in concentration of 500 ng/mL.

Analytical recoveries were calculated by comparing the peak areas obtained by adding the quality control samples and the IS in the extract of blank prior to and after the extraction procedure.

For an evaluation of matrix effects, the peak areas of extracted blank spiked with QC samples after the extraction procedure were compared to the peak areas of pure diluted substances.

Confirmation of the efficiency of the extraction procedure

Since corticosteroids in creams are usually present in the oily part, extraction efficiency was further tested through the recovery test by direct incorporation of standards in powder form into the blank. The spiked validation samples were prepared according to the art of the pharmacy profession by mixing the blank with 10 mg of each analyte. This mixture was transferred to a 10 mL volumetric flask, spiked with IS and extracted with about 5 mL by ultrasonication at 50 °C for 15 min. After cooling to room temperature, it was filled up to volume. The solution was diluted with acetonitrile to obtain concentrations of 20, 200 and 2000 ng/mL and 2.5 mL

of these solutions was transferred to a 5 mL volumetric flask filled up with 0.1% formic acid to volume. This solution was then filtered through a 0.45 µm filter paper into a 2 mL vial. The same procedure was conducted for dexamethasone separately. IS was added to a 10 mL flask prior to extraction in order to obtain a final concentration of 100 ng/mL. Final concentrations of corticosteroids were 10, 100 and 1000 ng/mL. This procedure was carried out on two different matrices. The first one was the blank used for the validation, which encompassed a large number of common ingredients of different types of creams which make it hydrophilic, o/w cream. The second matrix was the lipophilic, water in oil (w/o) cream containing only w/o emulsifying agents. Recoveries were calculated in the same manner as described in the previous section.

Sample preparation

One gram of the cosmetic sample was weighed into a 10 mL volumetric flask, and spiked with IS to obtain a final concentration of 100 ng/mL. This was diluted with about 5 mL acetonitrile, and then ultrasonicated at 50 °C for

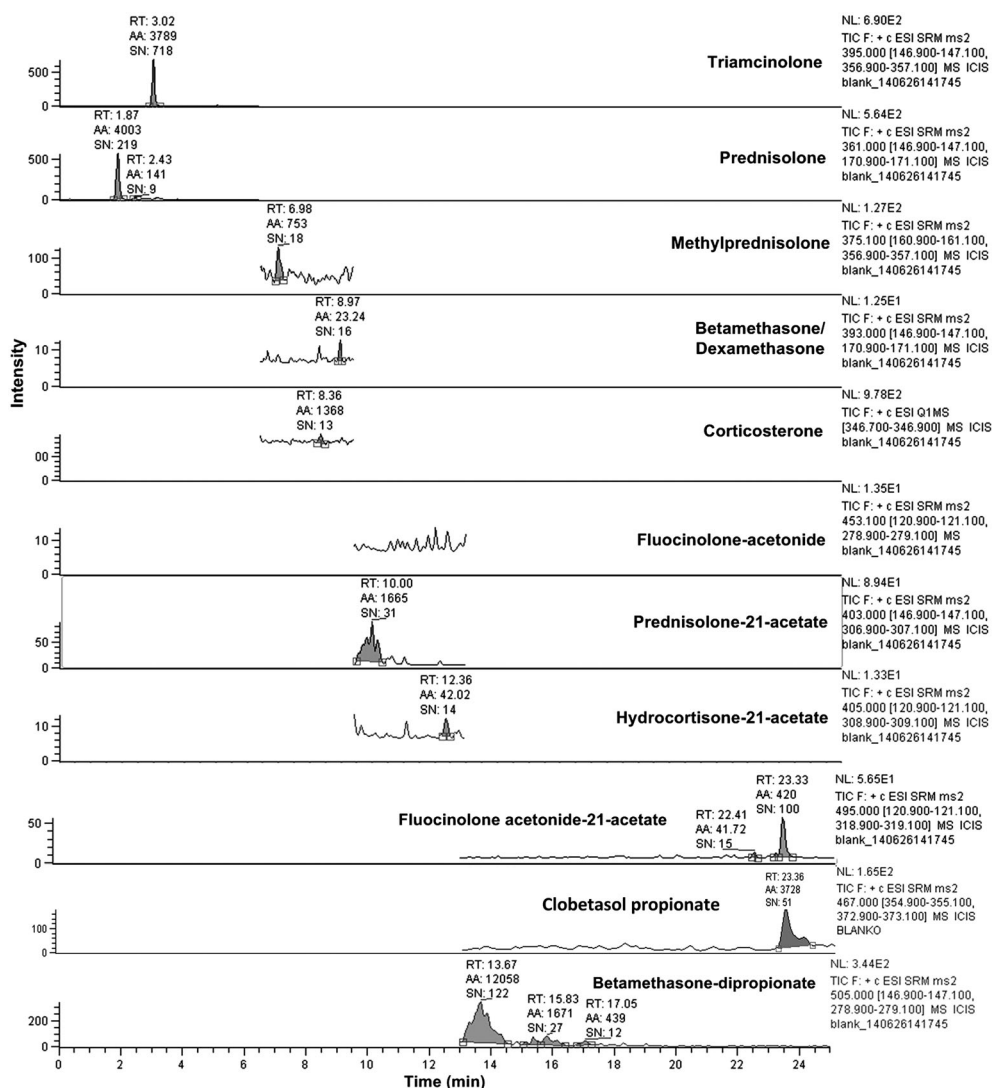


Figure 3. MRM chromatograms of the blank.

15 min. After cooling to room temperature, the flask was filled up to volume. 2.5 mL of this solution was transferred to a 5 mL volumetric flask and filled up with 0.1% formic acid in water to volume. This solution was then filtered through a 0.45 μm filter paper and transferred into a 2 mL vial.

RESULTS AND DISCUSSION

MRM optimization and confirmation of analytes by LC/MS/MS

MRM transitions used for confirmation of analyzed corticosteroids and corresponding collision energies are presented in Table 2. All the fragments were identified as typical for 1,4-diene-3-keto corticosteroids and hydroxyl-1,4-diene-3-keto steroids and are already known in the literature.^[8,9,23–26] The fragment ions at m/z 121, 171 and 279 can be explained by cleavages in the B, C and D rings. Two other fragment ions at m/z 147 for compounds without substitution in C_6 , and at m/z 161 for 6 α -methyl compounds, appear to be specific for Δ^1 -corticosteroids.^[9,24–26] Further, loss of water can lead to the fragment ion at m/z 357 from methylprednisolone, while loss of HF with consecutive loss of water can lead to the fragment ion of m/z 357 from triamcinolone.^[9,23,25,26] Loss of HF and propionic acid from clobetasol propionate led to a fragment ion at m/z 373, while additional loss of water led to a fragment ion at m/z 355.^[17] Fragment ions at m/z 307, 309 and 319, corresponding to $[M+H-96]^+$ and $[M+H-176]^+$, have also been reported in the literature.^[8]

Chromatographic method development

The gradient-elution method seems indispensable regarding very similar physicochemical properties of the analytes and internal standard. The initial composition and gradient slope were varied slowly in order to improve separation of analytes and enable method segmentation. Eventually, standards and samples were separated by initial mixture of 0.1% formic acid in water (solution A) and acetonitrile (solution B) in the ratio 60:40 (v/v) and subsequent gradient up to 50% of solution B in 10 min and continued until there was 100% of solution B by 20 min. Then the percentage of solution B was decreased to 40% by 21 min. The column was reequilibrated for 4 min, giving a total run time of 25 min. The column temperature was set at 25 $^{\circ}\text{C}$, while the flow rate was 0.6 mL/min. Method segmentation is presented in Table 2. It should be pointed out that dexamethasone and betamethasone were not completely resolved on the baseline. They certainly can be distinguished based on the difference in retention time. It is not expected that a cream contains both of them illegally. Their simultaneous presence can be observed by peak overlapping. In that case probably some of the chromatographic methods that resolves them completely can be additionally employed.^[10,11]

MRM chromatograms of standard substances at concentration of 500 ng/mL are presented in Fig. 2.

Validation results

The method specificity was determined by checking the chromatograms obtained from the blank, and no endogenous interferences were encountered under the chromatographic

Table 3. Validation data

Analyte	R^2	Regression equation	LOD (ng/mL)	LOQ (ng/mL)	Precision – intraday (RSD*, n = 5)			Precision – interday (RSD, n = 15)			Accuracy (Bias%)*			Recovery (%)		
					QC1**	QC2**	QC3**	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3
Triamcinolone	0.993	$y^{1/2} = -0.0303 + 0.0854x^{1/2}$	0.25	0.75	4.9	5.0	1.5	8.7	7.1	8.4	-8.7	6.4	6.0	94.4	109.4	100.3
Prednisolone	0.991	$y^{1/2} = -0.0332 + 0.1316x^{1/2}$	1	3	7.9	7.6	3.5	12.2	10.4	11.3	-7.11	6.45	3.16	108.2	100.7	99.5
Methylprednisolone	0.991	$y^{1/2} = 0.0250 + 0.2048x^{1/2}$	0.3	1	6.0	4.4	5.6	13.1	12.2	7.8	-8.9	6.89	3.0	109.9	100.7	97.8
Betametasone	0.994	$y^{1/2} = -0.06 + 0.0999x^{1/2}$	0.3	1	9.8	6.7	6.0	14.7	13.2	10.3	-5.4	4.4	2.5	95.3	99.3	99.9
Dexamethasone	0.999	$y^{1/2} = -0.0127 + 0.0888x^{1/2}$	0.25	0.75	9.0	4.5	2.5	12.5	10.5	8.9	4.28	5.66	1.2	101.9	110.7	97.8
Fluocinolone acetonide	0.995	$y^{1/2} = -0.0451 + 0.0565x^{1/2}$	1	3	6.5	7.9	5.0	14.2	8.5	6.2	4.78	0.3	1.5	88.7	102.2	104.7
Prednisolone-21-acetate	0.991	$y^{1/2} = -0.028 + 0.1547x^{1/2}$	0.25	0.75	5.1	4.7	3.5	13.8	11.2	6.7	-9.0	7.0	2.0	94.5	104.8	96.8
Hydrocortisone-21-acetate	0.993	$y^{1/2} = -0.0471 + 0.063x^{1/2}$	1	3	11.2	3.3	2.0	13.2	9.3	5.5	-0.6	3.7	0.6	104.0	108.4	106.2
Fluocinolone acetonide-21-acetate	0.993	$y^{1/2} = -0.0302 + 0.0816x^{1/2}$	1	3	12.8	9.2	8.3	14.0	12.5	8.4	-3.2	3.8	2.3	103.3	108.8	106.1
Clobetasol propionate	0.999	$y^{1/2} = -0.0884 + 0.2922x^{1/2}$	0.25	0.75	4.8	0.94	0.77	7.2	2.5	3.0	10.3	4.9	-1.7	108.5	99.1	95.3
Betametasone dipropionate	0.993	$y^{1/2} = -0.1102 + 0.2073x^{1/2}$	1	3	2.8	6.8	4.5	11.3	9.4	9.6	-6.0	-2.89	0.3	97.6	87.0	89.7

*RSD (%) = (SD/mean C_{obs}) $\times$ 100.

**QC1-10 ng/mL, QC2-100 ng/mL, QC3-1000 ng/mL.

***Accuracy (Bias %) = $[(C_{\text{obs}} - C_{\text{nom}})/C_{\text{n}}]$.

conditions (Fig. 3). The calibration curves for detection of the target compounds were obtained in the range of 10–1000 ng/mL by performing a linear regression analysis of analyte and internal standard peak area ratios against the analyte concentrations. Considering the broad concentration range, a weighting factor was necessary.^[27] In this case the square root ($x^{1/2}$) was found to be an appropriate weighting factor. Good linearity was obtained for all analytes with coefficients of determination (R^2) higher than 0.990. Their limits of detection were 0.25–1 ng/mL and limits of quantitation were 0.75–3 ng/mL. Average recoveries of each compound at three spiking levels ranged

from 91.4% to 106.1%. As an evaluation of the method precision for quantitation, RSDs for all the analytes were less than 15% at QC levels, and 20% at LOQ levels, respectively. Also, the quantitation accuracy for all the analytes were within the range of $\pm 15\%$ (Bias %) at QC levels, and 20% at LOQ levels, respectively. The comparison of the chromatograms of the standard solution and the same standard solution in matrix added prior to injection showed a very small matrix effect on the analytical response. For all the analytes the responses in matrix ranged from 90% to 110% of the responses in solution. The validation data are summarized in Table 3.

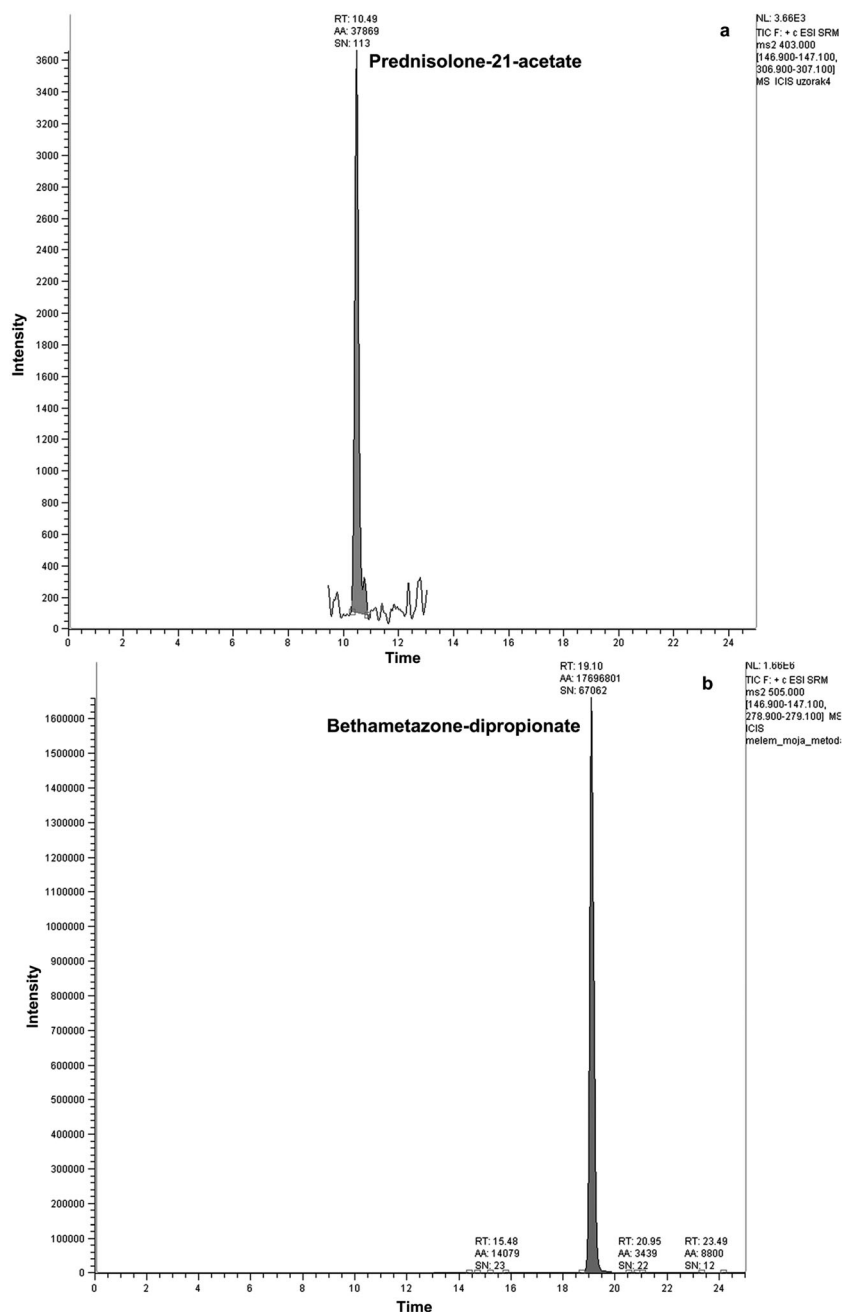


Figure 4. MRM chromatograms of cosmetic samples found to contain undeclared corticosteroids: (a) prednisolone acetate and (b) betametasone dipropionate.

Efficiency of the extraction procedure

Average recoveries, obtained according to the procedure described in the section "Confirmation of the efficiency of the extraction procedure", of each compound at three spiking levels for both types of matrix (o/w and w/o) were between 85% and 115% which was considered sufficient for this type of concentration and matrix.^[28–31] This confirmed the adequacy of the validation procedure in terms of QC and calibration sample preparation. Good recovery obtained for a lipophilic blank confirmed the ability of the proposed method to be applied to both types of creams.

Analysis of cosmetic samples

Twenty cosmetic products, intended for the treatment of atopic dermatitis or very dry skin, were analyzed using the developed method. Two samples were positive to corticosteroids – prednisolone acetate (Fig. 4(a)) and betamethasone dipropionate (Fig. 4(b)). Both products were prescribed in dermatological clinics. Found concentrations were 2.8 ppm of prednisolone acetate and 0.023% betamethasone dipropionate. Although the level of prednisolone acetate found in the tested cream was low, frequent long-term application of these creams can lead to adverse effects. The concentration of betamethasone dipropionate was dangerously high, especially for children. Unfortunately, that cream was labelled to be used for children, twice a day with no specified period of time. Necessary steps for the examination of these products by regulatory bodies have been made.

CONCLUSIONS

An LC/MS/MS method for the rapid and simultaneous determination of low levels of eleven topical corticosteroids in creams was developed, optimized and validated. Analyzed corticosteroids are prednisolone, methylprednisolone, prednisolone-21-acetate, fluocinolone acetonide, fluocinolone acetonide-21-acetate, hydrocortisone-21-acetate, dexamethasone, betamethasone, betamethasone dipropionate, clobetasol propionate and triamcinolone. The developed method was validated using pooled blank creams and the results obtained from the validation met EU guidelines. The parameters studied in the validation study were the following: linearity, specificity, precision, accuracy, recovery, limits of detection and limits of quantification. The proposed method enables analysis of eleven corticosteroids in cosmetic creams. The run time of 25 min is quite acceptable bearing in mind the large difference in polarity of the investigated drugs and the number of the drugs. Partial resolution of dexamethasone and betamethasone is good enough to enable identification of the two drugs based on the retention time of the standards. Segmentation of the method enabled a decrease in limits of detection and quantification compared to the existing methods. Consequently, the LC/MS/MS method was successfully applied to the products available on the market.

The method was applied to 20 products without labeled corticosteroids. Two of them were found to contain corticosteroids. The developed method can be used for testing

of different products indicated for the treatment of atopic dermatitis, including "natural products" and "herbal creams" with "miraculous effects".

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