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Analytical method development and validation for simultaneous determination of triamcinolone acetate, betamethasone valerate and clobetasol propionate in cream formulation by RP-HPLC

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ABSTRACT

Topical steroids are usually used for the treatment of inflammation and irritation of the skin; however, their inappropriate use results in serious side effects. Thus, the Thai Food and Drug Administration regulation forbade the use of corticosteroids in cosmetics. In this study, a method of high-performance liquid chromatography with a photodiode array detector was developed and validated for the simultaneous determination of three corticosteroids in cream formulation: triamcinolone acetonide (TA), betamethasone valerate (BV), and clobetasol propionate (CP). Chromatographic separation was performed on ACE® C18 (4.6 mm × 150 mm, 5 µm particle size) using a mobile phase consisting of water and acetonitrile in gradient elution. The detection wavelength was set at 254 and 240 nm. The validation results revealed the specificity and linearity of the proposed method with r≥0.999 within the concentration range of 2.5–50 µg/mL. The relative standard deviation (%) of the repeatability and intermediate precision of all corticosteroid samples at three different concentration levels were in the range of 0.04%–2.04% and 0.41%–4.93%, respectively, and the recovery (%) was 98.72 %–105.41%. The detection limits of TA, BV, and CP were 0.69, 0.46, and 0.48 µg/mL, respectively, and their quantitative limits were 2.08, 1.39, and 1.47 µg/mL, respectively. The validated method can be applied to quantify the amount of active compounds in commercial formulations and to determine the corticosteroid adulterants present in cream samples from online shops.

Keywords: high performance liquid chromatography; method validation; corticosteroids; triamcinolone acetonide; betamethasone valerate; clobetasol propionate

1. INTRODUCTION

Corticosteroids are mainly used as an anti-inflammatory and immunosuppressive drug. They are prescribed in many conditions such as rheumatoid arthritis, asthma, allergies, multiple sclerosis, and many other conditions. However, the long-term treatment or inappropriate use of corticosteroid may cause severe side effects, such as high blood pressure, Cushing's syndrome, increase risk of infection, suppressed adrenal gland hormone production, electrolyte imbalance, and osteoporosis. Topical corticosteroids have the indication for the treatment of atopic dermatitis, psoriasis, lichen simplex, discoid lupus erythematosus, lichen planus, urticaria, and eczema (Mehta et al., 2016). Because of the rapid anti-inflammatory effect on skin, corticosteroids are usually added to the local cosmetics in order to over claim them as whitening or anti-acne. The improper use of topical corticosteroids mostly caused local adverse effect such as skin atrophy, steroid face, corticoid acne, hypopigmentation, and allergic contact dermatitis (Brazzini and Pimpinelli, 2002). The Thai Food and Drug Administration (Thai FDA) announced the corticosteroids as forbidden substances in cosmetics (Prescribing substances prohibited to be used as ingredients in manufacturing cosmetics B.E. 2566, 2023). However, the corticosteroid adulteration in cosmetics has been still occurred in Thailand, especially the illegal cosmetics which did not register according to FDA regulations.

Various methods have been reported for the qualitative and quantitative determination of corticosteroids in creams or cosmetics. Some examples include qualitative or semiquantitative analysis by thin-layer chromatography (TLC) (Alam et al., 2015) and TLC with UV spectrophotometry (Bassuoni et al., 2016; Dolowy et al., 2017; Roychowdhury and Das, 1996). Although spectroscopic methods and TLC are simple and rapid, they have low sensitivity, accuracy, and specificity and might require sample pretreatment. Thus, they are incapable of accurate quantification on their own or require a densitometer for the quantitative aspect (Elgizawy et al., 2014). Specific methods, such as NMR spectroscopy, have also been used (McEwen et al., 2012); however, this technique requires sophisticated instruments and a special solvent.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a popular technique for determining corticosteroids in pharmaceutical products and cosmetics because of its speed, selectivity, sensitivity, and simplicity. The United States Pharmacopeia 43 (USP43) method for determining the amounts of triamcinolone acetonide (TA) (United States Pharmacopeia, 2020a) and betamethasone valerate (BV) (United States Pharmacopeia, 2020b) in the cream formulation is RP-HPLC with a mobile phase composed of water and acetonitrile in gradient elution and the assay method for clobetasol propionate (CP) in cream formulation is RP-HPLC with the mobile phase composed of water/acetonitrile/phosphate buffer in isocratic elution (United States Pharmacopeia, 2020c). However, these methods can only analyze one corticosteroid at a time and may not be suitable for cosmetics with more ingredients than pharmaceutical products. A specific RP-HPLC method has been developed for the simultaneous determination of various corticosteroids and other active compounds in creams and cosmetics (Gimeno et al., 2015; Hotha et al., 2020; Johnston et al., 2010; Shaikh et al., 2009). Most of the analyses are performed using acetonitrile and water as the mobile phase in either isocratic or gradient elution. For example, RP-HPLC with a UV detector was optimized for determining mometasone furoate, hydrocortisone acetate, fluocinonide, fluocinolone acetonide, betamethasone, betamethasone dipropionate, and TA (Ivković et al., 2022). The HPLC column was Inertsil® ODS-3V (250 × 4.6 mm, 5 µm), the mobile phase was water and acetonitrile in gradient elution, and the UV detector was set at 240 nm. The optimization results showed good separation of all corticosteroids, and the validation results conformed to the requirements. Another RP-HPLC method with a photo diode array detector (PDA) was developed and validated for determining hydroquinone, dexamethasone, TA, hydrocortisone acetate, BV, and retinoic acid in cream formulations (Rahmayuni et al., 2018). This study used the C18 column (4.6 mm × 250 mm, 5 µm) and acetonitrile and 0.1% formic acid as the mobile phase in the gradient system, and the PDA was set at 210–400 nm. The validated method demonstrated accuracy and precision and could be used for cosmetic and pharmaceutical formulation analysis.

To date, no analytical method has been reported for the simultaneous determination of the three corticosteroids representing medium/low potency (TA), medium potency (BV), and high potency (CP) in cream formulations. These corticosteroids have been reported as common adulterate corticosteroids in illegal cosmetics in Thailand. Therefore, a RP-HPLC method with PDA was developed to simultaneously determine these three corticosteroids. The chromatographic condition was developed in gradient elution to separate the peaks of corticosteroids from the peaks of other ingredients in the formulation. The analytical method was validated in accordance with the International Conference on Harmonization (ICH) guidelines using the following parameters: linearity and range, specificity, accuracy, precision, detection limit (DL), quantitation limit (QL), and system suitability. The acceptance criteria were adopted from AOAC guidelines.

2. MATERIALS AND METHODS

2.1 Materials

Corticosteroid standards (TA, BV and CP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were acquired from Merck Ltd., Germany. All solvents were of HPLC grade. A steroid test kit was obtained from B Smart Science, Nonthaburi, Thailand. The cream base used in this study was RAMA cream base, composed of water, mineral oil, stearyl alcohol, propylene glycol, cetyl alcohol, sodium lauryl sulfate, methyl paraben, and propyl paraben. Commercial medicinal TA, BV, and CP creams were purchased from the Faculty of Pharmaceutical Sciences, Burapha University, Thailand. The cosmetic samples were obtained from the online market.

2.2 Standard solution preparation

Standard stock solutions of TA, BV, and CP were prepared in methanol at a concentration of 1 mg/mL. The working standard solutions of these corticosteroids were then mixed to a final concentration of 0.1 mg/mL. This solution was further used for method validation. All the solutions were filtered through a 0.2–µm nylon filter (Anpel Laboratory Technologies (Shanghai) Inc., Shanghai) prior to HPLC analysis.

2.3 Spiked sample solution preparation

Spiked sample solutions were prepared by spiking the standard solution into the cream base at the designed concentration to determine the effect of the matrix on the analytical procedure. The spiked samples were diluted with methanol at a cream to methanol ratio of 1:10, and the mixtures were then vigorously mixed and sonicated for 15 min. The mixtures were cooled in an ice bath for 15 min and centrifuged at 10,000 rpm for 15 min to remove the insoluble wax and oil phase in the creams. The supernatant containing the corticosteroids was collected. All the solutions were filtered through a 0.2–µm nylon filter prior to HPLC analysis.

2.4 Sample solution preparation

In brief, 1.0 g of commercial corticosteroid cream (TA, BV, and CP creams) and cosmetic cream samples were accurately weighted, diluted with methanol, and vigorously mixed and sonicated for 15 min. The samples were cooled in an ice bath for 15 min and centrifuged at 10,000 rpm for 15 min. The supernatants were collected. For the TA, BV, and CP creams, the sample solutions were diluted with methanol to a concentration of 30 µg/mL. All sample solutions were filtered through a 0.2–µm nylon syringe filter prior to HPLC analysis.

2.5 Chromatographic condition

The HPLC system comprised a quaternary pump, an autosampler, a thermostat column compartment, and a PDA (Shimadzu Scientific Instruments, Kyoto, Japan). The HPLC column was ACE® C18 (4.6 mm × 150 mm, 5 µm particle size) (Advanced Chromatography Technologies Ltd.) with a C18 phase guard column (Phenomenex®, USA). Chromatographic separation was performed in gradient elution using a mobile phase consisting of water and acetonitrile (Table 1) at a constant flow rate of 1 mL/min. The mobile phase was filtered through a 0.45–µm nylon filter and degassed by sonication for 30 min before use. The injection volume was 10 µL and the PDA was set at 254 and 240 nm.

Table 1. Gradient elution for chromatographic separation

Time (min)	Water (%)	Acetonitrile (%)
0 – 10	70	30
20 – 30	50	50
40 – 45	30	70
55	70	30

2.6 Analytical method validation

The analytical method was validated in accordance with the ICH guidelines (ICH Q2(R1), 2005), and the acceptance criteria were adopted from the AOAC guidelines (Latimer, 2016).

2.6.1 Linearity and range

Linearity was determined for the standard solutions and spiked sample solutions. The working solution was diluted with methanol to generate at least five concentrations of corticosteroids in the range of 2.5–50 µg/mL. Calibration curves were constructed between peak areas versus concentration for the standard and spiked samples. Linearity was evaluated in terms of the coefficient of determination (r^2) over the specified range.

2.6.2 Accuracy

The standard solutions and spiked sample solutions were prepared at three different concentrations (2.5, 20, and 50 µg/mL) in triplicate and then analyzed by the HPLC system. The percentage recovery of TA, BV, and CP was calculated by comparing the recovered amount of the sample with the known amount of the standard added.

2.6.3 Precision

The repeatability and intermediate precision of the standard solutions and spiked sample solutions were examined. Repeatability was analyzed by injecting the samples at three different concentration levels (2.5, 20, and 50 µg/mL) in triplicate. Intermediate precision was studied using the same procedure for repeatability but on different days. The precision of the analytical method was demonstrated as the percentage relative standard deviation (% RSD).

2.6.4 Detection limit (DL) and quantitation limit (QL)

The DL and QL of the proposed analytical method were computed based on the standard deviation of response (σ) and the slope (S) of the calibration curve expressed as DL = 3.3 σ/S and QL = 10 σ/S, respectively. The DL was confirmed by subjecting the solution at the calculated concentrations to the HPLC system. The S/N values from the HPLC instrument were evaluated.

2.6.5 Specificity

The specificity of the analytical method was evaluated by comparing the chromatograms of cream bases in different formulations with the chromatograms of the standard solution and spiked sample. The specificity of the method was justified by the lack of other peaks in the cream bases appearing at the same retention time as the TA, BV, and CP peaks.

2.6.6 System suitability

The system suitability was assessed following the ICH guidelines by injecting the six standard solutions at a concentration of 20 µg/mL. Chromatographic parameters such as repeatability, theoretical plate, peak symmetry (tailing factor), and resolution were evaluated.

2.7 Application to commercial medicinal products and cosmetic cream samples

The developed method was applied for the determination of corticosteroids in commercial medicinal products (TA, BV, and CP creams) and cosmetic samples with whitening or antiacne claims. The amount of corticosteroids in the commercial creams was quantified by linear regression obtained from the calibration curve and compared with the

labeled claim. The corticosteroid adulterants in the cosmetic samples were primarily screened using the steroid test kit. The amount of corticosteroids was quantified using the regression, and the type of corticosteroids was confirmed by comparing the retention time of the peaks obtained from the samples with those obtained from the standard solutions.

3. RESULTS AND DISCUSSION

3.1 Chromatographic development

The RP-HPLC method was successfully developed for the determination of three corticosteroids in cream formulations. The mobile phase comprised of water and acetonitrile in gradient systems as mentioned in Table 1. The TA, BV, and CP peaks were separated from the adjacent peaks at retention times of 9.0, 27.8, and 32.0, respectively, as shown in Figure 1. The three corticosteroids were eluted in order from the most polar compound to the least polar compound because this study used a RP-HPLC system. Thus, TA with the highest polarity was first eluted, and CP with the lowest polarity was eluted after TA and BV (Osamura, 1982; Ponec and Polano, 1979). High response and peak area were recorded during the detection at 240 nm. Thus, further studies were conducted at 240 nm because it had the highest sensitivity for the corticosteroids. The total running time was set at 55 min to ensure the elution of other possible ingredients or degradants in the samples. However, the method could be further developed to minimize the analysis time while considering the peak separation between corticosteroids and other ingredients in the formulation. For example, the rate of gradient elution might be faster than that adopted in this study, or the ratio of organic solvent to water might be greater than 30:70 at the beginning of the analysis (Chan et al., 2021; Hotha et al., 2020; Ivković et al., 2022). In addition, high-level technologies, including UPLC and LC-MS/MS, can be used to improve the analytical efficiency and decrease the analysis time (Giaccone et al., 2017; Jian et al., 2021).

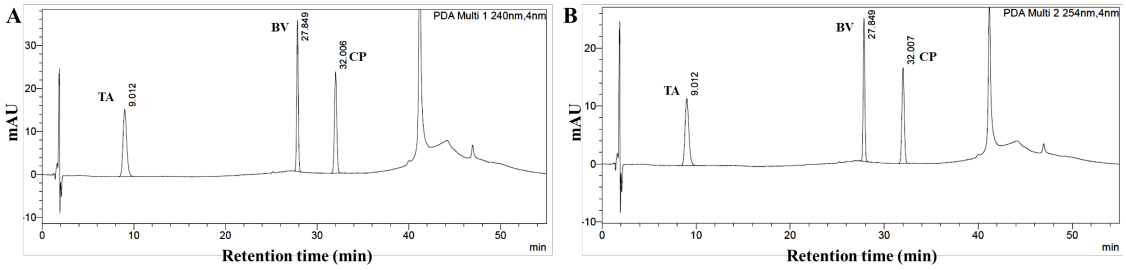

Figure 1. Chromatograms of standard solution at (A) 240 nm, and (B) 254 nm

3.2 Method validation

The analytical method for the simultaneous determination of TA, BV, and CP was validated following the ICH guidelines. The parameters, including specificity, linearity and range, accuracy, precision, DL, QL, and system suitability, were determined.

3.3 Linearity

Linearity was studied for the standard solutions and spiked sample solutions. The calibration curves were plotted between the peak areas and the concentration of corticosteroids over the range of 2.5–50 µg/mL (Figure 2). The linear regression equations are shown in the graph. The coefficient of determination (r^2) of all corticosteroids in the standard solutions and spiked sample solutions was greater than 0.999. This result revealed that the developed method had good linearity over the working range. The concentration used in this study was lower concentration with a wider range than the previous HPLC-UV or HPLC-PDA methods (Jakasaniya et al., 2014; Rahmayuni et al., 2018). In addition, the regression lines of the spiked sample solutions were identical to those of the standard solutions. This finding supported that the corticosteroid extraction procedure was suitable for recovering corticosteroids from cream formulations without interference from the other ingredients and revealed the specificity of the developed method.

3.4 Accuracy

The accuracy of the developed method was examined by spiking the standards to the cream base at three concentrations of 2.5, 20, and 50 µg/mL represented as the low, medium, and high concentrations, respectively. The recoveries of the corticosteroids were quantified as shown in Table 2. The percent recoveries at the three concentration levels were in the ranges of 99.88%–104.20%, 98.72%–101.00%, and 100.46%–105.41% for TA, BV, and CP, respectively. These values met the AOAC criteria (i.e., 80%–110%) at the designed concentration, indicating that the developed method was accurate for all three corticosteroids.

3.5 Precision

The precision of the analytical method was evaluated by repeatability (intra-day) and intermediate precision (inter-day). For repeatability analysis, three separate standard solutions and spiked sample solutions were prepared at three concentration levels (2.5, 20, and 50 µg/mL). The intermediate precision was evaluated by preparing the standard solutions and spiked sample solutions using the same method for the repeatability study but on three different days. The repeatability and intermediate precision were represented by the RSD percentages. As shown in Table 2, the intra-day RSD values of all three corticosteroids at three different levels were in the range of 0.04%–0.78% for the standard solutions and 0.09%–2.04% for the spiked sample solutions, indicating the good repeatability of the method. Meanwhile, the inter-day RSD values of all three corticosteroids were in the range of 0.41%–4.93% for the standard solutions and 1.24%–3.54% for the spiked sample solutions. According to the AOAC guidelines, the repeatability and intermediate precision at the designed concentration in this study must not be more than 5.3%. Thus, the results indicated that the developed method had good precision and met the criteria.

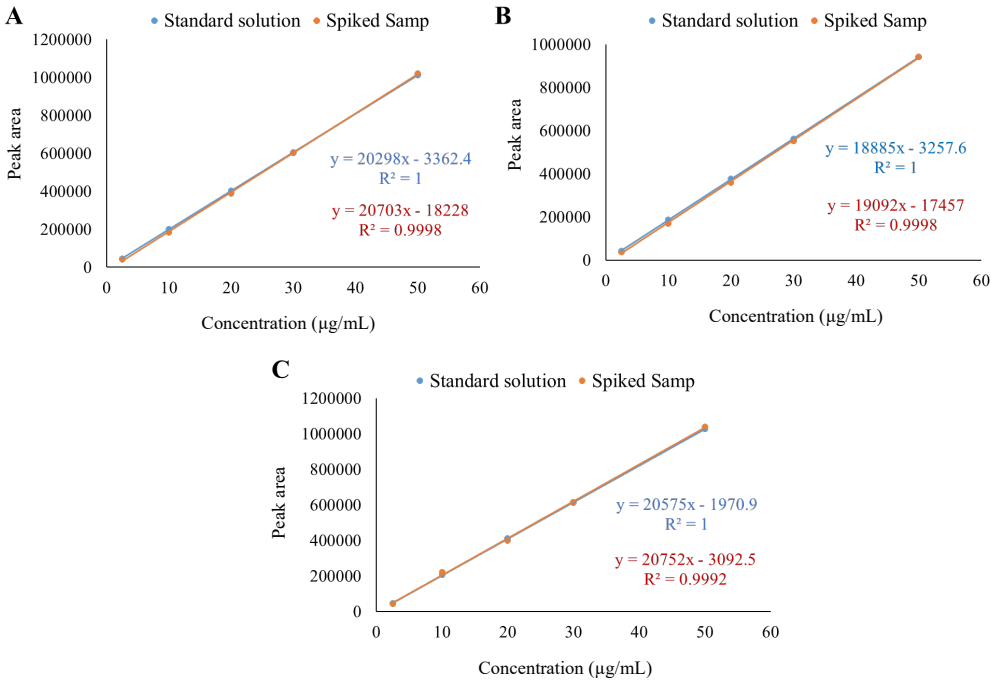

Figure 2. Calibration curves of standard solutions and spiked sample solutions with linear regression equations and coefficient of determination (r^2) of (A) TA, (B) BV and (C) CP

3.6 Detection limit and quantitation limit

The DL was calculated based on the standard deviation of the response (σ) and the slope (S) of the calibration curve, expressed as DL = 3.3 σ/S. The DLs of TA, BV, and CP were 0.69, 0.46, and 0.48 µg/mL, respectively. Standard solutions at these DL concentrations were prepared and subjected to the HPLC system to confirm the obtained DLs. The chromatographic reports from the HPLC instrument revealed that the S/N ratios of three corticosteroids were greater than 3:1. The QL was also computed similarly and expressed as QL = 10σ/S. The QLs of TA, BV, and CP were 2.08, 1.39 and 1.47 µg/mL, respectively. The DL and QL values indicated that the developed method could be used to determine the three corticosteroids in cream formulations at the microgram level. In addition, the DL and QL of this method were comparable with those in previous reports (Gimeno et al., 2015; Nam et al., 2011; Reepmeyer, 2001). However, the detectability of the analytical method was dependent on the sensitivity of the detector. Owing to its high separation efficiency, HPLC can achieve highly sensitive detection at the nanogram level when used with a mass spectrometer (LC-MS or LC-MS/MS) (Fiori and Andrisano, 2014; Golubović et al., 2015).

Table 2. Accuracy and precision of method represented in recovery (%) and RSD (%)

	Concentration (µg/mL)	Recovery ± SD (%)	Precision (%RSD) (Standard solution)		Precision (%RSD) (Spiked sample solution)	
			Repeatability	Intermediated Precision	Repeatability	Intermediated Precision
TA	2.5	104.20 ± 1.60	0.44	4.68	2.04	2.78
	20	100.93 ± 0.22	0.11	2.99	0.22	1.29
	50	99.88 ± 1.24	0.26	0.42	1.24	1.24
BV	2.5	101.00 ± 1.97	0.78	4.93	0.60	3.54
	20	100.02 ± 0.18	0.41	2.79	0.18	1.87
	50	98.72 ± 1.32	0.29	0.41	1.34	1.27
CP	2.5	105.41 ± 0.70	0.04	4.71	0.68	3.28
	20	101.71 ± 0.09	0.46	2.89	0.09	1.78
	50	100.46 ± 1.07	0.40	0.47	1.06	1.35

3.7 Specificity

The specificity of the developed method was determined by comparing the chromatograms of the standard solutions, spiked sample solutions, and cream base solutions. The chromatograms in Figure 3 show that no excipient peaks overlapped with the major peaks of TA, BV, and CP. The UV spectra containing the peaks of TA, BV, and CP obtained from the PDA were also examined to confirm the similarity in the spectra. The result indicated that the developed method was specific and suitable for the simultaneous determination of the three corticosteroids.

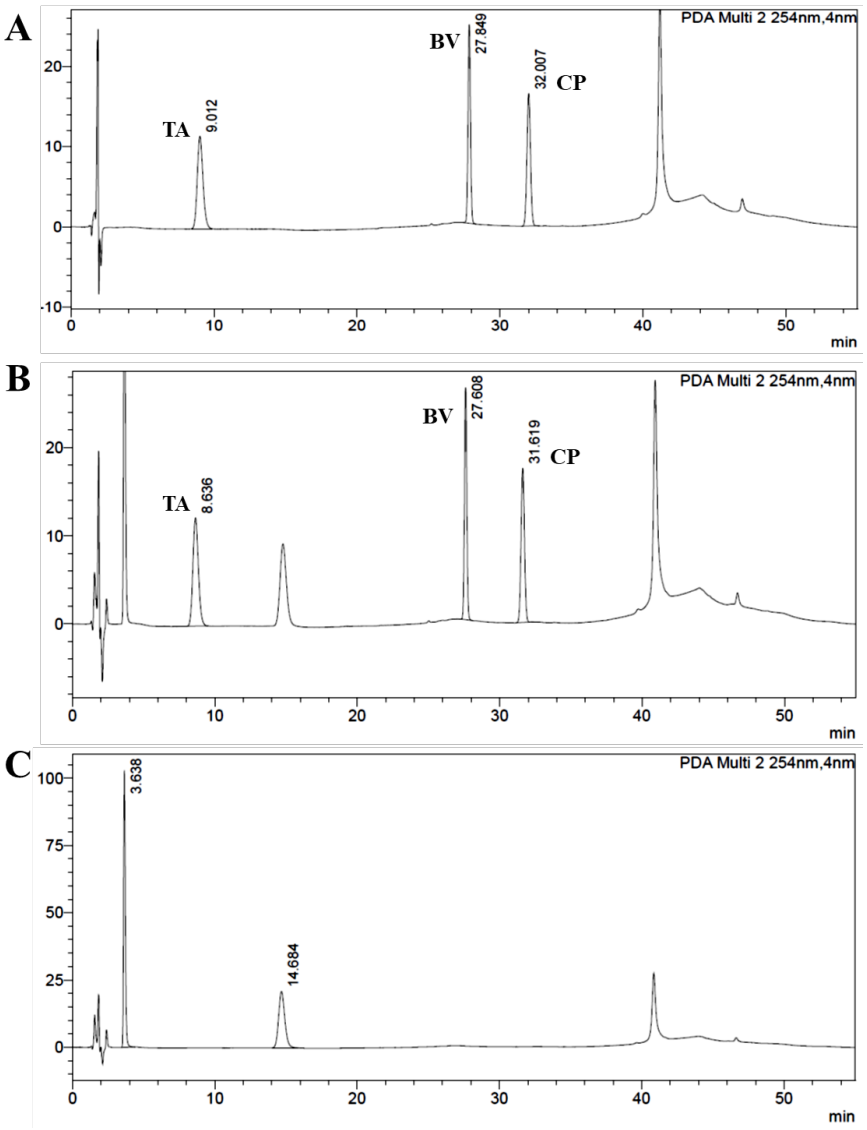

Figure 3. Chromatograms of (A) standard solution, (B) spiked sample solution, and (C) cream base solution

3.8 System Suitability

System suitability was examined to ensure the appropriate chromatographic conditions for analysis. The RSD of the peak responses of all corticosteroids from six injections at 20 µg/mL was less than 2%. Other system suitability parameters, including theoretical plate, tailing factor, and resolution, were also examined, as shown in Table 3. The results revealed that the chromatographic system was suitable for analysis and complied with the requirements.
Table 3. System suitability parameters.

Table 3. System suitability parameters

	RT ± SD (min)	RSD of peak responses (%)	Theoretical plate	Resolution	Tailing factor
TA	8.92 ± 0.10	0.28	2250.33	15.41	1.16
BV	27.78 ± 0.05	0.08	109868.33	8.66	1.06
CP	31.89 ± 0.09	0.31	72601.33	10.13	1.04

3.9 Applications: Commercial medicinal corticosteroid creams and cosmetic samples

The validated method was applied for the determination of three corticosteroids in commercial medicinal products: 0.1% TA cream, 0.1% BV cream, and 0.05% CP cream (Table 4). The percentages of drug amount, in the TA, CP, and BV cream, were 90.1%, 106.1%, and 100.1%, respectively. These results respected the label claimed within the USP requirements of 90.0–110.0% of the labelled amount of the active ingredient in samples.

The method was then applied for examining the corticosteroids in cosmetics, which claimed as whitening and anti-acne aspects because the determination of corticosteroid adulterants in cosmetic, is important and

beneficial for costumer protection. The improper or unintended use of these compounds might bring about the serious side effects. Firstly, the samples were tested with the available corticosteroid test kit for preliminary screening of corticosteroid adulterants. The commercial medicinal product provided the obvious color change from white to purple within 15 min. Two of the three cosmetics on the online market showed a color change, which indicated that they might contain the forbidden corticosteroids. However, the cosmetic samples did not clearly show the color alteration because the color of the samples interfered with the visual inspection. This was the limitation of the test kit because it was based on the colorimetric determination, which might be interfered by other compositions in the sample and could not specify the type of corticosteroids (Sefid-Sefidehkhan et al., 2022). In addition, the detection limit of the test was about 0.02–0.1%w/w, thus it could not detect the corticosteroid at the low concentration. In this case, the developed method also has the benefit for the formulation with the intense coloring agents. The samples were analyzed by the developed method, and the results was shown in Table 4. The types of corticosteroids found were identified by comparing the retention time of the peak obtained from standard solutions with those obtained from sample solutions, and the amounts of corticosteroids were computed by linear regression. The cream 2 might contain TA at 25.37±3.14 μg/g of sample, and the cream 3 might contain CP at 1.02±1.05 mg/g of sample.

However, in this study, we focused on three corticosteroids, which are usually found in cosmetic cream formulations in the local and online markets. Other corticosteroids are possibly added to the various formulations, such as ointments, lotions, or solutions. The simultaneous analytical method for diverse corticosteroids and the sample treatment in different cosmetic formulations should be further developed.

Table 4. Application of developed method in commercial medicinal products and online cosmetics

	Screening with test kit		Found corticosteroid	Found amount (per g of cream)
	Color of samples	Degree of color change after 15 min		
Cream base	White	-	-	-
TA cream	White	+++	TA	0.90 ± 0.00 mg
BV cream	White	+++	BV	1.06 ± 0.00 mg
CP cream	White	++	CP	0.50 ± 0.00 mg
Cream 1	White	-	Not detected	Not detected
Cream 2	Green	+	TA	25.37 ± 3.14 μg
Cream 3	Green	+	CP	1.02 ± 1.05 mg

Remarks: The symbols -/+ represented the degree of color change

4. CONCLUSION

RP-HPLC was successfully developed for the determination of TA, BV, and CP adulterants in medicinal creams and cosmetics. The corticosteroids were separated in the chromatographic system through gradient elution and were detected with PDA at 240 nm. The validation data indicated the specificity, accuracy, and precision of the developed method, with linearity over the desired working range meeting the acceptance criteria of the AOAC guidelines. The developed method was able to examine the corticosteroids at the microgram level, as indicated by DL and QL. Therefore, the RP-HPLC method was suitable for the simultaneous determination of the three corticosteroids in commercial medicinal creams and cosmetics from the local and online markets for customer protection.

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