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Development and validation of a novel HPLC-UV method for the determination of faradiol in creams including Marigold (*Calendula officinalis* L.) extract

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ABSTRACT: A straightforward, quick, and sensitive high-performance liquid chromatography (HPLC) technique was created to quantify the triterpene alcohol faradiol present in cosmetic creams that contain extract from marigold (*Calendula officinalis* L.). The mobile phase consisted of 95:5 methanol:water (2% o-phosphoric acid) with an isocratic elution and a flow rate of 1.3 mL/min. The stationary phase was a C18 (5 µm × 4.6 mm × 150 mm) column. Throughout the separation, a constant temperature of 35 °C was maintained. Thirty µL was the injection volume. At 6.38±0.20 min, the faradiol peak was eluted. The International Conference on Harmanization (ICH) criteria for linearity, limit of detection, limit of quantitation, selectivity, sensitivity, robustness, accuracy, and precision were followed in the validation process. The limits of quantification and detection were 0.01 µg/mL and 0.003, in respective order. The arrangement of linear expression was between 0.01 and 30 µg/mL. For both the daily and hourly analysis, the relative standard deviation values were less than 1.20%. A variety of cosmetic goods were analysed using the suggested approach. The technique is anticipated to be appropriate for regular triterpene alcohol faradiol analysis, quality assurance, and standardisation of cosmetic cream products.

KEYWORDS: Faradiol; Margiold creams; HPLC-UV; Validation

1. INTRODUCTION

In Europe and the US, marigold (*Calendula officinalis* L., Asteraceae) is a widely used pharmaceutical and cosmetic herb. The herbal remedy has been included in monographs in a number of national pharmacopoeias as well as the 1999 European Pharmacopei supplement [1]. *Calendula officinalis* L. flower heads were extracted using hot lard, which is similar to human skin fat, in addition to classical galenic formulations like infusa, tinctures, and fluid extracts in folk medicine. This allowed for the production of ointments for a variety of dermatological conditions, including burns, ulcers, eczemas, varicose veins, haemorrhoids, and wounds. The herb's ability to promote wound recovery and act as a topical anti-inflammatory, as well as pharmaceutical products developed from it, have received favourable reviews [2,3].

The parts of plants used in pharmaceutical and cosmetic products are indicated as dried flower heads or dried ligulate flowers (ray florets). Triterpene saponins, triterpene alcohols and their fatty acid esters, carotenoids, flavonoids, coumarins, essential oil, hydrocarbons, and fatty acids are all present in the ligulate flowers [4,5]. Triterpenoid fatty acid esters have been linked, via in vivo pharmacological testing, to the anti-inflammatory characteristics of calendula flowers [6,7]. The anti-inflammatory effect of 20-taraxastene variants appears to be mostly dependent on triterpenes, particularly on its main triterpenoid ingredient, faradiol (Figure 1). Triterpenes like ψ-taraxasterol, diols like faradiol and arnidiol, and triols like heliantriol A-C are all present. However, in this case, they are primarily found in the form of their 3-O-lauroyl, -myristoyl, and -palmitoyl esters [8-10].

Like pharmaceutical products, cosmetics are complex mixtures with precise compositions and specific properties. Chemical analysis or weighing applications are often only part of the manufacturing process but significantly impact the quality and integrity of the finished product. Essentially, in many countires, cosmetics must be safety tested and certified for compliance with GMP before being put on sale,

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and measurement of parameters such as pH, viscosity, specific gravity, and active ingredient is critical for consistency, homogeneity of mixtures and safety.

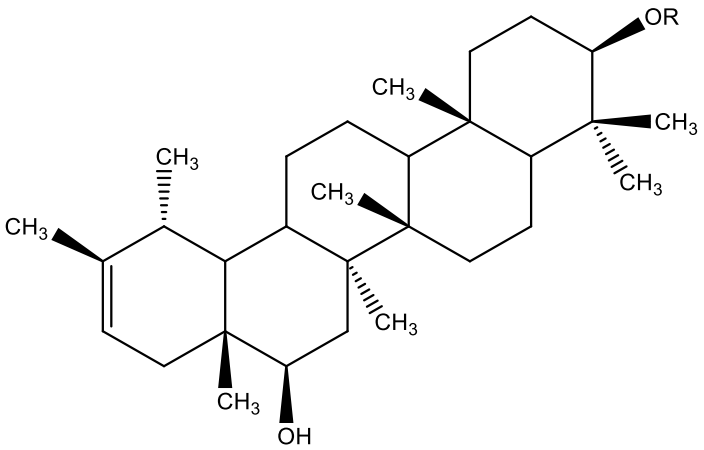

Figure 1. Structure of faradiol.

When we look at the literature, there are only a few methods available for the determination of faradiol by chromatographic methods (LC-APCI-QTOF-MS and TLC) [11,12]. Current methods are aimed at the determination and isolation of the active ingredient faradiol in plant extracts. Additionally, these methods have expensive detector requirements and long sample preparation procedures.

Nevertheless, there isn't a reliable technique available in the literature to measure faradiol in cream cosmetics that contain marigold (*Calendula officinalis* L.). The aim of this study is to ascertain the amount of faradiol present in creams for cosmetic items that contain marigold. Sensitive and targeted analysis is made possible by the proven HPLC technique. There isn't a single approach in the literature review that examines this range of products. Furthermore, despite the complexity of beauty product formulae, the straightforward extraction process is the first to be documented in the literature. Thanks to the proposed process, analysing cosmetic cream items containing marigold is simple and quick.

2. RESULTS AND DISCUSSION

2.1. Method Development

The best technique for determining the amount of faradiol in marigold creams, traditionally, is reverse-phase HPLC. The ideal chromatographic conditions were achieved by preliminary experiments. Tests were conducted on various kinds of columns at a variety of temperatures. At 35 ^{o}C, a higher resolution value (more symmetrical and sharper peaks) was obtained using a C18 column with the following dimensions: 4.6 mm I.D., 150 mm length, and 5 µm particle size. The chromatographic separation was carried out by isocratic elution at room temperature on a GL Sciences (Japan) C18 (ODS) column with methanol and water (2% o-phosphoric acid) (95:5, v/v) containing with a flow rate of 1.3 mL min^{-1}. The maximum absorption of faradiol was measured by UV spectrophotometer at 210 nm.

Under these circumstances, the retention period of faradiol is 6.38±0.20. The ideal conditions were determined by measuring peak areas and resolution values. Figure 2 (a-c) displays comparable chromatograms of the marigold cream sample (the genuine sample), the standard solution, and the undiluted solution. Table I displays the chromatographic separation procedure quality and the system appropriateness characteristics of the approach.

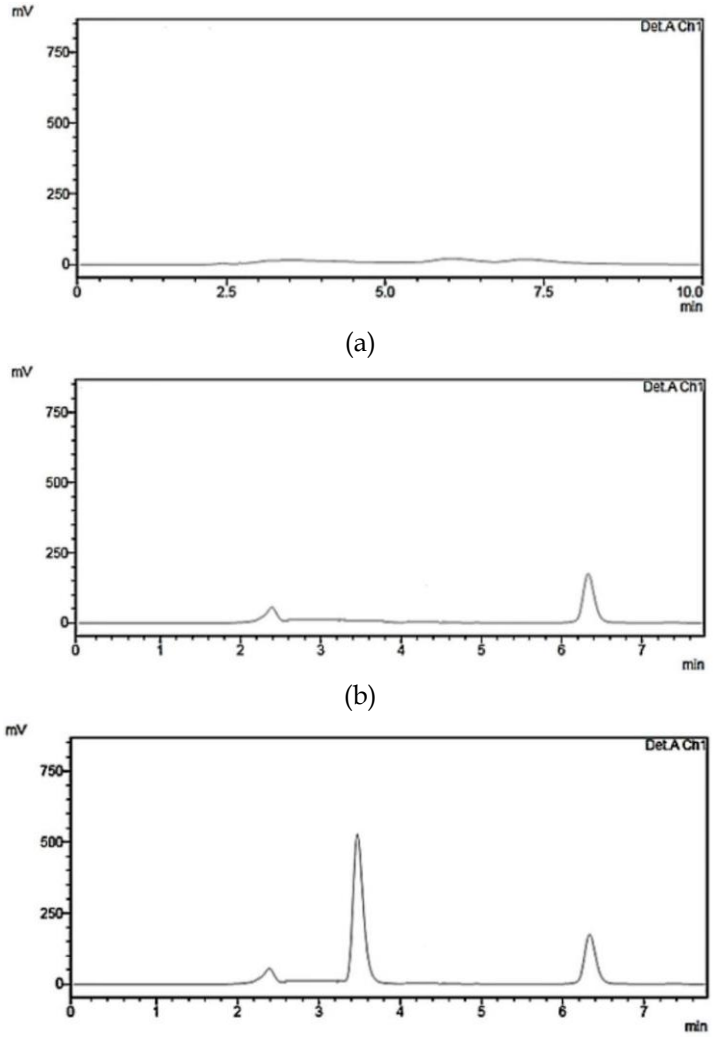

Figure 2. Aqueous medium (blank), standard solution (10 µg/mL standard faradiol solution), and genuine sample (cream sample) are shown as in a,b and c.

Table 1. Characteristics of the chromatographic system appropriateness.

Capacity factor*	Resolution*	HETP*	Tailing factor*	Asymmetry factor*	Theoretical plate numbers (N)
8.43	3.6	0.04	1.3	0.8	2528

*mean values of the parameters of all the points in calibration study are mentioned

2.2. Validation of the mehod

The following guidelines provided by the International Conference on Harmonisation (ICH) were followed in the validation of the approach [13,14].

2.2.1. Preparation of the calibration curve

The preparation of the calibration curve involved analysing standard solutions containing different quantities of faradiol, differentiating from 0.01 to 30 µg/mL. The calibration curve equation was calculated as y = 164951x - 181039, where x represents the concentrations of faradiol in 0.1-30 µg/mL and y shows the peak areas. This was done using linear least-squares analysis to determine the linear concentration ranges of the method (each levels has been examined as five duplicates).

2.2.2. LOD & LOQ

The formula used to calculate LOD and LOQ was LOD or LOQ=kSDa/b, where k=3 for LOD and 10 for LOQ, SDa standing for standard deviation of the intercept, and b for slope. The parameters for the analytical performance of the proposed method are compiled in Table 2.

Table 2. Illustration of parameters for the methods.

Parameters	Method
Concentration range[a] (µg/mL)	0.01-30
Regression equation[b]	y=164951x-181039
Slope±SD	164951±144
Intercept±SD	181039±128
Correlation coefficient, r^2	0.9963
LOD (µg/mL)	0.003
LOQ (µg/mL)	0.01

[a] Average of six determinations
[b] $y=xC + b$ where where C is the concentration in µg mL^{-1} and y is the peak area

2.2.3. Accuracy, precision and recovery

QC samples at three concentration levels were determined in order to evaluate accuracy and precision. QC samples comprising marigold cream specimens were made at three different concentrations (0.01, 10.0, and 30.0 µg/mL), which can be categorised as low, medium, and high concentration (n = 5). Recovery values were used to indicate accuracy, and RSD values of the recovery findings in five replicate trials were used to describe precision in the recovery research. By extracting spiked cream samples and comparing their peak areas with those received from the identical volumes of liquid, unextracted faradiol solutions, the 100% recovery of faradiol from marigold cream samples was investigated. When the amounts that are added to the spiked and measured by the calibration curve are compared, the mean relative recovery is determined to be 99.04%.

Five duplicate samples at every concentration were tested for accuracy and precision on a hourly basis in one day, and for daily results, five days. Every assay's RSD value, whether hourly or daily, was less than 2.62%. Good precision and accuracy were noted, based on all of these findings, which are compiled in Table 3. In comparison to the numbers obtained in our prior study, the percentage of recovery values are 99.40, 98.05, and 99.67 [15].

Table 3. Accuracy and precision of the method.

	Existing concentration	Added concentration	Found concentration (Mean±SD)	Percentange of Recovery	RSD of hourly variation	RSD of daily variation
Faradiol	10	0.01	9.95±0.28	99.40	0.62	1.75
	10	10	19.61±1.10	98.05	0.93	2.62
	10	30	39.87±1.91	99.67	0.45	1.88

n=3 for every concentration (given in µg mL^{-1})

2.2.4. Robustness

As mentioned in the validation section above, robustness was evaluated by determining the QC samples at three concentration levels (n=3). Adjusting the flow rate, column oven temperature, acetonitrile content, and mobile phase's aqueous phase contents allows for the measurement of the method's robustness. Aside from changing the column temperature from 35 °C to 40 °C and 30 °C, the mobile phase proportions were adjusted from 95:5 (methanol-water) to 97:3 and 93:7. The flow rate was also altered from 1.3 to 1.4 and 1.2 mL/min. Resolution and peak area were unaffected by these modifications. The method's robustness was demonstrated by low RSD values in Table 4.

Table 4. Results from robustness experiments.

Condition	Value	Recovery percent	RSD percent
Flow rate mL/min	1.2	96.44	2.19
Flow rate mL/min	1.4	95.13	2.65
Mobile phase composition	93:7	92.77	3.15
Mobile phase composition	97:3	93.62	3.08
Column temperature	30	98.60	1.84
Column temperature	40	99.03	1.76

n=3 for all QC sample levels.

2.2.5. Stability

Using spiking faradiol-containing marigold cream standards at 0.01, 10, and 30 µg/mL, which underwent four freeze-thaw cycles before to analysis, the effects of freezing and thawing of faradiol concentrations were investigated. It was also assessed how stable faradiol was in creams that were spiked with marigold and kept at room temperature for 24 hours and at 25 °C for four weeks. Faradiol stock solutions kept at -20 °C were durable for a minimum of 30 days. The concentration of faradiol in the marigolda creams wasn't decreasing following 30 days.

2.3. Extraction Process

The amounts of faradiol in marigold creams are listed in Table 5 following the technique and validation tests. From 94.95 to 82.80% were the recovery values. A cream containing marigold has a recovery value that is directly proportionate to the faradiol content (µg/mL).

Table 5. Concentrations of cream ingredients and reproducibility of the process.

Cosmetic product	Recovery	%RSD	Faradiol (µg/mL)
Sample 1	%82.80	2.61	14.90
Sample 2	%93.44	0.97	20.04
Sample 3	%91.62	1.25	19.25
Sample 4	%94.65	0.58	22.42
Sample 5	%90.34	1.57	18.89
Sample 6	%87.80	2.10	17.11
Sample 7	%84.62	1.93	24.02
Sample 8	%86.84	1.88	16.47
Sample 9	%83.40	2.33	15.38
Sample 10	%89.20	1.72	17.74

For each concentration n=5

4. CONCLUSION

Because cosmetic creams are widely used, an approach to measure the amount of faradiol in the formulations is presented. By measuring the amount of faradiol in cosmetic creams containing marigold (*Calendula officinalis* L.), the gap in the literature was filled. The HPLC-UV analysis period in earlier research (using plant extracts) was through thirty and thirty-two minutes. But the analytic duration for this work is merely 6.38 minutes. Additionally, one significant benefit of the approach is our use of isocratic elution. A detection is employed, possessing highly selective and sensitive characteristics. One benefit of the approach is the quick implementation of sample preparation. The primary benefit of this methodology is its appropriateness for examining several cosmetic formulas. This is also a quick, easy, and affordable process. This is the initial approach to identify cosmetics containing faradiol. It is anticipated that this procedure will be utilised for the regular examination, supervision, and uniformity of cosmetic items that include faradiol. The approach can be practically used in ordinary examination in this fashion.

5. MATERIALS AND METHODS

5.1. Chemicals and Reagents

Acetonitrile, methanol, ethanol, and faradiol (≥95.0% HPLC) were acquired from Sigma Aldrich. Merck provided the orthophosphoric acid (Darmstadt, Germany). A Human system (Japan) provided water purification.

5.2. Solutions

Standard solutions ranging from 0.01 to 30 µg/mL were procured by diluting a stock solution of 200 µg/mL of faradiol with methanol. Working standard solutions were aliquoted in 30 µL volumes for HPLC analysis. The chromatograms were analysed using the peak areas versus the concentration of the faradiol components.

5.3. Preparation of the Calibration Curves

By analysing working standard solutions of faradiol at different concentrations, a calibration curve was created. Linear least-squares regression calculations were used to determine the linear concentration ranges (each concentration was investigated as five repetitions) of the technique for the two pharmaceuticals.

The calibration curve's equations have been obtained as y = ax+b, where x represents the drug substances' concentrations in µg/mL and y represents the peak areas.

5.4. Instrumentation and Chromatographic Conditions

The Shimadzu (Japan) liquid chromatography system, which included an LC-20AT pump, SIL AT-HT autosampler component, SPD-20A HT UV spectrophotometric detector set at 210 nm, and CTO 10 AC column oven, was utilised for the HPLC analyses. To achieve the most effective chromatographic separation, a range of mobile phase, column types, and stationary phase size combinations were tested with varying flow rates and column temperatures. On a GL Sciences (Japan) C18 (ODS) column with the dimensions of 4.6 mm I.D., 150 mm length, and 5 µm particle size, chromatographic separation was achieved isocratically at 35 ^{o}C. Methanol makes up mobile phase A, while 2% o-phosphoric acid in water, with a flow rate of 1.3 mL/min, makes up mobile phase B.

5.5. Extraction Process for Application the Method to Cosmetic Products

Different extraction methods, like liquid-liquid extraction (LLE) and solid phase extraction (SPE), are established for the purpose of extracting faradiol out of cosmetic cream products. The liquid-liquid extraction (LLE) approach was initially investigated using a range of extraction solvents (methanol-water, ethanol-water, methanol-acetonitrile, ethanol-acetonitrile, chloroform-water, chloroform-acetonitrile, dimethylsulfoxide-water, dimethylsulfoxide-acetonitrile), combinations (1:1 and 1:2) and extraction solvent volumes (0.5 mL, 1.0 mL and 1.5 mL) in order to create an efficient method of extracting faradiol from creams. Employing the solid phase extraction (SPE) technique with various cartridges types and sizes (C18-N, C8, NH_2, C18 Resprep cartridges; 6 mL, 1.000 mg), experiments were conducted using various elution procedures employing reversed phase and normal phase sorbents. Following these trials, which demonstrated higher recovery values and increased efficiency from LLE, the following procedure was chosen to be used.

0.5 g of cream samples were weighed, and 1.5 mL of an ethanol:acetonitrile (1:1) mixture was added to the Eppendorf tube. After 30 seconds of vortexing, the mixture went through a centrifuge for 10 minutes at 4000 rpm. Using 0.45 µm polyethersulfone filters (Dainippon Seiki, Kyoto, Japan), 800 µL of the supernatant was filtered, and the liquid was then transferred to a 1.5 mL HPLC vial. For every duplicate, 30 µL of the liquid from the vial was administered.

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