Research Article

High-performance Liquid Chromatography Method Development and Validation for the Determination of Ellagic Acid in Topical Cream Formulations as Hyperpigmentation Treatment

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Abstract

Skin hyperpigmentation is a dermatological condition that cause a change in skin coloration. In this condition an overproduction of melanin happens in skin melanocytes. Ellagic Acid (EA) is a pharmaceutical substance that found in many fruits and plants which is quite useful to apply in skin products due to its antityrosinase and antioxidant activity. In this study a topical cream of EA was prepared and methanol was employed as extraction solvent to determine the amount of EA in the formulation. For this aim we have developed and validated a High-Performance Liquid Chromatography (HPLC) method in order to evaluation the amount of EA in cream. The developed method was validated against criteria such as linearity, specificity, precision, accuracy, Limit of Detection (LOD) and Limit of Quantification (LOQ) due to the International Council for Harmonization (ICH) guidelines, Q2. Our results showed high specificity, linearity ($R2=0.9973$), sensitivity and acceptable precision and accuracy rate. By our developed HPLC method the concentration of EA in topical cream was calculated, and it was found to be 9.81 mg/ml. Also, this method can be used for EA determining in lipid nanoparticles, such as liposomes, solid lipid nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs).

Keywords: Ellagic Acid determination; Antityrosinase activity; HPLC Method validation; Topical cream

Abbreviation: EA: Ellagic Acid; HPLC: High-Performance Liquid Chromatography; LOD: Limit of Detection; LOQ: Limit of Quantification; ICH: International Council for Harmonization; SLN: Solid Lipid Nanoparticle; NLC: Nanostructured Lipid Carrier.

Introduction

Skin hyperpigmentation establishes as a change in skin coloration that it becomes mostly darker. This condition is a result of many factors including drug effects, ultraviolet exposure, inflammation, hormonal changes, acne and skin damages that make an excess of melanin producing in skin melanocytes and cause pigmentation [1]. As first line therapy many substances such as kojic acid, hydroquinone, retinoic acid and azelaic acid are employed as skin lightening agents. According to the adverse effects of these substances like cytotoxicity and irritation, applying natural products as lightening agents is increasing [2].

Ellagic Acid (EA) ($C14H6O8$) is a phenolic active substance which found in pomegranates, strawberries, blackberries and many other plants [3]. EA is considered as a useful agent for hyperpigmentation treatment because it affects the melanin biogenesis path in which tyrosinase catalyzes the hydroxylation of monophenols to o-diphenols and their consequent oxidation to the o-quinones, which are then changed to melanin [4-7]. Also, many studies mention its antioxidant activity due to this substance scavenging of free radicals ability [8-11].

Basically, for detection of a drug and evaluation its amount in a pharmaceutical formulation, developing an analytical method and

validate the developed method are necessary. Method validation includes a sequence of processes that approve the reproducibility and reliability of developed method [12].

Many Ultraviolet-Visible (UV) –spectrophotometry methods have been developed to evaluate the amounts of EA while few studies use HPLC method for determining EA amount. UV–spectrophotometry methods are easy to use, rapid and simple with some disadvantages such as low sensitivity and not reliability whereas High-Performance Liquid Chromatography (HPLC) is more reliable, repeatable and high-sensitive [12-14].

The aim of this study is determining EA amount in topical creams by a HPLC method that develop and validate according to the International Council for Harmonization (ICH) guidelines.

Materials and Methods

Materials

EA bulk powder, stearic acid and cetyl alcohol were purchased from Sigma-Aldrich (Germany). Tween® 80, propylene glycol and isopropyl myristate were obtained from Uniqema (Belgium). Pure DMSO and methanol was purchased from Merck (Germany).

Preparation of Standard Solutions

EA powder (1 mg) was weighted exactly and was completely dissolved in 5 mL methanol. Standard solutions with following concentration: 5, 10, 15, 20, 25 and 30 μg/ml were prepared by serial-dilution of first solution with methanol.

Topical Cream Preparation and Evaluation Amount of EA

EA-Cream was produced by dissolving EA (1% w/v) in lipid phase including stearic acid, cetyl alcohol and isopropyl myristate and pre-heating to 80°C. Separately, the deionized water, propylene glycol and Tween® 80 as aqueous phase was heated up to the same temperature. Then the aqueous phase was quickly supplemented to the lipid phase and the cream was obtained by a high-speed stirrer (Ultra-Turrax IKA T10; IKA Werke GmbH & Co. KG, Staufen, Germany) for 5 min at 5000 rpm. The resultant formulation was cooling down to room temperature. The cream without EA was also prepared by the same method that mentioned above.

To quantify the concentration of EA in topical cream, 100 mg of formulation was diluted in 5 ml absolute methanol and the concentration of EA was determined by using HPLC method. This method was performed by a HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a C18 reverse-phase column (250 × 4.6 mm, 5 μm; Teknokroma Co., Barcelona, Spain). The mobile phase in this method was a mixture of deionized water (50%), methanol (30%), acetonitrile (20%) and phosphoric acid (0.1%) with 0.8 min/ml flow rate and the wavelength was set up at 254 nm. The retention time of EA in these conditions was 5.8 minutes.

Method Validation Studies

To validate the developed method, studies were set up due to ICH guidelines, Q2. The evaluated parameters were linearity, specificity, precision, accuracy, Limit of Detection (LOD) and Limit of Quantification (LOQ) (15).

Linearity

The prepared standard solutions were evaluated by the developed HPLC method in triplicate. The calibration diagram was drawn by the concentrations and peak areas of them. The linearity parameter was validated using correlation Coefficient (R^2) of the obtained linear regression.

Specificity

By comparison the peak areas of reference solution and EA-cream, the influence of cream ingredients on quantification of EA was evaluated and the specificity parameter got validated.

Precision

By evaluating the repeatability of the developed method on one day (intraday precision) and on three different days (interday precision), the precision parameter was validated. With the aim of assess repeatability, three different standard solutions (10, 20 and 30 μg/ml) were assayed and mean, standard deviation (SD) and percentage of relative standard deviation (RSD %) were determined. Three replicates of each concentration were evaluated.

Accuracy

To validate the accuracy of method, three different standard solution (24, 30 and 36 μg/ml) were prepared and spiked in empty cream. Then by developed HPLC method the peak areas of each sample were assayed and the percentage of recovery, mean and SD were assessed.

LOD and LOQ

These parameters were obtained by determining the slope of calibration diagram (S), SD of y-intercepts of the regression line (δ) and the following equations:

$$LOD = 3.3\delta/S \qquad LOQ = 10\delta/S$$

Results and Discussion

The aim of this study was the development and validation of a HPLC method to determining the amount of EA in topical cream. This validation was done by investigating in terms of linearity, specificity, precision, accuracy, LOD and LOQ according to ICH guideline, Q2.

Method Validation Results

Linearity and Specificity

First, a peak was obviously identified in the chromatogram of EA standard solution at 5.8 min (Figure 1a). As shown in Figure 1b this peak was also recognized at the same retention time in EA-cream sample. Next, calibration curve was obtained by plotting peak areas against six EA standard concentrations with three injection per concentration (Figure 2) and also correlation coefficient was calculated (Table 1).

Precision and Accuracy

In order to validate the precision of developed method, three concentrations of EA were selected and analyzed three times on the same day and three times on different days. Results showed acceptable intraday and interday variability and calculated RSD% values were lower than 2% (Table 2).
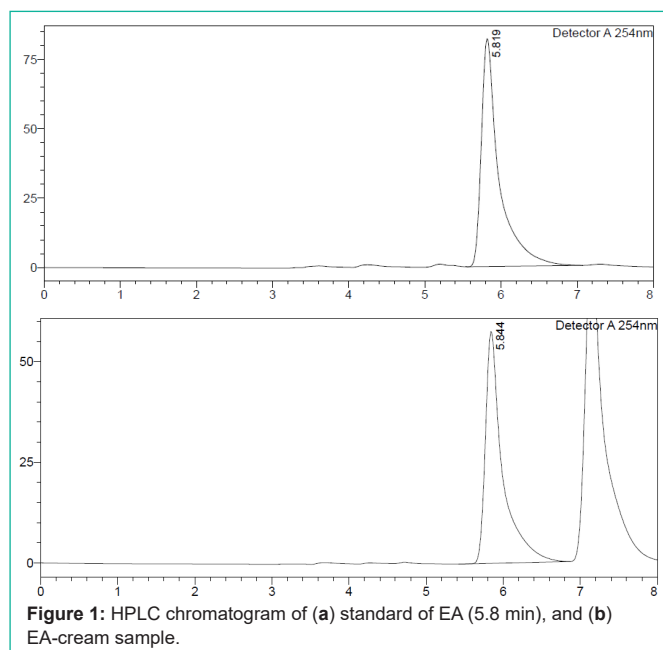
Figure 1: HPLC chromatogram of (**a**) standard of EA (5.8 min), and (**b**) EA-cream sample.
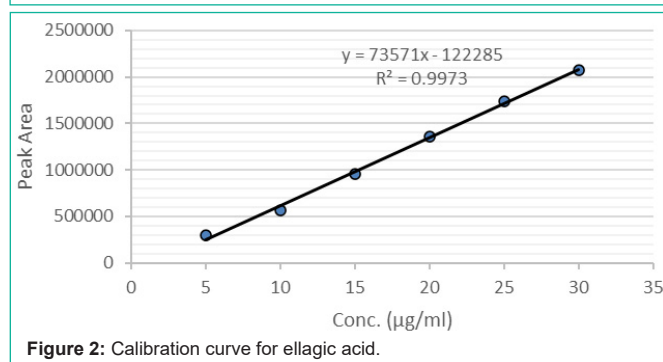

Figure 2: Calibration curve for ellagic acid.

Table 1: Results of linearity regression, correlation coefficient, LOD, and LOQ for ellagic acid.

	Regression Equation	R2	Residual STD (s)	Calibration Curve Slope (S)	LOD (µg/ml)	LOQ (µg/ml)
Ellagic acid	73014x - 132342	0.9974	37357	73571	1.67	5.07
	78086x - 145105	0.9937				
	69612x - 89407	0.9986				
Integration (n=3)	73571x - 122285	0.9973				

Table 2: Intraday and interday variabilities of ellagic acid.

	Conc. (µg/ml)	Intraday Variability (n = 3)		Interday Variability (n = 3)	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Ellagic acid	10	9.93 ± 0.19	1.91	9.56 ± 0.16	1.67
	20	20.02 ± 0.08	0.40	20.35 ± 0.20	0.98
	30	30.53 ± 0.34	1.11	30.32 ± 0.54	1.78

Table 3: Results of accuracy validation for ellagic acid.

	Conc. (µg/mL)	Observed (µg/ml) Mean ± SD	Recovery (%) Mean ± SD
Ellagic acid	24	24.21 ± 0.25	100.84 ± 1.04
	30	30.67 ± 0.36	102.24 ± 1.20
	36	36.23 ± 0.43	100.63 ± 1.19
	Average (n=9)		101.23 ± 1.14

To validate the accuracy of method, drug recovery percentage of standard solutions spiked in empty cream were determined and the mean recovery percentage was confirmed to be 101.23% (Table 3). Accordingly, by our results the both precision and accuracy of developed method were validated.

LOD and LOQ

The calculated LOD and LOQ values for developed method were found to be 1.67 and 5.07 µg/ml, respectively that confirm the sensitivity of this method (Table 1).

Evaluation Amount of EA in Topical Cream

Finally, the validated method was applied to quantify the concentration of EA in topical cream. First the samples (10 ml) were prepared as described above and then by the developed HPLC method, the concentration of EA in formulation was calculated, and it found to be 9.81 mg/ml.

Conclusion

The purpose of this study was development and validation of a HPLC method in order to determine the EA amount in topical cream formulation.

The developed method was validated against criteria such as linearity, specificity, precision, accuracy and sensitivity. Also, the quantification of EA in topical cream was done successfully by the validated method. Consequently, this method is reliable and can be applied not only for conventional topical creams but also for various formulations especially lipid nanoparticles, such as liposomes, SLNs and NLCs.

Author Statements

Declarations

The authors state that they have no known financial interests or personal relationships that could have affected the work described in this paper.

Conflict of Interest Statement

All authors declare that there is no conflict of interests in this study.

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