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A new HPLC-UV method for the simultaneous measurements of α-escin and β-escin in creams containing *Aesculus hippocastanum* L. extract

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Abstract: Amount of α-escin and β-escin in cosmetic cream formulations were determined by simple reverse phase HPLC method by using the C18 column at 220 nm. The mobile phase is composed of acetonitrile-water (60:40) at pH 2.4. The method validation study was carried out based on the ICH recommendations and linear regression equations were determined as y=1548.3x+340.7 and y=1521.3x-91.9, for the analytes, respectively. While LOD values of the method were determined for α-escin and β-escin as 0.003 µg/mL and 0.027 µg/mL, LOQ values were determined as 0.01 µg/mL and 0.08 µg/mL, respectively.

Keywords*:* Horse chestnut; α-escin; β-escin; HPLC-UV; validation. © 2023 ACG Publications. All rights reserved.

1. Introduction

The main active component of horse chestnut (*Aesculus hippocastanum* L.) seed extracts is escin (aescin), a blend of triterpene saponins. Hemorrhoids, inflammation, cerebral ischemia injury, and chronic venous insufficiency are among the illnesses for which escin has historically been prescribed [1-3]. Its exceptional efficacy in preventing and treating a range of peripheral vascular diseases stems from multiple molecular mechanisms, such as inhibition of neutrophil adhesion, sensitization of calcium channels, enhancement of venous tension and capillary sealing, elastase and other enzymes inhibition and releasing PGF2α [4]. α-escin and β-escin are the two forms of the saponins found in escins (Figure 1). The separation of them is done through the melting point, hemolytic index, specific rotation and solubility in water [4]. β-escin the main active component in extracts made from horse chestnut seeds. As a result of several different research, it is clear that β-escin can inhibit proliferation and/or induce apoptosis in numerous cancer cell types such as A549 cell, HT-29 cell, HCC cell, OVCAR3 cell and H-Ras 5RP7 cell [5,6].

As a result of the studies, β-escin has shown possibilities to be used as a chemopreventive agent, particularly for malignant growths related to an inflammatory response. Even though α-escin is the minor component of escin, it shows bioactivity but at a lower rate compared to β-escin. Because of the lower bioactivity, only a few studies were dedicated solely on the pharmacological activity of α-escin. Even though the number of the studies are low in quantities, these studies have demonstrated significant improvement for the treatment of osteopenia in rats. Despite the difference in pharmacological activity, α- and β-escin are used together in production of commercial samples [7,8].

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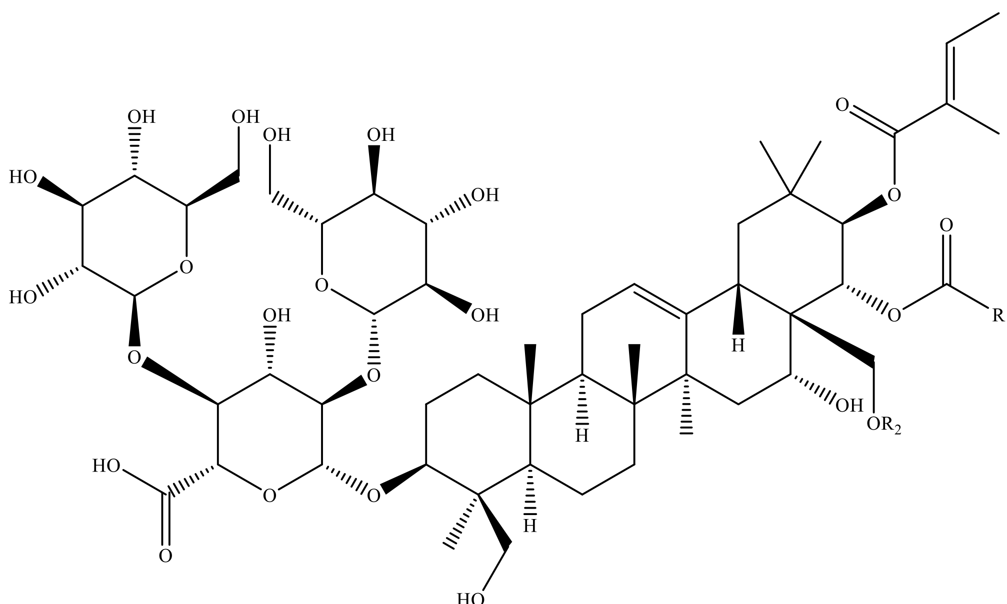

Figure 1. Chemical structure of α-escin (R_1=H and R_2=$COCH_3$) and β-escin (R_1=$COCH_3$ and R_2=H)

Numerous analytical techniques were published for the estimation the level of escin including thin-layer chromatography [9], enzyme immunoassay [10], high-performance liquid chromatography (HPLC) [11], radioimmunoassay (RIA) [12,13] and LC-MS [14]. Nevertheless, the listed methods are generally used in the applications of herbal medicinal products, seed extracts or in vitro studies of transport through the skin. The two separate research done by Schrödter and Kuntz using radioimmunoassay was solely focused on the measurements of total β-escin after one or multiple dosages of human plasma have been administered [12,13]. Despite the widespread preference for horse chestnuts among patients, healthcare professionals, and the general public, there is currently no scientific method displayed to measure α- and β-escin in creams containing horse chestnut.

The use of creams containing horse chestnut (*Aesculus hippocastanum* L.) is for the aim to develop and validate a high-performance liquid chromatography (HPLC) method that will allow analysis of α-escin and β-escin together without interfering with other components. According to the developed method, the analysis of cream products claimed to contain horse chestnut will be carried out easily and quickly in routine laboratories. According to the validation studies, it was shown that this method is sensitive, selective, precise, and accurate.

2. Experimental

2.1. Instrumentation and Reagents

A Shimadzu (Japan) LC-20 liquid chromatograph, equipped with an SPD-20A HT UV detector with a wavelength set at 220 nm, an LC-20AT pump, a SIL AT-HT autosampler component, and a CTO 10 AC column oven, was utilized for the HPLC studies. On a GL Sciences (Japan) C18 (ODS) column with a measurement of 4.6 mm I.D., 150 mm length, and 5 µm particle size, chromatographic separation was accomplished isocratically at 25℃. With a flow rate of 1.2 mL/min, the mobile phase consisted of acetonitrile-water (0.1% phosphoric acid) (pH 2.4) containing 1 mL/L triethylamine. All chemicals used in this study were of analytical reagent grade. α-escin (≥95 %) and β-escin (≥95 %) were acquired through Sigma Aldrich, St. (Louis, Missouri, USA). Acetonitrile, orthophosphoric acid, and triethylamine were obtained from Merck (Darmstadt, Germany). Water was purified by the Human (Japan) ultrawater purification system.

2.2. Methods

The stock standard solutions of α-escin and β-escin were dissolved in water at 100.0 µg/mL. In order to produce a working standard solution in different concentrations before measurements, stock solutions were diluted with water. Working standard solutions were aliquoted in 20 µL volumes for HPLC analysis. The peak areas versus concentration of the cosmetic raw components were used to analyze the chromatograms.

2.3. Preparation of the Calibration Curves

The preparation of calibration curves involved the examination of α- and β-escin standard solutions for use at different concentrations. The proportional concentration ranges of the approach for the two substances were found using linear least-squares regression analysis. Each concentration level was examined in five replicates, and the calibrating slope calculations were computed as follows: $y = ax + b$, where x represents the measured concentration of the cream's content in µg/mL and y represents the maximum area.

2.4. Uncertainty Assesment

The uncertainty from the purity of the compound ($u_{standard}$), weighing ($u_{weighing}$), calibration curve parameters ($u_{calibration}$), recovery ($u_{recovery}$), and repeatability ($u_{repeatability}$) were evaluated for the main uncertainty contributions. The combined uncertainty ($U_{Combined}$) was calculated using the formulas described in the literature [15-20]. The overall expanded uncertainty ($U_{expanded}$) was determined at 95% confidence level by multiplying the achieved combined uncertainty with coverage factor (k) equal to 2.

2.5. Extraction Process for Application the Method to Cosmetic Products

Various extraction procedures have been developed for the extraction of α-escin and β-escin from cosmetic products, including solid phase extraction (SPE) and liquid–liquid extraction (LLE). In order to provide efficient extraction of α-escin and β-escin from creams, the liquid-liquid extraction (LLE) technique was first tested with various extraction solvents, mixtures and different volumes of extraction solvents. Experiments were carried out using different elution techniques with normal phase and reverse phase sorbents using the solid phase extraction (SPE) technique. As a result of these trials, LLE showed greater efficiency with higher recovery values, and it was decided to apply the following method.

0.1 g of cream samples weighed 1.5 mL of Eppendorf tube and 1 mL of ethanol:acetonitrile (1:1) mixture was added. The mixture was vortexed for 30 seconds. centrifuged at 4000 rpm for 10 min. 800 µL of the supernatant was filtered through 0.45 µm polyethersulfone filters (Dainippon Seiki, Kyoto, Japan) and the liquid transferred to 1.5 mL of HPLC vial. 20 µL of the liquid from the vial was injected for each replicate.

3. Results and Discussion

3.1. Method Development

Regarding the concurrent identification of α-escin and β-escin in cream products, a reverse-phase HPLC approach was chosen. Tests were conducted on several kinds of C8 and C18 HPLC columns at different temperature (20°C, 25°C and 30°C) levels. Higher resolution (sharper and more symmetrical peaks) was achieved on C18 column (150 mm, 4.6 mm, 5 µm) at 25ºC. *o*-phosphoric acid solution (0.1%) including containing 1 mL/L triethylamine was chosen four aqueous part of the mobile phase. The organic modifier of choice being acetonitrile, a mobile phase composition of acetonitrile–phosphoric acid (pH: 2.4), 60:40 (v/v) at a flow rate of 1.2 mL/min was employed in order to obtain an exceptionally precise result. Based on UV pattern of the analytes the detection wavelength for quantification was selected at

220 nm. The cream materials' retention periods within such circumstances are 5.62 ± 0.04 and 7.21 ± 0.03 for α-escin and β-escin, respectively (Figure 2).

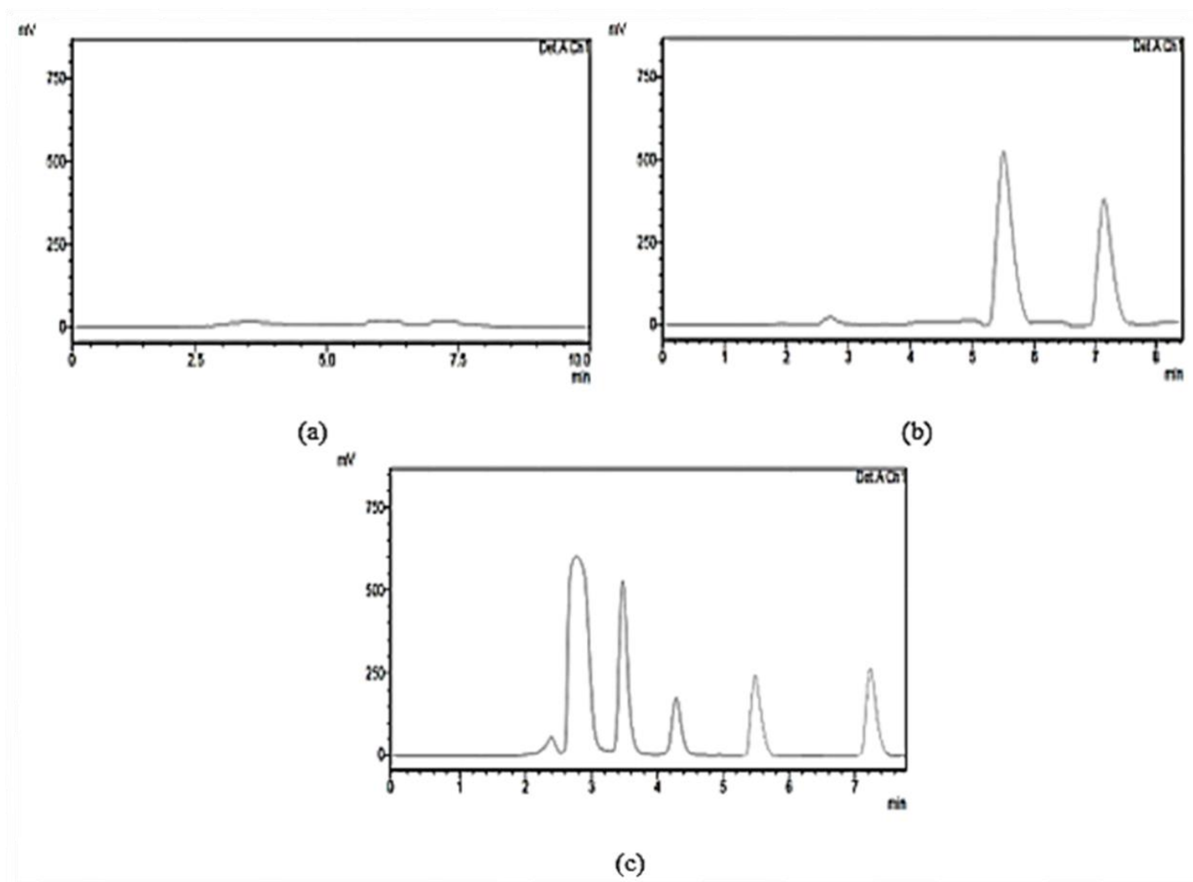

Figure 1. Blank (aqueous medium) **(a),** standard solution (10 μg/mL standard α-escin and β-escin solutions) **(b),** cream sample **(c)**

3.2. Method Validation

The International Conference on Harmonization (ICH) provided the subsequent specifications, which were followed during the technique's verification [21].

3.2.1. Linearity, LOD and LOQ

The resulting linearity limits were 0.01-50 for α-escin and 0.08-20 μg/mL for β-escin and linear regression equations were obtained $y = 1548.3x + 340.7$ (R^2=0.9915) and $y = 1521.3x - 91.9$ (R^2=0.9924), respectively (Table 1).

Limits of detection (LOD), limit of quantification (LOQ) the method were calculated using the following equation, the LODs of the approach for distinct substances were determined: LOD or LOQ = κSDa/b, where κ = 3 for LOD and 10 for LOQ. While LOD values of the method were determined for α-escin and β-escin as 0.003 μg/mL and 0.027 μg/mL, LOQ values were determined as 0.01 μg/mL and 0.08 μg/mL, respectively.

Table 1. Analytical variable findings relating to the suggested approach

Parameter	α-Escin	β-Escin
Linearity range* (µg/mL)	0.01-50	0.08-20
Regression equation	*y=1548.3× + 340.7*	*y=1521.3× - 91.9*
Slope±SD	1548.3	1521.3
Intercept±SD	340.7	91.9
Correlation coefficient, R^2	0.9915	0.9924
LOD (µg/mL)	0.003	0.027
LOQ (µg/mL)	0.01	0.08

*n=5 matches the replicated analysis for every stage.

3.2.2. Precision

Three degrees of concentrations (low, medium, and high) have been assessed for the daily and hourly accuracy measurements by analyzing cream specimens for seven distinct days (each with n = 5). Significant reproducibility of the method was shown by the RSD values, which were found to be 0.22–1.84% for hourly accuracy and 1.66–2.43% in daily consistency.

3.2.3. Recovery

Recovery experiments were carried out to determine the accuracy of the method for the quantification of α-escin and β-escin. Recovery of the method was checked at three different concentrations by spiking of the standards to the cream formulations which are given in Table 2 and the following equation was used for the recovery experiments.

$$Recovery\ \% = ((C_t - C_u))/C_a)\times 100$$

Where C_a represents the proportion of the purified analyte introduced to the formulation, C_u represents the proportion of the analyte contained within the formula, and C_t is the overall concentration of the analyte discovered. Table 2 displays the findings from the recovery research and the examination of the industrial cream specimen types.

Table 2. Findings from recovery experiments using the standard addition method

	Existant concentration (µg/mL)	Added concentration (µg/mL)	Found concentration (µg/mL) (Mean±SD)	Recovery (%)	RSD of intraday variation	RSD of interday variation
α-escin	25	0.01	24.98±0.66	99.88	0.96	1.95
	25	10	34.11±0.18	97.45	1.01	1.23
	25	25	49.33±0.22	98.46	1.46	1.53
β-escin	10	0.08	10.01±0.73	99.30	0.63	2.19
	10	1	10.88±0.34	98.90	0.58	1.83
	10	10	19.28±0.88	96.40	1.10	1.66

*For each concentration n=5

3.2.4. Stability

This feature for the working standard mixtures of the cream samples was assessed under various storage conditions, including room temperature in the dark, in autosampler conditions for 48 hours, and kept cooled at 4ºC for a month. The results of the stability studies indicated that the samples remained stable under these conditions. Specifically, the samples were seen to be steady when stored at room temperature for 48 hours, as well as when kept in autosampler conditions for the same duration. Additionally, refrigeration at 4ºC for 1 month did not compromise the stability of the samples. In all tested storage conditions, both α-escin and β-escin were determined to be stable. I suggest that the method can reliably analyze these cosmetic raw materials without concerns about sample degradation during storage.

3.2.5. Robustness

The robustness of the approach was assessed by varying a few important factors, including the flow rate, column oven temperature, and the proportions of acetonitrile and the acidic solution in the mobile phase. Specifically, the mobile phase composition was modified from the initial 60:40 (acetonitrile–acidic solution) to two different ratios: 55:45 and 65:35. The column temperature was adjusted from the initial 20ºC, 25ºC and 30ºC, while the flow rate was altered from 1.2 to 1.1 and 1.3 mL/min. It's noteworthy that these changes in the method's parameters did not have any significant impact on the peak areas. Low relative standard deviation (RSD) values, as indicated in Table 3, demonstrate the robustness of the method. This suggests that the method can provide consistent and reliable results even when slight variations in these parameters are introduced, which is an essential characteristic for its practical applicability.

Table 3. Results from robustness experiments

Condition	**Value**	**Recovery**		**RSD (%)**	
		α-escin	β-escin	α-escin	β-escin
Flow rate mL/min	1.1	99.18	100.11	0.61	0.85
	1.3	99.56	98.81	1.35	1.74
Mobile phase composition	55:45	97.44	98.37	1.80	2.24
	65:35	98.39	99.24	1.98	2.54
Column temperature	20	99.01	99.66	0.28	1.23
	30	98.55	98.78	0.36	1.30

*For each concentration *n*=5

3.3. Extraction Process

After the method and validation studies, the concentrations of α-escin and β-escin in cream products containing horse chestnut (*Aesculus hippocastanum* L.) are given in Table 4.

Table 4. Creams material concentrations and method reproducibility

Cosmetic product	**Recovery**	**%RSD**	**α-escin**	**β-escin**
Sample 1	%92.60	1.20	33.90	12.85
Sample 2	%89.36	2.83	26.54	9.11
Sample 3	%90.88	1.62	29.47	10.66
Sample 4	%93.41	0.98	36.80	13.38
Sample 5	%95.55	1.36	39.21	16.23
Sample 6	%87.63	2.10	22.61	7.32
Sample 7	%90.22	1.58	28.95	9.80

*For each concentration *n*=5

3.4. Estimation of Uncertainty Budget

The results were expressed in percentage (%) at 95% confidence level for both analytes in the calculated parameters. The results noticed in the study were presented in Table 5 which suggests that the results were acceptable. The result related to the uncertainty due to sample weighing was noticed to be negligible and hence were not presented.

Table 5. Combined and expanded uncertainity results

Analytes	$u_{standard}$	$uc_{alibration}$	$u_{recovery}$	$u_{repeatability}$	$u_{combined}$	$U_{expanded}$
α-escin	0.3	1.5	0.2	0.7	1.2	3.4
β-escin	0.3	1.3	0.3	2.0	2.4	4.8

k=2 (95 % confidence level)

In conclusion, a simple extraction and an HPLC method α-escin and β-escin in cosmetic cream formulation using a C18 column column at 220 nm. The method is simple, precise and accurate and it can be applicable for the routine measurements of the cosmetic laboratories within 8 min.

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