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A Novel HPLC technique for the determination of casticin in pharmaceutical preparations and human plasma

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ABSTRACT: This study introduces a combined HPLC and UV detection technique for the quantification of casticin in capsule and human plasma samples. The chromatographic separation was carried out utilizing a C18 column (150 mm × 4.6 mm × 5 µm) at a temperature of 25 °C. Isocratic elution with a mobile phase comprising 60:40 v/v (methanol-0.05% formic acid) was employed. Flow rate was adjusted 1 mL/min. The analyte was determined at a wavelength of 258 nm, with a retention time of 14.7±0.01 min. The developed method underwent validation according to ICH criteria, covering specificity, linearity, precision, accuracy, detection and quantitation limits, as well as robustness. The linear range was determined to be 10-60 ng/mL for both capsule and spiked plasma specimens. The suggested technique was performed to the analysis of casticin in spiked human plasma and pharmaceutical preparations, yielding a recovery of 106.04% and demonstrating precision through intra-day and inter-day experiments with the highest relative standard deviation (RSD %) value of 4.94. Consequently, the technique was performed to the quantifying of human plasma specimens from in a patient taking medication containing casticin.

KEYWORDS: Casticin; high performance liquid chromatography; validation; human plasma; capsule

1. INTRODUCTION

Casticin, also known as Vitexicarpin, is a major flavone component found in medicinal plants of the Vitex species, including *Vitex agnus-castus* L., *Vitex trifolia* L., and *Vitex rotundifolia* L. Its chemical name is 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one (Figure 1). In recent years, casticin has gained attention as the primary active chemical constituent in these plants, particularly for its potential in treating premenstrual syndrome (PMS) [1-3]. Casticin has demonstrated various pharmacological properties, including antitumor [4-10], antioxidant [3,11], anti-inflammatory [12-15], anti-hyperprolactinemia [1,2,16], and anti-nociceptive [2,17] effects. Due to these therapeutic activities, casticin holds promise as a potential new pharmaceutical agent for PMS. To develop a new method of quantification of casticin, it is crucial to determine a sensitive analytical technique for accurately quantifying casticin in biological samples.

Numerous techniques employing high performance liquid chromatography (HPLC) have been reported for the separation and quantification of casticin [18,19,20]. However, most of these methods have primarily focused on quantifying casticin in pharmaceutical forms, with limited works available on its analysis in biological specimens. Although there are studies analyzing casticin in animal plasma, data on the analysis of casticin in human plasma is lacking. For instance, one study performed liquid chromatography-mass spectrometry (LC-MS) technique for determining casticin in rat plasma specimens [21]. Another study performed plasma analysis in rats following the oral administration of Viticis fructus extracts utilizing ultra-performance liquid chromatography-tandem mass spectroscopy (UHPLC-MS/MS) [22]. In contrast to these animal studies, the aim of this study was to establish and validate a basic and fast HPLC-UV trial for quantifying casticin in both human plasma samples and pharmaceutical preparations.

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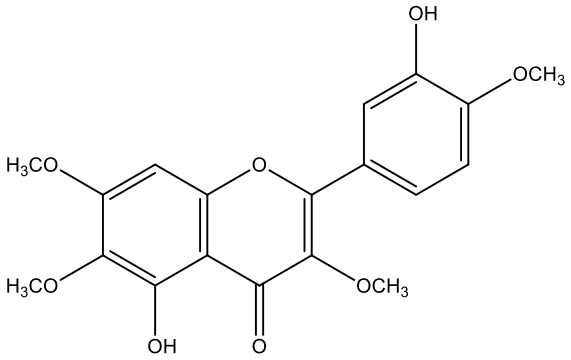

Figure 1. Chemical structure of casticin

2. RESULTS AND DISCUSSION

2.1. Chromatographic Process

The quantification of casticin in human plasma was performed utilizng an HPLC-UV detection method. Preliminary experiments were conducted to optimize the chromatographic conditions. Various column types were tested at different temperatures, and the most efficient results were obtained using a C18 column (Shim-Pack, Shimadzu Corporations-Japan) with 150.0 mm × 4.6 mm × 5.0 µm, operating at 25.0 °C. Different mobile phase solutions were evaluated at various flow. It was found that an acidic mobile phase provided satisfactory results, and thus a formic acid solution with a concentration of 0.05% (v/v) was chosen as the preferred acidic aqueous solution. For the chromatographic separation, an isocratic elution profile with a mobile phase composition of methanol-0.05% formic acid (60:40, v/v) was used, with a flow rate of 1.0 mL/min, to achieve high resolution. Detection was performed at a wavelength of 258 nm. Casticin was determined at 14.7±0.01 minutes. The chromatograms of blank plasma, spiked plasma, and standard solutions are illustrated in Figure 2 (a, b, c). The system suitability parameters, which indicate the quality of the chromatographic system, are presented in Table 1.

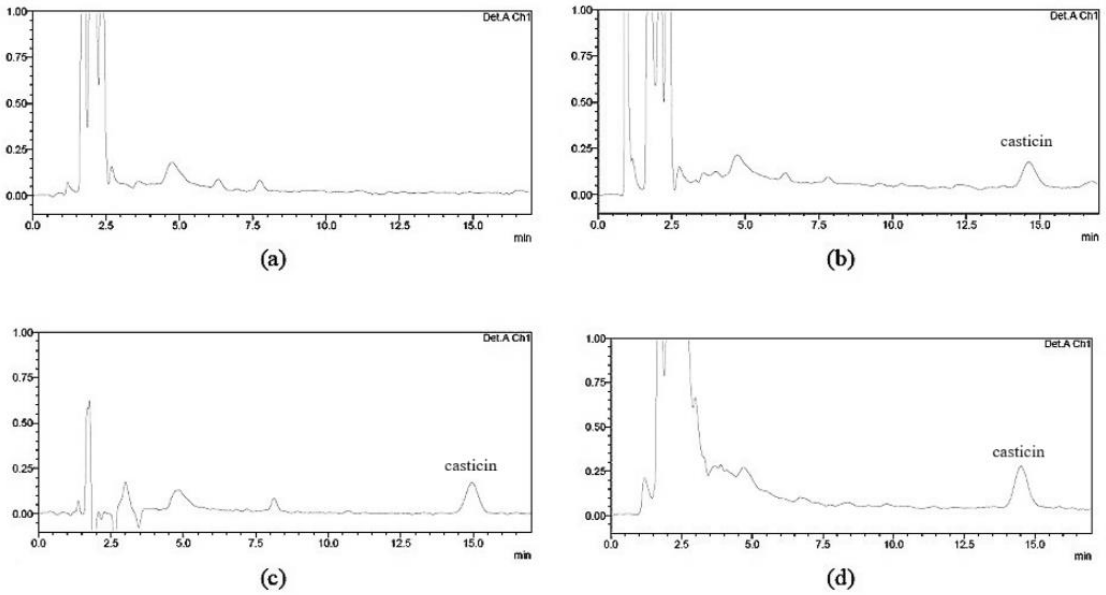

Figure 2. a: blank plasma b: 30 ng/mL casticin spiked to plasma c: 30 ng/mL standard solutions d: 37.5 ng/ml capsule methanol extract

Table 1. System suitability parameters for casticin

Sample	Resolution (>2) *	HETP (<1 mm) *	Tailing Factor (≤2 mm) *	k' (>1) *	N (>2000) *
Plasma	9.166	0.004	1.014	1.2	5728
Capsule	10.940	0.0005	1.073	1.6	5132

HETP: Height equivalent to theoretical plates
k': Capacity factor
N: Column efficiency
* Recommended value

2.2. Preparation of the Calibration Curve

for capsule analysis: The calibration curve was prepared by adding standard casticin solutions to plasma between 10 and 60 ng/mL. Each concentration was analyzed in five replicates. Linear least-squares regression analysis was applied on the obtained data to quantify the linear concentration range of the method. The calibration equation was found to be y = 148.15x - 454.73 (correlation coefficient = 0.9933).

for spiked plasma analysis: Plasma calibration solutions were made by spiking 0.5 mL of human plasma with casticin at concentrations ranging from 10 to 60 ng/mL. The spiked plasma specimens were then processed following the previously described sample preparation method for plasma. An aliquot of 20.0 µL from each prepared specimen was given into the HPLC system for analysis. Each concentration level was studied in five replicates during the analysis. The calibration equation was found to be y = 147.61x - 435.2 (correlation coefficient = 0.9928).

2.3. Validation Studies

The analytical method that was developed underwent validation following international guidelines. The validation process encompassed various parameters including accuracy, selectivity, recovery, precision, calibration curve, sensitivity, reproducibility, and stability of the analyte in capsules and spiked specimens. These guidelines were based on the International Conference on Harmonization (ICH) [23].

1. Specificity: The chromatograms depicted in Figure 2a and 2b do not exhibit any additional peak signals aside from casticin. This observation indicates that the developed method is specific to casticin and does not show interference from other constituents present in plasma and capsules.

2. Sensitivity: The limit of detection (LOD) and limit of quantitation (LOQ) for the suggested method were quantified utilizing the formula: LOD or LOQ = kSD / m. In this formula, k represents the desired multiplication factor, which is 3 for LOD and 10 for LOQ. SD refers to the standard deviation of the intercept, and m represents the slope of the calibration graph. The specific values for LOD and LOQ, along with other performance parameters of the suggested technique, are given in Table 2.

Table 2. Analytical parameters of the technique

Parameters	Plasma	Capsule
Concentration range [a] (ng mL^{-1})	10 - 60	10 - 60
Regression equation[b]	y = 147.61x - 435.2	y = 148.15x - 454.73
Intercept± SD	435.2 ± 56.84	454.73 ± 150.57
Slope± SD	147.61 ± 2.06	148.15 ± 2.89
Correlation coefficient (r^2)	0.9928	0.9933
LOD (ng mL^{-1})	1.16	3.05
LOQ (ng mL^{-1})	3.85	10.16

[a] Average of six
[b] y=xC +b where C is the concentration in ng/mL and y is the peak area

3. Accuracy and precision: These parameters were evaluated to assess the reliability of the developed method. Accuracy studies were conducted at three different concentrations (25, 35, and 55 ng/mL) for both plasma and capsule matrices, representing low, medium, and high concentrations. Precision studies were performed at concentrations of 15, 30, and 45 ng/mL. Accuracy was determined by calculating the recovery values, while precision was evaluated using the relative standard deviation (RSD) values from three replicate studies. The percentage of recovery was quantified through the standard addition technique. The mean relative recovery was found as 97.22% by adding casticin at QC levels to a plasma sample with a casticin concentration of 15 ng/mL, with the highest RSD observed at 5.67%. To demonstrate the precision of the technique, three different specimens were analyzed on the same day for intraday precision and on three days for interday precision. The RSD obtained from both intraday and interday analysis were below 4.94%. These results indicate that the technique exhibits great precision and accuracy. The detailed results can be found in Tables 3 and

Table 3. Recovery results

	Existant concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found Concentration (ng mL^{-1}) (Mean±SD[1])	Recovery (%)	RSD of recovery
Plasma	15	10	26.51 ± 0.78	106.04	3.29
		20	32.47 ± 0.69	92.76	2.43
		40	52.89 ± 1.61	96.18	3.39
Capsule	15	10	23.16 ± 1.07	92.66	5.67
		20	34.81 ± 0.77	99.46	2.43
		40	52.93 ± 0.72	96.24	1.48
		Mean relative recovery = 97.22			

For each concentration n=3

Table 4. Precision results

	Added concentration (ng/mL)	Found concentration of interday variation (ng/mL) (Mean±SD)	Found concentration of intraday variation (ng/mL) (Mean±SD)	RSD of intraday variation	RSD of interday variation
Plasma	15	14.98 ± 0.12	14.91 ± 0.27	1.78	0.81
	30	29.99 ± 1.48	29.89 ± 1.12	3.76	4.94
	45	43.95 ± 1.00	41.64 ± 0.24	0.58	2.28
Capsule	15	14.98 ± 0.12	14.57 ± 0.29	2.64	2.02
	30	29.99 ± 1.48	34.53 ± 0.35	0.76	1.01
	45	43.95 ± 1.00	39.19 ± 0.03	0.15	0.07

For each concentration n=3

4. Robustness: Robustness of the method was evaluated by analyzing the specimens at different concentrations. Several factors were altered to assess the robustness of the technique. These parameters included the flow rate, oven temperature, and the proportions of methanol. The mobile phase ratio, initially set at 60:40 (methanol-0.05% formic acid) was changed to 62:38 and 58:42. The flow rate was changed from 1.0 mL/min to 0.8 mL/min and 1.2 mL/min, and the oven was adjusted from 25 °C to 23 °C and 27 °C. Despite modifications, no substantial impact on the chromatogram was observed, indicating the robustness of the technique. The low values of relative standard deviation (RSD) further support the robustness of the technique. These values are summarized in Table 5.

Table 5. Robustness results

	Value	Recovery %		RSD %	
		Plasma	Capsule	Plasma	Capsule
Flow rate mL/min	0.8	100.25	98.58	3.82	1.71
	1.2	93.00	100.02	4.33	4.93
Mobile phase composition (methanol:aqueous phase)	62:38	98.05	96.57	1.97	1.55
	58:42	98.39	97.75	1.46	3.96
Column temperature	23 °C	94.18	100.24	2.28	3.82
	27 °C	95.67	103.39	2.68	3.91

n=3 for all QC specimen

5. Stability results: The stability of the casticin solutions was assessed under various storage conditions and freeze-thaw cycles, with three replicates at each quality control (QC) level. The tested storage conditions included keeping the solutions in the dark at room temperature for 24.0 hours, in autosampler conditions for 24.0 hours, and refrigerated at 4.0 °C for 1.0 month. Additionally, four freeze-thaw cycles were performed. The recovery values of the plasma samples under the tested conditions were found to be 94.62%, 95.57%, and 97.41%, respectively. The highest relative standard deviation (RSD) value observed in these experiments was 2.58%. Similarly, the recovery values of the capsule samples were determined to be 97.24%, 95.86%, and 100.81%, respectively, with the highest RSD value of 5.06%. Casticin remains stable under all tested conditions. The low RSD values further indicate the stability of casticin in both plasma and capsule samples.

4. CONCLUSION

Casticin is a prominent secondary metabolite naturally found in Vitex species and is considered one of the principal active constituents responsible for the therapeutic effects in the management of premenstrual syndrome. Owing to its diverse pharmacological activities, casticin is commonly included in various herbal preparations and pharmaceutical extracts. Although several studies in the literature have reported the quantification of casticin in plant extracts and herbal products, data regarding its determination in biological matrices are limited. Specifically, only a few investigations have examined its concentration in rat plasma, and to date, no validated HPLC–UV method has been reported for the quantification of casticin in human plasma.

The HPLC–UV method developed in this study provides a rapid, practical, and cost-effective analytical approach for the quantification of casticin in both human plasma and capsule formulations. The method requires only a UV detector, involves a simple sample preparation procedure, and consumes low amounts of solvent, making it suitable for routine analyses. The low LOD value obtained in plasma (1.16 ng/mL), along with acceptable accuracy, precision, and high repeatability, further supports the applicability of the method in clinical research and pharmaceutical quality control.

When compared with similar methods reported in the literature, this study exhibits important differences. For instance, Xu et al. [21] utilized an LC–MS system to quantify casticin in rat plasma and reported a wider linear range (14.06–7187 ng/mL) along with a higher correlation coefficient ($r > 0.9996$). However, LC–MS–based methods are costly, require specialized equipment, and may not be readily accessible in all laboratories. In contrast, the proposed HPLC–UV method is more accessible in terms of instrumentation and still provides a low LOD value (1.16 ng/mL) for plasma, indicating adequate sensitivity. HPLC-DAD or UPLC-DAD methods reported for plant materials are primarily intended for quality control purposes, and their applicability to biological matrices is limited. Therefore, the present study offers one of the first examples of a validated HPLC–UV method for the quantification of casticin in human plasma, representing a significant methodological contribution.

In the study conducted by Chen et al. (2021) [22], plasma analysis was performed in rats following the oral administration of Viticis fructus extract, and absorbed constituents were evaluated using pharmacokinetic and network pharmacology approaches. Although this work provides valuable insights into multi-component absorption and potential mechanistic pathways, it does not present a targeted quantitative method for casticin in human plasma. Conversely, the method developed in the present study focuses on the selective determination of a single analyte and offers a practical, economical, and easily applicable analytical strategy suitable for both human plasma and pharmaceutical formulations. The obtained linear range (10–60 ng/mL), sensitivity, and recovery values (%92.66–106.04) confirm the suitability of the method for routine quality control and clinical applications.

In conclusion, the proposed method provides a rapid, sensitive, selective, and economical analytical approach for the quantification of casticin in human plasma and pharmaceutical dosage forms. Its reliable performance in terms of accuracy and repeatability enables the precise determination of casticin in both biological fluids and pharmaceutical formulations, thereby filling an important gap in the current literature.

3. MATERIALS AND METHODS

3.1. Used Chemicals

Casticin (Merck, USP Reference Standard CAS No: 479-91-4) was acquired from Sigma Aldrich (St. Louis, USA). Used methanol of HPLC quality, formic acid, and dichloromethane were purchased by Merck (Darmstadt, Germany). Ultrapure water used in the experiments was purified using the Human (Japan) ultrawater purification system. Casticin pharmaceutical preparation (Castonex®) containing 375 mg of *Vitex agnus-castus* L. extract per capsule was obtained from Anti (Ankara, Turkey).

3.2. Solutions

To prepare the stock solution of casticin, a concentration of 0.1 mg/mL was achieved. This prepared solution was then further diluted using methanol to generate standard solutions with concentrations ranging from 10 to 60 ng/mL.

3.3. Instrumentation

Spectral analysis was conducted utilizing a Shimadzu UV-160 A spectrophotometer equipped with a 1 cm quartz cuvette. The instrument was controlled using Software Vision 32-bit Version 1.10. The absorption spectrum of the specimen solutions was obtained, and the maximum absorbances were observed at 258 nm.

The HPLC experiments were done on a Shimadzu (Japan) LC 20 liquid chromatograph with a LC-20AT pump, a SPD-20A HT UV spectrophotometric detector, SIL AH-HT autosampler part and CTO 10 AC column oven. In order to achieve optimal chromatographic separation, various of mobile phase, stationary phase combinations were tried. Additionally, varied flow rates and oven temperatures were explored to determine the most effective conditions for the chromatographic analysis.

3.4. Chromatographic conditions

The optimum chromatographic separations were attained by employing isocratic elution with a mobile phase ratio of 60:40 v/v (methanol-0.05% formic acid) at a flow rate of 1.0 mL/min. These conditions were maintained at a column temperature of 25 ^{o}C.

3.5. Sample preparation procedure for capsules

To prepare the capsule extract, ten capsules were weighed and mixed in the mortar. An amount of powder equivalent to 375 mg of capsules was then weighed and transferred into a 100 mL flask. Next, 75 mL of methanol was placed to the flask, and the mixture was shaken for 15 minutes to ensure thorough extraction. The volume was then made up to with methanol, ensuring proper mixing, and the resulting mixture was filtered. The obtained capsule extract had a concentration of 3.75 mg/mL. To obtain working concentrations, the capsule extract was appropriately diluted with methanol.

3.6. Sample preparation procedure for spiked plasma

Venous blood specimens were collected from a healthy volunteer, following the necessary protocols and informed consent obtained as per ethical committee approval. The blood was collected into plastic tubes including disodium ethylenediaminetetraacetic acid and then centrifuged at 4000 × g for 15 minutes. The resulting plasma specimens were stocked at -20 ^{o}C for further analysis. Different concentrations of casticin were added to the plasma samples and mixed by vortex for 1 minute. To extract casticin from the plasma specimens and minimize potential interferences, a liquid-liquid extraction technique was employed. For each extraction, 0.5 mL of plasma specimen was mixed with 5.0 mL of dichloromethane utilizing a vortex for 1 minute. The prepared mixture was then centrifuged at 4000 x g for 15.0 minutes to separate the aqueous and organic layers. The aqueous phase was eliminated, and the organic phase was subjected to evaporation a stream of nitrogen gas at 25 ^{o}C until dryness. Afterward, 1.0 mL of a methanol was added to the residue, and the solution was mixed using a vortex for 1.0 minutes. The resulting mixture was filtered with a syringe filter, and 20.0 µL of the specimen was given into the HPLC device for analysis.

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Conflict of interest statement: The author declare no conflict of interest.

Ethical approval: All procedure performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Bezmialem Vakıf University approved by the Clinical Trials Ethic Commitee (No: 12/6).

Informed consent: Informed consent was obtained from all individual participants included in the study.

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