DOI: https://doi.org/10.31351/vol35iss3pp46-55

Development and validation of RP-HPLC for the Determination of Caspofungin in the Ufasomes Dispersion, Gel and Rat Plasma

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Received 16/9/2025, Accepted 12/2/2026, Published 28/9/2026

Abstract

Caspofungin is a lipopeptide echinocandin antifungal and its mechanism involves inhibition of (1,3)-β-D-glucan synthase, disrupting fungal cell wall integrity. This study presents a validated RP-HPLC method for detecting and quantifying caspofungin diacetate (CSP) in ufasomes dispersion and rat plasma, providing a reliable and reproducible approach for pharmaceutical analysis. The method employed a C18 column with a mobile phase of phosphate buffer (pH 2.5) and acetonitrile (65:35, v/v). The CSP standards and samples were prepared in methanol and plasma and filtered prior to analysis. Method validation followed ICH Q2 and FDA guidelines, assessing linearity, specificity, precision, accuracy, matrix effects, limit of detection (LOD) and limit of quantification (LOQ). The method showed excellent linearity in methanol and plasma (R^2 = 0.9999), high specificity against excipients, recovery of 97.9% with RSD 1.21,0.36%, and low detection limits (LOD = 1 ng/mL, LOQ = 3 ng/mL). Matrix effects caused longer retention times in plasma compared to ufasomes dispersion, but peak resolution remained clear.
Keywords: Caspofungin diacetate, Analytical method, RP-HPLC, Validation, Ufasomes

Introduction

Caspofungin is a lipopeptide antifungal drug developed by Merck & Co., Inc. in 2001 and approved by the United States Food and Drug Administration (FDA) as Caspofungin diacetate (CSP), Cancidas® for fungal infection disease. It consists of large lipopeptide molecules derived from fungal fermentation and subsequently modified synthetically. It is an amphiphilic cyclic hexapeptide possessing both hydrophilic and hydrophobic regions, characterized by an N-linked acyl lipid side chain. The antifungal activity of CSP largely depends on the position and conformation of its N-linked acyl lipid side chains. These side chains are believed to interact with the fungal cell membrane and blocking cell wall production [(1)] , (Figure. 1) describes the structure of caspofungin and its salt CSP.

The CSP is an echinocandins, working by inhibiting the enzyme (1,3)-β-D-glucan synthase and thereby disturbing the integrity of the fungal cell wall [(2)]. It is slightly soluble in water, soluble in methanol and dimethyl sulfoxide (DMSO), but practically insoluble in acetone, ethanol, and other non-polar solvents. It has a large molecular size (its molecular weight 1213.42 g/mol), which is the reason it is approved only for intravenous administration (IV) [(3)]. The IV delivery can cause local reactions, requires a functional cannula, and is generally more costly and labor-intensive. In addition, cannulation can be uncomfortable for patients and carries a risk of infection [(4,5)]. The detection of CSP is difficult to determine by Ultraviolet radiation (UV) alone, Therefore, reversed Phase- High Performance liquid chromatography (RP-HPLC) was used. There are previous method studies for determining and validated of CSP using liquid chromatography–tandem mass spectrometry (LC-MS/MS) [(6,7)] and high-performance liquid chromatograph (HPLC) [(8,9)] . The sample preparation must be simple, consistent specially at low concentrations or in complex biological matrix because complicated sample procedures improved the variability. In addition, the usage of an internal standard help to minimize errors and enhance recovery [(10,11)].

Developing a new drug delivery of the CSP that targets and disrupts the fungal cell wall could be a promising area in drug treatment. The ufasomes are lipid bilayer vesicles that represent an effective drug delivery system by enhancing CSP stability and skin penetration. They are composed of unsaturated fatty acids forming closed bilayer structures, where hydrophobic chains align outward and carboxyl groups face the aqueous environment, producing ionized soap-like assemblies at pH 7–9. The ufasome is influenced by factors such as fatty acid type, cholesterol content, buffer composition, and pH. Compared with liposomes, ufasomes offer greater stability, lower cost, and improved encapsulation of both hydrophilic and lipophilic drugs [12] .Formulating CSP in ufasomes offers a targeted approach for skin antifungal therapy, potentially reducing systemic toxicity and improving patient adherence in the treatment of fungal infections specifically candida skin infections with minimizing side effects [13]. The aim of the study is to develop and validate method to determine CSP *in vitro* and *in vivo*.

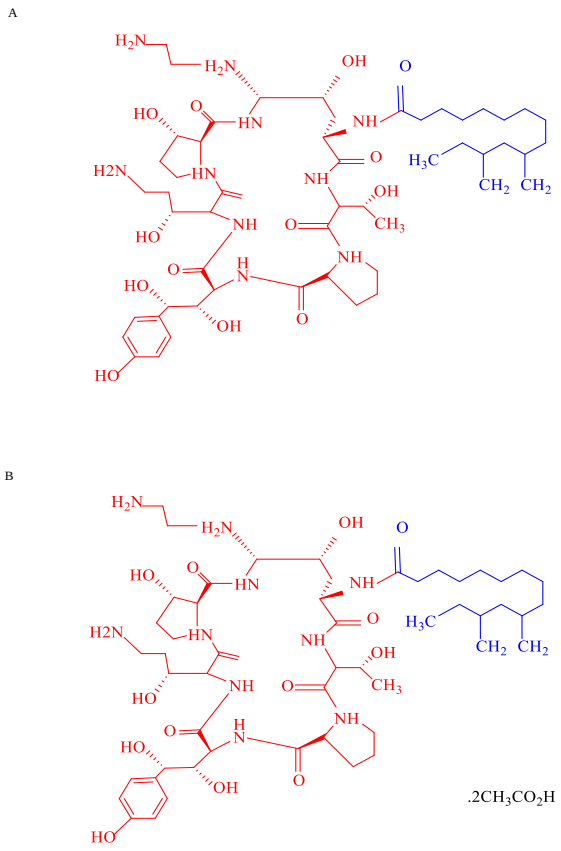

Figure 1. The structure of A) caspofungin and B) caspofungin diacetate (CSP) modified from [14]. Red color represents the hydrophilic groups, and the blue color represents the hydrophobic groups of caspofungin and caspofungin diacetate

Materials and Methods

Materials

Caspofungin diacetate (CSP), cholesterol, palmitoleic acid and fluconazole were purchased from Shanghai Macklin Biochemical Technology Co., China. Methanol was purchased from Analytical Chemlab, Belgium. Fluconazole was purchased from Shanghai, China. The Wistar Albino rats were obtained from the Animal House of the College of Pharmacy, University of Baghdad.

Instrumentation

The main instruments used in this study are the reversed phase- high performance liquid chromatography RP-HPLC system (Sykam®, Germany), rotary vacuum evaporator (BUCHI Corporation, United States), probe homogenizer (Q125 Sonicator®, USA) and centrifuge (Hettich, Germany)

The Sample preparation of CSP

CSP-loaded ufasomes were formulated using the thin-film hydration method with modifications [15–17]. 500 mg of palmitoleic acid,5 mg of CSP, and 10 mg of cholesterol were placed in 50 mL of methanol in a 500 mL rotary flask. The mixture was rotated using a rotary vacuum evaporator (BUCHI Corporation, United States) for 1 hour at 150 rpm, 244 mbar, and 50 ◦C (below the boiling point of methanol, 64.96◦C [18,19]) under vacuum until the methanol evaporated and a thin film formed. Then, to hydrate the film, 50 mL of borate buffer (pH 8.5) was added to the flask, and the rotary evaporator was operated at 150 rpm at 40 ◦C without vacuum for another hour, ensuring complete hydration. The resulting dispersion was homogenized for 3 min using a probe homogenizer 2:2 with amplification 30 % (Q125 Sonicator®, USA) to ensure uniformity and smaller dispersion particle size [20].

About 1.5% gel of CSP loaded ufasomes was prepared by using 0.75 gm of carbopol 934 gradually added to 50 mL of CSP-loaded ufasomal dispersion in a beaker. Then, a few drops of triethanolamine and 0.1% methyl paraben was added as preservative, then the mixture was stirred continuously using a magnetic stirrer at 500 rpm and increase gradually to 800 rpm until a clear gel is formed [21,22]. The 18 male Wistar rats, each approximately three months old and weighing around 200 ± 10 gm, were used in this study. The animals had free access to food and water throughout the experiment. All *in vivo* procedures were approved by the Research Ethics Committee for Experimental Investigations at the College of Pharmacy, University of Baghdad, Iraq, under

protocol number RECO3202511A. The rats were randomly divided into two equal groups. The rat skin was shaved and about 1 g of gel applied topically and covered with adhesive plaster to ensure the gel remained in place during the sampling period. The rats were anesthetized and about 2 mL of blood samples was collected into EDTA tubes (containing the anticoagulant) then the plasma was separated by centrifugation at 4000 rpm for 10 minutes (Hettich, Germany). The clear plasma layer (0.5 mL) was carefully transferred into Eppendorf tubes and stored in a freezer (−20 °C) for subsequent analysis [23]

The RP-HPLC Condition (Chromatography)

The isocratic separation was carried out on a C18-Octadecylsilane (ODS) column (4.6 mm × 250 mm, 5 µm particle size). The mobile phase was composed of a solution of 20 mM phosphate buffer (pH 2.5) and acetonitrile in a 65:35 volume ratio. The flow rate was 1.0 mL/min at 30ºC. The detection by a photodiode array (PDA)/fluorescence detector with excitation and emission at 224 nm and 304 nm and run time is 20 min. 20 µL of CSP-loaded ufasomes dispersion, control rat plasma CSP rat plasma and fluconazole (internal standard) injected into RP-HPLC [24,25]. (Table 1).

Table 1. The RP-HPLC chromatographic conditions of samples *in vitro* and *in vivo*

The criteria	The optimal condition
Mobile Phase	20 mM phosphate buffer (pH 2.5) and acetonitrile (65:35)
Stationary Phase (Column)	C18-Octadecylsilane (ODS) column (4.6 mm × 250 mm, 5 µm particle size
Flow rate	1 ml/min
UV detection	224 nm
Injection volume	20 µL
Temperature	30°C
Run Time	20 min

Method validation

The selected method of CSP analysis was validated in accordance with ICH Q2-EMA and FDA `guidelines, assessing parameters such as linearity, detection and quantification limits, specificity, repeatability, recovery and robustness [26–28] .

Selectivity and matrix effect

The matrix effect was assessed by comparing CSP concentrations measured in rat plasma with those obtained from direct injections of CSP prepared at the same concentrations in ufasome dispersions [29]. Fluconazole was used as the internal standard in this study because it is stable, has similar chemical behavior and extraction properties to caspofungin, and does not interfere with caspofungin's signal due to its structural and mechanism of action [30].

Linearity

Multiple concentrations of CSP in ufasomes dispersion and plasma between 2-10 µg/ml and between 5-20 ng/ml were prepared by diluting the stock solution. Each sample was then run three times on an HPLC device. Finally, a calibration curve was created by plotting the peak response (area) against the corresponding concentration [31,32]

Precision (repeatability)

Precision was evaluated by injected 5 µg/mL CSP rat plasma. Six injections of the same solution were carried out, three within a single day (intra-day) and three across three consecutive days (inter-day). The relative standard deviation (RSD%) of the obtained responses was calculated, reflecting the reproducibility of the method when the same sample is analyzed multiple times [33,34].

$$RSD\% = \frac{Standard\ deviation\ (StD)}{mean} * 100\%$$

Specificity

The specificity validation is done by preparing different samples of CSP containing the same amount of CSP and different amounts of oil in ufasome dispersion (5 µg/ml CSP/ 100 mg oil, 5 µg/ml CSP/ 300 mg oil, 5 µg/ml CSP/ 500 mg oil) and different samples of CSP containing different concentration of CSP (5 ng/ml, 25 ng/ml) in rat plasma [35].

LOD and LOQ

The limit of detection (LOD) refers to the smallest quantity of drug that can be identified by the analytical method, whereas the limit of quantification (LOQ) represents the lowest concentration that can be measured with acceptable precision and accuracy [36]. The LOD obtained directly from the instrument and LOQ is measured according to the equation: LOQ =3*LOD [37]

Accuracy and Recovery

Accuracy refers to how closely the measured value of a substance obtained by the proposed method matches its true value. From the precision test, the obtained results were then compared with the actual concentrations to determine the method's accuracy, as illustrated by the following equation [38]

$$\textbf{Recovery}\ \% = \frac{\textbf{measured value (concentration)}}{\textbf{true value (concentration)}} * \mathbf{100}\ \%$$

Results and Discussion

Selectivity and matrix effect

A sharp and well-defined peak of CSP appeared in ufasomes dispersion and rat plasma with a retention time of 3.01 and 6.00 min respectively, (Figure. 2). In this study, fluconazole was employed as the internal standard and showed clear separation from CSP peak. All the samples showed no interfering peaks and eluted within an appropriate timeframe, indicating consistent retention times across ufasomes dispersion and rat plasma without any matrix interference (Figure. 3). This is in accordance with Fatiguso et al., 2017 and Al-Momani et al., 2023 findings [39,40]

The difference in CSP retention time between ufasomes dispersion and rat plasma is mainly attributed to matrix effects, as plasma is a complex medium rich in proteins, lipids, salts, and metabolites that can alter CSP's polarity, binding state, and chromatographic behavior. The CSP has high protein binding (>95%) further contributes to variability during sample preparation, since free and bound forms may elute differently. Unlike the relatively the CSP in ufasomes dispersion, plasma samples require deproteinization or extraction, leaving residual components that may cause peak broadening, co-elution, or minor shifts [41].

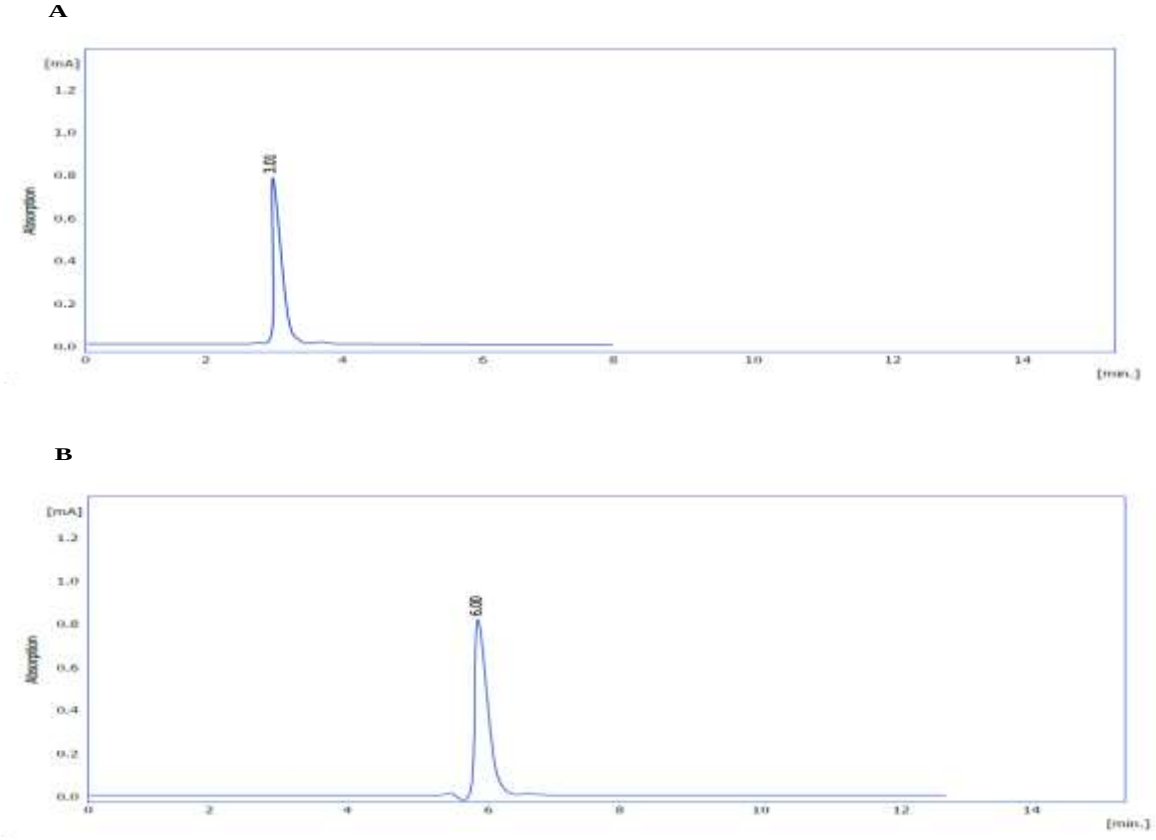

Figure 2. Chromatograms of the CSP in (a) ufasomes dispersion and (b) rat plasma

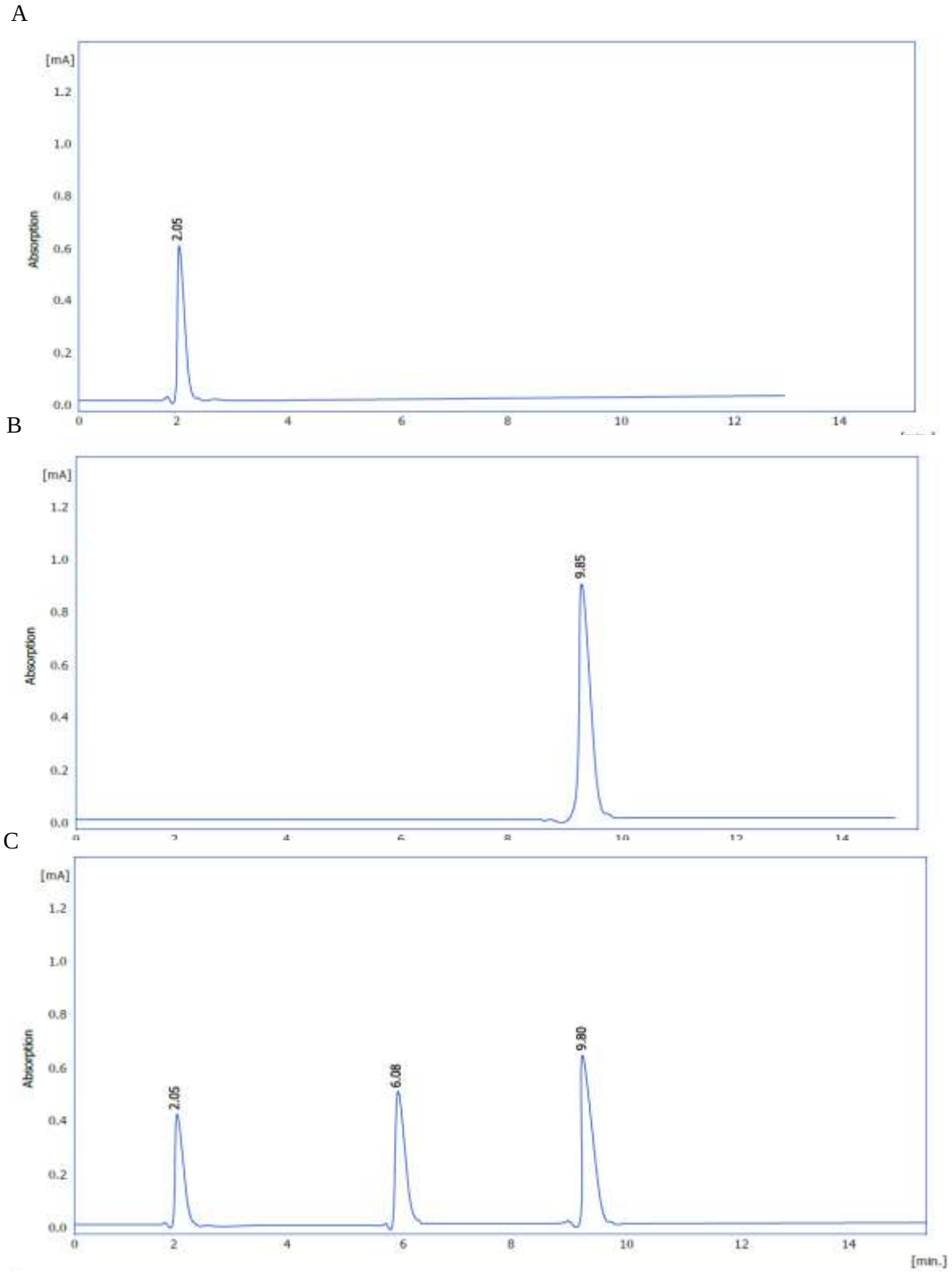

Figure 3. Chromatograms of the CSP in (A) Rat Plasma (Control), (B) Fluconazole (internal standard), and (C) Rat Plasma incorporated with CSP-loaded ufasomes gel

Linearity

Linearity was evaluated by plotting peak area versus concentration in methanol yielding the equation Y = 40.78182X+ 2.18049 (R^2) of 0.9998 (Figure. 4) and in plasma, yielding the equation Y = 92.66667X + 5.7735 with a correlation coefficient (R^2) of 0.9999 (Figure. 5), confirming excellent linearity across the tested range. These results highlight the method's reliability and adaptability for quantifying CSP in pharmaceutical formulations of varying doses and strengths, with high reproducibility. This result aligns with the findings of Neo et al. (2013), who demonstrated excellent linearity for CSP in the concentration range of 50–800 µg/mL with a correlation coefficient (r^2 = 0.9997 ± 0.0002), confirming that detector response was directly proportional to concentration and suitable for quantitative analysis. Similarly, Wu et al. (2025) reported strong linearity for CSP in human plasma, with r^2 values exceeding 0.990 across all tested ranges, indicating that the method is reliable for therapeutic drug monitoring, as the calibration ranges covered the clinically relevant concentrations [42,43].

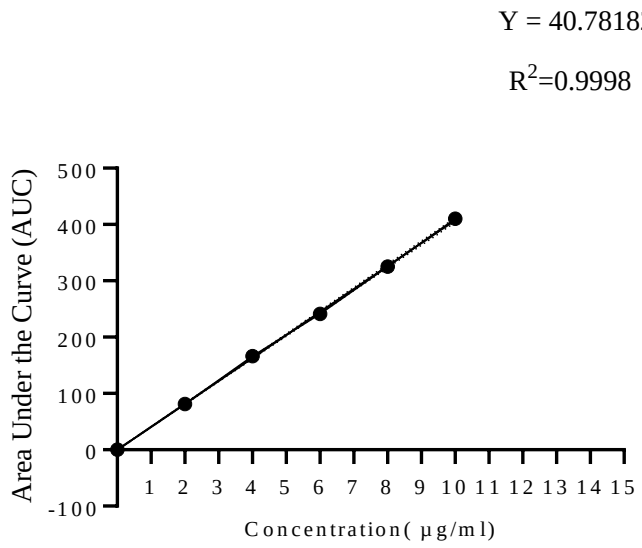

Figure 4. The calibration curve of CSP in ufasomes dispersion

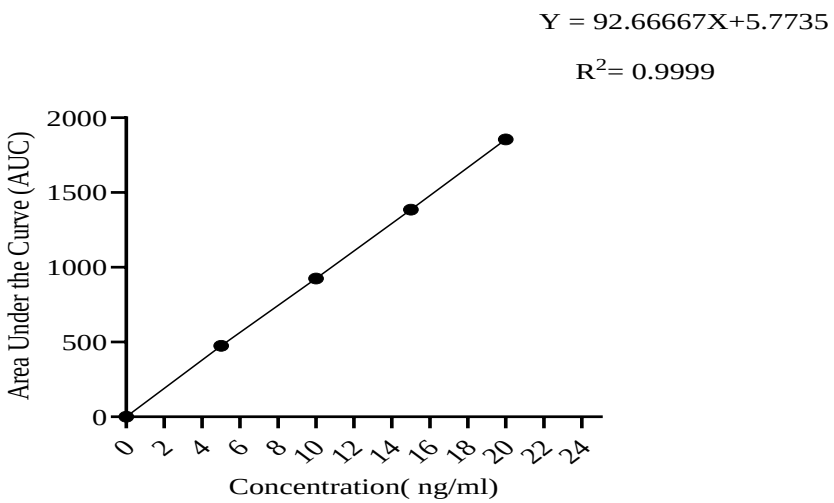

Figure 5. The calibration curve of CSP in rat plasma

Precision (Repeatability)

Table 2 presents the percentage relative standard deviation (%RSD) values, reflecting the precision of the method. The %RSD was 1.21, 0.36% for both intraday and interday analyses, well below the ICH limit of 2%, reflecting consistent and reliable repeatability of the method [44,45]. These findings are consistent with the results of S. Chitlange et al. (2008), who reported intra-day %RSD values of 0.138% for amlodipine and 0.129% for metoprolol, and inter-day %RSD values of 0.590% for amlodipine and 0.414% for metoprolol, all well below 2%, confirming the high precision of the method. Similarly, Mujawar et al. (2022) demonstrated that both metoprolol and benidipine exhibited excellent intra- and inter-day precision by RP-HPLC, with low standard deviations reflecting the method's accuracy and reproducibility [46,47] .

Table 2. The Precision results of CSP in rat plasma

Injection Time	Run number	Retention time (min)	Response area	Mean	Std	%RSD
Intra Day	**1**	6.00±0.1	475.09±0.2	474.12	3.62	1.21
	2	6.05±0.2	477.15±0.2			
	3	6.07±0.1	470.11±0.3			
Inter Day	**4**	6.03±0.4	477.12±0.1	475.90	1.07	0.36
	5	6.05±0.2	475.45±0.3			
	6	6.02±0.2	475.14±0.1			

Specificity

Table 3 and 4 showed high specificity of CSP in ufasomes dispersion and rat plasma with very low variability across repeated measurements of the same sample followed Hamid et al., 2024 findings [48].

Table 3. The specificity of CSP in ufasomes dispersion

Run Number	Sample components	Retention time (min)	Response area
1	5 µg /ml CSP/ 100 mg oil	3.01±0.1	3589.08±0.4
2	5µg /ml CSP/ 300 mg oil	3.06±0.1	3985.00±0.3
3	5µg /ml CSP/ 500 mg oil	3.07±0.2	3695.28±0.2

Table 4. The specificity of CSP in rat plasma

Sample ID	Sample components	Retention time (min)	Response area
1	5 ng /ml	6.08±0.1	475.09±0.2
2	25 ng /ml	6.01±0.3	477.14±0.1

LOD and LOQ

The instrument determined the LOD and LOQ for CSP as 1 ng/mL and 3 ng/mL, respectively. These low values demonstrate the method's high sensitivity, enabling detection of trace amounts and precise quantification with good accuracy. Such sensitivity makes the method well-suited for pharmaceutical analysis, particularly in quality control and stability studies, even for formulations containing very small amounts of the drug**.**

Accuracy and Recovery

The developed method for CSP determination showed a recovery of 97.9%. These results confirm the method's suitability for pharmaceutical analysis, including routine quality control and research applications, aligning with previous findings that the recovery accepted range (95%-100%) with acceptable RSD indicates robust performance [49,50]

Conclusion

The RP-HPLC method was successfully developed and validated for the CSP determination in ufasome dispersions and gel formulation in rat plasma. The method includes all validation criteria, such as linearity, specificity, precision, robustness and accuracy, ensuring its reliability for routine analysis. The selected chromatographic conditions enabled efficient separation without interference from formulation excipients or plasma constituents. Overall, the validated method proves reliable for use in formulation optimization, routine quality assessment, and pharmacokinetic evaluation of CSP-loaded ufasomes and other topical delivery preparations.

Acknowledgment

The authors gratefully acknowledge the dedicated staff of the College of Pharmacy, University of Baghdad, for their invaluable support and guidance throughout the course of this work. Special thanks are extended to Dr. Ali AL-Tameme

for his significant contribution in developing the analytical method.

Conflicts of Interest

No conflict of interest.

Funding

No funds are available.

Ethics Statements

The study adhered to ethical guidelines approved by the Research Ethics Committee of the College of Pharmacy, University of Baghdad (protocol code RECO3202511A).

Author Contribution

Both authors participate in study conception, data collection, writing, reviewing and approved the final version of the manuscript

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تطوير والتحقق من صحة طريقة باستخدام جهاز كروماتوغرافيا السائل عالي الأداء ذات الطور العكسي لتحديد كمية الكاسبوفنجين في اليُوفاسومات والجل وبلازما الفئران

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الخلاصة

الكاسبوفنجين هو مضاد فطري من فئة الإيكينوكانديناتِ الليبوببتيدية. يعمل الدواء عن طريق تثبيط إنزيم (١،٣)-β-D-غلوكان سينثاز، مما يؤدي إلى خلل في جدار الخلية الفطرية. تقدم هذه الدراسة طريقة جهاز الكروماتوغرافيا السائلة عالية الأداء ذات الطور العكسي الأداء مُعتمدة للكشف عن الكاسبوفنجين (CSP) وقياس كميته في مُشتَّت اليُفاسومات وفي بلازما الفئران، موفرةً طريقة موثوقة وقابلة للتكرار للتحليل الصيدلاني. استخدمت الطريقة عمود C18 مع طور متحرك مكون من محلول فوسفات (pH 2.5) وأسيتونتريل بنسبة ٣٥:٦٥ (حجم/حجم). تم تحضير معايير وعيناتCSP في الميثانول والبلازما وترشيحها قبل التحليل. وتم التحقق من صحة الطريقة وفق إرشادات ICH Q2 وFDA، من خلال تقييم الخطية، والنوعية، والدقة، والصحة، وتأثيرات المصفوفة (تأثير مكونات الوسط)، وحد الكشف (LOD) وحد التقدير الكمي (LOQ). أظهرت الطريقة خطية ممتازة في الميثانول والبلازما (R^2 = 0.9999)، ونوعية عالية تجاه المواد المساعدة، واسترجاع بنسبة ٩٧,٩٪ مع RSD ١,٢١٪، ٠,٣٦٪، وحدود كشف منخفضة (LOD = 1 نانوغرام/مل، LOQ = 3 نانوغرام/مل). أدت تأثيرات المصفوفة إلى زيادة أوقات الاحتفاظ CSP في البلازما مقارنة بمُشتَّت اليفوسومات، لكن كانت ذروة التحليل واضحة. بشكل عام. تُعد طريقة جهاز الكروماتوغرافيا السائلة عالية الأداء ذات الطور العكسي المطورة حساسة ودقيقة وموثوقة، ومناسبة لمراقبة الجودة الروتينية والتطبيقات البحثية للكاسبوفنجين في التركيبات الصيدلانية.

الكلمات المفتاحية: كاسبوفنجين ثنائي الأسيتات، طريقة تحليلية، الكروماتوغرافيا السائلة عالية الأداء ذات الطور العكسي، التحقق، يوفاسومات