ARTICLE

Development and validation of an HPLC-PDA method for the quantification of fluconazole and application to fluconazole-loaded buccoadhesive film

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ABSTRACT

Fluconazole is a widely used antifungal drug against *Candida* infections, including oral candidiasis. To enhance its bioavailability for oral candidiasis management, buccoadhesive dosage forms are being suggested as alternatives to oral dosage forms. This study aimed to develop a simple and reliable high-performance liquid chromatography-photodiode array (HPLC-PDA) method for the detection and quantification of fluconazole in buccoadhesive film formulation. Chromatographic analysis was performed using C18 column (250 mm × 4.6 mm, 5 µm particle size, 100 Å pore size) under isocratic conditions, with mobile phase consisting of water and acetonitrile at a ratio of 75:25. The method used a 10 µL injection volume, a flow rate of 1.20 mL/min, detection at 257 nm under ambient temperature of around 25°C and was run for 6 min. The fluconazole peak appeared at 3.655 ± 0.019 min. The number of theoretical plates was 28238.43 ± 301.34, and the tailing factor was 1.31 ± 0.03, both meeting the acceptable criteria. Method validation confirmed linearity with a coefficient of determination (r^2) of 0.9999, good accuracy with a percentage recovery range of 96.28 - 103.57%, and good precision with a percent coefficient of variation below 15%. The acceptable accuracy and precision were also observed when the method was applied in the assay of fluconazole incorporated in buccoadhesive film. Based on the results, the developed HPLC-PDA method proved to be suitable for the detection and quantification of fluconazole in buccoadhesive film formulation.

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KEYWORDS

buccoadhesive film, fluconazole, HPLC-PDA, method development, method validation

INTRODUCTION

Oral candidiasis is a common fungal infection of the oral mucosa caused predominantly by *Candida* species. Among antifungal agents, fluconazole, a triazole compound, remains the first-line treatment due to its broad-spectrum activity, favorable safety profile, and high efficacy in eradicating *Candida* infections (Kuriyama et al. 2005; Xiao et al. 2022). Fluconazole exerts its antifungal effect by inhibiting C-14 demethylase, a key enzyme in the biosynthesis of ergosterol, a crucial component of the fungal cell membrane. Additionally, it has been reported to reduce *Candida* adhesion to buccal epithelial cells (Lyon and de Resende 2007), further aiding in infection resolution. Despite its effectiveness, conventional oral fluconazole formulations such as tablets and capsules face several limitations. These include significant hepatic first-pass metabolism, which reduces systemic bioavailability, and potential drug-drug interactions with tacrolimus, warfarin, and cisapride, among others (Pathak et al. 2016; Scorzoni et al. 2021). These formulations may not be ideal for patients with severe oral mucosal lesions, who often experience dysphagia and pain during swallowing (Taha Al-Nuaimy et al. 2025). These challenges underscore the need for localized drug delivery systems that can enhance therapeutic outcomes while minimizing systemic side effects.

Buccal drug delivery systems, particularly buccoadhesive films, have gained attention as promising alternatives. These films adhere to the mucosal surface, allowing the drug to be released at the site of infection over an extended period. Their ability to bypass first-pass metabolism and maintain therapeutic levels locally makes them especially suitable for drug delivery in the oral cavity (Hanif et al. 2015; Russo et al. 2016). As a result, numerous buccoadhesive films have been developed using a variety of polymers for drugs belonging to anesthetics, antihypertensives, and antimicrobials (Boroujeni et al. 2020; Hassan et al. 2023).

A critical component in the development of such systems is the establishment of a validated, reliable, and sensitive analytical method for drug quantification. Accurate assay methods are essential for evaluating drug content, release profiles, and ensuring product quality during formulation development and quality control (Pramod et al. 2013). A validated assay method is also vital for ongoing and future development of buccal delivery systems incorporating drugs like fluconazole. Hence, the present research work aimed to develop and validate a simple and rapid high-performance liquid chromatography-photodiode array (HPLC-PDA) method tailored specifically for the detection and quantification of fluconazole in simulated saliva and in a κ-carrageenan-based buccoadhesive film. The method is intended for use in both research and quality control settings, ensuring the accurate measurement of drug content during formulation development. In addition, this work demonstrated the method's applicability by successfully assaying fluconazole in a fabricated buccoadhesive film, thereby offering a practical analytical tool for buccal drug delivery research.

MATERIALS AND METHODS

Chemicals and reagents

Fluconazole (>98.0%) was procured from Tokyo Chemical Industries Co., Inc. (Tokyo, Japan), while κ-carrageenan and polyethylene glycol 400 (PEG 400) were procured from Sigma-Aldrich (Missouri, USA). Acetonitrile and water used as mobile phase were HPLC grade (Daejung Chemicals and Metals, Siheung-si, South Korea). Ortho-phosphoric acid (Ajax Finechem, New South Wales, Australia) and all other reagents for the preparation of simulated saliva were of analytical grade or equivalent.

Chromatographic instrumentation

An HPLC-PDA method was developed for the detection and quantification of fluconazole in simulated saliva. Waters Alliance e2695 HPLC system and 2998 PDA (Milford, Connecticut, USA) detector were used, equipped with Kinetex C18 column (Phenomenex, California, USA) with the following specifications: 250 mm long, 4.6 mm internal diameter, 5 µm particle size, and 100 Å column pore size.

Chromatographic method development

The appropriate chromatographic parameters for the analysis of fluconazole were explored, which include mobile phase composition, injection volume, elution mode, flow rate, and UV wavelength.

Stock solutions, calibration standards, and quality control samples

Simulated saliva was first made by dissolving KH_2PO_4 (0.19 g/L), NaCl (8.00 g/L), and Na_2HPO_4 (2.38 g/L) in distilled water, and the pH was adjusted to 6.8 using dilute orthophosphoric acid (Suharyani et al. 2024). This was used in the preparation of stock solutions, calibration standards, and quality control samples. A stock solution of fluconazole was prepared by carefully weighing and dissolving 30 mg in 100 mL of simulated saliva. The stock solution was used to obtain calibration standards. Three quality control samples, namely, high quality control (HQC) – 50 µg/mL, medium quality control (MQC) – 30 µg/mL, and low quality control (LQC) – 15 µg/mL, were likewise prepared.

Chromatographic method validation

The HPLC-PDA method was validated for system suitability, specificity, linearity, carry-over effect, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ) (FDA 2018).

System suitability

To ensure the reliability of the HPLC-PDA, system suitability tests were conducted. The system suitability was assessed via the analyses of six replicates of fluconazole standard (200 µg/mL). The responses of analyses were evaluated according to the number of theoretical plates, tailing factor, and percent coefficient of variation (% CV) of the peak area.

Specificity

To ascertain that the simulated saliva and the excipients used in the formulation of buccoadhesive film caused no interference in the detection and quantification of fluconazole, an assessment of the specificity of the HPLC-PDA method was conducted. To perform this assessment, chromatograms of blank simulated saliva (without drug or excipients), blank buccoadhesive film dissolved in simulated saliva (prepared without fluconazole but containing κ-carrageenan and PEG 400), and fluconazole standard solution in simulated saliva were analyzed. Each sample was injected into the HPLC system utilizing the developed chromatographic conditions. Specificity was confirmed by examining the chromatograms for any co-eluting peaks at the retention time of fluconazole. The method was considered specific if no peaks appeared at or near the fluconazole retention time in either blank simulated saliva or blank film matrix. Furthermore, the fluconazole peak in the standard solution should appear as a well-defined, symmetrical peak without interference.

Carry-over

Carry-over effect was analyzed by injecting a blank simulated saliva immediately after the analysis of the upper standard at a concentration of 200 µg/mL. The absence of the fluconazole peak in the chromatogram of the blank simulated saliva sample indicates a lack of transfer or carry-over (Noga et al. 2023).

Linearity
For linearity, calibration curves were constructed using linear regression analysis, plotting peak area versus fluconazole concentration across the validated range (1 - 200 µg/mL). The linearity of the method was assessed by calculating the coefficient of determination (r^2).

Limit of detection (LOD) and limit of quantitation (LOQ)
The calculation of LOD and LOQ for the method developed was according to the standard deviation of the response and slope derived from the linearity analysis. Equations 1 and 2 were employed for the calculation of LOD and LOQ, respectively:

$$LOD = 3.3\sigma/S \quad (1)$$

$$LOQ = 10\sigma/S \quad (2)$$

where σ is the standard deviation of the response, while S is the slope of the calibration curve (Lobo et al. 2024).

Accuracy
The accuracy of the developed method was assessed by recovery studies using the HQC, MQC, and LQC samples, and was expressed as percent recovery. Intraday and interday accuracy were performed in triplicate for each QC sample.

Precision
The intraday and interday precision of the developed method was evaluated via triplicate analyses of HQC, MQC, and LQC samples. The results were expressed as % CV.

Formulation of fluconazole-loaded buccoadhesive film
Fluconazole-loaded buccoadhesive film was prepared using the solvent casting method (Landová et al. 2013). A 2% (w/v) κ-carrageenan solution was first prepared in distilled water under continuous magnetic stirring (LMS-3003, Daihan Scientific, Wonju-si, South Korea) for 24 h to ensure complete polymer hydration and dispersion. Subsequently, PEG 400, a commonly used plasticizer, was added at 20% (w/w) relative to the polymer weight and stirred for an additional 30 min to improve film flexibility and reduce brittleness. Fluconazole was then incorporated into the mixture at a final drug concentration of 10 mg/mL and stirred for 15 min to ensure uniform distribution. The resulting viscous solution was poured onto a glass petri dish lined with parchment paper and dried at 45 °C for 24 h in an oven (Thermotec, Contherm Scientific Ltd., Hutt City, New Zealand). Once dried, the film was carefully peeled off and stored in a desiccator until further analysis. A placebo film was prepared following the same procedure, excluding the addition of fluconazole, to serve as a control.

Assay of fluconazole in buccoadhesive film
Buccoadhesive film containing 10 mg/mL fluconazole was dissolved in simulated saliva to obtain a solution having a concentration of 0.20 mg/mL. The resulting solution was filtered using a 0.45 µm syringe-driven nylon filter disk. Samples, including a blank κ-carrageenan film, were analyzed using the developed and validated HPLC-PDA method. Analysis was performed in triplicate.

Statistical analysis
Chromatographic data were processed using the Empower™ 3 chromatography software (Waters Corporation, Milford, Connecticut, USA). Retention time, number of theoretical plates, and tailing factor were expressed as mean ± standard deviation. Accuracy and precision were reported as mean percent recovery and % CV, respectively.

RESULTS AND DISCUSSION

The HPLC-PDA method was first developed by identifying the ideal chromatographic conditions for the detection and quantification of fluconazole in simulated saliva. The ideal chromatographic conditions summarized in Table 1 were obtained through the evaluation of various parameters, such as the mobile phase ratio of water:acetonitrile, injection volume, elution mode, flow rate, and UV wavelength.

Table 1: Chromatographic conditions for the detection and quantification of fluconazole in simulated saliva.

Parameter	Condition
Mobile phase composition	Water:Acetonitrile (75:25)
Elution mode	Isocratic
Injection volume	10 µL
Flow rate	1.20 mL/min
UV wavelength	257 nm

Using these chromatographic conditions, the data from the system suitability test revealed that the average retention time of the fluconazole peak was 3.655 ± 0.019 min (Figure 1), having a % CV of 0.51%, while the peak area of the injections had a % CV of 1.32%. These were within the acceptable criteria of % CV ≤ 2.00%, indicating good results of repeatability (Khan et al. 2016). The number of theoretical plates was 28238.43 ± 301.34, which was above the recommended value of > 2000 (Gupta et al. 2024), proving a good column efficiency. Tailing factor was 1.31 ± 0.03, which was also within the recommended value of < 2.0 (Gedawy et al. 2018). This value is considered minor tailing, which can still be improved if desired through various techniques such as reducing the injection volume to prevent column overloading or by adding mobile phase modifiers, such as acids, to minimize interactions between the analyte and the stationary phase, thereby reducing excessive retention (Setyaningsih et al. 2021). In light of the system suitability data, method optimization through the use of a shorter column and an increased flow rate may enable a faster analysis time (Schuster et al. 2013). Nevertheless, it is important to verify that all system suitability parameters remain within the specified limits. This approach is particularly advantageous in the pharmaceutical industry, wherein efficiency is necessary.

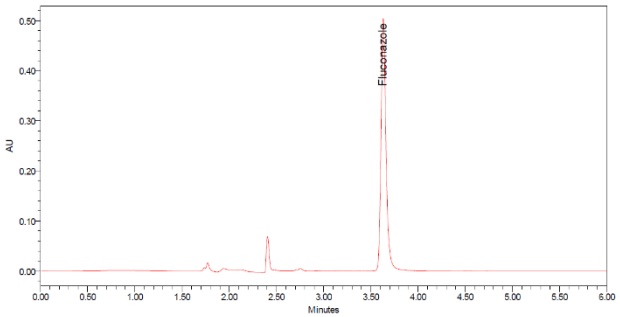

Figure 1: Representative chromatogram of fluconazole using the developed and validated HPLC-PDA method.

The method developed was validated to confirm its reliability, repeatability, and suitability for its intended analytical application. Validation of the method developed in terms of specificity, linearity, carry-over effect, accuracy, precision, LOD, and LOQ was performed systematically in the HPLC-PDA instrument. The specificity study confirmed that neither the blank simulated saliva nor the excipients used in the blank κ-carrageenan film produced peaks at or near the retention time of fluconazole. Chromatograms showed a single, well-defined fluconazole peak in the standard solution with no overlapping peaks in either blank sample, indicating that the method is highly specific and free from matrix interference. Simulated saliva was used as the medium to appropriately mimic the buccal

environment. A linearity study was performed by measuring the peak areas of non-zero calibrator standards (Figure 2A), and a standard curve was generated (Figure 2B). Linear regression equation was calculated by peak area and was y = 8840.3x + 12983. Furthermore, the r^2 showed a strong linear relationship with a value of 0.9999, supporting the method's reliability for determining fluconazole. The developed method demonstrated no significant carryover effect, as evidenced by the absence of a detectable peak at the fluconazole retention time in the blank simulated saliva, analyzed immediately after a 200 µg/mL sample run. The LOD and LOQ, which correspond to the minimum level of fluconazole that can be detected and quantified, respectively, were established. Using the standard deviation of the response and slope derived from the linearity, the value of LOD was found to be 4.86 µg/mL, while LOQ was 14.73 µg/mL. These values indicate the method's sensitivity for detecting low concentrations of fluconazole.

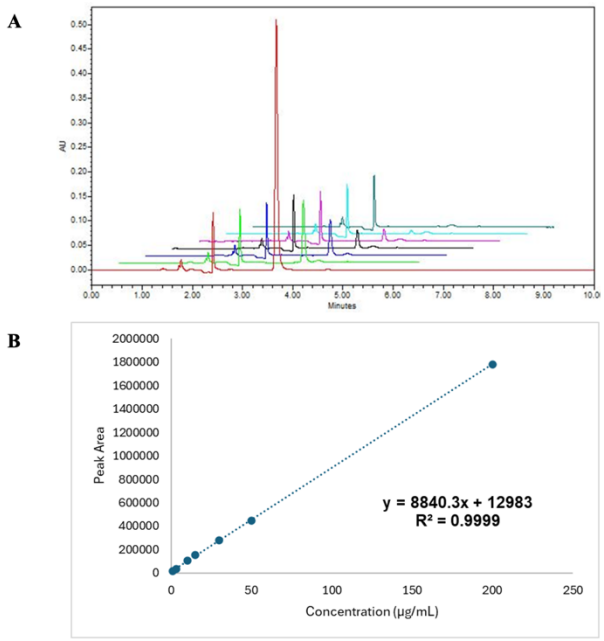

Figure 2: (A) Chromatograms of non-zero calibration standards, 1 - 200 µg/mL. **(B)** Standard curve of fluconazole showing the equation of the line and r^2 value.

The intraday and interday accuracy and precision at the corresponding QC concentrations are summarized in Table 2. The data from the QC samples that were collected showed that the method has good accuracy and precision. The accuracy according to the measured concentration was within the allowed deviation from the nominal concentration of ± 15% (FDA 2018). Likewise, the precision in terms of % CV was also within the acceptable limits of not exceeding 15%. Based on the results of validation, the method was deemed reliable, repeatable, and suitable for the detection and quantification of fluconazole in simulated saliva.

Table 2: Accuracy and precision of the HPLC-PDA method for the detection and quantification of fluconazole in simulated saliva (n = 3).

Quality Control Sample	Nominal Concentration (µg/mL)	Intraday analysis			Interday analysis		
		Measured concentration (µg/mL)	% Recovery	% CV	Measured concentration (µg/mL)	% Recovery	% CV
HQC	50	48.14	96.29	1.21	48.58	97.16	3.18
MQC	30	31.07	103.57	6.69	29.61	98.69	4.82
LQC	15	15.31	102.03	9.94	15.21	101.39	6.09

To further check the applicability of the developed method, an 8-mm fluconazole-loaded buccoadhesive film (Figure 3) was analyzed for drug content. A simple buccoadhesive film was made from κ-carrageenan and PEG 400. κ-carrageenan, a sulfated polysaccharide, is known for being highly safe, biocompatible, biodegradable, and non-toxic. Its widespread use in buccal drug delivery systems is largely due to the sulfate groups, which interact with mucin in the buccal mucosa to enhance adhesion (Kristó et al. 2024). Meanwhile, PEG 400, a polymeric plasticizer, improves the film's flexibility, reduces brittleness, and further augments buccoadhesion. In the present study, these two excipients served as the primary components of the buccoadhesive film, acting as the carrier for fluconazole. Fluconazole loaded in the buccoadhesive film was quantified using the method developed and validated. The results revealed 101.77 ± 1.22% recovery with 1.12% CV. The findings demonstrated that the method can accurately and precisely quantify fluconazole in polymeric matrices. The lack of interference from film excipients and the acceptable recovery further validated the method's utility in formulation analysis and quality control.

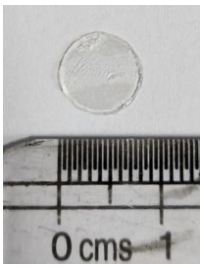

Figure 3: Representative photo of the formulated fluconazole-loaded buccoadhesive film, approximately 8 mm in diameter.

The present study used simulated saliva, an aqueous buffer at pH 6.8, for the preparation of a standard solution for the assay of fluconazole in buccoadhesive film. Unlike the United States Pharmacopeia and National Formulary (USP-NF) assay methods for fluconazole and other related formulations, which uses organic solvents, this study prioritized a physiologically relevant solvent. The choice of aqueous buffer at pH 6.8 was crucial, as it not only simulates saliva but also enhanced the dissolution of fluconazole, a drug that has poor water solubility. A decrease in pH of the medium compared to pure water

improves the solubility of fluconazole (Chaturvedi and Sahu 2025). It was notable that the formulated buccoadhesive film was soluble in the simulated saliva, which facilitated the assay via the developed method. The enhanced solubility of the formulation can be partly attributed to the presence of PEG 400, which has been used to improve the solubility of poorly water-soluble drugs (Nayak and Panigrahi 2012; Shakeel et al. 2017).

The developed method provides a practical tool for the quantification of fluconazole in buccoadhesive delivery systems. However, it is limited to assay applications and does not include forced degradation studies or in vitro release kinetics, which are important for comprehensive formulation development. Further studies should aim to expand the method for stability-indicating purposes and in vitro dissolution or permeation studies.

CONCLUSION

A simple and reliable HPLC-PDA method was successfully developed and validated for the detection and quantification of fluconazole in simulated saliva and buccoadhesive film formulation. The method demonstrated specificity, linearity, precision, and accuracy, complying with validation criteria. With a short analysis time and minimal use of complex solvents, the method offers a practical approach for the detection and quantification of fluconazole, even in minute amounts. Furthermore, the successful application of the method to κ-carrageenan-based buccoadhesive films confirms its suitability for formulation development and dosage form evaluation. This work provides an essential analytical foundation for future studies involving drug release, stability testing, and in vitro-in vivo correlation (IVIVC) of buccoadhesive antifungal therapies. Future directions should include the extension of this method in stability-indicating studies and in vitro release assessments, enabling a more comprehensive characterization of buccal drug delivery systems intended for the treatment of oral candidiasis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

GLTD: Conceptualization and design of work, data gathering, acquisition, analysis, and interpretation of data, and writing the original draft. BPIB: Design of work, analysis and interpretation of data, supervision, writing, review, and editing. JAPN: Analysis and interpretation of data, supervision, writing, review, and editing. JFCC: Conceptualization and design of work, analysis and interpretation of data, supervision, writing, review, and editing. All authors approved the final version of the manuscript.

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