Development and *in vitro* Evaluation of Voriconazole Nanoparticle Formulation for Mucosal Application

Mukozal Uygulama için Vorikonazol Nanopartikül Formülasyonunun Geliştirilmesi ve *in vitro* Değerlendirilmesi

**ABSTRACT**

Objectives: This study aimed to prepare and evaluate mucoadhesive nanoparticle formulations of voriconazole, an antifungal drug, for mucosal application. It was also aimed to develop and validate a HPLC method of voriconazole.

Materials and Methods: In this study, mucoadhesive nanoparticles containing voriconazole were prepared using a coating polymer of chitosan. The obtained nanoparticles were characterized via particle size, polydispersity index, zeta potential measurement, and mucoadhesion studies. Drug loading capacity was tested for determination of the voriconazole amount in the nanoparticles. *in vitro* drug release was also examined. The HPLC method was validated for linearity, accuracy, precision (repeatability and reproducibility), specificity, stability, limits of detection (LOD), and limit of quantification (LOQ).

Results: *in vitro* characterization results of the mucoadhesive nanoparticle formulation containing voriconazole was found to be appropriate with a small particle size of 217.1±4.2 nm, a narrow polydispersity index of 0.335±0.042, 99.052±0.424% drug loading, and a positive zeta potential value of +26.82±0.4 mV. According to a mucoadhesive study, it can be concluded that the nanoparticle was able to interact with mucin due to ionic interaction. Also, the turbidity of nanoparticle/mucin dispersion was higher than the turbidity of mucin dispersion itself. Based on the *in vitro* drug release, no burst effect was observed, indicating that voriconazole was homogeneously dispersed in the nanoparticle dispersion and that no significant amount of drug was adsorbed onto the nanoparticle surface. The release was found to follow a non-Fickian diffusion mechanism with first-order drug release. The proposed HPLC method was simple, highly sensitive with good linearity, accurate, precise, specific, and stable, showing that the method is useful for routine quality control.

Conclusion: This study has shown that the mucoadhesive nanoparticle formulation containing voriconazole reported here is a promising candidate for the local treatment of mucosal diseases. The developed HPLC method can be successfully applied to pharmaceutical preparations containing voriconazole.

**KEY words:** Voriconazole, mucoadhesive nanoparticle, chitosan, local application, HPLC

**ÖZ**

Amaç: Bu çalışma, mukozal uygulama için antifungal bir ilaç olan vorikonazolun mukoadezif nanopartikül formülasyonunu hazırlamayı ve değerlendirme amacını almıştır. Ayrıca vorikonazolun HPLC yöntemi geliştirilmiş ve valide edilmiştir.

Gereç ve Yöntemler: Bu çalışmada, kitozan kaplama polimerini kullanılarak vorikonazol içeren mukoadezif nanopartikül hazırlanmıştır. Elde edilen nanopartikülleri, partikül boyutu, polidispersite indeksi, zeta potansiyeli ölçülmüş ve mukoadezyon ölçümleri ile karakterize edilmiştir. İlaç yükleme kapasitesi nanopartikülde vorikonazol miktarının belirlenmesi için gerçekleştirilmişdir. *In vitro* ilaç salınımı da incelendi. HPLC yöntemi doğrulanmış, doğru, kesinlik (tekrar edilebilirlik ve tekrar elde edilebilirlik), özgürük, stabilite, LOD ve LOQ ile valide edilmiştir.

Bulgular: *In vitro* karakterizasyon sonuçları mukoadezif nanopartikül formülasyonu, 217.1±4.2 nm küçük partikül boyutu, 0.335±0.042 dar polidispersite indeksi, %99.052±0.424 ilaç yükleme kapasitesi, +26.82±0.4 mV pozitif zeta potansiyel değerle, mukoadezyonunun, iyonik etkileşim nedeniyle müsinle etkileşime girdiği sonucuna varılmıştır. Ayrıca nanopartikül/müsin dispersiyonunun, müsin dispersiyonunun bulanıklığına daha fazla bulanıklık göstermiştir. *In vitro* ilaç salınımına göre, vorikonazolun nanopartikül dispersiyonunda homojen olarak dağılmış olduğu ve nanopartikül yüzeyinde önemli miktarda ilaçın adsorbe ettiği göstermuştur. 1. dereceden verici bir salınım oldu ve HPLC yöntemi, vorikonazolun farmasötik preparatlar için başarılı bir şekilde uygulanabilir.

Anahtar kelimeler: Vorikonazol, mukoadezif nanopartikül, kitosan, lokal uygulama, HPLC

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INTRODUCTION

Voriconazole (VRZ) ((2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butan-2-ol) is a novel broad spectrum triazole antifungal agent for the treatment of serious infections caused by Aspergillus, Fusarium, Scedosporium, and resistant Candida species. Since its release by the United States Food and Drug Administration in May 2002, VRZ has established itself as the first-line treatment for invasive aspergillosis and proven useful in other fungal infections that are resistant and refractory to standard antifungal therapies. In Europe, the drug has been approved by the European Medicines Agency (EMA) since March 2002 for the treatment of invasive aspergillosis, fluconazole-resistant strains of Candida species, and serious infections caused by Scedosporium spp. and Fusarium spp. (EMEA 2002). VRZ is a synthetic derivative of fluconazole, one of the triazole moieties in fluconazole is replaced by a fluoropyrimidine group and an alpha methyl group is also introduced. It is more active than fluconazole and itraconazole against Candida species. The drug has reported adverse effects of visual disturbances, hepatic toxicity, headache, and dermatologic reactions, as well as serious cytotoxicity. Because of the well-reported adverse effects, drug interaction, and the ominous risk of drug resistance of systemic VRZ, a local formulation without the aforementioned risks is needed in clinical practice.

Local drug delivery is frequently used for the treatment of localized disorders. The main advantages of local administration are the ability to deliver the active agent directly to the site and the maintenance of the required concentration of active substance at the site for a prolonged period. A great deal of attention has been devoted to the development of mucoadhesive drug delivery systems. Mucoadhesive nanoparticle (NP) formulations have raised widespread interest for their recognised potential for improving the bioavailability of drugs and maintaining the local effect in the targeted area. The increased contact time and localization of the drug is due to applying nanoparticles (NPs) of VRZ, which are made mucoadhesive, thus enhancing its delivery. This approach prolongs the activity of the active substance as well as reduces the frequency of administration. A possible added advantage of mucoadhesive NP is that particulates have the advantage of being relatively small and are thus accepted by patients.

NPs coated with chitosan (CSH) have attracted a special interest for drug delivery through the mucosal routes because of their ability to interact with the negatively charged sites on the mucosa surface, prolonged retention time, mucoadhesive properties, and increased local concentration of NPs. CSH, which is strong mucoadhesive, nontoxic, cationic, biocompatible, biodegradable, and has mucoadhesiveness as well as antibacterial, antifungal, and antitumor activity, is a suitable polymer for mucoadhesive drug formulations. These functional properties provide suitability and extensive pharmaceutical applications; therefore, CSH-coated NPs have reached an important position in the arena of drug delivery.

The objective of this study was to prepare a mucoadhesive NP formulation of VRZ. CSH was used as coated polymer to provide the mucoadhesive property to the NP system. In vitro characterization of the prepared mucoadhesive NP formulation was performed. An additional aim was to develop and validate a simple, rapid, and economic high-performance liquid chromatography (HPLC) method for the analysis of VRZ as per International Council for Harmonisation of Technical Requirements (ICH) guidelines.

MATERIALS AND METHODS

Materials

VRZ were obtained from Sigma-Aldrich (St Louis, MO, USA). Eudragit (EUD) RS 100 as gifts from Karadeniz Chemical Company (Karadeniz, Turkey). High-molecular-weight CSH (Brookfield viscosity [1%, m/V, in 1% acetic acid solution at 25°C]; 800,000 mPa.s, 75% deacetylated) and HPLC grade acetonitrile were purchased from Sigma-Aldrich. All other materials were of analytical grade.

HPLC system

HPLC was conducted using a Hewlett Packard series 1100-1200 HPLC apparatus (Santa Clara, CA, USA) equipped with an UV detector set at 255 nm using a C18 column (5 μm, 4.6x250 mm). The injection volume was 50 μL. The mobile phase, fluxed at 1.5 mL/min, was a mixture of acetonitrile:water (35:65 v/v). The flow rate was maintained at 25±1°C.

Preparation of stock solutions and standard working solution

Stock solution of VRZ was prepared by dissolving 1 mg of drug in 10 mL methanol. VRZ concentrations in the working solution chosen for the calibration curves were 2.5, 5, 10, 15, 20 and 30 μg/mL. All samples were filtered through an aqueous 0.2-μm pore-size membrane filter before injection.

Validation of HPLC method

The developed HPLC method was validated according to ICH guidelines including the determination of linearity, calibration curve, accuracy, precision (repeatability and reproducibility), specificity, stability, limit of detection (LOD), and limit of quantification (LOQ) of analysis in spiked samples.

Specificity

The specificity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. To evaluate the specificity of the analytical method, the VRZ test solution, methanol, and mobile phase were injected into the chromatographic system. These parameters were determined by comparing the chromatograms of the VRZ standard, methanol, and mobile phase.

Linearity

The linearity between the peak area and concentration was analyzed using a calibration curve obtained from standard solutions of VRZ (2.5 to 30 μg/mL). In addition, it was evaluated using linear regression analysis, which was calculated by the least-square regression analysis.
Accuracy and recovery
The accuracy study is the closeness of test results obtained by the method to the true value and is defined recovery. The prepared three standard solutions (10, 15, 20 μg/mL) were injected six times at different levels as a test sample.

Precision
The precision of the assay was determined with repeatability and reproducibility. Repeatability was calculated from six replicated injections of freshly prepared VRZ test solution in the same equipment at a concentration value of 10 μg/mL of the intended test concentration value on the same day. For reproducibility study, 10 μg/mL sample was prepared and injected into HPLC system as per the test procedure. The peak area of VRZ was determined and precision was reported as % RSD.

LOD and LOQ
LOD and LOQ tests for the procedure are performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyte that can be reproducibly quantitated above the baseline noise that gives S/N = 10.

Stability
For a short-term stability study, a sample solution of VRZ was prepared and analyzed initially and also at the end of 48 hours by keeping the solution at room temperature.

Preparation of mucoadhesive NP formulation
The NPs were prepared by adapting the spontaneous emulsification technique previously described by Bodmeier et al. The final preparation method was established in accordance with Rençber et al.'s study. In brief, 2.5% of EUD RS polymer and 0.05% VRZ were dissolved in 25 mL ethanol. The alcoholic solution mixture was added dropwise (3 mL/min) to 50 mL of 0.025% w/v aqueous chitosan solution under continuous magnetic stirring at 800 rpm. The formed CSH-coated NP was further stirred for 48 hours at room temperature.

Measurement of particle size (PS), polydispersity index (PI) and zeta potential (ZP)
The PS, PI and ZP were measured at 25°C using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK). The PS and PI values were obtained by averaging ten measurements at an angle of 173° using disposable cells. The ZP was calculated from the electrophoretic mobility using the Helmholtz-Smoluchowski equation under an electrical field of 40 V/cm. The processing was performed using the software included within the system (n=5).

Mucoadhesive evaluation: ZP determination and turbidimetric measurement
Two in vitro methods were used to assess the mucoadhesive evaluation of NP. In the first method, the mucoadhesive property of NP with VRZ was evaluated by measuring the changes of ZP on interaction with negatively charged mucin. The mucoadhesive NP containing VRZ was incubated at 37°C in 0.1% mucin dispersion. The ZP of the NP was measured over 6 hours. The alteration of ZP of the CSH-coated NP with VRZ indicates interaction with mucin.

Turbidimetric measurements of mucoadhesive NP containing VRZ was compared with mucin dispersion at 650 nm by ultraviolet-visible spectrophotometer. The accurately mucoadhesive NP (5 mL) was added to 5 mL aqueous mucin dispersion and stirred at 200 rpm. The turbidity of the dispersions was measured at certain time intervals over 6 hours and compared with the turbidity of the mucin dispersion. The increase in turbidity of mucin: mucoadhesive NP dispersion with VRZ indicated mucoadhesive property.

Drug loading (DL) capacity
The DL capacity of NP was determined by dissolving 0.04 mL of the NP in 8 mL methanol with vortex, followed by a validated HPLC assay for VRZ. The DL capacity was calculated according to the following equations:

\[
DL = \frac{\text{Total amount of VRZ}-\text{The amount of free VRZ}}{\text{Total amount of formulation components}} \times 100
\]

In vitro drug release studies
The release of VRZ from 4 mL of NP was assessed using a dialysis bag (cellulose membrane, 12,000-14,000-molecular-weight cutoff Spectrum Labs, Rancho Dominguez, CA, USA into phosphate buffer saline (PBS) at 37°C±0.5°C, and stirred continuously with a magnetic stirrer at 300 rpm for 24 hours. The amount of the drug in the receiving solution was analyzed using the validated HPLC method. Sink conditions were maintained in the receptor compartment during in vitro release studies (n=5).

Determination of drug release mechanism
The dissolution data were fit to the Peppas equation, and best-fit parameters were calculated to determine the release mechanism of the tablets.

RESULTS
HPLC system
An HPLC method for quantitative analysis of VRZ in mucoadhesive NP formulation was developed and validated. To evaluate the specificity of the analytical method, the VRZ test solution, methanol, and mobile phase were injected into the chromatographic system. There was no interference from the methanol and mobile phase at VRZ peaks. The chromatogram of the VRZ standard presented a peak in the retention time of 13.669 min; the total analysis time was 15 min (Figure 1).

A 6-point calibration curve was constructed covering a concentration range from 2.5 to 30 μg/mL for standard solution of bulk VRZ. The determination correlation coefficient (R²) for a regression line is 0.9931 with the linear regression equation y=42.537x-46.906. The analyses of calibration are shown in Figure 2. The HPLC area responses for accuracy determination were evaluated and the mean recovery data of VRZ were within the range of 89.702 and 105.500% (Table 1). The mean % relative
standard deviations (R.S.D.) were between 0.112 and 0.542%, thereby satisfying the acceptance criteria for the study (R.S.D. maximum 2.0%). Hence, the accuracy of the method was confirmed, this method can be used for further studies. As Table 2 shows, the percentage of mean recovery values of precision study were found as 100.541%-100.616%, with R.S.D. ranges 0.028-0.392.

The determined values of LOD and LOQ were 0.804 µg/mL and 1.608 µg/mL, respectively.

During the storage of the solution at room temperature, the solutions were stable for 48 hours (Table 3). 

Preparation and characterization of mucoadhesive NP formulation

In our study, mucoadhesive NP formulation was successfully prepared adapting the spontaneous emulsification technique in accordance with Rençber et al.'s study. The PS, PI and ZP were found as 217.1±4.2 nm, 0.335±0.042, and 26.82±0.4 mV, respectively.

To obtain insight into the mechanism of mucoadhesive NP with VRZ: mucin interaction, ZP measurements of their dispersions were performed and the results are shown in Figure 3A. As seen in Figure 3B, the absorbance of 0.1% aqueous mucin dispersion at λ=650 nm was used as a reference for the turbidimetric study.

The DL capacity of the mucoadhesive NP formulation was found as 99.052±0.424%. The in vitro VRZ release from the NPs using a dialysis bag for 24 hours is shown in Figure 4. The values of n, log k, and r² calculated using the Peppas equation were found as 0.7477, 0.1946, and 0.9962, respectively.

\[
\log \left( \frac{M_t}{M_\infty} \right) = \log k + n \log t
\]

where \( M_t/M_\infty \) is the fractional release, k is the diffusional constant, and n is the diffusional exponent that characterizes the drug release mechanism.
DISCUSSION

HPLC methods have been widely employed in pharmaceutical analysis due to the ease of performance, specificity, sensitivity, and the analysis of samples of complex nature. In this study, HPLC was proposed to quantify VRZ in mucoadhesive NP formulation. Mobile phase selection was based on peak parameters, run time, ease of preparation, and cost. All samples were maintained at 25°C in the autosampler prior to injection. A volume of 50 μL of each sample was directly injected into the HPLC system. Acceptable separations (Figure 1), with a retention time of 13.669 min for VRZ was obtained using a C-18 column and a mobile phase composed of acetonitril:water (35:65). The column temperature was maintained at 25°C. Monitoring of VRZ was realised with UV detection at a wavelength of 255 nm.

To be considered specific, an analytical method should demonstrate that it can separate and quantify the drug from impurities, degradation products, and excipients. To evaluate the specificity of the analytical method, the VRZ test solution, methanol, and mobile phase were injected into the chromatographic system. There was no interference from the methanol and mobile phase at VRZ peaks.

The precision is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to analyte concentration in samples within a given range.\textsuperscript{23,24} Linearity data indicate that the VRZ peak areas are linear over a concentration range of 2.5-30 μg/mL. A linear relationship between the peak area and concentration of VRZ was observed. A correlation coefficient was equal to 0.99, which indicated a strong linear relationship between the variables. The standard deviation of the slope and intercept were low.

To be considered precise, an analytical method has to reproduce its results under the same conditions. In terms of the method precision study of our experiment, 10 µg/mL solutions were injected six times into the system and the percentage of recovery was evaluated. Because the percentage of recovery has been found almost 100 and the R.S.D. value less than the acceptance criteria, which is 2%, the analysis system for the determination of the assay was verified. The low values of standard deviation denote very good repeatability of the measurement. Thus it was shown that the equipment used for the study were correct and the developed HPLC method was highly repetitive.

The LOD and LOQ tests for the procedure were performed on samples containing very low concentrations of analyses. LOD is
defined as the lowest amount of analyte in a sample that can be detected above baseline noise, but not necessarily quantitated as an exact value. LOQ is defined as the lowest amount of analyte in a sample that can be reproducibly quantitated above the baseline noise, which gives the signal-to-noise ratio (S/N).  

Samples should be tested over at least a 48-hour period and quantitation of components should be determined through comparison with freshly-prepared standards. The stability of VRZ in 10 μg/mL standard solutions was determined by storing the solutions at ambient temperature (25±1°C).

The mucoadhesive NP was prepared by adapting the spontaneous emulsification technique previously described by Rençber et al., which was the avoidance of using toxic organic solvents. This system prolongs the residence time of the dose form at the site of application and facilitates an intimate contact of the dose form with the underlying mucosal surface, and thus contributes to improved and/or better therapeutic performance of the VRZ. The prepared mucoadhesive NP was characterized in terms of PS, PI, ZP, and DL capacity. The mucoadhesive property with two methods of NP was determined. In vitro drug release of CSH-coated NP was performed.

The PS of the NP affects its important properties. The most important properties influenced in pharmaceutical technology are increased saturation solubility and adhesiveness to surfaces/membranes. Pharmaceutically positive effects justify the definition of NPs with a size below 1000 nm, and this size limit should be considered when defining a classification system. Also, PS has a direct relevance to the stability of the formulation. Larger particles tend to aggregate to a greater extent compared with smaller particles, thereby resulting in sedimentation. The mean size of the prepared mucoadhesive NPs was found as 217.1±4.2 nm. Previously, Mazzarino et al. produced CSH-coated NPs for buccal drug delivery and the PS was found around 200 nm. The PI of the formulation was low (PI <0.5), showing that this method of preparation resulted in a uniformly high NP. ZP is an important parameter that is key for the potential stability of colloidal systems. Prepared VRZ loaded mucoadhesive formulation was positively charged, with ZP values with 26.82±0.4 mV. Positive ZP may give rise to a strong electrostatic interaction with a negatively charged mucosal surface. Therefore, we used positively charged NPs for mucoadhesive drug delivery systems as its positively charged surface can be in favour of adhesion to the cell mucosa, which are normally negatively charged.

Two in vitro methods were used to demonstrate the interaction between mucoadhesive NP and mucin. Electrostatic interaction is the most expectable mucoadhesive mechanism. After 6 hours of incubation with mucin, there was a decrease in ZP for mucoadhesive NP, which can probably be attributed to interactions between negatively charged sialic groups in the mucin layer and positively charged surface layer of mucoadhesive NP. Therefore, it can be concluded that the mucoadhesive NP was able to interact with mucin due to ionic interaction. The turbidity of mucoadhesive NP–mucin aqueous dispersion was examined to obtain information about mucoadhesiveness. The absorbance of the mucin-free dispersion of the NPs did not significantly deviate from 0.4. Changes in the turbidity of mucoadhesive NP with VRZ.mucin dispersion should be considered as an indication for an eventual interaction between NP and mucin, and not due to the motion of particles. The turbidity of mucoadhesive NP–mucin dispersion was higher than that of mucin dispersion itself (Figure 3). This phenomenon could be explained due to the greater thickness of the CSH layer around these particles, as discussed earlier. Yoncheva et al. reported that CSH-coated poly(lactide-co-glycolide) NP–mucin dispersion demonstrated greater turbidity than mucin itself.

Mucoadhesive NPs with VRZ had high DL capacity (~99%). In vitro drug release of mucoadhesive NPs in PBS was studied. The drug release from NPs and subsequent biodegradation are important for developing successful formulations. The release rate of NPs depends upon the desorption of the surface-bound/adsorbed drug, diffusion through the NP matrix, diffusion through the polymer wall, NP matrix erosion, and a combined erosion/diffusion process. Thus, diffusion and biodegradation govern the process of drug release. Figure 4 shows that prepared mucoadhesive NP formulation significantly extended the release. No burst effect was observed, indicating that VRZ was homogeneously dispersed in the NP dispersion and that no significant amount of drug was adsorbed onto the NP surface. The theory of the determination of drug release mechanism from NPs is based on an empirical equation (Ritger & Peppas, 1987). The exponent n has been proposed as indicative of the release mechanism. In this study, the value of n=0.7477 indicates anomalous (non-Fickian) diffusion. Non-Fickian behavior requires two parameters to describe the coupling of diffusion and relaxation phenomena.

CONCLUSION

This study has described the mucoadhesive NP formulation of VRZ using a coating polymer of CSH. The method of prepared NPs was consistent and reproducible, and able to obtain colloidal solutions with adequate PS, PI, ZP, acceptable VRZ loading capacity, and appropriate in vitro drug release. The developed HPLC method for VRZ is very simple and specific because all peaks and results confirmed suitable accuracy, specificity, and precision. Therefore, the method could be useful for both routine analytical and quality control assays of VRZ in pharmaceutical formulations. This study has shown that the described mucoadhesive NP formulation containing VRZ is a promising candidate for the local treatment of mucosal diseases. The developed formulation containing VRZ has been found worthy of in vivo studies.

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