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RESEARCH ARTICLE

Validation Of Rp-Hplc Uv Method for Determination Ketoconazole in Rabbit Plasma: an Application to The Pharmacokinetic Study

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Validation of RP-HPLC UV method for determination ketoconazole in rabbit plasma: An application to the pharmacokinetic study

SUMMARY

The validated method for determining ketoconazole in plasma rabbit is not yet reported. The HPLC-UV method is simple, rapid, cost-effective, sensitive, and only requires a small blood sampling. The chromatographic system used a mobile phase consist of NaH2PO4:Acetonitrile (30:70) and stationary phase as a reversed-phase C18 column (250 x 4.6 mm, 5 µm) at a flow rate of 1 ml/ min and detection wavelength of 240 nm, and the retention time of about 5 minutes for ketoconazole and 11 min for itraconazole as internal standard. The peak of ketoconazole can separate from other peaks and has no interference from the diluent, indicating this method was selective to detect ketoconazole. The calibration curve presented linearity in the 0.05-8 µg/ml with R2=0.9969, which showed good linearity. Precision and accuracy of the method were obtained. The result is 9.47 %diff and 10.13-12.08 RSD% for LLOQ and 0.59-3.94 %diff and 1.82-13.56 %RSD <20% for low, medium, and high levels. The LLOQ in this method is 0.05 µg/mL. Plasma stability under storage in a freezer (-200C) for three days was studied. The validated analytical method was successfully applied to determine the pharmacokinetics parameter of KTZ after a single oral administration.

Key Words: *Chromatography, imidazole, plasma, pharmacokinetic, validation method*

Tavşan plazmasındaki ketokonazol'un belirlenmesi için RP-HPLC UV yönteminin doğrulanması: Farmakokinetik çalışma için bir uygulama

ÖZ

Tavşan plazmasında ketokonazol tayini için doğrulanmış yöntem henüz bildirilmemiştir. HPLC-UV yöntemi basit, hızlı, uygun maliyetli, hassastır ve yalnızca küçük bir kan örneği gerektirir. Kromatografik sistem, NaH2PO4:asetonitril (30:70) içeren bir mobil faz ve sabit faz olarak ters faz bir C18 kolon (250 x 4.6 mm, 5 µm) kullanıldı. işlemde akış hızı 1 ml/dk, saptama dalga boyu 240 nm olarak ayarlandı ve dahili standart olarak alıkonma süresi yaklaşık 5 dakika olan ketokonazol ve 11 dakika olan itrakonazol kullanıldı. Ketokonazol piki diğer piklerden ayrılabilir olması ve dilüe ediciden etkilenmemesi bu yöntemin ketokonazolü saptamak için seçici olduğunu gösterir. Kalibrasyon eğrisi, iyi doğrusallığı gösteren R2=0.9969 ile 0.05-8 µg/ml'de doğrusallık gösterdi. Yöntemin kesinliği ve doğruluğu elde edildi. Sonuç, LLOQ için %9.47 fark ve %10.13-12.08 RSD ve düşük, orta ve yüksek seviyeler için %0.59-3.94 fark ve %1.82-13.56 RSD <%20'dir. Bu yöntemdeki LLOQ 0.05 µg/mL'dir. Üç gün boyunca bir dondurucuda (-20°C) depolama altında plazma stabilitesi incelenmiştir. Doğrulanmış analitik yöntem, tek bir oral uygulamadan sonra KTZ'nin farmakokinetik parametresini belirlemek için başarıyla uygulandı.

Anahtar Kelimeler: *Kromatografi, imidazol, plazma, farmakokinetik, validasyon yöntemi*

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INTRODUCTION

Ketoconazole (KTZ) is an imidazole broad-spectrum antifungal agent used for systemic and local infections (Hamdy & Brocks, 2009). KTZ is reported to have the ability to inhibit CYP3A4 and P-gp, which could inhibit several other tyrosine kinase inhibitors' substrates (Wang et al., 2022). It is potent inhibitor biosynthesis in *Candida albicans* of ergosterol that major sterol to synthesizing the fungal cell wall and other complexes (Oji, 1982). It is used as oral administration to control systemic mucocutaneous fungal infection. KTZ has poor water solubility (0.04 mg/ml), highly degraded *in vivo*, and eliminated through the bile-solubilized feces pathway (Aljurbui et al., 2022).

High-performance liquid chromatography (HPLC) is one of the most effective techniques for separating various mixtures and determining the number of compounds in many samples (Ban & Jinno, 2001). The HPLC method has been reported for determining ketoconazole in plasma (Bajad et al., 2002). There were HPLC-UV (Bajad et al., 2002; Hamdy & Brocks, 2010; Vertzoni et al., 2006), HPLC coupled with fluorescence detection (Alvinerie et al., 2008; Han et al., 2019), and HPLC tandem MS-MS (Chen et al., 2002). The HPLC with the fluorimetric detector and tandem MS-MS has a higher cost than UV (Ranjan et al., 2013). The HPLC-UV method is simple, rapid, cost-effective, sensitive, and only requires a small blood sampling (Bajad et al., 2002; Hamdy & Brocks, 2010).

In the HPLC-UV method, the internal standard (IS) is crucial for extraction efficiency and recovery (Chen et al., 2002). In the previous study, the IS that was used took a long elution time (>20 min). There was phenothiazine (Pascucci et al., 1983), R41300 (Swezey et al., 1982), terconazole (Turner et al., 1986), and clotrimazole (Riley & James, 1986). With a long retention time, the IS makes analysis inefficient, causing high costs and a wasteful mobile phase. An ideal method should have cost-effective by short total elution time of analyte and IS (Chen et al., 2002). In the early 2000s, the HPLC-UV methods for determining ketoconazole were still limited. Vertzoni et al. presented a technique that used a reversed-phase Hypersil BDS-C18 column with mobile phase methanol, water, and diethylamine (74:26:0.1), used 9-acetyl anthracene as IS with an elution time about 7-10 min use canine/dog plasma (Vertzoni et al., 2006). Hamdy et al. published the method to separate midazolam and ketoconazole, used a Symmetry C18 column with the mobile phase as Acetonitrile and KH_2PO_4 (45:55), and diazepam as IS (run time was 10 min for rat plasma and 19 min for human plasma) (Hamdy & Brocks, 2010). Bajad et al. described a method that used HPLC to determine ketoconazole and piperine in rat and human plasma simultaneously. The column used Water's Symmetry C18 with Acetonitrile: KH_2PO_4 (50:50) as mobile phase, without internal standard (Bajad et al., 2002).

The pharmacokinetic information for ketoconazole is relatively limited. The bioavailability data of ketoconazole is available only for rats, dogs, monkeys, and humans following oral doses was 35.8%, 50%, 22%, and 81.2%, respectively (CHMP, 2014). The data for rabbits have not been reported yet. Due to inter-subjects in several species, data have variability, so it is important to be researched. Before conducting a pharmacokinetic study, it is essential to validate the method for determining the analyte. In addition, no validation method of HPLC-UV for the determination of ketoconazole in rabbit plasma has been reported to date.

This study aims to validate a simple, sensitive, less time utilizing, and reliable RP-HPLC method with UV detection to determine ketoconazole in rabbit plasma according to the EMA guidelines. We present a rapid, selective, and sensitive HPLC-UV way with simple pretreatment procedures to determine KTZ in rabbit plasma with 12 min elution time and itraconazole as IS. To the best of our knowledge, this is the first method for the pharmacokinetic study of KTZ in

rabbits that has been thoroughly validated.

MATERIAL AND METHODS

Materials

Ketoconazole and itraconazole standard were bought from BPOM, Indonesia. Ketoconazole's active component was obtained from PT. Kimia Farma, Indonesia. The sterile water for injection was manufactured by Ikapharmindo, Indonesia. Deionized water was supplied from CV. Alfa Kimia. Acetonitrile gradient grades for HPLC (Merck, Germany), NaH_2PO_2 (Merck, Germany), and NaOH (Merck, Germany).

Animals

Three male New Zealand White rabbits, 3-4 months, weight 2,5-3 kg, were purchased from rabbit breeder Yogyakarta, Indonesia. Three rabbits were kept in a clean caught (Laboratory Animal, Pharmacology Department, Faculty of Pharmacy, Indonesia) with a 12-h light and dark cycle, with diet food of Vital Rabbit (Citrafeed, Indonesia) and water available ad libitum. Ethical clearance of the study was approved by the Faculty of Veterinary, Universitas Gadjah Mada, Indonesia (approval number 039/EC-FKH/Eks./2022).

Blood sampling and sample preparation

Rabbits fasted overnight before the experiment. The rabbits were administered a 14 mg/kg KTZ (equivalent to 400 mg KTZ human doses). The drug was put into the rabbit's mouth through the tube. An aliquot of approximately 2 ml blood samples was collected from the ear marginals vein at 0 (before administration), 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, and 420 after the oral dosing. The sample was collected into an EDTA tube containing 3 mL EDTA. Blood samples were centrifuged for 10 min at 3000 x g rpm to get plasma. The supernatant as a plasma sample was transferred to a 1.5 mL microtube. The samples were kept at -18^{0}C until assayed.

The IS (100 µL) was added to 150 µL plasma sample rabbit plasma in a 1.5 ml microtube. Then the sample was deproteinized with 300 µL acetonitrile. After vortexing for 1 min, the plasma was separated by centrifugation at 12,000 x g for 10 min, and 100 µL supernatant was transferred to a clean 1.5 mL microtube. Before analysis, the analyte was filtered by a 0.45 µm membrane filter. The concentration in the supernatant was determined by the RP-HPLC method.

Chromatographic condition

The HPLC system consisted of Elite LaChrom HPLC, Hitachi UV-Vis detector L-2420, and Hitachi pump L-2130. The utilized column was Phenomenex Luna (250 x 4.6 mm, 5 µm). The UV wavelength was set at 240 nm. The mobile phase consisted of a mixture of 0.02 sodium dihydrogen phosphate pH 7.0 adjusted with 1 M sodium hydroxide and acetonitrile (30:70) at a 1 ml/min flow rate. The injection volume was 20 µl. The mobile phase was filtered using the cellulose 0.45 µm membrane filter. The retention time for ketoconazole and itraconazole (as IS) took around 5 and 11 min, respectively.

System suitability test

Six replicates of the system suitability test were injected at 4 g/mL. The retention time, peak area, theoretical plates, and tailing factor were calculated. To be considered acceptable, the relative standard deviation (%RSD) of retention time should be less than 2%, the peak area should be less than 20%, the tailing factor should be 2, and the theoretical plates of the column (N) should be 2000.

Preparation of stock solution

The stock solution of ketoconazole was made by dissolving 10.0 mg of ketoconazole, then diluted with 100 mL methanol to get the final concentration of 500 µg/mL. The Acetonitrile diluted the stock solution to make 20 µg/mL.

Linearity

Linearity was performed by seven series of standard solution with concentrations of 0.05; 0.1; 0.4; 0.5; 2; 4, and 8 µg/mL as follows: in microtube containing 135 µL blank plasma, 15 µL ketoconazole working solutions (0.5-80 µg/mL) were transferred. After

vortexing for the 30s, add 100 µL IS working solution (25 µg/mL) and 300 µL Acetonitrile. After vortexing for the '30s, the samples were centrifuged for 10 min at 12.000 rpm, at 4^0C. The supernatant was filtered with a nylon filter of 0.22 µm and then injected into the HPLC system. The plotting of concentration (x) and peak area (y) was obtained to get the calibration curve. The linear regression results calculated the intercept, slope, and correlation coefficient.

Specificity

Specificity was determined with a sample of blank plasma 25 µm/ml IS and ketoconazole spiked plasma standard (4 µm/ml).

Accuracy and Precision

Accuracy and precision were determined in spiked plasma standards at four levels (LLOQ, low, medium, and high), which were 0.05, 0.4, 4, and 8 µg/mL, respectively, measured three replicates (n=3) for accuracy and intraday precision, and measured three days consecutively for interday precision.

Stability study

The stability of KTZ was evaluated using a calibration standard of KTZ 8 µg/ml. The condition used in stability testing is a freshly prepared sample and storage in a freezer (-20^0C) for three days.

Data and statistical analysis

Peak-area ratios of the analyte and IS were used for calculation. One compartmental analysis (PKSolver-an add of Microsoft Excel) was used to estimate the pharmacokinetic parameters: the total area under the curve from time zero to infinity ($AUC_{0-\infty}$), Kel, Kab, $t_{1/2}$, Vd, Cl, $C_{max,}$ T_{max}. The data of the parameter result was analyzed using the One Sample T-test. Differences with *p* value less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The chromatographic method was a reversed-phase mode that used a C-18 column (250x4.6 mm, 5 µm particle size) as a stationary phase. The mobile phase consisted of a mixture of 0.02 sodium dihydrogen phosphate pH 7.0 adjusted with 1 M sodium hydroxide and acetonitrile (30:70) at a 1 ml/min flow rate. The injection volume was 20 µl. Absorption was measured at 240 nm. The elution times for ketoconazole and itraconazole (as IS) were approximately 5 and 11 min, respectively.

HPLC with UV detection was chosen due to its simple, fast, and good separation method for determining KTZ. The optimal chromatographic conditions were achieved as described above. The mobile phase composition is essential for separating observed compounds from the other analyte. The best result mobile phase is Acetonitrile and NaH_2PO_4 pH 7 (70:30). Acetonitrile was utilized to obtain peaks with better resolution and symmetry. Alaa et al. observed that using methanol to detect the plasma samples resulted in the band broadening with more retardation (Khedr, 2008). Generally, increasing the concentration of organic solvent in the mobile phase impacts reducing the distance between the solute molecule and the terminal carbon atoms (C18) in the ODS ligand column, which causes a decrease in the retention time (Sankalia et al., 2007)

Itraconazole has been chosen for IS because of its similar properties and chemical structure. It can be obtained in the same condition as sample preparation, chromatographic system, and detected using a UV detector at the same wavelength with KTZ. The IS demonstrates stable ionization efficiency and adequate and reproducible extraction recovery using the sample pretreatment method in this study (Gu et al., 2016). The retention time of KTZ and IS were eluted at about 5 and 11 min, respectively. The resolution between KTZ and IS has been good.

Validation method

Acetonitrile was used for protein precipitation to obtain satisfactory values for recovery KTZ and IS. The increase in Acetonitrile led to an improvement in extraction recovery. However, diluted samples with

extensive acetonitrile will reduce sensitivity (You et al., 2005). In this study, the optimal ratio of plasma sample to acetonitrile is 1:2. The chromatography system condition shows the assay's ability to separate the KTZ and the IS from the plasma sample without interference from any endogenous compounds.

The HPLC system was optimized to demonstrate the system's suitability, including the retention time, tailing factor, number of plates (N), retention factor (k'), and peak area (%RSD) (Table 1). In the system suitability test, the %RSD result of retention time was less than 2%, and the peak area was less than 20%, indicating that the system is suitable for analysis. Ketoconazole separated well from the front solvent and formed a symmetrical peak with a tailing factor of 1.38 (good if 2). The number of theoretical plates (N) at 7813 indicates that the criteria of N (>2000) are acceptable. The retention factor (k') is found to be 514. It meets the requirements that should be > 2.

Table 1. System suitability test result

System parameters	Acceptance criteria	Result
Retention time (min)	RSD < 2%	5.15±0.02 RSD: 0.32%
The ratio of Peak area	RSD < 20%	0.24±0.009 RSD: 4.09%
Tailing factor	< 2	1.38±1.37
Theoretical plates	>2000	7813±116
Retention factor (k')	>2	514±1.37

Selectivity has been evaluated by blank plasma, standard internal (IS), and a spiked plasma standard (4 µm/ml) containing 25 µm/ml IS and standard internal (Figure 1). The ketoconazole retention time was found to be 5.15 minutes. The peak of ketoconazole can separate from other peaks and has no interference from the diluent, indicating this method was selective to detect ketoconazole.

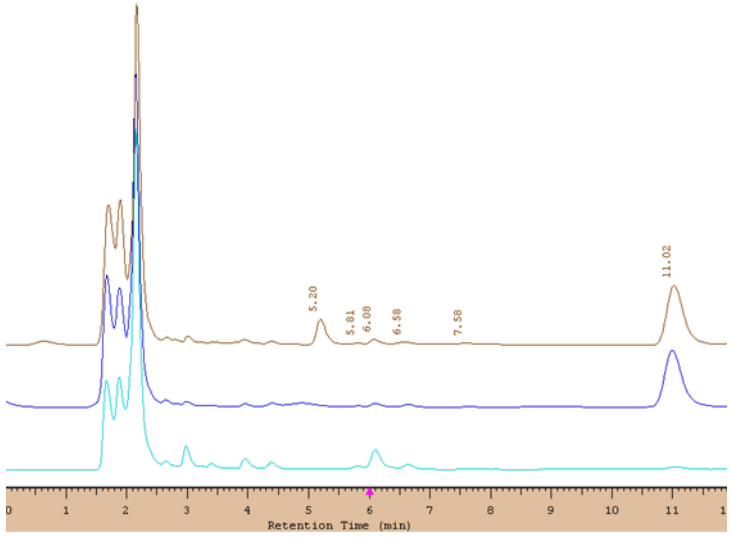

Figure 1. Specificity chromatogram of blank plasma (A), Internal Standard (IS) (B), and ketoconazole spiked plasma standard (4 µm/ml) containing 25 µm/ml IS (C), obtained for ketoconazole with mobile phase used NaH_2PO_4 pH 7:Acetonitrile (30:70)

C

Figure 2 depicts the standard calibration curve with concentration ranges of 0.05 to 8 µm. The intercept (a), slope (b), and coefficient (r2) were used to evaluate linearity. The method's capacity to proportionate correlation concentration in the sample is known as linearity. Sample concentration was linearly correlated with calibration curves. All chemicals' coefficients of determination (R2) were 0.9969, indicating that the approach has a solid linear relationship between peak area and concentration.

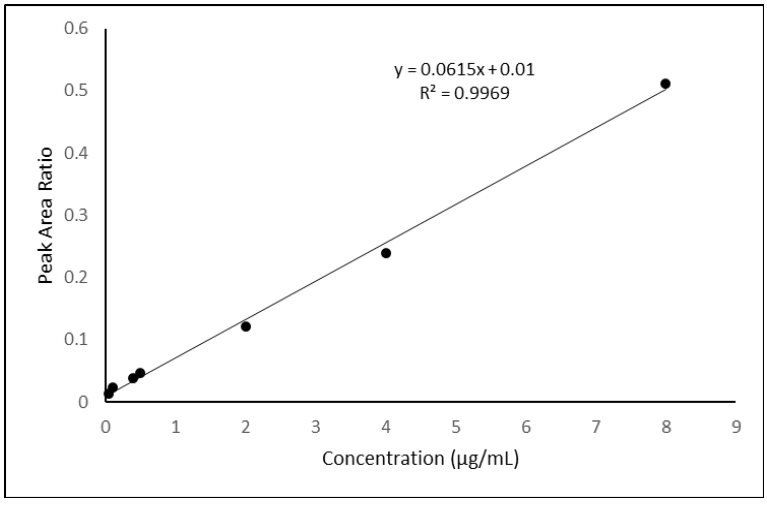

Figure 2. Calibration curve of with mobile phase used NaH_2PO_4 pH 7.0:Acetonitrile (30:70)

The evaluation of accuracy (Table 2) and precision (Table 3) used the standard addition method to calculate the %diff and %RSD for four concentration levels. There were LLOQ, Low, Medium, and High, with three replicates from each concentration. The result of LLOQ was 9.47 for %diff and 12.08 for %RSD. Other levels' results were <3.94 for %diff and 13.56 for %RSD. These values are within the acceptable range, indicating that the method is accurate and precise.

Table 2. Accuracy data of the proposed method

Level	Cons. standard (µg/mL)	Cons. detected (µg/mL)	%Diff
LLOQ	0.05	0.055±0.01	9.47
Low	0.4	0.402±0.05	0.59
Medium	4	3.842±0.12	3.94
High	8	8.208±0.15	2.59

Table 3. Precision data of the proposed method

Level	Cons. standard (µg/mL)	Intraday RSD(%)	Interday RSD (%)
LLOQ	0.05	12.08	10.13
Low	0.4	11.21	13.56
Medium	4	3.07	7.91
High	8	1.82	2.39

The stability of KTZ in different storage conditions was summarized in Table 4, which indicated that KTZ was stable for three days and kept in a freezer (-20^{0}C) without changes in the concentrations tested.

Table 4. Stability data of the proposed method

Condition	Cons. observed ± SD (n=3)	Diff (%)	RSD(%)
Freshly	8.177±0.64	2.21	7.83
Frezeer day 1	8.322±0.79	4.02	9.53
Frezeer day 3	8.240±0.84	3.00	10.26

Pharmacokinetic application

The procedure described above was used to measure the plasma concentration of KTZ following an oral dosage experiment on three rabbits. Chromatogram of ketoconazole in rabbit plasma after oral administration showed in Figure 3.

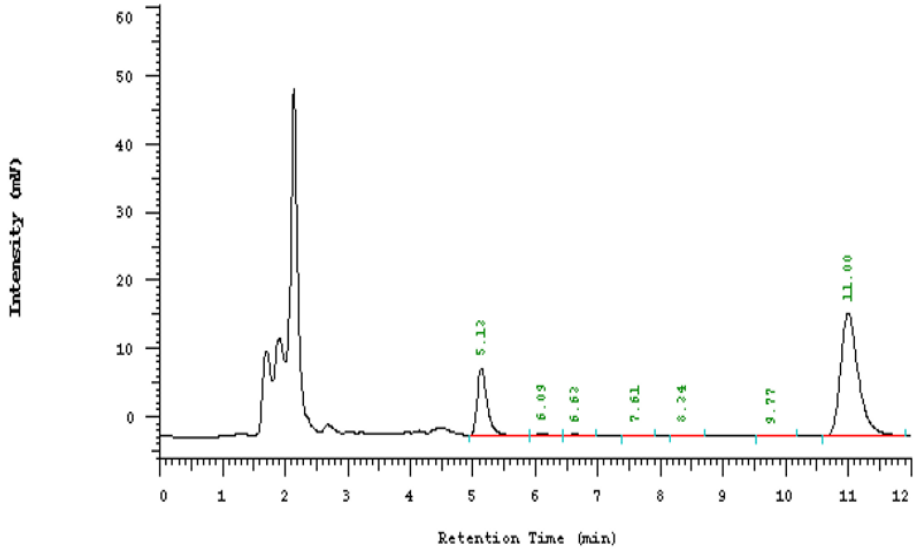

Figure 3. Chromatogram of ketoconazole in rabbit plasma after oral administration

Figure 4 shows three individual pharmacokinetic profiles of ketoconazole in rabbit plasma after oral administration of powder KTZ. Biological variations in the animal cause the difference in the data of the three replications.

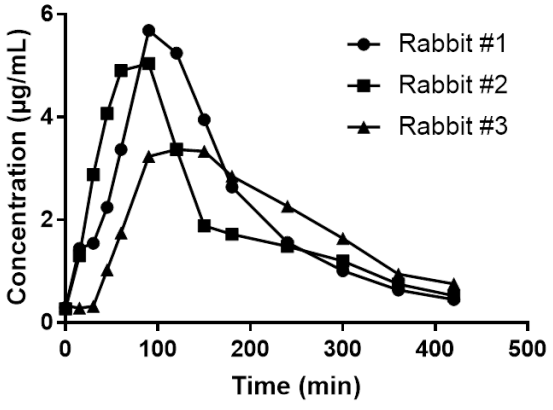

Figure 4. Pharmacokinetic profiles of ketoconazole in rabbit plasma after oral administration

Table 5 presents the corresponding individual pharmacokinetic parameters. The PK parameters were calculated using one-compartmental analysis by computing the particular value of the concentration at each time point. After formulation administration, a peak concentration of 3.74±0.67 μg/mL (C_{max}) was reached at 99.06±28.39 min (T_{max}). The peak concentration of the drug is defined as its highest concentration in blood plasma. It is one of the most important pharmacokinetic parameters for assessing how the medicine affects. The plasma levels may have different results based on dose, route of administration, ease of absorption, the distribution of the drug throughout the body, the bioavailability, and the effectiveness metabolized of the drug (Webb, 2011). The half-life was found to be 78.09±16.74. The half-life typically refers to eliminating the drug in plasma levels that fall to 50% of the initial dose. Different drugs have varying half-lives; the half-life value helps determine any particular drug's excretion rates and steady-state concentrations. The half-life of elimination can be used to predict the length of elimination of the drug. For the first order, the relationship between $t_{1/2}$ and elimination is after 3.3x half-lives, 90% of the given drug will be eliminated, and after 4-5x half-lives, elimination can be considered complete (Hallare & Gerriets, 2022). The area under plasma concentration ($AUC_{0-\infty}$) was 972±104. The AUC is commonly used to assess how much a drug reaches the bloodstream in each period following dose administration. The data can be used to evaluate pharmacodynamic responses (Scheff et al., 2011).

Table 5. Pharmacokinetic parameters of ketoconazole after oral administration

Pharmacokinetic parameter	Rabbit #1	Rabbit #2	Rabbit #3	Avarage±SD (n=3)	P value
C_{max} (μg/mL)	4.15	4.29	2.79	3.74±0.67	0.016
T_{max} (min)	93.73	67.26	136.19	99.06±28.39	0.039
$AUC_{0-\infty}$ (μg/mL*min)	1057	825	1034	972±104	0.006
$T_{1/2}$ (min)	66.98	65.54	101.76	78.09±16.74	0.022

The concentration of ketoconazole in plasma was obtained with a peak area ratio of ketoconazole to internal standard from calibration curves. The method had good linearity with R^2=0.9969, good selectivity, good precision within and interday (%RSD) for 4 level concentrations (LLOQ, low, medium, and high), good accuracy (%diff) for 4 level concentrations (LLOQ, low, medium, and high), and the plasma sample can be used for three days kept in the freezer (-20^{0}C) due to its stability. The LLOQ for this validated method is 0.05 μg/mL for plasma. It meets the criteria for detecting KTZ in rabbit plasma after oral dosing cause the lowest concentration obtained is 0.4 μg/mL. The *p*-value of the parameters is less than 0.05, indicating significant differences between the three replicates of rabbits. The differences in the results of pharmacokinetic data are primarily due to biological variations present in the animal.

CONCLUSION

The HPLC-UV method was successfully developed and comprehensively validated for application to the pharmacokinetic study of ketoconazole. Linearity, selectivity, sensitivity, accuracy, precision, recovery, and stability were measured using the HPLC-UV method. The calibration curve presented linearity in the 0.05-8 μg/ml with R^2=0.9969, which indicated good linearity. Intra-day and inter-day repeatability studies obtained the method's precision, and accuracy was examined by %diff. The result is <15% for LLOQ and <20% for low, medium, and high levels. Plasma stability under storage in a freezer (-20^{0}C) for three days was studied. As a result, the analysis method proposed in this work could prove to be a promising alternative for preclinical pharmacokinetic studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept (VA, AEN), Design (VA), Supervision (TNSS, AKN, AEN), Resources (VA, TNSS, AKN), Materials (AEN), Data Collection and/or Processing (VA), Analysis and/ or Interpretation (VA, TNSS, AKN), Literature Search (VA), Writing (VA), Critical Reviews (TNSS, AKN, AEN)

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