Validation of RP-HPLC method for quantification of ketoconazole in two-stage model of biorelevant dissolution: application to supersaturation study

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Received: 14 June 2023
Revised: 11 August 2023
Accepted: 18 August 2023
Published: 22 November 2023

Egyptian Pharmaceutical Journal 2023, 22:676–682

Background
Supersaturation may occur in weakly base drugs due to the physiological pH in the gastrointestinal (GI) tract cause their pH depends on solubility. The biorelevant medium used in this study were simulated gastric fluid (SGF) and Fasted State Simulated Intestinal Fluid (FaSSIF).

Aims
The *in vitro* evaluation of the supersaturation study measures the drug concentrations in solution as a function of time (concentration-time profiles). The method for analysis of concentration ketoconazole in supersaturation study has been validated.

Settings and design
A Two-Stage model of biorelevant dissolution is one of the methods to create the supersaturation condition. The method approach aims to simulate the condition of the drug in the gastrointestinal tract from gastric to intestine using a modification of the United States Pharmacopeia (USP) dissolution procedure and biorelevant medium.

Material and methods
The chromatographic system consisted of a reversed-phase C18 column (250×4.6 mm, 5 μm) at a flow rate of 1 ml/min, a detection wavelength of 232 nm, and a retention time of about 3 min for ketoconazole.

Results
The linearity of the calibration curves in the concentration range was good ($R^2 = 0.9995$). The method was accurate with recoveries in the 100-103% range and precise (% relative standard deviation [RSD] of intraday variation was 0.85-1.57 and 0.3-1.61). The result of LOD was 0.230 μg/ml, and LoQ was 0.698 μg/ml.

Conclusion
The proposed method was selective, accurate, precise, and sensitive. So the method can be used to analyze the concentration of ketoconazole in supersaturation conditions induced by pH-shift in medium biorelevant.

Keywords:
ketoconazole, precipitation, RP-HPLC, supersaturation, validation method, weak base drugs

Egypt Pharmaceut J 22:676–682
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1687-4315

Introduction

The supersaturation process is thermodynamically unstable and tends to precipitation. Supersaturation may occur in weakly base drugs due to the physiological pH in the gastrointestinal (GI) tract causing their pH-dependent solubility Tanaka and colleagues[1]. One of the weak base drugs researched to observe the supersaturation phenomenon is ketoconazole Ruff and colleagues, Kambayashi and colleagues, Kataoka and colleagues, Psachoulias and colleagues, Higashino and colleagues, Jede and colleagues, Auch and colleagues [2–8]. Ketoconazole is a weakly basic drug with pH-dependent dissolution and two pH values (both alkaline), which are 2.94 and 6.51 Vertzoni and colleagues [9]. It is not soluble in water and base pH but it has good solubility in acid solutions Zhou and colleagues [10]. The level and duration of supersaturation and the precipitation rate influence the bioavailability of ketoconazole Tsume and colleagues [11].

Two-Stage model biorelevant dissolution is one of the methods to create the supersaturation condition. The method approach aims to simulate the condition of the drug in the GI tract from the gastric to the intestine using a modification of the United States Pharmacopeia (USP) dissolution procedure. A peristaltic pump is used to transfer the solution from

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the donor compartment to the acceptor. The selection of a dissolving medium has a significant impact on supersaturation results. Common media do not represent the pH of the stomach and intestine, e.g., buffer and HCl, due to the lack of similarity in media composition Annisa and colleagues [12]. The FaSSIF (Fast state simulated intestinal fluid) media is commonly used to simulate an alkaline condition in the small intestine. This medium was prepared as lecithin as a bile salt and phospholipid representative Dressman and Reppas [13]. The SGF (Simulated gastric fluid) media is used to simulate an acidic condition in the stomach. This medium contains pepsin, bile salts, and lecithin Jantratid and colleagues [14].

The *in vitro* evaluation of supersaturation study measures the drug concentrations in solution as a function of time (concentration-time profiles). The quantitative *in vitro* assay to evaluate supersaturation is determined by measuring the drug concentration after supersaturation inducing using the spectrophotometric UV/Vis or High Performance Liquid Chromatography (HPLC) method Bevernage and colleagues [15]. The HPLC method has some advantages, including good sensitivity, reproducibility, and specificity Mendez and colleagues, Hurtado and colleagues [16,17]. The validation methods of ketoconazole analysis using HPLC that previous studies have carried out to determine the solubility of ketoconazole in buffer pH Annisa and colleagues [18], determine the stability of ketoconazole in tablets Tina and colleagues [19], determine the dissolution of ketoconazole tablets in gastric fluid Shah and colleagues [20], detect the concentration of ketoconazole in pharmaceutical dosage forms, such as tablets, capsule, and topical dosage form Popovska and colleagues, Lima and colleagues, Amrutiya and colleagues, Martin and colleagues [21–24]. The validation method for determining the concentration of ketoconazole in a supersaturation study has not been found yet. The previous research articles only discuss the HPLC system used to analyze ketoconazole's precipitation profile, not the validation of the method Ruff and colleagues, Kambayashi and colleagues, Auch and colleagues, Annisa and colleagues, Tina and colleagues, Shah and colleagues, Popovska and colleagues, Lima and colleagues [2,3,8,18–22].

Therefore, this study aimed to validate the HPLC method with UV detection for the determination of ketoconazole in supersaturation study with a small-scale transfer two-stage model from the gastric compartment that used SGF to an intestine compartment that used FaSSIF.

Materials and methods

Materials

Materials for HPLC: Standard of ketoconazole was purchased from BPOM, Indonesia. Ketoconazole was purchased from PT. Kimia Farma, Indonesia. Water for injection (sterile) was manufactured by PT. Ikapharmindo, Indonesia. Deionized water was supplied by CV. Alfa Kimia. Acetonitrile gradient grades for HPLC, TEA and orthophosphoric acid were manufactured by Merck, Germany. Materials for biorelevant medium as FaSSIF: Lecithin soya (manufactured by Himedia, India), sodium taurocholate (manufactured by Himedia, India), NaCl, NaH_2PO_4, KH_2PO_4, NaOH (manufactured by Merck, Germany) and deionized water (supplied by CV. Alfa Kimia) and materials for biorelevant medium as SGF: pepsin (manufactured by Himedia, India), HCl (manufactured by Merck, Germany) and deionized water (supplied by CV. Alfa Kimia). All other chemicals were of analytical reagent grade.

Chromatographic condition

The chromatography system consisted of an Elite LaChrom HPLC, a Hitachi UV-Vis detector L-2420, and a Hitachi pump L-2130. A Phenomenex Luna column (250×4.6 mm, 5 m) was employed. Acetonitrile: water with TEA 0.15% (50 : 50). The injection had a 20 µl volume. The detecting wavelength was 232 nm, and the flow rate was 1 ml/min. All samples were filtered using a 0.45 µg/ml nylon filter before analysis.

Preparation mobile phase

The mobile phase was prepared by a combination of acetonitrile: water with TEA 0.15% adjusted to pH 3.3 (50 : 50) using orthophosphoric acid. The mobile phase was filtered with a 0.45 µm membrane filter.

Preparation of stock solution

A 50,0 mg of ketoconazole was weighed and then diluted with 100 ml methanol for a 500 µg/ml concentration. The calibration curve was made from the stock solution that dilutes with acetonitrile to get a final concentration of 5-200 µg/ml.

Selectivity

The selectivity was evaluated by determining ketoconazole, degradation of ketoconazole, and impurities from base FaSSIF and acid SGF condition. The samples are ketoconazole standard, blank medium of supersaturation study, ketoconazole due to transfer from SGF to FaSSIF, ketoconazole in FaSSIF, and ketoconazole in SGF.

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System suitability test

6 replicates of 50 µg/ml were injected. The suitability test parameters are relative standard deviation (% relative standard deviation [RSD]) of retention time, % RSD peak area, theoretical plates, and tailing factor. The % RSD value should be less than 2%, the tailing factor should be less than 2, and the theoretical plates of the column (N) should be greater than or equal to 2000.

Linearity

The six series of standard solution with concentrations of 5, 10, 50, 100, 150, and 200 µg/ml were prepared as follows: from standard stock solution, appropriate aliquots 0.05, 0.1, 0.5, 1.0, 1.5, and 2.0 ml were pipette out to 5 ml volumetric flasks and diluted by acetonitrile. The slope, intercept, and correlation coefficient were determined by plotting concentrations (x) and peak area (y).

Precision

The method's precision was evaluated by running three concentrations standards at 50, 100, and 150 µg/ml to permit intraday and interday (3 separate days).

Accuracy

The standard addition method evaluated the accuracy using three levels (low, medium, and high) standard solution. The ketoconazole standard was mixed with sampling (1 : 1) and then vortexing. The recovery was assessed by comparing the ratio of the total amount of sample and the concentration sample without a standard solution.

LoD and LoQ

LoD and LoQ were found by successive dilution of standard solution (1, 5, 10, 15, 20, and 25 µg/ml). The LoD represents the smallest quantity of analyte in the sample that can be detected, whereas the LoQ represents the lowest amount of analyte. LoD and LoQ were calculated by the signal-to-noise ratio of 3 and 10 times, respectively.

In vitro supersaturation study of small scale-two stages transfer model

A two-stage model of biorelevant of dissolution with inducted by pH shift was carried out to investigate the supersaturation behavior of ketoconazole. Modifications to the original set-up were made for the small-scale *in vitro* transfer model. The medium volume was scaled down in both compartments from 250 ml for SGF (simulated gastric fluid) pH 2.0 and 500 ml for FaSSIF pH 6.5 to 125 ml and 200 ml, respectively. The paddle speed is 100 rpm, and the transfer rate is 5 ml/min with a peristaltic pump (customization by PT. Stechoq Robotika Indonesia, Indonesia). The scaled-down was done to the dose of ketoconazole that uses 100 mg. The 5 mL samples were collected from the donor compartment at 15, 20, 45, 60, 90, and 120 min.

Quantitative analysis

The excess solid of the sample was centrifuged by centrifugation at 80.000 rpm for 10 min Then the supernatant was diluted using acetonitrile 1 : 1. Before analysis, the analyte was filtered by a 0.45 µm membrane filter. The concentration in the supernatant was determined through analysis by the Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method.

Results and discussion

Optimization of chromatographic conditions

The chromatographic method was developed to detect the drug in a supersaturation study with pH-shift using biorelevant media. The chromatographic method was a reversed-phase mode that used the C-18 column (nonpolar) as a stationary phase due to high stability, reproducibility, and wide availability Soliman and colleagues [25]. Ketoconazole is a weakly basic drug with pKa 2.94 and 6.51, the mobile phase used in this study has succeeded in good peak symmetry and resolution with reducing tailing problems with acceptable system suitability test parameters. The optimized mobile phase was a mixture of acetonitrile and water with TEA 0.15% adjusted to pH 3.5 (50 : 50) at a 1 ml/min flow rate.

System suitability test

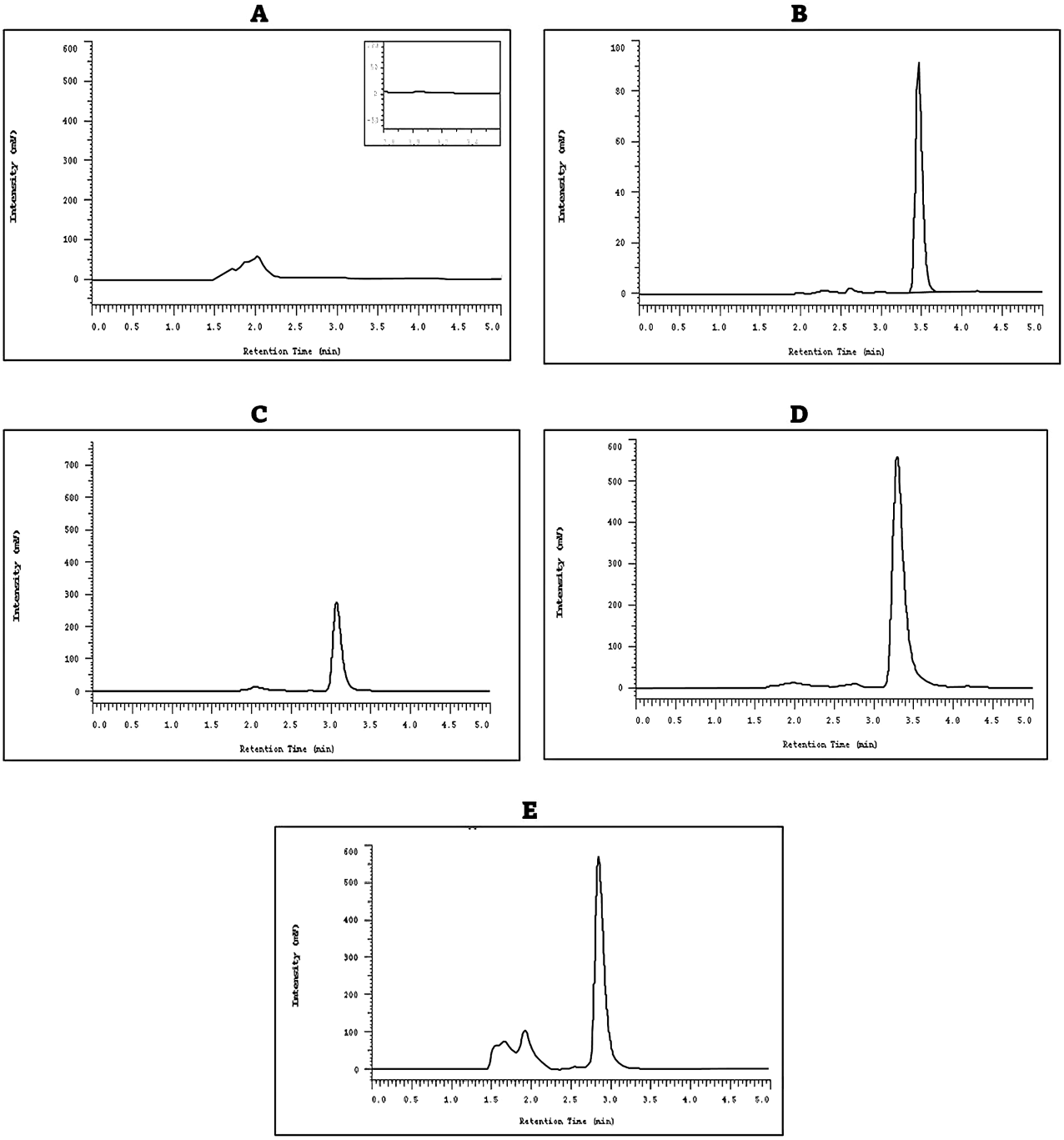
The HPLC system was optimized to demonstrate its suitability, and this included symmetry factor, number of plates (N), % RSD of retention time and peak area, and retention factor (k') (Table 1). The value of all parameters of the system suitability test met the acceptable criteria. There are % RSD of retention time and peak area less than 2%, tailing factor less than 2, N greater than 2000, and k' more than 2.

Table 1 System suitability test result of ketoconazole with mobile phase used acetonitrile: water with TEA 0.15% pH 3 : 3 (50 : 50)

System parameters	Acceptance criteria	Result
Retention time (min)	RSD <2	2.94±0.01 RSD: 0.25%
Peak area	RSD <2	2029278±15901 RSD: 0.78%
Tailing factor	<2	1.28±0.05
Theoretical plates	>2000	2222±107
Retention factor (k')	>2	293±0.69

*Mean of three determinations.

Figure 1



Selectivity chromatogram obtained for blank medium of supersaturation study (A), ketoconazole standard (B), ketoconazole as a result of transfer from SGF to FaSSIF (C), ketoconazole in FaSSIF (D), and ketoconazole in SGF (E) with mobile phase used acetonitrile: water with TEA 0.15% pH 3 : 3 (50 : 50).

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Selectivity

The separation of peak ketoconazole in the sample has no interference from the diluent. Therefore, this method was selective to detect ketoconazole (Fig. 1).

Linearity

The calibration curve was presented in Fig. 2 with the standard solution in the range of 5-200 µg/ml. The regression equation was calculated, and the result is y = 42947x-33086. The coefficients of determination (R^2) were 0.99, indicating that the technique has good linearity. The method's ability to proportionally provide correlation concentration in the sample is known as linearity. Concerning sample concentration, calibration curves were linear.

Accuracy

The recovery percentage in this method has been assessed using the standard addition method at three levels and three replicates from each concentration.

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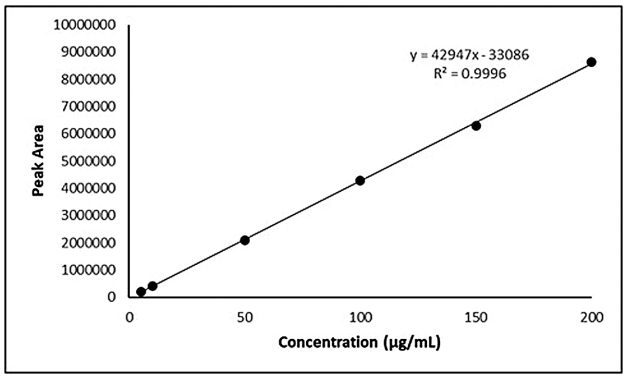
Figure 2



Calibration curve with mobile phase used acetonitrile: water with TEA 0.15% pH 3 : 3 (50 : 50).

Table 2 shows the recovery data result within the 100.7-103.2% range. According to the AOAC standard for the acceptable mean recovery, the recovery percentage for the concentration analyte at 100 g/ml was within the acceptable range of 90–107% [26].

Precision

The precision of intraday (Table 3) and interday (Table 4) was determined. The samples for intraday were evaluated on the same day at each QC level, and interday were studied for three days. The result of % RSD was in the range of 0.30-1.61%. This value indicates the method is good precision because it less than 2%.

Table 2 Recovery data of proposed method of ketoconazole with mobile phase used acetonitrile: water with TEA 0.15% pH 3 : 3 (50 : 50)

Level of recovery	Conc. spiked (ppm)	Total conc. found (ppm)*±SD (*n*=3)	Recovery (%)±SD
Low	49.7	50.53±1.49	101.7±2.99
Medium	107.3	108.04±2.68	100.7±2.49
High	142.4	147.06±1.67	103.2±1.17

*Mean of three determinations.

LoD and LoQ

The result of LOD for ketoconazole was 0.230, which could be reliably detected. The result of LoQ for ketoconazole was 0.698 µg/ml, representing the lowest concentration of the analytes that can be quantified with acceptable accuracy and precision Amrutiya and colleagues [23].

Supersaturation of ketoconazole in biorelevant media

As a weak base drug, Ketoconazole has the potency to be supersaturated by the pH shift method. The medium used in this study was SGF pH 2.0 and FaSSIF pH 6.5 to mimic the stomach and intestinal environment, respectively. Ketoconazole dissolves completely in acidic pH, when it is transferred to the basic compartment, the concentration decreases. It may be assumed that the concentration decreased due to precipitation occurring after the pH shift from the acidic compartment to the basic compartment. Two hours after the pH shift, ketoconazole almost approached its thermodynamic equilibrium solubility. The degree of supersaturation correlates with the precipitation rate. The supersaturation profile has been evaluated, as shown in Fig. 3.

Ketoconazole is a weak base drug whose solubility depends on pH, the dissolution of ketoconazole increases at low pHs, such as in the pH of the stomach (pH 1-3) [26]. Ketoconazole that transferred from the gastric to the intestinal compartment resulted in supersaturation and precipitation of crystalline drug *in vitro* Amrutiya

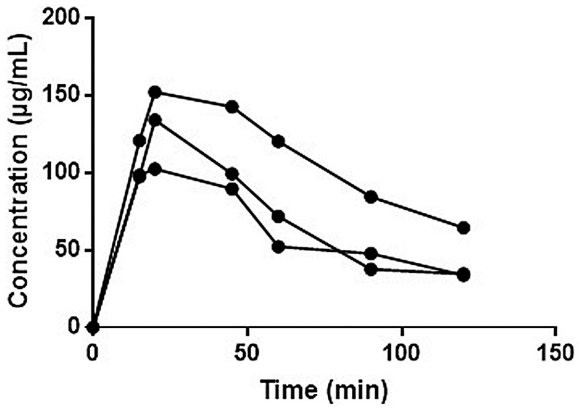
Table 3 Intraday precision data of the proposed method of ketoconazole with mobile phase used acetonitrile: water with TEA 0.15% pH 3 : 3 (50 : 50)

	Intraday (*n*=9)				
Conc. Stand. (ppm)	Rep 1	Rep 2	Rep 3	Mean±SD	%RSD
50	53.4	54.3	53.2	53.64±0.5	0.85
100	103.9	101.2	101.2	102.08±1.3	1.26
150	147.4	146.5	142.1	145.34±2.3	1.57

Table 4 Interday precision data of the proposed method of ketoconazole with mobile phase used acetonitrile: water with TEA 0.15% pH 3 : 3 (50 : 50)

	Interday (*n*=3)				
Conc. Stand. (ppm)	Day 1	Day 2	Day 3	Mean±SD	% RSD
50	55.3	55.8	56.0	55.70±0.29	0.53
100	93.7	90.9	94.3	92.95±1.49	1.61
150	163.2	163.8	162.6	163.17±0.49	0.30

Figure 3



Supersaturation profile of ketoconazole by the pH shift method detected by validated analytical method.

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and colleagues [23]. This phenomenon affects the number of drugs absorbed, so their bioavailability can decrease Sousa and colleagues [27]. The dissolution of ketoconazole in gastric solution has an essential role in the absorption of ketoconazole Roos and Lau-Cam [28].

The choice of testing media plays an important role in the test results. This biorelevant media resulted in *in vitro* and *in vivo* correlations closer than buffers [29]. Commonly used media such as buffers do not represent gastric or intestinal pH because they are not biorelevant to the GI composition, such as osmolarity conditions, ionic strength, buffering capacity, viscosity, bile and pancreatic secretions, and surface tension.

Tsume and colleagues has been studied about analysis of ketoconazole for prediction dissolution. The concentration analysis used the RP-HPLC system, the mobile phase was 0.15% TFA dan acetonitrile with 0.1% TFA (50 : 50), flow rate 2 ml/min during a 14 min, and the detection wavelength was 254 nm Tsume and colleagues [30]. The running time is longer than ours, so Tsume's method is less effective than ours.

Kambayashi and colleagues have studied the analysis precipitation profile of ketoconazole. The concentration analysis used the RP-HPLC system, the mobile phase was acetonitrile: phosphate buffer pH 6.9 (50:50), the flow rate at 1.00 ml/min, with a detection wavelength of 240 nm Kambayashi and colleagues, Mendez and colleagues [3,16]. Using basic pH for mobile phase is not recommended because it can cause the tailing result. In this study, the validation method used the mobile phase with pH adjuster up to 3.2 in order to reduce the number of silanophilic contacts. The basic groups of both ketoconazole and free silanol will be protonated in acid pH Sousa and colleagues [27]. TEA has been employed as a competitive base for masking accessible surface silanol groups due to its ability to decrease or completely remove silanophilic contact Roos and Lau-Cam [28].

In this study, the validation system used is inspired by Taupitz's, *et al.* study due to the similarity of condition and method of supersaturation study. Taupitz *et al.* did not validate the method, so we can not compare the validation result with our research. However, the gastric medium used in Taupitz, *et al.* study and our study differed. In this study, the simulated gastric fluid solution used pepsin and the volume was kept at 100 ml according to standard conditions (small-scale). In contrast, Taupitz, *et al.* used SGF without pepsin and used a lower volume of 40 ml. In this study, the final pH was still 6.5 because the FaSSIF we made was a double capacity buffer and the SGF in pH 2.0 so that the final pH at the intestine compartment did not drop drastically.

All parameters of the validation method, such as selectivity, precision, accuracy, and sensitivity, meet the requirements according to ICH guidelines. Therefore, the proposed validated method was successfully applied to determine the amount of ketoconazole in the supersaturation study.

Conclusion

Ketoconazole was determined using this HPLC-UV method with the optimized isocratic separation method. According to ICH requirements, all parameters meet the acceptable criteria: linearity, precision, accuracy, selective, and sensitivity. The calibration curves' linearity within the concentration range was good (R^2=0.9995). The approach was precise (%RSD of intraday variance was 0.85-1.57 and 0.3-1.61), with recoveries in the 100-103% range. The LOD value was 0.230 µg/ml while the LoQ value was 0.698 µg/ml. The suggested approach was sensitive, accurate, precise, and selective. As a result, the technique can be utilized to determine the concentration of ketoconazole under supersaturation conditions brought on by a biorelevant medium's pH shift.

Acknowledgements

The authors would like to acknowledge for financial support by Grant Programme of the Faculty of Pharmacy Universitas Gadjah Mada and the State

Ministry of Research and Technology under the Master Program of Education Leading to Doctoral Degree for Excellent Graduates (PMDSU) program, Indonesia. The authors would like to acknowledge too for the peristaltic pump instrument support by PT. Stechoq Robotika Indonesia.

Financial support and sponsorship
Nil.

Conflicts of interest
The authors declare that they have no conflict of interest.

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