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Validation of RP-HPLC method for determination of pH-dependent solubility of ketoconazole in phosphate buffer pH 6.8

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ABSTRACT: The solubility of ketoconazole is highly dependent on the pH of the medium. Solubility is an important physicochemical parameter needed to consider and predict to obtain the drug behavior in a physiological environment. Determination of solubility of ketoconazole in pH of intestine fluid (6-7.4) is not easy due to ketoconazole poor soluble in intestine fluid pH. The aim of this study was to validate an assay for the solubility of ketoconazole in phosphate buffer pH 6.8 using the RP-HPLC method. In addition to representing intestinal pH, phosphate buffer pH 6.8 is also generally used as an alkaline medium for the dissolution testing of several drugs. The method to analyze the solubility of ketoconazole has been developed and validated. The method was used an isocratic Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) using Phenomenex Luna (250 x 4.6 mm, 5 µm) column with a mobile phase consisting of acetonitrile: water with TEA 0.15% pH 3.3 in ratio 50:50, at the flow rate of 1 mL/min and UV detection at wavelength 232 nm. The correlation coefficients were obtained at r^2=0.9997 in the range of 0.5-12 µg/mL. The recovery % ranged within 100-101%. The intra and interday RSD% were less than 1.74%. LoD was 0.15 µg/mL and LoQ was 0.45 µg/mL. According to ICH guidelines, the method was validated for selectivity, linearity, accuracy, precision, LoQ, and LoD. The proposed method is suitable for determining the solubility of ketoconazole in phosphate buffer pH 6.8.

KEYWORDS: Solubility determination; ketoconazole analysis; HPLC; method validation; phosphate buffer; shake flask

1. INTRODUCTION

Ketoconazole (Figure 1) is an imidazole derivate indicated as an antifungal treatment [1]. According to the biopharmaceutics classification system, ketoconazole is classified as a Class II drug with low solubility and high permeability [2]. It has lower solubility at neutral pH and is easily soluble in water at acidic pH [3].

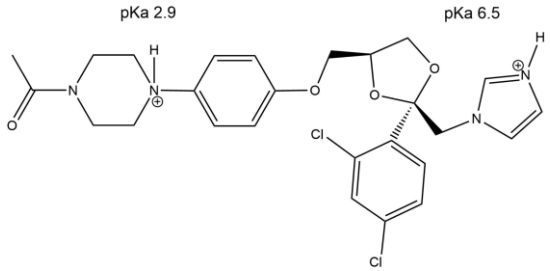

Figure 1. Ketoconazole Structure [40]

Ketoconazole has two pKa values, which are 2.94 from imidazole groups and 6.51 from piperazine groups [4]. Ketoconazole is a weakly basic drug. Its solubility is highly dependent on the pH of the medium. The aqueous environment of the gastrointestinal tract in the small intestine to the terminal ileum gradually has a pH value range of 6-7.4 [5]. The solubility of ketoconazole will decrease in this condition [5]. Solubility

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is an important physicochemical parameter to consider and predict to obtain the drug behavior in a physiology environment (in vivo studies) [6,7].

The quantification of ketoconazole can be determined by reversed-phase high-performance liquid chromatography (RP-HPLC) [6,8,9], spectrophotometry [10–13], colorimetry [14], voltammetric [15], and electrooxidation [11]. The RP-HPLC has been used to assay ketoconazole in pharmaceutical drugs [16]. The advantage of HPLC method is good sensitivity, reproducibility, and specificity [17,18]. The study about method validation for assay solubility of ketoconazole in basic pH as same as pH of the small intestine lacks' information. In many reports, we only found some studies about the method of analytical HPLC or only data of solubility without a method to obtain the result. Determination of ketoconazole's solubility in the intestine fluid's pH is not easy due to ketoconazole poor soluble in basic pH.

Soderlind, et al have been studied to determine the solubility of ketoconazole in a medium like a phosphate buffer pH 6.5, FaSSIF-1, FaSSIF-2, and HIF that used mobile phase contained water and methanol [19]. The analysis of base compounds by RP-HPLC has a problem due to poor peak shapes and acid-base interaction with free silanol groups (silanophilic interaction) on the C18 column. The silanophilic interaction is overcome by acidification of the mobile phase to decrease the ionization of free silanol groups [20].

The solubility study of ketoconazole at pH 6.8 is not reported yet. Several studies about the dissolution of ketoconazole used phosphate buffer pH as a dissolution medium [21]. The dissolution profiles have similar tendencies as the pH solubility profiles. However, the result of the dissolution study indicated a kinetic solubility, not equilibrium solubility [22]. Fung, et al studied the dissolution of ketoconazole powder that used USP type II apparatus with 500 mL phosphate buffer pH 6.8 [23]. Ullrich, et al studied the influence of polymer excipients on the dissolution of ketoconazole according to European Pharmacopeia conditions that used phosphate buffer pH 6.8 as the dissolution medium [24]. Kumar, et al studied the dissolution of solid dispersions of ketoconazole that used USP XXII apparatus with 900 mL phosphate buffer pH 6.8 [25].

The basic method for measuring solubility has remained the classical saturation shake-flask method. Shake-flask technique is based on simple procedures, but it is time-consuming and requires lots of manual work. However, there is no accepted standard to carry out this method.

The aim of this study was to validate an assay for the solubility of ketoconazole in phosphate buffer pH 6.8 using RP-HPLC.

2. RESULT AND DISCUSSION

2.1. Method validation

2.1.1 Chromatographic system

The chromatographic system that has been used in this study was reversed-phase (RP) HPLC. The RP mode is used most frequently in currently HPLC practice. In the RP-HPLC method, the stationary phase is non-polar (usually an alkyl-silica type bonded phase), and the mobile phase is a polar solvent [26]. Most of the silica-based columns (C18 columns) have residual silanols on the surface that are an acidic character. These silanol groups are generally strongly retained with basic compounds by ion-exchange electrostatic interaction or hydrogen bonding at a higher mobile phase pH [26]. The ionized surface silanols (SiO^-) will interact with positively ionized amine base groups (NH_2^+) from ketoconazole, then causing tailing and peak asymmetry [27]. Using an acidic mobile phase can decrease silanophilic interactions because both basic groups of ketoconazole and free silanol are protonated so that they are less able to interact with each other [28]. The mobile phase used was acetonitrile: water with TEA 0.15% and adjusted using orthophosphoric acid to get a final pH of 3.3 (50:50). The pH of the mobile phase has a significant influence on the retention time for an acid-based drug. The measurement of the pH mobile phase ensures that the analyte is in the desired acid-base form. However, an accurate measure of the pH of the mobile phase is not needed, but the most important is an appropriate buffer capacity after dilution with the organic modifier. TEA has been used as a competing base for masking accessible surface silanol groups, which is TEA can reduce or eliminate silanophilic interaction [29]. The overall results of the method validation parameters are represented in Table 1.

Table 1. Validation parameters for determination solubility of ketoconazole in phosphate buffer pH 6.8

Parameters	Values
Linearity (r^2)	0.9997
Calibration Equation	y=45687-1521.5
Accuracy (recovery %)	99±0.47 to 101±0.86
Precision (RSD%)	Intraday: 3.87±0.04 to 12.09±0.13
	Interday : 3.87±0.01 - 12.05±0.08
LoD (µg/mL)	0.15
LoQ (µg/mL)	0.45

2.1.2 *System suitability test*

HPLC system was optimized to demonstrate the suitability system, including symmetry factor, retention factor, number of plates, and peak area (RSD%) (Table 2). The RSD% result in the system suitability test was found within 2%, indicating the system is suitable for analysis. Ketoconazole formed a symmetrical peak with a tailing factor < 2 and adequately separated from the solvent front. Determination can be performed rapidly at the retention time observed (3.56 min), with minimum solvent consumption (cost-effectively). Number of theoretical plates (N) at 7919 that indicate acceptable with criteria of N (>2000).

Table 2. System Suitability test result

System suitability parameters	Result±SD	RSD
Retention time (min)	3.56±0.05	0.31
Peak area	366093±1411.88	0.38
Theoretical plates	7918±52.79	0.66
Tailing factor	1.29±0.02	1.67

2.1.3 *Specificity*

Specificity has been evaluated by comparing ketoconazole in phosphate buffer pH 6.8 solution (Figure 2). The retention time of ketoconazole was found at 3.77 min. The peak of ketoconazole has the capability to separate from the solvent peak, which means this method was specific to detecting ketoconazole.

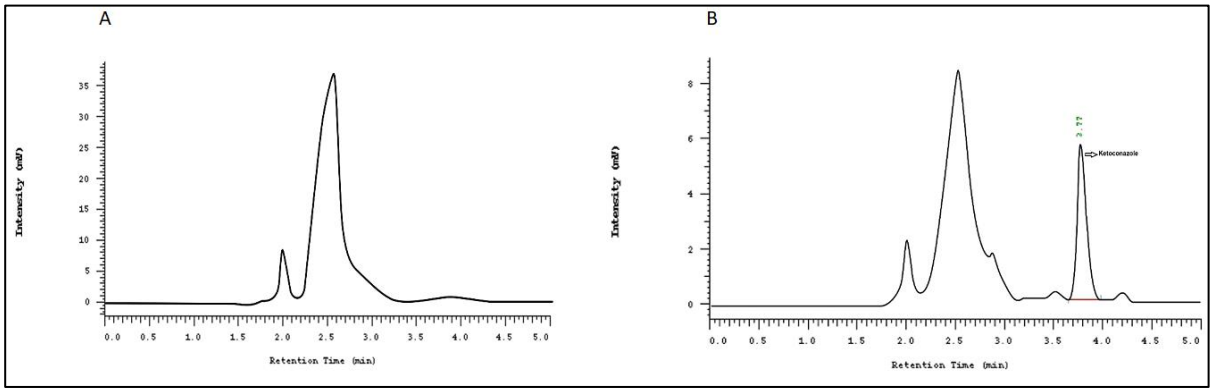

Figure 2. Method specificity chromatogram obtained for sample with mobile phase used acetonitrile: water with TEA 0.15% pH 3.3 (50:50). a) Phosphate buffer pH 6.8 as baseline, and b) Sample ketoconazole in phosphate buffer pH 6.8.

2.1.4 *Linearity*

The definition of linearity in ICH Q2 is the ability to generate a result directly proportional to the concentration of the analyte in the sample. Linearity can be determined by examining a plot of concentration series with a criteria minimum of six series concentrations and should be run minimum in triplicate [30]. The result of the calibration curve should be proportional between concentration and area under peak [31].

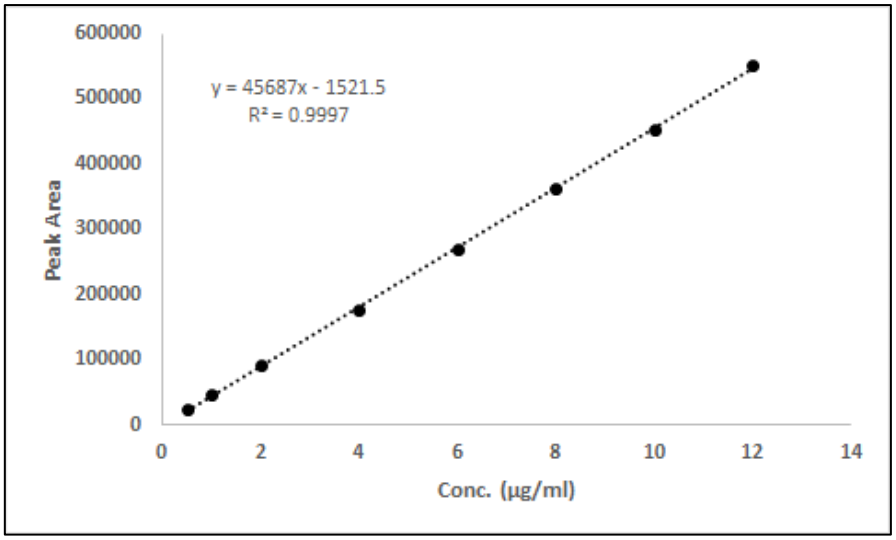

Figure 3. Calibration curve of ketoconazole used RP-HPLC method with mobile phase used acetonitrile: water with TEA 0.15% pH 3.3 (50:50)

The calibration curve was shown in Figure 3. using standard calibration in concentration 0.5-12 µg/mL, which is presented in Table 3. Evaluation of linearity was determined by intercept (a), slope (b), and coefficient (r^2). The linearity is the ability of the method to give correlation concentration proportionally in the sample. Calibration curves were linear with sample concentration. The coefficients of determination (r^2) of all compounds were >0.9995, indicating good linearity between the peak area and the concentration.

Table 3. Standard calibration of ketoconazole in phosphate buffer pH 6.8

Sample	Concentration (µg/mL) (n=24)								Slope	Intercept	r^2
	0.5	1	2	4	6	8	10	12			
	Area Under Peak										
Mean	23689	45653	92214	175088	270815	362571	454448	550727	45687	-1521.5	0.9997
SD	1976	2166	1984	2077	4638	6331	5708	6087			
RSD%	8.34	4.74	2.15	1.19	1.71	1.75	1.26	1.10			

2.1.5 Accuracy

The trueness of an analytical method is defined as the degree of agreement of the test result between the actual value obtained and with true value or an accepted reference [32]. The standard addition method has been used to evaluate recovery % in this method at 3 levels ((80%, 100%, and 120%) with 3 replicates from each concentration. Results within the range of 100-101% are presented in Table 4. The recovery % was acceptable within 80-110% for 10 µg/mL analyte concentration according to the AOAC standard of the acceptable mean recovery % [33].

Table 4. Recovery data for ketoconazole in phosphate buffer pH 6.8

Accuracy (n=9)			
Level of recovery	**Conc. standard (µg/mL)**	**Recovery%±SD**	**RSD%**
80%	8	99±0.47	0.47
100%	10	101±0.86	0.85
120%	12	99±1.19	1.20

2.1.6 Precision

Precision is the degree of agreement among individual results between measurements obtained from sampling replication of the same homogeneous sample [34]. It should be measured by the scatter of individual results from the mean as expressed as the relative standard deviation (RSD)% [32].

The precision of the method for intraday and inter-day analysis was determined. For intraday, the analyzed samples at each QC level were assessed on the same day, whereas for inter-day, samples were analyzed on three consecutive days. The intraday and interday precision study results are summarized in Table 5. The relative standard deviation (RSD%) value was less than 2% for intraday and interday, indicating good precision. The statistical analysis of the mean interday precision was conducted using a one-way ANOVA test. There is no significant difference in the concentration over three days.

Table 5. Precision data for ketoconazole in phosphate buffer pH 6.8

	Precision			
Conc. standard (µg/mL)	**Intraday ±SD (n=9)**	**RSD%**	**Interday ±SD (n=9)**	**RSD%**
4	3.86±0.04	1.18	3.87±0.01*	0.06
8	7.97±0.14	1.74	7.93±0.04*	0.48
12	12.09±0.13	1.10	12.05±0.08*	0.64

* Statistically no significant different according to the ANOVA test (p>0.05)

2.1.7 LoD and LoQ

The LoD and LoQ were determined to obtain the sensitivity of the developed method. The LoD refers to the smallest amount of analyte in a sample. The detection limit in chromatography is the injected sample that results in at least two or three times as high as the baseline noise level at a peak height. The LoQ refers to the minimum injected amount that provides satisfactory accuracy and precision measurement. The precision will get less than 10-15% RSD if the peak heights are 10-20 times higher than baseline noise [32,35].

LOD for ketoconazole was 0.15 µg/mL, which could be reliably detected. LoQ for ketoconazole was 0.45 µg/mL, representing the lowest concentration of the analytes that can be quantified with acceptable accuracy and precision [36].

2.2. Solubility determination

The solubility of ketoconazole was determined using phosphate buffer pH 6.8 after shaking using a shaker water bath. The average result was 1.842±0.04 µg/mL, and RSD% was 2.23% (Table 6). However, the RSD% value is still acceptable due to the requirement for concentration analyte with 10 µg/mL is ≤7.3%, according to the AOAC standard of the acceptable precision [33].

Table 6. Determination of sample concentration

Sample	**Peak Area**	**Cons. (µg/mL) x dilution factor**	**Mean±SD**	**RSD%**
1	39331	1.79		
2	40713	1.85	1.84±0.04	2.23
3	41616	1.89		

Solubility at a particular pH is defined as the mass balance sum of the concentration of all the species dissolved in the aqueous phase [37]. The pKa value representing the ketoconazole is 6.5 than 2.9 because the basic character from the imidazole ring is stronger than the piperazine group [38]. Ketoconazole is all ionized at pH 1-4 and will continue to decrease according to the increase in pH. Several studies have been reported about the solubility of ketoconazole in base pH. Solubility in phosphate buffer pH 6.5 is 6.9 µg/mL [4] whereas at water or pH 7 is 0.29 µg/mL [6]. In this study, the solubility of ketoconazole in phosphate buffer pH 6.8 was 1.8 µg/mL that close to solubility in water (pH 7) instead of phosphate buffer pH 6.5. These results are directly proportional to the percentage of ionization of ketoconazole (based on Henderson-Hasselbalch equations) at pH 6.5, 6.8, and 7, which are 50%, 33%, and 24%, respectively. That is because pH's effect on ionizable solubility is extensive [39].

The solubility values can be explained by the effect of particle size, crystallinity, other molecular features of the sample, or the differences in the applied experimental conditions. The applied experimental conditions have a significant effect on the variation result of solubility, including stirring time, sedimentation time, the composition of the aqueous buffer, temperature, amount of solid excess, and the phase-separation technique [39].

3. CONCLUSION

The HPLC-UV method with the optimized isocratic separation method was successfully used to determine ketoconazole in phosphate buffer pH 6.8 solution using a reverse-phase column. The method was validated in accordance with ICH guidelines for solubility studies, which require a linear, accurate, precise, specific, and sensitive measurement. This procedure was proven to be rapid, easy, highly sensitive, reliable, and effective.

4. MATERIALS AND METHODS

4.1. Materials

Standard of ketoconazole was purchased from BPOM/The National Agency of Drug and Food Control of Indonesia as Farmakope Indonesia Chemical Reference Substances (purity 100.10%), the active ingredient of ketoconazole was purchased from PT. Kimia Farma, Indonesia, water for injection (sterile) was manufactured by PT. Ikapharmindo, Indonesia, acetonitrile gradient grade for HPLC was manufactured by Merck, Germany. Buffer materials (potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH) and all other chemicals (methanol, TEA, orthophosphoric acid) were of analytical reagent grade and manufactured by Merck, Germany.

4.2. Method validation

4.2.1 Chromatographic condition

The HPLC method was performed by Elite LaChrom HPLC, Hitachi UV-Vis detector L-2420, Hitachi pump L-2130. The column used was Phenomenex Luna (250 x 4.6 mm, 5 µm). The mobile phase used was acetonitrile: water with TEA 0.15% and adjusted using orthophosphoric acid to get final pH of 3.3 (50:50). The injection volume was 20 µL. The flow rate was set to 1 mL/min, and detection was at 232 nm.

4.2.2 Preparation mobile phase

The mobile phase composition was acetonitrile: water with TEA 0.15% and adjusted using orthophosphoric acid to get final pH of 3.3 (50:50). The cellulose 0.45 µm membrane filter has been used to filter the mobile phase.

4.2.3 System suitability test

Six replicates were injected to assess the system suitability test at 8 µg/mL. The result included theoretical plates, retention time, peak area, and tailing factor. The relative standard deviation (RSD%) should be less than 2%, the tailing factor should be ≤ 2, and theoretical plates of the column (N) should be≥2000 to be considered acceptable.

4.2.4 Preparation of stock solution

The ketoconazole stock solution was prepared by accurately weighing 50,0 mg of ketoconazole and then diluting it in 50 mL methanol to obtain the final concentration of 1000 µg/mL. Then, it was diluted to a final concentration of 100 ppm again in a 50 mL volumetric flask using the mobile phase. The mobile phase was used to dilute the stock solution to obtain calibration solution in a concentration between 1-20 µg/mL.

4.2.5 Linearity

Linearity was performed by 6 series of standard solution with concentration 0.5, 1, 2, 4, 6, 8, 10, and 12 µg/mL as follows: from standard stock solution, appropriate aliquots 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mL were pipette out tp 5 mL volumetric flasks and diluted by mobile phase. The measurements were determined in triplicate (n=24). All values are reported as means ±standard deviation. The calibration curve plotting was plotted using concentration (x) and peak area (y). The result of linear regression was used to obtain the intercept, slope, and coefficient of correlation.

4.2.6 Accuracy

Accuracy was determined by the standard addition method at three levels (80%, 100%, and 120%). The standard addition method was prepared by adding ketoconazole standard at 8, 10, and 12 µg/mL concentrations to the sample. The recovery was carried out by calculating the total amount of sample ratio and the additional standard solution compared with the concentration sample without a standard solution.

***4.2.7** Precision*

Repeatability (intra-day) and intermediate precision (inter-day) were used to determine the precision test. In intraday, precision was assessed by analyzing 3 standard solutions at concentrations of 4, 8, and 12 µg/mL measured three replicates a day (n=9). In contrast, for interday, samples were examined for three days consecutively.

***4.2.8** Limit of detection (LoD) and quantification (LoQ)*

LoD and LoQ were found by successive dilution of standard solution (0.5, 1, 2, 4, 6, 8, 10, and 12 µg/mL). The LoD represents the smallest quantity of analyte in the sample that can be detected, defined as the analyte concentration that produces a signal equal to $y_b + 3.3_{s_b}$, where y_b is the blank signal and s_b is the standard deviation. The LoQ represents the lowest amount of analyte that can be quantitatively determined, which is defined $y_b + 10_{s_b}$.

4.3. Solubility studies

The shake flask method has been used to determine the equilibrium solubility of ketoconazole. In a 25 mL phosphate buffer pH 6.8 solution, an excess amount of ketoconazole (50,0 mg) was then shaken in a shaking water bath at 37^{0}C and 100 rpm. Aliquots were withdrawn at the end of the experiment (24 h) as equilibrium was reached. It was then filtered through 0.45 µm to avoid insoluble particles. Assays were performed in triplicate.

4.4. Statistical Analysis

The difference in precision interday were analyzed by one-way variance analysis (ANOVA) using IBM SPSS Statistics Version 24.

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