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Analytical Method Validation and Simultaneous Determination of Ketotifen Fumarate and Cyproheptadine Hydrochloride in Divided Powder by RP-HPLC-UV Method

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ABSTRACT

Ketotifen fumarate and cyproheptadine HCl on divided powder dosage form are the most popular prescription for children at "X" hospital in Yogyakarta. The interest for studying this divided powder was related to the quality test towards human health. If the drug doesn't meet the requirement of good quality drug, it can cause damage to the quality of life. Hence, it was important to develop a validated analytical method to quantify content of active drug from samples. The samples were prepared by a pharmacist at "X" hospital in Wonosari, Yogyakarta. Tablets from each compound were blended and divided by pulverizer into powder bags then were sealed. In this study, a reversed phase HPLC Ultraviolet (RP-HPLC-UV) method was optimized and validated then applied for determining the content of ketotifen fumarate and cyproheptadine HCl in divided powder. The optimal condition for HPLC separation with mobile phase of methanol were 0.04M Na_2HPO_4 buffer (60:40) and flow rate 0.8 mL/min. According to AOAC International 2019, the developed RP-HPLC-UV method was good and met the requirement of selectivity and sensitivity. The calibration curve formulas of ketotifen fumarate and cyproheptadine hydrochloride were y = 27714x-79111 (r=0.994) and y = 26324x+72581 (r=0.993) in the range of 10.9-54.5 and 10.6-53.0 µg/mL, respectively. Accuracy and intermediate precision were assessed on both standard solution and samples spiked with standard solution of ketotifen fumarate and cyproheptadine hydrochloride. It was found that both standard solution and the spiked samples solution met the requirement of recovery of 80-110% and RSD of less than 7.3% for intraday and interday analysis. Content of ketotifen fumarate and cyproheptadine hydrochloride in sample (n=7) were 16.574±0.098 and 30.640±0.035 µg/mL, respectively. It can be concluded that the RP-HPLC-UV method was successfully validated then applied in the determination of ketotifen fumarate and cyproheptadine HCl in divided powder.

Keywords: analytical**;** cyproheptadine hydrochloride; ketotifen fumarate; HPLC; validation

INTRODUCTION

Allergic diseases are the most common chronic conditions that affect the quality of patient's life (Kuna *et al*., 2016). Asthma is the most prevalent chronic respiratory disease worldwide. This disease affecting more than 300 million people of all ethnic groups at all ages (Soriano *et al*., 2017). It is the most common chronic disease especially in children, imposing an increasingly consistent burden on the health system (Asher & Pearce, 2014). The recent Global Asthma Study (GAN) phase reported that the global prevalence of asthma was 11% in children aged 6–7 years and 9.1% at 13–14 years (Marcos *et al*., 2022). Asthma has accounted for missed school days among children in Asia-Pacific (16–61%),

Europe (34–68%) and the United States (43%) and has been affected to lower academic achievement (Triasih *et al*., 2023). Antihistamines are the most commonly used in the therapy of allergies (Kuna *et al*., 2016). First and second generation of antihistamines providing effective relief of allergic conditions such as ketotifen fumarate and cyproheptadine hydrochloride (El-Kommos *et al*., 2015). Ketotifen fumarate has been administered to prevent allergic disease such as asthma and anaphylactic reaction (Moreno *et al*., 2020). Cyproheptadine has been used in chronic allergic and pruritic conditions (Chakraborty, 2019; Vardanyan, 2017). As the most popular prescription for children at "X" hospital in Yogyakarta, ketotifen fumarate and cyproheptadine hydrochloride were chosen to be analyzed in this study. It was stated in previous studies that the prescription of ketotifen fumarate and cyproheptadine hydrochloride from "X" hospital in Yogyakarta were frequently prescribed with the total amount of 87 from 291 prescription (the five most prescribed formula) (Yuliani *et al*., 2020). However, there were limited studies reported the analytical method development to ensure the quality of compounded drugs, especially divided powder. Hence, it is important to develop a validated analytical method to analyze ketotifen fumarate and cyproheptadine hydrochloride content in divided powder to ensure the quality and stability of this compounded drug.

High performance liquid chromatography (HPLC) method was commonly applied for the separation, quantification, and identification of compounds present in a mixture (Basharat *et al*., 2021). Several compounded preparation analysis including ketotifen fumarate or cyproheptadine hydrochloride has been using this HPLC method (Abdelrahman *et al*., 2021; Aldewachi & Omar, 2022; Gebretsadik *et al*., 2023; Kabra *et al*., 2014; Kesharbai, 2014; Mohammed *et al*., 2021; Muralidharan *et al*., 2012; Peikova *et al*., 2022; Pozharani *et al*., 2022; Prasad *et al*., 2022; Rajput & Sathe, 2019; Sahai & Devanna, 2021; Sethiya & Rathore, 2022; Tokumura *et al*., 2018; Yilmaz *et al*., 2022). However, studies analyzing ketotifen fumarate and cyproheptadine hydrochloride in divided powder combination are still limited. An analytical method validation should be performed to ensure that the analytical method is applicable to achieve the appropriate results (Mennickent & de Diego, 2019). The aim of this study was to validate the analytical method of RP-HPLC-UV and apply the validated method to quantify content of ketotifen fumarate and cyproheptadine hydrochloride in divided powder. The RP-HPLC-UV method was validated in several validation parameters including selectivity, linearity and range, precision, accuracy, sensitivity such as LOD and LOQ according to the AOAC Official Methods of Analysis (AOAC International, 2019).

MATERIALS AND METHODS

Materials and Chemicals

The reference standards of ketotifen fumarate and cyproheptadine hydrochloride were purchased from Indonesian Food and Drug Authority (FDA). The solvent used in this research was methanol for liquid chromatography grade (LiChrosolv®). The sodium hydrogen phospate for buffer solution was purchased from Merck Millipore. Ketotifen fumarate and cyproheptadine hydrochloride tablets were obtained from a drugstore in Yogyakarta, Indonesia.

Instrumentation and Software

A system of HPLC Shimadzu LC-2010 with UV/Vis detector equipped with C18 column Luna Phenomenex® (250×4.6 mm, i.d. 5 µm) endcapped was used in this study. Analytical balance OHAUS® PA 413, Gast® vacuum pump, Retsch® ultrasonicator, sterile syringe filter with a 0.45 µm pore size hydrophilic PTFE membrane (Merck Millipore), and a set of Socorex® micropipettes were also used in this study. Data from HPLC instrument was subsequently analyze using Excel (Microsoft Inc., USA) for generating linear calibration model and other data calculations.

HPLC Conditions

An isocratic RP-HPLC-UV method was applied with the mobile phase of methanol:0.04 M Na_2HPO_4 buffer pH 4.8 (60:40) and flow rate was adjusted to 0.8 mL/min. Column temperature was set to default 26.5°C. Wavelength detection was optimized and adjusted at 287 nm. Volume injection for each sample was 10 µL.

Preparation of standard solution

Accurate weights of 10.6 and 10.9 mg for ketotifen fumarate and cyproheptadine hydrochloride, respectively, were put into different 10 mL volumetric flask. The concentration of ketotifen fumarate was 1060 ppm and cyproheptadine hydrochloride 1090 ppm.

Preparation of sample solution

The preparation of sample solution was adapted from Kesharbai, 2014. An accurate weight of 225 mg of divided powder sample were placed in beaker glass and diluted by methanol into the volume of 10.0 mL volumetric flask. The working sample solution was prepared by transferring a 1.5 mL sample diluted solution into a 5 mL volumetric flask followed by dilution with methanol to volume. This solution was filtered using a sterile PTFE membrane before injection into the HPLC system.

Preparation of spiked sample solution

An accurate weighed of 225 mg ketotifen fumarate and cyproheptadine hydrochloride divided powder were placed in beaker glass and diluted by methanol into the volume of 10.0 mL volumetric flask, respectively. The spikedsample solution was prepared by transferring 85,95,105 µL cyproheptadine HCl and 110, 130,150 µL ketotifen fumarate solution into a 5 mL volumetric flask followed by dilution with methanol to volume. This solution was filtered using a sterile PTFE membrane before injection into the HPLC system.

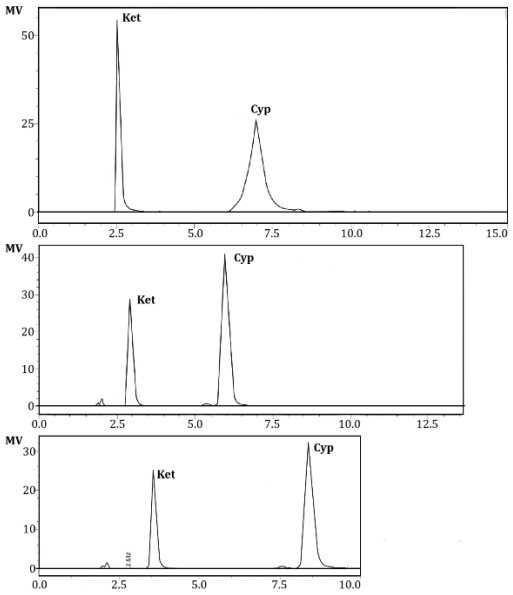

Figure 1. HPLC optimization using three variations of mobile phase for mixture standard solution of ketotifen fumarate (Ket) and cyproheptadine hydrochloride (Cyp). Wavelength detection at 287 nm. Notes: A: mobile phase = methanol : redistilled water : acetic acid (45:40:15), flow rate = 0.8 mL/min; B: mobile phase = buffer : methanol (65:35); flow rate = 0.8 mL/min; and C: mobile phase = buffer : methanol (60:40), flow rate = 0.8 mL/min.

Optimization of Analytical Method

Optimization of the chromatographic separation was performed by variation of mobile phase. Wavelength detection was set to 287 nm in which two analytes were intersecting wavelengths. The volume injection for each run was 10 µL. The composition of each run were methanol : redistilled water : acetic acid (45:40:15), buffer : methanol (35:65), buffer : methanol (40:60) with the same flow rate 0.8 mL/min (Figure 1).

Validation of Analytical Method

The HPLC method was validated for selectivity, linearity and range, precision, accuracy, sensitivity such as LOD and LOQ according to the AOAC Official Methods of Analysis (AOAC International, 2019).

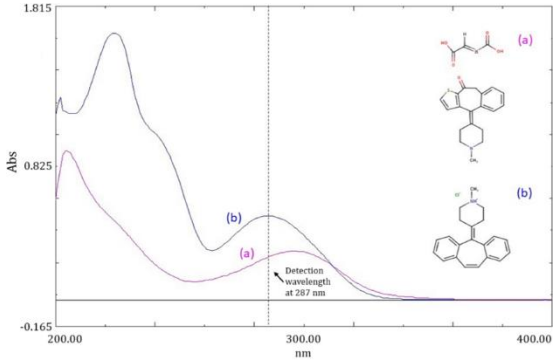

Figure 2. Chemical structures and UV spectra profiles of ketotifen fumarate (a) and cyproheptadine hydrochloride (b) in methanol solvent

RESULTS AND DISCUSSION

Determination of Wavelength Detection

Wavelength detection should be stated at the initial stage of HPLC-UV method development. It was important to choose an optimal wavelength for detecting several compounds in a mixture matrix (Figure 2). In this study, detection of the analytes was performed at 287 nm. At this wavelength, ketotifen fumarate and cyproheptadine hydrochloride can be optimally detected. The extensive overlapping spectra of analytes may become the limitation while applying the UV spectroscopy for a simultaneous quantification (Dzulfianto *et al*., 2017). However, by applying HPLC method, these two analytes can be detected simultaneously at the same wavelength and resulted separation profile for each analyte due to the interaction of the analyte itself both with mobile and stationary phase at the various interaction strength (Snyder *et al*., 2010).

Table I. Results of system suitability test (n=6)

Analytes	Retention Time		Area	
	Mean	RSD (%)	Mean	RSD (%)
Ketotifen Fumarate	3.475	1.551	271556.8	0.973
Cyproheptadine Hydrochloride	8.373	1.114	584726	0.887

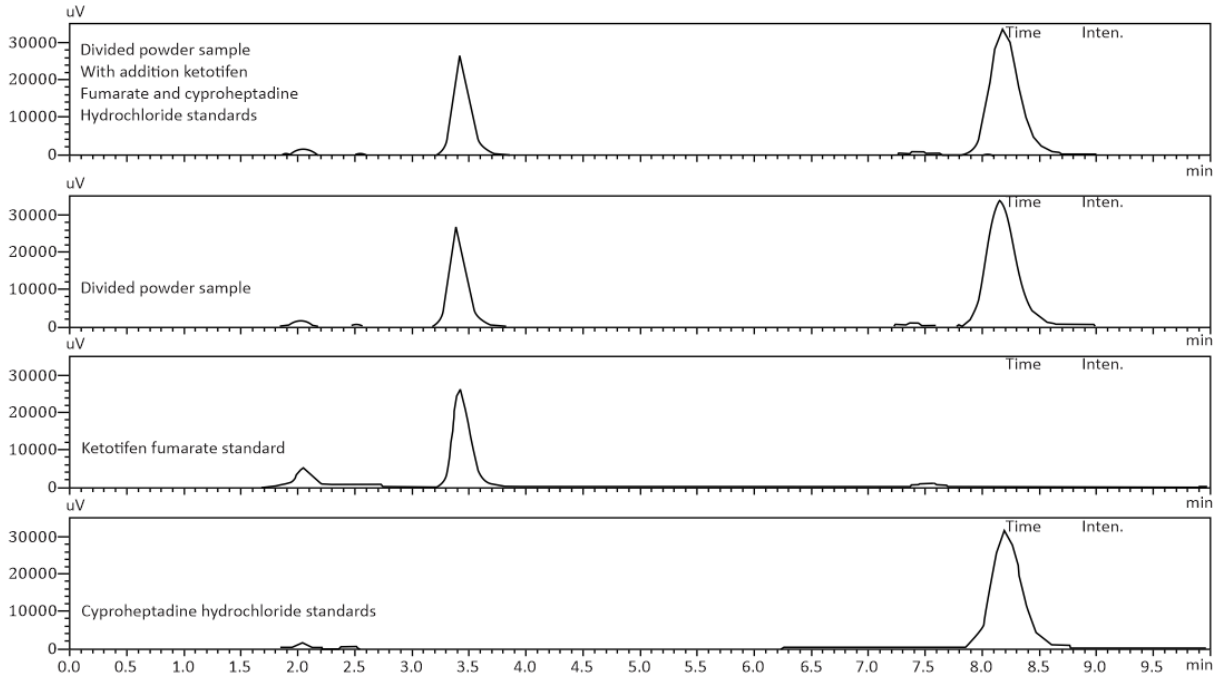

Figure 3. HPLC separation profiles ketotifen fumarate and cyproheptadine hydrochloride, divided powder sample, and divided powder sample with standard addition of ketotifen fumarate and cyproheptadine hydrochloride. Mobile phase: methanol:buffer (60:40 v/v). Column: C18 Luna Phenomenex® (250×4.6 mm, i.d. 5 µm) endcapped. Flowrate: 0.8 mL/min. Column temperature: 26.5 °C. Wavelength detection at 287 nm. Volume injection: 10 µL

System Suitability Test

The system suitability test is necessary to verify and ensure the continued performance of systems and analysis methods. HPLC separation properties are retention time and area. The relative standard deviation of retention time and area met the acceptance criteria (RSD ≤ 2%) (Snyder *et al*., 2010) (Table I).

Selectivity

Selectivity is a method to quantify the analyte from interference. This method can be held by a peak purity test. The peak should be single without another analyte. This HPLC condition met the criteria of resolution > 1.5, tailing factor < 2.0, theoretical plate number > 2000, and resulted minimum deviation (≤ 2%) both for retention time and peak area for each analyzed compound (AOAC International, 2019). The HPLC separation profiles of ketotifen fumarate and cyproheptadine hydrochloride, divided powder sample, and divided powder sample spiked with standard of ketotifen fumarate and cyproheptadine hydrochloride (Figure 3). Retention time of ketotifen fumarate and cyproheptadine hydrochloride were 3.444 and 7.981 min, respectively.

Linearity and Range

The data of peak areas versus concentration of standard solutions series from each compound were plotted to generate multiple point calibration curves for quantifying the content of ketotifen fumarate and cyproheptadine hydrochloride.

Table II. Accuracy and Precision Study of Standard Ketotifen Fumarate and Cyproheptadine Hydrochloride Solutions (n=3).

Compounds	Level	Added amount (µg/mL)	Found amount (µg/mL)	SD	RSD (%)	Recovery (%)
		Intraday				
Ketotifen Fumarate	Low	10.9	12.6	0.02	0.17	86.34
	Medium	21.8	21.2	0.06	0.31	102.67
	High	32.7	32.7	0.55	1.69	99.89
Cyproheptadine Hydrochloride	Low	10.6	12.2	0.03	0.27	86.29
	Medium	21.2	20.7	0.03	0.15	102.19
	High	31.8	31.6	0.05	0.17	100.52
		Interday				
Ketotifen Fumarate	Low	10.9	12.7	0.08	0.65	85.64
	Medium	21.8	21.2	0.08	0.41	102.48
	High	32.7	32.5	0.22	0.70	100.62
Cyproheptadine Hydrochloride	Low	10.6	12.3	0.05	0.47	85.55
	Medium	21.2	20.8	0.12	0.58	101.67
	High	31.8	31.9	0.56	1.74	99.65

Table III**.** Accuracy and Precision Study of Spiked Samples (n=3)

Compounds	Level	Added amount (µg/mL)	Found amount (µg/mL)	SD	RSD (%)	Recovery (%)
		Intraday				
Ketotifen Fumarate	Low	15	15.52	0.13	0.86	103.48
	Medium	13	11.35	0.18	1.59	87.34
	High	11	11.19	0.15	1.37	101.73
Cyproheptadine Hydrochloride	Low	21	22.05	0.21	0.96	105.04
	Medium	19	20.90	0.29	1.42	110.01
	High	17	17.50	1.69	0.86	102.95
		Interday				
Ketotifen Fumarate	Low	15	15.58	0.15	0.99	103.88
	Medium	13	11.36	0.17	1.50	87.44
	High	11	10.49	0.40	3.90	95.41
Cyproheptadine Hydrochloride	Low	21	22.08	0.17	0.77	105.17
	Medium	19	20.8	0.26	1.24	109.60
	High	17	17.71	0.24	1.38	104.19

Table IV. Results of Determination of Ketotifen Fumarate and Cyproheptadine Hydrochloride in Divided Powder

Replication	Analyte concentration (µg/mL)	
	Ketotifen Fumarate	**Cyproheptadine Hydrochloride**
1	16.56	30.62
2	16.56	30.57
3	16.60	30.62
4	16.64	30.64
5	16.36	30.65
6	16.63	30.68
7	16.63	30.66
Mean (µg/mL)	16.57	30.64
SD	0.09	0.03
RSD (%)	0.59	0.11

Calibration curve equation of ketotifen fumarate and cyproheptadine hydrochloride were y = 27714x-79111 (r=0.9945) and y = 26324x+72581 (r=0.9935), respectively. These curves were linear in the range of 10.9-54.5 and 10.6-53.0 µg/mL for ketotifen fumarate and cyproheptadine hydrochloride, respectively.

Limit of Detection and Limit of Quantification

LOD and LOQ as the parameters of sensitivity were determined using the standard deviation approach (Miller *et al*., 2018). LOD for ketotifen fumarate and cyproheptadine hydrochloride were 2.889 and 2.615 µg/mL, respectively. LOQ for ketotifen fumarate and cyproheptadine hydrochloride were 9.6312 and 8.7196 µg/mL, respectively.

Accuracy and Precision of Standard Solutions

Accuracy and intermediate precision of standard solution were assessed by determining concentration of ketotifen fumarate and cyproheptadine hydrochloride at three concentration levels (low, medium, high). Each concentration level was analyzed triplicate within three different days (interday) and at the same day (intraday). It was found that the developed RP-HPLC-UV method met the acceptance criteria of RSD < 7.3% and recovery in the range of 80-110% (AOAC International, 2019) (Table II) . It can be stated that this analytical method was accurate and precise for analyzing standard solution mixture of standard ketotifen fumarate and cyproheptadine hydrochloride both for intraday and interday evaluations.

Accuracy and Precision of Spiked Samples

Accuracy and intermediate precision of spiked samples were also performed in this study. Blank sample solutions were added by standard solution of ketotifen fumarate and cyproheptadine hydrochloride at three concentration levels. Each concentration level was analyzed triplicate within three different days (interday). Table III presented the results of accuracy and precision study of sample solutions with the addition of ketotifen fumarate and cyproheptadine hydrochloride standards. It was found that the developed RP-HPLC-UV method met the acceptance criteria of RSD < 7.3% and recovery in the range of 80-110% (AOAC International, 2019). It can be stated that this analytical method was accurate and precise for analyzing divided powder samples with the presence of standard ketotifen fumarate and cyproheptadine hydrochloride both for intraday and interday evaluations.

Determination of Ketotifen Fumarate and Cyproheptadine Hydrochloride in Divided Powder

The validated RP-HPLC-UV method was applied for determining content of ketotifen fumarate and cyproheptadine hydrochloride in divided powder samples. Table IV presented the results of determination of ketotifen fumarate and cyproheptadine hydrochloride in divided powder sample. The precision of method was assessed and proven by the percentage of RSD less than 7.3% in seven replicates of determination (AOAC International, 2019).

CONCLUSION

A validated isocratic RP-HPLC-UV method was successfully developed. This method was selective, linear, sensitive, accurate, and precise to quantitatively analyze ketotifen fumarate and cyproheptadine hydrochloride in different samples of divided powder combination to provide contextual information in hospital and drugstore. This method could be applied for routine analysis quality of drug in Indonesia. The good quality of drugs can directly impact to patient's health.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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