Tropical Journal of Pharmaceutical Research July 2014; 13 (7): 1141-1147
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v13i7.19

Original Research Article

Development and Validation of a Stability-Indicating LC-UV Method for Simultaneous Determination of Ketotifen and Cetirizine in Pharmaceutical Dosage Forms

Prachi V Kabra[1]*, LVG Nargund[1] and MS Murthy[2]
[1]Nargund College of Pharmacy, Dattatreyanagar, IInd Main, 100ft. Ring Road, BSK IIIrd Stage, Bangalore-560085, [2]Vignan Institute of Pharmaceutical Sciences, Vignan Hills, Deshmuki Village, Pochampally Mdl, Nalgonda District-508284, India

For correspondence:* ***Email:* *prachi.v.kabra@gmail.com*

Received: 15 October 2013 *Revised accepted: 10 May 2014*

Abstract

Purpose: *To develop and validate stability-indicating reversed phase high performance liquid chromatographic method for simultaneous determination of ketotifen fumarate and cetirizine dihydrochloride in solid dosage forms.*
Methods: *Chromatographic separation was achieved on Grace Smart C18 column (250 × 4.6 mm, 5 µm) using an isocratic mobile phase that consisted of acetonitrile and 10 mM disodium hydrogen phosphate buffer (pH 6.5) in a ratio of 45:55 % v/v at a flow rate of 1 mL/min. Detection was carried out at 230 nm. Salbutamol sulphate was used as an internal standard. The drugs were exposed to hydrolytic (acid and alkaline), oxidative, reductive and neutral stress conditions, and the stressed samples analyzed by the proposed method. Validation of the method was carried out as per International Conference of Harmonization (ICH) guidelines.*
Results: *The retention time for ketotifen, salbutamol and cetirizine was 2.05, 5.37 and 6.77 min, respectively. In stress studies, it was observed as cetirizine was more labile in acidic, oxidative and neutral conditions than ketotifen. Both the drugs were found comparatively stable in alkaline, neutral condition and labile in reductive condition. The method was linear in the concentration range of 1 - 30 µg/mL and 10 - 300 µg/mL for ketotifen and cetirizine, respectively.*
Conclusion: *The developed method is specific and stability-indicating as no interfering peaks of degradants and excipients were observed. Thus, the method is suitable for application in the simultaneous quality control of both drugs.*

Keywords: *Ketotifen, Cetirizine, Stability indicating method, Stressed conditions, Validation*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Asthma is a disorder that causes the airways of the lungs to swell and narrow, leading to wheezing, shortness of breath, chest tightness and coughing. An estimated three hundred million people worldwide suffer from asthma, with two hundred and fifty thousand (250,000) annual deaths attributed to this disease. Workplace conditions, such as exposure to fumes, gases or dust are responsible for 11 % of asthma cases worldwide. About 70 % of asthmatics also have allergies. It is thought to be caused by a combination of genetic and environmental factors [1].

Ketotifen fumarate (KETO) is a relatively selective, non-competitive histamine antagonist (H1-receptor) and mast cell stabilizer [2]. Cetirizine dihydrochloride (CET), a human

metabolite of hydroxyzine, is an antihistamine; its principal effects are mediated via selective inhibition of peripheral H1 receptors [3]. Chemical structure of KETO and CET is shown in Figure 1.

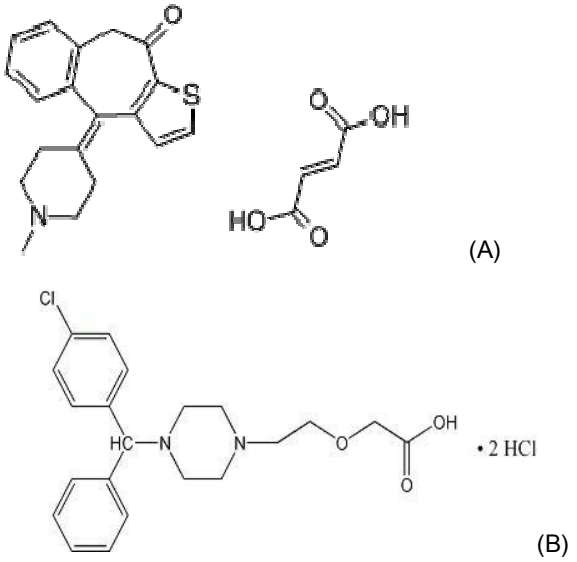

Figure 1: Chemical structure of (A) ketotifen fumarate and (B) cetirizine dihydrochloride

Recently a combination of ketotifen and cetirizine is launched in the market which can be used as antihistamine and anti-allergics for the treatment of asthma and chronic urticaria. Chronic urticaria is a condition where an itchy rash persists on and off for six weeks or more [4].

Stability testing attempts to ensure the quality of the drug product not only during manufacturing, production and packaging even during storage till the patient consumes it. During the course of storage the drug product may get exposed to adverse environmental conditions such as extreme temperature, varying humidity and intense lights. Considering these aspects, many regulatory bodies including ICH [5] and WHO [6] framed specific guidelines to ensure the quality of the drug product during the storage conditions. This mainly involves exposing the drug product to adverse environmental conditions: acidic, basic, oxidative, reductive and neutral conditions and also to develop an analytical method for the estimation of active constituent or constituents in a pharmaceutical formulation in the presence of the degradants [7].

Literature survey revealed that few analytical methods are reported for the estimation of ketotifen alone or with other drugs. Spectrophotometric methods using multicomponent mode of the instrument [8], using λmax of 298 nm when in methanol solution [9], colorimetric method [10], estimation based on ion pair formation [11] are available in literature. Several HPLC methods for estimation of ketotifen in pharmaceutical dosage form [12] and in biological fluids [13] are also reported. Stability indicating HPLC method is reported for ketotifen where drug is exposed to heat, oxidative media and UV light [14].

Various analytical methods such as spectrophotometric methods [15], HPLC method [16] and HPTLC methods [17] are reported for estimation of cetirizine alone or in combination with other drugs in pharmaceutical formulation. LC/MS/MS method is reported for estimation of cetirizine from human plasma using positive electrospray ionization method [18]. One stability-indicating HPLC method has been reported where the stability of cetirizine was checked at 40 and 50 °C at 75 % relative humidity for six months [19].

No stability-indicating reversed phase HPLC method for simultaneous determination of ketotifen and cetirizine was found in literaure. Thus, the objective of the present work was to develop and validate a stability-indicating reversed phase HPLC method for the simultaneous determination of ketotifen and cetirizine in pharmaceutical formulations.

EXPERIMENTAL

Materials

KETO and CET were procured as gift samples from East West Pharma, Haridwar and Micro Labs Pvt. Ltd., Bangalore respectively. HPLC Grade acetonitrile, orthophosphoric acid and analytical grade disodium hydrogen phosphate buffer, hydrochloric acid, sodium hydroxide, hydrogen peroxide, sodium bisulphate were purchased from Merck, Mumbai. Mastifen-C Tablets (KETO-1 mg, CET-10 mg; East West Pharma, Haridwar) were purchased from local pharmacy.

Chromatographic conditions

Chromatographic separation was performed on Grace Smart C18 column (250 mm × 4.6 mm, 5 µm) using mobile phase consisted of acetonitrile 10 mM disodium hydrogen phosphate buffer in proportion of 45:55 % v/v where pH of phosphate buffer was adjusted to 6.5 using 1 % orthophosphoric acid. The mobile phase was filtered through 0.22 µm Super 200 membrane

filter and degassed in ultrasonic bath for 10 min before use. The flow rate was maintained at 1.0 mL/min and wavelength of UV detector was set at 230 nm.

Preparation of standard solution

Standard stock solution of KETO and CET (1 mg/mL) were prepared separately in the HPLC grade water and after suitable dilution these solutions were used for mobile phase optimisation and peak identification. For the construction of the calibration curve, a combined standard solution of KETO (0.1 mg/mL) and CET (1 mg/mL) was prepared in HPLC grade water. Further, this stock solution was diluted with mobile phase to a concentration in the range of 1 - 30 µg/mL for KETO and 10 - 300 µg/mL for CET, with 10 µg/mL of salbutamol sulphate (SAL) as an internal standard (IS).

Instrumentation

A Shimadzu HPLC system that consisted of an isocratic pump LC 20 AT, rheodyne injector (20 µl) with SPD 20A UV visible detector was employed. HPLC data was processed using Spinchrome CFR software, version 2.1.4.93.

Analysis of dosage form

Twenty tablets of KETO and CET (Mastifen-C) were weighed and crushed to obtain fine powder. An accurately weighed tablet powder equivalent to about 2.5 mg of KETO (25 mg of CET) was transferred to 25 ml volumetric flask. About 10 ml of HPLC Grade water was added and the solution was sonicated for 15 min. The volume was made up to the mark with the same solvent to get concentration of 100 µg/mL of KETO and 1000 µg/mL of CET. The resulting solution was filtered through Whatman filter paper no. 41. From this solution 1 mL of the aliquot was transferred to a 10 ml volumetric flask along with this 1 mL of SAL solution (100 µg/mL) (IS) was added. The volume was made up to the mark with mobile phase to obtain a solution with final concentration of 10 µg/mL of KETO, 100 µg/mL of CET and 10 µg/mL of SAL. Similarly the combined standard stock solution, with IS was diluted in mobile phase to get the concentration of 10 µg/mL of KETO, 100 µg/mL of CET and 10 µg/mL of SAL. Content of both the drugs present in tablet were analysed using single point analysis method using equation 1.

$$C1 = R1(C2/R2) \quad \ldots\ldots (1)$$

where, C1 and C2 are concentration of sample and standard solutions, respectively, R1 = peak area ratio of drug to internal standard of sample solution, and R2 = peak area ratio of drug to internal standard of standard solution

Forced degradation study

In order to establish whether the developed analytical method was stability indicating, forced degradation of standard KETO and CET in combination was carried out under acid – base hydrolytic, oxidative, reductive and neutral conditions at 80 °C. As both the drugs are water soluble, solutions of 0.5 M HCl, 0.5 M NaOH, 3 % H_2O_2 and 1 % $NaHSO_3$ were prepared in water. Both the drugs were dissolved in these solutions to achieve the concentration of 100 µg/mL of KETO and 1000 µg/mL of CET. At specified time the samples of the reaction mixtures (1 mL) were withdrawn, cooled to room temperature and neutralized. To each sample, 1 mL of the internal standard, SAL (100 µg/mL) was added and the volume was made up to 10 mL with mobile phase to get concentration of 10 µg/mL of KETO, 100 µg/ mL of CET and 10 µg/mL of SAL.

Acid and base hydrolysis was performed using 0.5 N HCl and 0.5 N NaOH respectively and refluxed at 80 °C for 1 h. Oxidative studies were performed in 3 % H_2O_2 and refluxed at 80 °C for 30 min. Reductive studies were carried out in 1 % $NaHSO_3$ and refluxed at 80 °C for 1 h. For study in neutral solution, the drugs were dissolved in water and refluxed at 80 °C for 10 h.

Method validation

The analytical method was validated for various parameters according to ICH guidelines as follows.

Linearity

The linearity of the method was determined at ten concentration levels ranging from 1-30 µg/mL of KETO and 10-300 µg/mL of CET (n=6). The peak area ratio of KETO to SAL vs KETO concentration and peak area ratio of CET to SAL vs CET concentration were plotted and observed for correlation coefficient.

Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ parameters were determined from the regression equation of KETO and CET.

LOD = 3.3 SD/S, LOQ = 10 SD/S; where SD is the standard deviation of Y-intercept and S is the average slope value.

Accuracy

Recovery studies were carried out by standard addition method by adding known amounts of KETO and CET (reference standard) to the preanalyzed sample at three different concentration levels, i.e., 80, 100, and 120 % of assay concentration and % recovery was calculated.

Precision

The precision of the method was determined by repeatability, intermediate precision (intra-day, inter-day, variation by different analyst) and expressed as % relative standard deviation. Repeatability study was performed by analysing six times the same concentration of drugs (KETO 10 µg/mL, CET 100 µg/mL) while intra-day precision was determined by performing analysis of triplicate injections of three different concentration of combination of the drugs (4, 10, 20 µg /mL of KETO and 40, 100, 200 µg /mL of CET) on the same day at different time intervals and three different days for inter-day precision. Variation of results by different analyst was studied by performing assay in triplicate by Analyst I and Analyst II and the results compared for significant difference by F-test and Students t-test.

Robustness

Robustness of the method was evaluated by varying experimental conditions (± 2 %) such as pH, flow rate and organic phase content of the mobile phase. For each parameter change, its influence on the retention time, number of theoretical plates, tailing factor and resolution were evaluated.

RESULTS

The retention time of KETO, SAL (IS) and CET were found to be 2.05, 5.37 and 6.77 min, respectively. Chromatogram of the sample solution is shown in the Figure 2.

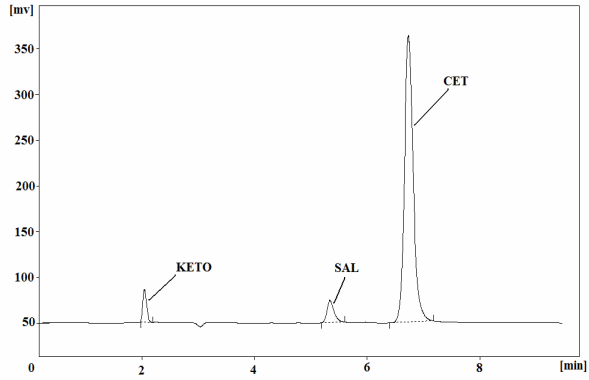

Figure 2: Chromatogram of sample solution

Amount of drugs present in the marketed formulation (Mastifen-C) were calculated using single point analysis method. Mean drug content was 99.43 and 100.50 % for KETO and CET, respectively. The result of the assay of the tablet formulation is shown in Table 1.
The drugs were subjected to different stress conditions and the outcomes of stress studies are tabulated in Table 2.

Linearity, LOD and LOQ

The calibration plot was linear over the concentration range of 1-30 µg/mL for KETO and 10-300 µg/mL for CET (n = 6) with RSD values ≤ 2.76 %, across the concentration range studied. The LOD values for KETO and CET were found as 0.13 and 1.60 µg/mL respectively. The LOQ values were observed as 0.40 µg/mL for KETO and 4.85 µg/mL for CET. Results system suitability and linearity data for the proposed method is shown in Table 3.

Table 1: Assay results for tablet formulation

S/N	Claimed amount (mg/tab)		Amount found (mg/tab)		Actual strength (%)	
	KETO	***CET***	***KETO***	***CET***	***KETO***	***CET***
1	1	10	0.9965	9.9985	99.65	99.99
2	1	10	0.9904	10.1101	99.04	101.10
3	1	10	0.9994	10.0424	99.94	100.42
4	1	10	0.9918	10.0333	99.18	100.33
5	1	10	0.9936	10.0261	99.36	100.26
6	1	10	0.9938	10.0886	99.38	100.89
Mean			0.9943	10.0498	99.43	100.50
± SD			0.00326	0.0416	0.3259	0.4161
%RSD			0.3278	0.4141	0.3278	0.4141

Table 2: Results of forced degradation studies

Stress condition	Degradation (%)			
	Standard		Sample	
	KETO	***CET***	***KETO***	***CET***
Acidic/0.5 M HCl/1h /80 ºC	7.519	29.896	17.037	29.436
Basic/0.5 M NaOH/1h /80 ºC	11.545	3.971	10.789	9.182
Oxidative/3 % H_2O_2/30min/80 ºC	0.293	30.007	3.516	29.630
Reductive/1 % $NaHSO_3$/1h/80 ºC	29.138	29.898	29.875	26.357
Neutral/H_2O/10h/80 ºC	12.710	25.245	14.016	29.285

Table 3: System suitability and linearity data for the proposed method

Parameter	KETO	CET
Retention time (min)(% RSD)	2.05 (0.20)	6.77 (0.19)
Tailing Factor (% RSD)	1.52 (1.46)	1.42 (1.59)
Plate Count ((% RSD)	4210 (1.76)	11557 (1.89)
Linearity and Range (µg//mL)	1-30	10-300
Slope (± SD)	0.090 (0.0007)	0.209 (0.0009)
Intercept (± SD)	0.0059 (0.0036)	0.693 (0.101)
Correlation Coefficient	0.9999	0.9995
LOD (µg/mL)	0.13	1.60
LOQ (µg/mL)	0.40	4.85

Accuracy and precision

Percent recovery of KETO and CET was in the range from 98.77 - 99.62 % and 99.39 - 100.41 %, respectively. Precision of the method was determined by % RSD found among intra-day precision, inter-day precision, repeatability. It was found to be less than 1 %.Variation of results by two different analyst was determined by preparing and measuring the sample solutions of KETO (10 µg/mL) and CET (100 µg/mL) by Analyst 1 and Analyst 2, separately. The values obtained were evaluated using F-test and t-test to verify their precision. Calculated value for t-test was 0.5748 for KETO and 0.0185 for CET, which are less than the tabulated or standard value (1.533), and hence no significant difference was observed between the results of the two analysts at a probability value of 0.10. The results of recovery study are shown in Table 4 while the results of variation by the two analysts are given in Table 5.

Robustness

For robustness study, the effect of change in the pH (2 %) of mobile phase, organic phase ratio (2 %) and flow rate (2 %) on the retention time, asymmetry factor, theoretical plates and resolution were studied. Combined standard solutions of KETO (10 µg/mL), CET (100 µg/mL) and SAL (10 µg/mL) were prepared and analyzed at different pH (6.37, 6.5, 6.63) of the mobile phase, at different organic phase ratio (44.1:55.9, 45:55, 45.9:54; 1 % v/v) and at different flow rate (0.98, 1.0, 1.02 mL/min). Percentage RSD of retention time, tailing factor, number theoretical plates and resolution of peak in all three variables was found to be less than 5 %.

Table 4: Results of recovery study

DRUG	Amount added (µg/mL)	% Recovery, %RSD
KETO	08	99.40, 0.016
	10	99.05, 0.258
	12	99.48, 0.114
CET	80	99.39, 0.013
	100	99.92, 0.164
	120	100.27, 0.123

DISCUSSION

All reported methods for KETO and CET as individual drug or in combination with other drugs, used C8 and C18 column as a stationary phase. Hence attempts were directed towards development of chromatographic method on

Table 5: Results of variation by different analyst

Drug	(% Drug content*±SD)		F-test value	t-test value	Inference
	Analyst 1	***Analyst 2***			
KETO	99.54±0.460	99.30±0.108	0.0557	0.5748	No significant difference
CET	100.50±0.562	100.49±0.341	0.3701	0.0185	No significant difference

commonly used C18 column for simultaneous estimation of the prescribed drugs. To optimize mobile phase composition for KETO and CET, different proportions of the mobile phase at different pH were tested. Considering the pKa value of KETO (8.43) and CET (1.6, 2.9, 8.3), different pH values of the mobile phase were tried in the range of 4.0 to 6.5. Final decision on the mobile phase composition and flow rate was done on the basis of peak area, resolution between the peaks of the drug and their degradation products, tailing factor, number of theoretical plates and time required for the analysis. The chromatographic conditions were finally optimized on Grace Smart C18 Column (250 × 4.6 mm, 5 µm) using 1 mL/min flow rate of 45:55 % v/v of acetonitrile: 10 mM disodium hydrogen phosphate buffer solution (pH 6.5 adjusted with 1 % ortho-phosphoric acid) as mobile phase. An analytical wavelength was selected considering the good absorbance of both the prescribed drug and IS. Hence, detection was done at 230 nm where all the drugs showed absorbance. Under these conditions the peak of KETO and CET were well defined and free from tailing.

Preliminary trials on the individual drugs and in combination were conducted to optimize various stress conditions. Conditions used for the stress studies were attenuated to achieve degradation in the range of 10 – 30 % for establishing stability indicating nature of the method. Samples were withdrawn at different time intervals, to monitor rate of degradation and the stress conditions were optimized. It was observed that, CET is more labile in acidic, oxidative and neutral conditions as compared to KETO. CET was found to be degrading after 4 h of heating in 0.1 N HCl but KETO showed insufficient degradation under the same condition, hence further stress studies for drug combination were performed in 0.5 N HCl. Both the drugs were found comparatively stable in alkaline condition and labile in reductive condition. Degradation of both the drugs were found to be extensive in 2.5 % of sodium bisulphate ($NaHSO_3$), hence further study was done in 1 % of $NaHSO_3$. Rapid degradation was observed when CET was exposed to 3 % H_2O_2 as compare to KETO. Hence, it was refluxed only for 30 min. Blank oxidative reagent, i.e., 3 % H_2O_2 showed a peak at 2.9 min, hence, it was not considered a degradant peak. Both drugs were stable in neutral conditions and therefore, the drugs were heated for 10 h to obtain a sufficient level of degradation.

CONCLUSION

Stability-indicating reversed phase HPLC method for simultaneous estimation of KETO and CET has been developed and validated. The method is specific and stability-indicating. The proposed method has ability to separate the two drugs and their degradation products and common excipients used in tablet dosage forms. Thus, it can be applied to the analysis of routine quality control samples as well as samples obtained in stability studies.

ACKNOWLEDGEMENT

The authors are grateful to East West Pharma, Haridwar and Micro Labs Ltd, Bangalore, India for providing ketotifen and cetirizine pure samples free of charge, and to Nargund College of Pharmacy, Bangalore for providing laboratory and research facilities for this work.

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