

RP-LC GRADIENT ELUTION METHOD FOR SIMULTANEOUS DETERMINATION OF RELATED SUBSTANCES OF ZALTOPROFEN AND PARACETAMOL AND APPLICATION FOR DRUG EXCIPIENT COMPATIBILITY STUDY

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Received: 20 Dec 2013, Revised and Accepted: 10 Feb 2014

ABSTRACT

Objective: A reverse phase liquid chromatography method in gradient mode has been developed for quantitative determination of impurities, related substances, degradation products of Zaltoprofen and Paracetamol simultaneously.

Methods: Method uses a 250 X 4.6 mm, 5 μ m Hypersil BDS C8 column with a mobile phase composition of ammonium acetate buffer pH 5.0: methanol in gradient mode. The flow rate was 1.0 mL min⁻¹ and wavelength was set at 240 nm.

Results: Five related substances of Zaltoprofen and three of Paracetamol were well separated with the developed method. Resolution between closely eluting peaks was more than 4.0. The method was validated for selectivity, linearity, accuracy, precision and robustness. The limit of detection and limit of quantitation for all available impurities were below 0.015% and 0.046% respectively. The method was linear in the range of limit of quantitation to 150% of specified concentration. The correlation coefficient for all components was more than 0.995.

Conclusion: The stress studies showed that the method was specific, selective to study related substances of Zaltoprofen and Paracetamol simultaneously. The peak purity of analyte showed that unknown degradation products formed during stress studies did not interfere with the determination of all the studied analytes. The mass balance was achieved for Zaltoprofen and Paracetamol for degraded samples. The excipient compatibility study concluded that all the studied excipients are compatible with both drugs.

Keywords: Zaltoprofen, Paracetamol, Impurities, Related substances, Chromatography.

INTRODUCTION

Zaltoprofen, 2-(10, 11-dihydro-10-oxodibenzo [b,f] thiepin-2-yl)pro-pionic acid is a potent non-steroidal anti-inflammatory drug (NSAID) (ZP). It has powerful anti-inflammatory and analgesic effects on inflammatory pain, which preferentially inhibits COX- 2 activity. It inhibits bradykinin-induced pain responses without blocking the receptors [1]. Paracetamol (Acetaminophen) is a non-opiate, non-salicylate, centrally and peripherally acting analgesic agent. Comparative effect of paracetamol and NSAIDs or their combination in postoperative pain management, rheumatoid arthritis and short term treatment of cancer pain has been reported [2-4]. Combined paracetamol treatment may increase the effect and decrease the dose-dependent side-effects of NSAIDs [5].

Addition of paracetamol to NSAID is well tolerated and effective in the treatment of osteoarthritis flare pain [6]. Therefore a novel combination of zaltoprofen with paracetamol will be a potent analgesic and anti inflammatory drug for future in the pain management.

A few analytical methods like LC [7-11], voltammetry [12], LC-MS-MS [13], TLC [14] and spectrofluorometry [15] have been reported for the determination and identification of PC either alone or in combination with other analgesics. Literature survey shows very few analytical testing methods are available for ZP. The drug can be estimated by HPLC in plasma [16-18]. There is a chiral HPLC method for enantioselective analysis [19]. ZP is official in JP [20].

In a regulated market, drug product compliance to ICH requirements for specification and impurities or degradation products is a mandatory requirement [21]. However to the best of our knowledge no method has been developed for simultaneous determination of ZP and PC and their impurities from formulation.

The aim of the present study is to develop a related substances method for the determination of impurities and degradation products of ZP and PC simultaneously.

MATERIALS AND METHODS

Chemicals and Reagents

Working standard of PC (99.4 %) purity and ZP (99.3 %) purity and impurity standards were obtained from Ipca laboratories, Mumbai, India. Marketed formulation of Zaltoprofen tablets 80mg and Paracetamol tablets 500 mg were purchased from local market and used for the study. LC grade methanol, orthophosphoric acid and ammonium acetate were procured from Merck Mumbai, India. High purity deionized water was obtained from Merck-Millipore Milli Q plus water purification system (Milford, USA).

Instrumentation

The LC system used were PDA 2996, Waters and equipped with quaternary gradient pumps with auto sampler and auto injector (Alliance 2695, Waters, Milford, MA, USA) connected with a photo diode array detector (PDA 2996, Waters, Milford, MA, USA) controlled with Empower software (Waters).

Chromatographic Conditions

The separation was achieved on 250 X 4.6 mm, 5 μ m Hypersil BDS C8 column with a mobile phase composition of 0.05mM ammonium acetate buffer adjusted to pH 5.0 : methanol in gradient mode. The buffer was filtered through a 0.45 μ m nylon membrane and degassed with sonication for 5 min. The final gradient program started with buffer: methanol composition 80:20 (v/v) and maintained at same composition for 6 mins. This composition was linearly changed to 70% methanol within 15 min and the isocratic gradient with buffer: methanol composition 30:70 (v/v) was run upto 35 min and then linearly changed to original composition 80:20 within next 5 minutes (40 min) and allowed to stabilize to original composition till next 5 min so as to complete the run time of 45 min.

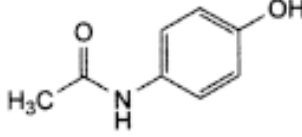
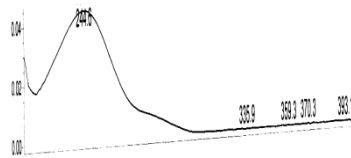
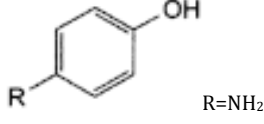
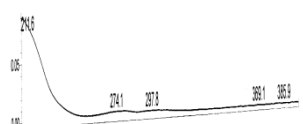
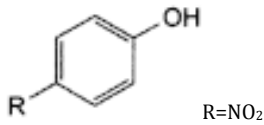

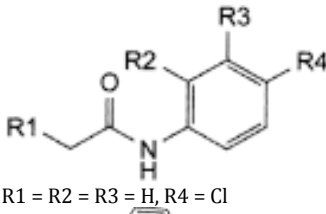
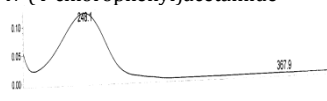
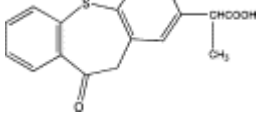
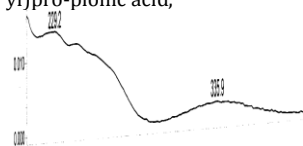
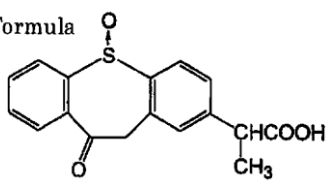
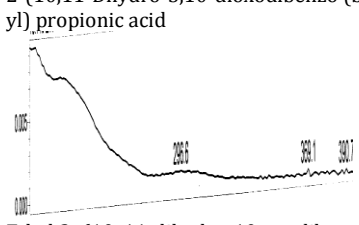
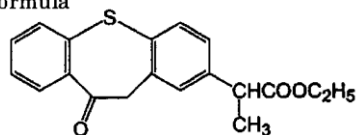
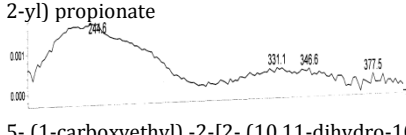
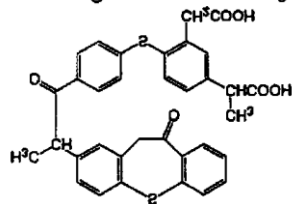
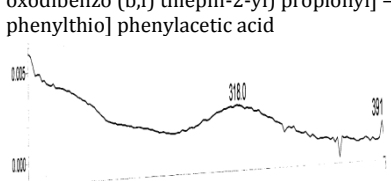
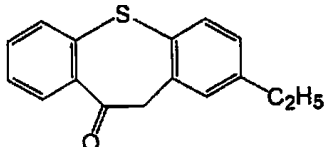
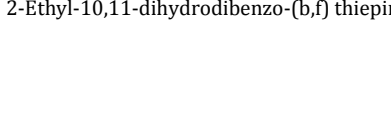
The mobile phase flow rate was 1.0 mL min⁻¹ and wavelength 240 nm. The injection volume was 20 μ L. Diluent used during the preparation of the standard and test sample was mixture of water: methanol (50: 50, v/v).

Preparation of Standard Solutions

A stock solution of ZP (0.8 mg mL⁻¹) and PC (5.0 mg mL⁻¹) was prepared by dissolving an appropriate amount in diluent. Working solutions containing 0.8 µg mL⁻¹ ZP and 5 µg mL⁻¹ PC were prepared from this stock solution, also stock solutions of impurities were

added to the working solution to contain 5 µg mL⁻¹ of 4A, 4N, 4C and 0.8 µg mL⁻¹ of ZA, ZB, ZC, ZD, ZE.

IUPAC names of impurities and drug are stated in (fig.1)

S. No.	Structure	IUPAC names and UV spectra
1	Paracetamol 	<i>N</i> -(4-Hydroxyphenyl)acetamide. 
2	4 aminophenol 	4 aminophenol 
3	4 nitrophenol 	4 nitrophenol 
4	4 chloroacetanilide 	<i>N</i> -(4-chlorophenyl)acetamide 
5	Zaltoprofen 	(±)-2-(10,11-dihydro-10-oxodibenzo[b,f]thiepin-2-yl)pro-pionic acid, 
6	Zaltoprofen impurity A Formula 	2-(10,11-Dihydro-5,10-dioxodibenzo (b,f) thiepin-2-yl) propionic acid 
7	Zaltoprofen impurity B (Ethyl ester) Formula 	Ethyl 2- (10, 11-dihydro-10-oxodibenzo (b,f)thiepin-2-yl) propionate 
8	Zaltoprofen impurity C 	5- (1-carboxyethyl) -2-[2- (10,11-dihydro-10-oxodibenzo (b,f) thiepin-2-yl) propionyl] - phenylthio] phenylacetic acid 
9	Zaltoprofen impurity D (Ethyl substance) 	2-Ethyl-10,11-dihydrodibenzo-(b,f) thiepin-10-one 

10

Zaltoprofen impurity E

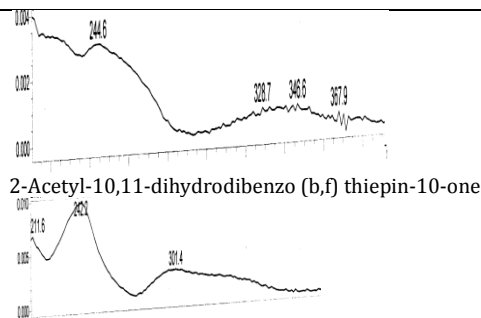
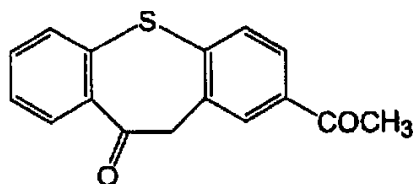


Fig. 1: Structure, UV spectra and IUPC names for impurities and drugs

Sample Solutions

Ten whole tablets of each drug were weighed, transferred to a 1000 mL volumetric flask, 10 mL of water was added to disperse the tablets. 700ml of diluent was then added to the flask and was shaken for 30 min using a wrist action shaker for complete extraction of analytes. The solution thus prepared was sonicated for 30 min and diluted to volume to give a solution containing 5000 $\mu\text{g mL}^{-1}$ of PC and 800 $\mu\text{g mL}^{-1}$ of ZP. This solution was filtered through a 0.45 μm pore size PVDF syringe filter and used for related substances determination.

RESULTS AND DISCUSSION

Method development and optimization

The HPLC method was optimized with a view to develop a SIM. Literature survey showed pKa value to be 4.21 for ZP and 9.86 for PC. Initially, method development felt to be challenging primarily due to wide range of pKa value. But PC being neutral molecule did not have much effect of mobile phase pH on the chromatographic retention. The amount of buffer affected the retention of PC. To retain PC, more quantity of buffer was required but ZP and its impurities eluted very late and also peak shapes were broad. Therefore it was necessary to use gradient elution to achieve the desired separation of drugs having wide range of polarities. Gradient elution refers to any kind of intentional variation of mobile phase composition with time during the course of an analysis. Solvent programming was designed such that the solvent strength increases with time. Therefore migration velocity of ZP and its impurities increased as a result of gradient. Gradient elution was advantageous as it gave sharp peaks and better detection limits for late eluting peaks as well. For initial run, a theoretical gradient program designed wherein initially solvent strength was very low and it increased after elution of PC. 0.05mM ammonium acetate buffer was selected for mobile phase. ZP being acidic molecule, it retained more with acidic mobile phase and retention decreased as pH of mobile phase increased towards basic side. The pH of buffer was optimized to 5.0 which gave well resolved peaks for all known impurities and unknown degradation products of ZP (acid degradation at rrt 1.34 and basic degradation at rrt 0.72).

The flow rate was maintained at 1 mL min⁻¹ and wavelength set at 240 nm which is the maximum wavelength for almost all components. At this wavelength, the response factors are obtained in a range of 0.2 to 5 which is well acceptable as per European pharmacopoeia for an impurity profile method. Injection volume 20 μL for column and HPLC system was found to be best for analysis. Diluent as mixture of water: methanol 50: 50 (% v/v) was confirmed for use as it could easily extract both the analytes from the whole tablets with shaking and sonication for 30 mins. The sample concentration was finalized based on LOD and LOQ values obtained. The quantitation limit should be well below the reporting threshold of impurities.

The final decision of chromatographic conditions thus was based on suitability of mobile phase, sensitivity to assay, suitability for stability studies, time required for analysis, ease of preparation and readily available cost effective solvents (Fig 2a).

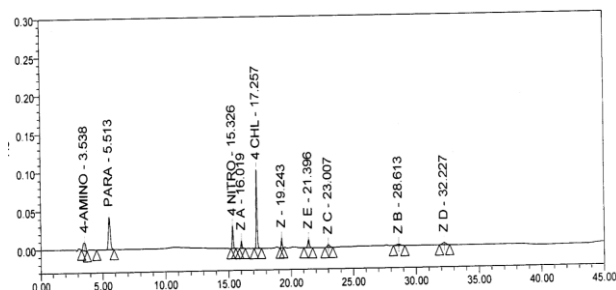


Fig. 2 a: Typical chromatogram

The specificity of a method is its suitability for analysis of a substance in the presence of potential impurities. Stress testing of a drug substance can help identify likely degradation products, which can in turn, help establish degradation pathways and the intrinsic stability of the molecule. The specificity of the LC method for ZP and PC was determined in the presence of eight impurities and degradation products. Forced degradation of ZP and PC was also performed to provide an indication of the stability-indicating properties and specificity of the method. The tablet formulations were exposed to stress studies along with active pharmaceutical ingredients. The stress conditions chosen to achieve degradation included acid hydrolysis (1M HCl), basic hydrolysis (1 M NaOH) and oxidation (30% H₂O₂) along with light (conducted as stipulated in ICH Q1B) and heat at 60 °C. For studies of the effects of heat and light the study period was about 7 to 10 days whereas for acidic, basic, and aqueous hydrolysis and oxidation it was about 2 h. The purity angle is less than the purity threshold limit obtained in all stressed samples by using Waters Empower software ensured analyte peak homogeneity as required by ICH Q2R (2). The peak purity obtained from stressed samples using PDA detector confirmed method to be stability indicating.

When ZP and PC were exposed to heat, degradation was not observed. Similarly exposure to photolytic degradation, PC did not show any degradation but ZP showed degradation with change in physical appearance from white to yellow. Also Drug product exposed to photolytic studies showed change in color from white to yellow. ZP was found to degrade in acidic hydrolysis, basic hydrolysis forming unknown impurities at rrt1.34 and rrt0.72 respectively. The carboxylic group in ZP is prone to esterification in presence of alcohol ie methanol used in diluent. The probable impurity generated in acid hydrolysis is methyl ester of ZP which eluted before impurity ZB ie ethyl ester of ZP.

ZP also showed degradation in oxidation forming impurity ZA. The thiol group in ZP underwent oxidation to form dioxoderivative of ZP ie impurity ZA which was confirmed by spiking imp ZA in degraded samples.

Stress studies on PC in basic hydrolysis, acidic hydrolysis and oxidation concluded that imp 4A to be one of the major degradants during acidic hydrolysis. (Fig 2b, 2c, 2d).

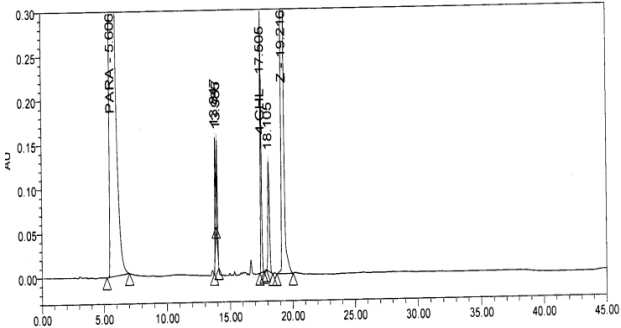
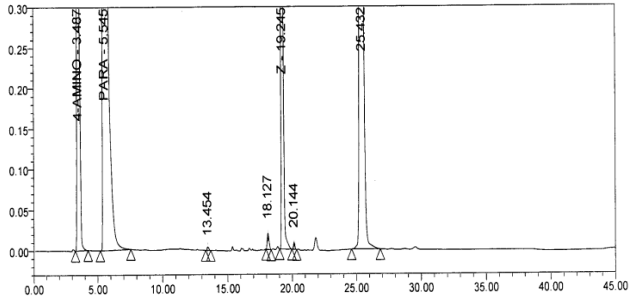


Fig. 2 b: Acid degradation combined sample.(1hr, 1M HCl)

Fig. 2 c: Basic degradation combined sample (1hr, 1N NaOH)

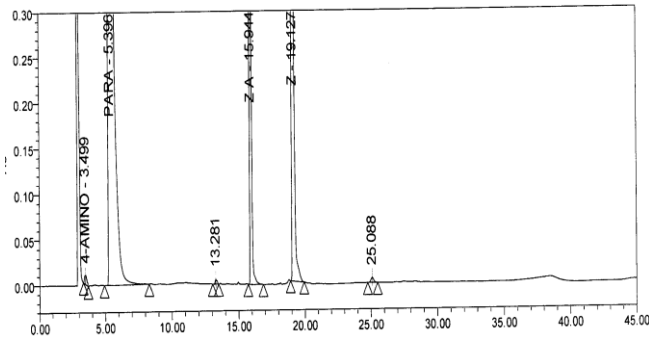


Fig. 2 d: Oxidation combined sample (1hr, 30% H₂O₂)

Method validation

Method validation was carried out as per ICH guidelines for parameters such as Precision, linearity, accuracy, Limit of detection and quantitation, robustness, response factor and stability in solution [22].

Precision

The precision of the related substance method was checked by six fold analysis of tablet sample spiked with 0.1% of each of the four impurities. The RSD (%) of peak area for each impurity which was within 5% confirmed precision of the method.

Limit of detection (LOD) and quantification (LOQ)

LOD and LOQ for all the impurities and both the drugs were estimated as the amounts for which the signal-to-noise ratios were 3:1 and 10:1 respectively. The study was performed by injecting a series of dilute solutions of known concentration within the developed chromatographic conditions. The limit of detection for all studied impurities was below 0.01 % and limit of quantitation was below 0.04 % of the test concentration. Table (1).

Linearity

Solution for testing linearity for the related substances was prepared by diluting the impurity stock solution to seven different concentrations from the LOQ to 150 % of the permitted maximum level of the impurity. The correlation coefficient, slope, and y-intercept for each impurity and analytes assured linearity in the studied range Table (1).Table 1: Summary of validation study Response factor is a relative term, being the response of equal weights of one substance relative to that of another in the conditions described in the test. The RRF value for given impurity as the ratio of response of peak at a particular concentration to that of analyte peak at that concentration. The RRF values less than 0.2 and more than 5.0 are not acceptable as per European pharmacopoeia. In such cases there is a need for change in chromatographic parameters like wavelength or different method of visualization is used. The RRF obtained for studied impurities are within 0.2 to 5.0. The extrapolation of linearity study was done to determine RRF and CF. Table (1).

Robustness

To determine the robustness of the method the experimental conditions were deliberately altered and checked for the system suitability criteria ie resolution between closely related peaks. The chromatographic conditions which were altered are flow rate ($\pm 0.2 \text{ mL min}^{-1}$; of mobile phase pH (± 0.2 units). The method was found to be robust for above variations as the resolution between closely eluting peaks was more than two and impurity profile, the elution pattern of impurities did not change

Table 1: Summary of validation study to include

Parameters	4A	PC	4N	ZA	4C	ZP	ZE	ZC	ZB	ZD
Retention time (min)	3.56	5.40	15.14	15.91	17.14	19.13	21.25	22.64	28.21	31.67
Relative retention time (wrt ZP)	0.2	0.3	0.80	0.83	0.90	1.0	1.11	1.19	1.49	1.67
Tailing factor	1.2	1.1	1.0	0.98	1.1	0.98	0.96	1.1	0.97	1.1
Resolution (USP)	NA	5.9	44.3	4.9	8.4	13.8	11.3	4.8	12.7	6.2
Linearity range ($\mu\text{g mL}^{-1}$)	0.7 - 7.5	0.1 - 7.5	0.1 - 7.5	0.08 - 1.2	0.1 - 7.5	0.08 - 1.2	0.04 - 1.2	0.16 - 1.2	0.32 - 1.2	0.24 - 1.2
LOD %	0.005	0.001	0.001	0.006	0.001	0.003	0.002	0.007	0.015	0.01
LOD ($\mu\text{g mL}^{-1}$)	0.243	0.043	0.045	0.048	0.030	0.027	0.016	0.054	0.121	0.080
LOQ %	0.015	0.003	0.003	0.018	0.002	0.010	0.006	0.020	0.046	0.030
LOQ ($\mu\text{g mL}^{-1}$)	0.739	0.130	0.137	0.147	0.089	0.082	0.048	0.162	0.366	0.244
Slope (b)	1098832	89210	34884	72707	109832	78463	117715	61726	39628	82074
Intercept (a)	-1621	-1050	-1360	-670	-1621	-2656	-1977	-2082	6478	-245
Correlation coefficient	1.00	1.00	1.00	0.9990	1.00	0.9997	0.9999	0.9988	0.9929	0.9975
Residual sum of squares (r^2)	1.00	1.00	0.9999	0.9998	1.00	0.9995	0.9950	0.9975	0.9858	0.9950
Response factor	0.3	1.0	0.4	0.9	1.2	1	1.5	0.8	0.5	1.04
Correction Factor	3.0	1.0	2.5	1.1	0.8	1	0.7	1.3	2.0	0.96

Relative response factor (RRF) and correction factor (CF) study

Response factor is a relative term, being the response of equal weights of one substance relative to that of another in the conditions described in the test. The RRF value for given impurity as the ratio of response of peak at a particular concentration to that of analyte peak at that

concentration. The RRF values less than 0.2 and more than 5.0 are not acceptable as per European pharmacopoeia. In such cases there is a need for change in chromatographic parameters like wavelength or different method of visualization is used. The RRF obtained for studied impurities are within 0.2 to 5.0. The extrapolation of linearity study was done to determine RRF and CF. Table (1).

Table 2: Results of Accuracy and Precision study

Parameters	Related Compounds							
	4A	4N	ZA	4C	ZE	ZC	ZB	ZD
Mean value (%) of spiked samples at 0.1% of test conc (CV, %) (n = 6)	0.098	0.096	0.155	0.097	0.114	0.101	0.179	0.099
(CV, %) (n = 12)	7.7	0.22	0.37	0.10	1.00	1.4	4.4	4.1
Accuracy /Recovery								
at 80 % level	95.3	100.1	99.9	99.5	104.9	105.9	109.9	97.5
at 100 % level	89.5	99.0	103.1	98.5	106.2	101.5	102.6	99.8
at 120 % level	88.2	99.3	105.9	97.8	108.3	101.4	98.3	103.5
mean	91.0	99.5	103.0	98.6	106.5	102.9	103.6	100.3

Table 3: Results of Excipient Compatibility Study performed for three weeks

Samples	Condition	4a	ZA	ZB	RRT 0.84	Total impurities
PC:ZP (6.25:1)	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.09	0.21
	40°C, 75% RH	0	0.05	0.09	0.1	0.24
Maize starch	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.1	0.22
	40°C, 75% RH	0	0.05	0.09	0.1	0.24
Pregelatinised starch	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.05	0.08	0.09	0.22
	40°C, 75% RH	0	0.06	0.09	0.11	0.26
Magnesium stearate	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.03	0.08	0.09	0.2
	40°C, 75% RH	0	0.04	0.09	0.09	0.22
Steric acid	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.06	0.08	0.09	0.23
	40°C, 75% RH	0.015	0.07	0.09	0.11	0.285
Cross carmelose sodium	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.08	0.2
	40°C, 75% RH	0	0.04	0.08	0.1	0.22
Sodium starch glycolate	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.09	0.21
	40°C, 75% RH	0	0.04	0.09	0.1	0.23
Titanium dioxide	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.09	0.21
	40°C, 75% RH	0	0.05	0.08	0.11	0.24
Hypromellose	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.1	0.22
	40°C, 75% RH	0	0.05	0.08	0.1	0.23
Sodium lauryl sulphate	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.05	0.08	0.09	0.22
	40°C, 75% RH	0	0.04	0.09	0.1	0.23
Tween 80	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.05	0.08	0.09	0.22
	40°C, 75% RH	0	0.06	0.09	0.1	0.25
Gelatin	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.04	0.08	0.1	0.22
	40°C, 75% RH	0.017	0.05	0.09	0.1	0.257
Crosspovidone	Initial	0	0.03	0.08	0.09	0.2
	55°C	0	0.08	0.08	0.1	0.26
	40°C, 75% RH	0	0.09	0.09	0.11	0.29

Accuracy

For the impurities, recovery was determined in triplicate for 0.08, 0.1, and 0.12 % of the analyte concentration (5000 µg mL⁻¹ PC and 800 µg mL⁻¹ for ZP) and % recovery of the impurities were calculated found within 85 to 115% Table (2).

Stability in Solution

The stability of test solution was determined by keeping test solutions of the sample and spiked sample solution volumetric flasks at room temperature for 72 h at bench top and withdrawn at regular time intervals to assay the analytes.

The absolute difference of individual impurity value within 0.05% from initial value concluded solution stability for 12 h.

Application of method

The related substances method was successfully applied to study the drug is to excipient compatibility as a part of preformulation. The increase in degradant impurities exponentially during the scheduled storage of 3 weeks was indicative of incompatibility of the particular excipient in the selected ratio. Both the drug in a ratio of 6.25: 1; PC:ZC are found compatible with each other and also found compatible with most of the excipients selected. The data is presented in (Table 3).

CONCLUSION

The isocratic RP-LC method for simultaneous determination of related substances of ZP and PC in simultaneously is precise, accurate and specific. The results obtained from the method were satisfactory. Stress study revealed degradation pathways of few impurities. The method is stability indicating and can be used for routine analysis of production samples and stability studies and also successfully used to study drug excipient compatibility study.

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