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## Simultaneous Quantitative Determination of Imidazole Antimycotics in Human Urine by using RPLC Technique

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### ABSTRACT

Imidazoles are well known heterocyclic compounds having important feature of a variety of medicinal agents. Studies with imidazole containing compounds are great importance in the clinical use of drugs. The presence of imidazole group in the molecules of Itraconazole (LCZ) and butoconazole (BCZ) is very important for its chemical and analytical behavior. Target analytes were efficiently separated with a Pinnacle DB Cyano column (5  $\mu\text{m}$ , 4.6 x 250 mm I.D.) and oxiconazole (OCZ) used as an internal standard. In this study, correlation between the retention factors of the solutes and pH of the mobile phase was determined at ten pH values in acetonitrile-water binary mixture. In optimization condition, quantification of these drugs were performed in human urine. The linear responses were observed over a wide concentration range ( $6.25 \cdot 10^{-6}$ - $3.13 \cdot 10^{-5}$  mol L<sup>-1</sup> for LCZ and  $6.32 \cdot 10^{-6}$  -  $2.53 \cdot 10^{-5}$  mol L<sup>-1</sup> for BCZ) in human urine. The method were validated by determining the limits of detection (LODs,  $2.01 \cdot 10^{-6}$  -  $5.50 \cdot 10^{-7}$  mol L<sup>-1</sup>), the limits of quantification (LOQs,  $6.08 \cdot 10^{-6}$  -  $1.67 \cdot 10^{-6}$  mol L<sup>-1</sup>), recoveries (99.14%, 99.93%) and intra-day and inter-day relative standard deviations (RSDs, <1%). The data presented in this report demonstrate a rapid, sensitive and accurate method for the simultaneous analysis of LCZ and BCZ in human urine for pharmacokinetic studies.

**Keywords:** imidazole antimycotics, RPLC, method validation, human urine

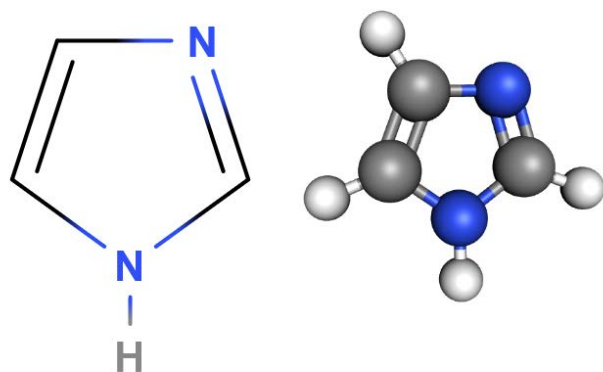
### INTRODUCTION

Antimycotics are a group of pharmaceuticals with increasing consumption rates. Imidazoles constitute the most important class of antimycotic drugs which have broad-spectrum antifungal activities against wide range of fungi that cause many of mycotic infections. This aromatic heterocyclic structure is a "1,3-diazole" and is classified as an alkaloid (**Figure 1**). Imidazole ring is amphoteric, because it functions as an acid as well as a base. Basic pK<sub>a</sub> of imidazole ring is about 6.90. [1].

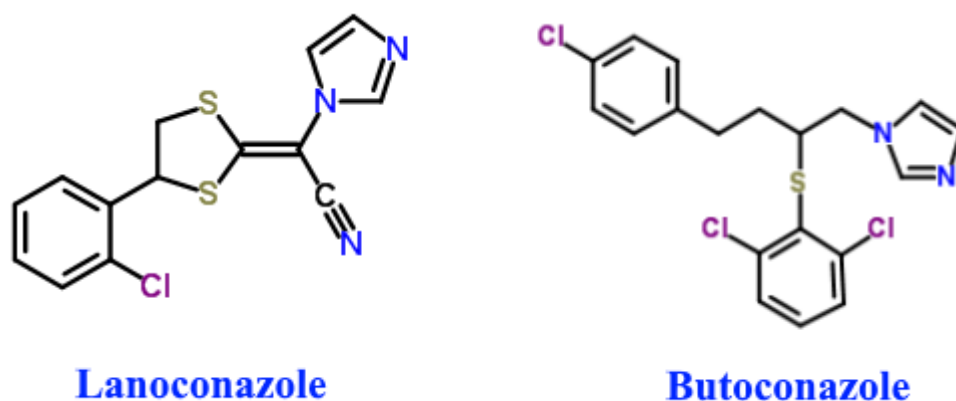
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**Figure 1.** Chemical structure of imidazole ring



**Figure 2.** Chemical structures of LCZ and BCZ

The use of imidazoles and their derivatives in chemical processes is becoming increasingly important. Derivatives of these strongly polar compounds are widely used in pharmacology. On the basis of various literature surveys, imidazoles and their derivatives show various pharmacological activities such as antibacterial activity, anticancer activity, antitubercular activity, antifungal activity, analgesic activity and anti-HIV activity. In addition to these efficiencies, these drugs have a good safety profile, sustained cutaneous retention and low systemic absorption, all of which make it ideal for topical applications [2].

There are several methods used for the analysis of imidazole compounds and also their various structure reactions offer enormous scope in the field of analytical chemistry. In this study, reversed phase liquid chromatography (RPLC) method was used to obtain determination and separations of two imidazole drugs: Lanoconazole (LCZ) and butoconazole (BCZ) (**Figure 2**). Lanoconazole is a racemic imidazole antimycotic which chemically has a dithiolane ring in addition to the imidazole ring. Butoconazole is an imidazole derivative that

has fungicidal activity and has been demonstrated to be clinically effective against vaginal infections [3].

Chromatographic techniques have been very useful for clinical analysis, with advantages of simultaneous measurements of different components and elimination of interfering species. RPLC is the most popular mode among these techniques. Almost 90% of all analysis of low molecular weight sample is carried out using RPLC [4]. In addition to this, the RPLC method intended to be applied for the pharmaceutical or industrial environment, the analysis time is usually optimized simultaneously without losing resolution [5]. Mobile phase pH and composition are among the main parameters used to control RPLC retention of most pharmaceutical compounds and to optimize separations. Developing and optimizing an isocratic RPLC method is a complex procedure that requires simultaneous determination of several factors such as the type and composition of the organic phase, column temperature, flow rate, pH, type of the stationary phase etc. The simultaneous analytical analysis provides specificity and assurance for the identification of the chemical entities in the biological fluids or pharmaceutical formulation. Also, simultaneous quantification is user-friendly due to its advantages of less solvent consumption and operation time [4].

Literature reviews indicate that many different methods including HPLC, gas chromatography, capillary electrophoresis for BCZ and LCZ determination [6-13]. However, isocratic RPLC method has not been evaluated in determination of BCZ and LCZ. Moreover, no references have been found for simultaneous optimization study using chromatographic behaviours of LCZ and BCZ. The difference of this study, combined effect of acetonitrile content and pH of the mobile phase on the retention behavior of LCZ and BCZ were used.

## EXPERIMENTAL

### Chemicals and Reagents

All reagents and chemicals used in this study were analytical grade. Lanconazole (LCZ) and butoconazole nitrate (BCZ) were purchased from Sigma-Aldrich (USA). Oxiconazole nitrate (OCZ) was chosen as internal standard (IS) and purchased from United States Pharmacopeia (USP) Reference Standard (Sigma-Aldrich, USA). Acetonitrile (as organic modifier), orthophosphoric acid, sodium hydroxide and uracil were bought from Sigma-Aldrich (USA). Potassium hydrogen phthalate (standard buffer) was obtained from Merck (Darmstadt, Germany).

### Equipment

The HPLC system (Shimadzu Technologies, Kyoto, Japan) consisted of a LC-20AD pump, equipped with a manual injector, variable UV detector (SPD-M20A), column oven (CTO-10AS VP) and a degasser unit (DGU-20A3). Data acquisition was carried out by computer assistance using the LC solution Software. pH measurements of the mobile phase were carried out with a Mettler Toledo MA 235 pH/ion analyzer (Schwerzenbach,

Switzerland) using M-T combination pH electrode. Chromatographic separation was carried out on a Pinnacle DB Cyano column (5  $\mu\text{m}$ , 4.6 x 250 mm I.D.), Restek Corporation (Bellefonte, PA).

### Chromatographic Procedure

RPLC study was performed under isocratic conditions and the mobile phase used was acetonitrile:water binary mixture (45:55, v/v). Studied mobile phase were prepared in different pH values. The pH of the mobile phase containing 25 mM o-phosphoric acid was adjusted between 3.0 and 7.5 by adding 1 M sodium hydroxide. Chromatographic measurements were done at 25 °C with an eluent flow rate of 1 mL min<sup>-1</sup>. The detection was performed at 202 nm for all compounds. Three injections (20  $\mu\text{L}$ ) of each solutions were injected into the chromatographic system. The retention factor ( $k$ ) was calculated as  $(t_R - t_0)/t_0$ , where  $t_R$  is the retention of the analytes and  $t_0$  is the retention time of the non-retained compound (uracil).

### Preparation of Reference Standard Solutions

A primary stock solution containing about  $3.13 \cdot 10^{-4}$  mol L<sup>-1</sup> of the LCZ and  $2.11 \cdot 10^{-4}$  mol L<sup>-1</sup> of the BCZ and  $2.03 \cdot 10^{-4}$  mol L<sup>-1</sup> of OCZ (IS) in mobile phase were prepared. Working standards in mobile phase were prepared from the above stock solution for the related compound determination. All solutions were kept at 4°C and brought to room temperature before use.

### Preparation of the Calibration Standards

Standard calibration graphs were constructed for LCZ and BCZ by plotting the ratio of the peak area of the drug to that of IS against the drug concentration. Calibration standards were prepared by spiking urine with the appropriate amounts of stock solutions. Urine calibration solutions were prepared by adding LCZ at the concentration  $6.25 \cdot 10^{-6}$ ;  $9.38 \cdot 10^{-6}$ ;  $1.25 \cdot 10^{-5}$ ;  $1.56 \cdot 10^{-5}$ ;  $1.88 \cdot 10^{-5}$ ;  $2.50 \cdot 10^{-5}$  and  $3.13 \cdot 10^{-5}$  mol L<sup>-1</sup> and BCZ at the concentrations  $6.32 \cdot 10^{-6}$ ;  $1.05 \cdot 10^{-5}$ ;  $1.26 \cdot 10^{-5}$ ;  $1.68 \cdot 10^{-5}$ ;  $1.90 \cdot 10^{-5}$ ;  $2.11 \cdot 10^{-5}$  and  $2.53 \cdot 10^{-5}$  mol L<sup>-1</sup> into blank human urine. Each concentration was analyzed by triplicate injections. During the experiment, the concentration of IS was kept at a constant level of  $2.03 \cdot 10^{-6}$  mol L<sup>-1</sup>.

### Procedure to Analyze LCZ and BCZ in Human Urine

Twentyfour hour drug-free human urine samples were collected from healthy individuals and stored at -20°C before analysis. The urine samples were collected over a 4-h period in plastic containers before analysis. Potentially interfering compounds need to be removed before analysis. Protein precipitation of urine samples was carried out by addition of mobile phase. After thawing to ambient temperature, 1 mL of urine was mixed with 9 mL of acetonitrile in order to precipitate proteins from the specimen, vortexed for 3 min. The resulting different concentrations of LCZ and BCZ and  $2.03 \cdot 10^{-6}$  mol L<sup>-1</sup> of IS were added to

analysis. Spiked urine samples were filtered through the 0.45  $\mu\text{m}$  Millipore-Millex HN membrane filters. A 20  $\mu\text{L}$  volume of each sample was injected into the HPLC system.

### Method Validation

The developed method was evaluated with validation parameters recommended by International Conference on Harmonisation (ICH) [14] such as linearity, accuracy, precision and sensitivity. Linearity was checked by constructing the calibration curves using spiked drug-free urine samples, at concentration levels in the range between  $6.25 \cdot 10^{-6}$ - $3.13 \cdot 10^{-5}$  mol L<sup>-1</sup> and  $6.32 \cdot 10^{-6}$ - $2.53 \cdot 10^{-5}$  mol L<sup>-1</sup> for LCZ and BCZ, respectively. Calibration curves for LCZ and BCZ with the respective correlation coefficient were calculated by linear regression analysis of the peak area ratio of each compound to IS. The limits of detection (LOD) were calculated using the equation  $LOD = 3.3 \times \sigma/S$  and the limits of quantification (LOQ) were calculated by the equation  $LOQ = 10 \times \sigma/S$ , where  $\sigma$  is the standard deviation of regression analysis and  $S$  is the slope of linear calibration curve. The intra-day and inter-day precision of method was carried out by peak area ratio, then RSD% was calculated. To ensure the reliability and accuracy of the method recovery studies were performed by adding the known amount of pure drug to pre-analyzed samples of human urine samples.

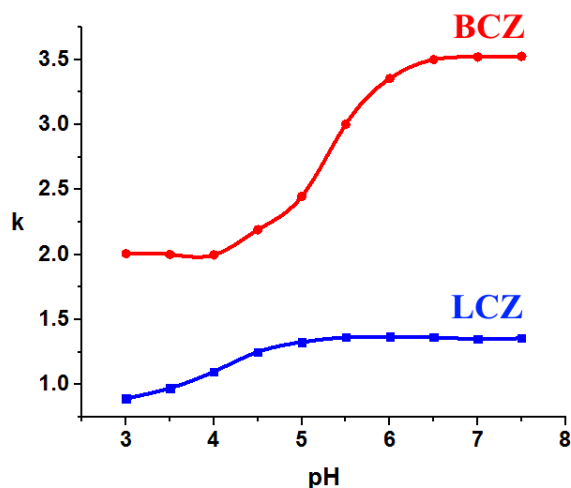
## RESULTS AND DISCUSSION

### Optimization of Chromatographic Separation

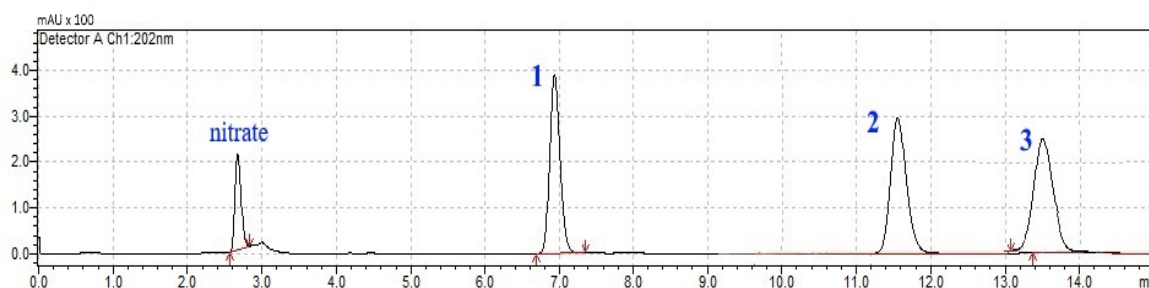
Variation of the mobile phase pH is a powerful parameter to enhance the chromatographic selectivity and retention for basic compounds. Mobile phase pH affects the analyte ionization. Knowing the pK<sub>a</sub> of the analytes permits an effective choice of mobile phase pH. If the target analyte is ionizable, the pK<sub>a</sub> of the analyte should be determined or obtained. The optimal pH to commence method development is at a pH that is at least 1-2 units from the analyte pK<sub>a</sub> in the particular hydroorganic mixture that is employed.

In this work, for optimization of separation of LCZ and BCZ was used correlation between retention factors and pH of mobile phase. Concentration of organic modifier such as acetonitrile was kept constant. The effect of the pH of mobile phase on chromatographic separation was studied. Retention factors ( $k$ ) were determined in 45% (v/v) acetonitrile-water binary mixture and studied pH values.  $k$  values were calculated to pH range of 3.0-7.5. In the literature, C18 and C8 columns have been used for determination of studied drugs. In this study, the best results were obtained by use of a cyano column. Pinnacle DB Cyano column was selected for method optimization and validation. This column is ideal for analyzing a wide range of compounds, from acidic to basic. As shown in **Figure 3**, the experimental data obtained at 45% (v/v) mobile phase mixture ( $k$  vs pH) are plotted for LCZ ( $3.13 \cdot 10^{-4}$  mol L<sup>-1</sup>) and BCZ ( $2.11 \cdot 10^{-4}$  mol L<sup>-1</sup>), demonstrating the sigmoidal curves.

Experiments were with varying mobile phase pH, while keeping all other parameters constant. It was observed from **Figure 3** that these compounds were completely resolved.



**Figure 3.** Values of retention factor versus pH of the mobile phase for LCZ and BCZ



**Figure 4.** Chromatogram of standard mixture (1) LCZ ( $1.56 \cdot 10^{-5}$  mol L<sup>-1</sup>), (2) IS ( $2.03 \cdot 10^{-6}$  mol L<sup>-1</sup>), (3) BCZ ( $1.68 \cdot 10^{-5}$  mol L<sup>-1</sup>). HPLC conditions; flow rate: 1 mL min<sup>-1</sup>, Cyano column (250 x 4.6 mm I.D.), UV detector (202 nm), injection volume: 20  $\mu$ L

According to the peak values determined by the ICH, the retention factor must be between 1 and 10. Likewise, the selectivity factor should be greater than 1.15 and the resolution should be greater than 1.5. These criteria have been taken into account in selection of optimum chromatographic condition. Moreover, mobile phase pH was selected at least one unit away from the pK<sub>a</sub> values of the drugs. So the optimum pH condition of the mobile phase was determined at pH 7.

The parameters of optimum separation condition were calculated and all compounds are well separated in an analysis time of about 14 min. Typical chromatogram of optimal condition are shown in **Figure 4**. Using the described analytical method, an optimal resolution of the analytes was achieved.

### System Suitability

The system suitability tests were carried out as per ICH requirements. The system suitability was assessed by five replicate analyses of the standard solutions of LCZ ( $1.56 \cdot 10^{-5}$

**Table 1.** System suitability parameters for RPLC analysis of LCZ, BCZ and IS

Name of Compound	Retention Time	Retention Factor	Selectivity	Resolution	Tailing	Plate Count
LCZ	7.192	1.413	-	-	1.247	11533
OCZ (IS)	11.796	2.958	2.093	11.269	1.321	13339
BCZ	13.578	3.556	1.202	3.896	1.236	14102

**Table 2.** Linear regression analysis parameters for the determination of LCZ and BCZ

Compounds	Concentration Range (mol L <sup>-1</sup> )	Linear Regression Parameters (n=7)		
		Slope ± S.D.	Intercept ± S.D.	r
LCZ	6.25.10 <sup>-6</sup> -3.13.10 <sup>-5</sup>	206273 ± 5804	0.197 ± 0.109	0.997
BCZ	6.32.10 <sup>-6</sup> -2.53.10 <sup>-5</sup>	180951 ± 1649	-0.072 ± 0.032	0.999

mol L<sup>-1</sup>) and BCZ (1.68.10<sup>-5</sup> mol L<sup>-1</sup>) and the parameters obtained with 20 µL injection volume. Under optimal conditions, retention times and system suitability test parameters (retention factor, selectivity, resolution, tailing, symmetry factor and plate count) were also evaluated for satisfying validation requirements. For example system suitability may be specified as:

1. Resolution of  $\geq 1.5$  for all components in a mixture,
2. Selectivity factor of all compounds  $\geq 1.15$ ,
3. Plate count of  $>2000$  for all components,
4. Tailing factor of all peaks  $\leq 2$ ,
5. Retention factor range 1-10.

The obtained results (**Table 1**) confirmed that the method is highly suitable for its intended purpose of separation LCZ and BCZ and its simultaneous determination in urine.

### Linearity of Calibration Standards

The peak area ratios of the drugs to IS were linear in the range 6.25.10<sup>-6</sup>-3.13.10<sup>-5</sup> mol L<sup>-1</sup> and 6.32.10<sup>-6</sup>-2.53.10<sup>-5</sup> mol L<sup>-1</sup> for LCZ and BCZ, respectively. The slope, intercept and correlation coefficient (r) obtained from regression analysis are shown in **Table 2**. The correlation coefficients were all greater than 0.99, indicating high degrees of correlation and good linearity of the method.

### Sensitivity (Detection and quantification limits)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. LOD values were found 2.01.10<sup>-6</sup> and 5.50.10<sup>-7</sup> mol L<sup>-1</sup> for LCZ and BCZ, respectively. The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. LOQ values were found 6.08.10<sup>-6</sup> and 1.67.10<sup>-6</sup> mol L<sup>-1</sup> for LCZ and BCZ, respectively. These results indicate that the method provided adequate sensitivity.

**Table 3.** Intra-day and inter-day precision of LCZ and BCZ

Compound	Concentration (mol L <sup>-1</sup> )	Intra-day RSD%	Inter-day RSD%
LCZ	9.38.10 <sup>-6</sup>	0.683	0.838
	2.50.10 <sup>-5</sup>	0.747	0.945
BCZ	1.05.10 <sup>-5</sup>	0.485	0.977
	2.11.10 <sup>-5</sup>	0.220	0.329

**Table 4.** Summarized results of recoveries of standards added to human urine at different concentrations

Concentration Spiked (mol L <sup>-1</sup> )	Recovery % ± S.D.	RSD %
<b>LCZ</b>		
9.38.10 <sup>-6</sup>	99.319 ± 0.836	0.677
1.25.10 <sup>-5</sup>	98.756 ± 0.343	0.280
1.56.10 <sup>-5</sup>	99.342 ± 0.358	0.290
<b>BCZ</b>		
1.05.10 <sup>-5</sup>	98.781 ± 0.420	0.343
1.26.10 <sup>-5</sup>	100.738 ± 0.992	0.901
1.68.10 <sup>-5</sup>	100.280 ± 0.811	1.187

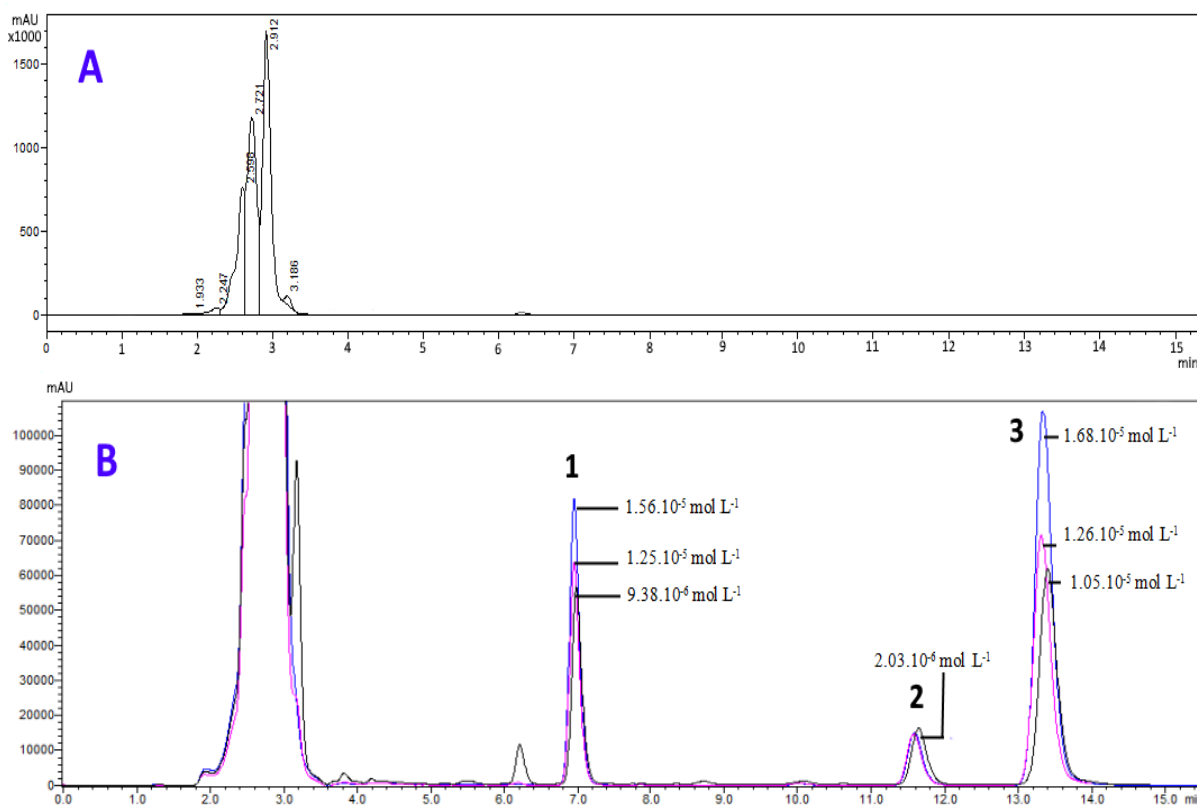
### Precision

Intra-day and inter-day variations were chosen to determine the precision of the improved assay. Intra-day precision was validated with two concentrations of mixed standard solutions under the optimized conditions for five times within 1 day. Inter-day precision was validated with the mixed standard solutions used above once a day for 3 consecutive days. Inter-day and intra-day precisions for investigated compounds were expressed as relative standard deviation (RSD%) (Table 3). Based on the obtained RSD values, it can be said that the precision of the method is quite sufficient.

### Accuracy and Recovery Studies

The accuracy of the method was verified by means of recovery studies, adding known amounts of LCZ and BCZ standard solutions to a known amount of urine and subjecting the mixture to the usual preparation procedure (n=5) for each concentration tested. Human blank urine was spiked with 9.38.10<sup>-6</sup>; 1.25.10<sup>-5</sup> and 1.56.10<sup>-5</sup> mol L<sup>-1</sup> of LCZ and 1.05.10<sup>-5</sup>; 1.26.10<sup>-5</sup> and 1.68.10<sup>-5</sup> mol L<sup>-1</sup> of BCZ. The peak areas of LCZ and BCZ were compared with those obtained of calibration curves constructed with blank urine. Typical chromatogram of the blank human urine and urine sample spiked with LCZ, BCZ and IS are shown in Figure 5. No interfering peaks were found at the retention times of LCZ, BCZ and IS. Table 4 demonstrate that the recoveries of the drugs from three spiked concentrations. The average recovery of this analytical method was 99.14% and 99.93% for LCZ and BCZ, respectively. The results provide evidence that there was no major loss during sample processing.





**Figure 5.** Chromatogram of blank urine (A) and chromatogram of urine spiked with three different concentrations of standard solutions (B). [1] LCZ, [2] IS, [3] BCZ

## CONCLUSION

This work represents the first study dealing with the investigate the influence of combined effect of pH and organic modifier concentration on the retention behaviour of LCZ and BCZ. RPLC method was optimized by changing mobile phase pH in constant amount of organic modifier. The method used was enabled easy prediction of the chromatographic behaviour of LCZ and BCZ. The main advantage of the method is the ability to analyze simultaneously the three compounds (LCZ, BCZ and OCZ) under similar conditions. The achieved method validation results show the reliability of the method. The high recovery values also showed that the drugs did not bind to urine proteins. The results obtained suggests that this method could be used suitably for simultaneous determination of LCZ and BCZ in urine.

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