Simultaneous Estimation of Terconazole and Benzoic Acid in Bulk and Creams Using RP-HPLC, Derivative Spectrophotometry and Chemometric Techniques

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ABSTRACT

Terconazole (TER) (anti-fungal) is commonly co-formulated with benzoic acid (BZO) (preservative) in pharmaceutical preparations. So, three simple, precise and selective analytical methods were developed and validated for estimation of TER and BZO simultaneously. The first method based on high performance liquid chromatographic (HPLC) separation of TER and BZO using a mixture of water [containing 0.2%, v/v TEA, pH adjusted to 3.5 with orthophosphoric acid]: acetonitrile: (30:70, v/v) as the mobile phase at a flow rate 1 mL min⁻¹ at ambient temperature on an Agilent TC-C18 (2) column (250 mm × 4.6 mm, 5 μm) with UV detection at 250 nm and 225 nm for TER and BZO, respectively. The linearity range was found to be (4-128 μg mL⁻¹) for TER and (4-128 μg mL⁻¹) for BZO. The second method based on first order derivative spectrophotometry. Trough amplitudes (D¹) of TER and BZO were measured at 320 nm and 283 nm, respectively using acetonitrile as solvent. The linearity range was found to be (5-40 μg mL⁻¹) for TER and (4-15 μg mL⁻¹) for BZO. The third method based on three multivariate calibration chemometric techniques, namely, classical least squares (CLS), principal component regression (PCR), and partial least-squares (PLS) using mixtures containing the two compounds in acetonitrile. Calibrations were constructed using the absorption data matrix corresponding to the concentration data matrix. The method is valid over concentration range (3-11 μg mL⁻¹) for TER and (1.4-5.6 μg mL⁻¹) for BZO. The three proposed methods were validated according to international conference on harmonization (ICH).

Keywords: Terconazole; Benzoic acid; RP-HPLC; Derivative spectrophotometry; Chemometry

INTRODUCTION

Terconazole (TER) (Figure 1a), is a triazole derivative that is thought to disrupt normal fungal cell membrane permeability. It is used in the local treatment of vulvovaginal candidiasis [1]. Chemically, it is 1-[4-[(2RS,4SR)-2-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy[phenyl]-4-(1 methylethyl) piperazine. It has a molecular formula of C₂₆H₃₁Cl₂N₅O₃ and a molecular weight 532.5 [2].
Benzoic acid (BZO) (Figure 1b) has antibacterial and antifungal properties. It is used as preservatives in pharmaceutical formulations [1]. It has a molecular formula of C₇H₆O₂ and a molecular weight 122.1 [2].

The literature review revealed that two spectrophotometric methods were applied to estimate TER in bulk and pharmaceutical dosage forms [3,4]. Besides, TER was estimated alone or in combination with other drugs using liquid chromatographic methods [5-8]. BZO was estimated in combination with salicylic acid using spectrophotometric method [9]. BZO was determined using HPLC methods [10-13].

The careful literature review revealed that no analytical method was reported to estimate TER and BZO simultaneously. Besides, the combination of TER with BZO was not officially reported in any pharmacopoeia. So the aim of this work was to develop and validate three alternative analytical methods to estimate TER and BZO simultaneously using RP-HPLC, derivative spectrophotometry and multivariate calibration chemometric techniques. The developed methods were validated according to ICH Guidelines [14]. The three proposed methods are suitable for the quality control analysis of bulk and dosage forms containing both compounds.

**Figure 1:** Chemical structures of (a) terconazole, (b) benzoic acid.

### EXPERIMENTAL SECTION

#### Instrumentation

A chromatographic system consisting of Agilent 1200 series (CA, USA); interface equipped with an Agilent quaternary pump G1311A, Agilent UV-visible detector G1314B, an Agilent manual injector G1328B equipped with (20 µl) injector loop, an Agilent degasser G1322A and an Agilent syringe, LC 50 µl. Separation and quantitation were made on an Agilent TC-C18 (2) column (5µm, 4.6 x 250 mm).

Spectrophotometer used was a double beam ultraviolet/visible spectrophotometer Shimadzu UV-1601 PC (Tokyo, Japan) connected to an IBM compatible computer and supported with UVPC software version 3.7 was used. The spectral bandwidth was 2 nm with quartz cell of 1 cm path length. A data processing program (Matlab™) version 7.10.0.499 and (PLS) Toolbox 2.0 was also used. The absorbance spectra of the test and reference solutions were measured in 1-cm quartz cell over the range 200-400 nm. Ultrasonic-degasser processor, Soniclean (Australia) and Elma S100 model KBK 4200 (Germany) were used.

pH meter; Jenway 3505, Essex-UK was used.

#### Materials and reagents

Pharmaceutical grade TER certified to contain 99.78%, was supplied by Multi- Apex pharma (Cairo, Egypt). Pharmaceutical grade BZO certified to contain 99.80 %, was supplied by Multi-Apex pharma (Cairo, Egypt). Gynoconazole 0.4%™ cream nominally containing 400 mg terconazole and 200 mg benzoic acid for each 100 gram cream (batch no. MR0160311), was supplied from Multi-Apex pharma (Cairo, Egypt). Acetonitrile HPLC grade (Scharlau, Spain) was used. Bi-distilled water was produced in-house (Aquatron Water Still, A4000D, U.K). Membrane filters 0.45 µm from Teknokroma (Barcelona, Spain) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise.

#### Solutions

**Standard solution preparation:**

**HPLC method:** A solution of 200 µg mL⁻¹ of each TER and BZO in the mobile phase (a mixture of water [containing 0.2%, v/v TEA, pH adjusted to 3.5 with orthophosphoric acid]: acetonitrile: (30:70, v/v)) was prepared. The required concentrations were prepared by serial dilutions with mobile phase.

**Derivative spectrophotometry method:** A solution of 100 µg mL⁻¹ of each TER and BZO in acetonitrile was prepared. The required concentrations were prepared by serial dilutions with acetonitrile.
Chemometry method: A solution of 25 μg mL\(^{-1}\) of each TER and BZO in acetonitrile was prepared. The required concentrations were prepared by serial dilutions with acetonitrile.

Laboratory-prepared mixtures:

**HPLC method:** Mixture solutions containing different concentrations of TER (10-120 μg mL\(^{-1}\)) and BZO (10-120 μg mL\(^{-1}\)) were prepared by transferring aliquots from their stock solutions into a series of 10-ml volumetric flasks and the volume of each was completed to the mark with the mobile phase.

**Derivative spectrophotometry method:** Mixture solutions containing different concentrations of TER (12-37 μg mL\(^{-1}\)) and BZO (5-14 μg mL\(^{-1}\)) were prepared by transferring aliquots from their stock solutions into a series of 10-mL volumetric flasks and the volume of each was completed to the mark with acetonitrile.

**Chemometry method:** Mixture solutions containing different concentrations of TER (3-11 μg mL\(^{-1}\)) and BZO (1.4-5.6 μg mL\(^{-1}\)) (training set) and of TER (4-10 μg mL\(^{-1}\)) and BZO (2-4.9 μg mL\(^{-1}\)) (validation set), were prepared by transferring aliquots from their stock solutions into a series of 10-mL volumetric flasks and the volume of each was completed to the mark with acetonitrile.

Sample preparation (cream sample preparation):

**HPLC method:** An amount of 2.5 grams of Gynoconazole 0.4%™ cream was accurately weighed in a conical flask and 40 mL of the mobile phase were added, and then the flask was placed in ultrasonic bath with aid of temperature not exceeding 40 °C for about 10 minutes. The flask was cooled and its content was transferred quantitatively to 50 mL volumetric flask and volume was completed to the mark with the mobile phase. The mixture was filtered through 0.45 μm membrane filters to obtain a sample solution of concentration equivalent to 200 μg mL\(^{-1}\) for TER and 100 μg mL\(^{-1}\) for BZO.

**Derivative spectrophotometry method:** An amount of 1.25 grams of Gynoconazole 0.4%™ cream were accurately weighed in a conical flask and 40 mL of acetonitrile were added, and then the flask was sonicated with the aid of temperature not exceeding 40°C for about 10 minutes. The flask was cooled and its content was transferred quantitatively to 50 mL volumetric flask and volume was completed to the mark with acetonitrile and mixed. The mixture was filtered through 0.45 μm membrane filters to obtain a sample solution of concentration equivalent to 100 μg mL\(^{-1}\) for TER and 50 μg mL\(^{-1}\) for BZO.

**Chemometry method:** An amount of 1.5625 grams of Gynoconazole 0.4%™ cream was accurately weighed in a conical flask and 200 mL of acetonitrile were added, and then the flask content was placed in ultrasonic with aid of temperature not exceeding 40 °C for about 10 minutes. The flask was cooled and its content was transferred quantitatively to 250 mL volumetric flask and volume was completed to the mark with acetonitrile. The mixture was filtered through 0.45 μm membrane filter to obtain a sample solution of concentration equivalent to 25 μg mL\(^{-1}\) for TER and 12.5 μg mL\(^{-1}\) for BZO.

Procedure

Construction of the calibration curves:

**HPLC method:** Accurately measured aliquots of standard stock solutions of TER and BZO equivalent to (40-1280 μg) and (40-1280 μg) respectively, were separately transferred into two series of 10 mL volumetric flasks and completed to volume with mobile phase. Chromatographic separation was achieved on an Agilent TC-C18 (2) column (250 mm x 4.6 mm, 5 μm) applying an isocratic elution. The flow rate was set at 1mL min\(^{-1}\). Analysis was performed at ambient column temperature and detection was programmed to be at 250 nm from 0-3.4 min and 225 nm from 3.4-5 min for TER and BZO, respectively. Twenty μL aliquot of each solution was injected in triplicates onto the chromatograph. Calibration curve was constructed by plotting the area under peak (AUP) against the corresponding concentrations (C) of each drug.

**Derivative spectrophotometry method:** Accurately measured aliquots of standard stock solutions of TER and BZO equivalent to (50-400 μg) and (40 -150 μg) respectively, were separately transferred into two series of 10 mL volumetric flasks and completed to volume with acetonitrile. The zero order absorption spectra of each solution was recorded against acetonitrile as a blank then The first order derivative zero crossing spectrophotometry technique was applied using Δλ=4 and scaling factor=100. Zero crossing first order derivative spectrophotometric method was
used and the trough amplitudes \((D^1)\) of the obtained first derivative spectra were measured at 283 nm for BZO where TER showed zero crossing and at 320 nm for TER where BZO showed zero level. A calibration curve was obtained for TER and BZO by plotting trough amplitude \((D^1)\) against the corresponding concentration \((C)\) of each drug.

**Chemometry method (Construction of the training set):** Fifteen binary mixtures of TER and BZO were prepared by transferring different volumes of their standard solutions into a series of 10 mL volumetric flasks and completed to the mark with acetonitrile (Table 1). The three multivariate calibration models (CLS, PCR, and PLS) were constructed using the data obtained. For the three techniques, the absorbance data matrix, were obtained by the measurement of absorbencies between 220 and 240 nm in the intervals of 0.2 nm. A training set design of the concentration data corresponding to TER and BZO mixtures was organized statistically to maximize the information content from the spectra and to minimize the error of multivariate calibrations.

**Assay of laboratory prepared mixtures and Gynoconazole 0.4%™ cream.**

**Laboratory prepared mixtures:**

**HPLC method:** Laboratory prepared mixtures containing different concentrations of TER \((10 \text{–} 120 \mu g \text{ mL}^{-1})\) and BZO \((10 \text{–} 120 \mu g \text{ mL}^{-1})\) prepared as directed under section (2.3.2) were injected onto the chromatogram, (Figure 2a).

**Derivative spectrophotometry method:** Laboratory prepared mixtures containing different concentrations of TER \((12 \text{–} 37 \mu g \text{ mL}^{-1})\) and of BZY \((5 \text{–} 14 \mu g \text{ mL}^{-1})\) prepared were scanned and processed as directed under section (2.3.2), (Figure 3a).

**Chemometry method (Construction of the validation set):** To evaluate the prediction performance of the proposed chemometric models, a set of six synthetic validation mixtures of TER and BZO containing \((4 \text{–} 10 \mu g \text{ mL}^{-1})\) and \((2 \text{–} 4.9 \mu g \text{ mL}^{-1})\), respectively, was prepared by transferring different volumes of their stock solutions into 10 mL volumetric flasks and processed as mentioned under section (2.4.1). The suggested models were applied to these mixtures to predict the concentrations of TER and BZO.

**Assay of Gynoconazole 0.4%™ cream:**

**HPLC method:** Sample solution prepared under section (2.3.2) was serially diluted with the mobile phase to get concentrations equivalent to \(15\text{–}96 \mu g \text{ mL}^{-1}\) TER and \(7.5\text{–}48 \mu g \text{ mL}^{-1}\) BZO. Samples were injected in triplicates (Figure 2b). Concentrations of TER and BZO were calculated using calibration equations.

**Derivative spectrophotometry method:** Sample solution prepared under section (2.3.2) was serially diluted with the acetonitrile to get concentrations equivalent to \(9\text{–}22 \mu g \text{ mL}^{-1}\) TER and \(4.5\text{–}11 \mu g \text{ mL}^{-1}\) BZO, (Figure 3b). Concentrations of TER and BZO were calculated using calibration equations.

**Chemometry method:** Aliquots of the filtered cream solution prepared under section (2.3.2) were serially diluted with acetonitrile to get concentrations equivalent to \(5\text{–}5.8 \mu g \text{ mL}^{-1}\) TER and \(2.5\text{–}2.9 \mu g \text{ mL}^{-1}\) BZO. The spectra of the prepared solutions were scanned then the developed multivariate models, CLS, PCR and PLS were applied to calculate the concentrations of TER and BZO.
RESULTS AND DISCUSSION

The literature review revealed that no analytical method was so far reported to analyze TER and BZO simultaneously. So, three different analytical techniques were developed and validated to estimate TER and BZO simultaneously using RP-HPLC, first order derivative spectrophotometry and multivariate calibration chemometric techniques (CLS, PCR and PLS).

Method development

HPLC method:
An isocratic mode was tried using various mobile phase compositions of water, methanol and acetonitrile, in different proportions and pH values. It was found that, an isocratic mode with at least 50% of acetonitrile and 50% of water was needed to elute TER and BZO but tailing of TER eluted peak was observed with low resolution between TER and BZO peaks. Increasing acetonitrile proportion up to 60%, much better resolution between TER and BZO peaks and tailing of TER eluted peak was minimized but still observed. Increasing acetonitrile proportion up to 70%, No tailing for TER peak was observed with reasonable analysis time and shape for TER and BZO eluted peaks. TEA was added to the aqueous component of mobile phase (0.2%, v/v) to minimize tailing of TER eluted peak. So best chromatographic separation was attained by using a mobile phase composed of water: acetonitrile: (30:70, v/v) adding triethylamine to aqueous component of mobile phase (0.2%, v/v) and at a flow rate 1 mL min⁻¹. Different pH values were tried at pH 3.5, pH 4.5, pH 5.5 and pH 7 and it was found that at pH 3.5, optimum resolution with reasonable peak shapes and retention times was observed. At pH values much higher than 3.5, excessive tailing for TER eluted peak was observed. So pH of aqueous component of mobile phase was adjusted at 3.5 using orthophosphoric acid solution. TER has the maximum absorbance at 250 nm, while BZO has maximum absorbance at 225 nm. So, a programmed detection was carried out at 250 nm from 0-3.4 min then at 225 nm from 3.4-5 min for the maximum sensitivity of eluted peaks. Best chromatographic separation was attained by using an Agilent TC-C18(2) column (250 mm x 4.6 mm, 5 µm). Changing column temperature had no effect on eluted peaks.

Derivative spectrophotometry method:
As zero order absorption spectra of TER in binary mixture with BZO shows overlapping between cited compounds (Figure 4) so, simple, accurate and precise zero crossing first order derivative spectrophotometric technique has been developed and validated to overcome problem of interference. For TER and BZO mixture the trough amplitudes of the obtained first derivative spectra were measured at 283 nm for BZO where TER showed zero crossing and at 320 nm for TER where BZO showed zero level (Figure 5).

Chemometry method:
Chemometric calibration techniques in spectral analysis is gaining importance in the quality control of drugs in mixtures and pharmaceutical formulations containing two or more drugs with overlapping spectra (Fig. 4) as these techniques do not need any separation procedure. TER and BZO were estimated simultaneously using three
chemometric techniques – classical least squares (CLS), principal component regression (PCR), and partial least-squares (PLS) using mixtures containing the two compounds in acetonitrile. So, three multivariate calibration models (CLS, PCR, and PLS) were constructed using the data obtained. For the three techniques, the absorbance data matrix for the training set concentration matrix, (Table 1), were obtained by the measurement of absorbencies between 220 and 240 nm in the intervals of 0.2 nm. Calibration or regression was obtained by using the absorbance data matrix and concentration data matrix for prediction of the unknown concentrations of TER and BZO in their binary mixtures and pharmaceutical formulations. CLS model was constructed with non-zero intercept. To build the CLS model, the computer was fed with the absorbance and concentration matrices for the training set. The calculations to obtain the K matrix were carried out. For the PCR and PLS models, the training set absorbance and concentration matrices together with PLS-toolbox 2.0 software were used for calculations. To select the optimum number of factors in the PLS and PCR algorithms, a cross-validation method leaving out one sample at a time [15] was employed using calibration set of fifteen calibration spectra. PLS and PCR calibration on fourteen calibration spectra were performed and, using this calibration, the concentration of the sample left out during the calibration process was predicted. This process was repeated fifteen times until each training sample had been left out once. The predicted concentrations of the components in each sample were compared with the actual concentrations in this calibration samples and Root-Mean-Square Error of Cross-Validation (RMSECV) was calculated for each method. It indicates both of the precision and accuracy of predictions. It was recalculated upon addition of each new factor to the PLS and PCR models. Visual inspection was used for selecting the optimum number of factors. The main advantages of proposed chemometric techniques are the higher speed of processing data concerning the values of concentrations and absorbencies of compounds with strongly overlapping spectra. Besides, the errors of calibration model are minimized by measuring the absorbance values at many points in the wavelength range of the zero-order or derivative spectra.

Table 1: The concentrations of different mixtures of TER and BZO used in the training set for the chemometric techniques

<table>
<thead>
<tr>
<th>Sample number</th>
<th>TER Conc. (µg/mL)</th>
<th>BZO Conc. (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2.6</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>3.4</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>3.5</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>4.4</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>4.6</td>
</tr>
<tr>
<td>13</td>
<td>11</td>
<td>5.4</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>5.5</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Figure 4: Zero order scan for spectrophotometric determination of TER (20 µg mL\(^{-1}\)) (-----) & BZO (10 µg mL\(^{-1}\)) (------) standard solutions in acetonitrile as solvent
Figure 5: First order derivative spectrophotometric spectra for determination of TER (20 μg mL\(^{-1}\)) (-----) & BZO (10 μg mL\(^{-1}\)) (-----) standard solutions at 320 nm and 283 nm, respectively in acetonitrile as solvent

System suitability tests (HPLC method)
System suitability tests are important tests of liquid chromatographic methods in order to reach optimized conditions of the proposed method [16]. They are mainly used to test the resolution and reproducibility and to verify that they are suitable for the analysis performed. The parameters of these tests include column efficiency (number of theoretical plates), capacity factor (K), tailing of chromatographic peak, and repeatability as % R.S.D of peak area for six injections of a solution of a 32 μg mL\(^{-1}\) and 16 μg mL\(^{-1}\) for TER and BZO, respectively and reproducibility of retention as % R.S.D of retention time. The results of these tests for the proposed method were listed in Table 2.

Table 2: System suitability tests for RP-LC method for the simultaneous determination of TER and BZO. (N: number of theoretical plates; T: tailing factor; K: capacity factor; α: separation factor; R: resolution factor)

<table>
<thead>
<tr>
<th>Item</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>11173</td>
<td>17109</td>
</tr>
<tr>
<td>T</td>
<td>1.14</td>
<td>1.095</td>
</tr>
<tr>
<td>K</td>
<td>1.346</td>
<td>1.846</td>
</tr>
<tr>
<td>α</td>
<td>1.371</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>6.66</td>
<td></td>
</tr>
<tr>
<td>% RSD of 6 injections of peak area</td>
<td>0.25</td>
<td>0.142</td>
</tr>
<tr>
<td>% RSD of 6 injections of retention time</td>
<td>0.058</td>
<td>0.069</td>
</tr>
</tbody>
</table>

Validation of the methods
Linearity and range:
**HPLC method:** In this study, six concentrations were chosen for each compound. Each concentration was analyzed three times. Good linearity of the calibration curve was verified by the high correlation coefficient. The analytical data of the calibration curve including standard deviations for the slope and intercept (Sb, Sa) were summarized in Table 3.

**Derivative spectrophotometry method:** Linearity was studied for TER and BZO analysis by the proposed derivative spectrophotometry method in range of 5-40 μg mL\(^{-1}\) and 4-15 μg mL\(^{-1}\), respectively. A linear relationship between trough amplitude (D\(^3\)) and concentrations (C) was obtained and the regression equation for both drugs was also computed, (Figure 6). Results are given in Table 4.

**Chemometry method:** Linearity was studied for TER and BZO analysis by the proposed chemometric technique through construction of the training set by preparing mixture solutions containing TER (3-11 μg mL\(^{-1}\)) and BZO (1.4-5.6 μg mL\(^{-1}\)), Table 1.

**Accuracy:**
**HPLC method:** Accuracy of the results was calculated by % recovery of 6 different concentrations (injected in triplicates) of TER and BZO combined in the laboratory prepared binary mixture. The results obtained including the mean of the recovery are displayed in Table 3.
Derivative spectrophotometry method: Accuracy of the results was calculated by % recovery of 6 different concentrations of TER and BZO combined in the laboratory prepared binary mixture. The results obtained including the mean of the recovery are displayed in Table 4.

![Figure 6: First order derivative spectrophotometric spectra of (a) TER for Calibration curve (5-40 µg mL$^{-1}$), (b) of BZO for calibration curve (4-15 µg mL$^{-1}$)](image)

Chemometry method: Accuracy of the results was calculated by % recovery obtained from the constructed validation set applied using CLS, PCR and PLS models. Results are shown in Table 5.

Table 3: Results obtained by the proposed RP-HPLC method for the simultaneous determination of TER and BZO; ($S_b$: slope standard deviation, $S_a$: intercept standard deviation, LOD: limit of detection, LOQ: limit of quantitation)

<table>
<thead>
<tr>
<th>Item</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min.)</td>
<td>3.05</td>
<td>3.7</td>
</tr>
<tr>
<td>Wavelength of detection (nm)</td>
<td>250</td>
<td>225</td>
</tr>
<tr>
<td>Range of linearity (µg mL$^{-1}$)</td>
<td>4-128</td>
<td>4-128</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$y = 39.7241x + 10.3912$</td>
<td>$y = 93.7449x + 217.0483$</td>
</tr>
<tr>
<td>Regression coefficient (R$^2$)</td>
<td>1</td>
<td>0.9977</td>
</tr>
<tr>
<td>LOD (µg mL$^{-1}$)</td>
<td>0.846</td>
<td>0.921</td>
</tr>
<tr>
<td>LOQ (µg mL$^{-1}$)</td>
<td>2.821</td>
<td>3.071</td>
</tr>
<tr>
<td>$S_b$</td>
<td>0.0952</td>
<td>2.258</td>
</tr>
<tr>
<td>$S_a$</td>
<td>5.742</td>
<td>136.237</td>
</tr>
<tr>
<td>Confidence limit of the slope</td>
<td>39.7241±0.2643</td>
<td>93.7449±6.268</td>
</tr>
<tr>
<td>Confidence limit of the intercept</td>
<td>10.3912±15.94</td>
<td>217.0483±378.194</td>
</tr>
<tr>
<td>Standard error of the estimation</td>
<td>10.098</td>
<td>239.572</td>
</tr>
<tr>
<td>Drug in binary mixture (%)</td>
<td>101.05±0.922</td>
<td>101.33±0.943</td>
</tr>
<tr>
<td>Drug in binary dosage form (%)</td>
<td>100.20±1.223</td>
<td>100.92±1.176</td>
</tr>
<tr>
<td>Drug added % (in binary dosage form)</td>
<td>99.82±0.936</td>
<td>99.52±1.301</td>
</tr>
</tbody>
</table>

Precision

HPLC method: The intra-day and inter-day of the method was assessed by using 3 concentrations in triplicates for three consecutive days for TER and BZO in binary mixtures (25.6/12.8, 32/16 and 38.4/19.2 µg mL$^{-1}$ of TER/BZO, respectively) representing 80%, 100% and 120%, respectively. The values of the precision (% RSD) for TER and BZO peak area were found to be less than 1% in the three concentrations, Table 6.

Derivative spectrophotometry method: The intra-day and inter-day of the method was assessed by using 3 concentrations in triplicates for three consecutive days for TER and BZO in binary mixtures (17.6/8.8, 22/11 and 26.4/13.2 µg mL$^{-1}$ of TER/BZO, respectively) representing 80%, 100% and 120%, respectively. Good values of the precision (% RSD) for TER and BZO trough amplitude were obtained, Table 7.

Chemometry method: The intra-day and inter-day of the method was assessed by using 3 concentrations in triplicates for three consecutive days for TER and BZO in binary mixtures (5.2/2.6, 6.5/3.25 and 7.8/3.9 µg mL$^{-1}$ of TER/BZO, respectively) representing 80%, 100% and 120%, respectively. Results are given in Table 8.
Table 4: Results obtained by the proposed derivative spectrophotometric determination of TER and BZO. (S_d: slope standard deviation, R^2: intercept standard deviation, LOD: limit of detection, LOQ: limit of quantitation)

<table>
<thead>
<tr>
<th>Item</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength of measurement</td>
<td>320 nm</td>
<td>283 nm</td>
</tr>
<tr>
<td>Calibration range (µg mL^{-1})</td>
<td>May-40</td>
<td>15-Apr</td>
</tr>
<tr>
<td>LOD (µg mL^{-1})</td>
<td>0.749</td>
<td>0.348</td>
</tr>
<tr>
<td>LOQ (µg mL^{-1})</td>
<td>2.271</td>
<td>1.16</td>
</tr>
<tr>
<td>Regression equation</td>
<td>D^2 = 0.0151C -0.0031</td>
<td>D^2 = 0.0925C -0.0233</td>
</tr>
<tr>
<td>Regression coefficient (R^2)</td>
<td>0.9999</td>
<td>0.9994</td>
</tr>
<tr>
<td>Standard deviation of intercept (S_d)</td>
<td>0.000084</td>
<td>0.001148</td>
</tr>
<tr>
<td>Standard deviation of the intercept (S_r)</td>
<td>0.002224</td>
<td>0.011575</td>
</tr>
<tr>
<td>Confidence limit of slope</td>
<td>0.0151± 0.00023</td>
<td>0.0925± 0.00319</td>
</tr>
<tr>
<td>Confidence limit of intercept</td>
<td>0.0031± 0.00617</td>
<td>0.0233± 0.03213</td>
</tr>
<tr>
<td>Standard error of estimation</td>
<td>0.00262</td>
<td>0.010728</td>
</tr>
<tr>
<td>Drug in binary mixture (%)</td>
<td>101.047±0.887</td>
<td>100.607±1.343</td>
</tr>
<tr>
<td>Drug in binary dosage form (%)</td>
<td>99.446±0.889</td>
<td>99.622±1.195</td>
</tr>
<tr>
<td>Drug added % (in binary dosage form)</td>
<td>101.098±1.068</td>
<td>99.760±1.827</td>
</tr>
</tbody>
</table>

Table 5: Results of the analysis of the mixtures of the validation set of TER and BZO using CLS, PCR and PLS chemometric techniques

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Concentration (µg/mL)</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLS</td>
<td>PCR</td>
<td>PLS</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>98.05</td>
<td>96.3</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>99.14</td>
<td>100.13</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>99.6</td>
<td>99.22</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>100.58</td>
<td>100.47</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>99.67</td>
<td>99.65</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>101.66</td>
<td>100.96</td>
</tr>
<tr>
<td>SD</td>
<td>±1.235</td>
<td>±0.688</td>
<td>±0.692</td>
</tr>
<tr>
<td>SE</td>
<td>±0.504</td>
<td>±0.281</td>
<td>±0.283</td>
</tr>
</tbody>
</table>

Table 6: Results for the determination of intra-day and inter-day for TER and BZO in laboratory prepared binary mixture by the proposed RP-LC method

<table>
<thead>
<tr>
<th>Item</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (% RSD) for binary mixture</td>
<td>0.240-0.994</td>
<td>0.142-0.774</td>
</tr>
<tr>
<td>Inter-day (% RSD) for binary mixture</td>
<td>0.571-0.998</td>
<td>0.327-0.933</td>
</tr>
</tbody>
</table>

Table 7: Results for the determination of intra-day and inter-day for TER and BZO in laboratory prepared binary mixture by the proposed derivative spectrophotometric method

<table>
<thead>
<tr>
<th>Item</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (% RSD) for binary mixture</td>
<td>0.531-0.754</td>
<td>0.308-0.535</td>
</tr>
<tr>
<td>Inter-day (% RSD) for binary mixture</td>
<td>0.314-0.413</td>
<td>0.151-0.722</td>
</tr>
</tbody>
</table>

Table 8: Intra-day and inter-day results for chemometric determination of TER and BZO

<table>
<thead>
<tr>
<th>Item</th>
<th>TER</th>
<th>BZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-day (% RSD) for binary mixture</td>
<td>0.532-1.023</td>
<td>0.605-0.997</td>
</tr>
<tr>
<td>Intra-day (% RSD) for binary mixture</td>
<td>0.470-0.980</td>
<td>0.498-0.894</td>
</tr>
</tbody>
</table>

Specificity
HPLC method: The proposed method could be applied to determine the intact compound and in laboratory prepared mixtures, good recovery results were obtained for cited compounds in presence of each other ensuring method specificity. The chromatogram of each compound in the sample solution was found identical to the chromatogram received by the standard solution at the wavelengths applied. Also, the chromatograms of the samples were checked for the appearance of any excipients’ peaks. These results demonstrate the absence of
interference from other materials in the pharmaceutical formulations (Figure 2), and therefore confirm the specificity of the method.

**Derivative spectrophotometry method:** The first order derivative spectrophotometry spectra of analysed compounds in the sample solution was found identical to the that received by the laboratory prepared mixture of the standard solutions at the wavelengths applied. Also, the first order derivative spectrophotometry spectra of the samples were checked for the appearance of any excipients' spectra. These results demonstrate the absence of interference from other materials in the pharmaceutical formulations (Figure 3), and therefore confirm the specificity of the method.

**Chemometry method:** The three proposed chemometric models (CLS, PCR and PLS) were applied successfully to Gynoconazole 0.4%™ cream, so assuring absence of interference from any co-formulated excipients.

**Limit of detection and limit of quantification (HPLC method):**
Limit of detection (LOD) which represent the concentration of the analyte at S/N ratio of 3 and limit of quantitation (LOQ) representing the concentration of analyte at S/N ratio of 10 was determined experimentally for the proposed method and results were given in Table 3.

**Robustness (HPLC method):**
Robustness is a measure of the method ability to remain unaffected by small variations in the method conditions and is an indication of the method reliability. The flow rate of the mobile phase was changed from 1 mL min\(^{-1}\) to 0.8 mL min\(^{-1}\) and 1.2 mL min\(^{-1}\). The organic strength was varied, as the acetonitrile proportion from 70% to 72% and 68%, meanwhile aqueous component was changed also from 30% to 28% and 32%. Also, a change of the mobile phase aqueous component pH value was changed from (pH 3.5) to (pH 3.3) and (pH 3.7). These variations did not have significant effect on chromatographic resolution by the method for TER and BZO, indicating good robustness of the proposed method (Table 9).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Flow rate (mL min(^{-1}))</th>
<th>pH</th>
<th>Organic composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.8</td>
<td>1.2</td>
<td>3.3</td>
</tr>
<tr>
<td>TER/BZO Resolution</td>
<td>7.1</td>
<td>6.6</td>
<td>6.5</td>
</tr>
</tbody>
</table>

**Statistical analysis of the results**
A statistical analysis of the results obtained by the proposed method and the reference methods for each analyte was carried out by “SPSS statistical package version 11”. Results obtained by the proposed methods for the determination of pure samples of TER and BZO were statistically compared to those obtained by the official reference methods [16]. The calculated values of (t-test) and (F-ratio) at p=0.05 were less than the corresponding tabulated ones, which revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the official ones. Results are given in Tables 10-12.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>100.098</td>
<td>101.053</td>
<td>100.827</td>
<td>101.338</td>
</tr>
<tr>
<td>± SD</td>
<td>0.965</td>
<td>0.922</td>
<td>0.99</td>
<td>0.943</td>
</tr>
<tr>
<td>± SE</td>
<td>0.394</td>
<td>0.376</td>
<td>0.404</td>
<td>0.385</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.964</td>
<td>0.912</td>
<td>0.982</td>
<td>0.931</td>
</tr>
<tr>
<td>Variance</td>
<td>0.931</td>
<td>0.85</td>
<td>0.98</td>
<td>0.889</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Student’s t-test</td>
<td>1.753(2.228)</td>
<td></td>
<td>0.916(2.228)</td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td></td>
<td></td>
<td>0.916(2.228)</td>
<td></td>
</tr>
<tr>
<td>F-ratio</td>
<td>1.095(5.05)</td>
<td></td>
<td>1.102(5.05)</td>
<td></td>
</tr>
</tbody>
</table>
Table 11: Statistical comparison between the recovery results of the proposed derivative spectrophotometric method and the reference method for the determination of TER and BZO

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>100.098</td>
<td>101.047</td>
<td>100.827</td>
<td>100.607</td>
</tr>
<tr>
<td>± SD</td>
<td>0.965</td>
<td>0.887</td>
<td>0.99</td>
<td>1.343</td>
</tr>
<tr>
<td>± SE</td>
<td>0.394</td>
<td>0.362</td>
<td>0.404</td>
<td>0.548</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.964</td>
<td>0.878</td>
<td>0.982</td>
<td>1.335</td>
</tr>
<tr>
<td>Variance</td>
<td>0.931</td>
<td>0.787</td>
<td>0.98</td>
<td>1.804</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Student’s t-test | 1.773(2.228) | 0.323(2.228) |

F-ratio | 1.183(5.05) | 1.841(5.05) |

Table 12: Statistical comparison between the recovery results of the proposed chemometric methods and the reference methods for the determination of TER and BZO

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>100.098</td>
<td>99.783</td>
<td>99.955</td>
<td>99.96</td>
<td>100.827</td>
<td>99.845</td>
<td>99.898</td>
<td>99.905</td>
</tr>
<tr>
<td>SD</td>
<td>0.965</td>
<td>1.235</td>
<td>0.688</td>
<td>0.692</td>
<td>0.99</td>
<td>0.816</td>
<td>0.612</td>
<td>0.621</td>
</tr>
<tr>
<td>SE</td>
<td>0.394</td>
<td>0.504</td>
<td>0.281</td>
<td>0.283</td>
<td>0.404</td>
<td>0.333</td>
<td>0.25</td>
<td>0.254</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.964</td>
<td>1.238</td>
<td>0.688</td>
<td>0.692</td>
<td>0.982</td>
<td>0.817</td>
<td>0.613</td>
<td>0.622</td>
</tr>
<tr>
<td>Variance</td>
<td>0.931</td>
<td>1.525</td>
<td>0.473</td>
<td>0.479</td>
<td>0.98</td>
<td>0.666</td>
<td>0.375</td>
<td>0.386</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Student’s t-test | 0.492 | 0.296 | 0.285 | 1.875 | 1.955 | 1.932 |

F-ratio | 1.638 | 1.968 | 1.944 | 1.471 | 2.613 | 2.539 |

CONCLUSION

The three proposed methods (RP-HPLC, derivative spectrophotometry and chemometry) are simple, accurate, selective, valid and reproducible for simultaneous determination of TER and BZO in bulk and creams. The proposed methods were validated as per ICH guidelines. The proposed methods are suitable for the quality control determination of the cited compounds in bulk and creams without any preliminary separation step.

REFERENCES

[16] Authority of the United States Pharmacopeial Convention, The United States Pharmacopoeia (USP 38), National Formulary (NF 33), Maryland, USA, **2015**.