Validated spectrophotometric methods for simultaneous determination of troxerutin and carbazochrome in dosage form

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Highlights
- Troxerutin (TXN) is co-formulated with Carbazochrome (CZM).
- No method was reported for determination of TXN and CZM in their mixture form.
- Four spectrophotometric methods were developed for their simultaneous determination.
- The methods were validated according to the ICH guidelines.
- The results were statistically compared to the manufacturer's method.

Abstract
Four simple, accurate, sensitive and precise spectrophotometric methods were developed and validated for simultaneous determination of Troxerutin (TXN) and Carbazochrome (CZM) in their bulk powders, laboratory prepared mixtures and pharmaceutical dosage forms. Method A is first derivative spectrophotometry (D1) where TXN and CZM were determined at 294 and 483.5 nm, respectively. Method B is first derivative of ratio spectra (DD1) where the peak amplitude at 248 nm for TXN and 439 nm for CZM were used for their determination. Method C is ratio subtraction (RS); in which TXN was determined at its λmax (352 nm) in the presence of CZM which was determined by D1 at 483.5 nm. While, method D is mean centering of the ratio spectra (MCR) in which the mean centered values at 300 nm and 340.0 nm were used for the two drugs in a respective order. The two compounds were simultaneously determined in the concentration ranges of 5.00–50.00 μg mL−1 and 0.5–10.0 μg mL−1 for TXN and CZM, respectively. The methods were validated according to the ICH guidelines and the results were statistically compared to the manufacturer’s method.

Introduction
Troxerutin (TXN) is chemically designated as 2-{3,4-bis(2-hydroxyethoxy)phenyl]-5-hydroxy-7-(2-hydroxyethoxy)-4-oxo-4H-chromen-3-yl6-o-(6-deoxy-β-α-mannopyranosyl)-β-α-glucopyranoside, it is a flavonol and known as vitamin P4 [1]. TXN has a considerable broad pharmacological activities; it improves capillary function, reduces capillary fragility and abnormal leakage. It is also used for reducing the occurrence of night cramps, treatment of varicose veins and hemorrhoids [2]. Carbazochrome (CZM) is chemically designated as [(3-Hydroxy-1-methyl-6-oxo-2,3-dihydroindol-5-ylidene)amino]urea and it is an oxidation product.
of adrenaline and used as anti-hemorrhagic agent [3]. Both drugs are co-formulated (TXN 150 mg and CZM 1.5 mg) and the dosage form was shown to have a good efficacy and safety profile in non-surgical patients with acute uncomplicated hemorrhoids. Their chemical structures are shown in Fig. 1.

Several methods were reported on each drug individually. For TXN, it was determined by spectrophotometry [4–6], capillary electrophoresis [7] and high-performance liquid chromatography (HPLC) [8–10], electrochemistry [11] and capillary electrochromatography [12]. While for CZM, it was determined by spectrophotometry [13], high-performance liquid chromatography (HPLC) [14,15] and Chemiluminescence [16].

No method was reported for determination of TXN and CZM in their mixture form. So, our aim was to develop and validate simple spectrophotometric methods for simultaneous determination of both drugs in their pure form, laboratory prepared binary mixture and pharmaceutical formulation.

Experimental

Instruments

A double beam UV–visible spectrophotometer (SHIMADZU, Kyoto, Japan) model UV-1650, pc with quartz cell of 1 cm path length, connected to IBM compatible computer operated with UV-probe personal spectroscopy software version 2.21. The spectral band width is 2 nm and wavelength scanning speed is 2800 nm/min. Mean centering computations were done using Mat-\textsuperscript{lab}\textsuperscript{6.5 with PLS-Toolbox.}

Samples

Pure samples of TXN and CZM were kindly supplied by Minapharm for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Both were certified to contain 99.90% w/w according to the manufacturer’s method. Fleboton\textsuperscript{a} ampoules, labeled to contain 150 mg of TXN and 1.5 mg of CZM were manufactured by Minapharm Pharmaceuticals and Chemical Industries, Egypt (batch No. 9DE0219) and were obtained from local market.

Chemicals and solutions

Methanol spectroscopic grade was used. Stock standard solutions of TXN and CZM (1.0 mg mL\textsuperscript{-1}) were prepared in methanol. Working standard solutions of TXN and CZM (0.1 mg mL\textsuperscript{-1}) were prepared by an additional dilution of their stock standard solutions with methanol. A set of laboratory prepared mixtures containing different ratios of TXN (10.0–50.0 \(\mu\)g mL\textsuperscript{-1}) and CZM (0.5–7.5 \(\mu\)g mL\textsuperscript{-1}) was prepared.

Procedures

Construction of calibration curves

Aliquots of TXN working standard solution (0.1 mg mL\textsuperscript{-1}) equivalent to 50.0–500.0 \(\mu\)g mL\textsuperscript{-1} and of CZM working standard solution (0.1 mg mL\textsuperscript{-1}) equivalent to 5.0–100.0 \(\mu\)g mL\textsuperscript{-1} were accurately transferred into a series of 10 mL volumetric flasks; the volume was completed to the mark with methanol. The zero order spectra of the prepared solutions were recorded using methanol as a blank in the range of 200–600 nm.

For D\textsuperscript{1} method

The D\textsuperscript{1} curves of the scanned spectra were recorded using \(D_k = 4\) and scaling factor = 10. Calibration curves were then constructed by plotting the values of the peak amplitude of D\textsuperscript{1} curves at 294 nm for TXN (corresponding to zero crossing of CZM) and 483 nm for CZM (corresponding to zero absorbance of TXN) versus the corresponding concentrations and the regression parameters were computed.

For DD\textsuperscript{1} method

The scanned spectra of TXN were divided by a standard spectrum of 10.0 \(\mu\)g mL\textsuperscript{-1} CZM while the spectra of CZM were divided by a standard spectrum of 40.0 \(\mu\)g mL\textsuperscript{-1} TXN and the first derivative of the ratio curves (DD\textsuperscript{1}) for each compound were then obtained with \(D_k = 4\) and scaling factor = 10. Calibration curves were constructed by plotting the peak amplitude at 248 and 439 nm of the DD\textsuperscript{1} curves versus the corresponding concentrations of TXN and CZM, respectively and the regression parameters were computed.

For ratio subtraction method (RS)

For the determination of TXN, a calibration curve was constructed relating the absorbance of zero order spectra of TXN at 352 nm to the corresponding concentrations and the regression

![Fig. 1. Chemical structure of (a) truxerutin and (b) carbazochrome.](image_url)
equation was computed while CZM is determined as previously mentioned in $D^1$.

For MCR method

The stored spectra of the prepared solutions of TXN were divided by the normalized absorption spectrum of CZM. Similarly, the zero-order spectra of the prepared solutions of CZM were divided by the normalized absorption spectrum of TXN. The obtained ratio spectra were then mean centered. The calibration curves for the two drugs were constructed by plotting the mean centered values at 300 nm and 340 nm versus the corresponding concentrations of TXN and CZM, respectively.

Application to laboratory prepared mixtures

The absorption spectra of the laboratory prepared mixtures were recorded. Then the procedures were followed as described under construction of the calibration curves. The concentrations of TXN and CZM were calculated by substituting in the corresponding regression equation.

Application to pharmaceutical formulation

Three Fleboton Ampoules were mixed and an accurate volume equivalent to 50 mg of TXN and 0.5 mg of CZM was transferred into a 100 mL volumetric flask. Dilution of active ingredients was carried out by addition of methanol. Suitable dilutions were made using methanol to prepare solution containing 50.0 μg mL$^{-1}$ TXN and 0.5 μg mL$^{-1}$ CZM.

Results and discussion

Truxerutin is co-formulated with Carbazochrome in ampoules for treatment of chronic venous insufficiency. By reviewing the literature in hand, there was no reported spectrophotometric method for their simultaneous determination. The absorption spectra of TXN and CZM show severe overlap (Fig. 2) which hinders their direct spectrophotometric determination. So, our aim was to develop simple, rapid, accurate and precise spectrophotometric methods for the simultaneous determination of TXN and CZM in dosage form.

Fig. 2. $D^0$ absorption spectra of 1.25 μg mL$^{-1}$ of CZM, and 50 μg mL$^{-1}$ of TXN in methanol.

$D^1$ method

It is a very useful analytical technique for eliminating spectral overlapping by using the first or higher derivatives of absorbance with respect to wavelength [17]. Upon applying $D^1$ method, TXN could be determined by measuring its peak amplitude at 294 nm (corresponding to zero-crossing of CZM), while CZM could be determined by measuring its peak amplitude at 483 nm (corresponding to zero-absorbance of TXN) (Fig. 3). In order to optimize $D^1$ method, different smoothing and scaling factors were tested, where a Δ$λ$ = 4 and a scaling factor = 10 showed a suitable signal to-noise ratio and the curves showed good resolution. A linear correlation was obtained between the peak amplitude and its corresponding concentration for TXN at λ = 294 nm and for CZM at 483 nm in the ranges of 5.0–50.0 μg mL$^{-1}$ and 0.5–10.0 μg mL$^{-1}$ for TXN and CZM, respectively. The parameters of the regression equations are shown in Table 1.

$DD^1$ spectrophotometric method

In order to improve the selectivity of the analysis of TXN and CZM, $DD^1$ was also applied and validated. The main advantage of this method is that the whole spectrum of the interfering substance is canceled [18]. In order to optimize the $DD^1$ method, several divisors were tested along with the normalized spectrum. The best results were obtained using each of 10.0 μg mL$^{-1}$ of CZM and 40.0 μg mL$^{-1}$ of TXN as a divisor for TXN and CZM, respectively. Peak amplitude at 248 nm and 439 nm for TXN and CZM, respectively (Figs. 4 and 5) were selected, plotted against the corresponding concentration. Good linearity was obtained for both drugs and the regression parameters were calculated and shown in Table 1.

Ratio subtraction method

The method was applied for determination of mixture of TXN (X) and CZM (Y), when the spectrum of (Y) extended than the other (X), as shown in Fig. 2. The determination of (X) could be achieved by scanning the absorption spectra of the laboratory prepared mixtures of X and Y in methanol, then dividing them by a carefully chosen standard spectrum of Y (10.0 μg mL$^{-1}$, Y = divisor) to produce a new ratio spectra that represents $X/Y$ + constant, as shown in Fig. 6. The values of these constant ($Y/Y$) were subtracted as shown in (Fig. 7), followed by multiplication of the obtained spectra by the divisor (Y') as shown in (Fig. 8). Finally, the original spectra of X were obtained and the absorbance values at 352 nm were plotted against the concentration, the corresponding regression equation could be calculated. This can be summarized as follows:

$$X + Y = \frac{X}{Y} + \frac{Y}{Y'} - \frac{X}{Y'} + \text{constant}$$

$$X \times \frac{Y'}{Y} + \text{constant} - \text{constant} = \frac{X}{Y'}$$

$$X \times \frac{Y}{Y'} = X$$

The constant can be determined directly from the curve $(X + Y)/Y'$ by the straight line which is parallel to the wavelength axis in the region where (Y) is extended. The correct choice of the divisor is a fundamental step, as if the concentration of the divisor increases or decreases, the resulting constant value will be proportionally decreased or increased [18]. A linear correlation was obtained between the absorbance and the corresponding concentration of TXN at its corresponding wavelength, the parameters of the regression equations are shown in Table 1. While, CZM was previously determined by $D^1$ method.
For further improvement of the selectivity, a new, simple recently developed method was applied. This is based on the mean centering of ratio spectra. It eliminates the derivative step and so the signal-to-noise ratio is therefore enhanced [19].

The MCR method was applied and was able to quantitatively determine both TXN and CZM in their laboratory-prepared mixtures and in their pharmaceutical preparation. As shown in Fig. 2, the absorption spectra of TXN and CZM in methanol are severely overlapped in the wavelength region of 250–500 nm. So, the absorption spectra of the standard solutions of the TXN with different concentrations were

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>D1</th>
<th>DD1</th>
<th>Ratio subtraction</th>
<th>MCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CZM</td>
<td>TXN</td>
<td>CZM</td>
<td>TXN</td>
</tr>
<tr>
<td></td>
<td>CZM</td>
<td>TXN</td>
<td>CZM</td>
<td>TXN</td>
</tr>
<tr>
<td>Range</td>
<td>0.5–10 μg mL⁻¹</td>
<td>5–50 μg mL⁻¹</td>
<td>0.5–10 μg mL⁻¹</td>
<td>5–50 μg mL⁻¹</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0492</td>
<td>0.00128</td>
<td>0.3815</td>
<td>0.0251</td>
</tr>
<tr>
<td>Intercept</td>
<td>−0.0048</td>
<td>0.0117</td>
<td>0.00822</td>
<td>0.0128</td>
</tr>
<tr>
<td>SE of the slope</td>
<td>0.305 × 10⁻³</td>
<td>0.086 × 10⁻³</td>
<td>1.839 × 10⁻³</td>
<td>0.208 × 10⁻³</td>
</tr>
<tr>
<td>SE of the intercept</td>
<td>1.590 × 10⁻³</td>
<td>2.855 × 10⁻³</td>
<td>0.012</td>
<td>6.918 × 10⁻³</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9998</td>
</tr>
<tr>
<td>LOD</td>
<td>0.167</td>
<td>0.762</td>
<td>0.174</td>
<td>0.956</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.508</td>
<td>2.308</td>
<td>0.526</td>
<td>2.897</td>
</tr>
<tr>
<td>Accuracy (mean ± SD)</td>
<td>100.79 ± 0.538</td>
<td>99.74 ± 0.510</td>
<td>99.87 ± 0.715</td>
<td>99.80 ± 0.894</td>
</tr>
<tr>
<td>RSD%</td>
<td>0.534</td>
<td>0.511</td>
<td>0.716</td>
<td>0.896</td>
</tr>
</tbody>
</table>

Fig. 3. D1 of 10 μg mL⁻¹ of CZM, and 50 μg mL⁻¹ TXN in methanol.

Fig. 4. (a) D0 of TXN (5–50 μg mL⁻¹) in methanol, using 10 μg mL⁻¹ of CZM as a divisor. (b) DD1 of (5–50 μg mL⁻¹) of TXN, using 10 μg mL⁻¹ of CZM as a divisor.

### MCR method

For further improvement of the selectivity, a new, simple recently developed method was applied. This is based on the mean centering of ratio spectra. It eliminates the derivative step and so the signal-to-noise ratio is therefore enhanced [19].
recorded in the wavelength range of 200–600 nm and divided by the normalized spectrum of the CZM. The ratio spectra were obtained. Mean centering of the ratio spectra was carried out and the concentration of TXN was determined by measuring the amplitude at 300 nm (corresponding to a maximum wavelength) (Fig. 9).

The spectra of the standard solutions of the CZM with different concentrations were recorded in the wavelength range of 200–600 nm and divided by the normalized spectrum of the TXN. The ratio spectra were obtained. Mean centering of the ratio spectra was carried out and the concentration of CZM was determined by measuring the amplitude at 340 nm (corresponding to a maximum wavelength) (Fig. 10).

A linear correlation was obtained between the mean centered values and its corresponding concentration for TXN at 300 nm and for CZM at 340 nm. The parameters of the regression equations are shown in Table 1.

The effect of divisor concentration on the analytical parameters such as slope, intercept and correlation coefficient of the calibration graphs was also tested. Different divisors were tested; a normalized spectrum of each of TXN and CZM was used as a divisor spectrum in the proposed method.

The specificity of the proposed methods was proved by the analysis of laboratory prepared mixtures of TXN and CZM in different ratios, as presented in Table 2.

All the proposed methods were successfully applied for the determination of TXN and CZM in Fleboton® Amp. (Table 3) and the results obtained were statistically compared with those obtained by the manufacturer method and there is no significant difference regarding both accuracy and precision as shown in Table 4.

**Conclusion**

The proposed methods were simple, rapid, sensitive and precise. They could be easily applied in quality-control laboratories for simultaneous determination of TXN and CZM. MCR method...
Fig. 7. Ratio spectra of laboratory prepared mixtures of CZM and TXN using 10 μg mL$^{-1}$ of CZM as divisor, after subtraction of the constant.

Fig. 8. Spectra of laboratory prepared mixtures of CZM and TXN after multiplying the subtracted ratio spectra by the spectra of 10 μg mL$^{-1}$ of CZM.

Fig. 9. MCR of ratio spectra of TXN using normalized CZM spectrum as a devisor.
Fig. 10. MCR of ratio spectra of CZM using normalized TXN spectrum as a divisor.

Table 2
Determination of CZM and TXN in laboratory prepared mixtures by the proposed spectrophotometric methods.

<table>
<thead>
<tr>
<th>Mixture no.</th>
<th>Concentration (µg mL⁻¹)</th>
<th>D¹ Recovery%</th>
<th>DD¹</th>
<th>Ratio subtraction</th>
<th>MCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CZM</td>
<td>TXN</td>
<td>CZM</td>
<td>TXN</td>
<td>CZM</td>
</tr>
<tr>
<td>1</td>
<td>7.5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* CZM was determined according to its regression equation in D¹.
** Dosage form ratio.

Table 3
Statistical analysis of the results of the proposed methods and manufacturer methods for TXN and CZM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CZM</th>
<th>TXN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D¹</td>
<td>DD¹</td>
</tr>
<tr>
<td>Mean</td>
<td>100.79</td>
<td>99.87</td>
</tr>
<tr>
<td>SD</td>
<td>0.538</td>
<td>0.714</td>
</tr>
<tr>
<td>Variance</td>
<td>0.289</td>
<td>0.509</td>
</tr>
<tr>
<td>Student's</td>
<td>1.66</td>
<td>0.67</td>
</tr>
<tr>
<td>t</td>
<td>(2.31)*</td>
<td>(2.31)*</td>
</tr>
<tr>
<td>F</td>
<td>1.43</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>(5.41)*</td>
<td>(9.01)*</td>
</tr>
</tbody>
</table>

* The values in parentheses are the corresponding tabulated values at P = 0.05.
** Two methods were used one for TXN (UV), D⁰ at 254 nm, and the second for CZM HPLC (using phosphate buffer pH 5.7: Methanol, 55:45 (v/v), C-18 column, flow rate 0.8 mL min⁻¹ and detection at 354 nm).

Table 4
Application of the proposed methods for the analysis of TXN and CZM in pharmaceutical dosage form.

<table>
<thead>
<tr>
<th>Product</th>
<th>D¹</th>
<th>DD¹</th>
<th>Ratio subtraction</th>
<th>MCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fleboton® ampoules 1.5 mg CZM and 150 mg TXN/ampoule B.N.9DE0219</td>
<td>100.41 ± 1.18</td>
<td>98.92 ± 0.66</td>
<td>100.41 ± 1.18</td>
<td>99.02 ± 0.72</td>
</tr>
</tbody>
</table>

* CZM was determined according to its regression equation in D¹.
** Average of 3 determination.
has the advantage of eliminating the derivative steps and therefore the signal-to-noise ratio is not degraded. They methods could be applied for the routine QC analysis in their pure bulk powders and in dosage form without any preliminary separation step.

References


