Simultaneous determination of Carbazochrome and Troxerutin in their binary mixture by HPLC and HPTLC-Densitometric methods

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Abstract Two accurate, precise and sensitive RP-HPLC and HPTLC-Densitometric methods have been developed for the determination of Carbazochrome and Troxerutin in their binary mixture without previous separation. Method (A) is RP-HPLC which depended on isocratic elution using C18 column and mobile phase consisting of water containing (0.2% triethylamine (TEA), 1% tetrahydrofuran (THF)):methanol (65:35, by volume) at a flow rate of 1.5 mL min⁻¹ and the effluent was monitored at 350 nm. Good resolution was obtained with t_R values of 2.069 and 5.174 min for Carbazochrome and Troxerutin, respectively. Method (B) is HPTLC-Densitometric method, using silica gel 60 F₂₅₄ HPTLC plates and methanol:chloroform:ammonia (40:60:7, by volume) as a developing system. The bands were scanned at 350 nm. The proposed methods have been validated as per ICH guidelines and their linearity was evident in the ranges of 0.25–10 and 5–50 µg mL⁻¹ for method (A) and 0.02–0.8 and 0.1–2 µg band⁻¹ for method (B) for Carbazochrome and Troxerutin, respectively. The developed methods have been applied for the determination of the above mentioned drugs in their pharmaceutical formulation where no interference from the excipients has been detected. Statistical comparison of the results obtained by the developed methods and those obtained by the reported HPLC methods showed no significant difference between them. The developed methods are sensitive, accurate and precise and can be easily used for quality control analysis of the studied drugs.

1. Introduction

Carbazochrome (CAR) is chemically designated as 3-hydroxy-1-methyl-5,6-indolinedione semicarbazone,¹ Fig. 1a. It is an oxidation product of adrenaline and has been given as a hemostatic.¹ Troxerutin (TRO) is chemically designated as...
2-[3,4-bis(2-hydroxyethoxy)phenyl]-3-[6-O-(6-deoxy-α-L-manno-pyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-7 (2-hydroxyethoxy)-4H-1-benzopyran-4-one (tris(hydroxyethyl)rutin). It is the principal component of oxerutin flavonoid which is a naturally occurring antioxidant that is widely distributed in plants. Preparations containing natural or semisynthetic flavonoids are thought to improve capillary function by reducing abnormal leakage. They have been given to relieve capillary impairment and venous insufficiency of the lower limbs and for hemorrhoids. Combination of CAR and TRO medication is indicated for capillary fragility and venous insufficiency.

The literature survey reveals few analytical methods for the determination of CAR such as fluorescence quenching and chromatographic methods. The British pharmacopoeia reported IR spectrophotometric and liquid chromatographic methods for an analysis of Troxerutin, also detailed literature survey reveals that spectrophotometric, micellar chemiluminescence, RP-HPLC and other chromatographic methods have been reported for the quantitative estimation of Troxerutin individually in various matrices such as human plasma, pharmaceutical dosage forms, bulk, rat urine, chicken plasma and food supplements. RP-HPLC methods have been reported for the determination of Troxerutin in combination with other flavonoids and other drugs. On the other hand, there are no pharmacopeial methods available for the analysis of Carbazochrome. Spectrophotometric methods have been reported for the simultaneous determination of Carbazochrome and Troxerutin in dosage form.

Reviewing the literature in hand, no chromatographic methods have been published for the determination of the studied mixture. So the aim of this work is to develop accurate, sensitive and selective RP-HPLC and HPTLC-Densitometric methods for simultaneous estimation of CAR and TRO in their binary mixture and in their pharmaceutical formulation.

2. Experimental

2.1. Instruments

2.1.1. Agilent technologies, 1200 infinity series LC supplied with 1260 UV–VIS Detector with 1260 infinity Agilent technologies (25 cm × 4.6 mm i.d., 5 μm particle size) Eclipse plus C18 column was used as a stationary phase for HPLC determinations.

2.1.2. A sample applicator for TLC linomat V with 100 μL syringe (Camage, Muttenz, Switzerland).

2.1.3. TLC scanner 3 densitometer (Camage, Switzerland) controlled by WINCATS software (V 3.15, Camage).

2.1.4. HPTLC aluminum plates (20 × 20 cm) coated with 0.25 mm silica gel 60 F254 (Merck, Germany).

2.1.5. UV lamp with short wavelength 254 nm (VL-6.LC, MARNE LA VALLEE cedex 1, FRANCE).

2.1.6. The following parameters were adjusted during HPTLC scanning.
- Scanning mode: absorbance.
- Source of radiation: deuterium lamp.
- Slit dimension: 6 × 0.45 mm.
- Scanning speed: 20 mm s⁻¹.

2.1.7. Sonix TV ss-series ultrasonicator (USA).

2.2. Materials and reagents

2.2.1. Pure standards
Carbazochrome (CAR) and Troxerutin (TRO) pure standards were kindly supplied by MINAPHARM Egypt IND., Egypt. Their purity was found to be 99.49% and 99.22%, respectively, according to the manufacturer certificate.

2.2.2. Pharmaceutical formulation
Fleboton® ampoules for I.M. injection (B.No. CCE0564) manufactured by MINAPHARM Egypt IND., Egypt, labeled to contain 1.5 mg CAR and 150 mg TRO mg per 3 mL ampoule.

2.2.3. Chemicals and solvents
All chemicals used throughout this work were of analytical grade and were used without further purification;
– deionized water (SEDICO pharmaceutical Co., 6th October City, Egypt),
– Tetrahydrofuran, triethylamine and methanol HPLC grade (CHROMASOLV® Sigma–Aldrich Chemie GmbH, Germany).

2.2.4. Solutions

2.2.4.1. Stock standard solutions of CAR and TRO (0.5 and 1 mg mL$^{-1}$), respectively. 50 and 100 mg of CAR and TRO respectively were accurately and separately weighed into 100-

2.2.4.2. Working standard solutions of CAR and TRO (0.1 mg mL$^{-1}$). They were prepared by diluting 20 mL and

2.2.4.3. Solutions

3. Procedure

3.1. Chromatographic conditions

3.1.1. HPLC method

Chromatographic separation was performed on Eclipse plus C$_{18}$ column (25 cm x 4.6 mm i.d, 5 μm particle size) using (methanol:(0.2% triethylamine (TEA), 1% tetrahydrofuran (THF) solution) (35:65, v/v) at a flow rate of 1.5 mL min$^{-1}$ at ambient temperature and the effluent was monitored at 350 nm then peak areas and retention times were recorded.

3.1.2. HPTLC-Densitometric method

It was performed using pre-coated silica gel HPTLC aluminum plates (20 x 10 cm). The plates were pre-washed with methanol and activated at 100 °C for 15 min prior to samples’ application. Samples were applied in the form of bands (6 mm length, 12 mm spacing, and 10 mm from the bottom edge of the plate). Linear ascending development was performed in a chromatographic tank previously saturated with methanol:chloroform:ammonia (40:60:7, by volume) for half an hour at room temperature to a distance of about 80 mm. The developed plates were air dried and then scanned at 350 nm.

3.2. Linearity and construction of calibration curves

3.2.1. HPLC method

Calibration graphs for CAR and TRO$^*$ were obtained by recording and storing the peak areas of different concentrations of each in the ranges of 0.25–10 μg mL$^{-1}$ and 5–50 μg mL$^{-1}$ for CAR and TRO, respectively, prepared by suitable dilutions of their respective working solutions. Calibration graphs were constructed by plotting relative peak area (using 1 μg mL$^{-1}$ CAR and 15 μg mL$^{-1}$ TRO as external standard solutions) versus the corresponding concentration and the regression equations were then computed.

3.2.2. HPTLC-Densitometric method

Accurate volumes of CAR and TRO$^*$ were separately transferred from their respective stock standard solutions (0.5 and 1 mg mL$^{-1}$, respectively), applied in triplicate on the pre-washed HPTLC plates in the form of bands to obtain the concentration range of 0.02–0.8 and 0.1–2 μg band$^{-1}$ for CAR and TRO, respectively. The procedure under chromatographic conditions was then followed. The integrated peak area was then recorded and a calibration curve for each component was constructed by plotting the relative integrated peak area (using 0.1 μg mL$^{-1}$ CAR and 0.6 μg mL$^{-1}$ TRO as external standard solutions) versus the corresponding concentration and the regression equations were then computed.

3.3. Application to pharmaceutical formulations

10 ampoules of Fleboton® have been mixed and an accurate volume equivalent to 1 and 100 mg of CAR and TRO, respectively was transferred to a 100 mL-volumetric flask; and the volume was completed to the mark with methanol to obtain a concentration of 10 and 1000 μg mL$^{-1}$ of CAR and TRO, respectively.

The procedure under linearity for each method was then followed on Fleboton® ampoule and the concentrations of CAR and TRO were determined using the computed regression equations.

4. Results and discussion

The main task of this work was to develop sensitive, selective and accurate RP-HPLC and HPTLC-Densitometric methods for the determination of CAR and TRO in their binary mixture, with satisfactory precision for good analytical practice (GAP).

4.1. RP-HPLC method

RP-HPLC has become the most versatile and widespread technique used by the pharmaceutical industries for quality control and analysis of drugs. It has many applications in the field of pharmaceuticals including the quantitative determination of drugs present either alone or in presence of other mixture components. Factors affecting chromatographic separation have been studied and optimized. Different mobile phases have been tested to achieve the best chromatographic separation among the studied drugs such as methanol:water (35:65 and 80:20, v/v), methanol:0.1% acetic acid solution (50:50, v/v), acetonitrile:water (40:60 and 60:40, v/v), methanol:(0.2% triethylamine (TEA), 1% tetrahydrofuran (THF) solution) (35:65, v/v). The use of the last system gave the best chromatographic resolution with sharp symmetric peaks. Effect of scanning wavelength on the sensitivity of the method was studied by testing different scanning wavelengths (225, 254, and 350 nm) where scanning at 350 nm gave the lowest LOD and LOQ values. After method optimization, chromatographic separation has been achieved on Eclipse plus C$_{18}$ column using...
(methanol: (0.2% triethylamine (TEA), 1% tetrahydrofuran (THF) solution)) (35:65, v/v) at a flow rate of 1.5 mL min\(^{-1}\) and the effluent was monitored at 350 nm. Two peaks were obtained at 2.069 and 5.179 min for CAR and TRO, respectively, as shown in Fig. 2. Calibration graphs were constructed by plotting the relative peak area (using 1 \(\mu\)g mL\(^{-1}\) CAR and 15 \(\mu\)g mL\(^{-1}\) TRO as external standard solutions) versus the corresponding concentrations of each drug and the regression equations were then computed and found to be:

\[
A_1 = 0.8516 C_1 + 0.1547 \quad r_1 = 0.9999 \quad \text{For CAR}
\]

\[
A_2 = 0.0680 C_2 - 0.0116 \quad r_2 = 0.9999 \quad \text{For TRO}
\]

where \(A_1, A_2\) are the relative peak areas, \(C_1, C_2\) are the concentrations in \(\mu\)g mL\(^{-1}\), and \(r_1, r_2\) are the correlation coefficients of CAR and TRO, respectively. Regression equation parameters are given in Table 1.

4.2. HPTLC-Densitometric method

Thin layer chromatography has become a well-established technique for the assay of drugs either in binary or in multicomponent mixtures.\(^{24,25}\) The proposed method is based on the difference in the retardation factor \((R_f)\) between the CAR and TRO. In order to achieve the best separation with sharp symmetric peaks, the method has been optimized. Different developing systems with different ratios were tried such as methanol:hexane (2:8, v/v), methanol:chloroform (4:6, v/v), methanol:chloroform:acetic acid (4:6:0.1, by volume), methanol:chloroform:triethylamine (4:6:0.5, by volume), methanol:chloroform:ammonia (4:6:0.7, by volume). On using the first system CAR and TRO had \(R_f\) values close to each other. Replacing hexane with chloroform enhanced the separation among the two drugs but with tailed peaks for TRO. Addition of either acetic acid or triethylamine to the developing system did not improve the peaks shape while using ammonia gave sharp symmetric peaks without affecting the separation among the two drugs. Complete separation of CAR and TRO was achieved by using methanol:chloroform:ammonia (4:6:0.7, by volume) as a developing system. Effect of scanning wavelength on the sensitivity of the method was also studied by testing different scanning wavelengths (225, 245, 290, and 350 nm) where scanning at 350 nm gave the optimum sensitivity with minimum noise for the studied components. After method optimization, compact, sharp and symmetric peaks were obtained for CAR and TRO with significantly different \(R_f\) values (TRO, \(R_f = 0.08\) and CAR, \(R_f = 0.75\), Fig. 3.

A linear relationship between the concentrations of CAR and TRO and the relative peak areas (using 0.1 \(\mu\)g mL\(^{-1}\) CAR and 0.6 \(\mu\)g mL\(^{-1}\) TRO as external standard solutions) was observed in the range of 0.02–0.8 \(\mu\)g band\(^{-1}\) and 0.1–2 \(\mu\)g band\(^{-1}\) for CAR and TRO respectively, and the regression equations were computed and found to be:

\[
A_1 = 8.9152 C_1 + 0.0973 \quad r_1 = 0.9999 \quad \text{For CAR}
\]

\[
A_2 = 1.2800 C_2 + 0.2141 \quad r_2 = 0.9998 \quad \text{For TRO}
\]

where \(A_1, A_2\) are the relative peak areas, \(C_1, C_2\) are the concentrations in \(\mu\)g band\(^{-1}\), and \(r_1, r_2\) are the correlation coefficients of CAR and TRO, respectively. Regression equation parameters are given in Table 1.

The validity of the proposed methods for the analysis of CAR and TRO was studied by assaying Fleboton® ampoule Table 2. It was further assessed by applying standard addition technique, which showed that there was no interference from excipients, as in Table 2.

Statistical analysis of the results obtained by applying the developed HPLC and HPTLC-Densitometric methods with those obtained by the reported HPLC methods,\(^{2,5}\) showed no significant difference within confidence limit of 95% regarding both accuracy and precision, Table 3.

5. Method validation

Method validation was performed according to ICH guidelines.\(^{26}\)

5.1. Linearity and range

Linearity of the proposed methods was evaluated and it was evident in the range of 0.25–10 and 5–50 \(\mu\)g mL\(^{-1}\) for HPLC

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{HPLC chromatogram of 10 \(\mu\)g mL\(^{-1}\) of Carbazochrome and 45 \(\mu\)g mL\(^{-1}\) of Troxerutin.}
\end{figure}
method and 0.02–0.8 and 0.1–2 μg band\(^{-1}\) for HPTLC-Densitometric method for CAR and TRO, respectively. Linearity was ensured in both HPLC and HPTLC data sets of CAR and TRO by the use of residual data plots and normal probability data plots as shown in Figs. 4 and 5.

Good linearity was evident from the high value of correlation coefficient and low value of intercept as shown in Table 1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HPLC</th>
<th>TLC-Densitometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAR</td>
<td>TRO</td>
</tr>
<tr>
<td></td>
<td>CAR</td>
<td>TRO</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.25–10 (μg mL(^{-1}))</td>
<td>5–50 (μg mL(^{-1}))</td>
</tr>
<tr>
<td>Slope</td>
<td>0.852</td>
<td>0.068</td>
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<tr>
<td>Intercept</td>
<td>0.155</td>
<td>−0.012</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>Accuracy (Mean)</td>
<td>100.82</td>
<td>100.27</td>
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<tr>
<td><strong>Precision (% RSD)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability(^a)</td>
<td>0.825</td>
<td>0.952</td>
</tr>
<tr>
<td>Intermediate precision(^b)</td>
<td>1.067</td>
<td>1.024</td>
</tr>
<tr>
<td><strong>Robustness parameters (% RSD)</strong></td>
<td></td>
<td></td>
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<tr>
<td>For HPLC method:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic solvent ratio ± 2%</td>
<td>0.087</td>
<td>0.851</td>
</tr>
<tr>
<td>Flow rate ± 0.05 mL min(^{-1})</td>
<td>0.109</td>
<td>0.102</td>
</tr>
<tr>
<td>Scanning wavelength ± 1 nm</td>
<td>0.076</td>
<td>0.687</td>
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<tr>
<td>For TLC-Densitometric method</td>
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<tr>
<td>Ammonia volume ± 0.05 ml</td>
<td>0.735</td>
<td>0.951</td>
</tr>
<tr>
<td>Saturation time ± 5 min</td>
<td>0.538</td>
<td>0.822</td>
</tr>
<tr>
<td>Scanning wavelength ± 1 nm</td>
<td>0.189</td>
<td>0.547</td>
</tr>
<tr>
<td>LOD(^a)</td>
<td>0.083 (μg mL(^{-1}))</td>
<td>1.67 (μg mL(^{-1}))</td>
</tr>
<tr>
<td>LOQ(^a)</td>
<td>0.25 (μg mL(^{-1}))</td>
<td>5 (μg mL(^{-1}))</td>
</tr>
</tbody>
</table>

\(^a\) Concentration of Troxerutin is calculated as Trihydroxyethylrutin.
\(^b\) LOD and LOQ values were calculated using a visual non-instrumental method.\(^36\)
\(^a\) The intraday (\(n = 3\)), average of three different concentrations repeated three times within day.
\(^b\) The interday (\(n = 3\)), average of three different concentrations repeated three times in three successive days.

**Figure 3** TLC chromatogram of a mixture of (a) Troxerutin and (b) Carbazochrome using methanol: chloroform: ammonia (4:6:0.7, by volume) as a developing system.

5.2. **Accuracy**

Accuracy of the method was checked by applying the proposed methods for the determination of different blind samples of pure CAR and TRO. The concentrations were calculated from the corresponding regression equations and the results are presented in Table 1. Accuracy of the method was further assured...
by applying the standard addition technique on pharmaceutical formulation where good recoveries were obtained revealing no interference from excipients, as given in Table 2.

5.3. Precision

Repeatability: Three concentrations (4, 6 and 8 \( \mu g \text{ mL}^{-1} \)) of CAR and (15, 20 and 25 \( \mu g \text{ mL}^{-1} \)) of TRO for HPLC and (0.02, 0.1 and 0.6 \( \mu g \text{ mL}^{-1} \)) of CAR and (0.2, 0.6 and 1 \( \mu g \text{ mL}^{-1} \)) of TRO for HPTLC method were analyzed three times intra-day using the proposed method. Good% RSD values were obtained confirming the repeatability of the method as given in Table 1.

Intermediate precision: The previous procedure was repeated inter-day on three different days for the analysis of the three chosen concentrations. Acceptable% RSD values were obtained as given in Table 1.

5.4. Specificity

Specificity of the methods was tested by how accurately and specifically the analytes of interest are determined in presence of other components (e.g., co-formulated drugs, excipients, impurities, degradation products, etc). This is evident from HPLC chromatogram and HPTLC-Densitogram in Figs. 2 and 3 proved the specificity of the proposed method.

5.5. Limit of detection and limit of quantitation

ICH recommendations\(^{26}\) were followed using a visual non-instrumental method to calculate the values of LOD and LOQ of the two studied components where LOD is the concentration at which the signal to noise ratio is equal to 3:1 while LOQ is the concentration at which the signal to noise ratio is equal to 10:1. Low values of both LOD and LOQ indicated the high sensitivity of the developed method, Table 1.

5.6. Robustness

Robustness of an analytical procedure is the capacity of the method to remain unaffected with small deliberate variations in method parameters and provides an indication of its reliability during normal usage\(^{26}\) e.g.: changing ammonia volume in the developing system ±0.05 mL and changing saturation time ± 5 min for HPTLC-Densitometric method and changing organic solvents ratios ± 2% and flow rate ± 0.05 ml min\(^{-1}\).

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### Table 2

<table>
<thead>
<tr>
<th>Pharmaceutical formulations</th>
<th>HPLC method Recovery ± SD</th>
<th>TLC method Recovery ± SD</th>
<th>Standard addition technique</th>
<th>HPLC method Pure added (( \mu g \text{ mL}^{-1} )) Recovery%</th>
<th>TLC method Pure added (( \mu g \text{ mL}^{-1} )) Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fleboton® B.N. ccE0564 Labeled tocontain 2.5 mg CAR and 250 mg TRO/3 mL ampoule</td>
<td>CAR 94.30 ± 1.099 95.51 ± 1.127</td>
<td>TRO 105.30 ± 1.061 105.72 ± 1.191</td>
<td></td>
<td>3 99.97 99.89</td>
<td>0.05 99.89</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>4 98.26 98.12</td>
<td>0.1 98.12</td>
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<td></td>
<td></td>
<td></td>
<td>5 100.23 100.24</td>
<td>0.2 100.24</td>
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<td></td>
<td></td>
<td></td>
<td>6 100.45 100.45</td>
<td>0.3 101.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ± SD 99.73 ± 1.001 99.82 ± 1.230</td>
<td></td>
<td>30 100.04 101.74</td>
<td>0.2 101.74</td>
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<td></td>
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<td>35 101.94 99.40</td>
<td>0.3 99.40</td>
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<td>40 101.57 101.79</td>
<td>0.4 101.79</td>
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<td></td>
<td>45 100.38 101.45</td>
<td>0.5 101.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ± SD 99.98 ± 0.915 101.10 ± 1.140</td>
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### Table 3

<table>
<thead>
<tr>
<th>Items</th>
<th>HPLC</th>
<th>TLC</th>
<th>Reported method</th>
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<tr>
<td>CAR</td>
<td>94.30</td>
<td>95.51</td>
<td>99.51</td>
</tr>
<tr>
<td>TRO</td>
<td>105.30</td>
<td>105.72</td>
<td>104.65</td>
</tr>
<tr>
<td>SD</td>
<td>1.096</td>
<td>1.077</td>
<td>1.953</td>
</tr>
<tr>
<td>% RSD</td>
<td>1.097</td>
<td>1.127</td>
<td>1.057</td>
</tr>
<tr>
<td>N</td>
<td>8</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Student’s t-test</td>
<td>1.233 (2.145)</td>
<td>0.975 (1.782)</td>
<td>1.779 (2.179)</td>
</tr>
<tr>
<td>F-value (3.972)</td>
<td>1.529 (3.787)</td>
<td>1.242 (3.972)</td>
<td>1.440 (3.972)</td>
</tr>
</tbody>
</table>

\(^*\) Figures between parenthesis represent the corresponding tabulated values of \( t \) and \( F \) at \( P = 0.05 \).

\(^{**}\) HPLC method for CAR determination: C18 Column, 5 \( \mu m \), 250 × 4.6 mm column, 1 mL min\(^{-1}\) flow rate, 0.12% ammonium dihydrogen phosphate (pH 3.0):ethanol (85:15) as a mobile phase, \( \lambda = 363 \text{ nm} \).

\(^{***}\) HPLC method for TRO determination: C18 Column, 0.5 mL min\(^{-1}\) flow rate, acetonitrile: NaH\(_2\)PO\(_4\) adjusted to pH 4.4 with dilute phosphoric acid (20:80) as a mobile phase, \( \lambda = 350 \text{ nm} \).
Figure 4  Normal probability and residual data plots for (a) Troxerutin and (b) Carbazochrome in HPLC method.

Figure 5  Normal probability and residual data plots for (a) Troxerutin and (b) Carbazochrome in HPTLC method.
7. Quan H, Bai H, Yang X. Determination of Troxerutin and trace rutin contents in Troxerutin tablets by double wavelength UV spectrophotometry. Taiyuan: Shanxi Medical University; 2005.


