Validated Stability Indicating TLC-Densitometric Method for the Determination of Diacerein

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This work presents an accurate, sensitive and selective thin-layer chromatography–densitometry method for the simultaneous determination of diacerein in the presence of rhein, the active metabolite and hydrolytic degradation product of diacerein, and emodin, the diacerein impurity, in bulk powder and pharmaceutical formulations. Chromatographic separation was performed on aluminium plates precoated with 60 F254 silica gel using hexane–ethyl acetate–acetic acid (60:40:0.8, by volume) as a developing system and with detection at 230 nm. The retention factor values of diacerein, rhein and emodin were 0.12, 0.44 and 0.6, respectively. The method was successfully applied for the determination of these compounds with high sensitivity; the linearity ranges were found to be 0.5–10 μg/band (for diacerein and rhein) and 0.5–7 μg/band (for emodin). The developed method was validated according to International Conference on Harmonization guidelines and was applied for the determination of diacerein in different pharmaceutical formulations. Moreover, a statistical comparison between the results of the developed method and those of the reported reversed-phase high-performance liquid chromatography method showed no significant differences. This method can be used for the routine analysis of diacerein, rhein and emodin in quality control laboratories.

Introduction

Diacerein (DIA) is chemically designated as 1,8-diacetoxy-3-carboxyanthraquinone (1, 2) (Figure 1A). It is used for osteoarthritis treatment and to prevent and treat vascular diseases (3). Rhein (RH) is chemically designated as 4,5-dihydroxyanthraquinone-2-carboxylic acid (2) (Figure 1B) and is reported to be the active metabolite (2) and the hydrolytic degradation product of DIA (4). RH inhibits the synthesis of interleukin-1 (IL-1), a cytokine that is involved in cartilage damage, and activates the synthesis of the primary components of cartilage, proteoglycans and hyaluronic acid. (5). It can easily be synthesized from aloin, the natural glucopyranoside. (4). Emodin (EMO) is chemically designated as 1,3,8-trihydroxy-6-methylanthraquinone (6) (Figure 1C) and is reported to be the primary impurity and related substance of DIA (6, 7). It has laxative and antitumor properties (8), but has no healing effect in osteoarthritis cases.

Despite the longstanding commercial distribution of DIA in oral capsule form, no official methods are available for the determination of DIA, either in bulk powder or in its pharmaceutical formulations. On the other hand, the literature survey reveals some methods for its determination, such as spectrophotometric methods (4, 9–18), flow injection chemiluminescence analysis (19), high-performance liquid chromatography (HPLC) methods for the quantitative estimation of DIA in bulk powder (20) and dosage forms (21–27), stability-indicating high-performance thin-layer chromatography (HPTLC) (28) and thin-layer chromatography (TLC)–densitometry (4) and colorimetric determination of DIA in bulk powder and pharmaceutical formulations (29–33). Two impurities from DIA bulk drug have been isolated and structurally elucidated by HPLC and liquid chromatography–mass spectrometry (LC–MS) methods, respectively (34). All of the developed stability-indicating methods have been used for the determination of DIA in the presence of RH.

In accordance with International Conference on Harmonization (ICH) guidelines Q1A (R2) (35) on the stability testing of new drug substances and products, DIA has been subjected to different stress conditions, including hydrolysis, oxidation and photolytic degradation. DIA was degraded under hydrolytic and oxidative degradation conditions to give one degradation product, RH, whereas the drug was found to be stable under photolytic degradation conditions. Thus far, no TLC–densitometric method has been reported for the simultaneous determination of DIA in the presence of RH and EMO. Accordingly, the objective of this work is to develop a selective, sensitive and specific TLC–densitometric method for the determination of DIA, RH and EMO, either in bulk powder or in pharmaceutical formulations. The developed method was successfully applied for the determination of RH and EMO with high sensitivity. The proposed method can be used as an alternative technique to the reported reversed-phase (RP)-HPLC methods; moreover, it has higher sensitivity and selectivity than the reported spectrophotometric and TLC–densitometric methods.

Experimental

Instruments

The instruments used in this study included a sample applicator for TLC Linomat V with a 100 μL syringe (Camag, Muttenz, Switzerland); a TLC Scanner 3 densitometer (Camag) controlled by winCATS software (version 3.15; Camag); TLC plates (20 × 10 cm) coated with 60 F254 silica gel (Merck, Darmstadt, Germany) with 0.2 mm thickness; an ultraviolet
(UV) lamp with short wavelength of 254 nm (VL-6.LC; Marne la Vallee, France). Parameters were adjusted during TLC scanning as follows. Scanning mode: absorbance; source of radiation: deuterium lamp; slit dimension: 6/0.45 mm; scanning speed: 20 mm/s.

Materials and reagents

Pure standards
DIA pure standard was supplied by Delta Pharm Co. (Cairo, Egypt), with a claimed purity of 99.69, according to the manufacturer’s certificate. EMO pure standard was supplied by Sigma-Aldrich Co. (Cairo, Egypt), with claimed purity of 97.83, according to the manufacturer’s certificate.

Pharmaceutical formulations
Osteocerein capsules (batch number Y 0017) were labeled to contain 50 mg of DIA per capsule and manufactured by Novartis Pharma Co. (Cairo, Egypt). DIA capsules (batch number 004497) were labeled to contain 50 mg of DIA per capsule and manufactured by Delta Pharm Co.

Chemicals and solvents
All chemicals used throughout this work were of analytical grade and were used without further purification. These included ethyl acetate, hexane, methanol (El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt), acetic acid and HPLC grade acetonitrile (Sigma-Aldrich).

Solutions
Stock standard solutions of DIA and EMO (1 mg/mL) were prepared as follows: 100 mg of DIA and EMO were accurately and separately weighed into two separate 100 mL measuring flasks; the volume was completed to the mark with acetonitrile. For the stock solution of RH (1 mg/mL), 100 mg of RH was accurately weighed into a 100 mL measuring flask; the volume was completed to the mark with acetonitrile.

Procedure

Forced degradation of DIA
For hydrolysis, two portions of DIA powder equivalent to 25 mg were separately dissolved in two flasks, one containing 25 mL of 0.1N NaOH solution and the other containing 25 mL of 0.1N HCl. The solutions were refluxed for 5 h until complete degradation was achieved, as confirmed by TLC using hexane–ethyl acetate–acetic acid (60:40:0.8, by volume) as a developing system. Acidic and alkaline degraded samples were neutralized to pH 7, the solutions were filtered and the precipitated degradates were washed with water (3 × 10 mL) and finally dried. The structure of the isolated degradation product was elucidated using MS and infrared (IR) analyses.

For oxidation, two portions of DIA powder equivalent to 25 mg were separately dissolved in two flasks, one containing 25 mL of methanolic solution of H₂O₂ (3%, v/v) and the other containing 25 mL of methanolic solution of H₂O₂ (30%, v/v). The solutions were kept for 8 h at room temperature in the dark to exclude the possible degrading effect of light. The resultant solutions were spotted on TLC plates every two hours along with standard DIA to investigate similar degradation pathways or the presence of other degradation products. The solutions were evaporated to dryness and washed with water (3 × 10 mL), and the produced degradate was then dried. The structure of the isolated degradation product was elucidated using MS and IR analyses.

For photolysis, 25 mg of DIA powder was dissolved in methanol and the solution was exposed to both full white and UV lamps (ICH Q1B guidelines; light source option II) for 24 h. The resultant solution was spotted on TLC plates along with standard DIA to investigate similar degradation pathways or the presence of other degradation products.

Chromatographic conditions
The TLC–densitometric method was performed using pre-coated silica gel TLC aluminum plates (20 × 10 cm). The plates were pre-washed with methanol and activated at 100°C for 15 min before sample application. Samples were applied in the form of bands (4 mm length, 8.9 mm spacing and 15 mm from the bottom edge of the plate). Linear ascending development was performed in a chromatographic tank previously saturated with hexane–ethyl acetate–acetic acid (60:40:0.8, by volume) for one hour at room temperature to a distance of approximately 90 mm. The developed plates were air dried and then scanned at 230 nm.

Linearity and construction of calibration curves
Accurate volumes of DIA, RH and EMO were separately transferred from their respective stock solutions (1 mg/mL) and applied in triplicate on the pre-washed TLC plates in the form of bands to obtain the concentration range of 0.5–10 µg/band for DIA and RH and 0.5–7 µg/band for EMO. The previously described chromatographic procedure was then followed. The peak area was recorded and a calibration curve was

Figure 1.
Chemical structures: DIA (A); RH (B); EMO (C).
constructed for each component by plotting the mean integrated peak area versus the corresponding concentration; the regression equations were then computed.

**Application to pharmaceutical formulations**

The contents of 10 capsules each of osteocerein and DIA were emptied, accurately weighed and mixed well. An accurate weight of the mixed powder of each pharmaceutical formulation equivalent to 100 mg of DIA was transferred separately to a 250 mL beaker; 50 mL of acetonitrile was added and the solutions were magnetically stirred for 15 min. The solutions were filtered into two separate 100 mL volumetric flasks and the volume was completed to the mark with acetonitrile to obtain a concentration of 1 mg/mL.

The previously described linearity procedure was followed on the prepared solutions of osteocerein and DIA (3 µg/mL). DIA concentrations were determined using the computed regression equations.

**Results and Discussion**

Planar instrumental chromatography with precise sample application and computer controlled quantification and evaluation of the chromatograms has been considered to be a reliable technique for purity control and for quantitatively testing drugs and (36). The proposed TLC–densitometric method is much more advantageous than the reported TLC–densitometric and spectrophotometric methods, because it offers higher sensitivity and selectivity. Also, it can be used in laboratories lacking the facilities to conduct HPLC techniques.

DIA was exposed to forced degradation conditions according ICH guidelines Q1A (R2) (35) on the stability testing of new drug substances and products. The densitograms obtained from the samples subjected to different stress conditions (hydrolysis, oxidation and photolysis) are shown in Figures 2A–E. DIA was degraded upon hydrolytic and oxidative conditions. This degradation was confirmed using TLC, in which only one spot was observed under acidic and alkaline hydrolytic and oxidative degradation, which did not correspond to DIA. The structure of the isolated degrade was elucidated using MS and IR analyses. The mass spectrum of the degradate showed a base peak at m/z = 284 corresponding to M+1 = 285, which corresponds to RH. Moreover, the IR spectrum of the degrade showed the disappearance of the carbonyl group at 1,770 cm⁻¹, indicating cleavage of the two ester groups. Only the carbonyl groups of the ring and the carboxylic acid are present at 1,695 cm⁻¹, whereas the broad bands (2, 968–3, 425 and 2, 642–3, 429) are present in the two IR spectra, indicating OH⁻ groups (Figures 3 and 4). On the other hand, DIA was stable upon exposure to photolytic degradation conditions. This was confirmed using TLC, in which only one spot corresponding to DIA was observed.

The first step in method development is to test all the published TLC–densitometric systems. Unfortunately, none of the methods were able to achieve good chromatographic separation among the three components. Experimental conditions such as developing system composition, band dimensions, scanning wavelength and slit dimensions were optimized to provide good chromatographic resolution.

**Developing system**

Different developing systems of different compositions were tried; the developing system of choice was hexane–ethyl acetate–acetic acid (60:40:0.8, by volume), which showed good resolution, with Retardation factor (Rf) values of 0.12, 0.44 and 0.6 for DIA, RH and EMO, respectively (Figure 5).

**Band dimensions**

Different band dimensions were tested to obtain sharp and symmetrically separated peaks. The optimum bandwidth was 4 mm with 8.9 mm interspace between bands.

**Scanning wavelength**

Different scanning wavelengths were tested to obtain the best sensitivity for all separated components, such as 210, 230, 250, 275 and 300 nm. The best scanning wavelength was 230 nm, which showed a good signal-to-noise ratio for all components, resulting in high sensitivity.

**Slit dimensions of scanning light beam**

The slit dimensions of the scanning light beam should ensure complete coverage of the band dimensions on the scanned track without interference from the adjacent bands. Different slit dimensions were tested and 4 × 0.45 mm proved to be the slit dimensions of choice, providing high sensitivity.

Calibration curves were constructed by plotting the integrated peak area versus the corresponding concentration in the range of 0.5–10 µg/band for DIA and RH and 0.5–7 µg/band for EMO. The concentrations of DIA, RH and EMO were calculated from the following equations:

\[
\text{For DIA, } Y_1 = 561.595C_1 + 1358.129 \quad r_1 = 0.9998 \\
\text{For RH, } Y_2 = 1521.234C_2 - 638.935 \quad r_2 = 0.9999 \\
\text{For EMO, } Y_3 = 933.112C_3 + 1281.799 \quad r_3 = 0.9999
\]

where \( Y_1, Y_2 \) and \( Y_3 \) are the integrated peak areas of DIA, RH and EMO, respectively; \( C_1, C_2 \) and \( C_3 \) are the concentrations of DIA, RH and EMO, respectively, in µg/band; and \( r_1, r_2 \) and \( r_3 \) are the correlation coefficients.

**Method Validation**

Method validation was performed according to ICH (35) guidelines. Table I shows the results of accuracy, repeatability and intermediate precision of the method. Other regression parameters in Table I show a good linear relationship in accordance with Beer’s law for the proposed methods, as revealed by the correlation coefficients; additionally, the limit of detection (LOD) and limit of quantification (LOQ) were visually obtained. System suitability parameters were also studied and satisfactory results were obtained (Table II). Studies concerning robustness were investigated and showed that the small change in the scanning wavelength and the composition of the mobile phase had no effect on the results.
Figure 2. Densitograms taken after 2 h: DIA (peak 1) (A); DIA under acidic conditions (B); DIA under alkaline hydrolysis (C); DIA under oxidative hydrolysis (D); DIA under photolytic conditions (E).
Figure 3. IR spectra: DIA (A); RH (B).

Figure 4. Mass spectrum of RH.
The results given in Table III show that the suggested method is valid and applicable for the analysis of DIA in pharmaceutical formulations (osteoceratin and DIA), with acceptable percentage recoveries. Furthermore, the validity of the proposed method was assessed by applying the standard addition technique, which showed no interference from excipients (Table III).

Statistical analysis of the results, obtained by applying the developed TLC–densitometric method and a reported HPLC method (21), showed no significant differences regarding both accuracy and precision within a probability of 95% (Table IV).

Table I
Regression Parameters of the Proposed TLC–Densitometric Method for the Determination of DIA, RH and EMO

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DIA</th>
<th>RH</th>
<th>EMO</th>
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</thead>
<tbody>
<tr>
<td>Linearity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.5–10 µg/band</td>
<td>0.5–10 µg/band</td>
<td>0.5–7 µg/band</td>
</tr>
<tr>
<td>Slope</td>
<td>581.595</td>
<td>1,521.234</td>
<td>933.112</td>
</tr>
<tr>
<td>Intercept</td>
<td>13.58129</td>
<td>–638.935</td>
<td>1,281.800</td>
</tr>
<tr>
<td>Accuracy</td>
<td>99.999</td>
<td>0.9999</td>
<td>0.9999</td>
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Table II
System Suitability Testing Parameters of the TLC–Densitometric Method

<table>
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<th>Parameters</th>
<th>Obtained value</th>
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<tr>
<td></td>
<td>DIA</td>
</tr>
<tr>
<td>Symmetry factor (Rs)</td>
<td>0.96</td>
</tr>
<tr>
<td>Resolution (Rs)</td>
<td>3.68</td>
</tr>
<tr>
<td>Capacity factor (K')</td>
<td>1</td>
</tr>
<tr>
<td>Selectivity factor (α)</td>
<td>3.4</td>
</tr>
<tr>
<td>N</td>
<td>23.04</td>
</tr>
<tr>
<td>H</td>
<td>32.55 × 10⁻² cm</td>
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Table III
Determination of DIA in Commercial Capsules and Application of the Standard Addition Technique by the Proposed TLC–Densitometric Method

<table>
<thead>
<tr>
<th>Pharmaceutical formulations</th>
<th>Claimed taken (µg/mL)</th>
<th>Recovery (% ± relative standard deviation (RSD))</th>
<th>Standard addition technique</th>
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<tr>
<td></td>
<td>Pure added (µg/mL)</td>
<td>Recovery (%)</td>
<td></td>
</tr>
<tr>
<td>DIA capsules (batch number 004497), labeled to contain 50 mg DIA/capsule</td>
<td>3</td>
<td>94.72 ± 0.502</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Mean ± RSD</td>
<td>99.96 ± 1.046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoceratin capsules (batch number Y0017), labeled to contain 50 mg DIA/capsule</td>
<td>3</td>
<td>104.87 ± 0.507</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Mean ± RSD</td>
<td>101.19 ± 1.002</td>
<td></td>
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Table IV
Statistical Comparison of the Results Obtained by Applying the Proposed Method and the Reported HPLC Method as a Reference Method for the Determination of DIA in its Pure Form

<table>
<thead>
<tr>
<th>Items</th>
<th>TLC–densitometric method</th>
<th>HPLC method (20)</th>
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<tr>
<td>DIA</td>
<td>Osteoceratin</td>
<td>DIA Osteoceratin</td>
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<tr>
<td>Mean</td>
<td>94.72</td>
<td>104.18</td>
</tr>
<tr>
<td>SD</td>
<td>0.996</td>
<td>1.760</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.052</td>
<td>1.689</td>
</tr>
<tr>
<td>Student’s t-test</td>
<td>0.9178</td>
<td>0.0057</td>
</tr>
<tr>
<td>F-value</td>
<td>1.0589 (3.787)¹</td>
<td>1.0173 (3.787)¹</td>
</tr>
</tbody>
</table>

¹Figures between parentheses represent the corresponding tabulated values of t and F at ρ = 0.05.

Conclusion
The present work provides a sensitive, cost-effective and selective TLC–densitometric method for with high sensitivity the determination of DIA, RH and EMO in their ternary mixtures. The advantage of the TLC–densitometric method is that several samples can be run simultaneously using a small quantity of mobile phase, unlike HPLC, thus lowering analysis time and cost per analysis and providing high sensitivity and selectivity. The developed method can easily be applied for the routine analysis of the studied drug in quality control laboratories.

References
spectrodensitometric methods for the determination of diacerein in the presence of its degradation product; Drug Testing and Analysis (2011); 4: 221–227.


6. Zhang, H.X., Liu, M.C.; Separation procedures for the pharmaco-


