Stability-Indicating RP-HPLC Methods for the Determination of Fluorometholone in Its Mixtures with Sodium Cromoglycate and Tetrahydrozoline Hydrochloride

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Abstract

Two stability-indicating reversed-phase liquid chromatographic methods were developed and validated for the determination of fluorometholone (FLU) in its mixtures with sodium cromoglycate (SCG) and tetrahydrozoline hydrochloride (THZ). The first HPLC method (Method 1) was based on isocratic elution of FLU and SCG along with their alkaline degradation products on a reversed phase C18 column (250 × 4.6 mm id)—ACE Generix 5, using a mobile phase consisting of methanol–water (70 : 30, v/v), pH adjusted to 2.5 using orthophosphoric acid at a flow rate of 1.2 mL min⁻¹. Quantitation was achieved with UV detection at 240 nm. The second HPLC method (Method 2) was based on isocratic elution of FLU, its alkaline degradation product and THZ on a reversed phase C8 column (250 × 4.6 mm)—ACE Generix 5, using a mobile phase consisting of acetonitrile–50 mM potassium dihydrogen orthophosphate (40 : 60, v/v) at a flow rate of 2 mL min⁻¹. Quantitation was achieved by applying dual-wavelength detection, where FLU and its alkaline degradation product were detected at 240 nm and THZ was detected at 215 nm at ambient temperatures. Linearity, accuracy and precision were found to be acceptable over the concentration range of 5–50 and 10–500 μg mL⁻¹ for FLU and SCG (Method 1) and over the concentration range of 5–80 and 5–60 μg mL⁻¹ for FLU and THZ (Method 2), respectively. Besides, the FLU alkaline degradation product was verified using IR, NMR and LC–MS spectroscopy. The two proposed methods could be successfully applied for the routine analysis of the studied drugs either in their pure bulk powders or in their pharmaceutical preparations without any preliminary separation step.

Introduction

Fluorometholone (FLU) [9α-fluoro-11b,17α-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione] (Figure 1A) is a corticosteroid employed for its glucocorticoid activity (1). It is thought to act by the induction of phospholipase A2 inhibitory proteins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of their common precursor, arachidonic acid.

Sodium cromoglycate (SCG) [disodium-4,4′-dioxo-5,5′-(2-hydroxytrimethylenedioxy)-di(4H-chromene-2-carboxylate)] (Figure 1B) is believed to act primarily by preventing release of mediators of inflammation from sensitized mast cells through stabilization of...
mast cell membranes. It is used in the prophylaxis of many types of asthma (2, 3).

Tetrahydrozoline hydrochloride (THZ) [4,5-dihydro-2-(1,2,3,4-tetrahydro-1-naphthalenyl)-1H-imidazole hydrochloride] (Figure 1C) is a sympathomimetic agent with α-adrenergic activity. It acts as a local vasoconstrictor. Solutions and suspensions of THZ are used as a conjunctival decongestant (4).

The literature survey revealed determination of FLU alone colorimetrically (5), in its mixture with SCG using UV spectrophotometric (1, 6), TLC spectrodensitometric (1) and HPLC methods (7). Other UV spectrophotometric and HPLC methods were reported for its determination in the mixture with THZ (8) and with ofloxacin and prednisolone using HPLC (4). FLU purity was studied using HPLC (9). Furthermore, its determination in different biological fluids using LC–MS was reported (10–12).

Besides, several methods have been reported for the determination of SCG by spectrophotometric (13–15), TLC-densitometric methods (16, 17) and also by electrochemical methods (18, 19). Several HPLC methods were described for the determination of SCG using UV detection (7, 20–24), fluorescence detection (25) or tandem mass spectrometric detection (26–28). Moreover, two stability-indicating HPLC methods were reported for the determination of SCG in the presence of its related substances (29, 30).

Also, several analytical methods have been reported for the determination of THZ by UV spectrophotometry (8), HPLC (8, 31–35), ion pairing technique (36) and gas chromatography (37). Furthermore, a stability-indicating HPLC method was reported for the determination of THZ (32).

FLU degradation was not studied in any reported method. Moreover, all the reported spectrophotometric, TLC-densitometric and HPLC methods for the determination of the FLU and SCG binary mixture (1,6,7) and the reported HPLC and spectrophotometric methods for the determination of FLU and THZ (8) are not stability-indicating methods. The reported stability-indicating methods were only concerned with the drugs under investigation alone, but not in mixture. Thus, the aim of the this study was to develop simple, sensitive and selective LC methods for the simultaneous determination of FLU and THZ along with alkaline degradation products of both drugs and determination of FLU and THZ along with alkaline degradation product of FLU. Also, the structure of FLU alkaline degradation product was verified using IR, 1H NMR and mass spectrometry. Both methods are applicable for the determination of these drugs in their laboratory mixtures and pharmaceutical preparations.

Experimental

Instrumentation

Agilent 1260 series (Waldbronn, Germany) HPLC system was used, and the interface was equipped with an Agilent quaternary pump G1311C, Agilent UV-visible detector G1314F, an Agilent manual injector G1328C equipped with (20 μL) an injector loop and an Agilent degasser G1316A.

Separation and quantitation were made on a C18 column (250 × 4.6 mm id)—ACE Generix 5° for (Method 1) and on a C8 column (250 × 4.6 mm id)—ACE Generix 5° for (Method 2).

Reagents and reference samples

FLU was kindly supplied by Alcon-Couvreur (Puurs, Belgium) and was certified to contain 99.4%. SCG was kindly supplied by Amoun Company and THZ was kindly supplied by Orchidia Pharmaceutical and were certified to contain 100.2 and 99.6%, respectively. HPLC-grade acetonitrile and methanol were supplied by Scharlau (Barcelona, Spain). Ortho-phosphoric acid was supplied by Sigma-Aldrich (Fluka Analytical) (Seelze, Germany). Potassium dihydrogen orthophosphate was supplied by Honeywell Riedel-de-Haen (Seelze, Germany). Bi-distilled water was produced in-house (Aquatron Water Still, A4000D; UK). Membrane filters of 0.45 μm were purchased from Teknokroma (Barcelona, Spain), Fluka® sterile ophthalmic suspension, labeled to contain 20 mg of SCG and 1 mg of FLU per milliliter, was manufactured by Jamjoom Pharmaceuticals Company (Jeddah, KSA). Flumetol® eye drops nominally containing 0.25% w/v FLU and 0.1% w/v THZ were manufactured by Himont Pharmaceuticals (Lahore, Pakistan).

Experimental conditions

Method 1

Chromatographic separation was achieved on a C18 column (250 × 4.6 mm id)—ACE Generix 5° using a mobile phase consisting of methanol–water (70 : 30 v/v), pH adjusted to 2.5 with orthophosphoric acid at a flow rate of 1.2 mL min⁻¹. The column temperature was 25°C. UV detector was operated at 240 nm. The injection volume was 20 μL.

Method 2

Chromatographic separation was achieved on a C8 column (250 × 4.6 mm id)—ACE Generix 5° applying isocratic elution based on a mobile phase consisting of acetonitrile–50 mM potassium dihydrogen orthophosphate, pH 7.4 (40 : 60, v/v). The mobile phase was pumped through the column at a flow rate of 2 mL min⁻¹. Analysis was performed at ambient temperature, and detection was time programmed to be at 215 nm from 0 to 3 min for detection of THZ, then at 240 nm.
from 3 to 7 min for detection of FLU and its alkaline degradation product.

### Solutions

#### Stock standard solutions

**Method 1.** Stock standard solutions of FLU (0.5 mg mL\(^{-1}\)) and SCG (2 mg mL\(^{-1}\)) were prepared in methanol. Appropriate dilutions of these stock solutions were prepared in the mobile phase to use in linearity studies and assay purposes.

**Method 2.** Stock standard solutions of FLU (1 mg mL\(^{-1}\)) and THZ (1 mg mL\(^{-1}\)) were prepared in methanol. Appropriate dilutions of these stock solutions were prepared in the mobile phase to use in linearity studies and assay purposes.

#### Laboratory-prepared mixtures

**Method 1.** Solutions containing different ratios of FLU (5–20 µg mL\(^{-1}\)) and SCG (100–400 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.

**Method 2.** Solutions containing different ratios of FLU (15–75 µg mL\(^{-1}\)) and THZ (6–30 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.

#### Sample preparation

**Method 1.** Two milliliters were accurately transferred from Fluca® eye drops to a 25-mL volumetric flask and diluted to the mark with the mobile phase to get 80 and 1600 µg mL\(^{-1}\) of FLU and SCG, respectively. The prepared solution was filtered through a 0.45-µm Millipore syringe membrane filter.

**Method 2.** In a 50-mL volumetric flask, 3.2 mL of Flumetol® eye drops were accurately transferred and diluted to the mark with the mobile phase to get 160 and 64 µg mL\(^{-1}\) of FLU and THZ, respectively. The prepared solution was filtered through a 0.45-µm Millipore syringe membrane filter.

### Procedures

#### Construction of the calibration curves

**Method 1.** Aliquots equivalent to 5–50 µg mL\(^{-1}\) of FLU and 10–500 µg mL\(^{-1}\) of SCG were accurately transferred into two series of 10-mL volumetric flasks, and the volumes were completed to the mark with the mobile phase.

**Method 2.** Aliquots equivalent to 5–80 µg mL\(^{-1}\) of FLU and 5–60 µg mL\(^{-1}\) of THZ were accurately transferred into two series of 10-mL volumetric flasks, and the volumes were completed to the mark with the mobile phase.

A volume of 20 µL of each solution was injected in triplicates into the chromatograph under the specified chromatographic conditions described in “Method 1” and “Method 2”, under “Experimental conditions”, respectively. A calibration curve for each compound was obtained by plotting area under the peak (AUP) against concentration (C).

### Assay of laboratory-prepared mixtures

For **Method 1 and Method 2.** The solutions, described in “Method 1” and “Method 2” under “Laboratory-prepared mixtures”, were injected in triplicates into the chromatograph under the specified chromatographic conditions described in “Method 1” and “Method 2”, under “Experimental Conditions”. The concentration of each drug was calculated using the specified regression equation.

### Assay of pharmaceutical preparations

**Method 1.** The sample solution, described in “Method 1” under “Sample preparation”, was serially diluted to get concentrations equivalent to 8–16 and 160–320 µg mL\(^{-1}\) of FLU and SCG, respectively. Samples were then injected in triplicates, and concentrations of FLU and SCG were calculated using calibration equations.

**Method 2.** The sample solution, prepared in described in “Method 2” under “Sample preparation”, was serially diluted to get concentrations equivalent to 16–48 and 6.4–19.2 µg mL\(^{-1}\) of FLU and THZ, respectively. Samples were then injected in triplicates, and concentrations of FLU and THZ were calculated using calibration equations.

### Preparation of degradation products

#### Preparation of FLU alkaline degradation product

To 7.5 mL of FLU methanolic stock solution (1 mg mL\(^{-1}\)), an appropriate volume of 4 M NaOH (6.25 mL) was added and the mixture was diluted with methanol to 25 mL to reach 1 M NaOH and then the mixture was heated at 80°C in a water bath for 3 h. Solution was neutralized before incorporation in the analysis.

#### Preparation of SCG alkaline degradation product

(1-[2-[3-(2-acetyl-3-hydroxy-phenoxy)-2-hydroxy-propoxy]-6-hydroxy-phenyl]-ethanone) SCG (500 mg) was refluxed with 10% aqueous potassium hydroxide (10 mL) for 2 h. Subsequently, the solution was acidified with glacial acetic acid until complete precipitation of the degradation product. The precipitate was filtered and dried under vacuum. The stock solution of SCG degradation product was prepared by dissolving in methanol to prepare solution (0.6 mg mL\(^{-1}\)) (20).

### Results

#### System suitability tests

System suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed

### Table I. System Suitability Tests for the Proposed LC Method for Determination of FLU, SCG or THZ in Mixtures

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLU</td>
<td>SCG</td>
</tr>
<tr>
<td>Number of theoretical plates</td>
<td>8,097</td>
<td>1,942</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.03</td>
<td>1.13</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>4.385</td>
<td>0.926</td>
</tr>
<tr>
<td>Resolution</td>
<td>14.67</td>
<td>14.23</td>
</tr>
<tr>
<td>% RSD(^a) of six injections of Peak area</td>
<td>0.461</td>
<td>0.618</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>0.28</td>
<td>0.09</td>
</tr>
</tbody>
</table>

\(^{a}\)Relative to 9,11β epoxy fluorometholone.

\(^{b}\)Relative standard deviation.
Table II. Assay Parameters and Method Validation Obtained By Applying HPLC Method for the Simultaneous Determination of FLU and SCG or THZ in Mixtures

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLU</td>
<td>SCG</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>5.278</td>
<td>2.08</td>
</tr>
<tr>
<td>Wavelength of detection (nm)</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>Range of linearity (µg mL⁻¹)</td>
<td>5–50</td>
<td>10–50</td>
</tr>
<tr>
<td>Regression equation</td>
<td>Area = 39.646C_{µg/ml} + 21.595</td>
<td>Area = 57.115C_{µg/ml} + 108.84</td>
</tr>
<tr>
<td>Regression coefficient (r²)</td>
<td>0.9998</td>
<td>0.9999</td>
</tr>
<tr>
<td>LOD (µg mL⁻¹)</td>
<td>1.38</td>
<td>0.707</td>
</tr>
<tr>
<td>LOQ (µg mL⁻¹)</td>
<td>4.46</td>
<td>2.143</td>
</tr>
<tr>
<td>Sₐ</td>
<td>0.208</td>
<td>0.271</td>
</tr>
<tr>
<td>Sₐ</td>
<td>5.868</td>
<td>71.25</td>
</tr>
<tr>
<td>Confidence limit of the slope</td>
<td>39.646 ± 0.51</td>
<td>57.115 ± 0.664</td>
</tr>
<tr>
<td>Standard error of the estimation</td>
<td>8.371</td>
<td>132.1</td>
</tr>
<tr>
<td>Inter-day (% RSD)</td>
<td>0.21–0.49</td>
<td>0.28–0.66</td>
</tr>
<tr>
<td>Intra-day (% RSD)</td>
<td>0.22–0.89</td>
<td>0.49–0.77</td>
</tr>
<tr>
<td>Drug in bulk</td>
<td>100.73% ± 0.697</td>
<td>100.87% ± 0.857</td>
</tr>
<tr>
<td>Drug in dosage form</td>
<td>101.22% ± 0.499</td>
<td>101.977% ± 0.445</td>
</tr>
<tr>
<td>Standard added</td>
<td>100.374% ± 0.977</td>
<td>100.816 ± 0.381</td>
</tr>
</tbody>
</table>

a, Intercept; b, slope; Sₐ, standard deviation of intercept; Sₐ, standard deviation of slope.
method. They are used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters of these tests are column efficiency (number of theoretical plates), tailing of chromatographic peak, repeatability as percentage relative standard deviation (% RSD) of the peak area for six injections and reproducibility of retention as % RSD of retention time. Solutions of 25 and 250 μg mL⁻¹ of FLU and SCG for Method 1, and solutions of 50 and 30 μg mL⁻¹ of FLU and THZ for Method 2, respectively, were used. The results of system suitability tests for the proposed method are listed in Table I.

**Method validation**
Methods validation was carried out in accordance to ICH guidelines (38).
The linearity of the chromatographic methods for the determination of FLU, SCG and THZ were evaluated by analyzing a series of different concentrations of each drug. For Method 1, eight concentrations for FLU and SCG ranging from 5 to 50 and 10 to 500 μg mL⁻¹, respectively, were chosen while for Method 2, seven concentrations for FLU and THZ ranging from 5 to 80 and 5 to 60 μg mL⁻¹, respectively, were chosen. Each concentration was analyzed in triplicate. 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) are summarized in Table II.

### Accuracy
Accuracy of the results was calculated by % recovery of laboratory-prepared mixtures of six different concentrations of FLU, SCG or THZ and also by the standard addition technique for Fluca® and Flumetol® eye drops in the two methods. The results obtained including the mean of the recovery and standard deviation are displayed in Table II.

### Precision
The repeatability (intra-day precision) of the method was assessed by three determinations for each of the three concentrations representing 80, 100 and 120% for each drug. For Method 1, these concentrations were (20, 25 and 30 μg mL⁻¹) and (200, 250 and 300 μg mL⁻¹) for FLU and SCG, respectively. For Method 2, the concentrations (40, 50 and 60 μg mL⁻¹) and (24, 30 and 36 μg mL⁻¹) for FLU and THZ, respectively, were used. The repeatability of sample application and measurement of the peak area for active compounds were expressed in terms of % RSD. All experiments described in repeatability were repeated in three consecutive days by the same analyst to evaluate day-to-day ruggedness (inter-day precision), and the results are shown in Table II.

### Table III. Statistical Analysis of the Results Obtained by the Proposed Methods and the Reference Methods

<table>
<thead>
<tr>
<th>Statistical term</th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLU</td>
<td>SCG</td>
</tr>
<tr>
<td>Mean</td>
<td>100.73</td>
<td>100.06</td>
</tr>
<tr>
<td>SD</td>
<td>0.716</td>
<td>1.057</td>
</tr>
<tr>
<td>RSD</td>
<td>0.292</td>
<td>1.060</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Variance</td>
<td>0.513</td>
<td>1.117</td>
</tr>
<tr>
<td>t-value (2.23)</td>
<td>1.285</td>
<td>2.053</td>
</tr>
<tr>
<td>F-value (5.05)</td>
<td>2.177</td>
<td>1.793</td>
</tr>
</tbody>
</table>

*Reference method (manufacturer HPLC method for determination of FLU and SCG in mixture) (39).

*Reference method (HPLC method for determination of FLU and THZ in mixture) (6).

*Figures in parentheses are the corresponding values for theoretical t- and F-values at P = 0.05.
Specificity
Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences. No chromatographic interference from any of the degradation products was obtained by mixing alkaline degraded FLU and SCG solutions with FLU and SCG or mixing alkaline degraded FLU with FLU and THZ (Figures 2B and 3B). The chromatograms of the pharmaceutical formulation samples were checked for the appearance of any extra peaks. In addition, the
chromatograms of FLU and SCG or THZ in the sample solutions were found to be identical to the chromatograms obtained by the standard solution (Figures 4 and 5). Therefore, the proposed methods could be successfully applied for the routine analysis of the studied drugs in their dosage forms without any preliminary separation step. Results for determination of these drugs by the proposed method in their dosage forms along with the standard addition technique are displayed in Table II.

Limit of detection and limit of quantitation
Limit of detection (LOD), which represents the concentration of analyte at S/N ratio of 3, and limit of quantification (LOQ) at which S/N ratio is 10 were determined experimentally for the proposed methods and results are given in Table II.

Robustness
Robustness was performed by deliberately changing the chromatographic conditions. For both mixtures, pH of the mobile phase was varied by ±0.2 units. The flow rate of the mobile phases was changed by ±0.2 mL min⁻¹. The organic strengths were varied by ±2%. These variations did not have significant effect on chromatographic resolution by the proposed LC methods.

Discussion
Method development
Method 1
Many different ratios of methanol and water were tried as mobile phase for the separation of FLU and SCG such as (45 : 55, 50 : 50, 60 : 40 and 70 : 30, v/v). It was found that the ratio of (70 : 30, v/v) at a flow rate of 1.2 mL min⁻¹ was the best for obtaining good peak shape, best number of theoretical plates and reasonable retention time for FLU and SCG along with their alkaline degradation products. Moreover, it was found that adjusting the pH of the aqueous component of the mobile phase to 2.5 was optimum for the elution and the separation of all peaks. Detection was carried out at 240 nm to obtain sufficient peak intensity for both drugs and degradation products (Figure 2A and B). The retention times were 3.385 and 1.925 min for FLU and SCG, respectively. FLU and SCG alkaline degradation products were eluted at 4.455 and 8.229 min, respectively.

Method 2
During the optimization cycle, several chromatographic conditions were attempted using various mobile phase compositions such as methanol with water, acetonitrile with water and methanol with water and glacial acetic acid, in different proportions, applying isocratic mode. These mobile phases were applicable for the elution of FLU but with deformed peak of THZ. Upon using a mobile phase composed of acetonitrile with 50 mM potassium dihydrogen orthophosphate in different ratios, it was found that the ratio of (40 : 60 v/v), with a flow rate of 2 mL min⁻¹ using a C18 column was optimum for the separation of FLU and THZ along with the FLU alkaline degradation product. UV detection was adjusted at 215 nm for THZ from 0 to 3 min and at 240 nm for FLU from 3 to 7 min (Figure 3A and B). The retention times were 1.667 and 5.357 min for THZ and FLU, respectively. The FLU alkaline degradation product was eluted at 4.338 min.

Identification of degradation products
Structure elucidation of the obtained FLU degradation product was confirmed using IR, NMR and mass spectroscopic techniques and proved to be 9,11β-epoxy derivative of FLU. The IR spectrum of FLU degradation product showed disappearance of C–F bond at
1354 cm\(^{-1}\) when compared with the IR spectrum of FLU (Figure 6A and B). Besides, mass spectrum showed the molecular ion peak of the obtained 9,11\(\beta\)-epoxy FLU at 356.2 (M\(^+\)) (Figure 7). Moreover, this finding was confirmed by \(^1\)H-NMR spectrum of FLU degradation product (Figure 8).

On the other hand, SCG was degraded based on a reported method (20) as it was the best method that produced the degradation product in high purity and good yield (Figure 9). According to the Spath and Gruber method (40–42), khellin, which is a naturally occurring chromone compound, can be hydrolyzed in 1% aqueous potassium
hydroxide to yield khellinone. Consequently, a successful attempt has been carried out for the preparation of the degradation product (1-{2-[3-(2-acetyl-3-hydroxy-phenoxy)-2-hydroxy-propoxy]-6-hydroxy phenyl}ethanone) from SCG through the application of the aforementioned Spath and the Gruber method based on the similarity of the chromone nucleus in both compounds (SCG and khellin). Besides, THZ was subjected to alkaline hydrolysis by heating it in 0.1 M NaOH at 75°C for 0.5 h, but the compound remained intact.

Statistical studies
Statistical comparison between the results of the proposed methods and those of the reference methods, obtained by using Student’s t-test and F-ratio, showed no significant differences. Method 1 was compared with the manufacturer’s HPLC method for the determination of FLU and SCG in mixture (42). Method 2 was compared with the published HPLC method for the determination of FLU and THZ in mixture (8). It can be concluded that the proposed analytical methods are sufficiently accurate and precise, and the results are given in Table III.

Conclusion
Two validated stability-indicating LC methods were developed for the simultaneous determination of (FLU, SCG) and (FLU, THZ) mixtures along with alkaline degradation products of both drugs in Mixture 1 and with alkaline degradation product of FLU in Mixture 2. The first proposed method has the advantage of novelty as there is no reported HPLC method for the determination of FLU and SCG in a mixture. Moreover, the second proposed method is a stability-indicating assay, where FLU, its alkaline degradation product and THZ can be determined simultaneously. The proposed methods could be successfully applied for the routine analysis of the studied drugs either in their pure bulk powders or in their dosage forms without any preliminary separation step.

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