Article

Stability-Indicating HPTLC Method for Simultaneous Estimation of Flurbiprofen and Chloramphenicol in Ophthalmic Solution

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Abstract

A specific, accurate and reproducible stability-indicating high performance thin layer chromatography (HPTLC) method was developed for the estimation of flurbiprofen and chloramphenicol in the presence of their degradation products. Degradation studies of both the drugs were carried out in acidic, alkaline, neutral, oxidative, photolytic and thermal stress conditions. Separation was performed on thin layer chromatography plate precoated with silica gel 60 F254 using ethyl acetate : n-hexane : methanol : tri-ethyl amine (5 : 4 : 2 : 0.5, v/v/v/v). Spots at retention factor 0.29 and 0.62 were recognized as flurbiprofen and chloramphenicol, respectively, and were quantified through densitometric measurements at wavelength 267 nm. Method was found to be linear over the concentration range 12–60 ng/spot with correlation coefficient of 0.9997 for flurbiprofen and 200–1,000 ng/spot with correlation coefficient of 0.9977 for chloramphenicol. The proposed method was applied to the estimation of flurbiprofen and chloramphenicol in commercial ophthalmic formulation. The developed HPTLC method can be applied for routine analysis of flurbiprofen and chloramphenicol in the presence of their degradation products in their individual as well as combined pharmaceutical formulations.

Introduction

Flurbiprofen (FLU) (Figure 1A) (1–3) is a nonsteroidal anti-inflammatory drug (NSAIDs) of the propionic acid class. It exhibits anti-inflammatory, analgesic and antipyretic activities. It is used before and after eye surgery (such as cataract removal). Chloramphenicol (CHL) (Figure 1B) (1–3), an antibiotic, possesses broad-spectrum antibacterial activity and is used for the treatment of rickettsial and chlamydial diseases, gram-positive and gram-negative bacterial infections and topically for superficial conjunctival infections. A number of NSAIDs as well as corticosteroid and antibiotic combinations are frequently used as antibacterial agents to cure infections particularly associated with the eye. These combinations are available in different formulations such as eye ointment, eye drops and ophthalmic suspensions. Ophthalmic preparation of FLU with CHL is widely prescribed for the treatment of superficial eye infections.

For estimation of FLU from human plasma high performance liquid chromatography (HPLC) methods (4, 5), stereoselective determinations (6, 7), chiral separation (8), separation from ocular fluid (9) and also from pharmaceutical dosage forms (10, 11) have been reported. Spectrofluorimetric determination (12), electrochemical methods (13, 14), supercritical fluid chromatographic method (15) and titrimetric assay method (16) have also been reported.

Spectrophotometric methods (17–19), HPLC methods (20–26), electrochemical methods (27–32) and a luminescence method (33) have been reported for the estimation of CHL in pharmaceutical preparations, cosmetic creams, plasma, urine and serum. High performance thin layer chromatography (HPTLC) methods have also been reported for the determination of FLU (34, 35) and few HPTLC methods have also been reported for the determination of CHL in combination with 2-amino-1-(4-nitrophenyl) propane-1,3-diol (36) and with different corticosteroids like hydrocortisone acetate (37), prednisolone acetate (38) and dexamethasone sodium phosphate (39). No report for the stability-indicating method for simultaneous estimation of FLU and CHL in ophthalmic solution has been found so far.
The HPTLC stability-indicating assay method is fast, reliable and accurate and involves simultaneous analysis of many samples using small quantity of mobile phase, thus minimizing analysis time and cost per analysis. Stress testing provides evidence for the variation of quality of a drug substance with time under the influence of various environmental factors (temperature, light, humidity, etc.) and helps to establish shelf life and recommended storage conditions for the drug.

The present study describes a simple and validated HPTLC method for the simultaneous estimation of FLU and CHL in presence of their degradation products formed under the applied stress conditions. The validated HPTLC method demonstrates no interferences of degradation products with the assay of active drug components and can be used in the estimation of these components in routine analysis as well as stability studies.

**Experimental**

**Chemicals and reagents**

FLU and CHL were provided by FDC Ltd, Raigad, Maharashtra, India and Century Pharmaceuticals Ltd, Vadodara, Gujarat, India, respectively, as gift samples. Ophthalmic solution containing FLU (0.03% w/v) and CHL (0.5% w/v) in combination (Flubichlor eye drops) was gifted by Dilipkumar & Co., Mumbai, Maharashtra, India. The solvents and chemicals used in the study were of AR grade (S D Fine-Chem Ltd).

**Preparation of solutions**

**Standard stock solutions of FLU and CHL**

Accurately weighed FLU (25 mg) and CHL (25 mg) were transferred to separate 25-mL volumetric flasks, dissolved in and diluted up to the mark with methanol giving the concentration of 1,000 µg/mL of each drug. A 1.0 mL aliquot of standard stock solution of FLU was diluted to 10 mL with methanol (100 µg/mL) to prepare working standard solution of FLU (100 µg/mL). To make a mixed working standard solution, a 0.3 mL aliquot of working standard solution of FLU and a 0.5 mL aliquot of standard stock solution of CHL were transferred to a 10-mL volumetric flask and diluted to mark with methanol to get solution of concentration 3 µg/mL of FLU and 50 µg/mL of CHL.

**Preparation of samples for forced degradation**

Forced degradation of FLU and CHL was carried out under acidic, alkaline, neutral, oxidative, photolytic and thermal stress conditions using solutions of individually as well as in mixture of both drugs. Separate stock solutions as well as mixed standard stock solution of FLU and CHL containing 1,000 µg/mL of each drug in methanol were used for degradation.

**Degradation in acidic condition.** A 5 mL aliquot of each standard stock solution of FLU and CHL was diluted separately to 10 mL with 1 M HCl. Similarly, a 5 mL aliquot of mixed standard stock solution was diluted to 10 mL with methanol. The three resultant solutions were transferred to separate stopped conical flasks and heated for 2 h at 80°C in a thermostatically controlled water bath. The solutions were allowed to cool and 2 mL aliquot of each was transferred to a 10-mL volumetric flask, neutralized with 0.01 M HCl and diluted up to the mark with methanol.

**Degradation in neutral condition.** A volume of 5 mL of each standard stock solution of FLU and CHL was diluted to 10 mL with double distilled water. Similarly, 5 mL aliquot of mixed standard stock solution was diluted to 10 mL with double distilled water. The resultant solutions were transferred to separate stopped conical flasks and heated for 4 h at 80°C in water bath. The solutions were allowed to cool and 2 mL aliquot of each was transferred to a 10-mL volumetric flask and diluted to mark with methanol.

**Degradation in alkaline condition.** A 5 mL aliquot of each standard stock solution of FLU and CHL and mixed standard stock solution were separately diluted to 10 mL with 6% v/v hydrogen peroxide. The resultant solutions were transferred to separate stopped conical flasks and heated for 4 h at 80°C in water bath. The solutions were allowed to cool and 2 mL aliquot of each was diluted further to 10 mL with methanol.

**Photolytic degradation.** To study the effect of sunlight, 5 mL aliquot of individual standard stock solutions of FLU and CHL and mixed working standard solution were diluted to 10 mL with methanol. The resultant solutions were exposed to direct sunlight for 24 h. A 2 mL aliquot of each was further diluted to 10 mL with methanol. Dry powder of the two drugs was also exposed to sunlight and analyzed similarly.

**Thermal degradation.** About 50 mg of each drug individually and as physical mixture were placed in a petri dish in a hot air oven at 80°C for 2 h. Accurately weighed 20 mg of each was dissolved in and diluted to 10 mL with methanol. A 0.5 mL aliquot of each was further diluted to 10 mL with methanol.

**Instrumentation and chromatographic conditions**

Planar chromatography was performed by spotting the sample on thin layer chromatographic (TLC) plate, precoated with silica gel 60 F254 (20 × 10 cm, Merck, Germany) with the aid of Hamilton microliter syringe using automatic TLC Linomat V applicator (Camag, Switzerland). A constant sample application rate 130 µL/s was adopted, the distance between the two bands was 10 mm and the band width was 6 mm. Mobile phase (ethyl acetate : n-hexane : methanol : triethylamine (TEA), 5 : 4 : 2 : 0.5, v/v/v/v) was used for linear ascending development and chromatogram was allowed to move to a distance of 8 cm, in a twin trough glass chamber (Camag) previously saturated for 35 min. The developed TLC plate was dried with the help of air. Densitometric scanning was performed on Linomat scanner IV (Camag) in the absorbance/reflectance mode at wavelength 267 nm utilizing deuterium lamp as the source of radiation. Quantitative evaluation was performed via peak areas by winCats software (version 1.4.6). Densitometric scanning parameters were as follows: slit dimension: 4.00 × 0.30 mm, scanning speed: 20 mm/s.

**Calibration curve**

The method intends to estimate the two drugs simultaneously from marketed ophthalmic solution that contains FLU (0.03% w/v) and CHL (0.5% w/v). Hence, the calibration curves for the two drugs were prepared in the same ratio. Accurate volumes 4.0, 8.0, 12.0,
16.0 and 20.0 µL of mixed working standard solution containing FLU (3 µg/mL) and CHL (50 µg/mL) were applied to the TLC plate to deliver FLU 12, 24, 36, 48 and 60 ng/spot and CHL 200, 400, 600, 800 and 1,000 ng/spot, respectively. The plate was dried, developed and scanned as per chromatographic conditions described. The graphs of peak areas obtained vs. respective concentrations were plotted and the regression equations for the two drugs were computed.

**Method validation**
The developed method was validated as per International Conference on Harmonization (ICH) guideline (40).

**Linearity and range**
The linear responses for FLU and CHL at concentrations 12, 24, 36, 48 and 60 ng/spot for FLU and 200, 400, 600, 800 and 1,000 ng/spot for CHL were assessed in terms of slope, intercept and correlation coefficient values.

**Precision**
*Repeatability.* Repeatability of area measurement was determined by spotting mixed working standard solution (12 µL, 36 ng/spot of FLU and 600 ng/spot of CHL) on a TLC plate and analyzed by the proposed method. The separated spots of FLU and CHL were scanned seven times without changing plate position and percentage of relative standard deviation (%RSD) for peak area was calculated.

Repeatability of sample application was determined by spotting the mixed working standard solution (12 µL, 36 ng/spot of FLU and 600 ng/spot of CHL) seven times and analyzed by the method. The area of seven spots was measured and the %RSD of peak area was calculated.

*Intermediate precision.* Intraday precision was determined by analyzing mixed working standard solution over the entire calibration range three times on the same day and interday precision was determined by analyzing mixed working standard solution over the entire calibration range on three different days.

**Accuracy**
The accuracy was determined by standard addition method. To a fixed amount of preanalyzed sample of FLU and CHL, increasing amount of standard solution of FLU and CHL was added at 80, 100 and 120% of the target concentrations, i.e., 36 and 600 ng/spot, respectively. The recovery of FLU and CHL was calculated at each level (n = 3).

**Limit of detection and limit of quantitation**
The limit of detection (LOD) and limit of quantitation (LOQ) of the developed method were calculated from the standard deviation (SD) of the y-intercept and slope of the calibration curves of FLU and CHL using the equations LOD = 3.3 SD/slope and LOQ = 10 SD/slope, respectively.

**Specificity**
The spots of FLU and CHL from degradation solutions and ophthalmic solution were confirmed by comparing their retention factor (Rf) and absorbance/reflectance spectrum with that of respective standard. The peak purity of each drug was determined by correlating the spectra scanned at peak start (s), peak apex (m) and peak end (e) [i.e., \( r^2(s,m) \) and \( r^2(m,e) \)] positions of the spot.

**Stability of FLU and CHL in analytical solution**
The mixed working standard solution (12 µL, 36 ng/spot of FLU and 600 ng/spot of CHL) was analyzed, stored at 25 ± 2°C and analyzed again at 6, 12 and 24 h after preparation.

**Robustness**
Robustness of the proposed method was determined by making variations in the mobile phase composition (±0.1 mL) and chamber saturation time (±5 min). The effect of these changes on both the Rf values and peak areas was evaluated and %RSD was calculated.

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**Figure 1.** Chemical structure of (A) FLU and (B) CHL. This figure is available in black and white in print and in color at JCS online.
Analysis of marketed ophthalmic solution
Marketed ophthalmic solution was analyzed using developed method. A volume of 2.0 mL of ophthalmic solution (Flubichlor eye drops containing 0.03% w/v FLU and 0.5% w/v CHL) was diluted to 10 mL with methanol. Accurately measured 0.5 mL of the solution was diluted to 10 mL with methanol to get concentration corresponding to 3 µg/mL of FLU and 50 µg/mL of CHL. A volume of 12 µL of resultant solution was applied on TLC plate and analyzed by the proposed method.

Results
Optimization of HPTLC system
Solvent system was optimized with standards, samples and degraded drug solutions. Different solvent systems consisting of methanol, chloroform, toluene, n-hexane and ethyl acetate were tried in varying ratios. Suitable separation and resolution was achieved with ethyl acetate : n-hexane : methanol : TEA (5 : 4 : 2 : 0.5, v/v/v/v) as mobile phase. For the selection of analytical wavelength for the quantification of drugs, the standard spots were scanned and their overlain spectra were obtained. From the overlain UV spectra, wavelength 267 nm was selected as the analytical wavelength for further analysis. The densitogram showing standard FLU at Rf 0.29 and CHL at Rf 0.62 in mixture is presented in Figure 2.

Forced degradation studies
Forced degradation of FLU and CHL was performed in acidic, alkaline, neutral, oxidative, photolytic and thermal stress conditions. The degradation conditions were also optimized in such a way so as to produce sufficient degradation in both the drugs. The results of forced degradation study are summarized in Table I.

Destruction by hydrolysis
Both FLU and CHL were found to undergo acid degradation in individual as well as in combination solution. The reaction in 1 M HCl at 80°C for 2 h showed degradation for FLU with additional peaks at Rf values 0.19 and 0.91 and for CHL, additional peaks were observed with Rf values 0.01 and 0.14 (Figure 3). In alkaline hydrolysis (0.01 M NaOH/80°C/1 h), FLU showed extensive degradation with degradation peaks at Rf values 0.20, 0.90 and 0.95 and for CHL, at Rf values of 0.15, 0.20, 0.67 and 0.88 (Figure 4). Hydrolysis in double distilled water at 80°C for 4 h suggested that both FLU and CHL were prone to neutral degradation and showed degradation product for FLU at Rf value 0.82 and for CHL, at 0.11 and 0.85 (Figure 5).

Oxidative degradation
The drugs when subjected to oxidative degradation (6% v/v H2O2/80°C/4 h) showed FLU exhibited degradation product at Rf values 0.42, 0.44 and 0.86 and for CHL, degradation products were seen at Rf values 0.22, 0.23 and 0.80 (Figure 6).

Photolytic degradation
The FLU and CHL in solution exhibited degradation products at Rf values 0.21, 0.67, 0.97 and 0.01, 0.15, 0.21, 0.93, respectively, in direct sunlight for 24 h (Figure 7).

Table I. Summary of Stress Degradation Studies

<table>
<thead>
<tr>
<th>Degradation conditions</th>
<th>% Degradation of FLU</th>
<th>% Degradation of CHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids degradation (1 M HCl/80°C/2 h)</td>
<td>48.09</td>
<td>55.33</td>
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<tr>
<td>Alkaline degradation (0.01 M NaOH/80°C/1 h)</td>
<td>45.97</td>
<td>74.35</td>
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<tr>
<td>Neutral hydrolysis (double distilled water/80°C/4 h)</td>
<td>51.09</td>
<td>63.72</td>
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<tr>
<td>Oxidative degradation (6% H2O2/80°C/4 h)</td>
<td>67.01</td>
<td>61.29</td>
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<tr>
<td>Photolytic degradation (direct sunlight/24 h)</td>
<td>50.88</td>
<td>65.11</td>
</tr>
<tr>
<td>Thermal degradation (direct heat/80°C/2 h)</td>
<td>1.46</td>
<td>1.98</td>
</tr>
</tbody>
</table>

Figure 2. Densitogram of mixture of standards FLU (36 ng/spot) (Rf: 0.29) and CHL (600 ng/spot) (Rf: 0.62). This figure is available in black and white in print and in color at JCS online.
Thermal degradation
The thermal degradation studies suggested that both FLU and CHL were stable, when placed at 80°C for 2 h.

Method validation
The developed method was validated as per the requirements of the ICH guidelines. The linearity was evaluated by determining mixed working standard solution containing FLU (3 µg/mL) and CHL (50 µg/mL). Standard calibration curves of both FLU and CHL in the respective concentration range of 12–60 and 200–1,000 ng/spot (n = 5) were found linear with correlation coefficient of 0.9997 for FLU and 0.9977 for CHL. The average linear regressed equation for the corresponding curves were $y = 32.87x + 135.0$ for FLU and $y = 11.33x + 2,642.0$ for CHL (Table II).

The precision of the instrument was determined by repeated scan of the same spot of mixed working standard solution (12 µL, 36 ng/spot of FLU and 600 ng/spot of CHL) seven times without changing the plate position. The %RSD for measurement of peak area for FLU and CHL was found to be 0.32 and 0.35, respectively. The %RSD for repeatability of sample application for FLU and CHL was found to be 0.59 and 0.52, respectively. The values for %RSD in intraday and interday precision for FLU were 0.64–1.54 and 0.73–1.69%, respectively, while for CHL, 0.55–1.02 and 0.71–1.14%, respectively. Spiking studies showed recovery of FLU

Figure 3. Densitogram of mixture showing FLU (Rf: 0.29), its degradation products (Rf: 0.19 and 0.91) and CHL (Rf: 0.62), its degradation products (Rf: 0.01 and 0.14) observed after acidic degradation (1 M HCl/80°C/2 h). This figure is available in black and white in print and in color at JCS online.

Figure 4. Densitogram of mixture showing FLU (Rf: 0.29), its degradation products (Rf: 0.20, 0.90 and 0.95) and CHL (Rf: 0.62), its degradation products (Rf: 0.01, 0.20, 0.67 and 0.88) observed after alkaline degradation (0.01 M NaOH/80°C/1 h). This figure is available in black and white in print and in color at JCS online.
(99.29–100.17%) and CHL (98.64–99.92%) from the target concentration of 36 ng/spot of FLU and 600 ng/spot of CHL (Table III). For FLU and CHL, LODs were found to be 1 and 15 ng/spot, respectively, while LOQs were found to be 3 and 44 ng/spot, respectively (Table II). Specificity, evaluated by comparison of chromatograms of FLU and CHL in ophthalmic solution with respective standard, showed identical Rf values, i.e., 0.29 for FLU and 0.62 for CHL. The excipients and other components present in ophthalmic solution did not interfere in separation and resolution of FLU and CHL (Figure 8). The peak purity of sample spectra, determined by scanning at peak start (s), peak apex (m) and peak end (e) positions of individual spots of FLU and CHL, showed a high degree of correlation (above 0.99), thus confirming the purity of spots. The spectra of drugs were compared with spectra of respective standard drugs, which confirmed the identity of the spots.

Analytical solution stability was evaluated and there was no change in Rf and peak area of the two drugs, thus indicating that the solutions of FLU and CHL were stable for 24 h at 25 ± 2°C. The method was evaluated for robustness. The effect of deliberate variations in mobile phase composition and chamber saturation time was studied. Low RSD values (<1%) in peak areas and Rf values indicate that the method is robust (Table IV).

Figure 5. Densitogram of mixture showing FLU (Rf: 0.29), its degradation product (Rf: 0.82) and CHL (Rf: 0.62), its degradation products (Rf: 0.11 and 0.85) observed after neutral hydrolysis (double distilled water/80°C/4 h). This figure is available in black and white in print and in color at JCS online.

Figure 6. Densitogram of mixture showing FLU (Rf: 0.29), its degradation products (Rf: 0.42, 0.44 and 0.86) and CHL (Rf: 0.62), its degradation products (Rf: 0.22, 0.23 and 0.80) observed after oxidative degradation (6% H2O2/80°C/4 h). This figure is available in black and white in print and in color at JCS online.
Analysis of marketed ophthalmic solution

The marketed ophthalmic formulation was analyzed by the developed method. The chromatogram showed two peaks at Rf value 0.29 and 0.62 for FLU and CHL, respectively. The content of FLU and CHL was calculated by comparing peak areas of sample with that of the standard (Table V).

Discussion

The objective of this study was to develop an accurate and precise stability-indicating HPTLC method capable of accurately estimating simultaneously FLU and CHL in ophthalmic solution in the presence of all possible degradation products of FLU and CHL. The advantage of the present method over reported methods is in the HPTLC technique that offers fast, low-cost analysis without compromising accuracy, specificity and sensitivity. Moreover, the drugs were subjected to stress conditions separately as well as in mixture to confirm the source of all degradation products. The degradation conditions were optimized to produce adequate degradation of both the drugs. Six different stress conditions were applied to generate all possible degradation products. The spot of FLU was observed at Rf 0.29 and CHL at Rf 0.62. FLU and CHL were found to degrade in acidic, alkaline, neutral, oxidative and photolytic stress conditions, while both the drugs were stable in thermal stress condition when subjected to dry heat at 80°C for 2 h. Similar degradation products were observed individually.
as well as in mixture. Methods reported in literature are for the estimation of FLU or CHL individually and not simultaneously. A reported stability-indicating HPTLC method estimates only FLU (35) or CHL (36–39) alone. The proposed HPTLC method is stability-indicating for both FLU and CHL and has the advantage of their simultaneous estimation. The method was validated and found to be accurate, precise and specific. It was applied to the marketed ophthalmic solution and results of assay of the two drugs were in compliance with the label claim.

Conclusion

A validated HPTLC method for the estimation of FLU and CHL in ophthalmic solution has been developed. Stress degradation in acidic, alkaline, neutral, oxidative, photolytic and thermal stress conditions was performed. FLU and CHL were found to degrade in acidic, alkaline, neutral, oxidative and photolytic stress conditions. The developed stability-indicating HPTLC method is specific, accurate and reproducible and can be used for the simultaneous analysis of FLU and CHL as individual drugs and in combination dosage forms. It can also be applied for stability studies.

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Conflict of interest statement. None declared.

References
