Stability-Indicating Methods for Determination of Flubendazole and Its Degradants

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The resolution of flubendazole (FLB) and its degradants has been accomplished by using liquid chromatography (LC) using an octadecylsilane column and a mobile phase that consists of methanol and (0.1%) formic acid (75:25, v/v). Identification of FLB degradants was achieved with positive electron spray spectrometry detection. Furthermore, high-performance thin layer chromatography (HPTLC) separation was carried out using ethyl acetate:formic acid:acetic acid:water (9.5:0.11:0.11:0.28, v/v/v/v) as a mobile phase. Quantitation was achieved using densitometry at 254 nm. FLB was subjected to conditions of hydrolysis, photolysis, oxidation and thermal degradation. The proposed LC method determined that the kinetics of acidic and basic degradation followed a first-order kinetics, with an activation energy of 17.80 and 13.93 kcal mol−1 for acidic and alkaline degradation processes, respectively. The pH-rate profiles of degradation of FLB in Britton–Robinson buffer solutions within the pH range (2–12) were studied. The developed methods were validated within the corresponding linear ranges of 3–30 μg mL−1 with detection limits of 0.11 and 0.13 μg mL−1 for FLB and DG1, respectively, for the high-performance liquid chromatography method and 10–65 and 5–30 μg band−1 with detection limits of 0.22 and 0.30 μg band−1 for FLB and DG1, respectively, for the HPTLC method.

Introduction

Flubendazole (FLB) is a benzimidazole carbamate derivative that has been widely used for the treatment of various human and animal helminthoses (enterobiasis, ascariasis and hook-worm infections) (1).

FLB is a drug that contains an ester group, so it can easily be hydrolyzed. To the best of our knowledge, there is no stability-indicating method for the determination of FLB and its degradants. Only (2-amino-1H-benzo[d]imidazol-6-yl)(4-fluorophenyl) carbamic acid (DG2) was previously separated and mentioned as impurity for FLB in the British Pharmacopoeia (2). In the case of other degradants ([5-(4-fluorobenzoyl)-1H-benimidazol-2-yl] carbamic acid (DG1), and methyl(6-(4-fluorobenzoyl)-1-[(5-(4-fluorobenzoyl)-1H-benzo[d]imidazol-2-yl]carbamoyl)-1H-benzo[d]imidazol-2-yl]) carbamate (DG3)), it is first time to be separated and identified. Identification of FLB degradants were achieved with positive electron spray spectrometry (ESI(+)-MS) detection. The aim of this study is to develop several stability-indicating methods for determination of FLB and its degradants.

Furthermore, the developed HPLC method was used to investigate the kinetics of the acidic and basic degradation processes and to calculate the activation energy for FLB degradation. The pH-rate profile of degradation of FLB in Britton–Robinson buffer solutions within the pH range (2–12) was studied. Furthermore, the developed methods were validated to prove that the elaborated procedure is suitable for intended analytical purpose.

Experimental

Instrumentation

The HPLC (Hitachi LaChrom Elite, Tokyo, Japan) instrument was equipped with a model series L-2000 organizer box, a L-2300 column oven, a L-2130 pump with built-in degasser, and a Rheodyne 7725i injector with a 20 μL loop and a L-2455 diode array detector (DAD). The separation and quantitation were made on a 250 × 4.6 mm (i.d.), 5 μm octadecylsilane column (Inertsil, Tokyo, Japan). UV detection was performed under scan mode (in the range of 200–350 nm with 1 nm distance) and single wavelength chromotograms at a λmax of 296 nm were used for quantitative analysis.

A Camag HPTLC Scanner 3 (Switzerland) was used. Data collections and data analysis were conducted using an online computer with Camag HPTLC software (win CATS planar Chromatography Manager). The samples were applied to the plates using Camag Linomat IV. HPTLC plates (20 × 10 cm, aluminum plates precoated with silica gel 60 F254) were purchased from E. Merck (Darmstadt, Germany). A twin-trough glass development chamber, 20 × 20 cm (Camag), was used. The experimental conditions of measurements were λ = 254 nm; mode: absorbance/reflectance; and slit dimensions: 0.45 × 4 mm.

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A Solarbox 1500 (Co.fo.me.gra, Italy) was used to simulate the sunlight irradiation. The sample was irradiated with the light of a xenon arc lamp (1500 W) and outdoor UV filters that restrict the transmission of light with wavelengths below 290 nm. The irradiance was kept constant during all experiments at 55 W m⁻² (290–400 nm).

The IR spectrophotometer used was a Bruker Vector 22 (Germany). NMR spectra were recorded on a Varian Gemini 200 NMR spectrometer (200 MHz; USA). A Waters Acquity ultra performance LC/MS/MS-3100 (USA) was used to record the positive ion electrospray mass spectra (ESI(+)–MS) of the degraded FLB samples for its identification.

Materials and reagents
Pharmaceutical grade FLB, kindly supplied by the Egyptian International Pharmaceutical Industries Company (EIPICO, 10th of Ramadan City, Egypt), was used and certified to contain a purity of 99.8%. Methanol used was of HPLC grade (Sigma–Aldrich chemieGmbHD-8955, Steinheim, Germany). Water used was double distilled. Dimethyl sulfoxide, sodium hydroxide, phosphoric, formic, hydrochloric, acetic, citric and boric acids were of analytical grade (Sigma–Aldrich chemieGmbHD-8955, Steinheim, Germany).

Fluver® tablets (Alex. Co. for pharmaceuticals, batch no. 0139002) that contain 100 mg FLB per tablet and Fluver® suspension (Alex. Co. for pharmaceuticals; batch no. 1209025) that contains 100 mg FLB per 5 mL, were used for pharmaceutical dosage forms analyses.

Chromatographic conditions
The HPLC-UV mobile phase was prepared by mixing methanol and 0.1% formic acid (75:25, v/v). The mobile phase was filtered using a 0.45-µm disposable filter (Millipore, Milford, MA). The flow rate was 1 mL min⁻¹. All determinations were performed at ambient temperatures. The injection volume was 20 µL. Quantitation was achieved with UV detection at 225 nm, based on peak area.

Identification of FLB degradants was achieved with positive electron spray spectrometry (UPLC–ESI(+)+MS) detection. Samples were introduced into the ESI source using a syringe pump at a flow rate of 100 µL min⁻¹. The cone voltage was 30 V. The mass spectra were recorded in the range of 100–700 m/z.

For optimal sensitivity of the HPTLC method, solutions of the testing samples and standard were applied to the HPTLC plates as bands rather than spots. Bands were 6 mm long. The bands were separated by a distance of 8 mm apart and 10 mm from the bottom of the plate. The development chamber was saturated with mobile phase. The HPTLC plate was developed in the ascending way with ethyl acetate:formic acid:acetic acid:water (9:5:0.11:0.11:0.28, v/v/v/v/v) as a mobile phase. After developing over a distance of 8 cm, the HPTLC plate was air dried and scanned at 254 nm. The scan length and width were adjusted to cover the entire band.

Preparation of the stability testing solutions
Preparation of the alkali-induced degradation product
An accurately weighed 500 mg of FLB were first dissolved in 2 mL of 80% formic acid and refluxed with 98 mL of 0.2 M sodium hydroxide at 100°C (pH 11.8) for 1 h. Subsequently, the pH of the solution was adjusted to 1.5 using (1 M) hydrochloric acid to precipitate hydrolysis product of FLB. The precipitate was filtered, washed and dried under vacuum using a rotary evaporator and protected from air and light. The dried precipitate was analyzed by IR, NMR and mass spectroscopy and DG1 was found to be a degradation product of FLB in alkaline medium.

Preparation of the acid-induced degradation product
An accurately weighed 500 mg of FLB were first dissolved in 2 mL of 80% formic acid and refluxed with 98 mL of 2 M hydrochloric acid at 100°C for 14 h. Subsequently, the pH of the solution was adjusted to 1.5 using 1 M NaOH to precipitate the hydrolysis product of FLB. The precipitate was treated as mentioned before. It was found to be also DG1.

Effect of hydrogen peroxide on stability of FLB
An accurately weighed 100 mg of FLB were dissolved in 2 mL of 80% formic acid. Subsequently 1 mL of hydrogen peroxide (33.3%, v/v) was added, and the solution was made up to 100 mL with methanol and refluxed at 100°C for 2 h.

Effect of electrical light on stability of FLB
An accurately weighed 100 mg of FLB were dissolved in (1 mL) of 80% formic acid and diluted to 100 mL with distilled water. The concentration of formic acid did not exceed 1% (v/v) of the irradiated solution, in order to guarantee that formic acid does not influence the photo degradation, as recommended by the Organization for Economic Co-operation and Development guideline TG316 (25). The pH of the irradiated solutions was not adjusted and was not buffered to avoid the influence of the buffering agent in the photo degradation process. The solution was exposed to the electrical light (290–400 nm) for 4 days.

Effect of dry heat on stability of FLB
Susceptibility of the drug to dry heat was studied by exposing the drug to 70°C for 7 days. The powder and aqueous solution (without pH adjustment) were tested.

Standard solutions and calibration
Stock standard solutions were prepared by dissolving 25 mg of FLB and DG1 separately in 2 mL of formic acid and diluting to 50 mL with methanol. The standard solutions were prepared by diluting the stock standard solutions with methanol for the HPTLC method or with the mobile phase for the HPLC method to reach the concentration ranges of 3–30 µg mL⁻¹ for FLB and DG1 for the HPLC method and 10–65 and 5–30 µg band⁻¹ for FLB and DG1 for the HPTLC method, respectively.

The thin-layer chromatography method
Appropriate microliter aliquots of the stock standard solutions were applied to the HPTLC plate. Triplicate applications were made for each solution. The plate was developed using the previously described mobile phase. The peak area was plotted against the corresponding concentration to obtain the calibration graph for each compound.

The liquid chromatography method
Triplicate 20 µL injections were made for each concentration and chromatographed under the conditions described above...
with UV detection. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph of each compound.

**Sample preparation**
Ten tablets of Fluver tablets were weighed and finely powdered. A portion of the powder equivalent to about 25 mg of FLB was accurately weighed, dissolved in 2 mL of formic acid and diluted to 50 mL with methanol. Furthermore, certain volume of Fluver suspension equivalent to about 25 mg of FLB was transferred and diluted to 50 mL with formic acid. The sample solution was filtered. Further dilutions of the sample solution were carried out with methanol for the HPTLC method or with the mobile phase for the HPLC method to reach the linearity range specified for FLB. The general procedures described under calibration were followed and the concentration of FLB was calculated.

**Kinetic investigation of acidic and alkaline degradation**
An accurately weighed 15 mg of FLB was dissolved in 2 mL of formic acid and diluted to 100 mL with methanol. Separate (2 mL) aliquots of the above solution were transferred into separate stoppered conical flasks and mixed with 2 mL of 0.2 M sodium hydroxide or 2 M hydrochloric acid. The flasks were placed in a thermostatic oven at different temperatures (95, 90, 85, 80, and 75 °C for acidic degradation and 90, 85, 80, 70, and 60 °C for alkaline degradation) at different time intervals. At the specified time intervals, the contents of the flasks were neutralized to pH 7.0 using predetermined volumes of 1 M sodium hydroxide and 0.1 M hydrochloric acid solutions. The contents of the flasks were transferred into 10 mL volumetric flasks and made up to volume with the mobile phase. Aliquots of 20 μL of each solution were chromatographed under the conditions described previously and the concentration of the remaining FLB was calculated at each temperature and time interval.

**Results**

**Identification of the degradation products**
When FLB was boiled with 0.1 M sodium hydroxide for 1 h or 1 M hydrochloric acid for 14 h, DG1 could be isolated from the reaction mixture as degradation product of FLB in both acidic and alkaline mediums (Figures 1 and 2). Furthermore, degradation was observed on exposing the drug to the simulated sunlight irradiation for 7 h and DG1, DG2, and DG3 were formed. However, no degradation was observed either on boiling with hydrogen peroxide (0.33%, v/v) or exposing the drug to dry heat at 70 °C for 1 week (powder and aqueous solution) without pH adjustment.

The suggested pathway for the degradation of FLB in 0.1 M sodium hydroxide and in 1 M hydrochloric acid and the effect of electrical light are presented in Scheme 1.

**HPLC method**
The developed LC method has been applied for the separation of FLB and its degradants. It permits us to perform a stability study.
for the degradation kinetics of the drug. It also permits quantitation of FLB in commercial dosage forms. Figure 1 shows a chromatogram indicating good resolution of FLB, DG1, DG2 and DG3. The solution obtained was analyzed by HPLC with DAD and it provides useful information about the peaks present in routine samples, because it gains not only retention times of analytes, but also their UV spectra (Figure 3) and to check peak purity, by acquiring full-scan absorption spectra of the analytes.

The proposed method offers high sensitivity as about 0.11 µg mL\(^{-1}\) of FLB could be accurately detected. To optimize the HPLC assay parameters and to obtain well-defined symmetrical peak, the response of eluent was tested through experimental trials. Best results were obtained by using methanol and 0.1% formic acid (75:25, v/v) with a flow rate of 1 mL min\(^{-1}\) and at a temperature of 25\(^\circ\)C. The system suitability test results of the developed method are presented in Table I.

HPTLC method

The HPTLC-densitometric method was successfully applied for the determination of FLB in pure form, in the presence of its degradants and in different dosage forms. The HPTLC-densitometric method was characterized by separation of FLB from its degradation products. This method offers a simple way to quantify directly on a HPTLC plate by measuring the optical density of the separated bands and it was used for the quantification of the hydrolysis product of acid and base (DG1). The amounts of compounds are determined by comparing to a standard curve from reference materials chromatographed simultaneously under the same condition.

Analysis of pharmaceutical products

The proposed methods were applied to the determination of FLB in its commercial dosage forms. Seven replicate determinations were made. Satisfactory results were obtained for FLB in a good agreement with the label claims (Table II).

The results obtained for the determination of FLB were compared with the official method (2). Statistical comparison of the results was performed with respect to accuracy and precision using Student’s t-test and the F-ratio at 95% confidence level. There was no significant difference between the results (26) (Table II).

The mean percentage of declared contents (\(n = 7\)) of Fluver\textsuperscript{®} tablets was found to be 100.04% and 100.06% and of Fluver\textsuperscript{®} suspension was found to be 99.94% and 100.18%, determined by HPTLC and HPLC methods, respectively.

Kinetic investigation

The kinetics of degradation of FLB were investigated in only 0.1 M sodium hydroxide and 1 M hydrochloric acid because the decomposition rate of FLB at lower strengths of hydrochloric acid was too slow to obtain a reliable kinetic data. A regular decrease in the concentration of intact FLB with increasing time intervals was observed. At the selected temperatures, the acidic and alkaline degradation processes followed pseudo first-order kinetics (Figure 4). From the slopes of the straight lines, it was possible to calculate the pseudo first-order degradation rate constant and the half-life at each temperature for acidic and alkaline degradation processes of FLB. On plotting \(\log K_{\text{obs}}\) values versus \(1/T\), the Arrhenius plots (Figure 5) were obtained, which were found to be linear in the temperature range 75–95\(^\circ\)C for the acidic and 60–95\(^\circ\)C for alkaline degradation. The activation energy was calculated for FLB and found to be 17.80 kcal mol\(^{-1}\) for the acidic degradation process and 13.93 kcal mol\(^{-1}\) for the alkaline degradation process of FLB.

The pH-rate profiles of degradation of FLB in Britton–Robinson buffer solutions were studied at 85\(^\circ\)C (Figure 6). Britton–Robinson buffer solutions were used throughout the entire pH range in order to avoid possible effects of different buffer species. The pseudo first-order degradation rate constant and the half-life were calculated for each pH value.

FLB was found to be most stable at a pH of 2.0. The pH-rate profile curve from pH 2 to 12 had three main regions. The first one was above 7.0, where the drug showed ester hydrolysis through the attack of the \(\text{OH}^-\) group on the carbonyl carbon. The second one was below 5.0, where the drug showed also ester hydrolysis but through the addition of \(\text{H}_3\text{O}^+\) to the ester oxygen. The third region was from 5 to 7, where an inflection was observed at pH 6.

Validation of the method

Linearity

The linearity of the proposed methods was evaluated by analyzing a series of different concentrations of each compound. Seven concentrations were chosen, ranging between 3 and 30 µg mL\(^{-1}\) for FLB and DG1 for the HPLC method and between 10–65 and 5–30 µg band\(^{-1}\) FLB and DG1, respectively, for the HPTLC method. The assay was performed according to the experimental conditions previously established. Each concentration was repeated three times.

The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the small value of \(y\)-intercept.

Precision

The repeatability of the method was tested by choosing three concentration levels for each compound and analyzing them as
The results of the repeatability and intermediate precision experiments are shown in Table III. The developed method was found to be precise as the coefficient of variation (CV %) values for repeatability and intermediate precision studies were <2%, respectively.

**Range**

The calibration range was established through consideration of the practical range necessary, based on each compound concentration present in the pharmaceutical product, to give accurate, precise and linear results. The calibration range of each compound is as mentioned in the 'Linearity' section.

**Detection and quantitation limits**

According to the ICH recommendations (27), the approach based on the SD of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and the method has detection limits of 0.11 μg mL⁻¹ of FLB and 0.22 μg mL⁻¹ of DG₁ and quantitation limits of 0.34 μg mL⁻¹ of FLB and 0.68 μg mL⁻¹ of DG₁ for the HPLC method. Furthermore, the method has detection limits of 0.07 μg band⁻¹ of FLB and 0.09 μg band⁻¹ of DG₁ and quantitation limits of 0.28 μg band⁻¹ of FLB and 0.27 μg band⁻¹ of DG₁ for the HPTLC method.
System suitability tests
System suitability test parameters must be checked to ensure that the system is working correctly during the analysis. The following parameters are used for system suitability evaluation (2, 28): capacity factor ($K_0$), selectivity factor ($\alpha$), resolution ($R_S$), column efficiency (number of theoretical plates) and tailing factor ($T$) (Table I).

Selectivity
Method selectivity was achieved by preparing different mixtures of FLB and DG$_1$ within the linearity range concentration. The laboratory-prepared mixtures were analyzed according to the previous procedures using the proposed methods. Satisfactory results were obtained (Table II), indicating the high selectivity of the proposed method for determination of FLB and DG$_1$.

Accuracy
The accuracy study was performed by addition of known amounts FLB and DG$_1$ to a known concentration of the commercial tablets and suspension (standard addition method). The resulting mixtures were assayed and the results obtained for FLB and DG$_1$ were compared with the expected results. The excellent recoveries of the standard addition method suggest good accuracy of the proposed methods.

Robustness
To determine the robustness of the developed method, experimental conditions (ratio of organic modifier, flow rate and temperature) were purposely altered and the resolution between FLB and DG$_1$ was evaluated (Table IV).

Stability
The stability of FLB and DG$_1$ standard solutions in methanol or the mobile phase was evaluated by leaving the standard solutions in tightly capped volumetric flasks, protected from light, on a laboratory bench and in the refrigerator. The studied compound solutions in mobile phase exhibited no chromatographic changes for 24 h when kept at room temperature and for 3 days when stored in a refrigerator at 4°C.

Discussion
Identification of the degradation products
In general, the goal of this paper is to use the stability-indicating methods for determination of FLB and its degradants in...
pharmaceutical dosage forms. Three degradation products were observed. Acid and base hydrolysis produced only one degradation product (DG₁), but photodegradation produced three degradation products (DG₁, DG₂, and DG₃). All degradation products were identified by comparing (LC-ESI(+)–MS) spectra with the spectra of the respective FLB prior to the degradation process.

The degradation products of FLB were identified by comparing (LC–ESI(+)–MS) spectra with the spectra of the respective FLB prior to irradiation. The presence of each degradation product was confirmed based on the molecular ion peak for each isolated peak and investigated fragmentation pattern. Furthermore, the molecular formula and the structure of the products could be proposed and the degradation pathway was outlined based on these results (Scheme 1).

Table II

<table>
<thead>
<tr>
<th></th>
<th>Proposed HPLC method</th>
<th>Proposed HPTLC method</th>
<th>Official HPLC method (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory-prepared mixture</td>
<td>FLB</td>
<td>DG₁</td>
<td>FLB</td>
</tr>
<tr>
<td></td>
<td>99.71 ± 0.46</td>
<td>99.86 ± 0.67</td>
<td>100.03 ± 0.22</td>
</tr>
<tr>
<td>Fluver™ tablets</td>
<td>100.06 ± 0.34</td>
<td>100.04 ± 0.28</td>
<td>99.87 ± 0.38</td>
</tr>
<tr>
<td>Fluver™ suspension</td>
<td>100.18 ± 0.24</td>
<td>99.94 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>F for Fluver™ tablets</td>
<td>0.95</td>
<td>1.78</td>
<td>(2.45)</td>
</tr>
<tr>
<td>F for Fluver™ suspension</td>
<td>0.33</td>
<td>2.35</td>
<td></td>
</tr>
<tr>
<td>F for Fluver™ suspension</td>
<td>1.22</td>
<td>2.78</td>
<td></td>
</tr>
<tr>
<td>F for Fluver™ suspension</td>
<td>1.25</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>99.83 ± 0.34</td>
<td>99.82 ± 0.47</td>
<td>99.88 ± 0.39</td>
</tr>
<tr>
<td>FLB</td>
<td>99.80 ± 0.21</td>
<td></td>
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</tr>
</tbody>
</table>

*aMean and SD, percentage recovery from the label claim amount.

*bTheoretical values for t and F.

*cFor standard addition of different concentrations of FLB and DG₁.
The LC–ESI(+)–MS spectrum (m/z) of FLB represents a molecular ion peak at 313 Da corresponding to the molecular weight of FLB. While the mass spectrum revealed the presence of the proposed degradation products DG1, DG2, and DG3. All degradation products except one have lower molecular weight than the parent compound and photo-induced dimerization was observed.

The first degradation product (DG1) was observed again in the photodegradation sample and this was confirmed by the study of mass values in mass spectra that showed the presence of a molecular ion peak at 299 Da corresponding to the molecular weight of DG1. Further, similar retention time and retardation factor were observed by HPLC and HPTLC of DG1 formed under acidic and alkaline hydrolysis.

Table III

<table>
<thead>
<tr>
<th>Method</th>
<th>Component</th>
<th>Concentration (µg mL⁻¹)</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Recovery % ± SD</td>
<td>CV %</td>
</tr>
<tr>
<td>HPLC</td>
<td>DG1</td>
<td>10</td>
<td>100.03 ± 0.37</td>
<td>0.90 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>FLB</td>
<td>3</td>
<td>100.24 ± 0.76</td>
<td>0.76 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>DG1</td>
<td>5</td>
<td>99.56 ± 0.48</td>
<td>0.56 ± 0.56</td>
</tr>
<tr>
<td>HPTLC</td>
<td>DG1</td>
<td>5</td>
<td>99.56 ± 0.48</td>
<td>0.56 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>FLB</td>
<td>3</td>
<td>100.01 ± 0.46</td>
<td>0.49 ± 0.56</td>
</tr>
</tbody>
</table>

The third observed degradation product (DG3) was assigned as methyl(6-(4-fluorobenzoyl)-1-(6-(4-fluorobenzoyl)-1H-benzo[d]imidazol-2-yl)carbamoyl)-1H-benzo[d]imidazol-2-yl)carbamate with methanol (1:1) through photo-induced dimer formation. It showed a molecular ion peak at 626 Da corresponding to the molecular weight of DG3.

HPLC method

The proposed HPLC method offers a good resolution of FLB and its degradants with high sensitivity and reasonable retention time. The HPLC method development was done by testing one variable at a time to reach the best chromatographic conditions for better method optimization. The parameters tested were: detection wavelength, temperature, type and ratio of organic modifier and flow rate.

Choice of appropriate wavelength: The spectra obtained from the DAD was studied and the best wavelength was found to be 225 nm showing highest sensitivity and appreciable absorbance of the degradation products.

Choice of optimum temperature: Several temperatures were tested to obtain the optimum operating temperature. The results obtained are shown in Table IV.

Mobile phase composition: Several modifications in the mobile phase including the change of the type and ratio of organic modifier, the pH and the flow rate in order to study the possibility of changing the selectivity of the chromatographic system. The results obtained are shown in Table IV.

Type of organic modifier: Trials were carried out using acetonitrile and methanol and the lower cost methanol was the organic modifier of choice giving well-resolved symmetrical narrow peaks with no additional resolution obtained from using acetonitrile.

Ratio of organic modifier: The effect of changing the ratio of the organic modifier on the selectivity, asymmetry and retention time of the separated compounds was investigated using mobile phases containing 70–80% for methanol. Table IV shows that 75% methanol was the best one giving well-resolved and symmetric peaks with reasonable number of theoretical plates and retention time.

Ratios less than 70% resulted in peaks with very broad and long unacceptable retention time with highest tailing factor values, whereas ratios higher than 80% resulted in bad resolution of peaks.

Effect of flow rate: The effect of flow rate on formation and separation of peaks of the studied compounds was studied and a flow rate of 1 mL min⁻¹ was optimal for good separation in reasonable retention time.

HPTLC method

The developed HPTLC method gave a good resolution of FLB and its degradants. Studying the optimum parameters for maximum separation was carried out by trying different developing systems with different ratios but complete separation was achieved by ethyl acetate:formic acid:acetic acid:water (9.5:0.11:0.11:0.27, v/v/v/v) as a mobile phase which gave good resolution, sharp
and symmetrical peaks of FLB and its degradants as shown in Figure 2.

When these methods were compared with the official method, it became clear that the official method (2) is time consuming and also it does not reveal the presence of the degradation product.

**Analysis of pharmaceutical products**

The proposed methods were able to remove the interferences of the other excipients present in the pharmaceutical products and FLB was assessed with a high percentage of recovery.

**Kinetic investigation**

The alkaline and acidic degradation of FLB followed pseudo first-order kinetics with observed higher alkaline degradation rate compared to acidic degradation rate. The study of pH-rate profile showed that FLB is most stable in acidic medium (at a pH of 2.0). This is not against the fact that FLB is hydrolyzed in acidic medium. This can be explained through the previously mentioned three regions of the pH-profile curve.

At the first region (above 7), the high hydrolysis rate in the basic medium is attributed to the stabilization of the charge on carbonyl carbon by the resonance with the carbamate nitrogen atom. This process favors the attack of the OH⁻ group on the carbonyl carbon.

At the second region (below 5), the low hydrolysis rate in the acidic medium is attributed to the unavailability of the ester oxygen lone pair due to formation of intramolecular hydrogen bond between the ionized nitrogen in the imidazole ring (acidic medium) and the ester oxygen.

We observed that with decreasing the acidity (increase the pH), the degree of ionization of the imidazole nitrogen decreased and the ester oxygen was more available, so the rate of hydrolysis was increasing.

At the inflection region (between 5 and 7), there was a change in hydrolysis mechanisms between acid and alkaline hydrolysis and disappearance of the intramolecular hydrogen bond due to lack of ionization of the imidazole nitrogen at this near neutral pH.

**Validation of the method**

The performance of the proposed methods was validated according to the ICH guidelines. The linearity of the calibration graphs was tested by the high value of the correlation coefficient and the small value of y-intercept; additionally, the RSD values for inter- and intra-day repeatability indicated the precision of the method. The selectivity and accuracy of the method were assessed; the results showed that the proposed methods were able to analyze FLB from other endogenous interferences (degradants and excipients) with high selectivity and accuracy. Moreover, the stability of the analytical solution was checked; it was found that FLB and DG₁, in mobile phase were found to be stable.

**Conclusion**

The proposed HPLC and HPTLC methods provide simple, accurate and reproducible quantitative analysis for the determination of FLB and its degradants in pharmaceutical products, without any interference from the excipients. The suggested methods can be simply applied to accelerated stability study to predict expiry dates of FLB pharmaceuticals. FLB is rapidly degraded in alkaline medium while it is more stable in acidic medium. FLB is photo-sensitive and shows considerable degradation in sun light. The optimum stability of FLB was proposed to be at nearly pH 2.0.

**References**

14. Van De Steene, J.C., Lambert, W.E.; Validation of a solid-phase extraction and liquid chromatography-electrospray tandem mass spectrometric method for the determination of nine basic pharmaceuticals.


