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ORIGINAL ARTICLE

# Study of forced degradation behaviour of florfenicol by LC and LC-MS and development of a validated stability-indicating assay method

## Étude de la dégradation forcée du florfenicol par chromatographie liquide et chromatographie liquide couplée à la spectrométrie de masse, et développement d'une méthode validée indicatrice de stabilité

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**KEYWORDS**
Florfenicol;
Thiamphenicol;
Stress testing;
Stability-indicating assay method;
Validation;
LC-MS;
Degradation pathway

**Summary** A stability-indicating high performance liquid chromatography (HPLC) method was developed for the analysis of florfenicol in presence of its two available identified degradation products (thiamphenicol and chlorfenicol). The drug was subjected to different International Conference On Harmonisation (ICH) prescribed stress conditions (hydrolysis, oxidation and photolysis). The products formed under different stress conditions were investigated by liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC-MS). The LC method involved a Knauwer Eurospher C18 thermostated column at 25 °C; and ammonium acetate buffer 6.49 mM (pH adjusted to 4.5)—methanol (70:30 v/v) as mobile phase. The flow rate and detection wavelength were 1 ml/min and 225 nm respectively. The drug showed instability under acidic, alkaline and photolytic stress conditions mainly in solution state form; however, it remains stable in solid state form and under oxidative stress conditions. The developed method was validated for linearity, precision, accuracy and specificity. The degradation products were characterized by LC-MS. Through the mass/ionization (m/z) values and fragmentation patterns, two principal degradation products listed in bibliography have been shown: the florfenicol amine

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and thiamphenicol. Based on the results, a more complete degradation pathway of the drug could be proposed.


**MOTS CLÉS**
Florfénicol ;
Thiamphénicol ;
Tests de dégradation forcée ;
Méthode indicatrice de stabilité ;
Validation ;
CL-SM ;
Schéma de dégradation

**Résumé** Une méthode de chromatographie liquide indicatrice de stabilité a été développée pour l'analyse de florfénicol en présence de ces deux produits de dégradation connus et disponibles (thiamphénicol et chlorfénicol). La substance a été soumise aux différentes conditions de dégradation forcée prescrites par la Conférence internationale d'harmonisation (ICH) (hydrolyse, oxydation et photolyse). Les produits formés dans les différentes conditions de dégradation forcée sont analysés par chromatographie liquide (CL) et chromatographie liquide-spectrométrie de masse (CL-SM). La CL implique une colonne C18 Knauwer Eurospher, thermostatée à 25 °C, et comme phase mobile, tampon acétate d'ammonium 6,49 mM (pH ajusté à 4,5)—méthanol (70:30 v/v). Le débit et la longueur d'onde sont respectivement 1 mL/min et 225 nm. La substance médicamenteuse a été démontrée comme étant instable dans les conditions de dégradation forcée d'acide, basique et photolyse, notamment sous la forme d'une solution ; cependant, elle reste stable à l'état solide et dans les conditions de stress oxydatif. La méthode développée a été validée pour linéarité, fidélité, exactitude et spécificité. Les produits de dégradation ont été caractérisés par CL-SM. À partir du rapport masse—charge d'ionisation (m/z) et les modèles de fragmentation, deux principaux produits de dégradation inscrits en bibliographie ont été démontrés : le florfénicol amine et le thiamphénicol. En se basant sur ces résultats, un schéma de dégradation plus complet de la substance médicamenteuse peut être proposé.


## Introduction

The phenicols (florfenicol, thiamphenicol and chloramphenicol) constitute an important group of bacteriostatic agents. They are essentially bacteriostatic by inhibition of the bacterial protein synthesis. They have a wide spectrum of action towards the cocci and bacillus Gram+ and Gram− of which the anaerobes, *Rickettsia*, *Mycoplasma* and *Chlamydia* [1].

However, the use of this antibiotic class must be limited for the serious infections when the other medicaments are less efficient and more toxic [1].

The florfenicol is a synthetic antibiotic with a wide spectrum from the phenicol group, active towards the most Gram+ and Gram− isolated bacteria of domestic animals. It acts by inhibition of bacterial protein synthesis in ribosome and it is a bacteriostatic agent. However, a bactericide activity has been shown in vitro towards *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* when florfenicol is presented in concentrations more than the MIC for at least 12 hours [1].

In vitro, tests have shown that florfenicol is active towards the pathogenic bacteria, mainly the most implicated in the respiratory diseases, including *A. pleuropneumoniae* and *P. multocida* [1].

Several analytical methods have been reported for the quantification of florfenicol and its related compounds, for example gas chromatography, capillary electrophoresis, micellar electrokinetic chromatographic method and mass spectrometry [2—8].

Because residues of antibiotics in the food chain are of increasing concern due to their overall contribution to the increase of antibiotic resistance of pathogenic bacteria, as well as the potential allergenic reactions that they may illicit in certain individuals, it is absolutely necessary to do a severe quality control of veterinary commercials in terms of both qualitative and quantitative analysis [9].

Stability testing is an essential part of any drug development because the quality of a drug product changes with time under the influence of environmental factors such as temperature, humidity and light. The purpose of stability testing is to investigate those changes during storage that is likely to influence the quality, safety and efficacy and to establish a shelf life for the drug product and to recommend storage conditions [10].

Comprehensive LC and LC-MS studies of the degradation behavior of florfenicol under various ICH prescribed stress conditions [10—12] have been lacking. Thus, the aim of this work is to carry out forced decomposition studies according to the ICH requirements and to develop a selective and validated stability-indicating HPLC method. Then to identify new degradation products if any, and to postulate a complete degradation pathway of the drug.

## Experimental

### Samples

The florfenicol was obtained from Zhejiang Hisoar Pharmaceutical & Chemical Co., Ltd, and chloramphenicol from Gold Pharma. Thiamphenicol is a European Pharmacopoeia reference substance.

The N-2-methylpyrrolidone is obtained from Sintofarm Iberica, SA, and the polyethylene glycol from Sintofarm Iberica, SA.

The chemical structures of florfenicol, thiamphenicol and chloramphenicol are shown in Fig. 1.

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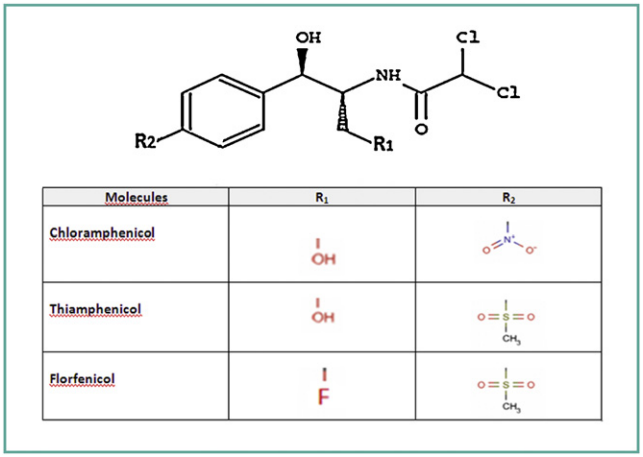


**Figure 1.** Chemical structure of chloramphenicol, thiamphenicol and florfenicol.
*Structure chimique de chloramphénicol, thiamphénicol et florfénicol.*

## Reagents

All solvents and reagents used in this study were analytical grade (HPLC grade). Solutions were prepared with deionised water (Milli-Q quality).

Acetonitrile, methanol, hydrochloric acid and glacial acetic acid were from Prolabo (France). The sodium hydroxide was from SDS, the hydrogen peroxide from Chemi-Pharma, and ammonium acetate from Quadrimex.

## Apparatus

For solution degradation studies, precision water baths were used.

Photodegradation was carried out in humidity and photostability chambers, set at 80 °C/95% RH (Bender Type KBF115).

The HPLC analysis for method development forced degradation studies, and method validation was performed with Agilent series 1100 system, which consisted of variable wavelength UV-VIS detector model 486, a quaternary gradient pump equipped with a solvent programmer, a rheodyne model 7125 injector with a 20 μl sample loop and a NEC 386/25 computer fitted with Agilent Baseline software. This system was used for the measurement and treatment of data.

The separations were achieved on a C18 (250 × 4.6 mm, particle size 5 μm) column (Zorbax Eclipse, Agilent technologies) and ammonium acetate buffer 6.49 mM (pH adjusted to 4.5 with HCl 1 M or NaOH 1 M)—methanol (70:30 v/v) as mobile phase.

The LC-MS for the identification of degradation products was carried out on a system in which LC part consist of 1200 series HPLC (Agilent technologies) comprising of an online degasser, binary pump, auto-injector, column oven and photodiode array (PDA) detector.

The MS system consists of a triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a nebulizer-assisted electrospray source.

The mass spectrometer was run in negative electron spray ionisation (ESI) mode with ultra-high purity (UHP) nitrogen as the nebulizer and drying gas at a flow rate of 50 L/h and 300 L/h respectively.

The capillary voltage is fixed at 3.5 KV. UHP argon was used as the collision gas with a collision-cell gas pressure of $1 \times 10^{-3}$ mBar. The source ionisation and desolvation temperatures were set at 300 °C and 70 °C respectively. The mass/charge (m/z) ratio is in the range of 200—450 m/z.

The potential values used to evaluate the pH of the mobile phase were measured with a Mettler Toledo Type Seven Multi pH meter.

Other equipments used were sonicater, a precision analytical balance (Mettler Toledo) and auto-pipettes.

## Stress studies

Stress studies were carried out under the conditions of hydrolysis, oxidation and photolysis as mentioned in ICH Q1A (R2) [10].

Hydrolytic decomposition was carried out in NaOH/20% acetonitrile, and HCl 1N/20% acetonitrile, on the drug solution at a concentration of 10 mg/ml, and on the finished product. The solutions were heated at 60 °C for a time till sufficient degradation was attained (20% of the drug initial amount).

For oxidative stress studies, florfenicol was dissolved in 10% $H_2O_2$ and kept for 24 h.

Photolytic studies were carried out by exposing the drug solutions and 5 g of the drug at solid state form in a photostability chamber set at 80 °C/95% RH for 12 days, and to sun lights for 12 h [13].

A minimum of four samples were generated for every stress conditions: the blank solution stored under normal conditions, the blank subjected to stress in the same manner as the drug, zero time drug solutions stored under normal conditions, and the drug solutions subjected to stress treatment.

## Preparation of phosphate buffer

The phosphate buffer was prepared by dissolving 0.5 g of ammonium acetate in 1 L of distilled water and adding 0.5 ml of glacial acetic acid. This solution were prepared daily and stored in a refrigerator until use.

## Preparation of samples for HPLC analysis

The samples from degradation studies were diluted 20 times with the mobile phase to yield a final concentration at 0.5 mg/ml of florfenicol.

The solid samples were suitably diluted in water.

All the solutions were passed through 0.22 μm filter before injections.

## Separation studies and development of a stability-indicating method

Attempts were made to develop a simple method on a C18 column with a better peak shapes. First HPLC studies were performed on all reaction solutions individually, and then on a mixture of degraded drug solutions.

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**Table 1** Retention times and relative retentions of various peaks.
*Les temps de rétention et les rétentions relatives des différents pics.*

| Peak | RT (min) | RR |
|---|---|---|
| I | 2.4 | 0.218 |
| II (thiamphenicol) | 5.7 | 0.518 |
| III (florfenicol) | 11 | 1 |

Different modifications like change in pH, different mobile phase compositions and column temperature adjustment were tried to get good separation between the drug and the degradation products, as well as between the degradation products.

## Validation of the developed method

Validation was done with respect to various parameters as required under ICH guideline Q2 (R1) [14—16]. To establish linearity and range, the drug at a concentration of 100 mg/ml was diluted to yield solutions in the concentration range of 40—60 mg/0.5 ml. The solutions were prepared in triplicate.

The intra-day and inter-day precision was established by analysing the drug solution in six injections on the same day and on consecutive days, respectively. The value of relative standard deviation (RSD) was calculated.

The study of accuracy was coupled to the linearity by fortifying the solutions with known concentrations of the corresponding drug. The percent recovery of the added drug was determined.

Specificity of the method was established by determining the peak purity using a PDA detector. Also the resolution factor for the drug and nearest resolving peak was determined. In fact, both peak purity as well as the resolution was determined for all the degradation products' peaks, in addition to the drug peak, to prove that the developed method was selective in nature.

## Characterization of degradation products by LC-MS

The obtained m/z values in negative ESI mode were compared to the molecular weights of the known degradation products. Based on the molecular weights and the fragmentation patterns, the presence of known degradation products was confirmed. The degradation pathway was outlined based on the results.

**Table 2** Retention time (RT) and resolution (RS) between the pairs of peaks in function of the mobile phase composition.
*Le temps de rétention (RT) et la résolution (RS) entre les pairs des pics en fonction de la composition de la phase mobile.*

| Mobile phase | Component | RT (min) | Pairs of peaks | RS |
|---|---|---|---|---|
| 1 | Degradation product (Deg) | 4.21 | — | — |
| Sol A 80%/Sol | Thiamphenicol (TAP) | 11.654 | $Peak_{TAP}$—$Peak_{Deg}$ | 41.20 |
| B 20% | Florfenicol (FF) | 25.64 | $Peak_{FF}$—$Peak_{TAP}$ | 82.26 |
| | Chloramphenicol (CAP) | 51.793 | $Peak_{CAP}$—$Peak_{FF}$ | 76.92 |
| 2 | Degradation product (Deg) | 2.4 | — | — |
| Sol A 70%/Sol | Thiamphenicol (TAP) | 5.772 | $Peak_{TAP}$—$Peak_{Deg}$ | 13.23 |
| B 30% | Florfenicol (FF) | 10.262 | $Peak_{FF}$—$Peak_{TAP}$ | 17.60 |
| | Chloramphenicol (CAP) | 23.151 | $Peak_{CAP}$—$Peak_{FF}$ | 30.32 |
| 3 | Degradation product (Deg) | 1.82 | — | — |
| Sol A 60%/Sol | Thiamphenicol (TAP) | 3.256 | $Peak_{TAP}$—$Peak_{Deg}$ | 5.63 |
| B 40% | Florfenicol (FF) | 4.998 | $Peak_{FF}$—$Peak_{TAP}$ | 6.83 |
| | Chloramphenicol (CAP) | 10.700 | $Peak_{CAP}$—$Peak_{FF}$ | 13.41 |
| 4 | Degradation product (Deg) | 1.61 | — | — |
| Sol A 50%/Sol | Thiamphenicol (TAP) | 3.094 | $Peak_{TAP}$—$Peak_{Deg}$ | 3.87 |
| B 50% | Florfenicol (FF) | 3.869 | $Peak_{FF}$—$Peak_{TAP}$ | 2.60 |
| | Chloramphenicol (CAP) | 6.308 | $Peak_{CAP}$—$Peak_{FF}$ | 5.73 |
| 5 | Degradation product (Deg) | 1.59 | — | — |
| Sol A 40%/Sol | Thiamphenicol (TAP) | 2.697 | $Peak_{TAP}$—$Peak_{Deg}$ | 2.89 |
| B 60% | Florfenicol (FF) | 3.032 | $Peak_{FF}$—$Peak_{TAP}$ | 1.57 |
| | Chloramphenicol (CAP) | 4.010 | $Peak_{CAP}$—$Peak_{FF}$ | 3.83 |
| 6 | Degradation product (Deg) | 1.51 | — | — |
| Sol A 30%/Sol | Thiamphenicol (TAP) | 2.520 | $Peak_{TAP}$—$Peak_{Deg}$ | 1.69 |
| B 70% | Florfenicol (FF) | 2.708 | $Peak_{FF}$—$Peak_{TAP}$ | 0.368 |
| | Chloramphenicol (CAP) | 3.161 | $Peak_{CAP}$—$Peak_{FF}$ | 1.33 |

* Solution A: ammonium acetate buffer; * Solution B: methanol.

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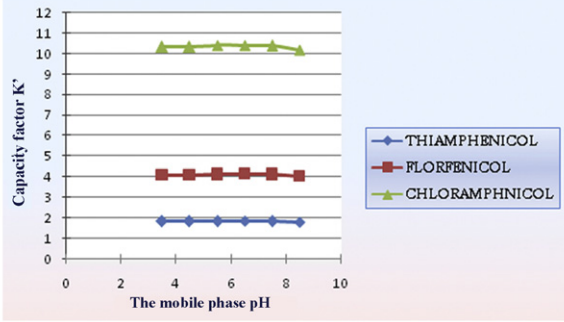


**Figure 2.** Effect of the mobile phase pH on the retention time of florfenicol, thiamphenicol and chloramphenicol.
*Effet de pH de la phase mobile sur le temps de rétention de florfénicol, thiamphénicol et chloramphénicol.*

## Results and discussion

### HPLC studies on the stressed solutions

HPLC studies on florfenicol under different stress conditions suggested the following degradation behaviour:

#### Hydrolytic conditions

The florfenicol degradation was observed to be more quickly in alkaline hydrolysis to acidic one. Indeed, the drug is totally degraded after 1 h in alkaline stress conditions, however in acidic one; the first degradation product is obtained after 1 h, and the second one after 2 h.

The products carry the notations I, II, III and IV in accordance with the sequence in which the peaks appeared from left to right on the HPLC Chromatogram.

Two degradation products have been observed (Table 1). The major peak continuous to degrade with time on heating.

#### Oxidative studies

No change was observed on florfenicol exposure to 10% $H_2O_2$ for 24 h, showing that it was stable against oxidative stress (either in solid state or in solution).

#### Temperature and humidity studies

The exposure of the drug, at solution state form, to 80 °C/95% RH for 10 days resulted in slight degradation, yielding to the two degradation products observed in hydrolytic conditions. However, the drug at solid state form remains stable against thermal stress.

#### Photolytic studies

Under light, similar behaviour was observed on the drug exposure to the temperature and humidity studies.

### Development of validated stability-indicating HPLC assay method

#### Development and optimization of the method

##### *Mobile phase composition*

The chromatographic conditions were optimized on florfenicol and its two available identified degradation products (thiamphenicol and chloramphenicol), with the aim of achieving good resolution, symmetrical peak shape and short analysis time for the drugs.

Preliminary experiments were performed with mobile phase consisting of ammonium acetate buffer 6.49 mM (pH 4.5)—methanol (20:80, v/v).

For economical reasons, we have chosen the methanol in place of acetonitrile and good results have also been obtained.

Some degradation products have not been retained with higher percentages of methanol, thus several assays have been done by decreasing the percentage of this organic solvent (Table 2).

The pH of mobile phase has also been studied. The increase of ammonium acetate buffer pH keeps no changes in the separation selectivity (Fig. 2), also the extremely values are not compatible with the nature of column. Thus, ammonium acetate buffer pH 4.5: methanol (70:30, v/v) was chosen as the final mobile phase since it provided the best separation and sufficient resolution for the degradation products, not only from the drug peak but also from one another, with a period of time not exceeding 25 min.

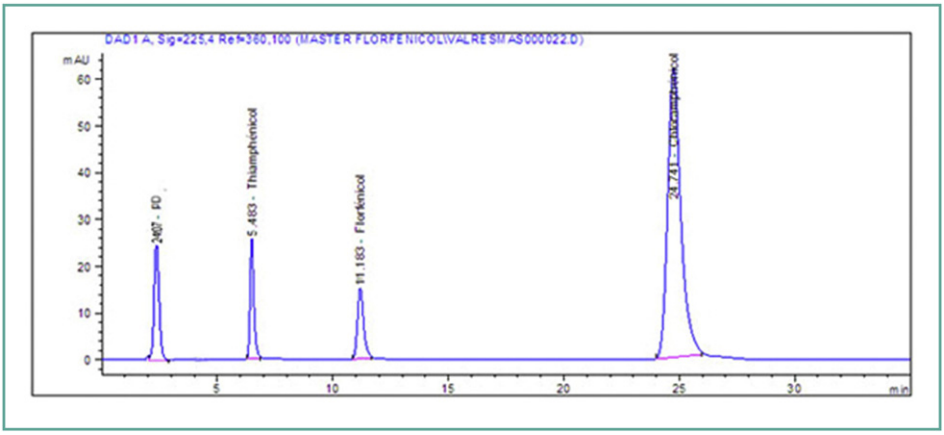


**Figure 3.** Chromatogram of florfenicol, and its major degradation products.
*Chromatogramme de florfénicol et de ces principaux produits de dégradation.*

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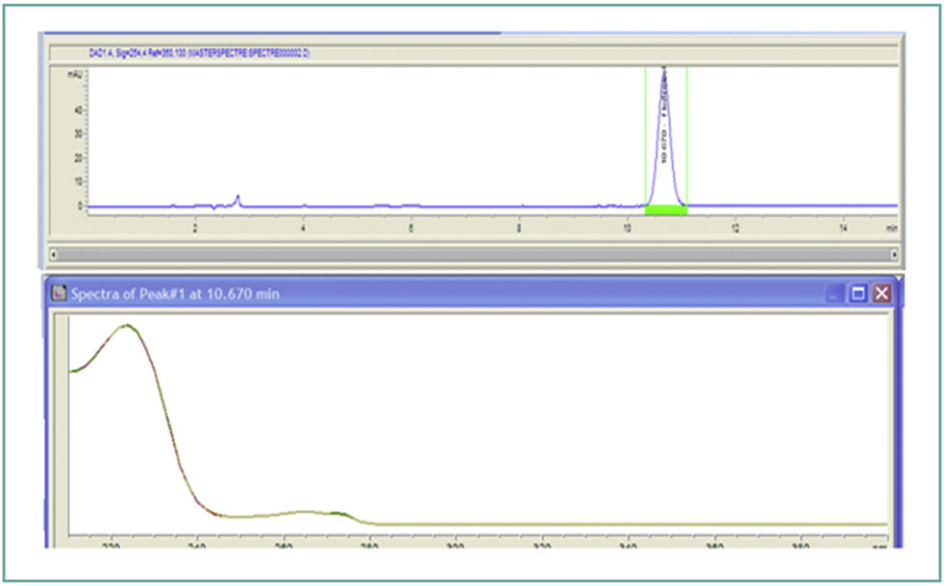


**Figure 4.** Purity spectra of florfenicol, a reference substance.
*Spectre de pureté de florfénicol, une substance de référence.*

### *Selection of the flow rate*

The influence of the mobile phase flow rate was studied. The retention times and chromatographic resolutions decreased when the flow rate increased. A flow rate of 1 ml/min was selected since we obtained a sufficient separation and an acceptable analysis time.

### *Selection of the column and its temperature*

Following the polarity of analysed molecules which are relatively polar, we have chosen a C18 column (inversed phase polarity phenomena). The influence of column temperature on separation has also been studied by using three different temperatures: 25 °C, 30 °C and 35 °C. The results obtained show that there is no influence on the chromatographic separation.

Thus, we have chosen a room temperature at 25 °C.

### *Selection of the wavelength*

A 225 nm was selected as suitable wavelength absorbance, since it is a compromise between the florfenicol and its principal's degradation products.

## Selected conditions

The chromatographic procedure is summarized in Table 3. The chromatogram of florfenicol and its principal degradation products in these conditions is shown in Fig. 3. Good

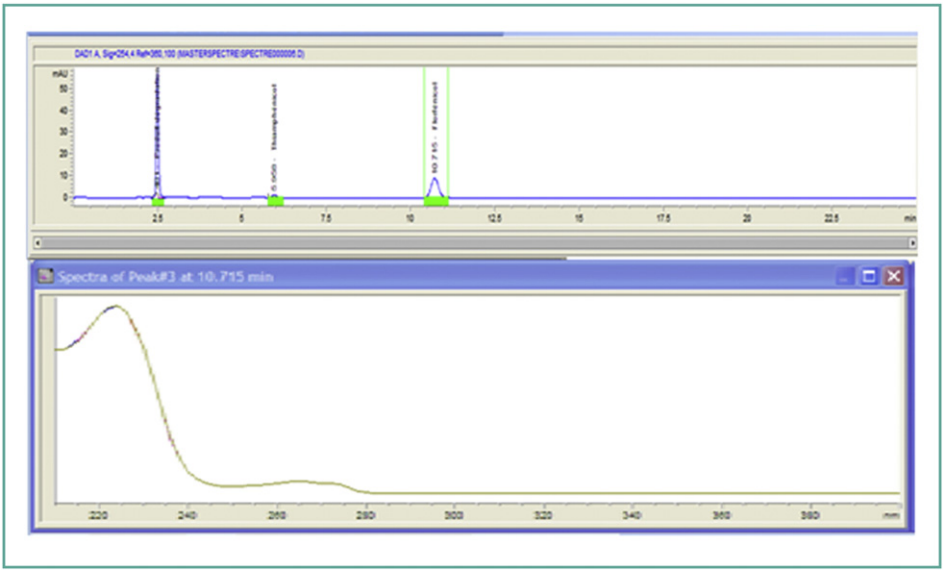


**Figure 5.** Purity spectra of florfenicol (RT = 11 min) obtained after acidic degradation.
*Spectre de pureté de florfénicol (TR = 11 min) obtenu après dégradation acide.*

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**Table 3** Chromatographic conditions selected.
*Les conditions chromatographiques sélectionnées.*

| | |
|---|---|
| Column thermostated at 25 °C | Knauer Eurospher 100-5 $C_{18}$ (250 × 4.6 mm; 5 μm) |
| Mobile phase | 6.49 mM ammonium acetate buffer (pH = 4.5): methanol (70:30, v/v) |
| Flow rate (ml/min) | 1 |
| Injection volume (μL) | 20 |
| Detection, UV-VIS (nm) | 225 |

resolution and peak shapes of every component can be seen. The purity of peaks was also proved through PDA detector (Figs. 4–8). The resolution factor for the drug peak from the nearest resolving peak was also checked to confirm the separation behaviour (Table 4).

The interpretation of classical, first and second derivative UV spectra, shows that florfenicol and the peak at RT = 11 min have the same spectral characteristics: the same maximum, minimum and zero-crossing points (Fig. 9). Also the same spectral characteristics have been observed for thiamphenicol and the degradation product at RT = 5.7 min (Fig. 10). Thus, we can conclude that florfenicol corresponds to the peak at RT = 11 min and thiamphenicol to the peak

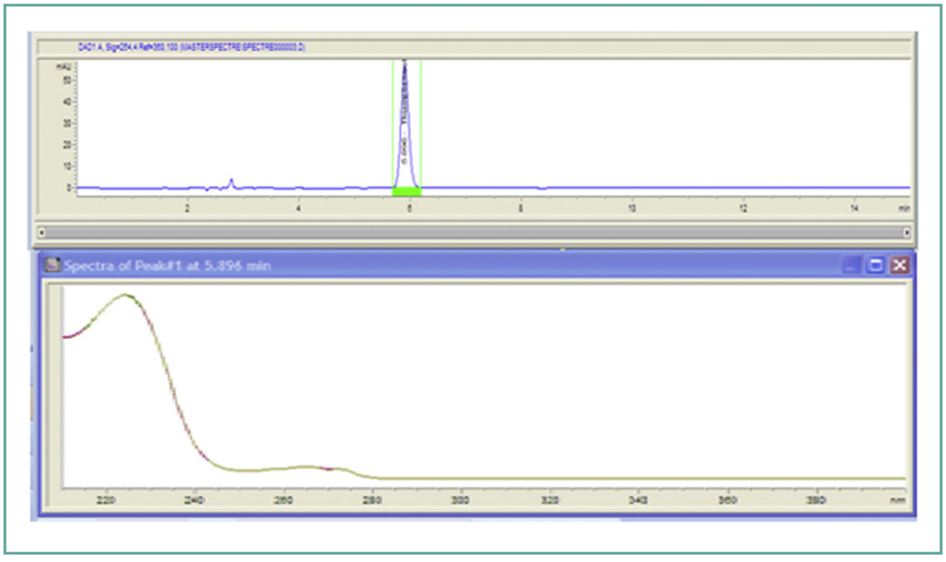


**Figure 6.** Purity spectra of thiamphenicol, a reference substance.
*Spectre de pureté de thiamphénicol, une substance de référence.*

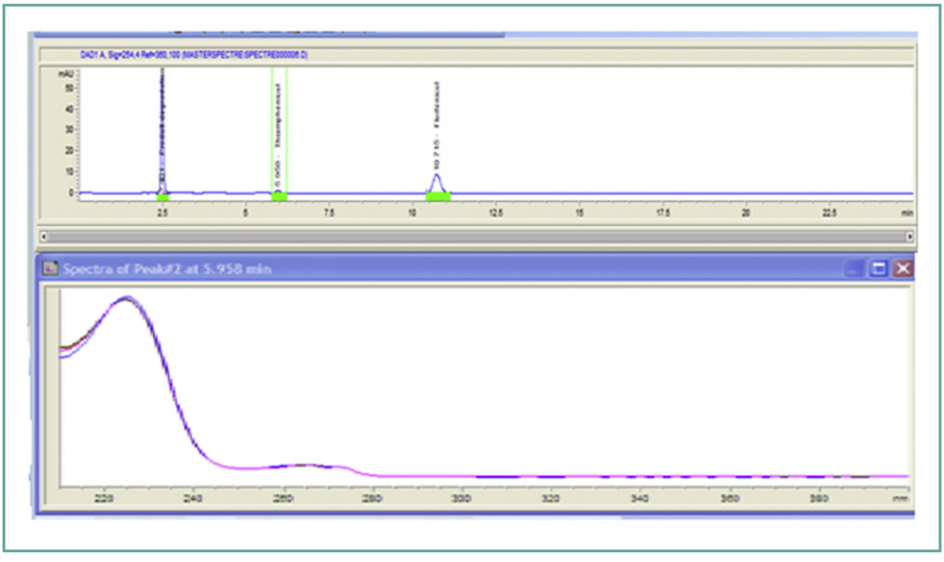


**Figure 7.** Purity spectra of the peak at RT = 5.7 min obtained after acidic degradation.
*Spectre de pureté du pic à TR = 5,7 min obtenu après dégradation acide.*

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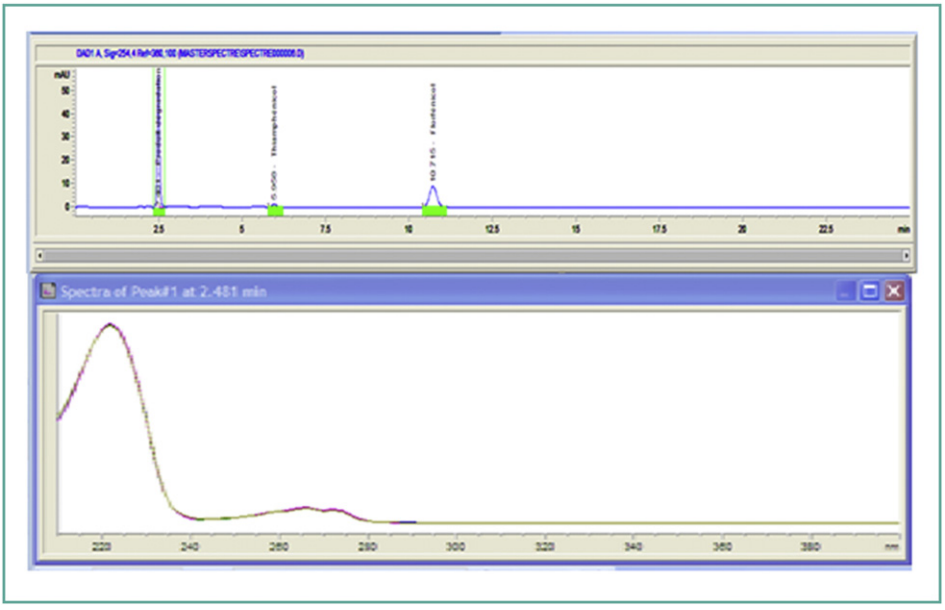


**Figure 8.** Purity spectra of the peak at RT = 2.4 min obtained after acidic degradation.
*Spectre de pureté du pic à TR = 2,4 min obtenu après dégradation acide.*

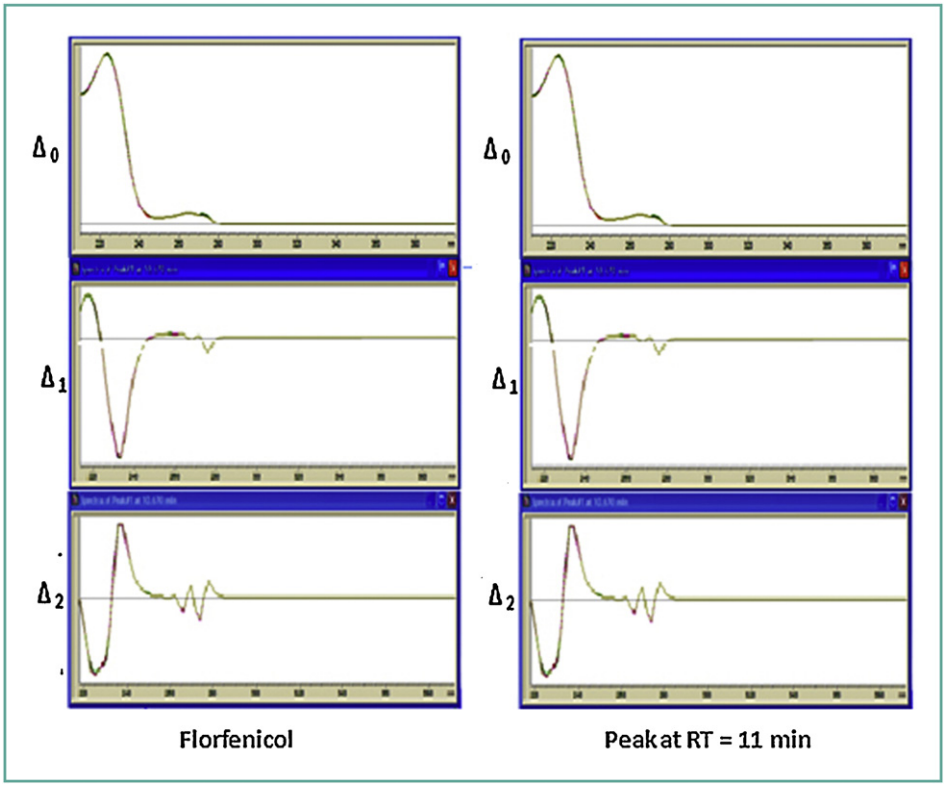


**Figure 9.** Classical spectra ($\Delta_0$), first ($\Delta_1$) and second ($\Delta_2$) derivative spectra of florfenicol, a reference substance, and the peak at RT ≈ 11 min.
*Spectre classique ($\Delta_0$) et spectre dérivé première ($\Delta_1$) et secondaire ($\Delta_2$) du florfénicol, une substance de référence, et de pic à TR ≈ 11 min.*

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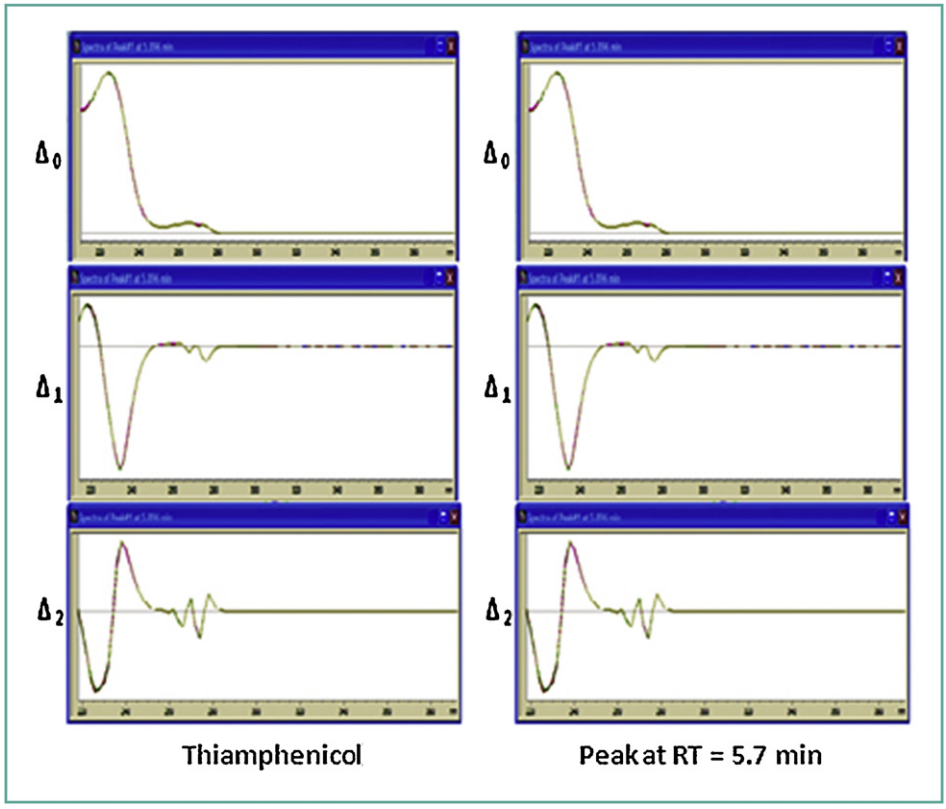


**Figure 10.** Classical spectra ($\Delta_0$), first ($\Delta_1$) and second ($\Delta_2$) derivative spectra of thiamphenicol, a reference substance, and the peak at RT ≈ 5.7 min.
*Spectre classique ($\Delta_0$) et spectre dérivé première ($\Delta_1$) et secondaire ($\Delta_2$) du thiamphénicol, une substance de référence, et de pic à TR ≈ 5,7 min.*

at RT = 5.7 min. This structure identification will be more confirmed through LC-MS studies.

Concerning the major product peak at RT = 2.4 min, the interpretation of classical, first and second derivative UV spectra, shows that the maximum, minimum and zero-crossing points are nearly similar to those observed with thiamphenicol and florfenicol (Fig. 11) and so the products have a near structure. Through LC-MS studies, the identification of this major degradation product structure will be more clarified.

## Validation of the developed stability-indicating assay method

### *Linearity*

A good linear relationship was obtained between concentrations and peak area showing that the method was linear with a correlation coefficient greater than 0.99. The statistical results are shown in Table 5. In all cases, the intercepts were considered as negligible by using the Student's *t*-test ($\alpha = 0.05$).

**Table 4** Resolution factor.
*Le facteur de résolution.*

| Peak | RT (min) | RR |
|---|---|---|
| I | 2.4 | – |
| II (thiamphenicol) | 5.7 | 13.23 |
| III (florfenicol) | 11 | 17.60 |
| IV (chloramphenicol) | 23.15 | 30.32 |

### *Precision*

The data obtained from precision experiments for intra- and inter-day precision studies shows that the % RSD values were less than 2.0%, confirming that the method was sufficiently precise.

### *Accuracy*

Percentage recovery was calculated from differences between the peak areas obtained for fortified and unfortified solutions. As shown from the data in Table 6, excellent recoveries were made at each added concentration.

### *Specificity*

The method proved to be specific to each peak, which was determined through the use of PDA detector (Figs. 4–8). Also, the resolution factor among various peaks was found to be superior to 2, proving good separation among all the peaks (Table 4).

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**Table 5** Statistical study of linearity.
*L'étude statistique de la linéarité.*

| | Florfenicol in reconstituted solution | Florfenicol standard solution | |
|---|---|---|---|
| Range of concentration | 40—60 (mg/0.5 mL) | 40.1—61 mg/0.5 ml | |
| Slope | 20.01 | 19.78 | |
| Intercept | −7.958 | −5.618 | |
| Correlation coefficient | 0.998 | 0.999 | |
| | | | Theoretical values |
| Comparison of intercept with 0 (*t*-test)[a] | 0.599 (NS)[c] | 0.713 (NS)[c] | $t_{(0.05;\ 13)} = 2.16$ |
| Homogeneity of variance (test of Cochran) | 0.4538 (NS) | 0.613 (NS) | $C_{(0.05;\ 5;\ 2)} = 0.68$ |
| Existence of a significant slope (*F*-test)[b] | 5469.479 (HS)[d] | 15,274.831 (HS)[d] | $F_{(0.05;\ 1;\ 13)} = 4.67$ |
| Intercept comparison test | 0.15 (NS) | | $t_{(0.05;\ 26)} = 2.056$ |
| Slope comparison test | 0.73 (NS) | | $t_{(0.05;\ 26)} = 2.056$ |

[a] Student's *t*-test.
[b] Snedecor's Fischer-test.
[c] NS: not significant.
[d] HS: highly significant.

**Table 6** Recovery studies.
*Les études d'exactitude.*

| Drug | Amount added(mg/0.5 mL) | Amount found (mg/mL) ± SD; RSD% ($n = 3$)[a] | Recovery (%) |
|---|---|---|---|
| Florfenicol in reconstituted solution | 40 | 49.84 ± 0.20; 0.40 | 99.68 |
| | 45 | 49.06 ± 0.08; 0.09 | 98.12 |
| | 50 | 50 ± 0.42; 0.84 | 100 |
| | 55 | 49.37 ± 0.42; 0.85 | 98.75 |
| | 60 | 50.47 ± 0.18; 0.35 | 100.94 |
| Florfenicol standard solution | 40.02 | 50.04 ± 0.31; 0.61 | 100.09 |
| | 44.83 | 48.88 ± 0.47; 0.48 | 97.77 |
| | 50.00 | 50.0 ± 0.44; 0.88 | 100 |
| | 54.93 | 50.06 ± 0.22; 0.44 | 100.11 |
| | 61.00 | 50.90 ± 0.05; 0.09 | 101.79 |

[a] *n*: number of determinations.

**Table 7** Observed m/z values for the [M-acetate] ions and major fragments of florfenicol and its different degradation products.
*Les valeurs m/z observées pour les ions [M-acétate] et les principaux fragments de florfénicol et de ces principaux produits de dégradation.*

| Peak | Observed m/z values | Major fragments |
|---|---|---|
| Florfenicol | 416.141 | 402; 384.19; 324.045 (100%); 326.018 (30%); 288.149; 270.074 |
| Thaimphenicol | 414.104 | 400.229; 322.072 (100%); M + 2 = 324.109 (30%); 286.113 |
| 11 min | 416<br>M = 408 (100%)<br>M + 2 = 410.031 (100%)<br>M + 4 = 412 (30%) | 374.071; M + 2 = 375.98; M + 4 = 377.95; 274.7; 276; 278; 280 |
| 2.22 min | M = 408 (100%)<br>M + 2 = 410.031<br>M + 4 = 412 (30%) | M = 374.071 (100%); M + 2 = 375.98 (65%); M + 4 = 377.95 (10.7%)<br>M = 274.7 (80%); M + 2 = 276 (100%); M + 4 = 278 (50%); M + 6 = 280 (10%)<br>M = 254.86 (80%); M + 2 = 256.89 (100%); M + 4 = 258.93 (50%); M + 6 = 260.84 (10%) |

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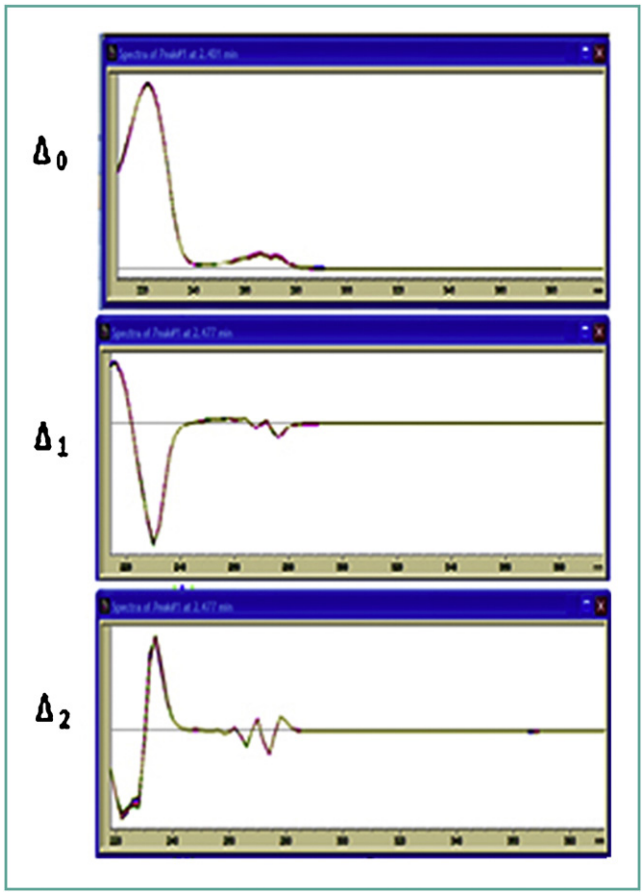


**Figure 11.** Classical spectra ($\Delta_0$), first ($\Delta_1$) and second ($\Delta_2$) derivative spectra of the impurity at RT = 2.4 min.
*Spectre classique ($\Delta_0$) et spectre dérivé première ($\Delta_1$) et secondaire ($\Delta_2$) de l'impureté à TR = 2,4 min.*

## LC-MS studies on forced decomposition samples

The mass spectra of the florfenicol and its principal degradation products are shown in Figs. 12—16. The observed m/z values for molecular ion peak and major fragments of the drug and its degradation products are listed in Table 7.

The mass spectra of florfenicol and the peak at RT = 11 min (Figs. 12 and 14) show that they are superposed. In fact, the same m/z values have been observed: 416, 402, 384, 324, 288 and 270. Thus, we can conclude, that the florfenicol is the peak at RT = 11 min.

For the peak at RT = 5.74 min (Fig. 15), the m/z obtained values are the same as those obtained with thiamphenicol (Fig. 13): 414, 400, 322 and 288. This confirms well that the degradation product obtained after hydrolysis is thiamphenicol resulting in fluorine atom substitution with hydroxyl group. This result is in conform with literature which shows that thiamphenicol is both a synthesis and impurity degradation product [17].

The mass spectra interpretation for the major degradation product (Fig. 16) obtained after florfenicol hydrolysis show that the drug is hydrolysed in the amide function to yield florfenicol amine, a degradation product having been demonstrated in some studies [18].

As a conclusion from the stress degradation studies of florfenicol, there is a major degradation product corresponding to florfenicol amine resulting in amide bond hydrolysis, and a minor degradation product corresponding to thiamphenicol resulting in the substitution of fluorine atom with hydroxyl group. Thus, a pathway for the formation of degradation products can be proposed (Fig. 17).

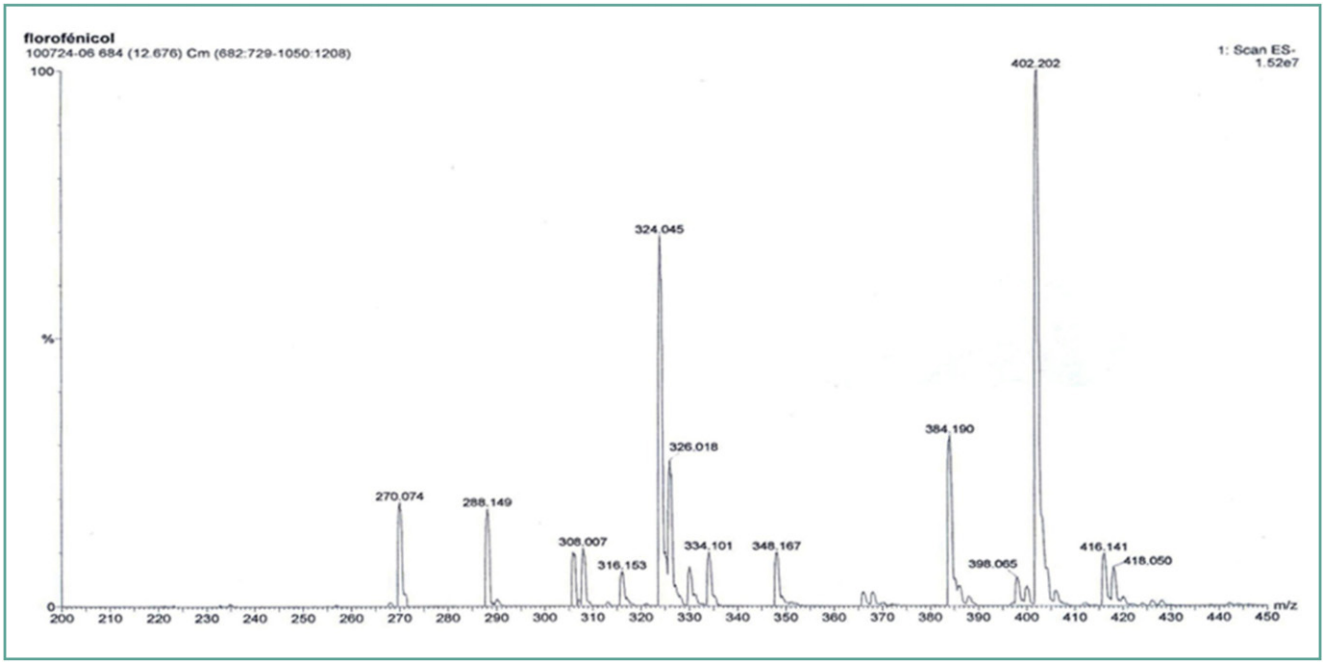


**Figure 12.** Mass spectra of florfenicol, a reference substance.
*Spectre de masse de florfénicol, une substance de référence.*

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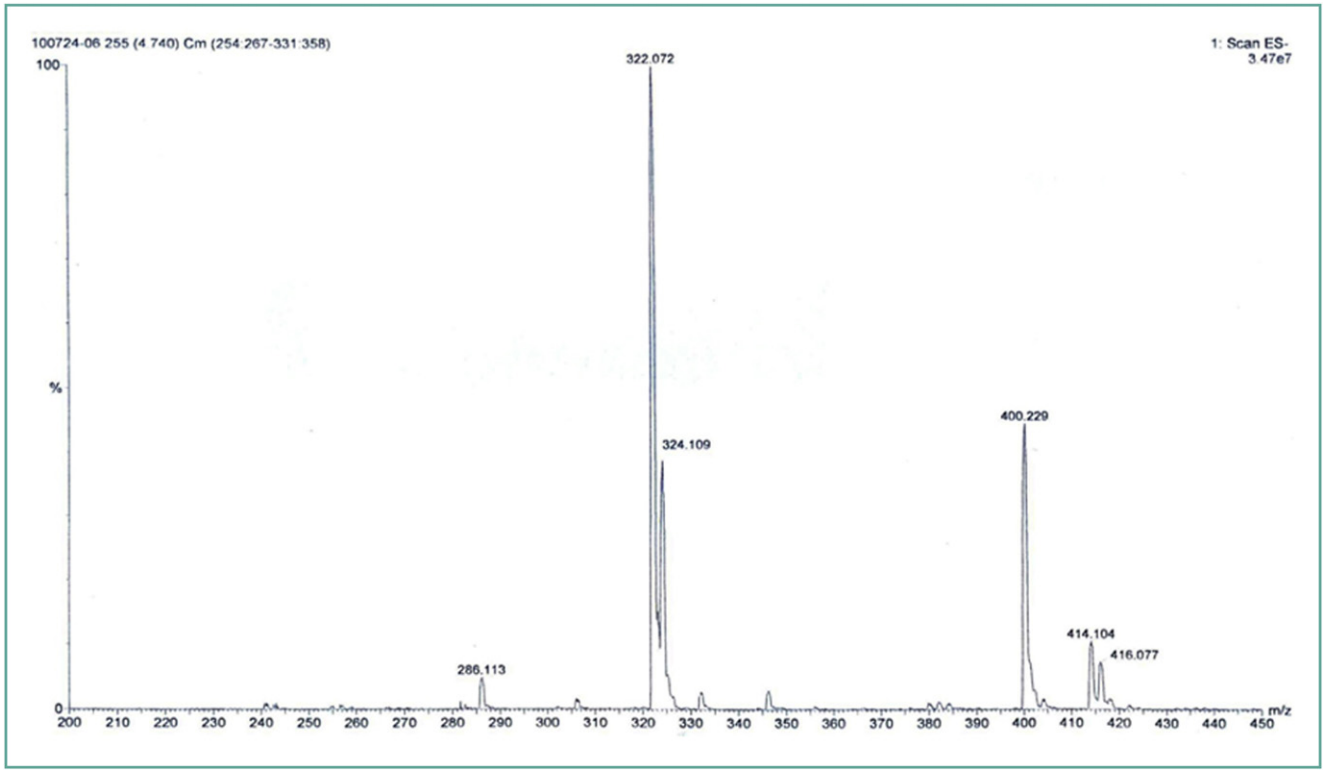


**Figure 13.** Mass spectra of thiamphenicol, a reference substance.
*Spectre de masse de thiamphénicol, une substance de référence.*

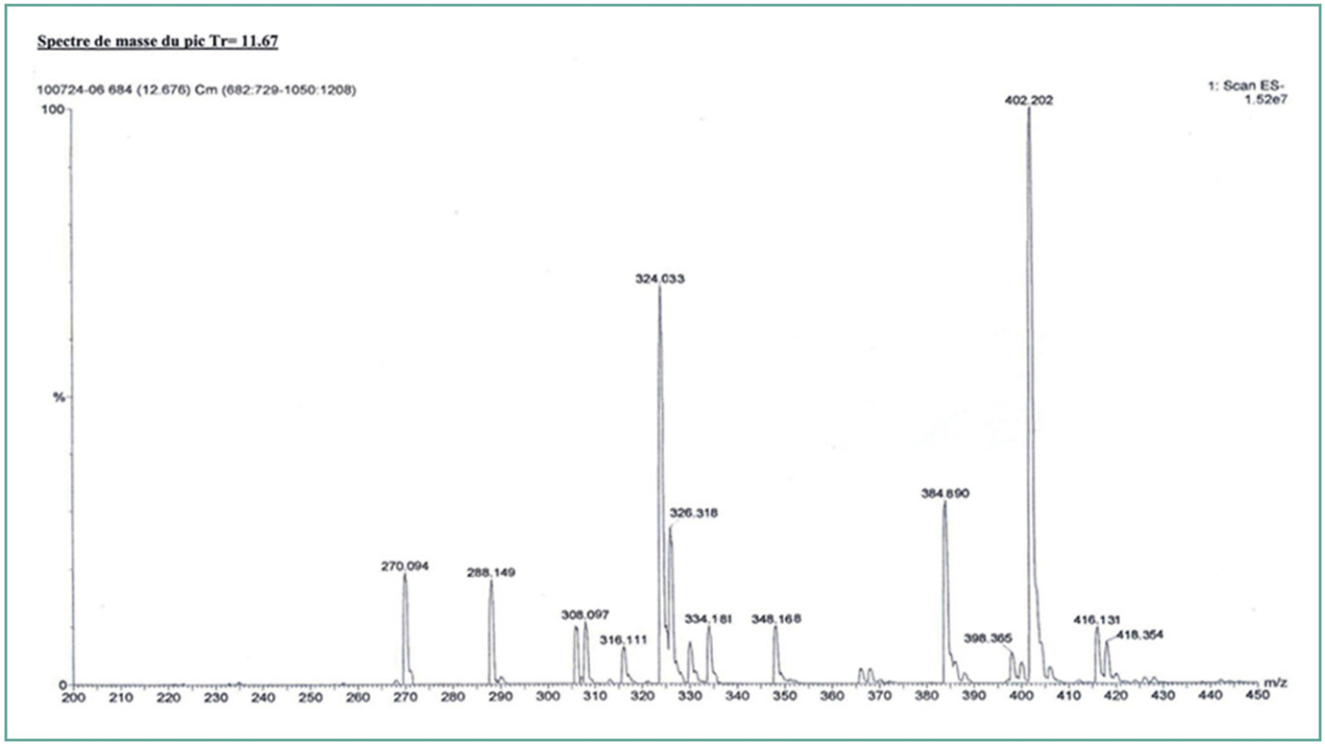


**Figure 14.** Mass spectra of the peak at RT = 11.67 min.
*Spectre de masse du pic à TR = 11,67 min.*

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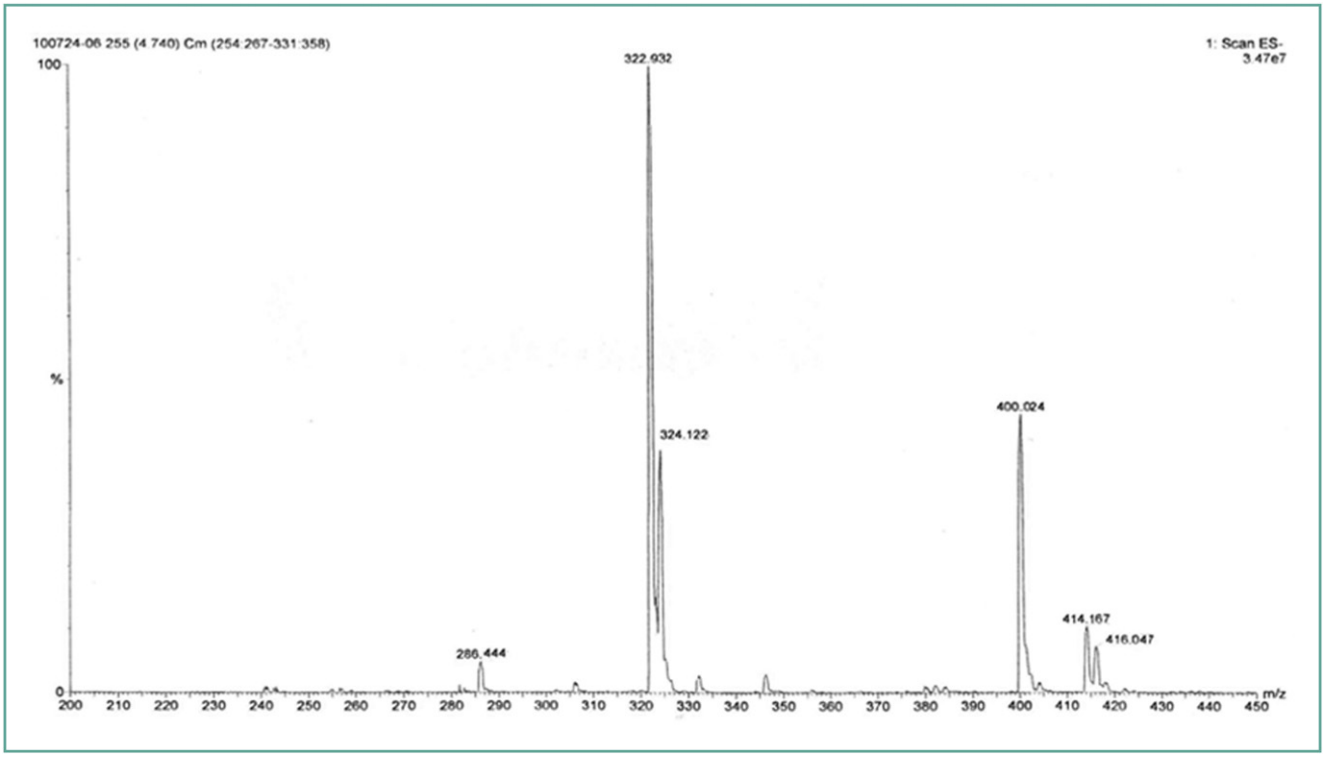


**Figure 15.** Mass spectra of the peak at RT = 5.74 min.
*Spectre de masse du pic à TR = 5,74 min.*

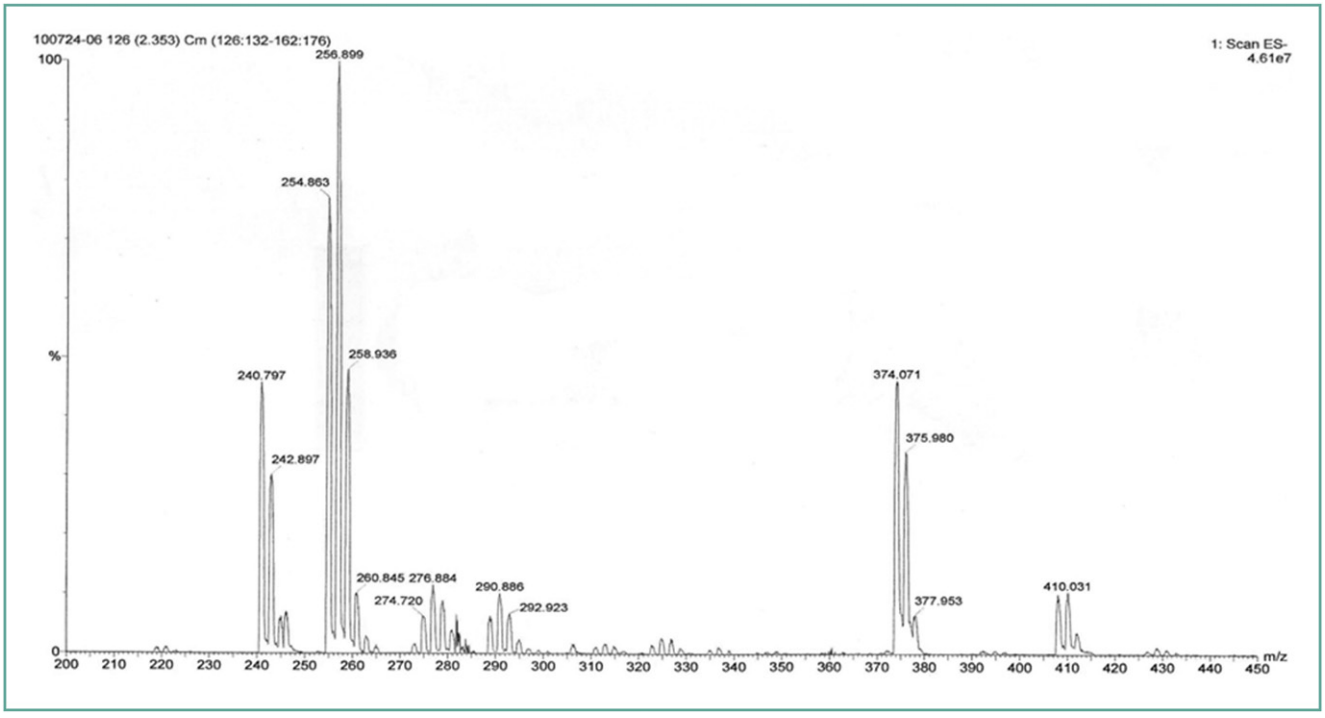


**Figure 16.** Mass spectra of the peak at RT = 2.22 min.
*Spectre de masse du pic à TR = 2,22 min.*

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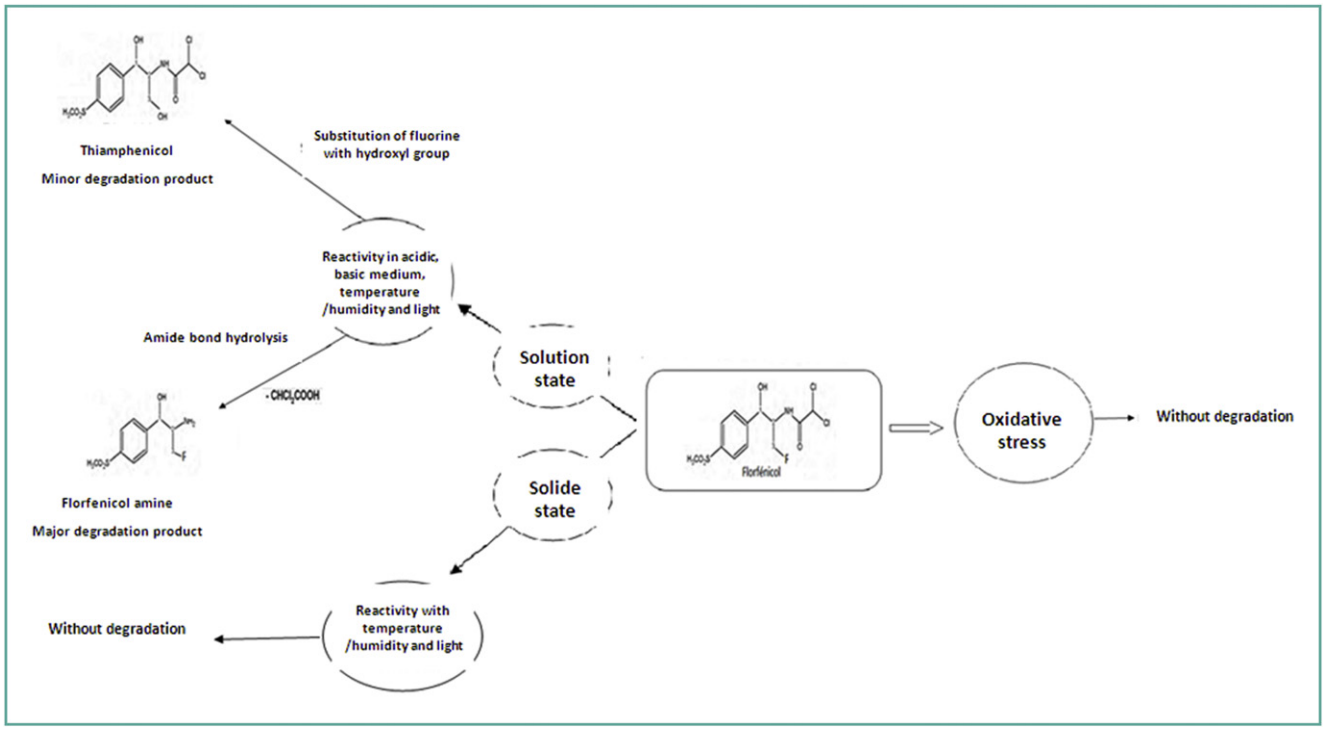


**Figure 17.** Degradation ways of florfenicol schema.
*Schéma de dégradation du florfénicol.*

## Conclusion

In this study, it was possible to develop a selective and validated stability-indicating HPLC assay method for florfenicol on a C18 column without gradient mode, which could separate the drug and its degradation products formed under a variety of stress conditions.

Florfenicol was found to be unstable to hydrolysis at the state of a solution form, whereas it remains stable in solid state.

The m/z values and fragmentation patterns obtained for the degradation products through LC-MS studies helped to confirm the presence of known products and to propose the structures of unknown compounds.

The results in totality helped to draw out a more extensive degradation route of the drug.

Indirectly the study highlights the benefit of the use of ICH stress testing approach in establishment of complete degradation pathways of drugs.

## Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

## Acknowledgement

The study sponsors had no such involvement.

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