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# Forced degradation behavior of two-drug combinations: Isolation and characterization of major degradation products by LC-MS

Rúbia Adrieli Sversut[a,b,*], James Cabral Vieira[a], Nájla Mohamad Kassab[a], Denise Brentan Silva[c], Hérida Regina Nunes Salgado[b]

[a] Universidade Federal de Mato Grosso do Sul (UFMS), Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição (FACFAN), Laboratório de Tecnologia Farmacêutica (LTF), Campo Grande, MS, Brazil
[b] Universidade Estadual Paulista (UNESP), Faculdade de Ciências Farmacêuticas, Araraquara, SP, Brazil
[c] Universidade Federal de Mato Grosso do Sul (UFMS), Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição (FACFAN), Laboratório de Produtos Naturais e Espectrometria de Massas (LaPNEM), Campo Grande, MS, Brazil

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ABSTRACT

Oxytetracycline (OTC) belongs to the antimicrobial class, diclofenac sodium (DICLO) and piroxicam (PIRO) are nonsteroidal anti-inflammatory drugs. Fixed-dose combinations of OTC with DICLO or PIRO, available as extended release injectable solutions, are widely indicated for animal use. These drugs were subject to forced degradation (alkaline, acid, neutral, oxidative photolytic conditions) as per ICH Q1 (R2) guideline and the kinetic of degradation reactions was investigated. OTC showed higher degradation under neutral, oxidative, alkaline and acid conditions and DICLO showed extensive photo degradation, while PIRO was the most stable drug under all degradation conditions studied. A total of seven degradation products (DPs) were observed and efficient chromatographic separations of drugs and their DPs were achieved on an InertSustain C8 column using a mobile phase composed by methanol-acetonitrile-water (40:35:25, v/v/v) at pH 2.5, adjusted with formic acid, in isocratic mode. Six DPs were isolated by HPLC-PDA and their chemical structures were proposed based on high resolution MS and MS/MS data. DP 1 to DP 5 had OTC as precursor drug, while DP 6 originated from DICLO photolysis. The chemical structures of DP 1, DP 4 and DP 5 are being reported here for the first time. The HPLC-PDA was adequately validated and it can be used in the quality control routine analysis as stability indicating method for quantification of drugs in pharmaceuticals and evaluation of their accelerated and long-term stability.

## 1. Introduction

Oxytetracycline (OTC) is a broad-spectrum antimicrobial widely used in human and veterinary medicine. OTC is often formulated in fixed-dose combinations with nonsteroidal anti-inflammatory drugs, mainly sodium diclofenac (DICLO) or piroxicam (PIRO). These pharmaceuticals are available as extended release injectable solutions, indicated to treat systemic infections accompanied by fever and other inflammatory conditions in cattle. Furthermore, these drugs are also available as a single dosage form in medicines for human and veterinary uses [1].

Forced degradation studies provides valuable information about the intrinsic stability of the drugs, including the establishment of the degradation reaction's kinetic, the identification of degradation products (DPs) and the comprehension of their mechanism of formation. The interpretation of these data allows predicting future problems of stability from the final product, suggesting the appropriate selection of pharmaceutical form, packaging material, logistics, and storage conditions [2].

Over the last years, relevant researches have been widely developed for the investigation of degradation behavior from drugs, as well as the identification of their DPs by liquid chromatography coupled to mass spectrometry (LC-MS) [3–8].

However, there are not reported studies about the forced degradation reactions of fixed-dose combinations composed by OTC and DICLO or OTC and PIRO. So far, there are some reports about the isolation from the OTC photochemical degradation by UV and $H_2O_2$ [9], the dimer formation of DICLO during UV photolysis [10] and the PIRO degradation behavior [11]. Recently, our research group published the developed and validated spectrophotometric methods for the

* Corresponding author at: UFMS, Av. Costa e Silva s/n, Campo Grande, MS 79070-900, Brazil.
*E-mail address:* rusversut@gmail.com (R.A. Sversut).

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simultaneous determination of these drugs in pharmaceuticals [12]. Nevertheless, these reports did not describe the fragmentation pathway, the formation mechanism of DPs and the kinetics of degradation reactions.

Hence, the aim of this present study was to investigate degradation behavior of two drug combinations OTC with DICLO and OTC with PIRO which involved: (i) to carry out stress degradation studies as per ICH guideline [13], (ii) to establish the kinetic models of degradation reaction, (iii) to development of HPLC method for simultaneous separation of drugs and their DPs, (iv) to isolate the major DPs by HPLC, (v) to identify the DPs by LC–MS, suggesting their chemical structures and mechanisms of formation and fragmentation pathway, (vi) to validate of the developed HPLC method as recommended by official guidelines [14–17], (vii) to show the applicability of HPLC method for simultaneous or isolated quantification of OTC, DICLO and PIRO in veterinary and human pharmaceuticals.

## 2. Experimental

### 2.1. Reference standard substances (RSS) and reagents

Oxytetracycline dihydrate (OTC, assigned purity 99.1%), diclofenac sodium (DICLO, assigned purity 99.3%) and piroxicam (PIRO, assigned purity 101.7%), used as RSS, were supplied from Pharmanostra (Rio de Janeiro, Brazil), Fagron (São Paulo, Brazil) and Valdequímica Chemical Products (São Paulo, Brazil), respectively. All RSS were provided accompanied by the laboratory analysis certificate and were used without previous purification. Analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide ($H_2O_2$) were purchased from LabSynth (São Paulo, Brazil). LC-MS grade methanol, acetonitrile and formic acid were procured from Sigma Aldrich (Missouri, USA). Ultrapure water was obtained from Milli-Q Gradient system (Massachusetts, USA) to prepare all solutions.

### 2.2. Instrumentation

The High Performance Liquid Chromatography (HPLC) analyses were performed on a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Massachusetts, USA), equipped with photodiode array detector (PDA) and quaternary gradient pump with an in-line degasser. Chromatographic data was acquired by Chromeleon 7.1 software. For the identifications of DPs, an UFLC LC-10AD Shimadzu (Kyoto, Japan) coupled to diode array detector and a mass spectrometer (MicrOTOF-Q III, Bruker Daltonics, Massachusetts, USA) with an electrospray ionization source (ESI) was used. The MS analyses were performed on positive ion mode (*m/z* 120–1300). Nitrogen was used as collision, nebulizer (4 bar) and drying gases (9 L $min^{-1}$ at 200 °C), applying the capillary voltage of 2500 Kv. The mass spectrometric data were processed by Data Analysis software (Bruker Daltonics, Massachusetts, USA).

An Ultracleaner 1400 ultrasonic bath sonicator from Unique (São Paulo, Brazil) was employed to homogenize the samples and a MS Tecnopon mPA210 pH meter (São Paulo, Brazil) was used to measure the pH of the mobile phase. A heating bath from Delta (Minas Gerais, Brazil) and a photostability chamber equipped with two lamps (Philips TLT 40W/75 RS UVB 290–350 nm and Philips® TL 40W/12 RS 400–700 nm, São Paulo, Brazil) were employed during forced degradation studies. The isolated DPs were concentrated using rotary evaporator Buchi R-3 vacuum pump V-700 (Zurich, Switzerland) and lyophilized (Christ Alpha 2-4 LD plus, Osterode am Harz, Germany). For the stability studies, a drying over (model BD216) equipped with a controller of temperature from Novus® (Rio Grande do Sul, Brazil) and a climatic chamber (model MA 835/UR) from Marconi® (São Paulo, Brazil) were used.

### 2.3. HPLC-PDA conditions

The chromatographic separations were optimized at room temperature (24 ± 2 °C) on an InertSustain HP C8 column (3.0 µm, 100A, 150 × 4.6 mm, GL Science). The mobile phase was methanol-acetonitrile-water (40:35:25, v/v/v) at pH 2.5, adjusted with formic acid, in isocratic mode. The photodiode array detector was set at 275 nm and 350 nm for the detection of drugs and their DPs. The flow rate and injection volume were 1.0 mL $min^{-1}$ and 20 µL, respectively.

### 2.4. Forced degradation studies

Stress degradation studies of the drugs in their respective binary mixture (OTC + DICLO and OTC + PIRO) were carried out as recommended by ICH guideline [13]. Different concentrations of stressors and exposure times were tested in order to establish optimal stress conditions that would provide a degradation range of 10 to 30% for the drugs, avoiding the generation of secondary DPs [18]. Then, the optimized stress conditions were: acid hydrolysis (0.1 M HCl, 60 °C, 8 h), alkaline hydrolysis (0.01 M NaOH, 60 °C, 4 h), neutral hydrolysis ($H_2O$, 60 °C, 30 min), oxidation (0.03% $H_2O_2$, 60 °C, 30 min) and photolytic degradation at room temperature (25 ± 2 °C) during 16 h, using a photostability chamber previously described in Section 2.2. The samples were dissolved and diluted with the mixture of methanol-acetonitrile-water (40:35:25, v/v/v) at pH 2.5, used as mobile phase, to reach a final concentration of 10.0 µg $mL^{-1}$ for OTC and 1.0 µg $mL^{-1}$ for DICLO and PIRO.

### 2.5. Isolation of major degradation products

The DPs obtained were isolated by developed HPLC-PDA method. There were collected 10 fractions corresponding to the peaks of major DPs from the degraded solutions containing 250.0 µg $mL^{-1}$ of each drug in their respective association (OTC + DICLO and OTC + PIRO). Then, the fractions collected were concentrated on Buchi® rotary evaporator at a temperature of 45 °C. Subsequently, the fractions were lyophilized on Christ® lyophilizer. Finally, the freeze-dried samples of DPs were solubilized in water:acetonitrile (80:20, v/v) and analyzed by UFLC-DAD-MS.

### 2.6. UFLC-MS conditions

The UFLC-MS analyses were performed using Kinetex C18 column (2.6 µm, 100A, 150 × 2.1 mm, Phenomenex) and mobile phase composed by water (A) and acetonitrile (B), both with formic acid 0.1% (v/v). The gradient program was: 0–2 min 3% of B, 2–25 min 3 to 25% of B, 25–40 min 25 to 80% of B and 40–43 min 80% of B. The oven temperature and flow rate were 50 °C and 0.3 mL $min^{-1}$, respectively. The isolated DPs were prepared at 1 mg $mL^{-1}$, filtered on PTFE filters (Millex 0.22 mm × 13 mm, Millipore) and 5 µL was injected into chromatographic system.

### 2.7. Method validation

In order to use the developed HPLC-PDA method for quantitative analysis, it was validated according to the following guidelines: Food and Drug Administration [14], International Conference on Harmonisation [15], Association of Official Analytical Chemists International [16] and Resolution of the Collegiate Board of Directors n° 166 [17]. The analytical parameters evaluated were: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness.

#### 2.7.1. Specificity

The specificity was determined by evaluation of interference of the excipients mixture (magnesium oxide, 2-pyrrolidone, benzyl alcohol

and ultrapure water) and the degradation products in the quantification of the drugs.

#### 2.7.2. Linearity, LOD and LOQ

The linearity of the present method was established by constructing three analytical curves for each drug (OTC, DICLO and PIRO), which were obtained from dilutions of the respective standard stock solutions. The linear equations were estimated by the Ordinary Least Squares method and statistically evaluated by the y-axis homoscedasticity analysis (Cochran's test), analysis of variance (ANOVA) of the linear regression and analysis of the residues (Shapiro-Wilk's test and visual analysis of the residues graphs). The LOQ and LOD were determined from analytical curves, using the standard deviation of the intercept (SD) and average slope (a). Thus, the LOD and LOQ were calculated by Eqs. (1) and (2):

$$\mathrm{LOD} = 3.3 \times {SD}/{a} \quad (1)$$

$$\mathrm{LOQ} = 10.0 \times {SD}/{a} \quad (2)$$

#### 2.7.3. Precision

The method precision was evaluated by the intra-day (repeatability) and inter-day (intermediate precision) tests, using commercial samples (injectable solutions) for animal use composed by fixed-dose association of OTC + DICLO (Oxitrat La Plus®, containing OTC 20.0% and DICLO 1.0%, Sample A) and OTC + PIRO (Terracam Plus®, containing OTC 22.0% and PIRO 1.2%, Sample B). Repeatability was determined by analyzing the drugs' content at one concentration within the linear range of the method, and the readings were performed at ten replicates in the same day. The intermediate precision was performed by analyzing the samples for three consecutive days by two different analysts, and the peak areas were obtained in ten replicates. The results of repeatability were expressed in terms of relative standard deviation (% RSD) and those of intermediate precision were statistically evaluated through one-way ANOVA with 95% confidence interval.

#### 2.7.4. Accuracy

The accuracy was determined through recovery assays performed by adding known amounts of standard solutions to commercial sample solutions [16]. The analyses were performed separately for each drug in order to contemplate the linear range of concentration of the method, using three concentration levels, i.e., 0.50, 0.75 and 1.00 μg $mL^{-1}$ for DICLO. The percentages of recovery were calculated by Eq. (3):

$$R = [C_F - C_U)/C_A] \times 100 \quad (3)$$

where CF represents the concentration of analyte measured in the fortified test sample; CU the concentration of analyte measured in the unfortified test sample; and CA the concentration of the analyte added to the fortified test sample.

#### 2.7.5. Robustness

Robustness was assessed through the Plackett-Burman factorial model, in which a matrix composed of 15 experiments is used, varying seven parameters in three levels: normal value of the procedure (0), upper value (1) and lower value (−1) [19]. The seven parameters selected were: methanol ratio in mobile phase, methanol supplier, mobile phase pH, flow rate, wavelength, column supplier and room temperature (Tables S1 and S2, Supplementary material). Commercial samples, diluted within the linear range of the method, were used in these experiments. The results were expressed in terms of Effect (E), which consists of a numerical value obtained from the difference among the average of the contents obtained from the analysis that employ the factors under normal and altered conditions. The significance of each factors' effect was obtained according with proposed by Nevado et al. [20]. For a method to be considered robust, the absolute values of E must be smaller than the critical value ($\sqrt{2S}$). This value was found by Eq. (4):

$$S = \sqrt{\frac{2}{7}\left(E_{A/a}^2 + E_{B/b}^2 + \ldots + E_{G/g}^2\right)} \quad (4)$$

where S is the internal shunt value, considering all results of the robustness test; E: the effect under normal (capital letters) and altered conditions (lowercase letters).

### 2.8. Method applicability

#### 2.8.1. Quantification of commercial samples

Besides commercial samples Oxitrat La Plus® (OTC + DICLO, sample A) and Terracam Plus® (OTC + PIRO, sample B), two others injectable solutions for animal use were quantified: Terramicina Mais® (sample C) and Ourotetra Plus La® (sample D), both composed by the fixed-dose association of OTC 20.0% and DICLO 1.0%.

With the same purpose of our previous research [12], the applicability of the HPLC-PDA method was evaluated for the quantification of the drugs from their isolated forms. Thus, the following human pharmaceutical products were analyzed: Terramicina® capsules, containing OTC hydrochloride 500 mg (Sample E); diclofenac sodium generic gastro-resistant tablets, containing DICLO 50 mg (Sample F); Diclofarma® intramuscular injectable solution, containing DICLO 25 mg $mL^{-1}$ (Sample G); piroxicam generic capsules, containing PIRO 20 mg (Sample H) and Feldene® intramuscular injectable solution, containing PIRO 20 mg $mL^{-1}$ (Sample I). All these commercial samples were diluted with mobile phase (methanol-acetonitrile-water, 40:35:25, v/v/v, at pH 2.5), contemplating the linear range of the method, and they were analyzed by HPLC-PDA developed method.

#### 2.8.2. Evaluation of accelerated and long-term stability

The developed HPLC-PDA method was also employed to evaluate the accelerated and long-term stabilities of the commercial sample A and B. The stability studies were performed according to Brazilian guideline for stability testing [21]. Thus, the commercial samples were kept in the primary packaging (100 mL amber glass vials) and, for the accelerated stability testing, they were stored in the drying oven set at 40 ± 2 °C for six months. For the long-term stability study, the samples were kept in the climatic chamber at a controlled temperature of 30 ± 2 °C for 24 months.

## 3. Results and discussion

### 3.1. Development and optimization of HPLC-PDA conditions

The development of HPLC-PDA method began with the selection of an appropriate column and mobile phase for simultaneous separation of the drugs associations (OTC + DICLO and OTC + PIRO) in the presence of their DPs. The results obtained with the C18 columns were not promising, probably due to the apparently irreversible adsorption of OTC in the residual silanols groups, resulting in high retention of this drug. Kahsay and coworkers [22], who developed and validated an HPLC method for the simultaneous determination of OTC and its related impurities using a C8 column, also found similar results. Thus, during the development and optimization of the HPLC-PDA method, we focused on the use of C8 columns because they have smaller alkyl chains bonded to silica, presenting lower retention of OTC.

There were tested five different C8 columns (InertSustain® HP, Phenomenex®, Merck Lichrosorb®, Thermo Hypersil® and Zorbax Eclipse XDB®). The InertSustain® HP C8 column provided better performance for analytical separation of the drugs and DPs, probably due to its technical advantages when compared to the other C8 columns. The InertSustain® HP C8 column has a special kind of silica gel called “Evolved Surface Silica” which provides both high inertness and durability. In addition, this column presents greater stability against the

wide pH range (1 to 10) and pressure of the chromatographic system (≤500 bar), which ensures a longer shelf-life and preservation of the chromatographic system. Another advantage of the InertSustain® HP C8 column is its small particle size (3 μm) which allowed higher mass transfer rate when compared to conventional columns (particle size ≥5 μm), resulting in efficient chromatographic separation with symmetrical peaks, good resolution and shorter analysis time.

The mobile phase composition was optimized based on the choice of the pH value and the organic modifier, which would provide symmetric peaks and an adequate resolution among drugs and DPs peaks. Different pH values of mobile phase were screened and suitable chromatographic separation was achieved in acid pH, specifically at pH 2.50, adjusted with formic acid. In this pH value, OTC molecule is in the zwitterionic form, i.e. with the protonated C-4 dimethylamino group and the ionized C-3 hydroxyl, and also in the form in which only the dimethylamino group is protonated. Consequently, there is the formation of ionic pairs between the acid anion contained in the mobile phase and the cation of the drug (protonated dimethylamino group), avoiding the strong adsorption of the drug in the reverse stationary phase. Methanol and acetonitrile were used as organic modifier because they produced a better resolution among binary mixture of the drugs and their DPs. Thus, a ternary mobile phase composed by methanol-acetonitrile-water (40:35:25, v/v/v) at pH 2.5, in an isocratic mode, was chosen for simultaneous analyses of the drugs and DPs.

### 3.2. Degradation behavior of drugs

The drugs and DPs were adequately separated as shown in Figs. 1 and 2. At least one DP appeared in each degradation condition, and for the OTC + DICLO association (Fig. 1) a total of 7 DPs was formed, whereas for the OTC + PIRO association were obtained 5 DPs (Fig. 2). According to Figs. 1 and 2, the DP4 ($t_R$ = 1.98 min) and DP5 ($t_R$ = 2.28 min) were repeated in both drugs associations, under neutral hydrolysis and oxidative degradation. In particular, the DP5 was also present in the photolysis. It is suggested that these DPs originated from OTC, since it is the common drug of the two associations. In addition, it is believed that DP6 and DP7 are obtained from the DICLO photo degradation, since they only appeared in the chromatogram of its respective association.

The drugs had different percentages of degradation due to their intrinsic stability. Almost all conditions provided an individual percentage of drugs degradation within 10 to 30% [18]. Only DICLO, in the optimized condition for photolysis, presented degradation percentage higher than this range (almost 61%). The stressed solutions became colored (particularly yellow in the photolysis of DICLO) in contrast to the colorless drugs solutions prior the degradation study. The summary of the degradation behavior of drugs is exposed in Table 1.

#### 3.2.1. Degradation kinetics of drugs

The zero, first and second order kinetic models were tested using the regression method in order to explain the degradation behavior of the drugs, once most of pharmaceutical products follow these models. The determination coefficients ($r^2$) of the kinetic models were calculated for the drugs in each degradation condition. The $r^2$ values closest to 1.0 indicated the order of the respective reactions [23] (Table S3, Supplementary material).

Therefore, the data suggested that all drugs obeyed the second-order kinetic model for acid and basic hydrolysis, which indicates that the reaction rate depends on the square of the drug concentrations. In neutral hydrolysis, only DICLO decayed according to the second-order model, while OTC and PIRO obeyed the first-order model, highlighting a reaction rate directly proportional to the remaining concentration of the drug in relation to time. For oxidative degradation, OTC degraded according to zero-order kinetics, which means that its degradation has a constant velocity and does not depend of the concentration of any reagent, DICLO and PIRO have obeyed the second order kinetic model. Finally, in photolysis, the mathematical models that most fit the decay of the OTC, DICLO and PIRO contents were, respectively, zero, first and second order.

In general, PIRO was the most stable drug, because it presented the highest values of half-life ($t_{50\%}$) and the lowest k values in the degradation conditions analyzed. OTC was the most unstable drug, once it showed the lowest values for $t_{50\%}$ and $t_{90\%}$ (shelf-life). Particularly, neutral hydrolysis ($t_{50\%}$ = 1.2 h) and oxidation ($t_{50\%}$ = 0.9 h) were the conditions where OTC showed higher instability. This result agrees that found by Xuan et al. [24], who observed that OTC hydrolysis was much faster at neutral pH than at acid or basic pH. In addition, the higher stability of OTC in acid hydrolytic ($t_{50\%}$ = 50 and $t_{90\%}$ = 5.6 h) than in

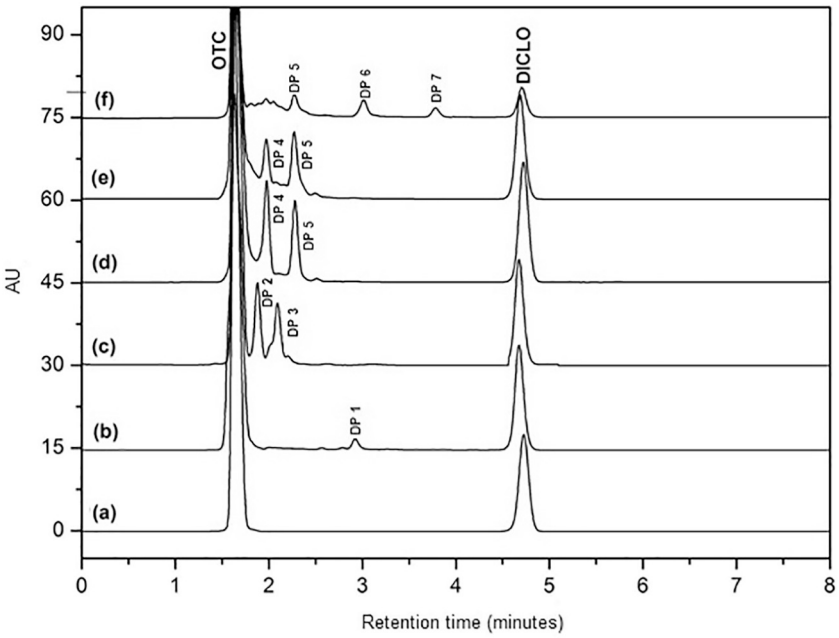


**Fig. 1.** Chromatograms of OTC + DICLO association and their degradation products. (a) without degradation, (b) alkaline hydrolysis (NaOH 0.01 M, 4 h), (c) acid hydrolysis (HCl 0.1 M, 8 h), (d) neutral hydrolysis ($H_2O$, 30 min), (e) oxidation ($H_2O_2$ 0.03%, 30 min) and (f) photolysis (UV/VIS light, 16 h).

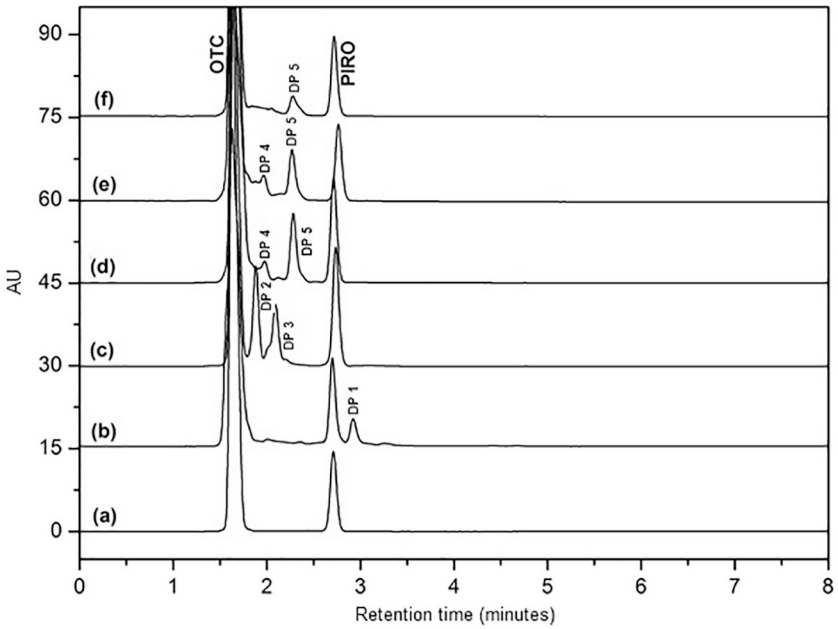


**Fig. 2.** Chromatograms of OTC + PIRO association and their degradation products. (a) without degradation, (b) alkaline hydrolysis (NaOH 0.01 M, 4 h), (c) acid hydrolysis (HCl 0.1 M, 8 h), (d) neutral hydrolysis ($H_2O$, 30 min), (e) oxidation ($H_2O_2$ 0.03%, 30 min) and (f) photolysis (UV/VIS light, 16 h).

**Table 1**
Summary of degradation behavior of drugs.

| Degradation condition | Stressor | Degradation products formed | Degradation (%) | | |
|---|---|---|---|---|---|
| | | | OTC | DICLO | PIRO |
| Alkaline hydrolysis | NaOH 0.01 at 60 °C for 4 h | DP 1 | 29.96 | 24.40 | 25.44 |
| Acid hydrolysis | HCl 0.1 M at 60 °C for 8 h | DP 2; DP 3 | 20.13 | 15.86 | 17.80 |
| Neutral hydrolysis | $H_2O$ at 60°C for 30 min | DP 4; DP 5 | 26.38 | 8.71 | 4.00 |
| Oxidation | $H_2O_2$ 0.03% at 60 °C for 30 min | DP 4; DP 5 | 12.99 | 9.44 | 6.78 |
| Photolysis | UV/VIS light for 16 h | DP 5; DP 6; DP 7 | 15.32 | 60.85 | 3.77 |

DP: degradation product; OTC: oxytetracycline; DICLO: diclofenac sodium; PIRO: piroxicam.

alkaline condition ($t_{50\%}$ = 9.7 and $t_{90\%}$ = 0.8 h) also is in agreement with that described in the literature [24]. Photolysis is the only degradation condition that OTC was not the most unstable drug. In this condition DICLO presented significant degradation ($t_{50\%}$ = 12.9 h and $t_{90\%}$ = 0.06 h), which corroborates with previous study (Keen et al., 2013) (Table S4, Supplementary material).

### *3.3. Structural characterization of drugs and their degradation products by UHPLC-MS*

#### *3.3.1. MS analyses of reference standard substances*

The OTC MS spectrum (Fig. S1a, Supplementary material) shows the $[M + H]^+$ peak at *m/z* 461.1569, corresponding to molecular formula $C_{22}H_{25}N_2O_9^+$, and a fragment ion at *m/z* 443.1458 $[M + H\text{-}H_2O]^+$ yielded by a charge-directed fragmentation from the protonation of C-6 hydroxyl group. The protonation of this group is pertinent and corroborate with the literature [25]. Then, as shown in the $MS^2$ spectra, the fragment ions at *m/z* 426.1211(neutral loss of $NH_3$, 17 Da and *m/z*381.0630 (subsequent neutral loss of dimethylamine group, 45 Da) were formed. From the ion *m/z* 426.1211, a charge retention fragmentation with the generation of the fragment ion at *m/z* 408.1102 (loss of $H_2O$, 18 Da). The ion at *m/z* 408.1102 can yield an ion at *m/z* 380.1130 because of the neutral loss of carbon monoxide (CO, 28 Da). Subsequently, the fragment ion at *m/z* 380.1130 can yield the product ion at *m/z* 337.0720 from a loss of $CH_3N{=}CH_2$ (43 Da). The chemical structures of fragment ions at *m/z* 283.0618 and 201.0552 are being proposed for the first time in our work and are we believed that they are derived from the neutral losses of $C_3H_2O$ (54 Da) and $C_4H_2O_2$ (82 Da), respectively (Fig. S2, Supplementary material).

The MS spectrum of DICLO (Fig. S1b, Supplementary material) showed a peak at *m/z* 296.0244 $[M + H]^+$, relative to molecular formula $C_{14}H_{12}Cl_2NO_2^+$, and the presence of chlorine isotopic ions ($^{35}Cl$ and $^{37}Cl$) confirmed the two chlorines atoms in DICLO [26]. The peak at *m/z* 318.0062 corresponds to the sodiated ion. The fragment ions at *m/z* 278.0141 correspond to loss of water ($H_2O$, 18 Da), which yielded the product ion *m/z* 250.0191 from neutral loss of carbon monoxide (CO, 28 Da) by ring contraction. Subsequently, this ion can loss an HCl molecule and a Cl radical, yielding the fragment ions *m/z* 214.0408 and at *m/z* 179.0757, respectively (Fig. S2, Supplementary material).

The MS spectrum from PIRO (Fig. S1c, Supplementary material) shows an intense ion at *m/z* 332.0667 confirming the molecular formula $C_{15}H_{14}N_3O_4S^+$ and the sodiated ion was also observed at *m/z* 354.0519. The cleavage of amide bond yielded the product ion *m/z* 238.0167, which occurs a keto-enol tautomerism with subsequent losses of carbon monoxide (CO, 28 Da) and carbon dioxide ($CO_2$, 44 Da), yielding the ions *m/z* 210.0224 and *m/z* 194.0266. The rupture of the dative bond between sulfur and oxygen (S → O) required lower energy than a rupture of covalent bond, thus this fragmentation was proposed and this heterolithic cleavage is common in gas phase or inert solvents [27]. Finally, the ion at *m/z* 332.0667 $[M + H]^+$ is the precursor of the ion at *m/z* 164.0818 by a retro-Diels-Alder fission (Fig. S4, Supplementary material).

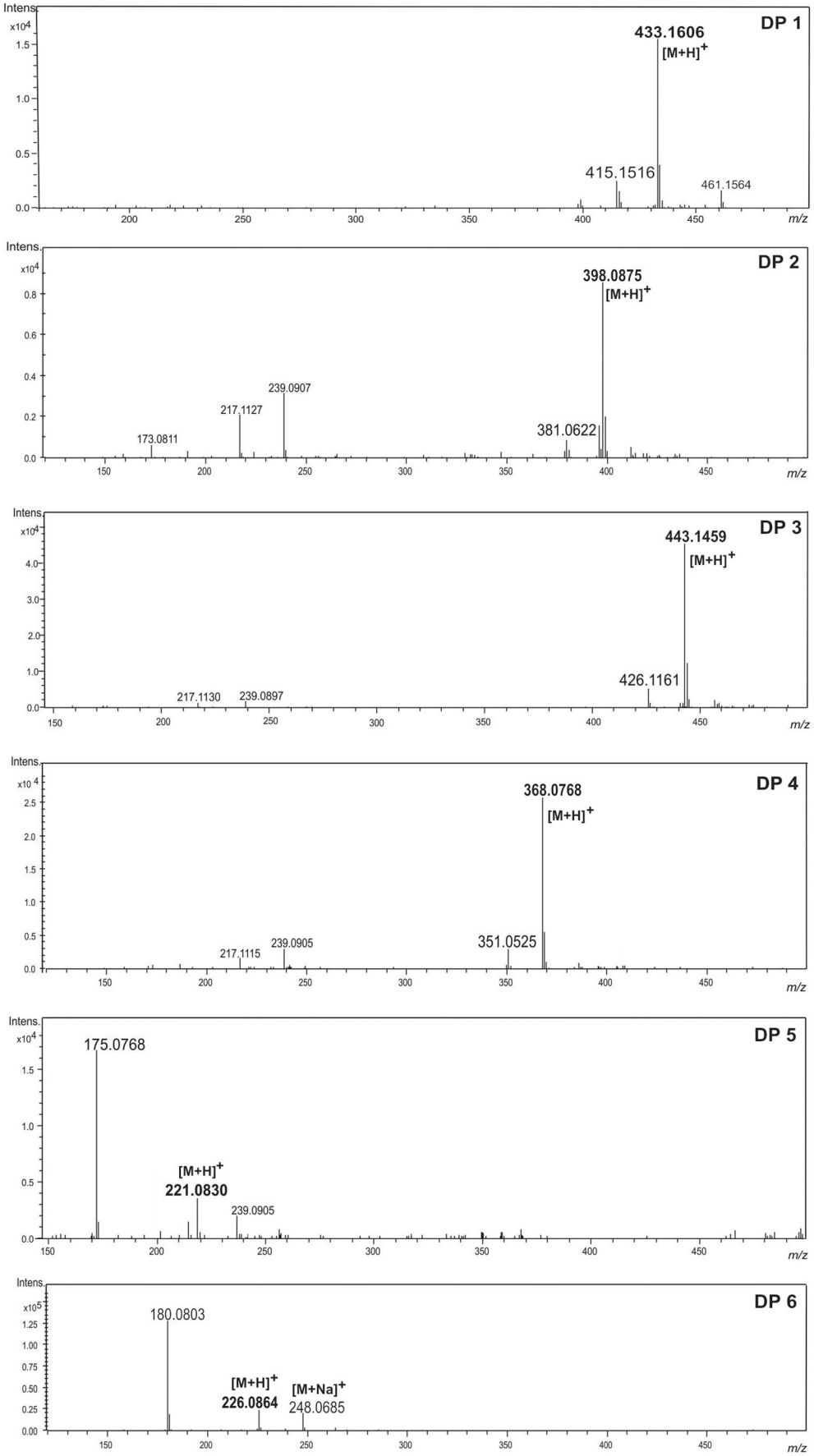


**Fig. 3.** MS spectra of DP 1 to DP 6.

**Table 2**
High resolution MS data of degradation products (DPs).

| Degradation products | Molecular formula | Experimental mass $[M + H]^+$ | Error (ppm) | Degradation condition | Precursor drug |
|---|---|---|---|---|---|
| DP 1 | $C_{21}H_{24}N_2O_8$ | 433.1606 | −0.1 | Alkaline hydrolysis | OTC |
| DP 2 | $C_{20}H_{15}NO_8$ | 398.0875 | −1.3 | Acid hydrolysis | OTC |
| DP 3 | $C_{22}H_{22}N_2O_8$ | 443.1459 | 2.2 | Acid hydrolysis | OTC |
| DP 4 | $C_{19}H_{15}NO_7$ | 368.0768 | −1.0 | Neutral hydrolysis and oxidation | OTC |
| DP 5 | $C_{12}H_{12}O_4$ | 221.0816 | −3.4 | Neutral, hydrolysis, oxidation, and photolysis | OTC |
| DP 6 | $C_{14}H_{11}NO_2$ | 226.0864 | 0.4 | Photolysis | DICLO |

*3.3.2. MS studies of degradation products*

The MS studies of DPs and their structural characterization were based on the proposed fragmentation mechanisms based on the observed from the precursor drugs. The high resolution MS spectra of isolated DP (DP 1–DP 6) are illustrated in Fig. 3 and the data are summarized on Table 2. DP 7 (Fig. 2f), probably formed from the DICLO's degradation by photolysis, was not identified possibly due to the formation of small amount, which complicated its isolation procedures and MS/MS acquisition.

DP 1 to DP 5, produced under different degradation conditions, had OTC as precursor drug, and DP 6 was obtained only from DICLO's photolysis. Moreover, DP 4 (*m/z* 368.0765 $[M + H]^+$) and DP 5 (*m/z* 221.0808 $[M + H]^+$) were formed in more than one degradation condition. Besides had been produced in the neutral hydrolysis, these DPs had also been originated from the oxidation and photolysis, which also contained water in the reaction medium added 0.03% hydrogen peroxide and UV/VIS light, respectively. In neutral and oxidative conditions, DP 4 and DP 5 were formed approximately the same proportion after 30 min of reaction; obeying different kinetic models (see Section 3.2.1). However, in the photolysis, performed at room temperature (25 ± 2 °C), only a minor proportion of DP 5 was detected after 16 h of reaction. Therefore, it is suggested that the water was the crucial stressor for the formation of DP 4 and DP 5 and the high temperature (60 °C) was the reaction catalyst.

Although PIRO had shown decay of content in the degradation conditions studied (Table 1), this drug was not the precursor of none degradation product identified. The non-mass balance can be justified by the potential loss of volatile DPs, retention of compounds in the column, or formation of early or extremely late eluents that could not be detected by the proposed HPLC-PDA method [2,28].

DP 2 (*m/z* 398.0875, $[M + H]^+$) and DP 3 (*m/z* 443.1459 $[M + H]^+$), derived from the acid hydrolysis of OTC, are predictable and described in the literature as terrinolidine (DP2) and α- or β-apo-oxytetracycline (DP 3), respectively [1]. Likewise, DP 6 (*m/z* 226.0864), from the DICLO's photodegradation, was reported in the literature [10], but its fragmentation pathway did not discuss. The chemical structures of DP 1 (*m/z* 433.1606), DP 4 (*m/z* 368.0768) and DP 5 (*m/z* 221.0816) were proposed for the first time in this research, as well as their fragmentation and formation pathways are discussed below.

*3.3.2.1. Identification of the degradation products (DPs).* The proposed chemical structures and fragmentation pathways of DP 1 to DP 6 are shown in Fig. 4.

Based on the described for OTC (Section 3.3.1), the suggested protonation site of DP 1 is also the C-6 hydroxyl group. Thus, from its ion at *m/z* 433.1606 $[M + H]^+$ was established the molecular formula $C_{21}H_{24}N_2O_8$, indicating the loss of CO in relation to OTC and a dehydration (neutral loss of $H_2O$, 18 Da), originating the fragment ion at *m/z* 415.1495, was observed in the MS/MS analyses.

The DP 2, DP 3 and DP 4 revealed the protonated ions at *m/z* 398.0875, 443.1459 and 368.0768 relative to the molecular formulae $C_{20}H_{15}NO_8$, $C_{22}H_{22}N_2O_8$ and $C_{19}H_{13}NO_7$, which confirmed the losses of $C_2H_9NO$, $H_2O$ and $C_3H_{11}NO$ from the OTC. From their protonated ions, neutral losses of ammonia ($NH_3$, 17 Da) were observed, yielding the fragment ions *m/z* 381.0622, 426.1161 and 351.0505 from DP 2, DP 3 and DP 4, respectively.

In addition, DP 5 was also yielded from OTC and revealed the protonated ion *m/z* 221.0816 ($C_{12}H_{12}O_4$), which produced fragment ions at *m/z* 203.0721 by the neutral loss of $H_2O$ (18 Da) and 175.0761 by neutral loss of CO (28 Da). This last fragment ion corresponds to the base peak displayed in the high resolution mass spectrum (Fig. 3).

The DP 6 is a product from DICLO, which showed the ions *m/z* 226.0864 $[M + H]^+$ and 248.0662 $[M + Na]^+$ and suggested the molecular formula $C_{14}H_{11}NO_2$. The fragment ion at *m/z* 180.0810 is yielded from a hydrogen migration in the ion *m/z* 226.0660 $[M + H]^+$ with subsequent neutral loss of formic acid (HCOOH, 46 Da) and the generation of four member ring from the nucleophilic attack of the nitrogen atom to the carbon.

*3.3.2.2. Proposed formation pathways of degradation products.* These DPs were obtained from alkaline (DP 1) and acid (DP 2 and DP 3) hydrolysis of OTC. This drug is highly susceptible to acid and alkaline hydrolysis due to the presence of hydroxyl (-OH) in C-6. Although some DPs from the alkaline hydrolysis of OTC are described in the literature (iso-oxytetracycline, terranoic acid, *N*-desmethyl and *N*-didesmethyl-oxytetracycline), the information provided by the MS analyses of DP1 did not coincide with these known DPs. Therefore, it is suggested that DP 1 was formed by the ring A contraction of OTC, in alkaline medium. In relation to the formation of DP 2 (terrinolidine) and DP 3 (α- or β-apo-oxytetracycline), it is believed that the –OH group at C-6 has favored the intramolecular cyclization reaction, resulting in the water elimination and the formation of the lactone group present in these both DPs. Under extremely acid conditions such as the one employed in this study (pH < 2), DP 3 (α- or β-APOTC) can degrade through an irreversible process, yielding terrinolidine (DP 2).

DP 4 and DP 5 had OTC as precursor drug and were formed under photolysis (only DP 5), neutral and oxidative conditions. It is presumed that these DPs are formed by oxidation reactions of the aliphatic tertiary amine and the alcoholic hydroxyls present in OTC. The proposed formation pathway of DP 4 is illustrated in Fig. S5 (Supplementary material), however the formation pathway of DP 5 is still unclear.

DP 6, originated from DICLO's photolysis, is chemically known as 9,9a-di-hydro-4-aH-carbazole-1-il acetic and it is formed due to the cleavage of C—Cl bond in the presence of UV light. As consequence, after hydrogen migration, occur the second dehalogenation and cyclization of the molecule (Fig. S6, Supplementary material).

*3.4. Method validation*

*3.4.1. Specificity*

The excipients did not show any eluted peak at the same retention time of the drugs analyzed. In addition, the peak purity values of drugs in the presence of all degradation products were higher than 990, indicating that homogeneous peaks were obtained and that the developed method was specific for the simultaneous or isolated determination of OTC, DICLO and PIRO.

*3.4.2. Linearity, LOD and LOQ*

The analytical curves were linear (r > 0.9996) in the concentration

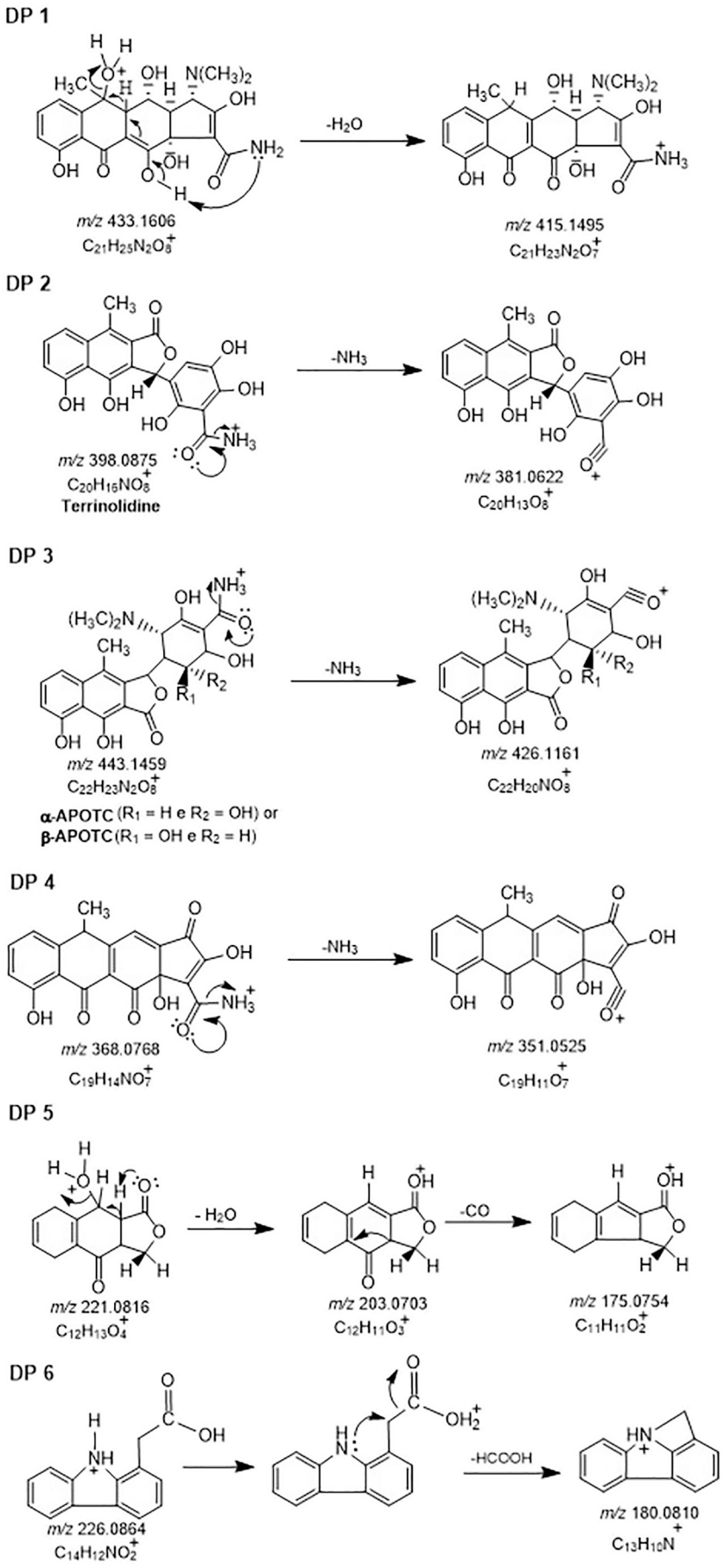


**Fig. 4.** Proposed chemical structures and fragmentation pathways of DP 1 to DP 6.

**Table 3**
Linearity, LOD and LOQ data of OTC, DICLO and PIRO.

| Data | OTC | DICLO | PIRO |
|---|---|---|---|
| Linear range ($\mu g\,mL^{-1}$) | 5.00–35.00 | 0.40–1.60 | 0.60–1.80 |
| Equation: y = ax + b | y = 0.8802x + 0.5687 | y = 0.8355x + 0.0175 | y = 0.9959x + 0.0102 |
| Slope (a) ± SD[a] | 0.8802 ± 0.0215 | 0.8350 ± 0.0164 | 0.9960 ± 0.0051 |
| Intercept (b) ± SD[a] | 0.5686 ± 0.1851 | 0.0185 ± 0.0108 | 0.00312 ± 0.0029 |
| Correlation coefficient (r) ± SD[a] | 0.9998 ± 0.00006 | 0.9998 ± 0.0001 | 0.9998 ± 0.0001 |
| LOD ($\mu g\,mL^{-1}$) | 0.694 | 0.043 | 0.042 |
| LOQ ($\mu g\,mL^{-1}$) | 2.103 | 0.129 | 0.126 |
| $C_{calculated}$ value[b] | 0.336 | 0.347 | 0.269 |
| Shapiro-Wilk p-value[c] | 0.081 | 0.457 | 0.357 |

[a] The values are discriminated as mean ± standard deviation (SD) of three analytical curves generated on three consecutive days (n = 3).
[b] Cochran's test (Y-axis homoscedasticity analysis with $C_{critical}$ = 0.561).
[c] Normality analysis of the residues.

range of 5.00–35.00 $\mu g\,mL^{-1}$ for OTC, 0.40–1.60 $\mu g\,mL^{-1}$ for DICLO and 0.60–1.80 $\mu g\,mL^{-1}$ for PIRO. The values of LOQ and LOD indicated that the developed HPLC-PDA have presented adequate sensitivity for the simultaneous or isolated determination of OTC, DICLO and PIRO. The results obtained for the Cochran's test allowed the application of the Ordinary Least Squares method and indicate that y-axis values (dependent variable) are homoscedastic, since the values of $C_{calculated} < C_{critical}$. The p-values of the linear regression, obtained by the ANOVA test of the analytical curves, were highly significant ($p < 0.0001$), which confirms the hypothesis of proportionality between the variables x (concentration) and y (signal). Moreover, all residues have had a normal distribution according to the results obtained in the Shapiro-Wilk test ($p > 0.05$) and the residues graphs showed values close to zero with a constant variance, indicating the absence of outliers. Table 3 summarizes the results of linearity, LOD and LOQ for each drug.

### 3.4.3. Precision

The RSD values obtained for repeatability were less than the recommended maximum limit of 1.0% for $n \geq 5$ [16]. The difference between variances obtained from ten determinations carried out on three consecutive days was shown to be non-significant ($p > 0.05$) by the ANOVA test. Therefore, as averages of the concentrations obtained on days 1, 2 and 3 did not differ significantly, it was confirmed the intermediate precision of the proposed method (Table S5, Supplementary material).

### 3.4.4. Accuracy

Percentages of recovery range for OTC, DICLO and PIRO were within the acceptable limits of 98–102% (RSD < 2.0%) for all three concentration levels analyzed, indicating that the developed method was accurate [16] (Table S6, Supplementary material).

### 3.4.5. Robustness

For the association of OTC and DICLO, the critical values ($\sqrt{2S}$) for OTC were 1.43 (level 1) and 0.78 (level −1) and for DICLO were 1.52 (level 1) and 1.27 (level −1). For the association of OTC and PIRO, the critical values ($\sqrt{2S}$) for variation levels 1 and −1 for OTC were, respectively, 1.74 and 1.82. For PIRO, the values were 1.82 (level 1) and 1.75 (level −1). Thus, no change in the parameters selected for the proposed methods resulted in absolute values of Effect above the critical values for each drug in both variation levels, demonstrating that the proposed method remained robust in face of the evaluated parameters (Fig. S7, Supplementary material).

## 3.5. Method applicability

### 3.5.1. Assay of commercial samples

The HPLC-PDA method is suitable for simultaneous and isolated determination of OTC, DICLO and PIRO from the veterinary and human pharmaceutical products (Table S7, Supplementary material). Moreover, none degradation product was identified by HPLC-PDA method in these commercial samples. Thus, all commercial samples analyzed presented contents of drugs within the limits recommended in their respective individual official monographs [29,30].

### 3.5.2. Evaluation of accelerated and long-term stability

After the accelerated and long-term studies, the color of commercial sample A, composed by OTC + DICLO association, was changed from yellowish brown to dark brown. However, the commercial sample B (OTC + PIRO) showed no change in color, keeping dark brown from the beginning to the end of the stability testing. In addition, the stability conditions did not cause significant changes in the pH values of the samples (pH 8–8.5).

The initial drug contents for each sample were found to within the recommended ranges. For the accelerated stability, OTC in the sample B presented the highest content decay percentage, i.e. approximately 36%, followed by PIRO (27.5%) and DICLO (20.5%). Nevertheless, in the sample A, the degradation of OTC was less expressive (18.2%) and it can be attributed to differences in the quality of the raw material, the adjuvants, in the production process and the storage conditions of the final products. Under long-term stability conditions, OTC was also the most degraded drug (degradation between 18 and 30%), while degradations of DICLO and PIRO were, respectively, 27% and 10% after 24 months of study. Despite the instability of the pharmaceutical formulations, no degradation products peaks were observed in their chromatograms (Table S8, Supplementary material).

The kinetic parameters of stability testing were calculated, and the drugs mostly were degraded under zero order kinetic model. Only the decomposition of OTC in the sample B, submitted to the long-term stability study, was according to the second-order kinetic model. Still in this sample, OTC showed the lowest half-life ($t_{50\%}$: 9.8 months) during the accelerated stability. The shelf-lifes ($t_{90\%}$) of samples were within the range of the labels claims (Table S9, Supplementary material).

## 4. Conclusion

Degradation behavior of three drugs (OTC, DICLO and PIRO) was investigated under different conditions of hydrolysis and photolysis as per ICH guidelines. The nonsteroidal anti-inflammatory drugs (DICLO and PIRO) were less susceptible to acid, alkaline, neutral and oxidative reactions than OTC. However, DICLO showed extensive photo degradation. A total of six degradation products were identified by LC-MS, being five providing from photolysis, oxidation, acid, alkaline and neutral hydrolysis of OTC (DP 1 to DP 5) and one from DICLO's photolysis (DP 6). Moreover, the chemical structures of DP 1, DP 4 and DP 5 are reported here for the first time. The developed and validated HPLC-PDA method was suitable for simultaneous and isolate determination of the drugs in the presence of their degradation product and for evaluation of accelerated and long-term stability. Thus, it can be used in the

quality control routine analysis of pharmaceuticals as a stability indicating method.

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## Appendix A. Supplementary data

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## References

[1] R.A. Sversut, A.A. Silva, T.F.M. Cardoso, N.M. Kassab, M.S. Amaral, H.R.N. Salgado, A critical review of properties and analytical methods for the determination of oxytetracycline in biological and pharmaceutical matrices, Crit. Rev. Anal. Chem. 57 (2017) 154–171.
[2] S. Singh, M. Junwal, G. Modhe, H. Tiwari, M. Kurmi, N. Parashar, P. Sidduri, Forced degradation studies to assess the stability of drugs and products, TrAC Trends Anal. Chem. 49 (2013) 71–88.
[3] D.A. Gawad, T.S. Belal, HPLC-DAD stability indicating determination of pentoxyverine citrate. Application to degradation kinetics and assay of syrup dosage form, Arab. J. Chem. 10 (2017) S2908–S28918.
[4] D. Swain, G. Samanthula, Study on the forced degradation behaviour of ledipasvir: identification of major degradation products using LC-QTOF-MS/MS and NMR, J. Pharm. Biomed. Anal. 138 (2017) 29–42.
[5] V.V.S.R.N.A.K. Mutha, S. Guduru, M. Kaliyaperumal, C.S. Rumalla, S.R. Maddi, R.B. Korupolu, S.B. Gajbhiye, Degradation study of irbesartan: isolation and structural elucidation of novel degradants, J. Pharm. Biomed. Anal. 157 (2018) 180–188.
[6] B.B. Chavan, V. Sawant, R.M. Borkar, S. Ragampeta, M.V.N.K. Talluri, Isolation and structural characterization of degradation products of afatinib dimaleate by LC-Q-TOF/MS/MS and NMR: cytotoxicity evaluation of afatinib and isolated degradation products, J. Pharm. Biomed. Anal. 166 (2019) 139–146.
[7] S.M. Baira, R. Srinivas, M.V.N.K. Talluri, Identification and structural characterization of hydrolytic degradation products of alvimopan by LC/QTOF/MS/MS and NMR studies, J. Pharm. Biomed. Anal. 165 (2019) 399–409.
[8] M.H. Abdel-Hay, M.A.A. Ragab, H.M. Ahmed, S.M. Mohyeldin, The use of Arrhenius kinetics to evaluate different hydrolytic stability of amiloride hydrochloride and cyclopenthiazide using chromatographic methods, Microchem. J. 147 (2019) 682–690.
[9] Y. Liu, X. He, Y. Fu, D.D. Dionysiou, Degradation kinetics and mechanism of oxytetracycline by hydroxyl radical-based advanced oxidation processes, Chem. Eng. J. 284 (2016) 1317–1327.
[10] O.S. Keen, E.M. Thurman, I. Ferrer, A.D. Dotson, K.G. Linden, Dimer formation during UV photolysis of diclofenac, Chemosphere 93 (2013) 1948–1956.
[11] D.T. Modhave, T. Handa, R.P. Shah, S. Singh, Successful characterization of degradation products of drugs using LC-MS tools: application to piroxicam and meloxicam, Anal. Methods 3 (2013) 2864–2872.
[12] R.A. Sversut, J.C. Vieira, A.M. Rosa, M.S. Amaral, N.M. Kassab, H.R.N. Salgado, Validated spectrophotometric methods for simultaneous determination of oxytetracycline associated with diclofenac sodium or with piroxicam in veterinary pharmaceutical dosage form, Arab. J. Chem. (2018), https://doi.org/10.1016/j.arabjc.2018.09.007.
[13] ICH, Stability testing of new drug substances and products Q1A (R2), International Conference on Harmonization, IFPMA, Geneva, 2003.
[14] FDA, Validation of Chromatographic Methods, Food and Drug Administration, Center of Drug Evaluation and Research Washington, 2004.
[15] ICH, Validation of analytical procedures: text and methodology, International Conference on Harmonization, IFPMA, Geneva, 2005.
[16] AOAC, Official Methods of Analysis, Association of Official Analytical Chemists, Washington, 2005.
[17] Brazil, Validação de métodos analíticos, Resolução n° 166 de julho de 2017, Agência Nacional de Vigilância Sanitária, Brasília, 2017.
[18] WHO, Technical Report Series 953, Annex 2, Stability Testing of Active Pharmaceutical Ingredients and Finished Pharmaceutical Products, World Health Organization, Geneva, 2009.
[19] R.L. Plackett, J.P. Burman, The design of optimum multifactorial experiments, Biometrica 33 (1944) 305–325.
[20] J.J.B. Nevado, M.J.V. Llerena, C.G. Cabanillas, V.R. Robledo, S. Buitrago, Sensitive capillary GC-MS-SIM determination of selective serotonin reuptake inhibitors: reliability evaluation by validation and robustness study, J. Sep. Sci. 29 (2006) 103–113.
[21] Brazil, Guia para Realização de Estudos de Estabilidade, Resolução n° 1, de 29 de julho de 2005, Agência Nacional de Vigilância Sanitária, Brasília, 2005.
[22] G. Kahsay, F. Shraim, P. Villatt, J. Rotger, C. Cassus-coussère, A. Van Schepdael, J. Hoogmartens, E. Adams, Development and validation of a reversed phase liquid chromatographic method for analysis of oxytetracycline and related impurities, J. Pharm. Biomed. Anal. 75 (2013) 199–206.
[23] L. Lachman, P. Deluca, M. Akers, Testes de estabilidade e fundamentos da cinética química, in: L. Lachman, H. Lieberman, J. Kanig (Eds.), Teoria e prática na Indústria Farmacêutica, Fundação Calouste Gulbenkian, Lisboa, 2001(pp.1277-1255).
[24] R. Xuan, L. Arisi, Q. Wang, S.R. Yates, K.C. Biswas, Hydrolysis and photolysis of oxytetracycline in aqueous solution, J. Environ. Sci. Health B 45 (2010) 43–81.
[25] A.M. Kamel, H.G. Fouda, P.R. Brown, B. Munsun, Mass spectral characterization of tetracyclines by electrospray ionization, H/D exchange, and multiple stage mass spectrometry, J. Am. Soc. Mass Spectrom. 13 (2002) 543–557.
[26] M.J. Galmier, B. Bouchon, J.C. Madelmont, F. Mercier, F. Pilotaz, C. Lartique, Identification of degradation products of diclofenac by electrospray ion trap mass spectrometry, J. Pharm. Biomed. Anal. 38 (2005) 790–796.
[27] V.I. Minkin, Glossary of terms used in theoretical organic chemistry (IUPAC Recommendations 1999), Pure Appl. Chem. 71 (1999) 1919–1981.
[28] R. Maheswaran, Scientific consideration of forced degradation studies in ANDA submissions, J. Validation Technol. 18 (2012) 92–96.
[29] USP 38, The National Formulary (NF 33), the United States Pharmacopeia, 38 ed., United States Pharmacopoeia Convention, Rockville, 2015.
[30] BP, British Pharmacopoeia, 1 The Stationary Office, 2013.