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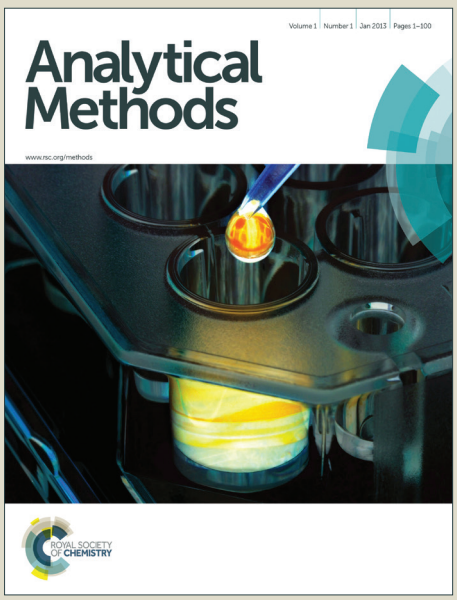

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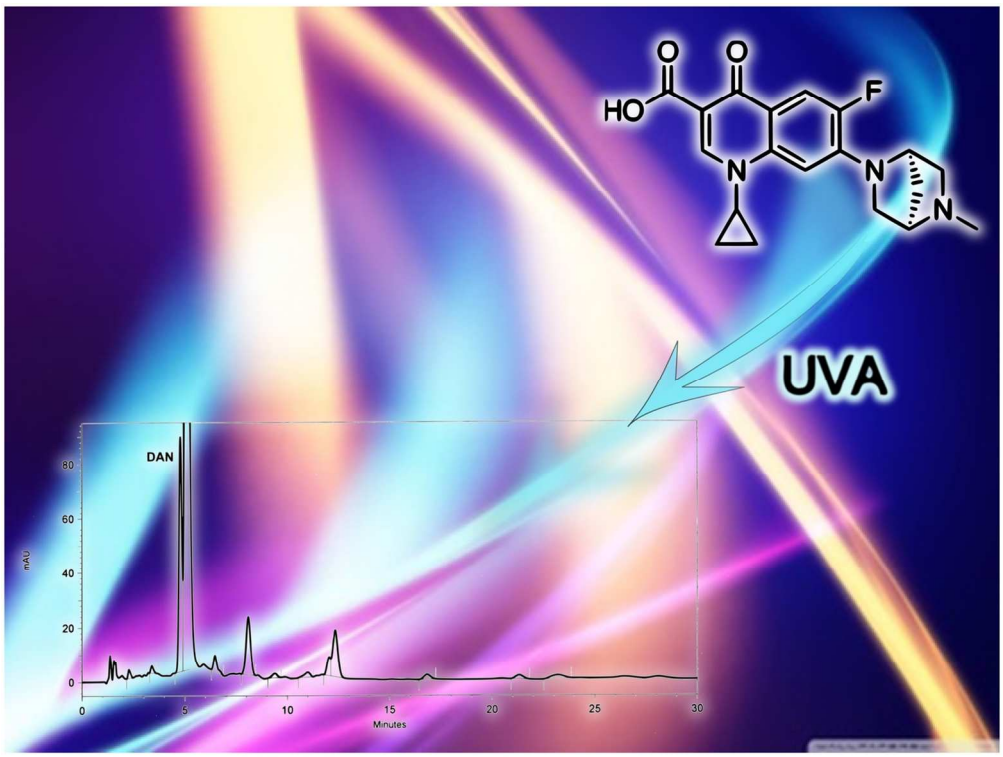

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Determination of danofloxacin and its photodegradation products by HPLC –DAD. Kinetic evaluation of degradation process and identification of photoproduct by mass spectrometry.

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Abstract

HPLC method was developed for determination of danofloxacin (DAN) in the presence of its photodegradation products. Chromatography was performed on a Gemini-NX C18 110A, 150mmx4.60mm, 3 μm particle size column with 0.025 M phosphate buffer (pH=5.00) : acetonitrile : methanol (95 : 10 : 30 v/v/v) as mobile phase at a flow rate of 1.2 mL min^{-1}. UV detection was performed at 280 nm. The column was thermostated at 25°C. The elaborated method meets the acceptance criteria for specificity, linearity, sensitivity, accuracy, and precision. The linear regression analysis for the calibration curve showed a good linear correlation over the concentration range 0.20 – 0.80 mg mL^{-1}, with determination coefficient, R^2, exceeding 0.9966. The method was shown to have good precision and intermediate precision, as reflected by the relative standard deviation values, lower than 2.21% and characterized by a recovery rate at three concentration levels from 98.0% to 101.70%. The limits of detection and quantification were respectively 0.0055 and 0.0167 mg mL^{-1}. The photodegradation process of DAN followed kinetics of the first order reaction for the substrate. Ten products of photodegradation were identified by UPLC/MS/MS.

Keywords: danofloxacin, determination, HPLC-DAD, UPLC/MS/MS, photodegradation, kinetic evaluation

INTRODUCTION

Danofloxacin (DAN) 1-cyclopropyl-6-fluoro-1,4 dihydro-7-[(1s,4s)-5-methyl-2,5-diazabicyclo[2.2.1]hept-2-yl]-4-oxo-3 quinolinecarboxylic acid belongs to fluoroquinolone antibiotics. Fluoroquinolones are an important group of synthetic antibacterial agents exhibiting high activity against a broad spectrum of Gram-negative and Gram-positive bacteria and a large number of licensed products containing these antibiotics are available for use in animal husbandry. Fluoroquinolones are used in the treatment of systemic infections including urinary tract, respiratory, gastro-intestinal and skin infections. In the veterinary field, they are used not only for the treatment of diseases but also as feed additives to increase the animal mass.[1]

A drawback of all fluoroquinolones is their photoreactivity. The existence of photoproducts can induce side effects and toxicity as well as loss of activity expected for the treatment. Exposure of a drug to irradiation can influence the stability of the formulation, leading to changes in the physicochemical properties of the product.[2, 3] Therefore stability testing of the drug substances and final preparation is very important and recommended by ICH guidelines.[4]

In the last years antibiotics including the fluoroquinolones have been considered as emerging pollutants and are thought to be potential threats to environmental ecosystems and human health and safety. The presence of these compounds in the environment can constitute a potential risk for aquatic and terrestrial organisms. Although present at vestigial levels, antibiotics may cause resistance in bacterial populations, making them, in the near future, ineffective in the treatment of several diseases. [5-8] The relatively high chemical stability of fluoroquinolones in solution makes them persistent pollutants.[9-11] Whereas, their photosensitivity causes that photodegradation process is an important degradation pathway for this group of antibiotics. [12] However, little is known about fluoroquinolones' photodegradation products that are formed under environmental conditions. It has not been

ascertained whether the photoproducts are more or less susceptible to biodegradation than the starting drug and whether these are toxic or promote bacterial resistance, especially when the active part of the parent molecule remains unaltered. Therefore there is a necessity to study the photodegradation and to develop new methods of separation and determination of fluoroquinolones as well as their photoproducts in solutions what leads to numerous publications in this field.[13-17]

Analysis of DAN photodegradation in solutions was investigated in several papers. The existing research concerning the photostability of DAN in solutions was performed using different irradiation conditions (usually sunlight or fluorescent light) and exposure time. Photodegradation of Dan was also tested in the presence of a suspended of TiO_2. [19] The methods used to analyze photodegradation process were HPLC with spectrophotometric in UV or fluorescence detection (FD) and LC-MS/MS and allowed for the determination of different number of degradation products (from 2 to 6). [18-21]

The purpose of this work was to develop a HPLC-DAD method for the determination of DAN and its photodegradation products. Additionally, the aim of this study was the kinetic evaluation of DAN photodegradation during exposure to UVA and identification of photoproducts by UPLC-MS/MS. The impact of iron and copper ions on the photodegradation process of DAN was also examined.

Iron was chosen for the research because it is one of the most important micronutrients for all organisms. It is required for enzymatic reactions, in particular for those involving electron transport. It also participates in the transport and storage of oxygen in tissues. Iron is present in hem-containing proteins (hemoproteins) such as: hemoglobin, myoglobin, cytochromes, cytochrome oxidases, catalazes and peroxidases. It is also a constituent of proteins which do not contain hem molecule: flavoproteins and mitochondrial aconitase. In addition, iron takes part in many metabolic processes, among others in synthesis and

catabolism of some hormones, synthesis of high-energy compounds and collagen, detoxification processes and immune reactions. [22]

Copper is a component of various enzymes involved in the oxidation-reduction processes, including cytochrome oxidase, lysyl oxidase, ascorbate oxidase, superoxide dismutase and ceruloplasmin. Copper is necessary for normal metabolism of connective tissue. It influences on the metabolism of lipids and cholesterol, and the properties of the myelin sheath of nerve fibers. Between the copper and the iron is synergism, which is beneficial particularly in the synthesis of hemoglobin. [23]

Due to their high importance for human health, deficiency of these micronutrients is combated by food fortification or pharmaceutical supplementation. The ubiquity of copper and iron ions in the human body, food and the environment creates a potential opportunity for interaction in the case of treatment with fluoroquinolones.

EXPERIMENTAL

Materials and Methods

Reagents

Methanol, acetonitrile, 85% orthophosphoric acid, glacial acetic acid of HPLC grade were purchased from Merck (Germany). Dipotassium phosphate of analytical grade was purchased from Sigma-Aldrich (Germany). HPLC grade water was obtained from HLP 5 (HYDROLAB Poland) apparatus and was filtered through 0.2 μm filter before use. Analytical grade copper (II) sulfate pentahydrate and iron (III) sulfate were manufactured by POCH (Gliwice, Poland).

Standard solutions and substance

Danofloxacin (DAN) - assay ≥98.0% (HPLC), cat no. 33700 Sigma-Aldrich (Germany). Solutions of DAN in methanol containing 1 mL of glacial acetic acid were prepared at

concentrations from 1.00 mg mL^{-1} to 2.50 mg mL^{-1}. On the basis of these solutions, by dilution with methanol, solutions with concentrations from 0.0084 to 0.75 mg mL^{-1} were prepared.

Metal salt solutions

To prepare the solution of metal ions at 0.1 M concentration were used: (1.2488 g) $CuSO_4 \cdot 5H_2O$ and (0.9988 g) $Fe_2(SO_4)_3$. Weighed portion of salts were transferred to 50 mL flask and filled with water to required volume. Directly for testing, the obtained solution was diluted with the same solvent to a final concentration of 0.025 M and 0.0025 M.

Preparation of samples for tests in solutions

Three milliliters of 0.50 mg mL^{-1} methanolic solution of DAN were measured off in quartz cuvettes of 1 cm diameter, and 0.25 mL of water or salt solution with different metal ions concentration (0.025 M or 0.0025 M) were added. The cuvettes were closed with a plastic nut. The solutions were exposed to UVA radiation for 3, 6, 24, 27, 48, 72 h and after that 5 μL were injected into a HPLC column. For each sample a dark control sample was prepared, which was protected with aluminum foil before irradiation.

Irradiation conditions

Irradiation was conducted in a climatic chamber KBF-ICH 240 APT.lineTM; (Binder GmbH, Tuttlingen, Germany) at 20°C and 60% humidity using UVA radiation (320–400 nm) with maximum emission at 365 nm. The intensity of radiation was determined by radiometer type VLX-3W, Vilber Lourmat, with a CX-365 sensor, to be each time of 0.25 mW/m^2. The distance between samples and radiation source was 13 cm.

HPLC conditions and equipments

The liquid chromatography system HIACHI High-Technologies Corporation (Tokio, Japonia) equipped with a solvent delivery pomp (L-2130), degasser, an autosampler (L-2200), a photodiode array detector (L-2455) and a column oven (L-2350) was used. The chromatographic analysis of DAN and its photodegradation products was performed on Gemini-NX 3u C18 110A column, Phenomenex (Torrance, USA) (150 x 4.6 mm, 3 μm particle size) coupled with guard-column. The column temperature was 25°C. The chromatographic separation was achieved using a isocratic elution. The mobile phase was 0.025 M phosphate buffer (pH=5.00) : acetonitrile : methanol (95 : 10 : 30 v/v/v). The flow rate of the mobile phase was 1.2 ml/min and injection volume was 5μL. Analysis time was 30 minutes. The samples were monitored at 280 nm.

UPLC/MS/MS analysis

The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C18 column; 2.1 × 100 mm, and 1.7 μm particle size. The column was maintained at 40°C, and eluted under gradient conditions from 95% to 0% of eluent A over 10 min, at a flow rate of 0.3 mL min^{-1}. Eluent A: 0.1% (v/v) formic acid in water; eluent B: 0.1% (v/v) formic acid in acetonitrile. Chromatograms were made using Waters eλ PDA detector. Spectra were analyzed in 200 – 700 nm range with 1.2nm resolution and sampling rate 20 points/s. MS detection settings of Waters TQD mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 350°C, desolvation gas flow rate 600 L h^{-1}, cone gas flow 100 L h^{-1}, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 m/z in time 0.5 s intervals. Collision activated dissociations (CAD)

analyses were carried out with the energy of 30 V, and all the fragmentations were observed in the source. Consequently, the ion spectra were obtained by scanning from 50 to 1000 m/z range. Data acquisition software was MassLynx V 4.1 (Waters).

Method Validation

The HPLC method was validated for specificity, linearity, limit of detection, limit of determination, accuracy, precision and robustness according to ICH guidelines. [24]

Specificity

Specificity of the method was assessed by comparing chromatograms of the pure standard substance, chromatograms of DAN solution after UVA exposure for 48 hours, blank chromatogram and chromatogram obtained for the mixture of solvents used to dissolve DAN. In all obtained chromatograms, the retention time (t_R) and resolution factor (R_s) values of the analyzed substances, peak areas, shape and purity of the peaks were taken into account.

System suitability

The system suitability parameters were defined with respect to tailing factor and resolution of the DAN and degradation product peaks using solution of DAN after exposure for 48 hours.

Linearity

The calibration plot for the method was constructed by analysis of five solutions containing different concentrations of DAN in the range 0.25 – 0.75 mg mL^{-1}. Solutions were injected into a column in the amounts of 5 µL. Further analytical procedure was as described in the *HPLC Conditions*. Linearity was assessed in duplicate on the basis of the relationship between peak areas and concentration, in milligrams per milliliter. Linear and quadratic

model were fitted to the calibration data. To evaluate the goodness of the fit, residuals were found and determination coefficient (R^2) was computed. Next, to determine whether the residuals have normal distribution, the Shapiro-Wilk statistical test was used. Statistical data were calculated and plotted using Statistica v. 10 and R-project open-source software. [25,26]

Limit of detection (LOD) and limit of quantification (LOQ)

For the determination of LOD and LOQ, calibration curve of low concentrations of DAN in the range 0.0084 – 0.0200 mg mL^{-1} was constructed. LOD and LOQ were calculated on the basis of the slope (a) of the calibration line and the standard error of the estimate (Se), using formulas LOD =3.3 Se/a and LOQ=10 Se/a.

Accuracy

The accuracy of the method was evaluated in triplicate (n=3) at the three concentrations of 0.40, 0.50 and 0.60 µg mL^{-1} (80%, 100% and 120%) of DAN solutions, and the recovery was calculated for each added (externally spiked) concentration.

Precision

The repeatability of the method was determined by analysis of five replicates of standard solutions from individual weightings. The study was done for concentration level of DAN: 0.50 mg mL^{-1}. The intermediate precision was obtained for the same concentration of freshly prepared solutions by different analysts who performed the analysis over a period of one week. The results were expressed as the relative standard deviation (RSD).

Robustness

Under conditions of the developed method, comparison of results obtained after changing of the pH of phosphate buffer in the range of 4.80 to 5.20 was done. The impact of small changes in the content of phosphate buffer in the mobile phase (± 5% from the initial composition) on the separation of DAN and its degradation product were also checked.

RESULT AND DISCUSSION

HPLC-DAD method was chosen to evaluate the photodegradation process of DAN without metal ions and in the presence of Cu (II) and Fe (III) ions. Test procedure was started with the development of the chromatographic method enabling the determination of DAN next to its photodegradation products. Satisfactory results have been achieved using a mobile phase composed of phosphate buffer pH = 5.00, acetonitrile and methanol in the proportions 95: 10: 30 (v / v / v) at a flow rate of 1.2 ml / minute and with the analysis time of 30 minutes. Retention time for DAN in the developed conditions was ~ 4.76 minutes. Detection of the peak area for DAN and its degradation products was carried out at 280 nm. The used type of detection was different from that used in two earlier publications devoted to photodegradation of DAN.[18,19] Moreover, the developed HPLC-DAD method allows to separate DAN and its eighteen photodegradation products. The amount of separated photodegradation products is the biggest comparing with previously used methods. [18-21]

Method Validation

For estimation of reliability of developed method according to ICH recommendations following parameters were determined, specificity, linearity, limits of detection and quantitation, recovery and robustness. [38]

The developed HPLC method was specific to DAN and guaranteed obtaining well shaped peaks both for active substance and coexisting photodegradation products. Peak of

DAN was well resolved from photoproducts in chromatograms and no interference that could have an influence on the obtained results was possible.

Regression analysis results obtained for examined fluoroquinolone are presented in Table 1. The correlation coefficients and determination coefficients (R^2) obtained for linear model for all examined fluoroquinolones were greater than 0.99. The y-intercept of the linear equation for DAN were statistically insignificant. The distribution of the residuals can well be approximated with a normal distribution as it is shown by p-value ($p>0.05$) of the Shapiro–Wilk normality test. Based on regression analysis, it was assumed that the calibration data fitted well to linear model. Linearity range, was observed in the wide concentration range 0.25 – 0.75 mg mL^{-1} for DAN.

Sensitivity of the method was good. The LOD and LOQ values were found to be 0.0055 mg mL^{-1} and 0.0167 mg mL^{-1}, respectively. Good precision and intermediate precision with% RSD less than 2.3% was observed. Recovery rate at three concentration levels was from 98.0% to 101.70%. Detailed results were presented in Table 1.

Variation of the phosphate buffer content in the mobile phase resulted in more than 10% change in retention times for DAN and its degradation products and caused the deterioration of peak symmetry. In contrast, changes in pH of the buffer had no effect on the retention parameters.

Table 1

Photodegradation of danofloxacin in solutions

In the next phase of the study the effect of UVA radiation on the stability of DAN in solutions was evaluated. It was found that under the applied conditions DAN undergoes photodegradation. Degradation of DAN increased with the increasing exposure time and reached 57.12% after 24 h, 90.76% after 48 hours and 100% after 72 h; also increase in the

number and concentration of photodegradation products was observed (Fig. 1A, B and C). No photodegradation products were observed in the dark control (Fig. 1D) suggesting that photodegradation of DAN occurred under the influence of UVA radiation.

Figure 1.

Then the impact of Cu (II) ions on the photodegradation process of DAN was assessed. During the analysis it was found that Cu (II) ions, depending on concentration, slow photodegradation process of the tested fluoroquinolone. After 72 h in a sample where 0.0025M of Cu (II) ions was added photodegradation of DAN was 80.27%, while in the sample with the addition of 0.025M Cu (II), only 5.58%. In the dark control samples of DAN containing 0.025M of Cu (II) ions small degradation of DAN has been observed, after 72 h was 2.33%, but two degradation products formed had different retention times than those registered in the tested samples. The degradation process was probably caused by the oxidation of the tested fluoroquinolone in the presence of Cu (II), which could be further encouraged by the slightly acidic reaction medium derived from acetic acid used for the preparation of the test solution. In the dark control samples of DAN containing 0.0025M of Cu (II) ions there was no degradation of the tested compound.

Then, the effect of the presence of Fe (III) ions on the photodegradation process of DAN under the influence of UVA was analyzed. As in the case of Cu (II) ions, the addition of Fe (III) ions slowed down the photodegradation process of the tested fluoroquinolone. In a sample irradiated for 72 hours with the addition of 0.0025M Fe (III) ions, the degradation of DAN was 81.99%, whereas in the sample with the addition of 0.025M of Fe (III) ions 31.14%. In the dark control samples, no degradation of the tested substance was observed.

In order to explain the inhibitory influence of metal ions on the photodegradation process of DAN absorption spectra were recorded in the range 350 - 550 nm for solutions of DAN without metal ions and with metal ions tested. It was found that in the case of solutions

containing Cu (II) or Fe (III) ions absorbance was significantly higher as compared to solutions of DAN without metal ions. The increase of absorbance may be associated with the d-d electron transition in the visible and near ultraviolet range characteristic for metal ions such as Cu(II) and Fe(III) forming complex compound. Furthermore, in the case of solutions containing metal ions at a concentration of 0.025 M additional absorbance maximum occurs in the recorded spectra that is shifted to less energetic longer wavelengths (Figure 2). It seems that the inhibitory effect of metal ions on the photodegradation process of DAN in solutions may be associated with their competitive absorption of actinic photons emitted by the lamp. These conclusions are confirmed by photodegradation studies of gatifloxacin and sarafloxacin in the presence of Fe (III) ions conducted by Ge et al. [20]

Figure 2

Kinetic evaluation

Kinetic evaluation of photodegradation process of DAN was performed for all tested samples. Using the plot of the natural logarithm of the concentration of DAN versus photodegradation time it was found that photodegradation reaction in all analyzed samples follows the kinetics of the first order reaction (Fig. 3). Degradation rate constant k for solutions of DAN without metal ions is higher compared to the degradation rate constants obtained for the solutions of DAN with metal ions. Calculated values of $t_{0.1}$ and $t_{0.5}$ indicate that the degradation process of DAN is substantially slower in the presence of Cu (II) and Fe (III) ions at a concentration of 0.025M and moderately slows down in the case of Cu (II) and Fe (III) ions at a concentration of 0.0025M (Table 2).

Figure 3

Table 2

Identification of photodegradation products.

The elucidation of structures of photodegradation products of DAN was performed on a basis of UPLC/MS/MS analysis. The proposed structures of photodegradation products are shown in Table 3. On a basis of the proposed structures of the observed photodegradation products it seems that the photodegradation may proceed on three different paths.

The main possible path of photodegradation seems to start with demethylation of nitrogen atom in 2,5-diazabicyclo[2.2.1]heptane substituent, yielding the most abundant product **DP-3**. Subsequent degradation may occur by hydroxylation of the substituent in position 7 of quinolone and its further degradation, yielding products **DP-8** (from **DP-3**), **DP-2** (from **DP-8**), **DP-13** (from **DP-2**), **DP-17** (from **DP-4**), hydroxylation of position 8 of quinolone, as it is observed for products **DP-4** (from **DP-8**), **DP-9** (from **DP-2**), substitution of fluorine in position 6 of quinolone with hydroxyl group, yielding product **DP-5** (from **DP-9**), **DP-11** (from **DP-3**), **DP-12** (from **DP-9**), and finally oxidation of amine moiety in position 7 of quinolone and subsequent substitution with hydroxyl group, leading to **DP-10** (from **DP-12** and **DP-13**), and hydroxylation of position 5 of quinolone leading to final product of photodegradation **DP-18**. It seems, that photodegradation of DAN by dealkylation of nitrogen atom in position 1 of quinolone is not preferred, since this process was observed only for **DP-5**, which is one of the minor products.

The other two proposed paths start with oxidation of 2,5-diazabicyclo[2.2.1]heptane substituent (product **DP-6**) or substitution of fluorine in position 6 of quinolone with hydroxyl group (product **DP-16**). These two paths seems to be much less preferable, because concentrations of **DP-6** and **DP-16** are over 40 fold lower than **DP-3**. Further degradation of these products involves similar processes as described above and leads to products **DP-7**, **DP-14** and **DP-15**, in case of **DP-6**, and **DP-11** in case of **DP-16**. Subsequent degradation of **DP-14** leads to **DP-9**, **DP-15** to **DP-2** and **DP-11** to **DP-12**. Further degradation proceeds as described for the first path.

The quantity of photodegradation products found is the largest compared with previously published papers. [12,13,34,35] The proposed chemical structures of photoproducts of DAN shown in Table 3 are new compared to those reported in earlier publications, with exception of product DP-1, DP-3, DP-13, DP-16.

Table 3

CONCLUSIONS

The elaborated method complies with the acceptance criteria for methods which can be useful for the determination of DAN in the presence of its photodegradation products. The method was completely validated showing satisfactory data for all the parameters tested. Executed studies have shown that photodegradation process of DAN followed kinetics of the first order reaction for the substrate. Calculated kinetic parameters such as rate constants k and the times $t_{0.1}$ and $t_{0.5}$ confirm higher stability of DAN solutions with copper and iron ions than in their absence. It was found that the inhibitory interaction of the tested ions on the photodegradation process of DAN is probably due to the fact that they act as radiation filters.

The studies performed by UPLC/MS/MS allowed determining the probable chemical structure of eighteen photodegradation products of DAN, which can be separated and determined using developed HPLC-DAD method.

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Figure legends

Figure 1. Chromatogram of separation of DAN and its photodegradation products after exposed to UVA radiation for: A- 24 h, B- 48h, C-72h, D- 72h dark control .

Figure 2. Absorption spectra of solutions of DAN before UVA irradiation: 1- DAN without metal ions, 2 - DAN with 0.025 M Fe(III), 3- DAN with 0.025 M Cu (II), 4- DAN with 0.0025 M Fe (III), 5- DAN with 0.0025 M Cu(II).

Figure 3. The ln c = f(t) graph of photodegradation of DAN.

Table 1. Validation of the method.

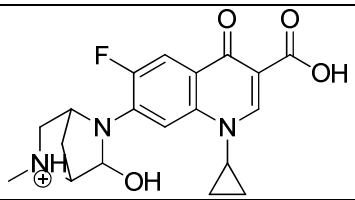
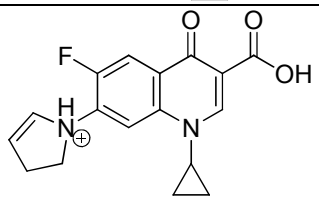
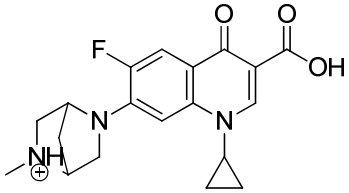
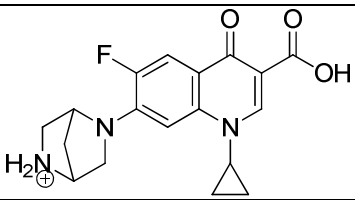
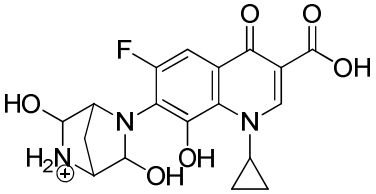
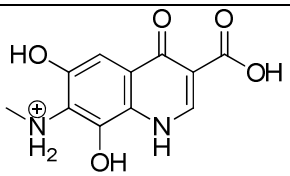
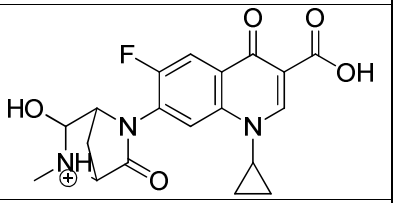
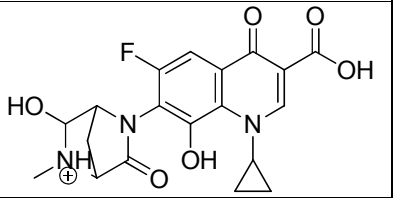
Parameter		DAN
t_R (min)[a]		4.76±0.024
LOD (mg mL^{-1})		0.0055
LOQ (mg mL^{-1})		0.0167
Linear range (mg mL^{-1})		0.25–0.75
Regression equation (y):		
Slope ($a \pm S_a$)		133073636 ± 2609370
Intercept ($b \pm S_b$)		2627673 ± 1383827
$t = b/S_b$		$1.90 < t_{\alpha,f}$ statistically insignificant
Normality of residuals[c] (Shapiro-Wilk test)		0.8968 (p=0.2021)
Correlation coefficient		0.9985
R^2 value		0.9966
Recovery	Level 80%	98.00%
	Level 100%	100.83%
	Level 120%	101.70%
Precision (% RSD)		1.79%
Intermediate precision (% RSD)		2.21%

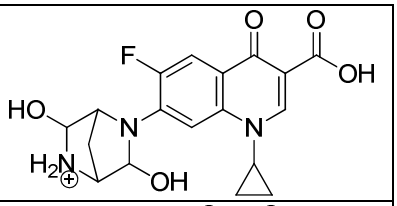
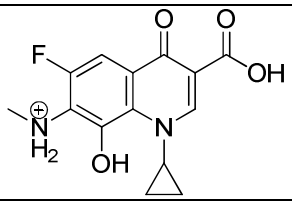
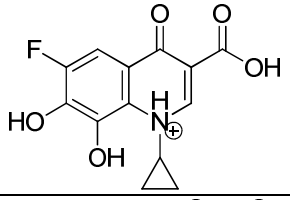
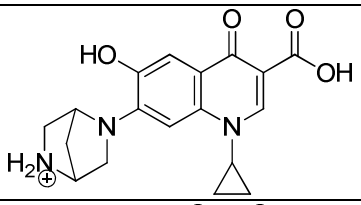
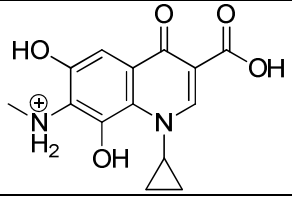
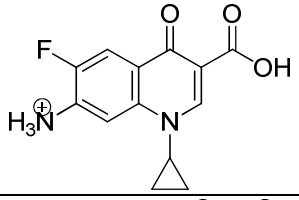
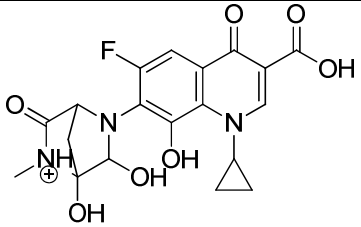
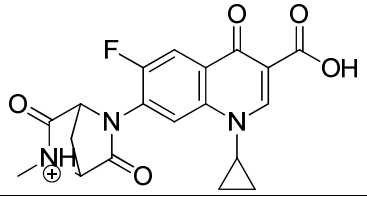
[a]Mean ± SD (n = 6).
[b]Resolutions were calculated between two adjacent peaks
Regression equation y=ac+ b; c - concentration of solution; y- peak area; S_a – standard deviation of slope; S_b - standard deviation of intercept, t- calculated value of Student's t-test, $t_{\alpha,f}$=2.306 critical value of Student's t-test for degrees of freedom f=8 and significance level α=0.05;
[c] normal distribution of residuals if p > 0.05

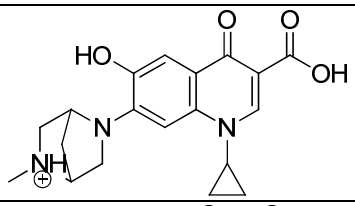
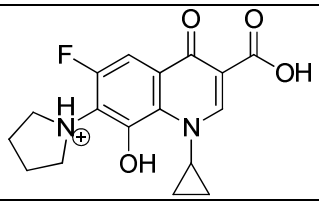
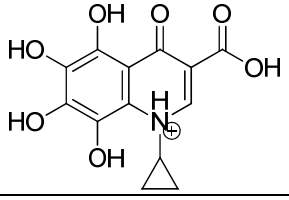
Table 2. Kinetic parameters of DAN photodegradation in solutions, with and without the presence of metal ions.

Component	Rate constant k [min^{-1}]	$t_{0.1}$ [min]	$t_{0.5}$ [min]	Correlation coefficient r
Without metal ions	4.82×10^{-2}	2.18	14.38	0.9839
Cu(II) 0.025M	8.00×10^{-4}	131.62	866.25	0.9576
Cu(II) 0.0025M	2.20×10^{-2}	4.79	31.50	0.9889
Fe(III) 0.025M	4.50×10^{-3}	23.40	154.00	0.8851
Fe(III)0.0025M	2.30×10^{-2}	4.58	30.13	0.9885

Table 3. Products of photodegradation of DAN

Product Id	t_R[a]	$[M+H]^+$	Proposed structure
DP-1	1.83	374.15	
DP-2	2.45	315.11	
DAN	2.60	358.16	
DP-3	2.68	344.14	
DP-4	2.78	392.12	
DP-5	2.82	251.07	
DP-6	2.97	388.13	
DP-7	3.01	404.12	

DP-8	3.18	376.13	
DP-9	3.29	293.09	
DP-10	3.39	280.06	
DP-11	3.46	342.14	
DP-12	3.49	291.10	
DP-13	3.64	263.08	
DP-14	3.83	420.12	
DP-15	3.97	386.11	

DP-16	4.10	356.16	
DP-17	4.30	333.12	
DP-18	4.53	294.06	

[a]t_R – retention times obtained in the UPLC-MS.

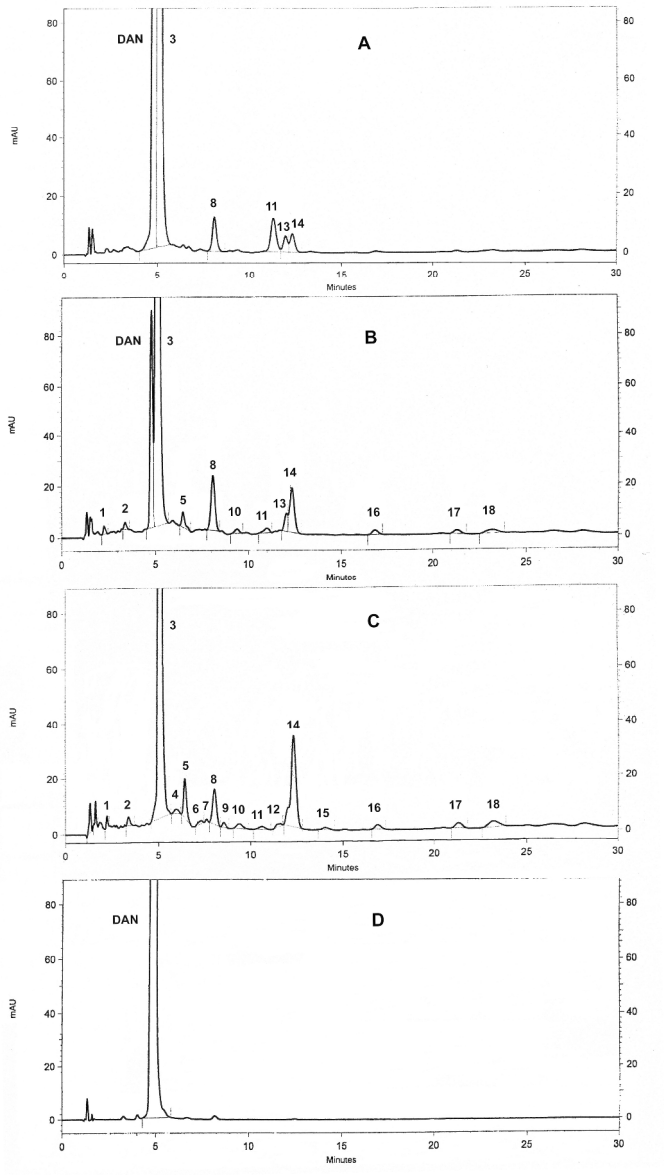


Figure 1. Chromatogram of separation of DAN and its photodegradation products after exposed to UVA radiation for: A- 24 h, B- 48h, C-72h, D- 72h dark control.
279x493mm (300 x 300 DPI)

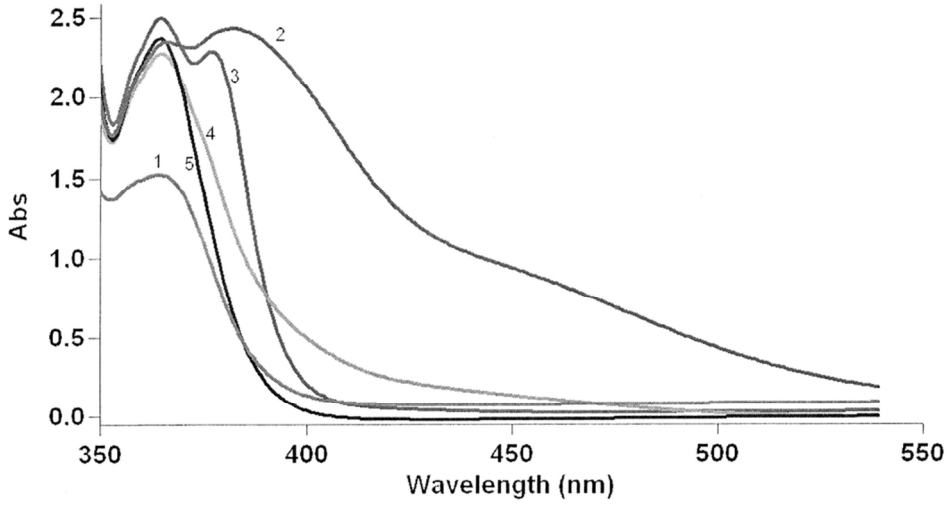


Figure 2. Absorption spectra of solutions of DAN before UVA irradiation: 1- DAN without metal ions, 2 - DAN with 0.025 M Fe(III), 3- DAN with 0.025 M Cu (II), 4- DAN with 0.0025 M Fe (III), 5- DAN with 0.0025 M Cu(II).
94x50mm (300 x 300 DPI)

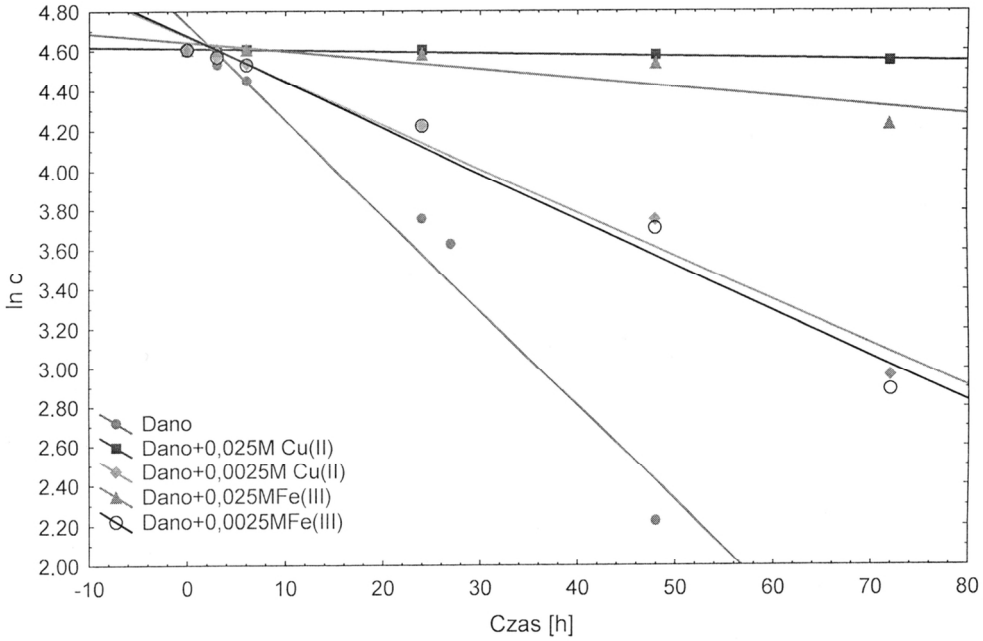


Figure 3. The ln c = f(t) graph of photodegradation of DAN.
112x74mm (300 x 300 DPI)