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Separation and characterization of ciprofloxacin, difloxacin, lomefloxacin, norfloxacin, and ofloxacin oxidation products under potassium permanganate treatment in acidic medium by UPLC-MS/MS

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ABSTRACT

A simple, sensitive and reproducible ultra-performance liquid chromatography method for determination of ciprofloxacin, difloxacin, lomefloxacin, norfloxacin and ofloxacin oxidation stability under permanganate treatment in acidic conditions at pH from 3.0 to 6.0, was developed. Chromatographic separations were carried out using the Acquity UPLC BEH C_{18} column; (2.1 × 100 mm, 1.7 μm particle size). The column was maintained at 40 °C, and eluted under isocratic conditions using 83% of eluent A and 17% of eluent B over 6.5 min, at a flow rate of 0.3 mL min^{-1}. Eluent A: water/formic acid (0.1 v/v%); eluent B: acetonitrile/formic acid (0.1 v/v%). An oxidation process followed kinetic of the second order reaction and depended upon solution acidity.

Oxidation of fluoroquinolones proceeded at piperazine moiety yielding respective hydroxy and oxo analogs, and remaining the quinolone fragment intact. Structures of products formed were assigned on a basis of UPLC/MS/MS fragmentation pathways.

1. Introdction

Ciprofloxacin (CIP), difloxacin (DIF), lomefloxacin (LOM), norfloxacin (NOR) and ofloxacin (OFL), the antibacterial synthetic drugs, belong to the second generation of fluoroquinolones. Mechanism of action of the fluoroquinolones in Gram-positive bacteria involves inhibition of DNA gyrase (a topoisomerase II), an enzyme responsible for supercoiling of bacterial DNA during DNA replication, while in Gram-negative bacteria, the primary target is topoisomerase IV, an enzyme responsible for relaxation of supercoiled circular DNA and separation of the inter-linked daughter chromosomes [1].

The fluoroquinolones exhibit increased antibacterial activity against the Enterobacteriaceae and other Gram-negative bacteria such as *Pseudomonas aeruginosa*, and exert some activity against certain Gram-positive cocci. Among them, difloxacin also shows notable efficacy against intracellular pathogens in experimental infections such as *Legionella pneumophila* and *Salmonella typhimurium* and has been approved for use in animals [2,3].

In stability studies of drugs, beside hydrolysis in acidic and basic solutions and verification of photostability, evaluation of an influence of oxidizing agents has been recommended [4]. It is worth noting, that the oxidation process of drugs, especially antibiotics, has focused more attention as an arising issue in the environmental protection.

The literature survey evidenced results of oxidation of ciprofloxacin, enrofloxacin, lomefloxacin, norfloxacin, ofloxacin, pipemidic acid and flumequine using MnO_2 followed by evaluation of the reaction kinetics and analysis of chemical structure of degradation products formed [5,6]. Similar studies were carried out for ciprofloxacin, levofloxacin, and norfloxacin using $KMnO_4$ in alkaline medium [7–9] and ciprofloxacin in acetate buffer at pH 7 [10,11].

Furthermore, an advanced oxidation, notably ozonation of ciprofloxacin, lomefloxacin, norfloxacin with a secondary wastewater effluent matrix was also reported [12]. Similar studies were carried out for ciprofloxacin in buffer solutions. The effects of ciprofloxacin concentration, diversified pH parameter (pH 3.0, 7.0 and 10.0), influence of temperature, or oxidizing agents' (ozone and H_2O_2) concentration were investigated; CIP oxidation products were characterized using HPLC/MS analysis [13].

Ciprofloxacin was also submitted to photooxidation of ciprofloxacin at pH 9.0. Identification of photo-oxidized products of ciprofloxacin formed in alkaline medium was performed by LC/MS/MS method. Structures of five products were attributed to

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photo-defluorination, photo-decarboxylation and loss of the piperazine moiety [14].

Another advanced oxidation processes, solar Fenton photocatalysis ($hv/Fe^{2+}/H_2O_2$) and heterogeneous photocatalysis with titanium dioxide (TiO_2), were studied for the chemical degradation of ofloxacin in secondary treated effluents [15].

Recently our interest focused on UPLC coupled with mass spectrometry technique for the separation and identification of oxidation products since the efficiency, sensitivity, and run time became an important factor in the pharmaceutical analysis. Literature indicates that ultra-performance liquid chromatography is a recent technique in liquid chromatography, which enables significant reductions in separation time and solvent consumption. Additionally, sensitivity of UPLC was much higher than that of conventional HPLC [16–19].

Herein, we report on the development of a new UPLC-MS/MS method for the determination of CIP, DIF, LOM, NOR, OFL and their oxidation products during reaction with $KMnO_4$ in solutions of different pH ranging from 3.0 to 6.0. The method was used for kinetic studies and identification of obtained degradation products of examined fluoroquinolones.

2. Chemicals and methods

2.1. Chemicals and reagents

Ciprofloxacin hydrochloride monohydrate Cat. No. 91033-1G Fluka, Difloxacin hydrochloride Cat. No. D2819-1G Sigma, Lomefloxacin hydrochloride Cat. No. D2906-1G Sigma, Norfloxacin Cat. No. 9890-1G Fluka, Ofloxacin Cat. No. O8757-1G Sigma. HPLC grade methanol, acetonitrile and formic acid (98%) were purchased from J.T. Baker. HPLC grade water was obtained from HLP 5 (HYDROLAB Poland) apparatus and was filtered through 0.2 μm filter before use.

2.2. Standard solution

The amount of 0.2 g of CIP, NOR, OFL, DIF and LOM, weighed with a precision of 0.1 mg, was dissolved in the volume of 50 mL of methanol, and filled up to the 100 mL with the same solvent. For method validation, solutions containing different concentrations of the examined fluoroquinolones in the range 0.04–2.00 mg mL^{-1} were prepared.

2.3. Oxidation study of the drug substance

The amount of 0.5 mL of methanol solutions of CIP, NOR, OFL, DIF, LOM (2.0 mg mL^{-1}), 2.5 mL demineralized water, 5.0 mL ammonium acetate buffer solution prepared according to *European Pharmacopeia* [20] with proper pH (3.0, 4.0, 4.5, 5.0 or 6.0) and 2 mL 0.002 M $KMnO_4$ was added to 10.0 mL flasks. The test solutions were incubated at room temperature and 1 μL of each reaction mixture was injected onto ACQUITY UPLC system after 15, 30 45 and 60 min, respectively.

Before the measurements of test samples, the analysis of solutions containing identical components as test samples but without $KMnO_4$ was done. The analyses were performed in triplicate.

2.4. 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) C_{18} column; 2.1 × 100 mm, and 1.7 μm particle size. The column was maintained at 40 °C, and eluted under isocratic conditions using 83% of eluent A and 17% of eluent B over 6.5 min, at a flow rate of 0.3 mL min^{-1}. Eluent A: water/formic acid (0.1 v/v%); and eluent B: acetonitrile/formic acid (0.1 v/v%).

Chromatograms were recorded using Waters eλ PDA detector. Compound concentration (%*i*) after oxidation induced by $KMnO_4$ was calculated from quotient of peak area (A*i*) to the sum of all peak areas (ΣA) on chromatograms according to formulation $\%i=(Ai/\Sigma A)100$ at $\lambda=294$ nm. Spectra were analyzed in 200–700 nm range with 1.2 nm 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, and cone potential 20 V. Nitrogen was used for both nebulizing and

Table 1
Validation of the method.

Parameter	CIP	DIF	LOM	NOR	OFL
RT (min)[a]	1.56 ± 0.025	3.10 ± 0.034	1.78 ± 0.024	1.43 ± 0.017	1.48 ± 0.017
Resolution[a,b]	2.32	0.73	2.81	3.28	0.75
LOD (mg mL^{-1})	0.07	0.05	0.07	0.08	0.04
LOQ (mg mL^{-1})	0.22	0.15	0.20	0.24	0.13
Linear range (mg mL^{-1})	0.22–2.00	0.15–2.00	0.20–2.00	0.24–2.00	0.13–2.00
Regression equation (*y*):					
Slope ($a \pm S_a$)	281675.4 ± 3708.0	280852.6 ± 2500.7	518232.2 ± 6116.4	346377.9 ± 4952.6	399707.8 ± 3173.7
Intercept ($b \pm S_b$)	−162.5 ± 4477.3	−207.1 ± 3019.6	631.0 ± 7385.5	−1308.4 ± 5980.2	6671.8 ± 3832.1
$t=b/S_b$	$-0.04 < t_{\alpha,f}$ statistically insignificant	$-0.07 < t_{\alpha,f}$ statistically insignificant	$0.09 < t_{\alpha,f}$ statistically insignificant	$-0.22 < t_{\alpha,f}$ statistically insignificant	$1.74 < t_{\alpha,f}$ statistically insignificant
Normality of residuals[c] (Shapiro–Wilk test)	0.8111 ($p=0.10$)	0.9453 ($p=0.70$)	0.9052 ($p=0.44$)	0.8647 ($p=0.25$)	0.9067 ($p=0.45$)
Correlation coefficient	0.9997	0.9999	0.9998	0.9997	0.9999
R^2 value	0.9993	0.9997	0.9994	0.9992	0.9998
Precision (% RSD)	0.56	0.89	0.51	0.82	0.48
Intermediate precision (% RSD)	1.10	1.21	0.93	1.18	1.01

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, $t_{\alpha,f}=2.776$ critical value of Student's *t* test for degrees of freedom $f=4$ and significance level $\alpha=0.05$

[a] Mean ± SD ($n=6$).
[b] Resolutions were calculated between two adjacent peaks.
[c] normal distribution of residuals if $p > 0.05$.

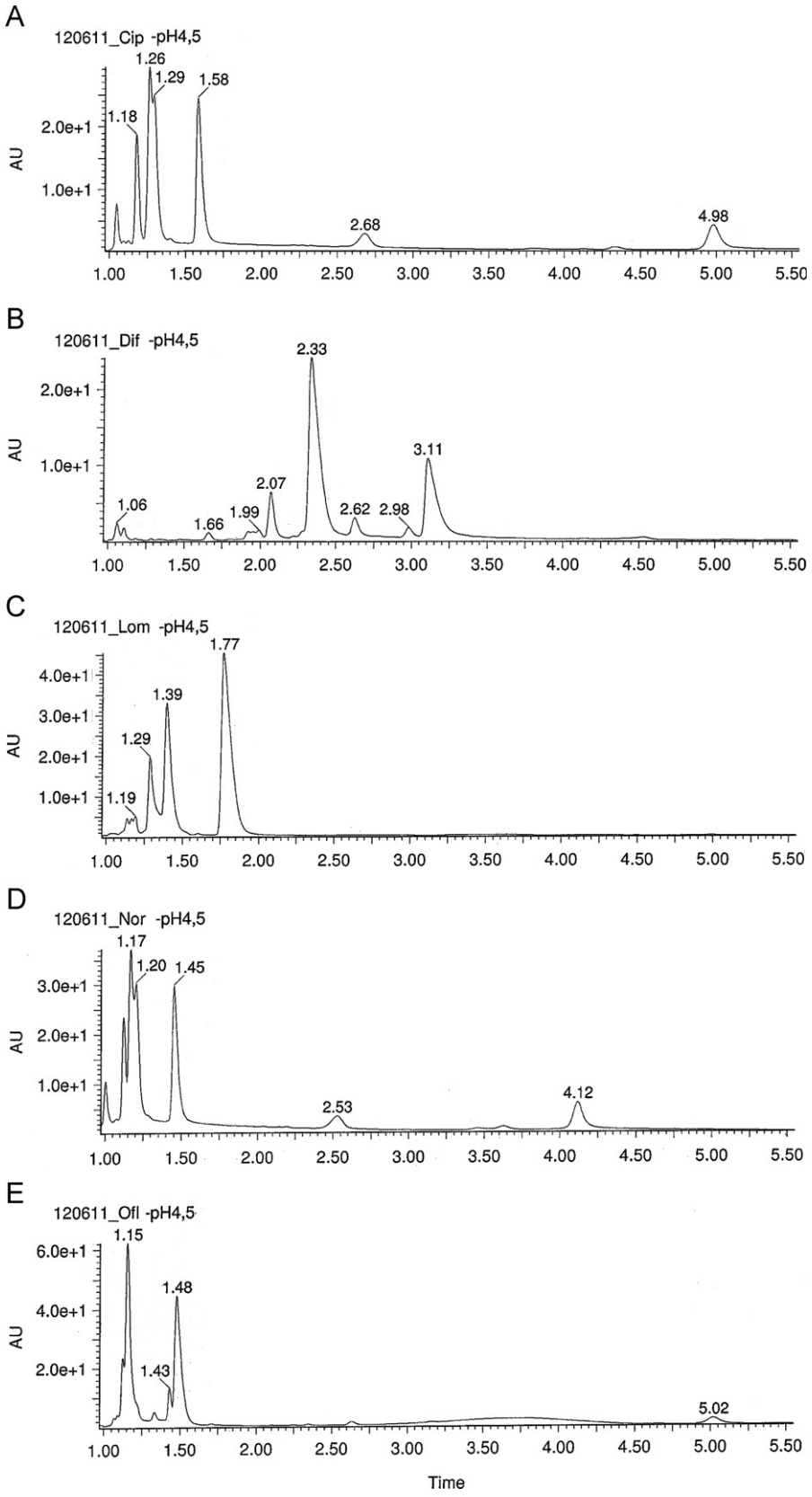


Fig. 1. UPLC chromatograms registered for fluoroquinolones after oxidation induced by $KMnO_4$ at pH 4.5: A, CIP RT=1.58; B, DIF RT=3.11; C, LOM RT=1.77; D, NOR RT=1.45; E, OFL RT=1.48. UPLC column: acquity UPLC BEH (bridged ethyl hybrid) C_{18} 2.1 × 100 mm, 1.7 μm; mobile phase A: water/formic acid (0.1 v/v%), mobile phase B: acetonitrile/formic acid (0.1 v/v%); isocratic conditions: 83% A and 17% B over 6.5 min; flow rate: 0.3 mL min^{-1}; column temperature: 40 °C, and injection volume: 1 μL.

drying gas. The data were obtained in a scan mode ranging from 50 to 1000 m/z in time 0.5 s intervals; eight scans were summed up to get the final spectrum.

Collision activated dissociations (CAD) analyses were carried out with the energy of 30 eV, and all the fragmentations were observed in the source. Consequently, the ion spectra were

obtained by scanning from 50 to 600 m/z range. Data acquisition software was MassLynx V 4.1 (Waters).

3. Method validation

The described method was validated for the determination of CIP, DIF, LOM, NOR and OFL in the presence of oxidation products by UPLC method according to ICH guidelines [21].

3.1. Specificity

To demonstrate the specificity of the developed UPLC method the solutions of CIP, DIF, LOM, NOR, OFL after oxidation stress was analyzed. Oxidation study was performed in 0.002 M $KMnO_4$ solution in ammonium acetate buffer at pH 4.0; the solution was left for 15 min at room temperature. Peak purity test was carried out for CIP, DIF, LOM, NOR, OFL peaks using MS detector in stressed sample.

3.2. System suitability

The system suitability parameters were defined with respect to tailing factor and resolution of examined fluoroquinolones peaks using solutions of CIP, DIF, LOM, NOR, OFL, after oxidation stress at pH 4.0 (Table 1).

3.3. Linearity

The linearity for CIP, DIF, LOM, NOR, OFL was assessed by injecting six separately prepared solutions covering the range of 0.13–2.00 mg mL^{-1}. The slope of regression line, *y*-intercept, standard deviation of slope and intercept, correlation coefficient, R^2 value and standard error of residuals of the calibration curve were calculated using the program Statistica *v*. 10. Next, to determine whether the residuals have normal distribution, the Shapiro–Wilk statistical test was used.

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

Based on the standard error of residuals (Se) and the slope (a) of the calibration plots and following the formula LOD = 3.3 Se/a and LOQ = 10 Se/a, the LOD and LOQ for examined fluoroquinolones were estimated.

3.5. Precision

The repeatability of the method was checked by a sixfold analysis of the concentration level 1.50 mg mL^{-1} of CIP, DIF, LOM, NOR, OFL solutions. The same protocol was followed for three different days to study the intermediate precision of the proposed method. Different analysts prepared different solutions on

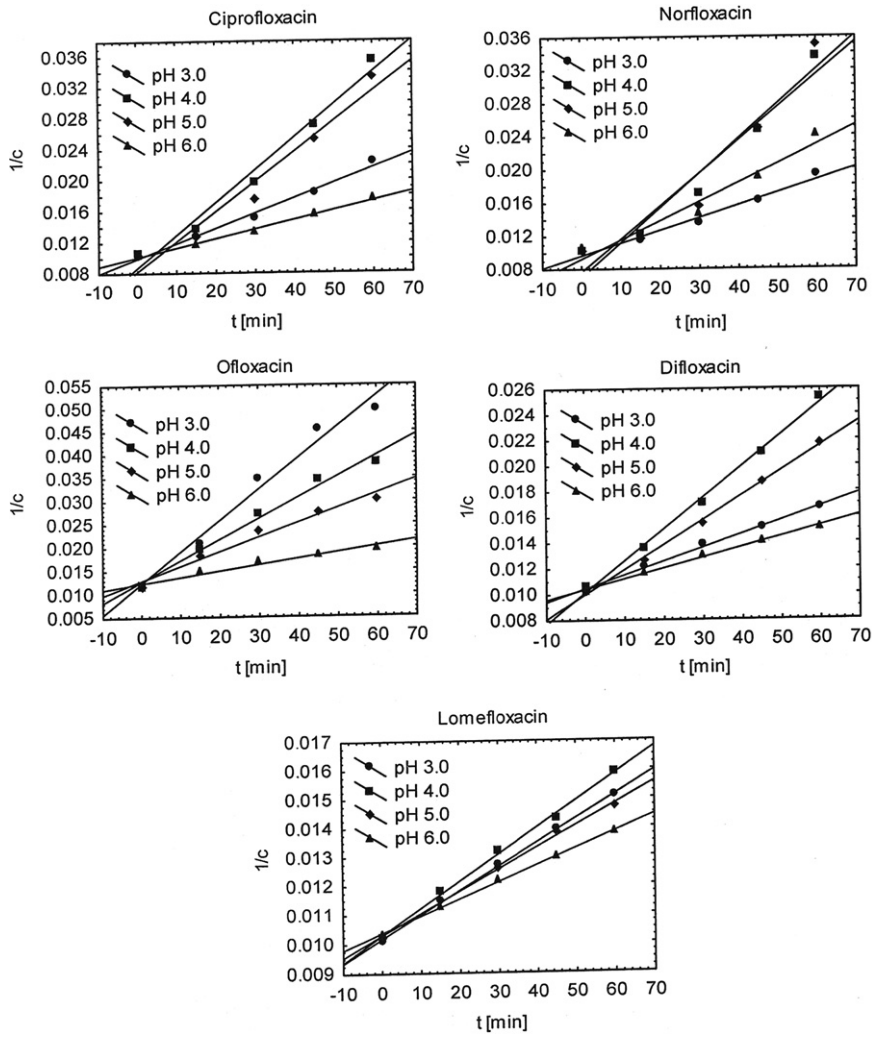


Fig. 2. The $1/c = f(t)$ graph of oxidation of CIP, DIF, LOM, NOR and OFL induced by $KMnO_4$ in acidic medium.

different days. The RSD (%) of the peak area of examined fluoroquinolones was calculated.

3.6. Robustness

To demonstrate the robustness of the method deliberate small changes of flow rate, content of acetonitrile and column temperature were made around the optimal values. The mobile phase flow rate was 0.30 mL min^{-1}; to study the effect of the flow rate on resolution, the flow rate was changed to 0.27 and 0.33 mL min^{-1}. The effect of the column temperature was studied at 36 °C and 44 °C (instead of 40 °C), and the mobile phase composition was changed +5% from the initial composition.

4. Result and discussion

Apart from hydrolysis and photostability assays, an influence of oxidizing agents in the process of forced degradation is an integral part of stress studies used for the stability evaluation of pharmaceutical products [4]. This effect may be evaluated by using validated analytical procedures enabling the determination of decreasing concentration of examined substance and detection of degradation products.

Herein we wish to develop a universal UPLC-MS/MS method for the separation and identification of examined constituents and for the kinetic studies of CIP, DIF, LOM, NOR and OFL in the presence of $KMnO_4$ in acidic solution.

Table 2
Oxidation reaction rate constant of fluoroquinolones in solution at different pH.

Component	pH	Rate constant k (min^{-1})	Correlation coefficient *r*
CIP	3.0	2.00×10^{-4}	0.9918
	4.0	4.00×10^{-4}	0.9872
	5.0	4.00×10^{-4}	0.9786
	6.0	1.00×10^{-4}	0.9965
DIF	3.0	1.00×10^{-4}	0.9982
	4.0	2.00×10^{-4}	0.9976
	5.0	2.00×10^{-4}	0.9987
	6.0	0.81×10^{-4}	0.9984
LOM	3.0	0.83×10^{-4}	0.9995
	4.0	0.93×10^{-4}	0.9983
	5.0	0.75×10^{-4}	0.9983
	6.0	0.58×10^{-4}	0.9987
NOR	3.0	2.00×10^{-4}	0.9866
	4.0	4.00×10^{-4}	0.9724
	5.0	4.00×10^{-4}	0.9547
	6.0	2.00×10^{-4}	0.9762
OFL	3.0	7.00×10^{-4}	0.9878
	4.0	4.00×10^{-4}	0.9933
	5.0	3.00×10^{-4}	0.9868
	6.0	1.00×10^{-4}	0.9754

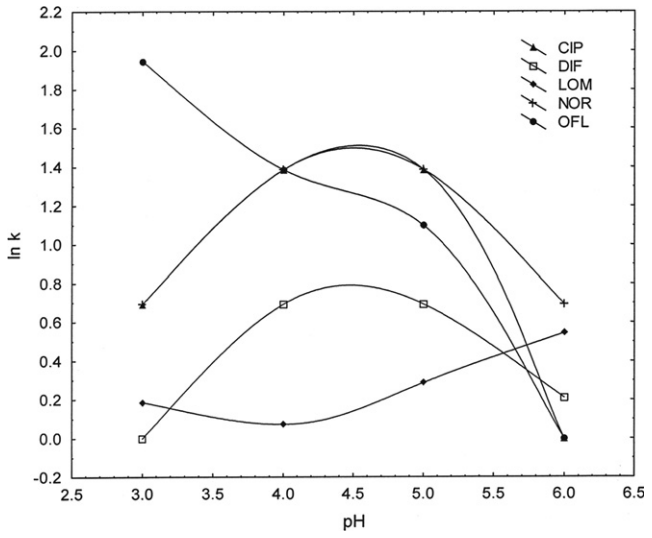


Fig. 3. The log $k = f(\mathrm{pH})$ graph of oxidation of CIP, DIF, LOM, NOR and OFL induced by $KMnO_4$.

Table 3
The kinetic results of investigated fluoroquinolones under $KMnO_4$ oxidation at pH 4.5 at room temperature.

Component	Rate constant *k* (min^{-1})	$t_{0.1}$ (min)	$t_{0.5}$ (min)	Correlation coefficient *r*
CIP	4.00×10^{-4}	2.78	25.00	0.9711
DIF	2.00×10^{-4}	5.56	50.00	0.9988
LOM	0.77×10^{-4}	14.43	129.87	0.9994
NOR	4.00×10^{-4}	2.78	25.00	0.9693
OFL	3.00×10^{-4}	3.70	33.33	0.9536

4.1. Optimization of chromatographic conditions

The main target of the chromatographic method was to achieve the separation of oxidation products and the main components CIP, DIF, LOM, NOR and OFL. To optimize the chromatographic separation, preliminary experiments were performed to test mobile phases containing different mixtures of acetonitrile and water (50/50, 83/17, 75/25 v/v; always the same amount of mobile phase additive was used. The solutions of examined fluoroquinolones after oxidation stress (buffer solution at pH 4.0, 30 min incubation) was analyzed. We took advantage of a mixture acetonitrile/water 83/17 v/v, with 0.1% of formic acid to obtain good peak resolution and symmetry.

4.2. Method validation

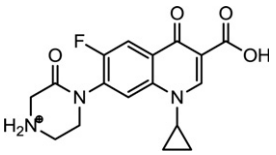
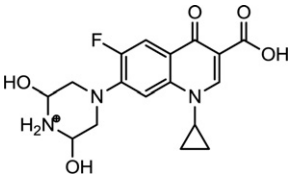
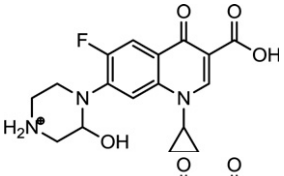
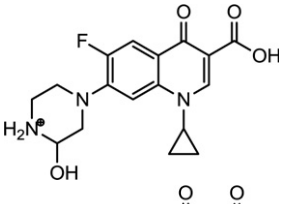
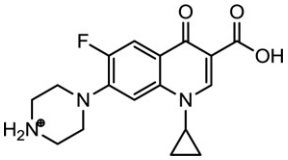
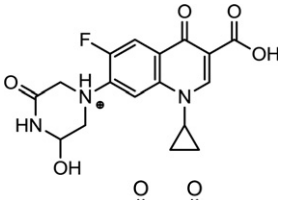
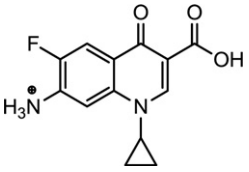
The developed UPLC method was specific to examined fluoroquinolones and guaranteed obtaining well shaped peaks both for active substances and coexisting oxidation products. Peaks of main components were well resolved from oxidation products in chromatograms and no interference that could have an influence on the obtained results was possible (Table 1). The main peak purity was examined with MS spectra using CODA algorithm (Waters Corporation, Milford, MA, USA). The investigated MS spectra uniquely contained signals corresponding to the examined fluoroquinolones and solvent.

Satisfactory resolution was also obtained for oxidation products, peaks appearing in chromatograms were sufficiently well resolved and could be analyzed by mass spectrometry (Fig. 1).

Regression analysis results obtained for examined fluoroquinolones 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*-intercepts of the linear equation for CIP, DIF, LOM, NOR and OFL were statistically insignificant. The distribution of the residuals can well be approximated with a normal distribution as it is shown by *p*-values ($p > 0.05$) 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 0.13–2.00 mg mL^{-1} for examined fluoroquinolones.

Sensitivity of the method was good. The LOD and LOQ values were found to be from 0.04 to 0.08 mg mL^{-1} and from 0.13 to 0.24 mg mL^{-1}, respectively. Good precision and intermediate precision with %RSD less than 2.0% was observed. Detailed results were presented in Table 1. In all the deliberately varied chromatographic

Table 4
Products of oxidation of CIP induced by $KMnO_4$ in acidic conditions.

Product Id	RT	$[M+H]^+$	Fragmentation ions	Proposed structure
CP-1	1.02	346.0	328.0, 302.0	
CP-2	1.16	364.0	346.0, 328.0, 306.0, 288.0, 262.0, 245.0	
CP-3	1.25	348.0	330.0, 313.0, 286.0, 258.0, 231.0	
CP-4	1.27	348.0	330.0, 313.0, 286.0, 258.0, 231.0	
CIP	1.56	332.0	314.0, 288.0, 245.0, 219.0, 205.0, 191.0, 179.0	
CP-5	2.65	362.0	344.0, 316.0, 298.0, 272.0, 244.0, 176.0, 136.0, 112.0	
CP-6	4.95	263.0	245.0, 205.0, 179.0	

conditions (flow rate, column temperature, mobile phase composition), examined fluoroquinolones and degradation products were adequately resolved, and the order of elution remained unchanged.

4.3. Oxidation of examined fluoroquinolones by $KMnO_4$ in acidic medium

Oxidative ability of $KMnO_4$ depends on the acidity of reaction solution. Oxidation potential of $KMnO_4$ decreases with increasing pH value and counts +1.23 V at pH 3, and at pH 5 reaches +1.07 V. In neutral or basic solution shows lower values equaling +0.58 V and +0.56 V, respectively [22].

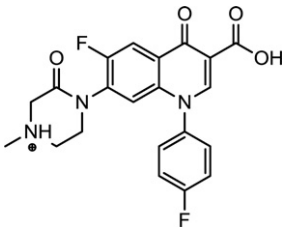
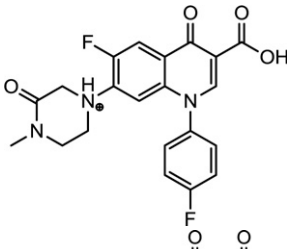
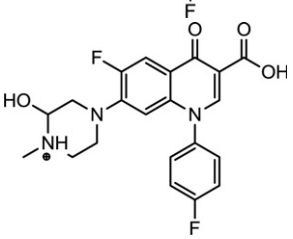
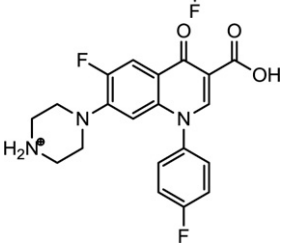
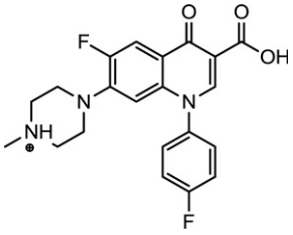
Chromatograms recorded for ammonium acetate buffer solutions with pH ranging from 3.0 to 6.0, and containing only fluoroquinolones studied, showed one peak (Supplementary Materials, Fig. S1). Whereas, in chromatograms recorded for test solutions containing $KMnO_4$ additional peaks of oxidation products of CIP, DIF, LOM, NOR and OFL were observed beside peaks of main components (Fig. 1).

The degradation process of CIP, DIF, LOM, NOR and OFL induced by $KMnO_4$ in studied conditions depended on the pH of solution, incubation time, and a kind of fluoroquinolone studied. Degradation of fluoroquinolones increased with the prolongation of incubation time and simultaneous decrease of pH (ranging from 6 to 4). In solution at pH 3.0 the degradation process decreased for CIP, DIF, NOR, and LOM, except OFL.

The number of oxidation products on chromatograms for incubated solutions in intervals from 15 to 60 min was different and depended on type of compound studied. Analysis of CIP revealed six additional peaks (CP-1–CP-6), NOR degraded to five products (NP-1–NP-5), DIF and OFL showed four oxidized products (DP-1–DP-4) and (OP-1–OP-4), and finally, LOM yielded only three products (LP-1–LP-3).

Percentage quota of main CIP degradation products, at pH 4 equals: CP-3 18.95%, CP-4 34.80%, while other oxidation products

Table 5
Products of oxidation of DIF induced by $KMnO_4$ in acidic conditions.

Product Id	RT	$[M+H]^+$	Fragmentation ions	Proposed structure
DP-1	1.93	414.0	397.0, 370.0, 273.0	
DP-2	1.97	414.0	397.0, 370.0, 273.0	
DP-3	2.37	416.0	398.0, 354.0, 338.0, 310.0	
DP-4	2.99	386.0	368.0, 342.0, 299.0, 273.0	
DIF	3.12	400.0	382.0, 356.0, 299.0, 273.0	

have not exceeded 8 % (CP-1 3.99%, CP-2 7.93%, CP-5 1.43% and CP-6 4.78%).

The main product of DIF oxidation was product DP-3 with a concentration of 54.11% at pH 4, whereas for OFL product OP-2 with reached concentration value 68.70% at pH 3. The sum of other oxidation products for DIF in above mentioned conditions was 6.44%, whereas for OFL was 11.23%.

Oxidation process of NOR and LOM led to formation of two main degradation products. For NOR products NP-2 and NP-3 appeared at pH 4 in amounts 25.59% and 33.65%, respectively, whereas for LOM two products LP-2 and LP-3 were obtained, in amounts 12.41% and 23.08%. The sum of percentage of other three oxidation products for NOR and LOM equaled 11.03% and 1.47%, respectively.

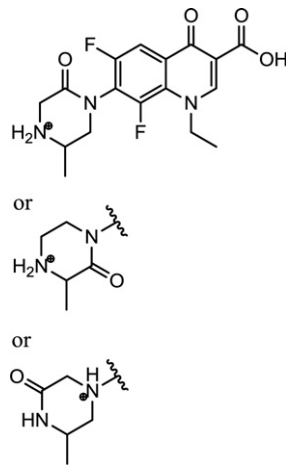
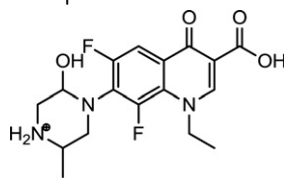
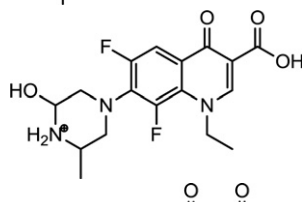
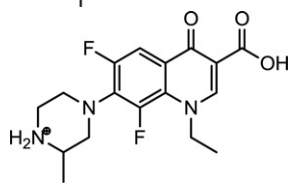
Data presented above were registered in chromatograms for solutions of investigated fluoroquinolones incubated for 60 min. During analysis only peaks with percentage above 0.5% were considered.

4.4. Kinetic evaluation

The analysis of the equation $1/c = f(t)$ for the oxidation of CIP, DIF, LOM, NOR and OFL induced by $KMnO_4$ in acidic solution at pH from 3.0 to 6.0 revealed that the process followed the kinetics of second order reaction (Fig. 2). Reaction rate constants k revealed to be pH-dependent and differed upon a kind of fluoroquinolone tested (Table 2).

The results for investigated fluoroquinolones enabled to plot profiles of observed changes, that were described according to equation $\ln k = f(\mathrm{pH})$, as well as to compare profiles shape and calculate $t_{0.1}$ and $t_{0.5}$ values. The curves for CIP and NOR were similar in shape and maximum value of reaction rate constants occuring at pH 4.5 (Fig. 3). Thus, it may be concluded that CIP and NOR did not differ significantly under oxidation process. Also the curve for DIF remained similar tendency, however k value was smaller in studied range of acidity. The curve for LOM in studied pH range has inversed a course in comparison with CIP, NOR and

Table 6
Products of oxidation of LOM induced by $KMnO_4$ in acidic conditions.

Product Id	RT	$[M+H]^+$	Fragmentation ions	Proposed structure
LP-1	1.17	366.0	348.0, 322.0, 264.0	
LP-2	1.28	368.0	350.0, 332.0, 306.0, 278.0, 236.0	
LP-3	1.40	368.0	350.0, 332.0, 306.0, 263.0, 236.0	
LOM	1.80	352.0	334.0, 308.0, 251.0	

DIF with minimum k value at pH 4.5. Different course was observed for OFL, with k value lower in the range of pH 3–6, and flattening in the range of pH 4–5 (Fig. 3).

The presented oxidation profiles for compounds under research, despite occurred differences did not exclude their common feature of maximum, minimum or flattening at pH 4.5. It was therefore deduced that values of $t_{0.1}$ and $t_{0.5}$ determined at pH 4.5 may properly describe the stability, i.e. time after which 10% or 50% of substrates is lost (Table 3).

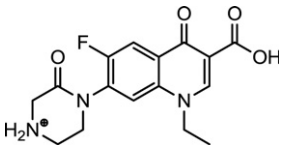
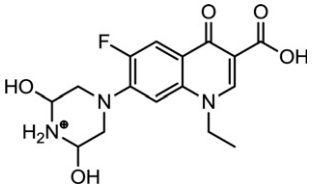
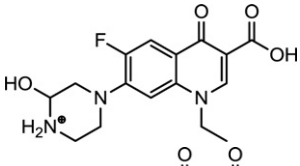
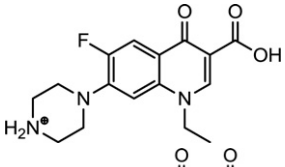
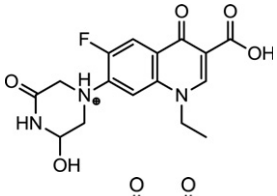
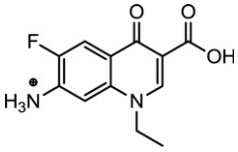
On a basis of the calculated $t_{0.1}$ and $t_{0.5}$ values for investigated fluoroquinolones, we determined their susceptibility for oxidation process, that followed the subsequent rank order: CIP = NOR > OFL > DIF > LOM.

4.5. Identification of oxidation products.

The identification of oxidation products of five fluoroquinolones (CIP, DIF, LOM, NOR, OFL) induced by $KMnO_4$ in acidic conditions was performed on a basis of UPLC/MS analysis and supported with fragmentation patterns obtained from MS/MS experiments. Oxidation products of examined fluoroquinolones are shown in Tables 4–8, for CIP, DIF, LOM, NOR, and OFL, respectively. The oxidation process was found to mainly affect 7-amine substituent of fluoroquinolone moiety, i.e. piperazine, 4-methylpiperazine, and 3-methylpiperazine, while the fluoroquinolone core, responsible for antibacterial activity, remained unchanged (Fig. 4).

Generally, the oxidation proceeded with hydroxylation in the close vicinity of *N*-1 and *N*-4 piperazine atoms to respective hydroxylated and successive oxidation to their oxo-counterparts. Since, the *N*-4 atom of piperazine is more susceptible for oxidation the most abundant oxidized products were 3-monohydroxylated products (e.g. CP-4, DP-4, LP-3, NP-3) except OFL, for which hydroxylated products were not detected. Consequently, in the case of OFL, a 3-oxo-derivative was the most abundant oxidation product observed. Although *N*-1 atom of piperazine is less reactive because of lower basicity, in the case of CIP, and LOM, respective 2-hydroxylated CP-3 and LP-2 products were also assigned. It was further found, that in the case of secondary amine containing fluoroquinolones–CIP and NOR–bihydroxylated products in positions 3 and 5 were detected (CP-2 and NP-2). Interestingly, these products were further transformed into their monooxo analogs yielding characteristic 3-hydroxy-5-oxo derivatives (hydroxylactams, CP-5 and NP-4). Such transformation was not observed in the case of LOM which contains methyl group in position 3 of piperazine. It further seems, that 3-methyl and more importantly two fluorine atoms in positions 6 and 8 of LOM increase stability of piperazine moiety towards oxidation induced by $KMnO_4$ under acidic conditions.

Table 7
Products of oxidation of NOR induced by $KMnO_4$ in acidic conditions.

Product Id	RT	$[M+H]^+$	Fragmentation ions	Proposed structure
NP-1	0.98	334.0	316.0, 290.0, 233.0	
NP-2	1.10	352.0	334.0, 316.0, 251.0, 236.0, 233.0	
NP-3	1.19	336.0	318.0, 300.0, 274.0, 246.0	
NOR	1.43	320.0	303.0, 276.0, 248.0, 233.0, 220.0, 205.0	
NP-4	2.51	350.0	332.0, 304.0, 286.0, 260.0, 232.0, 179.0	
NP-5	4.12	251.0	233.0, 207.0, 179.0, 151.0	

It was found, that one of oxidation routes of *N*-4 methylated fluroquinolones–DIF and OFL–proceeded to their demethylated analogs DP-4, and OP-3, respectively.

It is worth noting, that oxidation of piperazine moiety of CIP, NOR and OFL between oxidized centers and nitrogen atoms led to distinctive mass loss $m/z = 69$ and $m/z = 83$. This was attributed to ring opening, dealkylation, and deamination processes, which finally yielded 7-amino quinolone products CP-6, NP-5, and OP-4, respectively.

Structures of presented stable oxidation products were confirmed by Collisionally Activated Decomposition (CAD) experiments (Supplementary Materials, Figs. S2–S6). The fragmentation pattern involved loss of H_2O from the carboxylate group of quinolone (-18), then loss of carboxylate function (-46), dealkylation in *N*-1 position, followed by partial degradation of quinolone moiety. Dealkylation in *N*-1 position proceeded easily in case of CIP and NOR. In opposite, 4-F-phenyl moiety of DIF seems very stable in position *N*-1 of fluoroquinolone moiety. Dealkylation of LOM proceeded slowly in comparison to CIP and NOR. It may be attributed to an influence of additional electron-withdrawing fluorine substituent in position 8 of quinolone.

5. Conclusions

We have developed a new UPLC-MS/MS method for determination of stability of CIP, DIF, LOM, NOR and OFL and characterization of their oxidation products. The method meets acceptance criteria for validation and may be used for the kinetics analysis of CIP, DIF, LOM, NOR and OFL oxidation. The process followed kinetics of the second order reaction for the substrate, and the reaction rate constant depended on the solution pH.

It was found, that the most susceptible fluoroquinolones for oxidation were CIP and NOR with comparable $t_{0.1}$ and $t_{0.5}$ values. OFL and DIF displayed lower degradation rate, while LOM was the most stable among tested drugs. The proposed model of investigation of oxidation profile for presented fluoroquinolones may be regarded as a predictor of their stability, since observed reaction rate constants k at pH 4.5 change similarly like values calculated from kinetic equation.

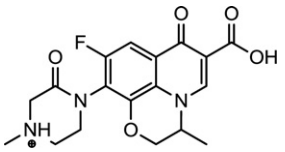
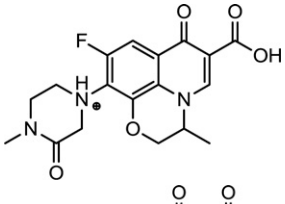
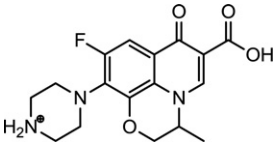
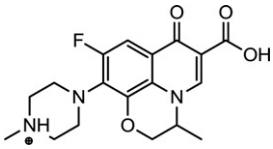
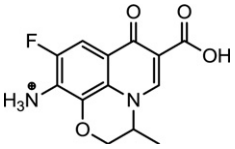
Degradation of fluoroquinolones mainly affected piperazine moiety giving respective hydroxy- and oxo-derivatives. Further oxidation of CIP, NOR and OFL, and not DIF and LOM, following dealkylation, and degradation of piperazine yielded 7-amino fluoroquinolones analogs.

It seems, that oxidation of model fluoroquinolones under KMnO4 in acidic medium, opens up the possibility for application in drug stability studies and environmental protection studies in the process of utilization of drug traces.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.055.

Table 8
Products of oxidation of OFL induced by $KMnO_4$ in acidic conditions.

Product Id	RT	$[M+H]^+$	Fragmentation ions	Proposed structure
OP-1	1.11	376.0	358.0, 332.0	
OP-2	1.14	376.0	358.0, 332.0, 264.0, 246.0	
OP-3	1.43	348.0	330.0, 304.0, 264.0, 246.0, 220.0	
OFL	1.49	362.0	345.0, 318.0, 264.0	
OP-4	5.06	279.0	261.0, 219.0	

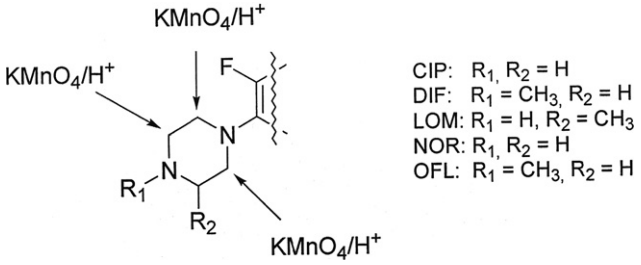


Fig. 4. Oxidation pathways of fluoroquinolones induced by $KMnO_4$ in acidic conditions.

References

[1] P.C.H. Sharma, A. Jain, S. Jain, Acta. Poloniae Pharm. – Drug Res. 66 (2009) 587–604.
[2] G. Carlucci, J. Chromatogr. A 812 (1998) 343–367.
[3] M. Martinez, P. McDermott, R. Walker, Vet. J. 172 (2006) 10–28.
[4] ICH-Q1A(R2) Stability Testing of New Drug Substances and Products, International Conference on Harmonization, Geneva, November 2003, 〈http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf〉.
[5] H. Zhang, C.-H. Huang, Environ. Sci. Technol. 39 (2005) 4474–4483.
[6] H. Zhang, W.-R. Chen, C.-H. Huang, Environ. Sci. Technol. 42 (2008) 5548–5554.
[7] A.A.P. Khan, A. Mohd, S. Bano, A. Husain, K.S. Siddiqi, Transition. Met. Chem. 35 (2010) 117–123.
[8] P.N. Naik, S.A. Chimatadar, S.T. Nandibewoor, Ind. Eng. Chem. Res. 48 (2009) 2548–2555.
[9] K.A. Thabaj, S.D. Kulkarni, S.A. Chimatadar, S.T. Nandibewoor, Polyhedron 26 (2007) 4877–4885.
[10] L. Hu, A.M. Stemig, K.H. Wammer, T.J. Strathmannn, Environ. Sci. Technol. 45 (2011) 3635–3642.
[11] L. Hu, H.M. Martin, T.J. Strathmannn, Environ. Sci. Technol. 44 (2010) 6416–6422.
[12] Ch. Liu, V. Nanaboina, G. Korshin, Chemosphere 86 (2012) 774–782.
[13] B. De Witte, K. Demeestere, H. Van Langenhove, J. Hazard. Mater. 161 (2009) 701–708.
[14] T.G. Vasconcelos, D.M. Henriques, A. König, A.F. Martins, K. Kümmerer, Chemosphere 76 (2009) 487–493.
[15] I. Michael, E. Hapeshi, C. Michael, D. Fatta-Kassinos, Water Res. 44 (2010) 5450–5462.
[16] R. Russo, D. Guillarme, D.T.-T. Nguyen, C. Bicchi, S. Rudaz, J.L. Veuthey, J. Chromatogr. Sci. 46 (2008) 199–208.
[17] D.T.-T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, J. Sep. Sci. 29 (2006) 1836–1848.
[18] A.D. Villiers, F. Lestremau, R. Szucs, S. Gelebart, F. David, P. Sandra, J. Chromatogr. A 1127 (2006) 60–69.
[19] S.A.C. Wren, P. Tchelitcheff, J. Chromatogr. A 1119 (2006) 140–146.
[20] European Pharmacopeia 6th ed., Council of Europe, European Directorate for the Quality of Medicines, Strasbourg, 2008.
[21] ICH-Q2 (R1) Validation and Analytical Procedures: Text and Methodology. International Conference on Harmonization: Geneva, November 2005, 〈http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf〉.
[22] R. Kocjan (Ed.), Analytical Chemistry, PZWL, Warszawa, 2000, pp. 518–519.