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Quantification of Isoxicam in the Presence of Related Compounds by TLC-Densitometry

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Abstract

A simple, sensitive, selective and precise TLC-densitometric determination of isoxicam as a drug was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60 F_{254} as a stationary phase. The mobile phase consisted of ethyl acetate : toluene : butylamine (2:2:1, *v/v/v*). The system was found to give good resolution for isoxicam (R_F value of 0.56). Densitometric detection was carried out at λ = 350 nm. The calibration plots showed good linear relationship in the working concentration range of 0.7 to 2.2 μg per band. The method was validated for precision (RSD < 1%), specificity, limit of detection and quantitation (0.22 and 0.67 μg per band, respectively). The drug was subjected to acidic and basic hydrolysis at different temperatures. All the peaks of the degradation products were well-resolved from the isoxicam with significantly different R_F values. The products formed were identified by their TLC retention times (R_F values), absorption spectra and HPLC-MS/MS analysis. The developed TLC-densitometric method can be applied for identification and quantitation of isoxicam in drugs, and it can be using as a screening method in pharmaceutical research. As the TLC method can effectively separate the drug from its degradation products it can be employed as a stability-indicating one and can be utilized to investigate the kinetics of degradation process.

Keywords: Thin-layer chromatography, Densitometry, Isoxicam, Stability, Degradation process

1. Introduction

Isoxicam (chemically 4-hydroxy-2-methyl-N-[5-mrthyl-3-isoxolyl-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide]) is a potent, orally-active, non-steroidal anti-inflammatory drug with prolonged activity, highly effective in relieving the symptoms of rheumatoid arthritis and degenerative joint disease. Side effects can occur with all medications, and the most important side effects that have been reported with isoxicam are hepatic and skin toxicity.[1,2]

Pharmacopoeias do not provide any monograph for isoxicam. A limited number of techniques exists for the determination of isoxicam in biological fluids, including liquid chromatography[3–6] and LC-tandem mass spectrometry.[7] The photostability of isoxicam has been reported using three different analytical methods (HPTLC, HPLC and capillary electrophoresis).[8] HPTLC analysis was performed on silica gel 60 F_{254} plates, which were prewashed before use with methanol : dichloromethane (1:1, v/v), using a horizontal developing chamber. A mixture of chloroform : 1-propanol : 96% acetic acid (9:0.5:0.5, v/v/v) was used as a mobile phase and densitometric detection was carried out at 280 nm.[8] A selective molecularly imprinted polymer (MIP) clean-up and pre-concentration approach was applied prior to spectrophotometric determination of isoxicam in complex matrixes, such as capsules and human serum samples.[9] In the available literature no papers were found on conventional TLC analysis of isoxicam and potential impurities or degradation products. The major advantage of TLC is that, unlike HPLC, several samples can be run simultaneously using a small amount of mobile phase, thus lowering analysis time and cost per analysis and protecting the environment.[10]

The aim of the present work was to develop a novel, specific and repeatable TLC method with densitometric detection for the identification and quantitative determination of isoxicam in presence of related compounds and possible degradation products, and for assessment of purity and stability of the drug. The proposed method was validated as per International Conference of Harmonization (ICH) guidelines.[11]

2. Experimental

2. 1. Chemical, Reagents and Solutions

Isoxicam was supplied by Sigma-Aldrich. All solvents used were of analytical grade and were purchased from POCh (Gliwice, Poland).

Standard solution was prepared by dissolving accurately about 1.5 mg of isoxicam in 10.0 mL of acetone to obtain a concentration 0.15 mg/mL.

2. 2. TLC conditions

The solutions were spotted on precoated silica gel aluminium TLC plates 60 F_{254} (13 × 10 cm, cut from 20 × 20 cm before use; Merck, Germany) using a Linomat V (Camag, Switzerland) sample applicator. All samples were applied (volume of 10 μL), as 10 mm width bands with a distance of 10 mm from the plate bottom, 10 mm from the edge, and a distance between two bands of 10 mm. The mobile phase consisted of ethyl acetate : toluene : butylamine (2:2:1, *v/v/v*). Chromatograms were developed at room temperature in a glass chamber (18 cm × 9 cm × 18 cm, Sigma-Aldrich) saturated with mobile phase for 15 min. The length of a chromatographic run was 9 cm. Subsequent to the development, TLC plates were dried in ambient air. Densitometric scanning and recording appropriate spots were done by using a Camag TLC Scanner3 with winCats4 software in the reflectance-absorbance mode at 350 nm. The slit dimension was kept at 8 × 0.45 mm and a scanning speed of 20 mm per s was employed.

2. 3. Validation of the Method

Validation of the optimized TLC method was done with respect to ICH guidelines, by linearity, specificity, limit of detection, limit of quantitation and precision parameters.[10]

2. 3. 1. Linearity

Linearity was checked in a working range 10 to 2.2 μg/mL using six replicate analyses. Peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

2. 3. 2. Specificity

The specificity of the method was ascertained by analyzing standard solutions. The spot for isoxicam was confirmed by comparing the retardation factor (R_F) values and spectra of the spots for different concentrations in the presence of a placebo. The peak purity of isoxicam was accessed by comparing the spectra at three different levels; peak-start, peak-apex and peak-end positions of the spot.

2. 3. 3. Limit of Detection and Quantification

Limits of detection (LOD) and quantification (LOQ) were estimated based on the standard error of the response (S_b) and the slope (a) of the calibration graph obtained by the method of least squares. For the estimation the following formulae were used: LOD = 3.3 S_b/a and LOQ = 10 S_b/a.

2. 3. 4. Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations of the drug (50, 100, 150 μg/mL) in triplicate on the same day. Intermediate precision of the method was checked by repeating analyses on three different days.

2. 4. Detection of the Related Impurities

The related impurities were determined by spotting higher concentrations of the drug so as to detect and identify them. Isoxicam (approx 5 mg) was dissolved in 10.0 mL of acetone, and this solution was termed as sample solution (0.5 mg/mL). 10 μL of the solution was applied on a TLC plate and the chromatograms were run as described in Section 2.2.

2. 5. Preparation of Acid- and Base-Induced Degradation Products

An amount of 12.5 mg of isoxicam was separately dissolved in 2.5 mL of solutions of 0.1, 0.5, 1.0 and 3.0 M HCl and NaOH. These solutions were kept at two temperatures (60 °C and 120 °C) for a set amount of time. Aliquot of 1.0 mL of above solutions were taken and diluted up to 2.0 mL with acetone. The resulting solutions were subjected to TLC plates triplicate and the chromatograms were run as described above. The measurements were made under conditions established for the method to determine the percentage decomposition by the internal normalization method. The percentage decomposition for the individual constituents (i[%]) was computed using the formula: i[%] = (A_i/!A)100; where: A_i is peak area of the determined constituent, and !A is the sum of the peak areas of all the constituents present in the densitogram.

The process of isoxicam degradation was rated by means of kinetic and thermodynamic parameters.[12] The order of the reaction, the reaction rate constants (k), the half-life ($t_{0,5}$), the time ($t_{0,1}$) at which the concentration of isoxicam is reduced by 10%, and the activation energy (E_a) were evaluated. Parameters were computed using the following formulae: $k = 2.303(\log c_1 - \log c_2) / (t_2 - t_1)$, $t_{0,5} = 0.635/k$, $t_{0,1} = 0.1053/k$, and $E_a = -2.303R(\log k_1 - \log k_2) / (1/T_1 - 1/T_2)$, where: R = 8,315 J/mol/K, $k_2 > k_1$, $T_2 > T_1$.

2. 6. Analysis of Degradation Products

To identify the isoxicam degradation products, besides densitometric analysis (R_F values and absorption spectra) also HPLC-MS/MS analysis were carried out.

The isoxicam solutions after hydrolysis in 1 M HCl and NaOH at 120Σ were separated on a chromatotron plate (model 8924, Harrison research, USA); the solution of the hydrolyzed substance in acetone was applied to the plate (prepared with Silica Gel PF_{254} and water). Based on the TLC analysis, appropriate solvents of increasing polarity and different proportions were investigated for optimal separation; and the best mixture was ethyl acetate : toluene : butylamine (2:2:1, *v/v/v*). After separation the solvent was evaporated and the products were identified.

2. 6. 1. HPLC-MS/MS Analysis

Liquid chromatography was performed using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) LC system. Chromatographic separation was carried out with an XBridge C18 analytical column (30 × 2.1 mm, 3.5 µm, Waters, Ireland) at 30 °C. Two solvent mixtures were used; solvent A: acetonitrile : formic acid (0.01%) and solvent B: H_2O : formic acid (0.01%). The following gradients were used: 0–5 min, 0–100% A; 5–7 min, 100% A; 7–8 min, 100–0% A; 8–20 min, 100% B. The flow rate was set at 0.6 mL/min. A sample volume of 20 ìL was injected onto the analytical column for compound analysis.

Mass spectrometric analyses were accomplished on an Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 2000 triple quadrupole mass spectrometer equipped with an electrospray ionization interface and performed in the positive ionization mode. A standard solution of polypropylene glycols was used for instrument tuning and mass calibration at unit mass resolution according to the Applied Biosystems manual. The mass spectrometer was operated with a dwell time of 200 ms, and a 5 ms delay between scans for each transition on the first quadrupole (Q1) in a range of 50 to 1000 Da. To find the optimal instrumental parameters for studied compounds the quantitative optimization was done by direct infusion of isoxicam at a concentration of 1 µg/mL and a flow rate of 10 µL/min using a Hamilton syringe pump (Hamilton, Reno, Nevada). The ion source parameters were as follows: ion spray voltage (IS): 5500 V; nebulizer gas (gas 1): 45 psi; turbo gas (gas 2): 45 psi; temperature of the heated nebulizer (TEM): 300 °C and curtain gas (CUR): 10 psi. Nitrogen (99.9%) from Peak NM20ZA was used as the curtain and collision gas. The ion path parameters for isoxicam were as follows: declustering potential (DP): 10V; focusing potential (FP): 350V; entrance potential (EP): 10V, electron multiplier (CEM): 2500V; collision cell entrance potential (CEP): 44V; and collision cell exit potential (CXP): 25V, respectively. Data acquisition and processing were accomplished using the Applied Biosystems Analyst ver.1.4.2 software.

3. Results and Discussion

The subject of the paper was to establish a new, simple and precise method for identification and quantitation of isoxicam. The conditions for the analysis were established experimentally, based on eluotropic series. Finally, determination was performed on TLC silica gel 60 F_{254} plates as a stationary phase, with ethyl acetate : toluene : butylamine (2 : 2 : 1; *v/v/v*) as a mobile phase. In the obtained chromatograms only the peak from the active substance ($R_{F(ISO)} \approx 0.56$) was visible at a concentration level of about 3 µg of isoxicam per band. At higher concentration levels an additional peak was observed at $R_{F(X)} \approx 0.40$. The chromatograms obtained from analysis of different concentrations of isoxicam are shown in Figure 1.

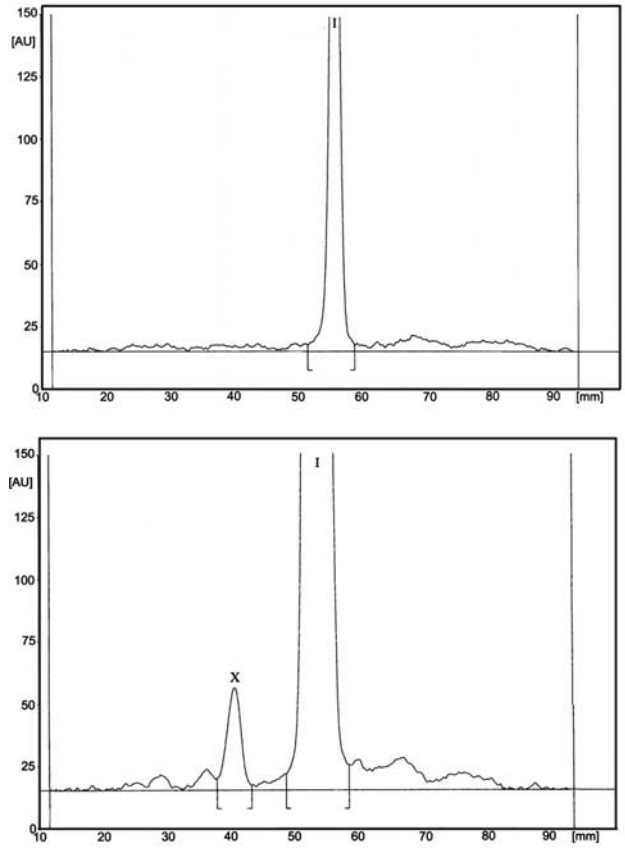

Figure 1. Typical densitograms for isoxicam (I) and its related impurity (X), obtained directly from the chromatogram.

To investigate the appropriate wavelength for determination of isoxicam, UV spectra in the range 200–400 nm were acquired from the chromatograms. The spot originating from isoxicam ($R_{F(ISO)} \approx 0.56$) shows the charac-

teristic maxima at 256 and 350 nm (Figure 2). For monitoring a drug the wavelength 350 nm was selected. It was observed that there was no interference from the mobile phase or baseline disturbance at 350 nm. Therefore, it was concluded that this wavelength was satisfactory for analysis of isoxicam next to its related impurities with suitable sensitivity.

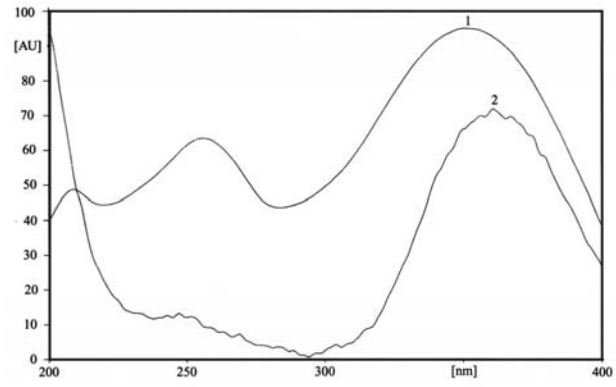

Figure 2. UV spectra for isoxicam (1) and the additional substance at $R_{F(X)} \approx 0.40$ (2), obtained directly from the chromatogram.

The linear regression analysis parameters for the calibration plot in the concentration range of 25–220 μg of isoxicam per mL showed a good linear relationship (correlation coefficient, r = 0.9944) with regard to peak area. The regression equation thus obtained from the calibration plot: y = 46.10x + 2433.0 (where y–peak area, x–concentration in μg/mL), was used for quantitative estimation of isoxicam in samples. The values of the standard deviation of the slope (S_a), standard deviation of the intercept (S_b) and standard error of estimate (S_e) were 2.44, 307.0 and 460.8, respectively.

The method developed is specific for studied components in the defined concentration range. There are no peaks in the recorded chromatograms from solvents or mobile phase. The peak purity for isoxicam was estimated by comparing the spectra at the peak start, peak apex, and peak end positions. Measuring spots showed overlaying spectra. This indicates that the spot was free of any interference that might be present in the analysis. R_F values for isoxicam were 0.56 (Figure 1).

The repeatability of sample application and measurement of peak area were expressed as %RSD. The developed method was found to be precise as the RSD values for

Table 1: The results of the determination for isoxicam and its degradation products in HCl and NaOH solutions at 60 °C.

	Concentration [%]					
	Time [day]	**Imp.A ($R_F \approx 0.12$)**	**Imp.B ($R_F \approx 0.30$)**	**Imp.C ($R_F \approx 0.41$)**	**Isoxicam ($R_F \approx 0.56$)**	**Imp.D ($R_F \approx 0.65$)**
3 M HCl	1	19.07	–	–	80.93	–
	7	18.23	–	11.76	69.45	0.56
	40	10.85	–	58.95	21.94	8.26
	60	–	9.40	76.15	4.16	10.29
1 M HCl	1	9.01	–	–	80.99	–
	7	18.63	–	4.76	76.61	–
	18	20.00	–	25.30	51.54	3.16
	40	0.82	17.07	34.24	36.61	11.26
	60	–	20.75	38.68	22.10	18.47
0.5 M HCl	1	3.98	–	–	96.02	–
	7	15.34	–	2.44	82.22	–
	18	10.22	6.12	7.33	68.42	7.91
	60	2.21	30.06	26.62	25.62	15.49
0.5 M NaOH	1	15.34	7.87	4.43	72.36	–
	7	16.44	10.40	9.54	63.08	0.54
	40	56.41	5.22	8.01	28.68	1.68
	60	78.71	–	7.49	11.23	2.57
1 M NaOH	1	17.79	–	–	82.21	–
	7	26.59	5.91	2.07	65.43	–
	18	43.65	8.28	5.28	41.18	1.61
	40	63.69	–	9.89	22.14	4.28
	60	77.89	–	12.79	2.42	6.90
3 M NaOH	1	3.17	–	–	96.83	–
	7	27.46	1.75	–	70.79	–
	18	40.75	3.06	14.23	51.96	–
	60	66.11	18.50	9.31	0.15	5.93

"0" – 100 % of isoxicam

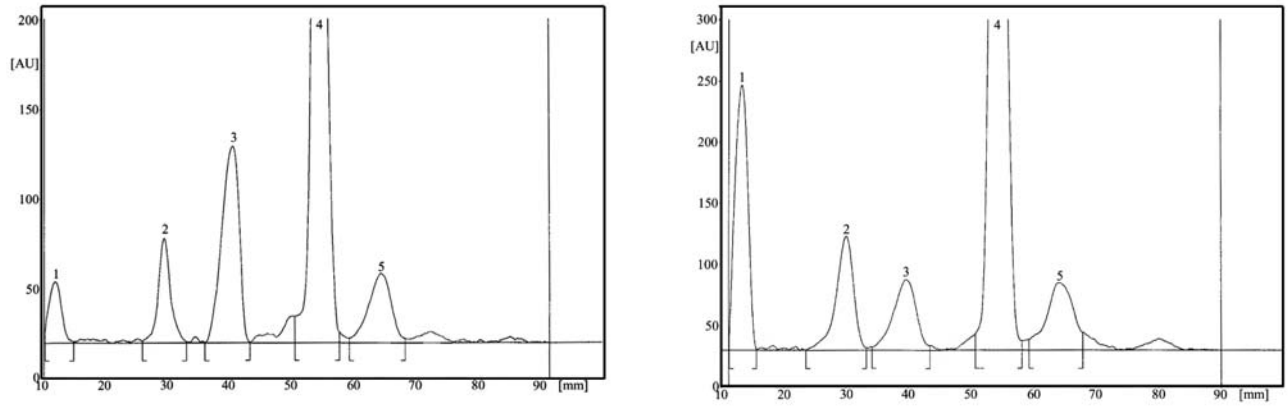

Figure 3. Typical densitograms obtained under stress conditions in acidic (a) and basic (b) environments (1-impurity A, 2-impurity B, 3-impurity C, 4-isoxicam, 5-impurity D).

repeatability and intermediate precision studies were in the range 0.18 to 0.32%. LOD and LOQ were found to be 0.22 and 0.67 μg per band, respectively. The low LOD and LOQ values indicated the high sensitivity of this method.

Such a conclusion led to further investigations focused on the behavior of isoxicam in different solutions at two temperatures for various incubation times, thus on the suitability of the novel method for degradation studies. TLC data obtained on stress testing of isoxicam suggested that pH, temperature and incubation time affect the rate of isoxicam degradation. The chromatograms obtained under stress conditions of the samples showed additional

Table 2: The results of the determination for isoxicam and its degradation products in HCl and NaOH solutions at 120 °C

	Concentration [%]					
	Time [h]	**Imp.A ($R_F \approx 0.12$)**	**Imp.B ($R_F \approx 0.30$)**	**Imp.C ($R_F \approx 0.41$)**	**Isoxicam ($R_F \approx 0.56$)**	**Imp.D ($R_F \approx 0.65$)**
1 M HCl	1	1.73	–	–	98.27	–
	4	3.91	–	–	96.09	–
	8	5.57	–	–	93.83	0.60
	24	6.76	–	–	91.11	2.13
0.5 M HCl	1	–	–	–	100	–
	2	1.16	–	–	98.84	–
	4	2.73	–	3.24	94.03	–
	8	4.34	–	7.34	88.32	–
	24	4.24	–	7.25	84.33	4.18
0.1 M HCl	1	–	–	–	100	–
	2	5.90	–	–	94.10	–
	6	5.86	–	0.12	94.02	–
	8	6.54	–	1.10	90.66	1.70
	24	8.12	–	1.98	82.28	7.62
0.1 M NaOH	1	5.86	–	17.85	76.29	–
	4	27.10	–	12.53	50.35	10.02
	8	98.22	–	–	0.07	1.71
	24	100.00	–	–	–	–
0.5 M NaOH	1	9.92	8.24	9.99	71.85	–
	2	29.59	10.47	22.24	21.08	16.62
	8	89.94	–	1.12	–	8.94
	24	95.03	–	–	–	–
1 M NaOH	1	4.35	10.95	4.40	40.07	40.23
	2	13.46	6.05	6.84	35.29	38.36
	8	99.88	–	–	0.12	–
	24	100.00	–	–	–	–

"0" – 100% of isoxicam

peaks with various R_F values, next to the peak of isoxicam (Figure 3).

The concentration of the drug was found to be changing from the initial concentration indicating that isoxicam undergoes degradation under acidic and basic and heat conditions. The spots of degraded products were well-resolved from the drug spot. Resolution factor values (R_s) were in the range from 0.9 to 2.1 implying that all spots were sufficiently separated. The level of degradation at different stress conditions, and the number of degradation products with their R_F values are shown in Tables 1 and 2.

Insight into the degradation process was determined based on the decreasing concentration of isoxicam and increasing concentration of degradation products. The natural log (ln) of percentage drug remaining when plotted against time gave a straight line, which indicated that the degradation process of isoxicam followed first-order kinetics at selected temperatures.

In the chromatograms obtained under conditions of the acid and basic degradation, four new products ($R_{F(IA)} \approx 0.12$, $R_{F(IB)} \approx 0.30$, $R_{F(IC)} \approx 0.41$ and $R_{F\ (ID)} \approx 0.65$) were observed. The number and amounts of the degradation products of isoxicam changed with hydrochloric acid and sodium hydroxide concentrations, temperature and incubation time (Tables 1, 2). It was found that within the studied HCl and NaOH concentrations, degradation of isoxicam in basic solutions happens more quickly than in acidic solutions. The obtained kinetic and thermodynamic parameters are presented in Table 3.

Isoxicam is completely decomposed in 1 mol l^{-1} NaOH after 8 h whereas shorter incubation times and lower basicity show less degradation at a slower rate. In HCl hardly any degradation takes place, independent of acidity and incubation time. The degradation products have been identified using characteristic parameters such as R_F values, UV spectra, and, additionally by HPLC-MS/MS analysis (Figures 1–4). The results indicate the products

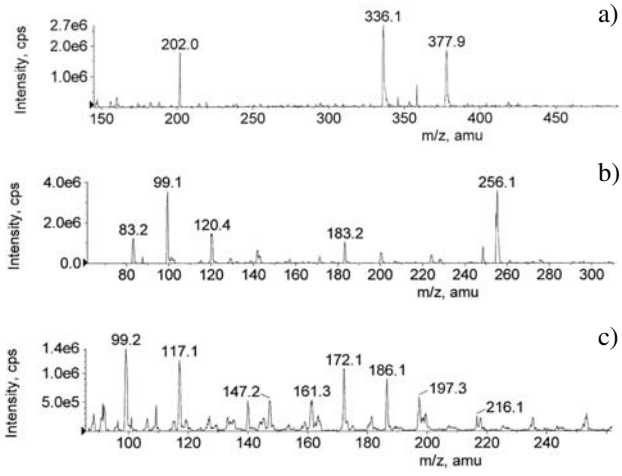

Figure 4. Product ion mass spectra of tested samples in ESI mode (a-alkaline solution; b, c-acidic solution).

Table 3: Kinetic and thermodynamic parameters describing degradation of isoxicam.

Temperature	HCl or NaOH concentration / Kinetic parameters	
60 °C	**3 M HCl**	**3 M NaOH**
	$k = 5.48 \cdot 10^{-4}$	$k = 2.95 \cdot 10^{-3}$
	$t_{0,1} = 192.15$	$t_{0,1} = 35.69$
	$t_{0,5} = 1264.60$	$t_{0,5} = 234.92$
	1 M HCl	**1 M NaOH**
	$k = 7.11 \cdot 10^{-4}$	$k = 2.40 \cdot 10^{-3}$
	$t_{0,1} = 148.10$	$t_{0,1} = 43.88$
	$t_{0,5} = 974.68$	$t_{0,5} = 288.75$
	0.5 M HCl	**0.5 M NaOH**
	$k = 1.06 \cdot 10^{-3}$	$k = 2.04 \cdot 10^{-3}$
	$t_{0,1} = 99.34$	$t_{0,1} = 51.62$
	$t_{0,5} = 653.77$	$t_{0,5} = 339.71$
120 °C	**1 M HCl**	**1 M NaOH**
	$k = 1.01 \cdot 10^{-2}$	$k = 8.41 \cdot 10^{-1}$
	$t_{0,1} = 10.43$	$t_{0,1} = 0.13$
	$t_{0,5} = 68.61$	$t_{0,5} = 0.82$
	0.5 M HCl	**0.5 M NaOH**
	$k = 1.40 \cdot 10^{-2}$	$k = 4.32 \cdot 10^{-1}$
	$t_{0,1} = 7.52$	$t_{0,1} = 0.24$
	$t_{0,5} = 49.50$	$t_{0,5} = 1.60$
	0.1 M HCl	**0.1 M NaOH**
	$k = 1.83 \cdot 10^{-2}$	$k = 3.03 \cdot 10^{-1}$
	$t_{0,1} = 5.75$	$t_{0,1} = 0.35$
	$t_{0,5} = 37.87$	$t_{0,5} = 2.29$
$E_a = 4.81\ \ 10^4$ (1 M HCl)		
$E_a = 10.63\ \ 10^4$ (1 M NaOH)		

k – the stability constants [h^{-1}]; $t_{0,1}$ – the time, concentration will after which about 10% [h];
$t_{0,5}$ – the time, concentration will decrease about 50% [h]; E_a – the energy of activation [J/mol K]

ion mass spectra obtained from collision-induced dissociation of the protonated molecular ions of isoxicam.

The positive ionization of isoxicam produced abundant protonated molecular ions $[MH]^+$. The protonated molecules at m/z 336 represent isoxicam. Under the experimental conditions, the ion mass spectra of degradation products of isoxicam produced an intense fragments at m/z 99 and 256 created by the division of an amide bond. The obtained MS spectra also indicate the presence of other ions, i.e. at m/z 186, 202, 216. Analyzing the degradation process of isoxicam by TLC revealed the presence of four additional peaks, next to the peak derived from isoxicam, i.e. Imp.A, Imp.B, Imp.C and Imp.D. The proposed degradation pathway for isoxicam so may lead to the creation of 5-methyloxazol-2-ylamine (m/z 99) and 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylic acid 1,1-dioxide (m/z 256), and other products, less persistent in term of the assay. In Figure 5 the possible isoxicam decomposition reactions have been summarized.

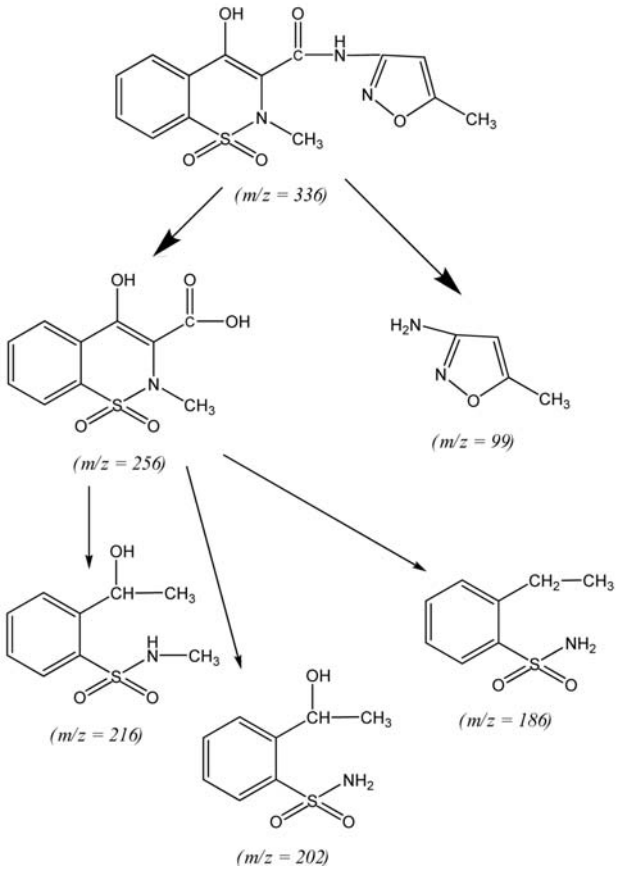

Figure 5. The scheme of the proposed degradation pathway of isoxicam in solutions under stress conditions.

As the reported methods could effectively separate the drug from its degradated products, it can be employed as a stability-indicating one. The system suitability tests performed verified the resolution, efficiency and repeatability of the chromatographic system.

4. Conclusion

The novel TLC-densitometry method developed is simple, rapid and specific for the simultaneous determination of isoxicam and its degradation products. It was demonstrated that this method allows for the selective determination of these analytes without any interference from the peaks of matrix components, and is useful for the routine application in the laboratory. The analyst can monitor any of the studied compounds with a single sample preparation protocol. The presented method can be used to determine the purity of the drug available from various sources by detecting the related impurities. The above results showed the suitability of the method for the kinetic study of isoxicam. As the method separates the drug from its degradation products, it can be employed as a stability-indicating one.

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Povzetek

Razvili in validirali smo enostavno, občutljivo in natančno TLC denzitometrično metodo za kvantitativno določanje izoksikama. Za ločbo na TLC silikagel 60 F_{254} ploščah smo kot topilo za razvijanje uporabili etil acetat : toluen : butilamin (2:2:1, v/v/v). Kvantitativno vrednotenje na osnovi denzitometrije smo izvedli pri λ = 350 nm. V okviru validacije smo testirali naslednje analizne parametre: natančnost (RSD < 1 %), specifičnost, mejo zaznave (0,22 μg / nanos) in meja določanja (0,67 μg / nanos), linearnost (0,7 do 2,2 μg / nanos). S to metodo smo produkte nastale po kisli in bazični hidrolizi izoksikama pri različnih temperaturah ločili od izoksikama in jih identificirali na osnovi R_F vrednosti, absorpcijskih spektrov in HPLC-MS/MS analize. Razvita TLC denzitometrična metoda se lahko uporablja za identifikacijo in določanje izoksikama v zdravilih, za študije stabilnosti in kinetike razgradnih procesov ter kot presejalna metoda v farmakoloških raziskavah.