Acta Poloniae Pharmaceutica – Drug Research, Vol. 70 No. 4 pp. 643–652, 2013
ISSN 0001-6837
Polish Pharmaceutical Society

DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING TLC-DENSITOMETRIC METHOD FOR DETERMINATION OF BETAXOLOL WITH LC-ESI/MS ANALYSIS OF DEGRADATION PRODUCT

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Abstract: The purpose of this work was to develop a sensitive stability indicating TLC-densitometric method for the determination of betaxolol (Bx) in pharmaceutical preparations and to study the stability of Bx in acidic solutions. The method was developed on TLC aluminium plates precoated with silica gel F_{254} using the mobile phase chloroform-methanol-ammonia 25% (18 : 4 : 0.2, v/v/v) which gives compact spots for Bx ($R_f \cong 0.64$) and its degradation product ($R_f \cong 0.39$). Densitometric analysis was carried out in UV at 280 nm. The developed method is highly sensitive (LOD = 66.6 ng/spot, LOQ = 200 ng/spot), precise (RSD = 2.73%) and accurate (mean recovery = 100.28% at 100% level). Bx was subjected to acidic and alkaline hydrolysis but degradation was observed only in acidic solutions. The degradation process was described with kinetic and thermodynamic parameters. Based on LC-ESI/MS analysis, it was found that Bx decomposes in acidic solution to produce ethoxyphenoxy-3-[(1-methylethyl)amino]propan-2-ol.

Keywords: betaxolol HCl, drug analysis, thin-layer chromatography, densitometric detection

Betaxolol (Bx) is a member of β-blockers, a group of drugs frequently used in therapy of cardiac arrhythmias, arterial hypertension, angina pectoris, myocardial infarction and related conditions. The cardioselective β_1-blocker betaxolol (1-{4[2-(cyclopropylmethoxy)ethyl]phenoxy}-3-(propan-2-ylamino)propan-2-ol) also has membrane-stabilizing action on myocardial muscle fibrils. Some of these drugs have been misused by athletes or horses participating in highly competitive events to decrease muscular convultions and palpitations. Bx as preparation for eye is currently used for treatment of glaucoma due to its high efficiency in intra-ocular pressure decreasing effect (1). Topically administered Bx enters the eye through local absorption, and it may reach concentrations in the pharmacologically active range within many anterior and posterior segment tissues.

Bx is a highly lipophilic compound (experimental log P = 2.4), freely soluble in water, soluble in ethanol and methylene chloride (2, 3).

A large number of reports have been published about the determination of Bx. Spectrophotometric methods have been applied for the determination of Bx in pharmaceutical preparations and in bulk drug (4-6). The screening analyses of multiple β-blockers in a single run have been mostly achieved by HPLC [7-11]. Bx and 15 other β-blockers presenting a wide range of lipophilicity have been determined in urine samples by GC/MS and LC/MS to compare the limits for doping tests [12]. HPLC with fluorescence detection has been applied for the determination of Bx in human aqueous humor [13]. Residues of Bx and 18 other β-blockers have been determined in animal tissues by HPLC coupled with tandem mass spectrometry [14]. Bx in ophthalmic solutions has also been determined by TLC-densitometric and videodensitometric method [15]. In our previous publications, we have investigated the influence of basic and acidic environment on the degradation process of atenolol, acebutolol and propranolol and the correlation between stability described by kinetic parameters and polarity of drugs [16, 17]. To the authors' knowledge no papers concerning degradation studies of Bx under different conditions with the establishing of degradation pathway are available in the literature.

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The aim of this work was the development and validation of a TLC-densitometric method for the determination of Bx in pharmaceutical preparations and in the presence of its degradation product. Additionally, Bx was forcibly degraded in acidic environment and the developed method was used to detect and quantitatively estimate the degradation product generated. The degradation product of Bx was subjected to LC-ESI/MS analysis to establish its chemical structure. Bx degradation process and identification of degradation product described in this work were not a subject of studies of any other authors.

EXPERIMENTAL

Equipment

Densitometer TLC Scanner 3 with Cats 4 software (Camag, Switzerland); sample applicator Linomat V (Camag, Switzerland); Silica gel aluminium TLC F_{254} plates No. 1.05554 (Merck, Darmstadt, Germany); TLC glass chamber of 18 × 8 × 15 cm in size (Sigma-Aldrich, St. Louis, USA); Incubator ECOCELL55-BMT (Brno, Czech Republic); Mass spectrometer API 2000 (Applied Biosystems MDX SCIEX, Concorde, Ontario, Canada); HPLC system with Xterra column Waters) and DAD detector (Agilent Technologies, Waldbronn, Germany).

Reagents and chemicals

Standard substance: betaxolol (1-{4[2-(cyclopropylomethoxy)ethyl]phenoxy}-3-(propan-2-ylamino)propan-2-ol) hydrochloride (LGC Standards, USP106990-3). Reagents: methanol, chloroform, formic acid, acetonitrile and water (Merck, Darmstadt, Germany), sodium hydroxide, ammonia and hydrochloric acid (POCh Gliwice, Poland).

Preparations used for analysis

The following preparations were analyzed: Lokren – tablets cont. 20 mg of betaxolol hydrochloride s. AR95HR (Sanofi Aventis, Rzeszów, Poland); Lokren - tablets cont. 20 mg of betaxolol hydrochloride s. 23001 (Synthelabo groupe, Tours, France); Optibetol 0.5% - eye drops cont. 5 mg/mL of betaxolol s. 01Y00108 (Warszawskie Zakłady Farmaceutyczne Polfa, Warszawa, Poland).

Solutions

Standard solutions were prepared by dissolving appropriate amount of betaxolol standard substance in methanol to give a concentration of 0.01% (w/v). Sample solutions of Lokren were prepared by grinding 10 tablets in a mortar, weighing the amount of tablet powder corresponding to 25 mg of Bx and shaking with 50 mL of methanol for 30 min. Then, the solution was placed in ultrasonic bath for 30 min, after that it was filtered and diluted with methanol at the ratio of 1 : 5, v/v.

Sample solutions of Optibetol were prepared by dilution of 1 mL of preparation solution with methanol at the ratio of 1 : 50, v/v.

TLC conditions

The conditions for the analysis of Bx were established by experimental selection of the appropriate stationary phase and mobile phase enabling the separation of active substance and its possible degradation products. Standard solutions in the amount of 20 μL were applied in the form of 10 mm bands, 10 mm apart, 10 mm from the lower edge of the plate to chromatographic plates TLC silica gel 60 with F_{254} fluorescence agent (Merck, Darmstad, Germany) 10 × 10 cm in size using sample applicator Linomat V equipped with 100 μL syringe. A constant application rate of 200 nL/s was used. Chromatograms were developed over a distance of 95 mm using chloroform-methanol-ammonia 25% (18 : 4 : 0.2, v/v/v) mobile phase in chromatographic chamber saturated for 15 min with mobile phase vapor at room temperature. Each chromatogram was developed separately and every time a freshly prepared mobile phase was used. After development, the TLC plates were dried at room temperature and analyzed densitometrically in UV at λ = 280 nm, that was chosen as the analytical wavelength on the basis of absorption spectra of Bx recorded directly from chromatogram. Densitometric scanning was carried out using a CAMAG TLC Scanner 3, equipped with deuterium lamp, controlled by CATS 4 Software resident in the system. The slit dimensions were 8 × 0.6 mm, the scanning speed 20 mm/s and data resolution 100 μm/step. Retardation factors R_s were used for the identification of Bx and possible degradation products.

Validation of the method

The developed method was validated by the determination of specificity, accuracy, precision, inter-day precision, linearity, limit of detection and quantitation according to ICH guidelines [18].

The specificity of the method was ascertained by analysis of standard solution and drug samples. The spots were identified by comparison of the R_f values and spectra of Bx with those obtained for the

standard. For extracted solution used for determination purposes, no additional spots were found and no visible interferences from excipients were observed.

The linearity was expressed as a relationship between peak area and analyte concentration within a specified measuring range. To determine linearity, a series of five solutions (three bands per concentration) at a concentration ranging from 0.2 μg/spot to 2.5 μg/spot for Bx were prepared. The regression plot, its regression equation and the correlation coefficient r = 0.9949 are indicative of linearity.

Limits of detection (LOD) and quantitation (LOQ) were determined on the basis of the standard deviation and slope of the straight line obtained from the equations: LOD = $3.3 \times S_e/a$, LOQ = $10 \times S_e/a$, where: S_e – the standard error of the estimate, a – the slope of a straight line.

The accuracy of the method was defined as % recovery of Bx added at three levels 80, 100 and 120% into a sample containing a known amount of Bx.

Precision of the method was carried out using six replicates of a sample solutions on the same day (intra-day precision). The degree of consistency of the results obtained for the analyte samples was checked by different analyst who made the analysis in one week interval (inter-day precision). The precision was estimated using peak areas and was evaluated as the standard deviation (SD) and relative standard deviation (RSD) values.

To examine robustness, the most significant chromatographic parameters were changed within the range 1-5% compared to those of the optimal conditions, while keeping the other parameters unchanged. The following parameters were changed: volume of chloroform, volume of methanol. The influence of stationary phase was also checked by application of silica gel aluminium HPTLC F_{254} plates (Merck, Germany; #1.05548.0001) instead of silica gel aluminium TLC F_{254} plates.

As a result of validation process analysis conditions were established, which were used for the determination of Bx.

TLC analysis of betaxolol in drugs

Solutions of Bx (20 μL of standard solution and 20 μL of sample solutions for determination of active substance) were applied with an applicator to the plates of 10 × 10 cm in size (cut from 20 × 20 cm plates before use). Chromatograms were developed to a distance of 95 mm with chloroform-methanol-ammonia 25% (18 : 4 : 0.2, v/v/v) as a mobile phase. Densitometric measurements were carried out by scanning chromatograms at λ = 280 nm. The concentration of betaxolol in tested preparations was computed by comparing the peak areas for standard and sample solutions.

Stability studies of betaxolol in acidic solutions

The influence of pH, temperature and incubation time on stability of Bx in solutions was investigated using newly developed and validated method [19]. For this purpose samples were prepared by mixing 2 mL of Bx stock solution 0.6% (w/v) with 2 mL of 2.00 M HCl, 1.00 M HCl and 0.50 M HCl in glass ampoules and heated in an electronic incubator up to the desired temperature. Temperature selection and incubation time were set experimentally. Incubation was performed at temperatures of 90, 100 and 110°C. The amount of 500 μL of incubated solutions were taken for the analysis. After cooling, the solutions were dissolved with methanol at 1 : 1 ratio. The amount of 20 μL of the resultant solutions were applied to the TLC plates in triplicate. The chromatographic-densitometric analysis was carried out according to the developed method. For quantitative determination in degradation studies, the scan areas of appropriate peaks were recorded, and the percent concentrations of each constituent were computed by employing the internal normalization method, according to the formula: $\%I = (x_i/Sx) \times 100$ where %I is constituent concentration, x_i is peak area for the determined constituent, and Sx is sum of peak scan areas in the chromatogram.

Analysis of degradation product

The obtained hydrolizates after incubation at 100°C were subjected to LC-ESI/MS analysis. Identification of studied drug and its degradation product was done from mass spectra obtained directly from chromatographic peaks in their appropriate retention times using a validated high-performance liquid chromatography tandem mass spectrometry method in the positive ionization mode.

Liquid chromatography was performed using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) LC system consisting of degasser, a gradient pump, an autosampler and a DAD detector. Chromatographic separation was carried out with a commercially available XBridge C18 column (30 mm × 2.1 mm, 3.5 μm, Waters, Ireland) set at 30°C. A sample volume of 20 μL was injected onto an analytical column. The mobile phase consisting of acetonitrile and water with an addition of 0.01% formic acid was set at a flow rate of 0.6 mL/min using a gradient elution 0–5 min water, 5–7 min acetonitrile, 7–20 min water.

Mass spectrometric detection was performed on an Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 2000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface, performed in the positive ion 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 from 50 to 600 amu.

The mass spectrometric conditions were optimized by continuously infusing the standard solution of Bx using a Harvard infusion pump set at 10 µL/min. Parameters of ion source were: ion spray voltage 5500 V, gas 1 – 20 psi, gas 2 – 25 psi, ion source temperature 550°C, curtain gas – 6 psi. Parameters of ion path were: declustering potential 61 V, focusing potential 360 V, entrance potential 12 V and electron multiplier 2300 V.

Data acquisition and processing were accomplished using the Applied Biosystems Analyst version 1.4.2 software.

Table 1. Summary of validation results for the determination of Bx

Parameter	Result
R_F	0.64
	Linearity [µg/spot] 0.2 – 2.5 P = a c + b a = 84.087; b = 352.5 r = 0.9949
LOD [ng/spot]	66.6
LOQ [ng/spot]	200
Accuracy [%] n = 6	Level 80%: x_{mean} = 98.27 SD = 0.84, RSD = 0.85% Level 100%: x_{mean} = 100.28 SD = 1.36, RSD = 1.36% Level 120%: x_{mean} = 98.95 SD = 1.20, RSD = 1.21%
Intra-day precision n = 6	x_{mean} = 2110.9 SD = 48.43, RSD = 2.29% µ = 2110.9 ± 124.51
Inter-day precision n = 6	x_{mean} = 2116.7 SD = 27.31, RSD = 1.29% µ = 2116.7 ± 70.21

P - peak area; c - concentration; r - correlation coefficient; SD - standard deviation; m - 95% confidence interval; RSD - relative standard deviation (%).

Table 2. The results of Bx determination in pharmaceutical preparations with statistical analysis.

Preparation	Declared content	Determined content		Statistical analysis (n = 6)
Optibetol 0.5%	5 mg/mL	4.69 4.75 4.82	4.85 4.91 4.83	x_m = 4.8083 SD = 0.0776 RSD = 1.61%
Lokren Sanofi Aventis	20 mg/tablet	18.51 18.20 19.31	18.85 18.74 19.31	x_m = 18.8200 SD = 0.4400 RSD = 2.34%
Lokren Synthelabo group	20 mg/tablet	19.57 18.34 18.85	18.72 18.95 18.24	x_m = 18.7783 SD = 0.4789 RSD = 2.55%

x_m - arithmetic mean, SD - standard deviation, RSD - relative standard deviation (%).

RESULTS AND DISCUSSION

According to the aim of this paper a new chromatographic-densitometric method has been developed for the determination of Bx in pharmaceutical preparations and for stability studies of Bx in acidic environment. By using TLC F_{254} silica gel coated plates as a stationary phase and chloroform-methanol-ammonia 25% (18 : 4 : 0.2, v/v/v) as the mobile phase chosen experimentally after testing several different mobile phases, dense, compact and well shaped bands of Bx were obtained (Fig. 1). No significant changes in the R_f of Bx were observed when the composition of the mobile phase was slightly changed. Densitometric analysis was carried out at λ = 280 nm, that was chosen as the analytical wavelength on the basis of absorption spectra of Bx recorded directly from chromatogram (Fig. 2). The

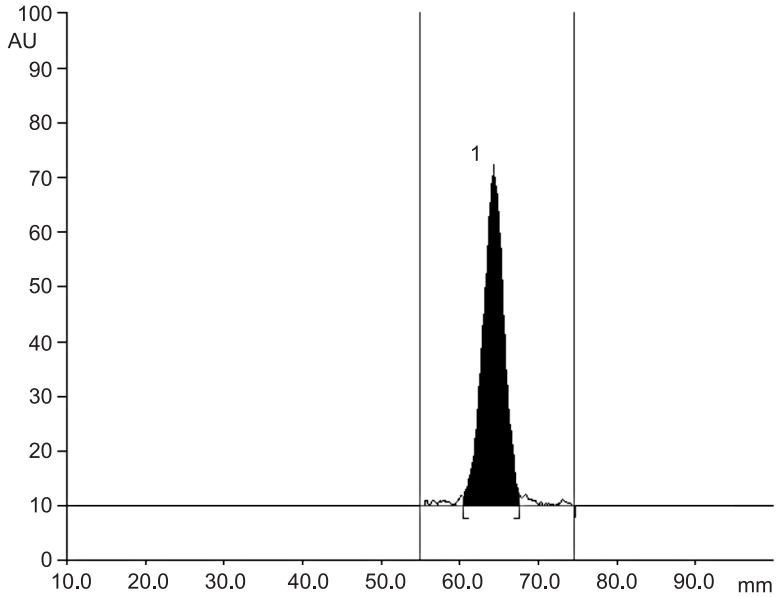


Figure 1. Densitogram registered for Bx standard solution

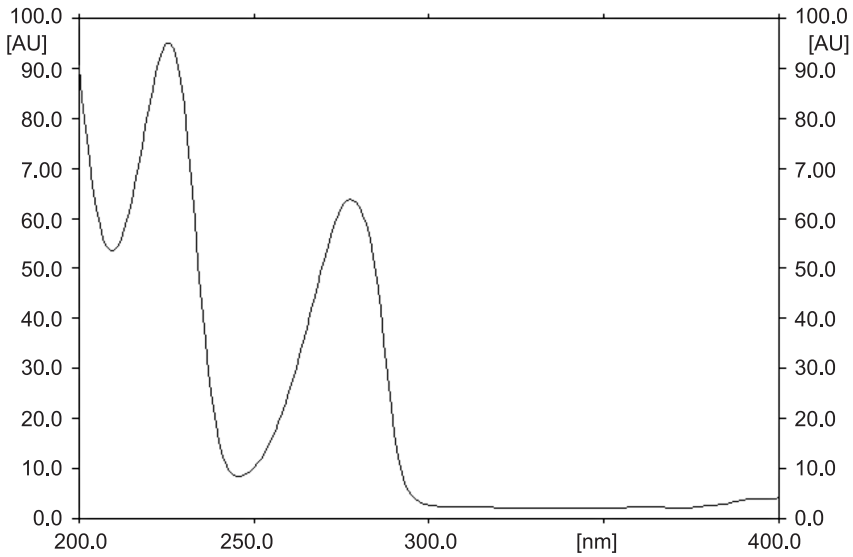


Figure 2. Absorption spectrum obtained from Bx standard solution registered directly from chromatogram

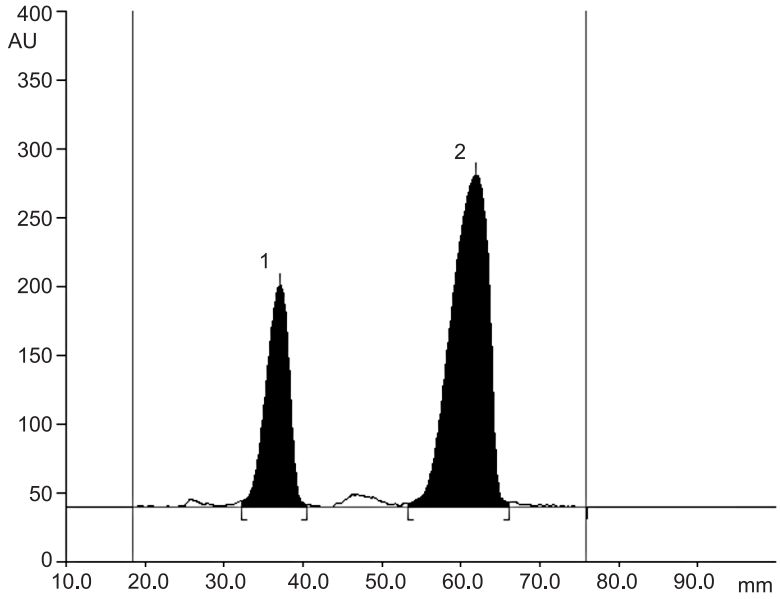


Figure 3. Densitogram of Bx (2) and its degradation product (1) after incubation for 3 h in 0.5 M HCl at 100°C

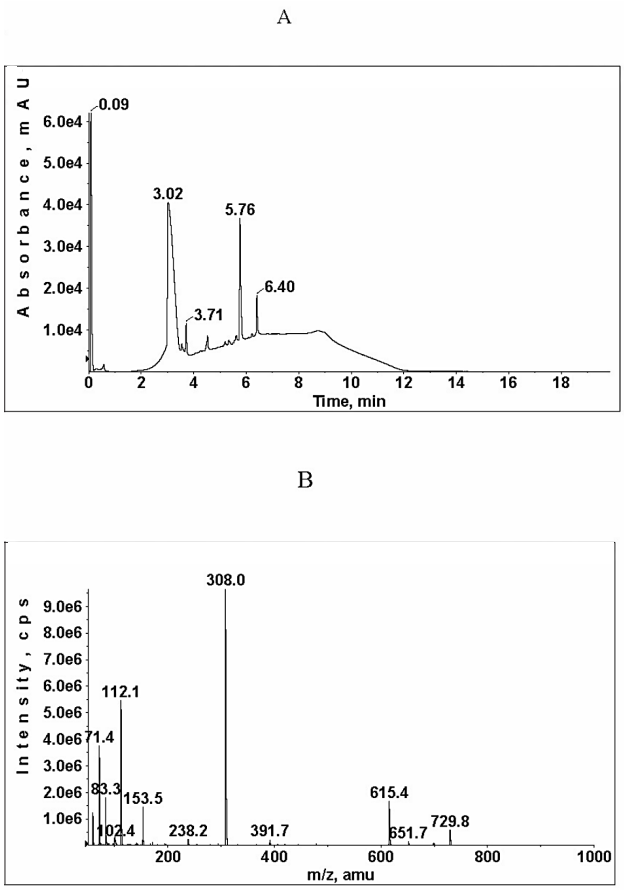


Figure 4. Chromatogram of control sample of Bx (A), mass spectrum of control sample of Bx at retention time of 3.02 min (B)

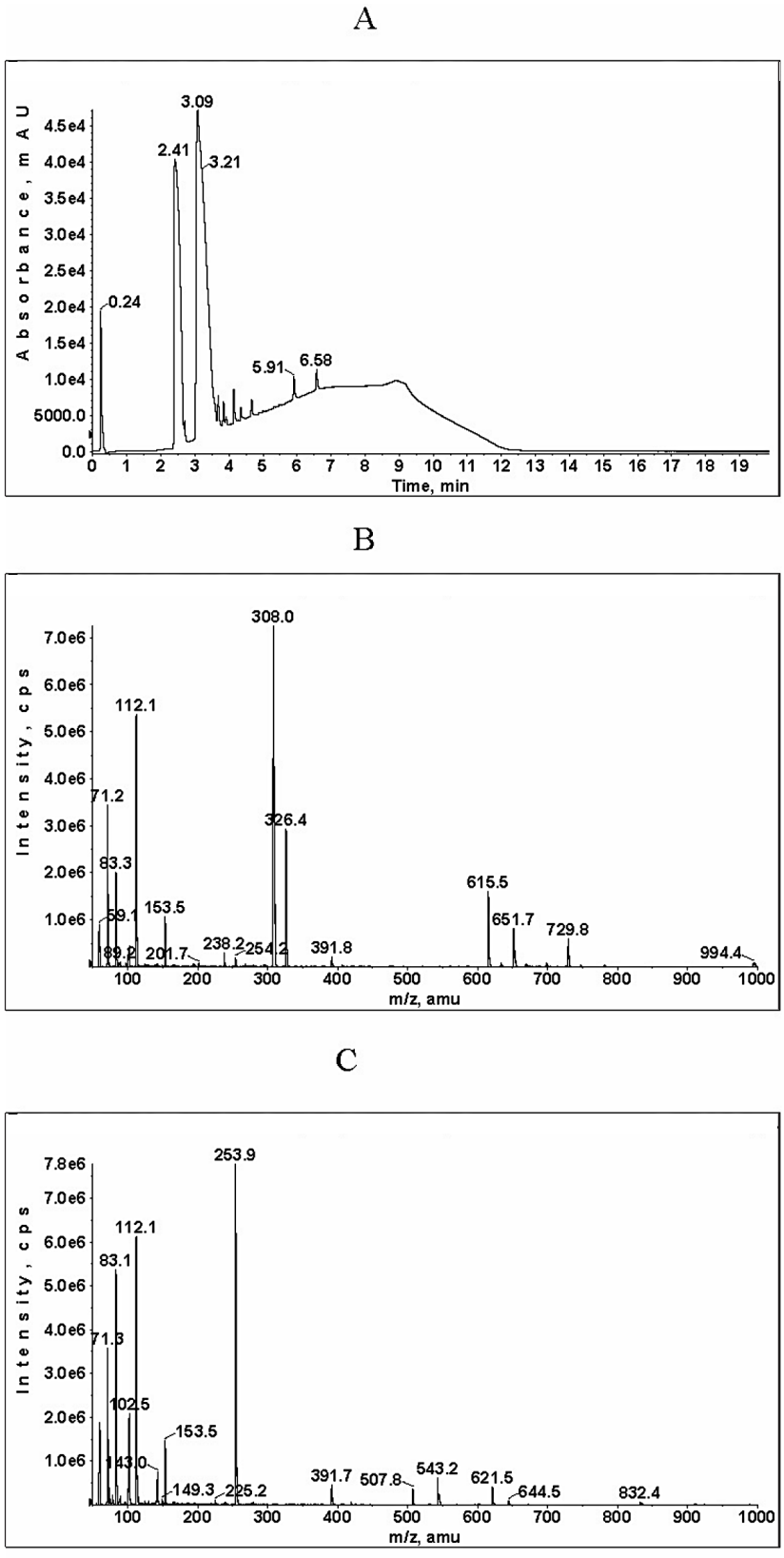
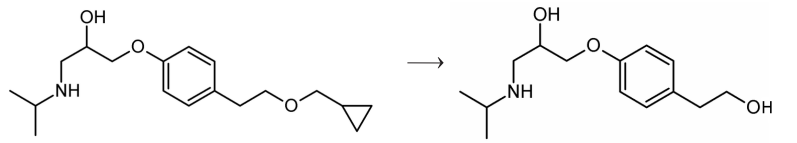


Figure 5. Chromatogram of Bx sample in acidic conditions (A), mass spectrum of studied sample of Bx at retention time of 3.09 min (B)



Betaxolol, m/z 308 amu ethoxyphenoxy -3-[(1-methylethyl)-amino]propan-2-o1, m/z 254 amu

Figure 6. The plausible degradation pattern of Bx in acidic conditions

Table 3. Results from Bx stability studies in acidic environment.

Bx mean concentration [%] n = 3				
HCl [mol/L]	Time [h]	90°C	100°C	110°C
0.25	0	100	100	100
	1	100	92.15	93.14
	2	98.59	91.44	80.08
	3	96.23	88.30	60.90
0.50	0	100	100	100
	1	95.86	84.60	86.70
	2	90.95	80.22	61.69
	3	84.72	75.09	42.38
1.00	0	100	100	100
	1	83.44	69.80	69.25
	2	73.84	58.84	31.53
	3	67.94	51.61	9.69

developed method was proven to have high sensitivity, wide linearity range, good accuracy and precision by the validation results (Table 1).

The determination results of Bx in available pharmaceutical preparations obtained by the developed method do not differ from those declared by manufacturers (Table 2).

The influence of pH, temperature and incubation time on stability of Bx in solutions was investigated using newly developed and validated method. Temperature selection and incubation time were set experimentally. Incubation was performed at temperatures of 90, 100 and 110°C for acidic solutions (Table 3). The densitogram presented in Figure 3 shows besides Bx (R_f = 0.64) additional well resolved peak representing degradation product (R_f = 0.39) appearing in acidic environment. The relationship between Bx concentration and time, log(c) = f(t) showed that degradation of Bx follows the kinetics of first order reaction. To obtain full characteristics of degradation process, kinetic parameters (the reaction rate constants k, half-life $t_{0.5}$ and the time $t_{0.1}$ in which the concentration of betaxolol is reduced by 10%) and thermodynamic parameters were calculated [20]. The fastest degradation of Bx was observed in acidic solution (1 M HCl) at 110°C k = 0.78 h^{-1} and the lowest degradation of Bx was in acidic solution (0.25 M HCl) at 90°C k = 0.012 h^{-1}. The results presenting kinetic evaluation of degradation studies of Bx are shown in Table 4.

The obtained degradation product was subjected to HPLC/ESI-MS analysis in order to identify its molecular weight. Figure 4A illustrates the HPLC diode array UV-VIS chromatogram of control sample of Bx obtained under the analytical conditions. In the HPLC/DAD chromatogram Bx was observed at retention time of 3.02 min. Figure 4B illustrates the full scan mass spectrum of control sample of Bx obtained under the LC-ESI/MS conditions. Based on mass spectra of Bx standard solution we could find out the parent drug and the occurrence of potential degradative products of Bx. For precursor ion

Table 4. Kinetic and thermodynamic parameters calculated for Bx degradation process in acidic environment.

Kinetic and thermodynamic parameters			
HCl [mol/L]	90°C 363 K	100°C 373 K	110°C 383 K
0.25	$k = 0.012\ h^{-1}$ $t_{0.5} = 57.75$ h $t_{0.1} = 8.78$ h	$k = 0.042\ h^{-1}$ $t_{0.5} = 16.50$ h $t_{0.1} = 2.51$ h	$k = 0.215\ h^{-1}$ $t_{0.5} = 3.22$ h $t_{0.1} = 0.49$ h
	$E_a = 166.80$ kJ/mol $\Delta H^{++} = 163.70$ kJ/mol		
0.50	$k = 0.06\ h^{-1}$ $t_{0.5} = 12.60$ h $t_{0.1} = 1.91$ h	$k = 0.124\ h^{-1}$ $t_{0.5} = 5.59$ h $t_{0.1} = 0.85$ h	$k = 0.287\ h^{-1}$ $t_{0.5} = 2.41$ h $t_{0.1} = 0.34$ h
	$E_a = 90.46$ kJ/mol $\Delta H^{++} = 87.36$ kJ/mol		
1.00	$k = 0.13\ h^{-1}$ $t_{0.5} = 5.37$ h $t_{0.1} = 0.82$ h	$k = 0.221\ h^{-1}$ $t_{0.5} = 3.13$ h $t_{0.1} = 0.48$ h	$k = 0.78\ h^{-1}$ $t_{0.5} = 0.89$ h $t_{0.1} = 0.14$ h
	$E_a = 103.57$ kJ/mol $\Delta H^{++} = 100.47$ kJ/mol		

k - reaction rate constant, $t_{0.5}$ - half-life time, $t_{0.1}$ - time in which concentration of Bx is reduced by 10%, E_a - activation energy, ΔH^{++} - activation enthalpy.

molecular mass, m/z 308 amu eluted at retention time of 3.02 min. was observed.

Figure 5A shows the HPLC diode array UV-VIS chromatogram of Bx after degradation in acidic solution at 100°C. In the chromatogram there are two main peaks observed at retention time of 3.09 and 2.41 min. Figure 5B confirms the existence of the parent drug with molecular mass m/z 308 amu. Moreover, in Figure 5C at retention time of 2.41 min we could observe the elution of the fragment with molecular mass m/z 253.9 amu, probably due to the loss of cyclopropylmethyl moiety from parent structure. The intensity of main degradation product in acidic conditions was very high. The analysis of chemical structure of the main degradation product revealed that Bx decomposes to ethoxyphenoxy-3-[(1-methylethyl)amino]propan-2-ol. The LC-ESI/MS analysis enabled to create the plausible degradation pattern of Bx under mentioned conditions that is presented in Figure 6.

CONCLUSIONS

The developed method may be applied for the determination of Bx in pharmaceutical preparations and for stability studies of Bx by quantitative determination of Bx in the presence of its degradation product. It was found that degradation of Bx depends on the acidic environment but in basic solutions Bx is very stable. Calculated kinetic and thermodynamic parameters describe the degradation process of Bx and HPLC/ESI-MS analysis enabled to establish chemical structure of the main degradation product.

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Received: 26. 10. 2012