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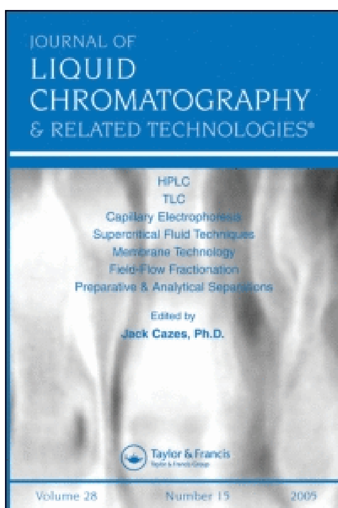

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Enantioselective HPLC Analysis and *In-vitro* Kinetic Study Decomposition of Potential *β*-Blocker Drug in Guinea Pig Serum

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Abstract: A method using off-line solid-phase extraction and liquid chromatography has been developed for the analysis of enantiomers of derivatives of aryloxyaminopropanol in guinea pig blood serum. The paper presents the results of HPLC enantioseparation of the derivative of aryloxyaminopropanol obtained by using of four chiral stationary phases [macrocyclic antibiotic (vancomycin, teicoplanin, teicoplanin aglycone, and methylated teicoplanin aglycone)] and the mixture of methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v) as mobile phase for all tested stationary phases. The presence of saccharide moieties in the chiral stationary phase (vancomycin and teicoplanin chiral stationary phases) plays an important role, together with charge interactions and steric interactions, in the separation of enantiomers of derivatives of aryloxyaminopropanol. The *in-vitro* degradation studies of individual (*R*)-enantiomer and (*S*)-enantiomer in blood serum demonstrated a difference in the final concentration of the enantiomers after the incubation at 37°C. It was found that the rate constant of the (*R*)-enantiomer is lower than the (*S*)-enantiomer.

Keywords: *β*-blocker, Degradation, Enantiomers, HPLC, Serum

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INTRODUCTION

A life is heavily dependent on chirality. The chiral discrimination is frequently encountered in the biological system. The chirality is also an important issue in the pharmaceutical industry due to the potential of different activities and toxicities of drug enantiomers.

β-blockers are clinically important drugs and are used in the treatment of disorders such as hypertension, congestive hearth failure, and abnormal heart rhythms, relieve angina, and prevent cardiac infarctions in humans. They work by blocking the effects of adrenaline on our body β-receptors, thereby slowing the nerve impulses to the heart and reducing its workload. β-Blockers of aryloxyaminopropanol type are drugs with a single stereogenic center and exhibit a chiral structure. From the viewpoint of β-adrenolytic activity, the (*S*)-enantiomer is several times more effective and, in many β-blockers, show different therapeutical indications. With the exception of penbutolol, levobutolol, or timolol,[1] all β-blockers are used in the form of racemic mixtures containing 50% of the more potent β-receptor blocking (*S*)-isomer and 50% of the (*R*)-isomer, which is less or mostly without β-blocking action.[2]

The studied derivative of aryloxyaminopropanol was synthesized as the analogue of the therapeutically used β-blocker drug celiprolol. The chemical structure of the studied drug is illustrated in Figure 1. From the relationship of the β-blocking activity on the structure of the molecule, we note that the derivatives containing the acyl group in the ortho position and with substitution in the para position are more pharmacologically effective, from the viewpoint of β-adrenolytic activity.[3]

Current analytical methods usually involve high performance liquid chromatography (HPLC) combined with electrochemical detection,[4] fluorimetric detection, and/or UV detection[5–7] for the determination of β-blockers. Several chiral stationary phases (CSPs) have been used for the separation of enantiomers of β-blocking drug substances: chiral

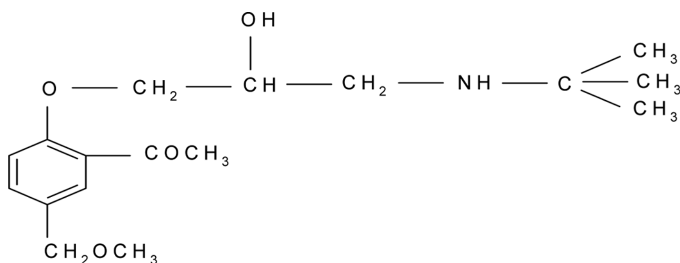

Figure 1. The structure of studied potential β-blocker of aryloxyaminopropanol type.

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phases consisting of immobilized proteins,[8–10] β-cyclodextrin,[11,12] Pirkle type phases,[13,14] and cellulose phases.[15,16]

This paper presents the study of the enantioseparation of aryloxyaminopropanol derivative on various types of chiral stationary phases, macrocyclic antibiotics-vancomycin, teicoplanin, teicoplanin aglycone, methylated teicoplanin aglycone, using high performance liquid chromatography combined with photodiode-array UV detection and the study of stability of enantiomers in blood serum.

EXPERIMENTAL

Reagents

The potential β-blocker, [2-(2-hydroxy)-(3-*tert*-butylaminopropoxy)-5-(methoxymethyl)phenyl]ethanone (Figure 1) was prepared by four step synthesis from 2-hydroxyacetophenone as starting material according to Čižmáriková and et al.[3]

The samples of guinea pig blood serum were prepared by centrifugation ($3000 \times g$) at $-5°C$. The serum was stored in a freezer at $-8°C$.

All HPLC grade solvents, methanol, acetonitrile, were obtained from Merck (Germany). Triethylamine, for synthesis and acetic acid, glacial were obtained also from Merck (Germany).

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Instrumentation and Liquid Chromatography Conditions

Chromatographic experiments were performed with a Hewlett Packard series 1100 HPLC system (Germany) consisting of a quaternary pump equipped with an injection valve Rheodyne model 7125 (USA), diode array detector, column thermostat, and polarimetric detector Chiralyser (IBZ Messtechnik, Germany).

The macrocyclic chiral stationary phases Chirobiotic T, 250×4.6 mm I.D. 5 µm, (Astec, USA), a Chirobiotic V, 250×4.6 mm I.D. 5 µm (Astec, USA), Chirobiotic TAG, 250×4.6 mm I.D. 5 µm (Astec, USA), Chirobiotic TAG-methylated, 150×4.6 mm I.D. 5 µm (Astec, USA), were used for the separation of enantiomers of studied compound.

The mobile phase was a mixture of methanol and acetonitrile to which acetic acid and triethylamine were added (methanol/acetonitrile/acetic acid/triethylamine 45:55:0.3:0.2, v/v/v/v). The separations were carried out at the flow rate of 0.8 mL/min and the column temperature was 25°C. The chromatograms were scanned at the wavelength of 247 nm. The injection volume was 20 µL. The analytes were dissolved in methanol and filtered with a 0.45 µm filter when necessary. The retention time of

solvent peak, methanol was used for the determination of the dead time for all types of column.

For the measuring of optical rotation of the fraction of enantiomers after semipreparative chromatography the polarimeter Jasco-1010 (Japan) was used. The operating temperature was 20°C and wavelength 589 nm.

Sample Preparation

The SPE column, OASIS HLB, 1 cc, 30 mg (Watrex, Germany) or Chromabond C18 ec, 100 mg (Macherey Nagel, Germany) was conditioned with 1 mL of methanol and 1 mL of water. Blood serum, 0.5 mL, spiked with the studied analyte, racemate, (*R*)-enantiomer, (*S*)-enantiomer, at concentration level 5 or 50 µg/mL was injected into the SPE cartridge. Subsequently, the sorbent layer was washed out with 1 mL of methanol/water (5:95, v/v). Analyte retained by the sorbent was eluted with 0.5 mL of methanol. The volume of 20 µL of eluate was injected into the chiral column.

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Chromatographic Characteristics

The selectivity coefficient was expressed as $\alpha = k_2/k_1$, where k_2, k_1 are the retention factors for the second and first eluting enantiomer. The retention factors k_1 and k_2 were calculated as follows: $k_1 = (t_1 - t_0)/t_0$ and $k_2 = (t_2 - t_0)/t_0$, where t_0, t_1, and t_2 are the dead elution time and elution time of enantiomers 1 and 2.

The resolution (R_s) of the first and second eluting enantiomers was calculated by the ratio of the difference between the elution times t_1 and t_2 to the arithmetic mean of the two peak widths w_1 and w_2: $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.

RESULTS AND DISCUSSION

HPLC Separation

The separation of potential β-blocker of the aryloxyaminopropanol type (Figure 1) was accomplished by macrocyclic antibiotics, vancomycin, teicoplanin, teicoplanin aglycone (without saccharide parts), and methylated teicoplanin aglycone chiral stationary phases. A common mobile phase consisting of methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v) was used in all cases for comparison purposes. In the acidic mobile phase, the ionization of basic analyte is assured and ion interaction of the stationary phase with functional groups (secondary amine group) of derivative of aryloxypropanol can be

expected. On the base previous studies with derivatives of aryloxyaminopropanols, it was expected that the charge interaction will have a positive influence on the separation of enantiomers.[17] The presence of methanol in mobile phase supports the production of hydrogen bounds and can effect the values of enantiomer resolution. The chromatographic characteristics, retention factor, selectivity coefficient, resolution, for separation of enantiomers are summarized in Table 1. It is evident that there is no significant difference in the separation of the enantiomers on the vancomycin and teicoplanin CSPs with the mobile phase tested. The similar values of retention factors obtained on both chiral CSP, vancomycin and teicoplanin, indicate similar sorption properties of analytes to the stationary phases. The values of resolution were not significantly higher on teicoplanin CSP. Comparing the separation of enantiomers on the teicoplanin column containing carbohydrate moieties (Chirobiotic T), teicoplanin column without carbohydrate moieties (Chirobiotic TAG), and methylated teicoplanin column without carbohydrate moieties (Chirobiotic TAG-methylated) the retention factors were increased in the order:

$$\mathrm{T} < \mathrm{TAG} < \mathrm{TAG\text{-}methylated}$$

The sequence of the values of resolution on the tested three columns was inversed to the retention factors. On the basis of the results, it is evident that the highest values of resolution were obtained on the column containing carbohydrate moieties.

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Table 1. Retention factors (k_1), selectivity coefficients (α), and resolution (R_s) of racemic mixtures of studied compound on macrocyclic antibiotics chiral stationary phases in the polar organic mode[1]

	k_1	α	R_S
V[2]	2.14 ± 0.03	1.09 ± 0.02	0.28 ± 0.06
T[3]	2.68 ± 0.04	1.12 ± 0.02	1.52 ± 0.06
TAG[4]	6.15 ± 0.10	1.03 ± 0.02	0.53 ± 0.02
TAG-methylated[5]	7.16 ± 0.11	1.04 ± 0.02	0.37 ± 0.02

[1]The mobile phase for all the separations was methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v). The flow rate was 0.8 ml/min. The detection wavelength was 247 nm and the operating column temperature was 25°C.

[2]V = Chirobiotic V (250 × 4.6 mm) column with vancomycin as chiral stationary phase, $t_0 = 4.40$ min.

[3]T = Chirobiotic T (250 × 4.6 mm) column with teicoplanin as chiral stationary phase, $t_0 = 4.70$ min.

[4]TAG = Chirobiotic TAG (250 × 4.6 mm) column with teicoplanin aglicon as chiral stationary phase, $t_0 = 4.00$ min.

[5]TAG-methylated = Chirobiotic TAG-methylated (125 × 4.6 mm) column with methylated teicoplanin aglicon as chiral stationary phase, $t_0 = 2.42$ min.

The teicoplanin chiral stationary phase and mobile phase composed of methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v) were finally used for the HPLC analysis of potential drug in guinea pig blood serum. The chromatograms of the separation of enantiomers of studied compound on vancomycin, teicoplanin, teicoplanin aglycone, and methylated teicoplanin aglycone chiral stationary phases are shown in the Figure 2.

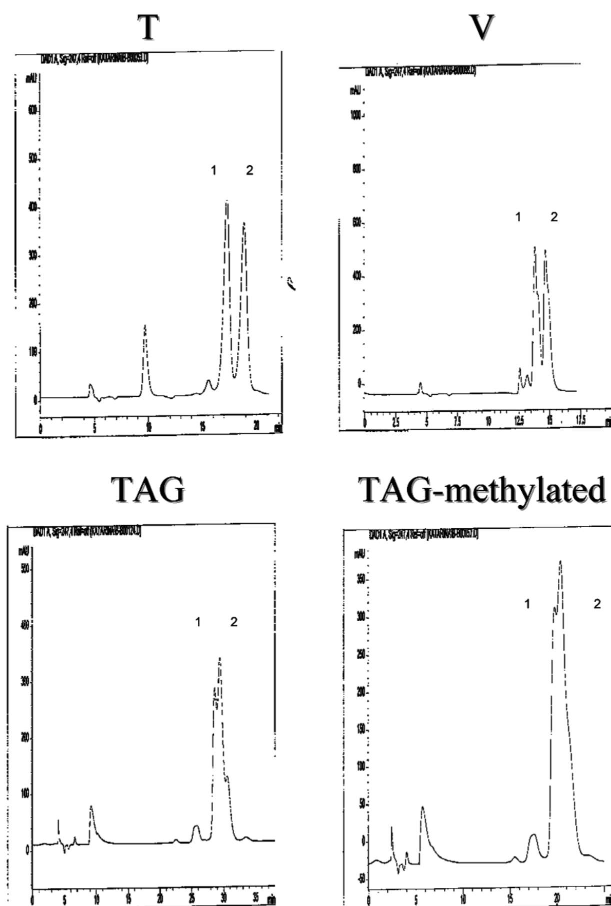

Figure 2. HPLC separation of studied potential β-blocker of aryloxyaminopropanol type on teicoplanin (T), vancomycin (V), teicoplanin aglycone (TAG), methylated teicoplanin aglycone (TAG-methylated) chiral stationary phases. Chromatographic conditions for all separations: mobile phase: methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v), flow rate: 0.8 mL/min, detection wavelength: 247 nm, column temperature: 25°C. Legend: *1*-first eluted enantiomer, *2*-second eluted enantiomer.

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Elution Order of Enantiomers

The elution order of enantiomers was determined by measuring optical rotation of fractions of enantiomers (in mobile phase) after semipreparative chromatography on teicopanin chiral stationary phases (Chirobiotic T). The chromatograms of fractions of enantiomers and racemate obtained using spectrophotometric detection are shown in Figure 3 (a, b, c). According to the results, the first eluted enantiomer has negative optical rotation and second eluted enantiomer has positive optical rotation. This corresponds with the chromatogram of racemate obtained from the separation on Chirobiotic T CSP with polarimetric detection (Figure 3d). The absolute configuration of the derivative of aryloxyaminopropanol corresponds with the optical rotation.[18] From these results the (*S*) configuration was assigne for the first eluted enantiomer and (*R*) configuration for the second eluted enantiomer.

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Solid-Phase Extraction of Potential Drug

The analytical method for selective extraction of potential drug has been developed in order to allow the quantitative determination of β-blocker in biological matrices. Several SPE procedures were available in the literature for the extraction of β-blocker from biological samples. Many of them involved the use of C18-based stationary phases such as the Octadecylsilica Isolute C18, the Bond Elut Certify LRC, Isolute IST HCX mixed-mode SPE cartridges, Oasis MCX (mixed-mode sorbent, reversed phase and cation exchanger).[19–22]

The sample of blood serum was purified by off-line solid phase extraction. Two types of sorbents were used for the sample cleanup, end-capped C18 cartridge (Chromabond C18ec) and a cartridge with hydrophilic-lipophilic properties (Oasis HLB).

To calculate the recovery of the SPE procedure, three replicates of spiked plasma samples at two different concentration levels (5 and 50 μg/mL) of racemate of β-blocker were used. These samples were compared with samples of blank plasma, which had been extracted following the same SPE procedure but spiked after the elution step. The recovery from guinea pig plasma ranged from 78.1 to 89.0% (Table 2). The recovery did not appear to be concentration dependent in the range under study. Repeatability of the extraction procedure, in terms of RSD did not exceed the 6%, as it is shown in Table 2.

The higher yields were obtained using sorbent prepared by a macroporous copolymer of lipophilic divinylbenzene and hydrophilic n-vinylpyrolidone (Oasis HLB) (84–89%). This type of cartridge was used for pretreatment of blood samples for kinetic studies.

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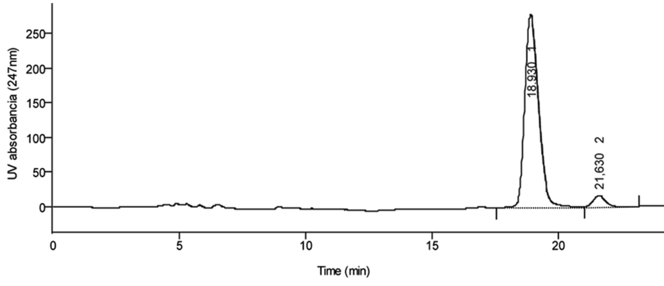
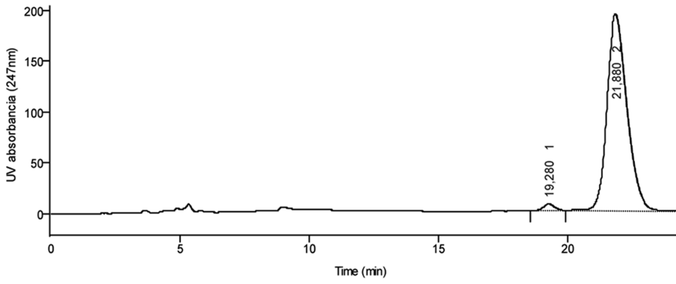


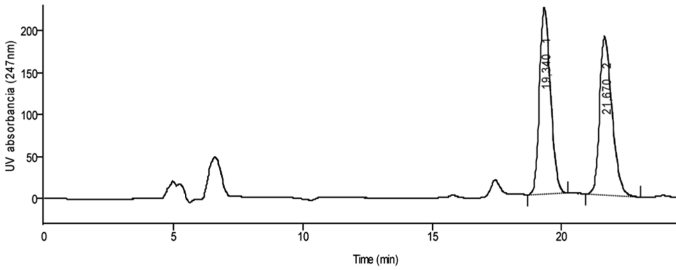
(S)-enantiomer

(a)



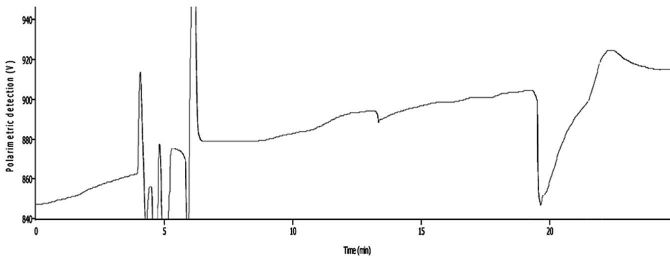
(R)-enantiomer

(b)



Racemate

(c)



Racemate

(d)

Table 2. The recovery of studied potential β-blocker from guinea pig plasma ($n = 3$)

	Concentration (μg/mL)	
	5.0	50
Chromabond C18 ec		
% Recovery	78.1	79.9
RSD (%)	5.8	4.6
Oasis HLB		
% Recovery	84.2	89.0
RSD (%)	2.5	2.7

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Method Performance: Precision, Accuracy, Detection and Quantification Limits, and Linearity

In this work, the precision was calculated for two concentration levels: 5 and 50 μg/mL of potential β-blocker (racemate). Precision was measured using three spiked plasma samples for both concentration level and each day. The evaluation of method precision was carried out in a day (intraday precision) and in three different days (interday precision) and evaluated by means of the RSD. The accuracy of the method was determined by replicate analysis of samples with standard addition of the analytes. It was tested by using three replicates for two concentration levels of potential β-blocker racemate (5 and 50 μg/mL) intra- and interday (in three different days). The results are shown in Table 3. The obtained values for the precision are less than 7% and for the accuracy less than 6%.

The limit of detection (LOD) was measured as the lowest amount of the analyte that may be detected to produce a response that is different from that of a blank (S/N = 3). The potential β-blocker enantiomer concentration obtained was 0.03 μg/mL. Limit of quantification (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise (S/N = 10). The potential β-blocker

Figure 3. HPLC chromatograms of fraction of enantiomers after semipreparative chromatography (a,b ; UV detection) and racemate (c; UV detection, d; polarimetric detection) of studied potential β-blocker. Chromatographic conditions: stationary phase: Chirobiotic T, mobile phase: methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v), flow rate: 0.8 mL/min, detection wavelength: 247 nm, polarimetric detection: range 4, column temperature: 20°C.

Table 3. The precision and accuracy obtained for the two β-blocker concentration levels ($n = 3$)

	Concentration level			
	5.0 μg/mL		50 μg/mL	
	Intra-day	Inter-day	Intra-day	Inter-day
Precision RSD (%)	5.7	6.8	4.9	6.1
Accuracy RSD (%)	4.5	5.8	3.6	5.6

enantiomer concentration obtained was 0.1 μg/mL. This concentration corresponds to the first point of the calibration curve.

The linearity of the analytical method was determined by means of the calibration curve. A regression line was fitted by applying the linear regression model based on the least square method. A blank plasma sample was spiked with the appropriate volume of the enantiomers of β-blocker to achieve concentrations of 0.1, 0.3, 0.7, 1.5, 2.9, 5.7, 11.5 μg/mL. All samples were treated and analyzed as is described in Sample Preparation. LC-DAD response (247 nm) was linear over the range from 0.1 to 100 μg/mL, with an $r > 0.998$ for enantiomers of the studied potential β-blocker.

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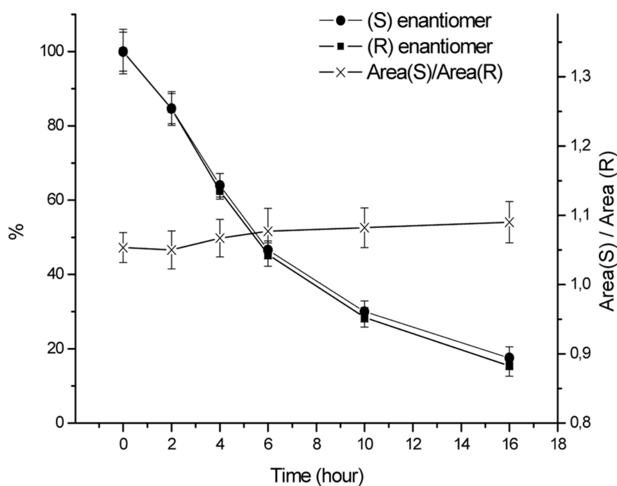
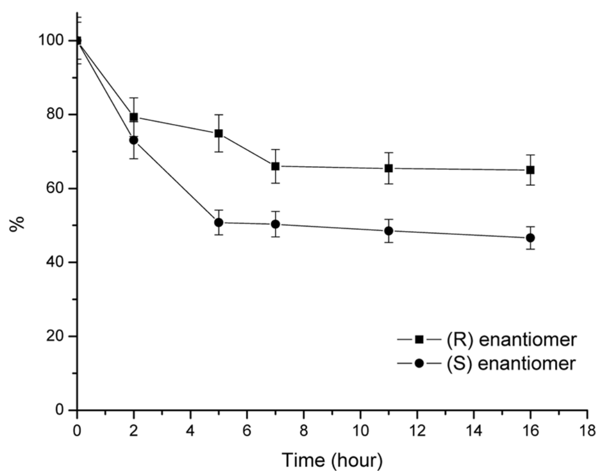
In-vitro Kinetic Study

The developed HPLC method was applied to investigate the kinetics of *in-vitro* enzymatic hydrolysis of enantiomers of potential β-blocker of the aryloxyaminopropanol type. The standard solution of β-blocker racemate and individual (*R*) and (*S*) enantiomeric forms, obtained from semipreparative chromatography, were separately added to the blood serum (t = 0 h) (concentration of 5 μg/mL) and the samples were incubated at 37°C during the time interval from 0 to 16 h. The time course of *in-vitro* degradation of racemate and pure enantiomers serum spiked with individual enantiomeric forms and after SPE analysed separately in blood serum are shown in Figure 4. From the dependencies (Figure 4a) it is evident that there is not a significant difference in the concentration of the enantiomers after the incubation treatment of serum spiked with racemate and, also, no significant change in the enantiomeric ratio with time (evaluated by means of ratio of areas of (*S*)- and (*R*)-enantiomers). During the first 6 h the decomposition is rather rapid, in the next 10 h it decreases slowly. After 16 h the concentration of enantiomers in blood serum is about 20% for both enantiomers in racemate. The time courses



(a)



(b)

Figure 4. The time courses of *in-vivo* degradation of enantiomers in racemate (a) and individual (*R*)-enantiomer and (*S*)-enantiomer (b) of studied potential β-blocker of aryloxyaminopropanol type.

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for the *in-vitro* degradation study of individual (*R*)-enantiomer and (*S*)-enantiomer serum spiked with individual enantiomeric forms (Figure 4b) also have hyperbolic trends, as in the case of the time courses of the serum spiked with racemate. The significant differences in the concentration of the enantiomers after the incubation treatment indicated that the decomposition of (*R*)-enantiomer is slower than the (*S*)-enantiomer.

Table 4. The rate constants (k) and the half-life times ($t_{1/2}$) of enzymatic hydrolysis of studied potential β-blocker of aryloxyaminopropanol type

	$k \cdot 10^5$ (s^{-1})	r	$t_{1/2}$ (h)
Racemate			
(*S*)-enantiomer	4.32	0.998	4.45
(*R*)-enantiomer	4.19	0.988	4.59
Individual enantiomers[1]			
(*S*)-enantiomer	5.34	0.979	3.60
(*R*)-enantiomer	2.57	0.987	7.48

[1] Blood serum spiked with individual (*S*)-enantiomer and (*R*)-enantiomer and analysed separately.

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After 16 h the concentration of enantiomers in blood serum is about 65% for (*R*)-enantiomer and about 45% for (*S*)-enantiomer. The results of the *in-vitro* kinetic study possibly indicate the differences in the biological activity of enantiomeric forms in blood serum.

The differences in time courses observed for the degradation of enantiomers in racemate and individual (*R*)-enantiomer and (*S*)-enantiomer in blood serum are probably caused by a synergic effect of enantiomers in racemate and probably racemization of enantiomers during the incubation at 37°C.

The results of *in-vitro* pharmacological studies of individual enantiomers of the studied derivative of aryloxyaminopropanol β-blocker on the isolated guinea pig atria indicated significantly higher antiisoprenaline activity of (*S*)-enantiomer than (*R*)-optic active isoform.[23]

The course of the enzymatic degradation of studied compounds corresponds to the reaction of the first order with regard to the change of the concentration of the enantiomers during study (the correlation coefficients were close to 0.99). The experimental rate constants (k) were determined using the linear dependencies $\ln(c/c_0) = f(t)$. Table 4 summarises the rate constants and the half-life times of enzymatic hydrolysis for the studied enantiomers. The calculated value of the rate constant of the (*R*)-enantiomer was lower than (*S*)-enantiomer.

CONCLUSION

In conclusion the method has been developed for the analysis of enantiomers of novel potential β-blocker of aryloxyaminopropanol type in blood serum by use of off-line solid-phase extraction and enantioselective liquid chromatography. The suitable phases for separation of enantiomers

were teicoplanin (Chirobiotic T) chiral stationary phase and mixture of methanol/acetonitrile/acetic acid/triethylamine (45:55:0.3:0.2, v/v/v/v) as mobile phase. It has proven to be an accurate, precise, and repeatable method for the quantitative determination of β-blocker of aryloxyaminopropanol type in blood serum.

The *in-vitro* degradation studies of individual (*R*)-enantiomer and (*S*)-enantiomer in blood serum demonstrated a difference in the final concentration of the enantiomers after the incubation at 37°C. It was found that the rate constant of the (*R*)-enantiomer is lower than (*S*)-enantiomer.

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