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ENANTIOSELECTIVE HYDROLYSIS OF 3-HYDROXY-1,4-BENZODIAZEPIN-2-ONE ESTERS BY PIG LIVER MICROSOMES

E.A. Shesterenko[a], I.I. Romanovska[a], O.V. Sevastyanov[a], S.A. Andronati[a], V.I. Pavlovsky[a], T.A. Yurpalova[a], B. Wicher[b], V.Ch. Kravtsov[c], A.A. Krysko[a]

[a]*Department of medical chemistry, A.V. Bogatsky Physico-chemical Institute, National Academy of Sciences of Ukraine, Lustdorfska dor., 86, Odessa, Ukraine*

[b]*Faculty of Chemistry, Adam Mickiewicz University, 60-780 Poznan, Poland*

[c]*Institute of Applied Physics, Academy of Sciences of Moldova, Chisinau, Republic of Moldova*

ABSTRACT

The method of enantioselective hydrolysis of 3-hydroxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one esters using pig liver microsomal fraction was developed. The S-enantiomers of three substrates were obtained with ee_s >97 % and yields 44-49 %, their absolute configurations were determined by X-ray crystallography. It was shown, that the S-enantiomers of 3-hydroxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one esters were 1.4-2.1 times more potent ligands of CBR than the corresponding racemates. Pig liver microsomal fraction was immobilized in calcium alginate beads. It was shown, that immobilized preparation has 3 times greater thermal stability at 50 ºC compared to the free microsomal fraction. Enantioselective hydrolysis of 1-methyl-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one using immobilized microsomal fraction was conducted for 12 cycles of use without loss of the esterase activity.

Key words: enantioselective hydrolysis, microsomal fraction, 3-hydroxy-1,4-benzodiazepin-2-one esters, immobilization.

1. Introduction

The configuration of chiral biologically active compounds plays an important role in processes of their transport, biotransformation and binding with biomembranes [1, 2]. Furthermore, pharmacokinetics profile of one enantiomer may be dependent on the presence of the other enantiomer [1].

Methods of asymmetric synthesis and resolution of enantiomers, such as spontaneous crystallization, usage of optically active solvents, via diastereomers, chromatography on chiral stationary phases are fraught with difficulties [3]. Thus development of economical preparative biotechnological methods of enantiomers resolution is prospective.

Carboxylesterases (EC 3.1.1.1) are the most studied enzymes, catalyzing the enantioselective hydrolysis of an exceptional range of acyclic [4, 5], carbocyclic [6-11] and heterocyclic [12, 13] compounds, including 3-hydroxy-1,4-benzodiazepin-2-one esters [14-16].

Benzodiazepine drugs are the rather wide class of medicines, which clinical effects include anxiolytic, anticonvulsant and hypnotic effects, muscle relaxation [17]. But the number of publications, devoted to the enantioselective hydrolysis of benzodiazepine derivatives is quite limited [14-16, 18-19].

Pig liver carboxylesterase is a member of an α,β-hydrolase- fold family and is localized in the endoplasmic reticulum and cytosol of hepatocytes [20]. The enzyme has many advantages: it is cheap, stable, does not require a coenzyme. Commercially available preparations are mixtures of isozymes, which exhibit essentially the same stereospecificity and can be used in asymmetric synthesis as a single enzyme [21]. Nevertheless, the limitations of the carboxylesterase usage are the high cost of commercial enzyme and its single usage.

Therefore, the application of more economical carboxylesterase as a component of pig liver microsomal fraction, as well as development of it immobilization on polymeric carriers [22, 23], such as calcium alginate [24, 25] are actual tasks.

Alginate is biocompatible, non-toxic, non-immunogenic and biodegradable biopolymer [26, 27]. It is an anionic copolymer, composed of D-mannuronic acid

and L-guluronic acid units, arranged in an irregular blockwise pattern [25]. Alginate forms a hydrogel in the presence of divalent cations, such as Ca^{2+}, which act as cross-linkers between the functional groups of alginate chains [26].

Present study describes the enantioselective hydrolysis of ester bonds in substrates **1-3**, catalyzed by pig liver microsomal fraction. Absolute configurations of S-enantiomers of the substrates **1a-3a** were determined by the X-ray crystallography method. The method of the microsomal fraction immobilization in calcium alginate beads was developed. Stability, esterase activity of the immobilized microsomal fraction and its application in the enantioselective hydrolysis of ester bonds in substrates **1-3** was investigated.

2. Materials and methods

2.1.Materials

Pig liver was obtained from local supermarket. Sodium alginate, hydroxylamine hydrochloride, silica gel (60-100 mesh) were purchased from Sigma Chem. Co, (Schnelldorf, Germany). Bovine serum albumin (BSA), potassium dihydrogen phosphate, sodium dodecyl sulfate, 1-naphthyl acetate and dimethyl sulfoxide, were purchased from Merck (Darmstadt, Germany). The other chemicals used in this work were of analytical grade from local sources.

2.2. Isolation of microsomal fraction, protein and activity assay

Isolation of microsomal fraction was performed by calcium sedimentation and low speed centrifugation [28] from pig liver. Protein concentration was determined according to modified Lowry method [29] using BSA as the standard protein. Esterase activity was determined by hydrolysis of 1-naphthyl acetate in potassium phosphate buffer (16 mM, pH 7.0) and 40 % (v/v) DMSO at 37ºC. Released 1-naphthol was quantified spectrophotometrically by reaction with p-nitrophenyl diazonium tetrafluoroborate at 490 nm [30]. One unit of activity (1 U)

was defined as the amount of enzyme (mg) releasing 1 μmol of 1-naphthol per minute under the assay conditions.

*2.3 Synthesis of the substrates **(2,3)***

*2.3.1. (R,S)-1-Methyl-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one **(2)***

(R,S)-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one **(1)** was synthesized according to the literature procedure [31]. In a 50 ml flask (R,S)-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**1**) (3.73 g, 10 mmol), iodomethane (1.24 ml, 20 mmol), tetrabutylammonium bromide (0.24 g, 1 mmol) and chloroform (20 ml) were mixed and saturated solution of K_2CO_3 was added. The reaction mixture was stirred for 3 h. Next, chloroform was separated, washed with water (10 ml x 3) and dried over $CaCl_2$. The solvent was removed under reduced pressure. The resulting oil was crystallized from acetonitrile.

Mass-spectra of synthesized compounds were recorded on mass-spectrometer VG 7070 at electron energy 70 eV and ion source temperature 200 ºC using direct injection system of sample. The exact mass of molecular ions were measured by the peak matching method using perfluorokerosene as internal standard at resolution about 10000. ^{1}H-NMR spectra were recorded on a Bruker spectrometer at 500 MHz frequency, in $CDCl_3$ and DMSO-d_6, internal standard TMS, at 25 ºC. Yield: 3.1 g (80 %); m.p. 267 – 268 ^{o}C; EI-MS m/z 386 [M^+]; HRMS calcd. for $C_{18}H_{15}BrN_2O_3$ [$M]^+$ m/z, 386.0266; found, 386.0276; ^{1}H-NMR: (500 MHz, $CDCl_3$) δ 7.64–7.74 (3H, m), 7.41–7. 52 (4H, m), 7.29–7.32 (1H, d), 5.94 (1H, s), 3.45 (3H, s), 2.33 (3H, s).

*2.3.2. (R,S)- 1-Ethyl-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one **(3)***

In a 50 ml flask (R,S)-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**1**) (3.73 g, 10 mmol), bromoethane (1.5 ml, 20 mmol), tetrabutylammonium bromide (0.24 g, 1 mmol) and chloroform (20 ml) were mixed and saturated solution of K_2CO_3 was added. The reaction mixture was stirred for 3 h. Next, chloroform was separated, washed with water (10 ml x 3) and dried over $CaCl_2$. The solvent was removed under reduced pressure. The resulting oil was crystallized from acetonitrile. Yield: 2.93 g (73 %); m.p. 202 – 205 ^{o}C; EI-MS m/z 400 [M^+]; HRMS calcd. for $C_{19}H_{17}BrN_2O_3$ [M]$^+$ m/z, 400.0423; found, 400.0434; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.61–7.70 (3H, m), 7.41–7.51 (4H, m), 7.33–7.35 (1H, d, J = 8.8), 5.94 (1H, s), 4.24-4.31 (1H, m), 3.71-3.78 (1H, m), 2.31 (3H, s), 1.11-1.13 (3H, t, J = 7.2);

2.4. General procedure for the enantioselective hydrolysis of 3-hydroxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one esters with the free or immobilized microsomal fraction

To the substrates **1-3** (0.37-0.40 g, 1 mmol) dissolved in potassium phosphate buffer (16 mM, pH 7.0) and 40 % (v/v) DMSO 13600-19040 U (20-28 mL) of free or immobilized microsomal fraction were added. The reaction mixture was incubated at 37 ºC for 2.5 h and extracted with chloroform (150 ml x 3). The combined organic layers were washed with water (300 ml x 2) and the solvent was removed under reduced pressure. Enantiomers of substrates **1a-3a** were purified by silica gel column chromatography (chloroform/ethyl acetate = 3/1 or 1.5/2.5, v/v). Quantitative analysis of enantiomers of substrates **1a-3a** was performed spectrophotometrically by ferric hydroxamate method [32].

Enantiomeric excesses of substrates (ee_s) were determined by HPLC using Shimadzu LC-8A pump with a chiral column ChiraDex 5 μm eluted with methanol/ water (70/30, v/v) at room temperature and a flow rate of 1 mL/min.

The retention times (tR) of peaks of the racemate **1** are 5.3 min - 49.93 % R-enantiomer and 7.8 min - 50.07 % - S-enantiomer.

tR of peaks of the obtained enantiomer **1a** are 5.3 min - 1.2 % R-enantiomer and 7.8 min 98.8 % - S-enantiomer. tR of peaks of **2** are 5.6 min - 48.9 % - R-enantiomer and 8.1 min - 51.1 % S-enantiomer. tR of peaks **2a** are 5.6 min - 1.3 % - R-enantiomer and 8.1 min 98.7 % S-enantiomer. tR of peaks of **3** are 7.6 min - 48.6 % - R-enantiomer and 9.6 min – 51.4 % S-enantiomer. tR of peaks **3a** are 7.6 min - 1.4 % - R-enantiomer and 9.6 min 98.6 % S-enantiomer.

The ee_s was calculated from the ratio (%) of [(S-form) − (R-form)]/[(S-form) + (R-form)].

*2.4.1. Compound **1a**: (S)-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one*

Yield; 49 %, ee_s > 97 %, $[\alpha]_D^{20}$ +116.9º (c = 1.0, $CHCl_3$).

*2.4.2. Compound **2a:** (S)-1-methyl-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4- benzodiazepin-2-one*

Yield; 47 %, ee_s > 97 %, $[\alpha]_D^{20}$ +195.3º (c = 1.0, $CHCl_3$).

*2.4.3. Compound **3a:** (S)-1-ethyl-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4- benzodiazepin-2-one*

Yield; 44 %, ee_s > 97 %, $[\alpha]_D^{20}$ +193.8º (c = 1.0, $CHCl_3$).

2.5. X-ray crystal structure analysis of **1a-3a**

The diffraction data for **1a** and **3a** were collected with a *Xcalibur E* single-crystal diffractometer using MoK_α radiation λ=0.71073 Å) and for **2a** data were collected with a *SuperNova* single-crystal diffractometer using micro-source radiation CuK_α λ=1.54184 Å). All experiments were carried out at room temperature 293K. Data collection and reduction were performed with CrysAlis^{Pro} software (versions 1.171.33.66 for **1a** and **3a** and 1.171.33 for **2a**). The data were

corrected for the Lorentz and polarization factors and for absorption. The structure was solved by direct methods refined by full-matrix least-squares method on F^2 with SHELXL-97 [33]. All non-hydrogen atoms were refined with anisotropic displacement parameters. The hydrogen atom positions were found in difference Fourier maps however, for further refinement H-atoms bonded with carbon atoms, were determined on the basis of molecular geometry (C–H 0.93-0.98 Å) and their displacement parameters were set equal to $1.5U_{eq}$(C) for methyl groups and $1.2U_{eq}$(C) for others. The absolute structure was determined from anomalous dispersion effects [34].

Molecules of **1a-3a** have S-configuration, the absolute structure Flack parameters are: **1a** -0.03(2), **2a**-0.00(1), **3a**-0.004(8).

Crystallographic data for the structure **1a**, **2a,** and **3a** reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Deposit Nos. CCDC 916962, 789193, and 916961, respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB12 1EZ, UK (fax +44 1223 336033 or e-mail:deposit@ccdc.cam.ac.uk).

2.6. [^{3}H] Flumazenil binding.

Affinity of compounds **1-3** and **1a-3a** for CBR of rat brain was determined and values of IC_{50} were evaluated as described previously [35, 36].

2.7. Immobilization of microsomal fraction in calcium alginate beads

The isolated microsomal fraction (1544 U or 15 ml) was mixed with 45 ml of a solution of sodium alginate and then dropped into 200 ml of calcium chloride solution (10 %) to form biocatalyst beads. The beads were washed with distilled water and stored at 4 ºC for its further use.

2.8. pH- and thermal dependence of microsomal fraction activity

The effect of pH on free and immobilized microsomes was studied by assaying the preparations at different pH values (3.0-10.0) using the following buffers: acetate (3.0-6.0), potassium phosphate (pH 6.0-8.0), Tris (8.0-9.0).

The effect of temperature on free and immobilized microsomal fraction was determined by changing the reaction medium temperature in the range from 10 to 50 ºC in potassium phosphate buffer (16 mM, pH 7.0).

2.9. Thermostability of microsomal fraction

Free and immobilized microsomal fraction were incubated in potassium phosphate buffer (16 mM, pH 7.0) at 50 ºC. Samples were taken after 0.25, 0.5, 1, 2, 3, 4 and 5 h. The residual activity was determined by hydrolysis of 1-naphthyl acetate. The thermal inactivation constants were calculated from the slope of residual activity value common logarithm vs. time dependence plot by the linear regression method.

2.10. Reusability of immobilized microsomal fraction

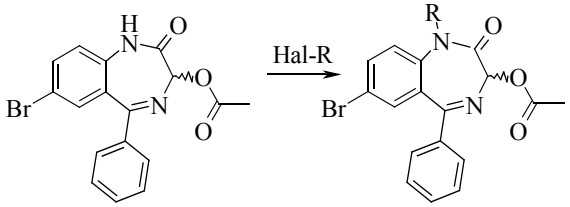
The reuse activity of immobilized microsomal fraction was studied in repeated batch experiments with hydrolysis of compound **2** (0.5 mM) dissolved in potassium phosphate buffer (16 mM, pH 7.0) and 40 % (v/v) DMSO at 37 ºC for 1 h. At the end of each cycle, the immobilized microsomes were filtered and washed thoroughly with distilled water.

3. Results and discussion

*3.1. Synthesis of (R,S)-esters **2-3***

(R,S)-1-Methyl-**(2)** and (R,S)-1-ethyl-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one **(3)** were synthesized by alkylation of (R,S)-3-acetoxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepine-2-one **(1)** with

iodomethane or bromoethane under phase-transfer catalysis conditions, respectively.



1 **2,3**

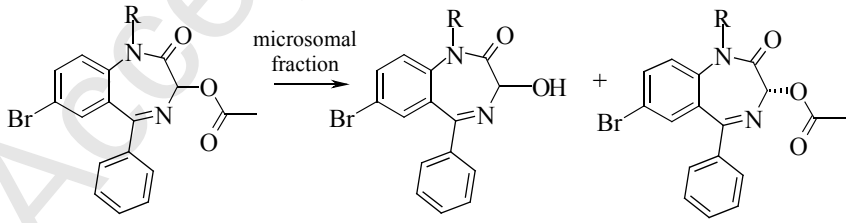
R: H (**1**), CH_3 (**2**), C_2H_5 (**3**)

Scheme 1. Synthesis of (R,S)-esters **2,3.**

The structure of (R,S)-esters **2,3** were confirmed by mass spectrometry and ^{1}H-NMR spectroscopy.

*3.2. Enantioselective hydrolysis of ester bonds in substrates **1-3** with free microsomal fraction*

Microsomal fraction was obtained from pig liver. Protein yield was 38.0 mg/g of liver tissue and esterase activity was 17.25 U/mg protein. Enantioselective hydrolysis of *ester bonds in compounds **1-3*** was conducted using the microsomal fraction (Scheme 2).



1-3 **4-6** **1a-3a**

R: H (**1,4**), CH_3 (**2,5**), C_2H_5 (**3,6**)

Scheme 2. Enantioselective hydrolysis of (R,S)-*3-hydroxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one* esters **1-3.**

The hydrolysis of **1** with the microsomal fraction was studied at different temperatures and pH to find the optimal reaction conditions. Table 1 shows, that the maximal conversion of **1** was observed at 37 ºC and pH 7.0.

Table 1

Effect of temperature and pH on enzymatic conversion of substrate **1**

Temperature (ºC)	Conversion (%)		pH	Conversion (%)	
	Free catalyst	Immobilized catalyst		Free catalyst	Immobilized catalyst
10	13.0	13.5	3.0	6.7	7.2
20	19.0	21.5	4.0	11.6	10.8
30	32.8	31.2	5.0	26.7	28.5
37	50.5	50.7	6.0	37.3	37.7
40	47.8	49.8	7.0	50.9	49.8
50	20.5	27.6	8.0	46.5	49.6
60	13.0	19.2	9.0	42.6	42.7

Next, we had investigated the effect of substituents in the first position of the benzodiazepine moiety on the microsomal carboxylesterase specificity (fig. 1). It was found, that to convert the compound **1** by 50 %, it was needed the least amount of the microsomal fraction – 100 U/ml. 50 % conversion of **2** and **3** was achieved with increasing the required amount of the catalyst [37].

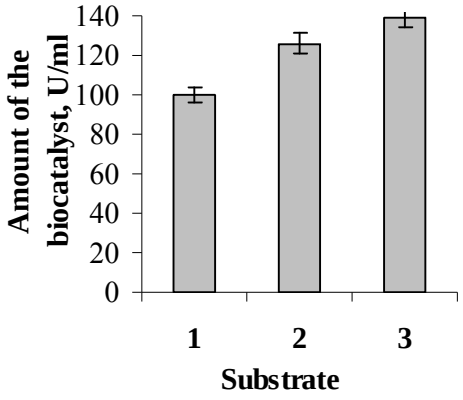


Fig.1. Effect of the substrate structure on the required amount of the microsomal fraction.

It was shown, that the products **4-6** underwent racemization during hydrolysis and subsequent isolation, which is consistent with the literature [38].

But the absolute configurations of the substrate enantiomers **1a-3a** were unambiguously determined by single crystal X-ray diffraction analysis as (S)-forms (ee_s > 97 %). Thus, the pig liver microsomal carboxylesterase is showing higher specificity to R-enantiomers of esters **1-3**.

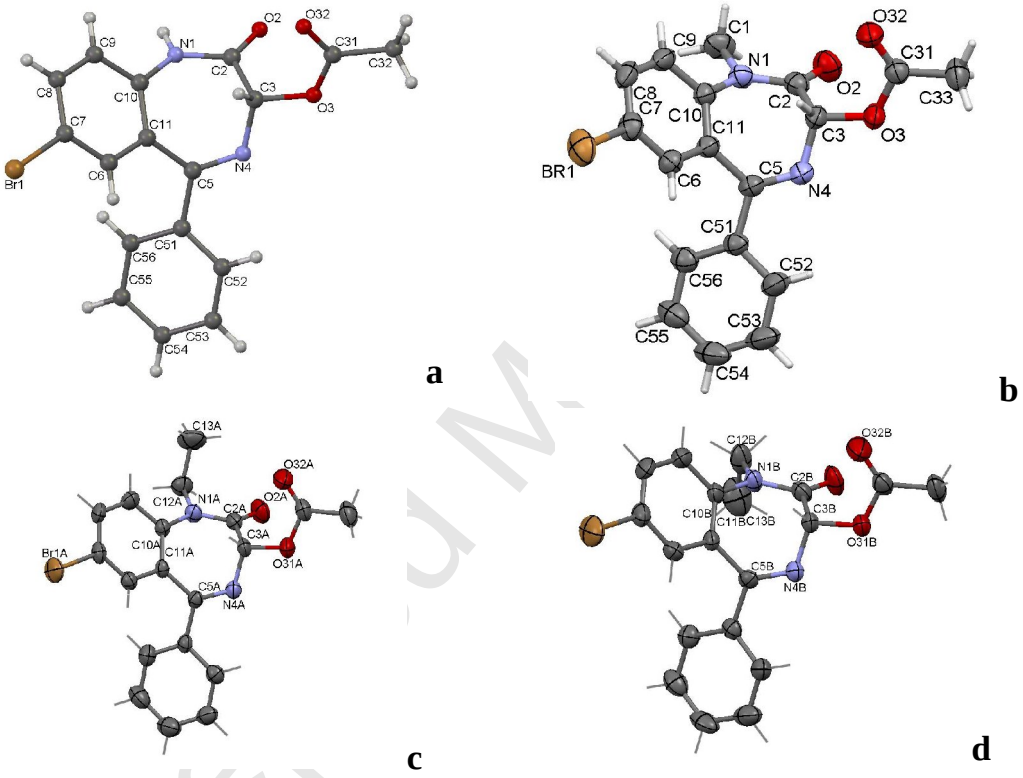


Fig.2. ORTEP view of molecular structure of **1a** (a), **2a** (b), and two conformers in the structure **3a** (c and d) illustrate their absolute configuration. Displacement ellipsoids are drawn at the 50% probability level.

The X-ray crystal structure analysis had shown the similar molecular conformation of **1a-3a,** Fig.2. Symmetrically independent molecules a and b in **3a** differ mainly by opposite orientation of ethyl substituent, torsion angle C(2)N(1)C(1)C(12) equals - 74.0° and 80.7° for molecule a and b. The seven-membered benzodiazepine moiety in all these compounds has a boat-like conformation. The deviation of atoms N(1), C(2), N(4) and C(5), which constitute

bottom of the boat, from the their mean plane is less than 0.004 Å in **1a** and **2a** and 0.022 Å in **3a**, while atoms C(3), C(10) and C(11) are displaced from this plane in the same direction by 0.75, 0.65 and 0.67Å in **1a,** 0.77, 0.73 and 0.73Å in **2a,** and 0.79/0.79, 0.75/0.80, and 0.70/0.76 Å in **3a.** A distortion of ring from an ideal cycloheptatriene "boat" with mirror symmetry C_s may be evaluated by Duix asymmetry parameter ΔC_s [39-40] which equal 3.32°, 6.85° for **1a, 2a,** and 2.33° and 7.14° for molecules a and b in **3a,** respectively and are well within the range found in 1,4-benzodiazepin-2-ones [40]. Bond lengths and angles in of **1a-3a** are in a good agreement. Distances N(4)-C(5) range within the values 1.276-1.294 Å and indicate the double character of this bond. Distances C(3)-N(4), N(1)- C(2), and N(1)-C(10) vary in the narrow intervals 1.435-1.442, 1.362-1.368, and 1.402-1.428Å, respectively.

Using radioligand binding methods, affinity of S-enantiomers **1a-3a** and racemates **1-3** for CBR of rat brain was determined and values of IC_{50} were evaluated. It was shown, that the S-enantiomers **1a-3a** are 1.4-2.1 times more potent ligands of CBR than the corresponding racemates **1-3**.

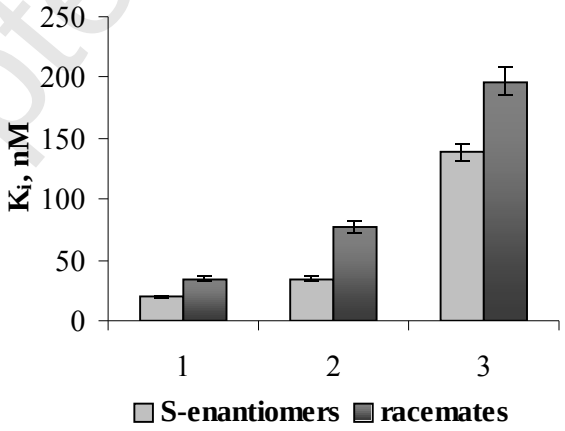


Fig. 3. In vitro binding affinity (K_i, nM) for CBR of compounds **1-3** and **1a-3a**

3.3. Immobilization studies

In this study, we have determined the optimal concentration of alginate and calcium chloride solutions (4 % and 5 %, respectively) to prepare the biocatalyst beads. Characteristics of the obtaining biocatalyst are shown in Table 2.

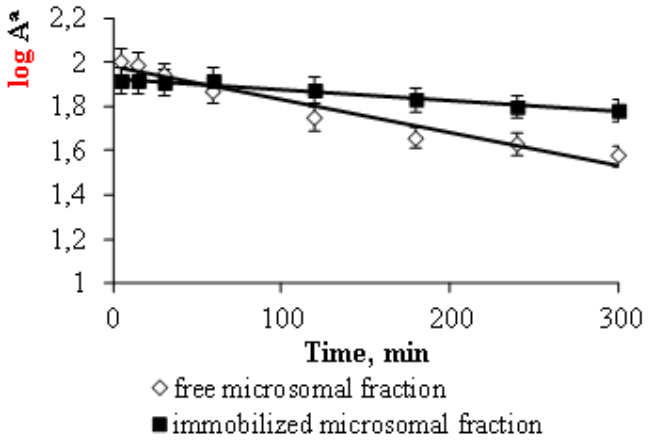
Table 2.

Characteristics of immobilized microsomal fraction

Biocatalyst characteristics	
Amount of protein, mg/g beads	19.2 ± 0.7
Esterase activity, U/ g beads	230.9 ± 10.3
Retention of initial activity, %	70.0
Beads diameter, mm	2.5 ± 0.5
Beads weight, mg	15 ± 0.45

As shown, there were no significant differences in pH and temperature profile of free and immobilized microsomal fraction (Table 1). But investigation of thermal inactivation of both biocatalysts showed an improvement of thermal stability of the immobilized microsomal fraction. The thermal inactivation constants were $0{,}8 \cdot 10^{-3}$ min^{-1} and $1{,}5 \cdot 10^{-3}$ min^{-1}, for the free and the immobilized biocatalysts, respectively (fig. 4).

Such enhancement of thermal stability seems to represent stabilization of microsomal vesicles by the carrier, which prevents the membrane-bound proteins denaturation, and as a consequence disaggregation of the vesicles [41].



[a]log A - common logarithm of residual esterase activity

Fig. 4. Thermal stability of free and immobilized microsomal fraction at 50ºC

*3.4. Enantioselective hydrolysis of ester **2** with immobilized microsomal fraction*

Enantioselective hydrolysis of ester **2** using immobilized microsomal fraction was conducted in optimized reaction conditions in a batch process (Fig. 5).

After 8 cycles, the beads of immobilized microsomal fraction have lost insolubility, because of the buffer solution influence, which contributes to the gradual release of calcium ions from the beads [27, 42].

So, the ability of distilled water application instead of the buffer solution was investigated. Application of distilled water made it necessary to increase the required amount of the biocatalyst at 18 %. But it was shown, that after 14 cycles of use the beads were not destroyed, and the 50 % conversion of **2** was found after 12 cycles of usage.

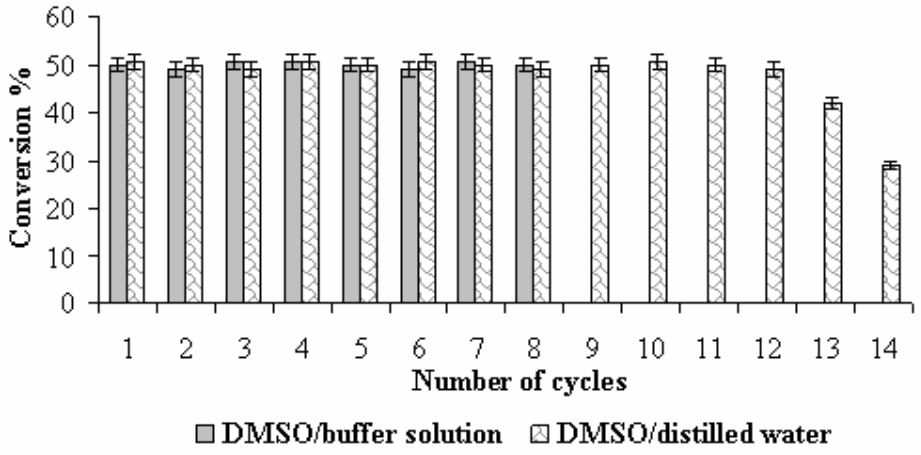


Fig. 5. Reusability of immobilized microsomal fraction in enantioselective hydrolysis of the ester **2.**

4. Conclusions

The optimized reaction conditions of enantioselective hydrolysis of the 3-hydroxy-7-bromo-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one esters using pig liver microsomal fraction were developed. Three S-enantiomers of substrates were obtained, so the pig liver microsomal carboxylesterase is showing higher specificity to R-enantiomers of esters **1-3**. It was found, that the S-enantiomers 1a-3**a** are the most potent ligands of CBR as compared to the racemates **1-3.** The biocatalyst with improved thermal stability was developed using the immobilization of microsomal fraction in calcium alginate beads. Obtained biocatalyst was used for the enantioselective hydrolysis of the ester **2** for 12 cycles of use in a batch process.

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Highlights:

The S-enantiomers of the 3-hydroxy-7-bromo-5-phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one esters were obtained (ee_s >97 %).

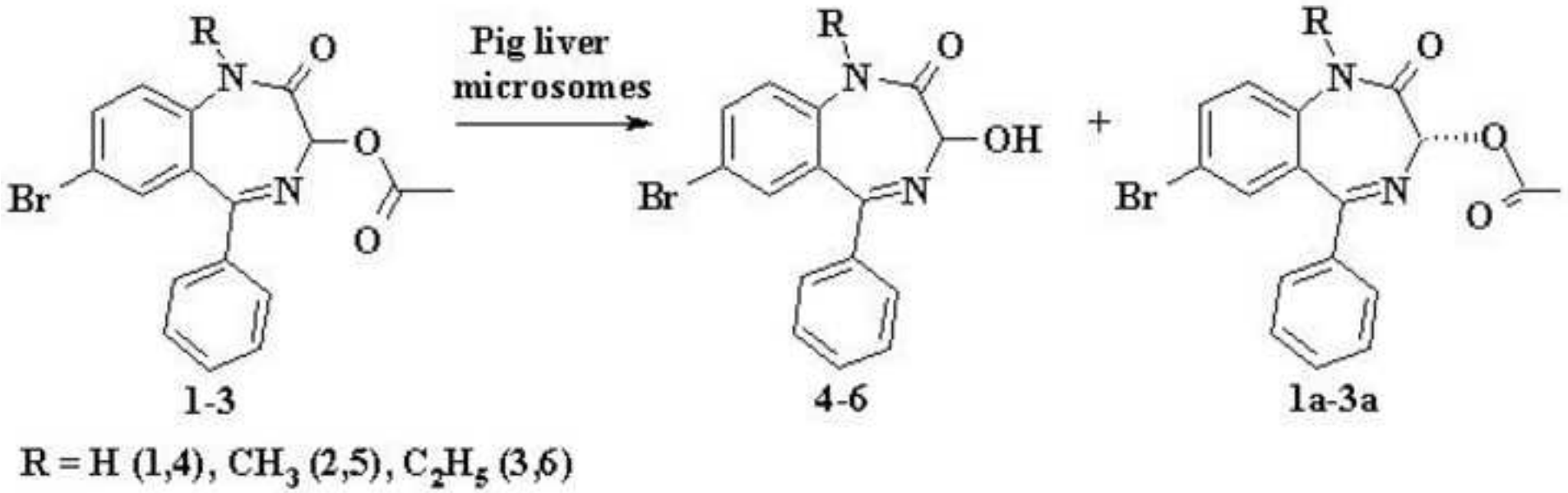
S-enantiomers were 1.4-2.1 times more potent ligands of CBR than the corresponding racemates.

Pig liver microsomal fraction was immobilized in calcium alginate beads.

Obtained biocatalyst has been applied for the enantioselective hydrolysis of the esters for 12 cycles of use.

[43]

***Graphical Abstract (for review)**



R = H (1,4), CH_3 (2,5), C_2H_5 (3,6)