**Kinetic resolution of β-adrenolytic drug with the use of lipases as enantioselective biocatalysts**

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**Abstract**

The study presented herein is focused on optimizing of kinetic resolution of racemic propranolol by screening of acetylating agent, reaction medium and enantioselective biocatalysts. The effects of acetylating agent and reaction medium onto efficiency of performed enantioselective biotransformation were investigated. Further, the catalytic activities on nine commercially available lipases were tested. Finally, the composition of reaction medium was selected, based on previously obtained results, providing the most efficient process of kinetic resolution of racemic propranolol.

**Keywords: enzyme, (*R*,*S*)-propranolol, kinetic resolution, enantioselective acetylation, *Candida rugosa* (OF) lipase.**

1. **Introduction**

Cardiovascular diseases and heart diseases, also known as cardiological diseases, are the most common cause of death in developed countries, including Poland. Additionally, cardiovascular diseases are the most widespread group of diseases not only among the elderly. The causes of cardiovascular problems are diverse, which is why medicines used to fight them belong to many groups. The most common cardiological diseases are: arterial hypertension, ischemic heart disease, arrhythmias or heart failure. The treatment strategies of these diseases consist in the use of antihypertensive drugs, i.e. those that lower blood pressure, drugs that improve heart function, operate antiarrhythmics or change blood properties (1).

Propranolol belongs to the β-adrenergic receptor antagonists (β-blockers) class, which are known as important classes of drugs widely used in the treatment of hypertension and cardiovascular disorders (2-5). Although propranolol is one of the oldest non-selective beta receptor blocking agent it has well known physical and chemical properties. Hence it can be use in compared study of the catalytic activity of different enzymes in biocatalysis reactions. Because β-blockers have an asymmetric carbon atom in their structure, they occur in two enantiomeric forms. Although the two enantiomers of β-blockers feature the same chemical and physical properties in an achiral environment, they differ in pharmacological activity. It has been reported by many studies that only the *S*-enantiomer of β-blockers has the desired therapeutic effect, whereas the administration of the racemate may cause dangerous side effects, such as bronchoconstriction or diabetes (6-9). Nevertheless, β-blockers are still commercially available drugs mainly used in medicine as racemates.

The main aim of the study presented herein was to obtain enantiomerically pure (*S*)-propranolol acetate, which is responsible for therapeutic effect.Additionally, enzyme screening and reaction medium selection were performed to select the best reaction conditions. The chiral compounds obtained as a result of enantioselective acetylation of (*R*,*S*)-propranolol were analyzed using chiral stationary phases and UPLC-MS/MS system. Moreover, to optimize the chiral chromatographic conditions, the type of stationary phase and mobile phase composition of the chromatographic process were selected.

1. **Materials and methods**
	1. **Chemicals**

(*R*,*S*)-propranolol, (*R*)-propranolol, *Burholderia cepacia* lipase, *Candida antarctica* lipase, *Candida rugosa* lipase, toluene, *n*-hexane, isopropenyl acetate, vinyl acetate, 2-propanol, acetonitrile, diethylamine, and acetyl chloride were purchased from Sigma-Aldrich Co. (Stainhaim, Germany). Anhydrous sodium sulfate, sodium sulfate decahydrate, 4 Å molecular sieves, and ethanol were purchased from POCH S.A. (Gliwice, Poland). Lipases OF and MY from *Candida rugosa,* lipase QLM from *Alcaigenes fecalis*, lipase PL from *Alcaigenes sp.*, lipase TL from *Pseudomonas stutzeri*, and lipase SL from *Burkholderia cepacia* were a gift from Meito Sangyo Co., Ltd. (Japan). The water used in the study was obtained using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). All incubations were performed at controlled temperature and number of rotations (250 rpm). Glassware was oven dried for several hours, assembled hot, and cooled in a stream of nitrogen.

* 1. **Instrumentation**

The Refrigerated CentriVap Concentrator was purchased from Labconco; the Inkubator +1000 and Unimax 1010 were purchased from Heidolph; and the Shimadzu UPLC-MS/MS system (Japan) was equipped with two LC-30AD solvent delivery pumps combined with gradient systems, a degasser model DGU-20A5, an autosampler model SIL-30AC, a column oven model CTO-20AC, a UV detector model SPD-M20A. A Lux Celullose-1 (LC-1) column with a cellulose tris(3,5-dimetylphenylcarbamate) stationary phase, a Lux Cellulose-2 (LC-2) column with a cellulose tris(3-chloro-4methylphenylcarbamate) stationary phase, a Lux Cellulose-3 (LC-3) column with a cellulose tris(4-methylbenzoate) stationary phase, a Lux Amulose-2 (LA-2) column with an amylose tris(5-chloro-2-methylphenylcarbamate) stationary phase and a Guard Cartridge System model KJO-4282 were purchased from Phenomenex Co.

* 1. **Chromatographic conditions**

To achieve satisfactory chromatographic resolution of (*R*,*S*)-propranolol and its esters, the effects of both the composition, flow rate of the mobile phase and the type of chiral stationary phase (CSP) were investigated. Five types of CSPs were tested in normal, reversed and polar/organic phase. Finally, the Lux Cellulose-1 column was chosen as an optimal stationary phase for chromatographic separation in the polar/organic phase mode (Figure 1). The mobile phase consisted of methanol/isopropanol/diethylamine/formic acid in a ratio of 99.8/0.2/0.1/0.1 (v/v/v/v). The chromatographic process was performed at 25 °C. The detection was performed with the use of mass spectrometry in MRM mode. To determine the optical purity and enantioselectivity of the enantioselective acetylation, previously used equations based on peak areas from a chromatogram obtained by the chromatographic separation of (*R*,*S*)-propranolol and its derivatives were applied (10, 11).

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**Figure 1.** Chromatogram of racemic propranolol and its esters after enantioselective biotransformation of (*R*,*S*)-propranolol with the use of *Candida rugosa* lipase OF: (*S*)-enantiomer of propranolol acetate (tR = 6.626), (*R*)-enantiomer of propranolol acetate (tR = 7.456), (*S*)-propranolol (tR = 9.131), (*R*)-propranolol (tR = 13.027).

* 1. **Chemical acetylation of (*R*,*S*)-propranolol**

The (*R*,*S*)-propranolol was acetylated according to the reported methodology (10, 12) with few modifications. Briefly, (*R*,*S*)-propranolol (0.02 g; 0.07 mmol) was refluxed with dichloromethane (20 mL), and acetyl chloride (8 µl; 0.076 mmol) was slowly added. Further, the reaction mixture was incubated at 30 ᵒC for 2 h and successively washed with equal volumes of brine and saturated aqueous sodium bicarbonate. Next, the organic layer was collected and evaporated under vacuum to afford propranolol acetate.Finally, the resulting derivate of propranolol was used as a standard to establish an optimal chromatographic method for quantitative and qualitive determination of racemic propranolol and its acetylated form.

* 1. **Enantioselective acetylation of (*R*,*S*)-propranolol**

Enantioselective biotransformation of (*R*,*S*)-propranolol using vinyl acetate or isopropenyl acetate as the acetylating agent resulted in the production of *N*-acetyl-propranolol
(Figure 2). Because the solubility of racemic propranolol is low in most organic solvents, the reaction was performed in 10 mL of the reaction medium. The reaction was performed with different solvents, e.g., acetonitrile, dichloromethane, disopropyl ether, *tert*-butyl-methyl ether, chloroform, tetrahydrofuran, toluene, and racemic propranolol (3.0 mg), and vinyl acetate or isopropenyl acetate (2-10 µL) as the acetyl donor. The reaction was begun by the addition of 10 mg of lipase in native form. The reaction mixture was shaken at 250 rpm at t = 37 °C. The process of enantioselective acetylation was monitored using chiral stationary phases and UPLC-MS/MS system. The samples were withdrawn at previously established time points (24, 48, 72 and 96h) and then evaporated and redissolved in pure acetonitrile, filtered, and injected into the UPLC-MS/MS system.



Figure 2. Enantioselective acetylation of racemic propranolol with the use of lipase as biocatalyst.

1. **Results and discussion**
	1. **Effect of acetylating agent**

The effect of acetylating agent was investigated in order to optimize the reaction conditions. The vinyl acetate as well as isopropenyl acetate were tested at different concentrations. The biocatalytic system was consisted of toluene as reaction medium, racemic propranolol (3.0 mg), lipase from *Candida rugosa* OF (10 mg) and proper acetylating agent. The reaction was carried out at ambient temperature for 96 h. As it is shown in Figure 3, the best reaction parameters was achieved by using isopropenyl acetate in concentration 0.018 mmol (2 µL). Additionally, the use of vinyl acetate resulted in obtaining many by-products, which could not be identified, therefore it was decided to use isopropenyl acetate in further steps of study optimization in case of the solvent and lipase screening.

**Figure 3.** Enzymatic parameters including enantioselectivity (E), enantiomeric excess of product (eep) and conversion (c) of different acetylating agents screened for the enantioselective acetylation of racemic propranolol. Reaction conditions: *Candida rugosa* lipase (OF) (10mg), (*R*,*S*)-propranolol (3mg), toluene (10mL), vinyl acetate or isopropenyl acetate (0.5-2.5µL), time – 96 h, temperature – 37°C, RPM – 250.

* 1. **Effect of organic solvent**

The effect of organic solvent onto properties of enzymatic kinetic resolution of racemic propranolol was investigated. It was decided to perform enantioselective biocatalysis in 10 mL of reaction medium due to the low solubility of racemic propranolol. Additionally, all of the tested solvents were organic, because of the characteristics of enantioselective biotransformation, which relied on reaction of transesterification. The reaction mixture in that step of study consisted of different reaction medium, i.e. acetonitrile, chloroform, tetrahydrofuran, *t*-butyl-methyl ether, diisopropyl ether or toluene, lipase from *Candida rugosa* OF (10 mg), isopropenyl acetate (0.018 mmol) as an acetylating agent and racemic propranolol (3.0 mg).

The catalytic properties of an enzyme are mainly affected by the hydrophobicity of the solvent. Therefore, the optimal choice of solvent as reaction medium is one of the most important parts of optimizing reaction conditions for achievement the best value of enantioselectivity. Among all tested solvents, only toluene turned out to be suitable for performing the enantioselective acetylation of racemic propranolol, which is shown in Table 1. Even though the solubility of propranolol is higher in other solvents than toluene, these solvents are not suitable for the kinetic resolution of (*R*,*S*)-propranolol, because the enantiomeric excess of product and enantioselectivity are low. Additionally, the use of toluene made it possible to obtain a high value of enantioselectivity, which was above 20. Based on abovementioned results, toluene as reaction medium was selected for further investigation of lipase screening.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Organic solvent** | **E** |  **ees [%]** | **eep [%]** | **c [%]** |
| Acetonitrile | 2,07 | 0,62 | 34,54 | 1,76 |
| Chloroform | 1,31 | 3,62 | 11,69 | 23,64 |
| diisopropyl ether | 7,36 | 13,95 | 73,07 | 16,03 |
| t-butyl-methyl ether | 4,96 | 0,76 | 66,23 | 1,14 |
| Tetrahydrofuran | 1,24 | 2,34 | 9,70 | 19,44 |
| Toluene | 23,56 | 21,38 | 90,06 | 19,19 |

**Table 1.** Enzymatic parameters including enantioselectivity (E), enantiomeric excesses of both substrate (ees) and product (eep) and conversion (c) of enantioselective acetylation of
(*R*,*S*)-propranolol with the use of lipase from *Candida rugosa* OF after 96 h of incubation.

* 1. **Screening of lipases**

Commercially available lipases from *Aspergilus niger*, *Burkholderia cepacia* (BCL, SL)*,* *Candida antarctica* (CALBY), *Candida rugosa* (MY, OF), *Alcaigenes fecalis* (QLM), *Alcaigenes sp.* (PL), and *Pseudomonas strutzeri* (TL) were tested for their catalytic properties in the enantioselective acetylation of racemic propranolol with isopropenyl acetate as acetylating agent, using toluene as the reaction medium. As it is shown in Table 2, among all tested enzymes, the most satisfactory results were obtained for *Candida rugosa* lipases (OF, MY). However, it should be emphasised that the enantiomeric ratio and enantiomeric excess of product were higher in reactions using lipase OF than using lipase MY. The other lipases tested did not provide any promising results, and their use resulted in obtaining significantly lower values of enantioselectivity and enantiomeric excesses of both substrate and product.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Lipase** | **E** | **ees %** | **eep %** | **c %** |
| Alcaigenes fecalis (QLM) | 1,12 | 1,71 | 5,07 | 25,17 |
| Alcaigenes sp. (PL) | 1,20 | 5,24 | 6,93 | 43,04 |
| Aspergilus niger | 1,24 | 8,34 | 7,06 | 54,16 |
| Burkholderia cepacia (BCL) | 3,22 | 6,55 | 50,23 | 11,53 |
| Burkholderia cepacia (SL) | 2,57 | 5,58 | 41,80 | 11,77 |
| Candida antarctica (CALBY) | 3,01 | 6,86 | 47,53 | 12,61 |
| **Candida rugosa (MY)** | **21,80** | **14,00** | **90,00** | **13,46** |
| **Candida rugosa (OF)** | **23,56** | **21,38** | **90,06** | **19,19** |
| Pseudomonas strutzeri (TL) | 2,78 | 2,09 | 46,25 | 4,32 |

**Table 2.** Enzymatic parameters including enantioselectivity (E), enantiomeric excesses of both substrate (ees) and product (eep) and conversion (c) of enantioselective acetylation of
(*R*,*S*)-propranolol with the use of various native lipases in toluene after 96 h of incubation.

1. **Conclusions**

The reported results confirmed that nine commercially available lipases have different effects on the kinetic resolution of (*R*,*S*)-propranolol. The type of lipase, the concentration of the acetylating agent and the reaction medium have significant impacts on the efficiency of the catalyst system. The most effective conversion and the highest enantioselectivity were obtained using lipase from *Candida rugosa*(OF). Among all tested reaction mediums, toluene proved to allow the highest enantioselectivity. The crucial point is that many different reaction factors influence the effectiveness of the enantioselective acetylation of β-blockers. Finally, the reaction mixture composed of *Candida rugosa*OF lipase, toluene as the reaction medium and isopropenyl acetate as the acetylating agent made it possible to obtain enantiomerically pure (*S*)-propranolol acetate with eep = 90.1 %. However, it is necessary to optimize the methods for the kinetic resolution of chiral drugs to obtain enantiomerically pure products, and thus it is not possible to find a universal catalytic system for all syntheses, as each enzymatic process requires an individual approach.

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**References**

1. Narina SV, Sudalai A. Enantioselective synthesis of (S)-timolol via kinetic resolution of terminal epoxides dihydroxylation of allylamines (vol 63, pg 3026, 2007). Tetrahedron 2007; 63(29): 6938-.

2. Carlberg B, Samuelsson O, Lindholm LJ. Atenolol in hypertension: is it a wise choice? Lancet 2004; 364(9446): 1684-9.

3. Lund IT, Bockmann PL, Jacobsen EE. Highly enantioselective CALB-catalyzed kinetic resolution of building blocks for beta-blocker atenolol. Tetrahedron 2016; 72(46): 7288-92.

4. Barbosa O, Ariza C, Ortiz C, et al. Kinetic resolution of (R/S)-propranolol (1-isopropylamino-3-(1-naphtoxy)-2-propanolol) catalyzed by immobilized preparations of Candida antarctica lipase B (CAL-B). New Biotechnology 2010; 27(6): 844-50.

5. Zelaszczyk D, Kiec-Kononowicz K. Biocatalytic approaches to optically active beta-blockers. Current Medicinal Chemistry 2007; 14(1): 53-65.

6. Escorcia AM, Daza MC, Doerr M. Computational study of the enantioselectivity of the O-acetylation of (R,S)-propranolol catalyzed by Candida antarctica lipase B. Journal of Molecular Catalysis B-Enzymatic 2014; 108: 21-31.

7. Dwivedee BP, Ghosh S, Bhaumik J, et al. Lipase-catalyzed green synthesis of enantiopure atenolol. Rsc Advances 2015; 5(21): 15850-60.

8. Escorcia AM, Molina D, Daza MC, et al. Acetylation of (R,S)-propranolol catalyzed by Candida antarctica lipase B: An experimental and computational study. Journal of Molecular Catalysis B-Enzymatic 2013; 98: 21-9.

9. Chiou TW, Chang CC, Lai CT, et al. Kinetic resolution of propranolol by a lipase-catalyzed N-acetylation. Bioorganic & Medicinal Chemistry Letters 1997; 7(4): 433-6.

10. Sikora A, Chelminiak-Dudkiewicz D, Ziegler-Borowska M, et al. Enantioseparation of (RS)-atenolol with the use of lipases immobilized onto new-synthesized magnetic nanoparticles. Tetrahedron-Asymmetry 2017; 28(2): 374-80.

11. Siodmiak T, Mangelings D, Vander Heyden Y, et al. High Enantioselective Novozym 435-Catalyzed Esterification of (R,S)-Flurbiprofen Monitored with a Chiral Stationary Phase. Applied Biochemistry and Biotechnology 2015; 175(5): 2769-85.

12. Sikora A, Chełminiak-Dudkiewicz D, Siódmiak T, et al. Enantioselective acetylation of (R,S)-atenolol: The use of Candida rugosa lipases immobilized onto magnetic chitosan nanoparticles in enzyme-catalyzed biotransformation. Journal of Molecular Catalysis B: Enzymatic 2016; 134, Part A: 43-50.