INCREASED RACEMATE RESOLUTION OF PROPRANOLOL ESTERS BY LIPASE IMMOBILIZED CATALYSIS

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***Abstract* - A screening of six different immobilized lipases (*C. rugosa, C. antartica, P fluorescens, M. miehie, R. niveus*) was done to determinate the best for the stereospecific hydrolysis of *RS*-O-BP. An experimental design (2^3) where the enzymatic activity was a function of temperature, buffer concentration and solvent was selected. *P fluorescens* showed the best results for *S(-)* isomer, with an increase of the stereoespecificity ratio of 1.5, using tetrahydrofuran as solvent, and 15mM buffer phosphate concentration at 37 °C. *C. rugosa* show-ed the best results for *R(+)* isomer, with an increase of the stereoespecificity ratio of 1.7, using acetone as solvent, and 25 mM buffer phosphate concentra-tion at 15 °C. The enantiomeric excess of *S(-)* propranolol with *P fluorescens* was 87%, higher than those reported elsewhere.**

***Keyword* - *Candida rugosa, Pseudomona fluorescens*, Enantioselectivity, Biotransformation, Lipase, Propranolol.**

I. INTRODUCTION

Propranolol (1-isopropilamino-3-(1-naphtoxy)-2-propanol) is a known beta-adrenergic blocking agent commonly used for the treatment of arterial hypertension (AHT) and some cardiovascular disorders (Barret, 1985). However, its use has been overpassed by other beta-blocking agents, Barret (1985) found the side-effects, mainly in asthma patients. Muscle contraction effects have been the cause of bronchoconstriction observed in those patients by Goodman and Gillman (1985). As propranolol is a mixture of two stereoisomers, *S(-)* and separately on AHT and bronchoconstriction. Barret (1985) demonstrated that such side effect could be attributed to the *R(+)* propranolol isomer, and there-fore proposed to develop enriched *S(-)* propranolol isomer galenic formulation of the highest possible "optical" purity to avoid the observed adverse effect of commercially available racemic mixtures of propranolol.

Yost and Holtzman (1979) proposed a highly attractive method of propranolol racemate resolution at preparative scale. Propranolol stereoisomers were separated by multiple-step recrystallization of its di-(p-toluyl) tartaric acid salts.

Silber and Riegelman, 1980; Matsuo and Ohno, 1985; Terao *et al.*, 1988 and Bevinakatti and Banerji, 1991, have proposed the preparation of *S(-)*-propranolol through immobilized catalysis using mainly several lipases as stereoespecific catalysts. The uses of immobilized enzymes versus other conventional chemical catalysis are being increasingly preferred by the pharmaceutical industry because of its higher selectivity and yield, with a less environmental impact.

Bevinakatti and Banerji (1991) have published examples about stereoselective esterification or hydrolysis of several drug racemates for its resolution using lipases. Chiral discrimination is a long-standing problem in the development, use and action of pharmaceutical agents. Since many drugs are chiral compounds and interact with a chiral receptor in the body, only one of the enantiomers will show the optimal therapeutic action. To avoid side effects of the unwanted enantiomer, the strongly regulated pharmaceutical industry increasingly demands drugs containing only the biologically active enantiomer. However, some synthetically produced chiral substances are still being sold in a racemic mixture. This is the case of Propranolol. Currently the commercially available preparation is a racemic mixture, in which only the *S(-)*-enantiomer has ß-adrenergic blocking activity (Barrett, 1985; Howe and Shanks, 1966; Potter, 1967; Rahn *et al.*, 1974; Walle *et al.*, 1984). Barrett (1985) indicated that its side effects on asthma patiens have been attributed to the *R(+)* isomer, which produces bronchoconstricction. Therefore, the resolution of commercial propranolol racemate in order to obtain the bioactive *S(-)* isomer at its highest purity is a matter of concern for a better treatment of AHT and asthma patients.

Several research groups (Silber and Riegelman, 1980; Hermansson and von Bahr, 1980; Hermansson, 1982; Thompson, *et al.*, 1982; Matsuo and Ohno, 1985; Terao, *et al.*, 1988; Bevinakatti and Banerji, 1991) have reported the preparation of (*S)*-propranolol by lipase catalysed reactions. However, the length of the procedure and the low overall yields precludes the industrial application of these methods.

Lipase catalysed reactions are superior to conventional chemical methods owing to mild reaction

conditions, high catalytic efficiency and the inherent selectivity of natural catalysts which results in much purer products, according to Dordick (1991).

The ability of enzymes to catalyse reactions in a selective manner has been used for many years by organic chemists to obtain enantiomerically pure compounds. Lipases (triacylglycerol hydrolases (EC 3.1.1.3) form a large group of enzymes frequently used in fine chemistry catalysis. They are stable in nonpolar solvents and they have the remarkable ability of assuming a variety of conformations to accommodate substrates of varying sizes and stereochemical complexities as Arroyo and Sinisterra (1994) reported. In organic media, there are examples related to enantioselective esterification or hydrolysis of racemic mixtures of drugs and agrochemical esters catalyzed by native or immobilized (Bevinakatti and Banerji, 1991) lipase but studies deal with the influence of structural variables on the enantioselectivity of immobilized enzymes are usually not reported in the organic chemistry literature.

The use of immobilized enzymes for such objective seems to be attractive in terms of the cost-benefit relationship and therefore a study conducted using several enzymes at different experimental conditions in order to establish a stereospecific procedure which may lead to obtain the propranolol *S(-)* isomer at the highest possible purity. The aim of this work has been the development of an enzymatic procedure to obtain the pure isomer from a propranolol racemic mixture and to establish the best conditions to achieve the biggest activity and enzymatic specificity.

II. METHODS

The *RS*-propranolol (*RS*-P) and the pure isomer were acquired from SIGMA. The screening were done with commercial lipases immobilized from *Candida rugosa (CR), Candida antartica (CA), Pseudomonas fluorescens (PF), Mucor mihei (MM), Rhizopus niveus (RN).* All reagents for synthesis were from Fluka and all the solvents were of analytical grade. ^{1}H and ^{13}C NMR spectra: Bruker Avance 400 instrument with $SiMe_4$ as internal standard, in $CDCL_3$; δ in ppm. HPLC-Mass Spectroscopy (LC-Mass): Agilent Technologies 1100 instrument LC/MSD Trap, equipped with ZORBAX Rapid resolution SB-C_{18}, (2.1 x 30 mm, 3.5μ) column; samples were dissolved in chloroform. HPLC (analytical and preparative): Agilent Technologies 1100 instrument equipped with UV detector, quaternary pump, thermostatized automatic injector and column compartment and Agilent ChemStation for LC systems; reverse phase column ChromSpher C18 (250 x 4,6 mm); flow 1 mL/min; mobile phase: Acetonitrile:H_2O:TEA; 60:40:0,14 (v/v/v).

A. Synthesis of *RS*-O-butyryl propranolol (*RS*-O-BP).

The *RS*-O-BP was synthetized follows the classical methodology of Gatterman (1961), in brief: *RS*-propranolol-HCl (1g; 3.8 mmol), was refluxed with dichloromethane (30 ml) and butyryl chloride (0.39 ml; 3.8 mmol) was added drop by drop. After two hours, the reaction mixture was washed successively with equal volumes of saturated aqueous sodium bicarbonate and brine. The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure to afford *RS*-O-BP. Yield: 85%; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.97(3H,t), 1.22(3H,d), 1.31(3H,d), 1.67(2H,m), 2.33(2H,m), 3.40(1H,q), 3.83(1H,q), 4.12(1H,m), 4.29(2H,m), 5.59(1H,m), 6.87-8.21(aromatics). ^{13}C NMR ($CDCl_3$) δ (ppm): 13.59, 18.33, 21.07, 21.80, 36.25, 41.33, 48.42, 68.51, 71.56, 104.82, 120.48, 121.89, 125.14, 125.52, 125.82, 126.32, 127.39, 134.42, 154.29, 173.80. ESI-MS m / z 352.4 [$(M + Na)^+$].

B. Procedure for lipase immobilization.

2ml of octil sepharose (volume of the packed support) was added to 8 U of lipase suspended in 2 mL of sodium phosphate tampon 25mM pH=7 at 4°C. A blanc suspension was also prepared adding 1ml of sepharose 4BCL. After 24h the immobilization process concluded filtering the suspension and washing the gel with abundant distilled water (Bastida *et al.*, 1998).

C. Enzymatic hydrolysis and effect of different parameters on its initial rate.

The starting conditions were: To a solution of *RS-O-BP* 0.5mM was added 20 % of organic solvent. For all the experiments the final reaction volume was 5 mL. The temperature of the mixture was adjusted to the selected value and 6.5U of immobilized lipase were added (the same procedure was used for pure isomers). The effect of different parameters on the initial rate was studied following the progress of the reaction with automatic titration (pH-stat) using a 25mM dissolu-tion of NaOH and withdrawing a 100 μl aliquot each 5 min. and diluting it with 100 μl of the elution buffer. Samples of 20 μl were injected in the chromatograph using as mobile phase acetonitrile: H_2O: trietylamine (60: 40: 0,14; v/v/v) (Ávila-González *et al*., 1997). The area peak corresponding to *RS*-O-BP was measured and converted into concentration by using a calibration curve that was constructed injecting known amounts of *RS*-O-BP in the same conditions. *RS*-O-BP concentration was plotted against time to obtain a curve of substrate consumption.

The initial rate was obtained calculating the slope of the linear part of this curve and was expressed as the decrease in the substrate concentration (μM) per minute.

C. Experimental Design

To optimize the hydrolysis a central composed design 2^3 was carried out; with three experiments in the central plane (Himmelblau, 1970)

As independent variables were studied: partition coefficient octanol/H_2O (Log P) ((-1.1)-(-0.49)); Temperature (T) (4-37 ^{0}C); Ionic Strength (IS) (10-50 mM). The enzymatic activity (EA) in *PF* and *CR* enzymes was chosen as dependent variable.

III. RESULTS AND DISCUSSIONS

As it was said previously lipases catalyze the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and synthetic esters, (Santaniello *et al.*, 1993). Khmelnitsky and Rich. (1999) signed that some of the lipase-catalysis applications are carried out in organic solvents, which due to their toxicity must be eliminated from the final product. Thus, enzymatic procedures for the synthesis of chiral drugs in semi aqueous systems would suppose a valuable alternative to the traditional methods.

A screening with several immobilized lipases was done following the starting conditions of hydrolysis above mentioned.

Table 1 show that the enzymes with greater activity and stereospecific ratio were those from *CR* and *PF*.

A later study showed the influence of pH on the activity and stereospecificity of both these enzymes. Two pH values were tested (7 and 9) for further work, because the EA at pH values, outside this range, decreased as Chin-Shih (1994) reported (Table 2).

A neutral pH value for the optimization studies of reaction parameters was chosen because greater stereo-specificity was obtained. The results show that the enzymatic hydrolysis occurs faster at pH 9 than that at pH 7, but the enantiomeric ratio is lower. Probably, the lipase recognize both isomers at the same time however when pH 7 is used the enantiomeric ratio is higher for both of them. This behaviour could be explained by the capability of the lipase to recognize the compounds by its ionization grade. When the substrate is no-ionized it is better recognized for the enzyme than when it is ionized, this increases the enzyme affinity and also the stereospecificity. Similar results were obtained by Fernandez-Lorente *et al.* (2004).

Table 1: Hydrolysis of *RS*-O-BP with different lipases.

Lipases	EA[a]	Enantiopreference	E[b]
PF	5.9	*S*	0.22*
CR	8.2	*R*	5.4
CA	0.5	*S*	0.6*
MM	0.02	*S*	0.5*
RN	0.2	*R*	2

[a] Enzymatic activity (EA) of *RS*-O-BP (U/mL gel).

[b] Stereospecificity calculated as the relationship of ***R(+)/S(-)***.
* High values for *S(-)*

Table 2: Influence of pH on the EA.

	pH 7			pH 9		
Lipases	*R(+)*	*S(-)*	E[b]	*R(+)*	*S(-)*	E[b]
PF	0.9	4.4	**0.2**	4.7	4.0	1.17
CR	6.3	1.2	**5.3**	3.5	4.2	1.2

[b] Stereospecificity calculated as the relationship of ***R(+)/S(-)***.

Considering the results obtained at the starting conditions, the behavior of the EA was modeled as a function of the independent variables using a statistical polynomial model of third order of magnitude.

$$Y=b_0+\sum b_i X_i+\sum b_{ij} X_i X_j+\sum b_{ii} X_i^2+\sum b_{iii} X_i^3+b_{ijk} X_i X_j X_k. \quad (1)$$

The Student *t*-test and the adjustment of the model evaluated the significance of the coefficients by the approach of Fisher for a 95% of confidence.

The results indicated that the influence of parameters on the EA was Temp>Log P>IS. In case of the *PF* enzyme the model did not show the expected adjustment. A total of 7 models were evaluated. The next equation showed the best result for the lipase from *PF*.

$$Y_1=\frac{Log\left(b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{23}X_{23}\right)}{Exp\left(b_1X_1+b_2X_2+b_3X_3\right)} \quad (2)$$

The analysis of significance of the parameters offered similar results to those obtained with the other enzyme: Temp>Log P>IS.

For both enzymes it was proven that the levels of significance of the Log P and the ionic strength were similar (8.5-6.0 and 30.7-26.05), while the temperature was superior. It was also observed that the temperature influenced in a different way the two enzymes.

For the *PF* enzyme the increase of the temperature caused an increase of the activity, however, in the case of *CR* it was inverse, the biggest activities were observed at low temperatures.

The denaturalization processes caused in some lipases by the increment of temperature could explain such results (Dordick, 1989).

With the purpose of determining the best conditions for *RS*-O-BP hydrolysis, it was used a software (MATLAB V 4.2) to search the global maximum of the system. The results show the conditions that guarantee the maximum stereospecificity in the reaction system (Table 3).

The experiments were carried out and they were compared with the calculated data. The following results were obtained (Table 4). The EA of the immobilized *PF* enzyme in the reaction of hydrolysis of the *RS*-O-BP was of 15.1 U/mL gel. With pure isomers, the activity with the *R(+)* isomer was 1.7

U/mL gel and for the *S(-)* isomer was 13.9 U/mL gel. The stereospecificity was 0.12.

For *CR* enzyme the experiment was not carried out to the exact conditions that were described due to the Log P maximum was obtained at –0.1 (Activity = 9.4), value that does not correspond to any solvent, so the nearest data-number was chosen, in this case acetone with –0.23. Under these new conditions the theoretical model was repeated and the value of 9.2 U/mL gel was obtained which do not have significative differences from the initial value.

Table 3: Theoretical results of the EA obtained from the optimization model.

Parameter	**Lipases from**	
	PF	***CR***
T (°C)	37	15
Log P	0.49	-0.1
IS (mM)	15	25
RS-O-BP EA (U/mL gel)	**22.7**	**9.4**

The experiment under these news conditions show-ed (Table 4) that the *CR* enzyme has an activity of 8.9 U/mL gel. With pure isomers, the activity with the *R(+)* isomer was 10.7 U/mL gel and for the *S(-)* isomer was 1.5 U/mL gel, the stereospecificity was 7.1.

Table 4: *Comparation* of the experimental results with the calculated values from optimization model.

O-BP	**EA (U/mL gel)**			
	PF		***CR***	
RS	22.7*	15.1	9.4*	8.9
R(+)		1.7		10.7
S(-)		13.9		1.5
E[b]		**0.12**		**7.1**

(*) Theoretical results.

[b] Stereospecificity calculated as the relationship of ***R(+)/S(-)***.

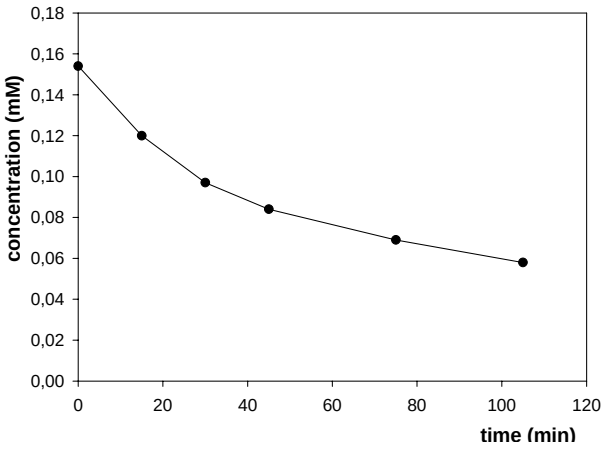

Fig. 1. Progress curve of the reaction with *PF* in the optimal conditions.

As it can be appreciated, *CR* enzyme model describes with more reproducibility the theoretical and practical results that *PF* enzyme. The results showed that the stereospecificity increased for both enzymes compared to the initial values. The enantiomeric excess of (*S*)-propranolol (ee_s) was determined at the end point of the reaction (0.058mM; 40% of substrate conversion) (Fig. 1). Under these conditions values of ee_s of 87% and E= 47 (Chen *et al.*, 1982) were obtained which are higher than that reported by Matsuo and Ohno (1985) for the enzymatic preparation of *(S)*-propranolol.

IV. CONCLUSIONS

The best results for the enzymatic hydrolysis of *RS*-O-BP were obtained with *PF* and *CR*. The better conditions were 37 °C, tetrahydrofuran and buffer phosphate 15mM for *PF* and 15 °C, acetone and buffer phosphate 25 mM for *CR*.

The immobilized enzyme *CR* is stereospecific for the hydrolysis of the *R*-O-BP and the *PF* for the *S*-O-BP. The values of stereospecificity increased for both enzymes compared to the values in the initial conditions. Under these conditions an *ee* 87% and E=47 were obtained.

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