CONTENTS

FREE LIPASES-BASED ENZYMATIC ACETYLATION OF RACEMIC ATENOLOL: A PRELIMINARY KINETIC RESOLUTION STUDY

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ABSTRACT

Since distomers of racemic drugs are frequently not used to heal diseases, utilization of single enantiomeric drugs not only decreases the drugs dosages and side effects but also reduces eco-toxicological problem. Since enzymatic membrane reactors (EMR) can be run continuously, observation on free-enzyme catalysis as a preliminary study before development of the EMR is needed. This paper describes acetylation of the racemic atenolol enzymatically using free lipases. The atenolol enantiomers reacted with vinyl acetate in water miscible organic compounds and phosphate buffer solutions. High conversions were obtained once the reactions were conducted in the organic media using PFL (X_R: 84.22%, X_S: 91.78%), Lipoprotein 62336 (X_R: 100%; X_S: 100%) and CALB (X_R: 77%, X_S: 51.82%). Reactions in PO_4 buffers produced low conversions. It seems the KR process was difficult to be developed through the acetylation pathway. During observations on the AT enantiomers' concentrations, the analytical protocols produced excellent selectivities. The highest selectivity was given by the slowest flow rate, which developed higher enantiomeric peak areas.

Keywords: Free-enzyme acetylation, organic and buffers media, racemic atenolol.

INTRODUCTION

The different effects of racemic drugs enantiomers emphasize that they should be used individually. As in β-blockers, the distomers give no β-blocking effects, reduce the overall drug selectivity, produce side-effects as the eutomers or possibly cause adverse effects. Since the distomers are frequently not used to heal diseases, uses of single enantiomers not only decrease the dosage and side effects but also reduce the eco-toxicological problem. The world consumptions on the single enantiomers increased from US$ 74.4 billion in 1996 [1] to US$ 225 billion in 2005 [2].

2-{4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl}acetamide or popularly known as atenolol (AT) is a famous β-blocker used for maintenance of blood pressure, angina pectoris and arrhythmia. It is available in the form of racemic and single enantiomeric compound. Its active isomer resides on (*S*)-enantiomer. This isomer avoids side effects generated by the racemate, while (*R*)-enantiomer does not show the β-blocking activity and has not lacked of the side effects. Switching from the racemate to the single enantiomer develops lesser side effects.

Highlight of the technology to generate (*S*)-AT are given in a previous literature [3]. It was synthesized asymmetrically from racemic or achiral feeds, which required presence of chiral metal ligand catalysts such as (*R,R*)-Co-(salen) complexes to do the hydrolytic kinetic resolution step [4]. The (*S*)-enantiomer was also prepared from catalytic reactions of achiral starting feeds without involvement of a chiral catalyst, which operated at relatively low temperatures, but a chiral addendum such as (*R*)- or (*S*)- epichlorohydrin must be available [5]. Although chromatographic resolution is difficult to appear in large scale operations, it had generated the active isomer using various chiral selectors and the existing racemic atenolol [6]. Microbial fermentation of the racemic atenolol and its derivates using *Rhizopus arrhizus* and *Geothricum candidum* gave the single enantiomer in high optical purities and chemical yields [7]. Enzymatic resolutions of the racemic AT using immobilised lipases were developed as well through enantioselective esterification and hydrolysis processes [8, 9]. Since enzymatic membrane reactors can be run continuously [10], observation on free-enzyme catalysis as a preliminary study before development of the EMR is required. In this report, enzymatic acetylation of the racemic atenolol in batch stirred reactors is described. To the best of our knowledge, no article has been found to resolve this racemate using free lipases.

METHOD

Materials

(*R,S*)-AT (99% pure), (*S*)-AT (97% pure) and (*R*)-AT (99% pure) were bought from Nanjing Chemlin Chemical Industry Co Ltd (China), Tocris Bioscience (England) and Sigma Aldrich (M) Sdn Bhd (Malaysia), respectively. The

racemate was dissolved in the reaction media according to the desired concentrations. Standard solutions of pure enantiomers were prepared by dissolving the crystals into dimethyl sulfoxide with concentrations of 9 mg/mL. All chemicals were of analytical grade except for analysis (of HPLC grade) and bought from EOS Scientific (M) Sdn Bhd, Fisher Scientific (M) Sdn Bhd, Merck Sdn Bhd and Sigma Aldrich (M) Sdn Bhd. The AT and other chemicals were used without purification. *Candida antarctica* lipase fraction B (Lipozyme® CALB L LCN02103 supplied by Science Technics Sdn Bhd, Malaysia), lipase basic kits (62323, 62327) from Fluka Analytical (supplied by Sigma Aldrich (M) Sdn Bhd, Malaysia), and Amano lipase PS from *Burkholderia cepacia* (supplied by Sigma Aldrich (M) Sdn Bhd, Malaysia) catalysed the enzymatic reactions. All lipases were used without initial treatment.

Preparation of Buffer Solution

Several 67 mM Sorensen buffers were prepared by mixing KH_2PO_4 and Na_2HPO_4 solution. A certain quantity of the KH_2PO_4 (Acros Organics, 99%+) and Na_2HPO_4 (Acros Organics, 98%+) were dissolved in doubly distilled water to form 133 mM buffers. Both solutions were then adjusted to the desired pH values and then diluted with the doubly distilled water to give the 67 mM buffers.

Lipase catalysed acetylation of (*R,S*)-AT

Reaction in organic media. 5 mg/mL racemic AT were prepared in DMSO, DMF and THF. 20 mL of each solution were mixed with vinyl acetate at ratio of 1:1.5 (mole/mole) in 100 mL Erlenmeyer Flasks. The mixtures were shaken in an orbital shaker (Max Q4000 Barnstead Lab-Line) at 200 rpm and 40°C for 30 minutes. Lipases were then added (1.5-50 mg). Enzymatic acetylations were carried-out at 200 rpm and 40°C (Protocol-01 used 35°C and 25 mL AT solutions) in the shaker for 6 hours.

Reaction in PO_4 buffers. Racemic AT was dissolved in Sorensen buffers (67 mM, pH 6.6–7.8) to form 10 mg/mL solutions. 25 mL of each solution were placed in reaction flasks where vinyl acetate was added (1:1 mole/mole). The flasks were shaken at 40°C and 200 rpm for 30 minutes. Lipase was then added (CALB: 665 µL, CRL: 7 mg). The enzymatic reactions were conducted in the shaker at conditions as stated previously for 10 hours. Variations of the operating factors were conducted using 30 mL solution of PO_4 pH 7.8 for 5 hours.

Samples Preparation and Analysis

Analysis of the AT enantiomers was performed on a Shimadzu UFLC (ultra fast liquid chromatography) LC-20A Prominence system. The system consist of two units LC-20AD dual plunger parallel flow solvent delivery pump, a SIL-20ACHT auto-sampler, a SPD-20A UV-VIS detector, a CTO-20AC column oven, a DGU-A3 degasser unit and the CBM-20A system controller. The UFLC was connected to a personal computer to operate the equipment using the Shimadzu LCsolution Real Time Application software.

After reactions, 1 mL sample was taken-out from each reaction flask and placed inside a 1.5 mL vial then kept in 4°C refrigerator prior to analysis. Before injection, all samples were centrifuged at 6,000 rpm using Profuge 6k centrifuge (supplied by Interscience Sdn Bhd, Malaysia) for 15-20 minutes where 500-650 µL of the centrifuged samples were taken out and transferred into 1.5 mL clean bottles. 1-2 µL of samples were injected automatically at a time into the Chiralcel® OD column (250 mm x 4.6 mm) with the mobile phase of hexane-ethanol-diethyl amine. The UV/Vis detector was set at the wavelength of 254-276 nm. The UFLC was operated at normal phase at 35°C. Qualitative and quantitative analysis were conducted on the resulted chromatograms via the Shimadzu LCsolution post-run analysis software based-on the standard procedure for the instrumentation.

RESULTS AND DISCUSSION

As racemic AT is a hydrophilic compound, buffer solutions and water miscible solvents were used in monophasic enzymatic reactions [7, 8]. Kinetic resolution of the racemate in hydrophobic solvents was also found [9], but the process required a biphasic system. Characteristics of the racemic AT mixtures in various solvents are given in Table 1. The racemate crystals dissolved in dimethylsulfoxide (DMSO) and dimethylformamide (DMF) formed clear bilayer mixtures when cyclohexane, heptane or isooctane was added to these AT solutions. The racemate in acetone, chloroform and 2-propanol created whitish suspensions when they were mixed with hexane, heptane, toluene, cyclohexane or isooctane in

proportional or high mixing ratio. Alcohols are excellent solvents for dissolving the racemic AT especially methanol and ethanol, but none of these liquids has been used to resolve the racemic AT.

Table 1: Combination of AT solutions and their co-solvents.

AT Solvent	Solution Concentration	Co-solvent	(Solvent : Co-solvent) Ratio	Remark
Acetone, 2-Propanol	5 mg/mL	Hexane, Cyclohexane, Heptane, Isooctane	1:1 – 1:3 (v/v)	Whitish suspension
Acetone, 2-Propanol	5 mg/mL	Toluene, Benzene	1:1 – 1:3 (v/v)	Clear solution
Chloroform	9 mg/mL	Hexane, Cyclohexane, Heptane, Isooctane, Benzene, Toluene	1:1 – 1:3 (v/v)	Whitish suspension
Ethanol	5 mg/mL	Hexane, Cyclohexane, Heptane, Isooctane, Toluene, Benzene	1:1 – 1:3 (v/v)	Clear solution
DMSO, DMF	5 mg/mL	Heptane, Isooctane, Benzene, Cyclohexane	1:1 – 1:3 (v/v)	Bilayer solution
DMSO, DMF	5 mg/mL	Toluene	1:1 – 1:3 (v/v)	Clear solution

All mixings were conducted at room temperature ; IPA: 2-Propanol

Enzymatic Acetylation of Racemic AT

Organic Solvents

The enzymatic processes were aimed to resolve the racemic AT through either kinetic resolution (KR) or dynamic kinetic resolution (DKR) to yield the active isomer of the AT compound. Results of the acetylation process in DMSO, DMF and THF are given in Table 2.

Both AT enantiomers reacted with the acetate compound in the reaction media. High conversions were obtained once the reactions were conducted in the media using PFL (X_R: 84.22%, X_S: 91.78%), Lipoprotein 62336 (X_R: 100%; X_S: 100%) and CALB (X_R: 41.23%; X_S: 46.85%) during the reaction time. Most lipases acetylated the (*R*)- and (*S*)-AT existing in DMSO and DMF. Although many lipases in THF showed poor activity, this medium was suitable as high conversions were developed by the enzymes. Since enzyme activity is high in non-polar and water immiscible solvents [19], combined media such as DMSO-Toluene and DMF-Toluene were also studied. Poor acetylation results were obtained in DMSO-Toluene, but reactions in DMF-toluene produced some high conversions for both enantiomers. It seems the KR process was difficult to be developed through the acetylation pathway.

Table 2: Results of the enzymatic acetylation in organic solvents.

Lipase		Conversion (%)									
		(*R*)-enantiomer					(*S*)-enantiomer				
		DMSO	DMF	THF	DMSO - Toluene	DMF - Toluene	DMSO	DMF	THF	DMSO - Toluene	DMF - Toluene
CRL	10 mg	21.88	3.86	0	2.92	6.73	32.87	4.67	0	8.35	10.28
PCL	10 mg	14.83	3.35	6.72	0	4.61	26.25	4.20	11.40	0	4.62
CCL	50 mg	13.54	12.77	4.88	0	6.25	23.76	14.74	5.37	0	9.64
LP 62336	10 mg	12.60	2.18	100.00	0	39.70	22.92	2.60	100.00	0	54.79
LP 62335	1.5 mg	50.75	0	0	0	1.28	19.45	0.53	0	0	2.12
LAPS	30 mg	13.63	2.27	0	0	0	24.19	3.21	0	0	0
MML	10 mg	12.05	0	0	0	0	21.15	0.83	0	0	0
RAL	30 mg	17.88	3.37	8.99	0	0	29.62	3.88	9.58	0	0
RNL	30 mg	10.59	0	2.10	0	76.99	20.72	0.17	2.99	0	100
HPL	30 mg	0	22.09	0	1.86	0	0	31.67	0	1.81	0
PFL	8 mg	16.13	3.20	84.22	0	0	23.23	3.63	91.78	0	0
MJL	35 mg	26.26	4.25	27.16	0	12.15	35.21	5.14	19.75	0	17.75
CALB	200 µL	29.82	18.17	41.23	0	9.33	42.82	19.57	46.85	0.77	13.55
AN	30 mg	27.80	6.56	17.25	0	0	38.60	6.48	18.26	0	0
AOL	20 mg	27.19	8.64	0	0	0	39.46	9.02	0	0	0
ROL	30 mg	23.87	10.62	7.63	2.32	0	33.62	11.52	7.66	3.37	0

CRL: *Candida rugosa*; PCL: *Pseudomonas cepacia*; CCL: *Candida cylindracea*; LP: Lipoprotein; LAPS: Amano Lipase from *Burkholderia cepacia* ; MML: *Mucor miehei* ; RAL: *Rhizopus arrhizus*; RNL: *Rhizopus niveus*; HPL: Hog Pancreatic; PFL: *Pseudomonas fluorescence*; MJL: *Mucor javanicus*; CALB: *Candida antarctica* fraction B ; AN: *Aspergilus niger*; AOL: *Aspergilus oryzae*; ROL: *Rhizopus oryzae*

Buffer Media

Results of the acetylation reactions in PO_4 solutions using CALB and CRL are shown in Fig. 1. Both lipases produced similar conversions of both enantiomers. In general, the results were in the range of 8.62-22.25% (mole/mole) where PO_4

buffer pH 7.4 (CRL) and 7.8 (CALB) produced high conversions. The same characteristic with the organic media were observed: the (*R*)- and (*S*)-AT enantiomers were acetylated. Lower results than the organic solvents were obtained, although high racemic AT concentrations were used.

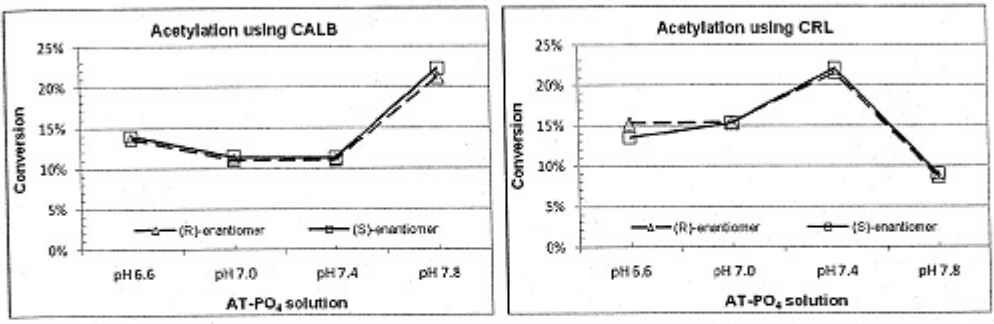

Fig. 1: Acetylation of the AT in buffer solutions.

Variations of some operational factors in PO_4 buffer pH 7.8 using CALB are shown in Fig. 2. Increasing the vinyl acetate quantity and agitation speed did increase the enantiomers conversions. High results were found at temperature of 35°C. The highest enzyme quantity tended to give high enantiomers conversions, but the enzyme quantity of 2000 IUN provide the results almost similar to the highest enzyme contents. The conversions of the (*S*)-enantiomer were found higher than the (*R*)-AT. The KR process through the acetylation pathway was found difficult to be developed.

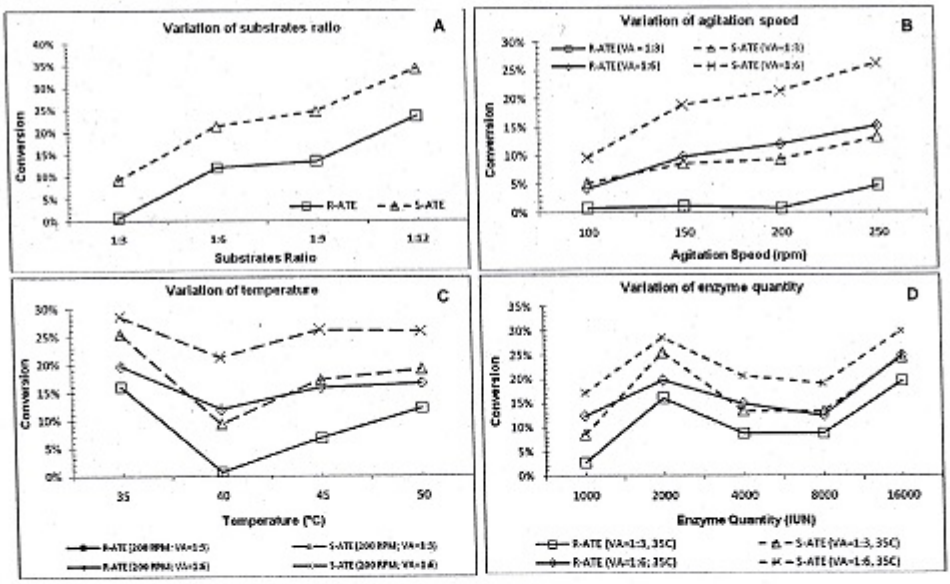

Fig. 2: Conversions of the enantiomers under various operating conditions: A) 380 µL CALB, 200 rpm, 40°C; B) 380 µL CALB, 40°C; C) 380 µL CALB, 200 rpm; D) 200 rpm.

The Analytical Protocol

Selection of an analytical protocol able to satisfy observations on the AT concentrations during the reaction times is essential as conversion, enantiomeric excess (ee) and enantiomeric ratio (E) of the resolution processes are based-on the UFLC area of the (*R*)- and (*S*)-enantiomer. Comparisons of the analytical protocols applied in the AT resolution studies are given in Table 3. The enantioseparation factors (selectivities) for all the tested protocols were excellent since the minimum

recommended α is 1.20 [11]. The highest selectivity was produced by Protocol 04, which operated at the lowest flow rate. Further works are required to improve the R_S because the results were low if compared with the standard baseline resolution (R_S = 1.5 [12, 13]).

Table 3: Characteristics of analytical protocols for the Chiralcel® OD column.

Protocol / [Ref.]	Mobile Phase (v/v/v)	Flow Rate (mL/min)	Wave length	UFLC Peak (min)		UFLC Results	
				(*R*)-AT	(*S*)-AT	α	R_S
01 / [14]	80 A / 20 B / 0.6 C	1.00	254 nm	8.5	10.3	1.33-1.37	0.73-1.07
02 / [15]	80 A / 20 B / 0.6 C	0.75	254 nm	10.1	11.8	-	-
03 / [16]	75 A / 25 B / 0.1 C	0.70	276 nm	9.9	11.7	1.36-1.52	0.62-0.82
04 / [17]	60 A / 0.1 C / 40 D	0.50	275 nm	12.4	15.4	1.44-1.93	0.82-0.94
05 / [18]	60 A/40 B/0.2 C /0.2 E	1.00	276 nm	-	-	-	-

A: Hexane; B: Ethanol; C: Diethylamine; D: 2-propanol; E: Acetic acid; (-): not examined; Ref.: Reference; α: enantioseparation factor; R_S: enantioresolution

Although Protocol 01-04 generally produced almost similar α and the high flow rate gave the quickest peak appearance, the slower flow rates developed higher peak areas as shown in Fig. 3. The Protocol-02 (low flow rate, high modifier compound, 2 µL injected volume) peak sizes were higher around 130,000 peak areas than the Protocol-01 (high flow rate, high modifier compound, 2 µL injected volume). The results obtained from the Protocol-03 were better than the Protocol-01 and -02. The third Protocol (low flow rate, low modifier compound) gave higher enantiomeric peak intensities (2-3 times) than the first protocol from a low samples volume (1µL). Protocol-04 (low flow rate, high alcohol content, and 1 µL injected volume) obtained the (*R*)-AT peak area slightly higher than the Protocol-03 result and three times higher than the first protocol. Lowering the flow rate did increase interaction between the analyte and chiral stationary phase.

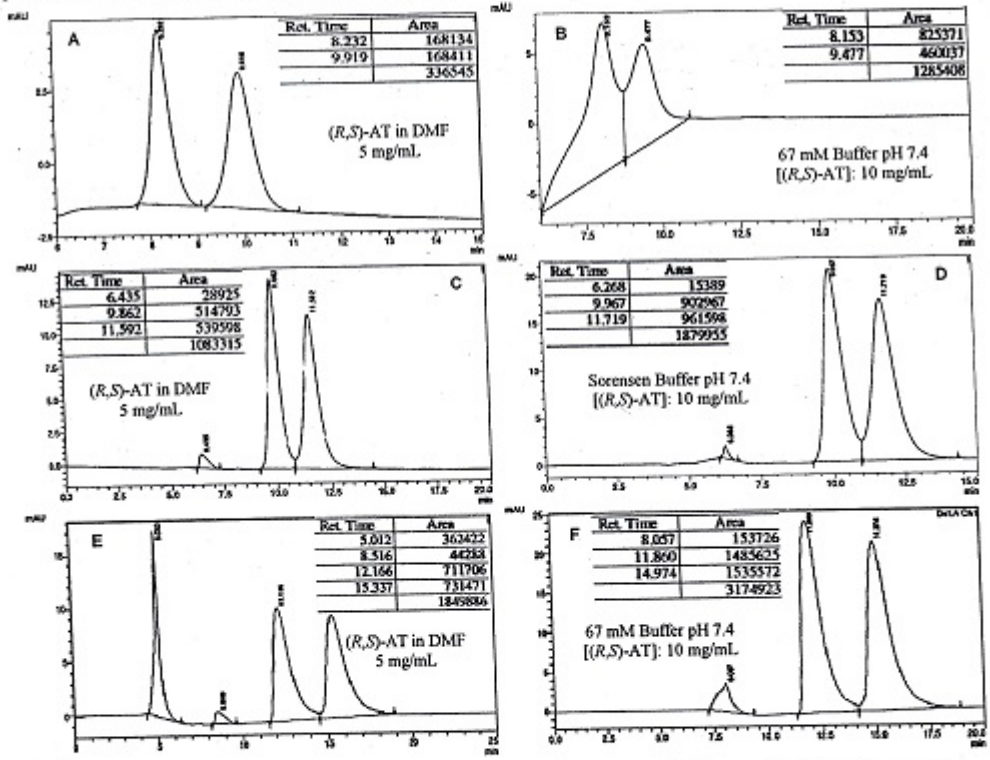

Fig. 3: Comparison of peak area sizes of each protocol (A,B: Protocol-01; C,D: Protocol-03; E,F: Protocol-04)

CONCLUSION

The AT enantiomers were acetylated either in buffer solutions or in organic solvents. High conversions were obtained once the reactions were conducted in the organic media using PFL (X_R: 84.22%, X_S: 91.78%), Lipoprotein 62336 (X_R: 100%; X_S: 100%) and CALB (X_R: 41.23%; X_S: 46.85%). Reactions in PO4 buffers produced low conversions. It seems the KR process was difficult to be developed through the acetylation pathway. During the reactions, selection of an analytical protocol able to satisfy observations on the AT enantiomers' concentrations in the reaction media is essential. The enantioseparation factors for all the tested protocols were excellent. The highest selectivity was produced by the slowest flow rate, which developed higher enantiomeric peak areas.

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