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Direct Enantioselective HPLC Monitoring of Lipase-Catalyzed Kinetic Resolution of Flurbiprofen

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ABSTRACT The solvent versatility of Chiralpak IB, a 3,5-dimethylphenylcarbamate derivative of cellulose-based chiral stationary phase, is demonstrated in the direct enantioselective HPLC monitoring of lipase-catalyzed kinetic resolution of flurbiprofen in nonstandard HPLC organic solvents. Nonstandard HPLC organic solvents were used as the reaction media for the lipase-catalysis and in mean time as diluent to dissolve the "difficult to dissolve" enzyme substrate (the acid) and as eluent for the simultaneous enantioselective HPLC baseline separation of both substrate and product in one run without any further derivatization. *Chirality 22:597–603, 2010.*

KEY WORDS: chiral separation; chiralpak IB; direct monitoring; enantioselective HPLC; flurbiprofen; HPLC; kinetic resolution; lipase

INTRODUCTION

Flurbiprofen (*R*, *S*)-[2-(3-fluro-4-phenyl) phenyl] propionic acid is one of the most prevalent nonsteroidal anti-inflammatory drugs (NSAIDs).[1,2] The S-enantiomer exhibits most of its anti-inflammatory activity, while the R-enantiomer enhances its gastrointestinal toxicity.[3] Furthermore, *R*-flurbiprofen blocks nociception in rats, indicating a different mechanism of analgesia and suggesting the use of this enantiomer, and possibly other *R*-aryl propionates, as analgesics.[4] Flurbiprofen is reported to be less prone to undergo chiral inversion in vivo.[5,6] Consequently, the resolution of flurbiprofen is particularly important.

To date, kinetic resolution is still one of the major strategies for resolution of racemic mixtures[7] using either chemical[8] or enzymatic[9] procedures. Indeed, enzymes are remarkable catalysts, capable of accepting a wide range of substrates, at the same time exhibiting highly chemo-, regio-, and enantioselectivity. Moreover, the price and availability of most classes of enzymes have significantly decreased over the last few years making them more economically attractive catalysts for the production of biologically active compounds.[10] Of particular interest, lipases were most commonly used as biocatalysts for the enzymatic resolution of racemates. Most lipases accept a broad range of non-natural substrates and are thus very versatile for applications in organic synthesis. They do not require cofactors and are commercially available in free and immobilized forms. In many cases, they exhibit good to excellent stereoselectivity.[11]

It is widely suggested that the lipase-catalyzed esterification be conducted in hydrophobic solvents.[12,13] However, the solubility of flurbiprofen is poor in the commonly used hydrophobic solvents, such as isooctane and *n*-hexane.[14] Thus, the reported lipase-catalyzed resolution of flurbiprofen has been performed in hydrophilic standard organic solvents, particularly, acetonitrile.[14–16] Nonstandard HPLC organic solvents are seldom explored in the lipase-catalyzed kinetic resolution of racemates due to the difficulty associated with the enantioselective analysis. Nevertheless, if analyzed, they are not directly monitored as both substrate and product and not analyzed in one run.[17,18] The latter render the calculated enantiomeric excesses of both substrate and product inaccurate. This is due to the different response factor of the detector towards the substrate (acid) and product (esters) in case of lipase-catalyzed esterification or vice versa in case of enantioselective hydrolysis.

Lipase-catalyzed kinetic resolution of flurbiprofen in nonstandard HPLC solvents such as dichloromethane (DCM), ethyl acetate (EtOAc), methyl *tert*-butyl ether (M*t*BE) and tetrahydrofuran (THF) might be the method of choice to overcome solubility problem of the substrate.[19] However, such nonstandard solvents might cause irreversible damage of the CSP by dissolving or swelling of the chiral selector.[20–22] Thus, reactions in nonstandard HPLC organic solvents are usually monitored off-line.[20]

Yet, a new generation of chiral packing materials for resolution of enantiomers by chromatography has been introduced by immobilizing the polysaccharide derivatives onto the chromatographic matrices.[23–28] Thus, series of immobilized polysaccharide-derived CSPs namely Chiralpak IA, Chiralpak IB and most recently Chiralpak IC became commercially available.[29,30] Such immobilization renders the polysaccharide derivatives insoluble in any

Dedicated to Professor Schurig on the occasion of his 70's birthday.

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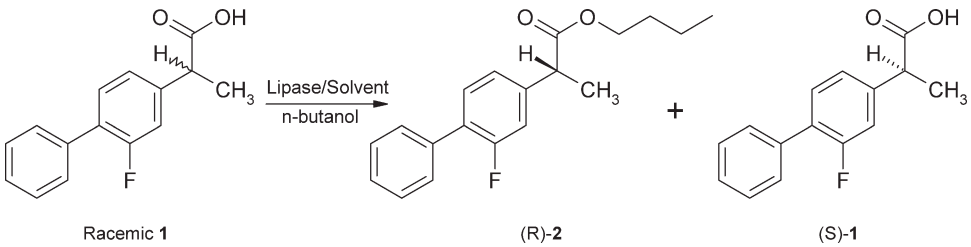

Fig. 1. Lipase-catalyzed enantioselective esterification of racemic flurbiprofen (**1**) using *n*-butanol in nonstandard HPLC organic solvents.

mobile phase solvent[21,22] and combines the benefits of the coated polysaccharide-type CSPs namely the broad application scope and the high preparative potential- with enhanced robustness, practically unlimited solvent compatibility and extended range of applications.[21–24] Subsequently, broadening the choice of solvents able to be used as mobile phase eluent or diluents.[19,20]

Here, we report on the first direct enantioselective HPLC monitoring of lipase-catalyzed enantioselective access to enantiomerically pure/enriched flurbiprofen using nonstandard HPLC organic solvents as diluent to dissolve the enzyme substrate and eluent to analyze both substrate and product in one run without further derivatization.

EXPERIMENTAL

Instrumentation

The mobile phase for HPLC was filtered through a Millipore membrane filter (0.45 ml) from Nihon Millipore (Yonezawa, Japan) and degassed via Waters in-line degasser AF. The HPLC system consisted of a Waters binary pump, Model 1525, (Milford, MA), equipped with a dual k absorbance detector model 2487 and an autosampler model 717plus. The UV-detector was set at 300 nm. Chiralcel OD (4.6 mm × 250 mm ID coated on 5 ml silica-gel) and Chiralpak IB (4.6 mm × 250 mm ID immobilized on 5 ml silica-gel) column was purchased from Chiral Technologies Europe (Illkirch, France). The enantiomeric excess ee of the substrate (ees) and the product (eep) as well as the conversion (Conv.) and enantioselectivity (*E*) were calculated as previously described.[31–33]

Materials

All solvents used as mobile phase in HPLC including 2-propanol were HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ). *n*-butanol used in the synthesis of ester was Analar grade and purchased from BDH chemicals (Poole, England). Lipases from *Asperigillus niger* (lipase AS), *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) free (lipase PS) and immobilized on ceramic particles (lipase PS-C) together with *Candida rugosa* (lipase AYS) were gifts from Amano (Nagoya, Japan). Immobilized *Candida antarctica* lipase B (Novozym435), its free form (Novozym525), and immobilized *Rhizomucor miehei* lipase (lipozyme RM IM) were from Novo Nordisk (Bagsvaerd, Denmark). Lipase type VII from *Candida rugosa* (CRL) was from Sigma (Steinhiem, Germany). Lipase from *Candida cylinderacea* (CCL), lipase immobilized in sol–gel-Ak on sintered glass from *Mucor miehei* (MML) and flurbiprofen were purchased from Fluka (Buchs, Switzerland).

Chromatographic Conditions

The mobile phase was consisted of *n*-hexane/methyl *tert*-butyl ether/2- propanol/trifluroacetic acid (85:15:0.4:0.1 v/v/v/v) and the flow rate was 1.3 ml/min. The column was operated at room temperature (24°C). UV detection was set at 254 nm.

Chemical Synthesis of Racemic Butyl Ester of Flurbiprofen (2)

Few drops of conc. sulfuric acid were added to 0.04 M racemic flurbiprofen (1) in benzene followed by the addition of 360 μl of *n*-butanol (0.04 mol, 3 M equiv.). The mixture was stirred under reflux over night, solvent was evaporated under vacuum and the residue was neutralized with 10% sodium hydrogen carbonate. The ester was extracted twice using 30 ml diethyl ether. The ether was dried over sodium sulfate anhydrous, filtered, and evaporated under vacuum to afford 0.46 g pale yellowish oil (76% chemical yield).

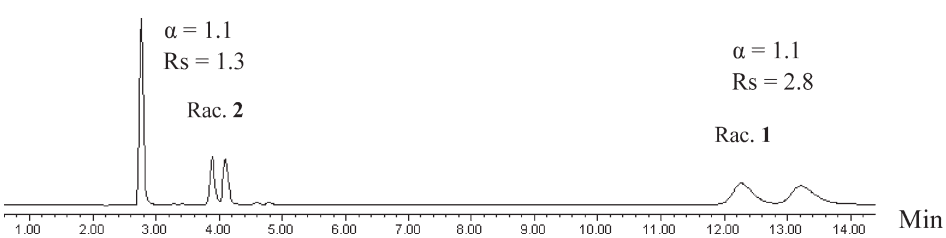

Fig. 2. Baseline separation of racemic flurbiprofen **(1)** and its corresponding *n*-butyl ester **2** on Chiralpak IB. Chromatographic parameters including the separation factor (α) and the resolution (Rs) are shown.

TABLE 1. Enzymatic parameters including enantiomeric excesses of both substrate 1 and product 2 ee_s and ee_p, respectively, conversion and enantioselectivity (*E*) of different lipases screened for the enantioselective esterification of flurbiprofen (1) using 1-butanol in acetonitrile

Lipase	Time (h)	ee_s (%)	ee_p (%)	Conv. %	*E*
Novozym435	168	29.2	72.3	28.8	8.2
Novozym525	168	13.3	60.4	18.0	4.6
Lipozyme	168	4.7	17.6	21.0	1.4
Lipase AS	168	5.1	33.3	13.2	2.1
Lipase PS	168	3.4	33.3	9.2	2.0
Lipase AYS	168	4.7	9.5	33.0	1.2
CRL	168	6.0	9.0	39.7	1.2
Lipase PS-C	168	6.0	42.8	12.2	2.6
CCL	168	4.0	14.2	21.8	1.3
MML	168	4.0	33.3	10.7	2.0

^{1}H NMR (CDCl3) δ 6.8–7.7 (m, 8H, aromatic), 3.9 (t, 2H), 3.6 (q, 1H), 1.5 (m, 2H), 1.4 (d, 3H), 1.2 (m, 2H), 0.8 (t, 3H).

^{13}C NMR (CDCl3) δ 130.7, 128.9, 128.4, 127.6, 126.9, 123.5, 115.4, 115.0, 64.8, 45.1, 30.6, 19.0, 18.3, 13.6, 12.2.

Lipase-Catalyzed Esterification of Flurbiprofen (1)

Ten milligrams (0.04 mmol) racemic flurbiprofen **1**, 10.8 μl *n*-butanol (0.12 mmol, 3 equiv.), molecular sieves, and 2.0 ml either standard (acetonitrile) or nonstandard HPLC organic solvent (such as toluene, MtBE, EtOAc, THF or DCM) were added and stirred in a 4 ml reaction vial. The reaction mixture was thermostated at 40°C. Sample (50 μl) was withdrawn and injected at zero time (control).

Lipase (50 mg) was added and 50 μl sample was withdrawn and directly injected to HPLC without dilution or workup at several time intervals.

Lipase-Catalyzed Hydrolysis of Flurbiprofen Butyl Ester (2)

Flurbiprofen butyl ester (12.2 mg; 0.04 mmol), 1.0 of ml solvent, and 1.0 ml of 0.1 M phosphate buffer (pH 7) were added to 4 ml reaction vial. The reaction mixture was thermostated at 40°C and 50 μl sample of the nonstandard HPLC organic solvent aliquot was withdrawn and injected at zero time (control). Lipase was (50 mg) added and 50 μl samples of the nonstandard HPLC organic solvent aliquot were withdrawn at several time intervals. The samples were filtered and used directly without dilution for direct enantioselective HPLC analysis.

RESULTS AND DISCUSSION

Organic solvents, as a reaction media, have an important role in determining the enantioselectivity in a lipase-catalyzed biotransformation reactions.[34,35] Moreover, versatility of the organic solvents in the mobile phase plays a crucial role in the achievement of the enantioselective HPLC baseline separation of the enzyme substrate and product in one run without further derivatization. These simultaneous baseline separations in one run is mandatory for the precise determination of the enantiomeric excesses of substrates and products. The latter is an important prerequisite for the detailed investigation of the enzyme-catalyzed enantioselective resolution of racemates.[19,20]

For the direct monitoring of the lipase-catalyzed enantioselective esterification of flurbiprofen **1** (cf. Fig. 1) in nonstandard solvents such as DCM, ethyl acetate (EtOAc), methyl *tert*-butyl ether (M*t*BE) and THF, the acid itself (**1**) and its corresponding ester (**2**) should be simultaneously baseline separated in their racemic synthesized form in one run without further derivatization using a chiral stationary phase that is compatible with these nonstandard organic solvents. Indeed, these solvents can not be used as mobile phase eluents in conventionally coated HPLC columns (e.g., Chiralcel OD).[20–22] Thus, the enantioselective HPLC separation of both synthesized racemic acid (**1**) and its corresponding butyl ester (**2**) was investigated using Chiralpak IB (the immobilized version of 3,5-dimethylphenylcarbamate derivative of cellulose-based chiral stationary phase, namely Chiralcel OD). Several investigations were performed using standard organic solvents consisting of *n*-hexane/2-propanol with different volume ratios. However, these combinations were not able to conduct the simultaneously baseline separation of both racemic acid (**1**) and its corresponding butyl ester (**2**) in one run. Thus, more investigations have been performed using the aforementioned nonstandard solvents solely or in combination with *n*-hexane in mobile phase composition using

TABLE 2. Enzymatic parameters including enantiomeric excesses of substrate 1 and product 2 ee_s and ee_p, respectively, conversion and enantioselectivity (*E*) of some selected lipases screened for the enantioselective esterification of flurbiprofen (1) using 1-butanol in several nonstandard organic solvents. Nr (no reaction)

Lipase	Solvent	Log *P*	Time (h)	ee_s (%)	ee_p (%)	Conv. %	*E*
Novozym435	Toluene	2.50	6	87.7	67.6	56.4	14.5
	MtBE	1.30	96	84.8	71.8	54.1	16.1
	Ethyl acetate	0.68	120	14.5	63.8	18.5	5.2
	THF	0.49	Nr				
	DCM	1.30	72	15.6	91.3	14.6	25.8
Novozym525	Toluene	2.50	16	96.1	81.8	54.0	39.0
	MtBE	1.30	120	75.7	98.2	43.5	263.5
	Ethyl acetate	0.68	120	8.8	69.9	11.2	6.1
	THF	0.49	Nr				
	DCM	1.30	120	12.6	46.2	21.4	3.0

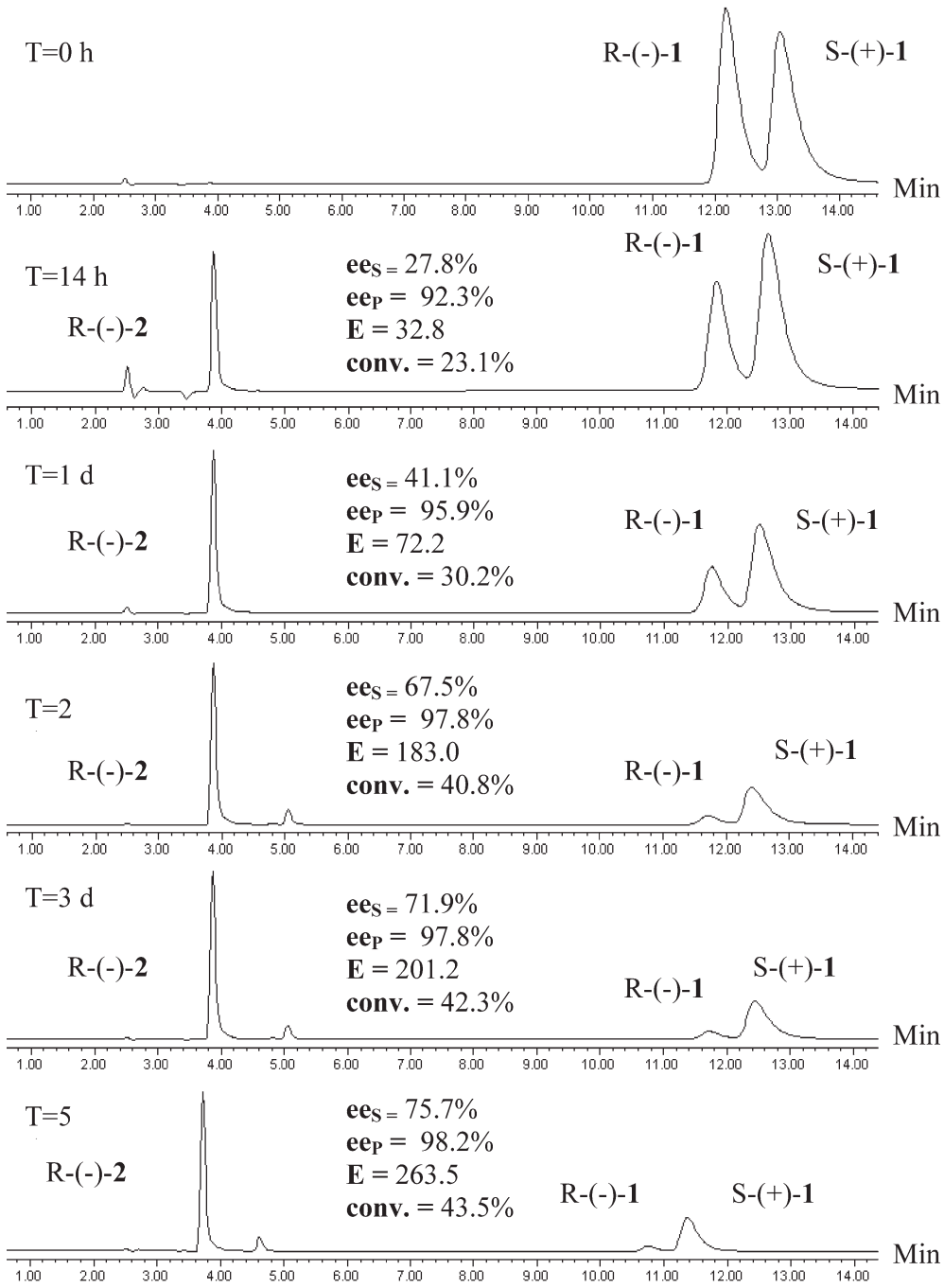

Fig. 3. HPLC chromatograms (from $T = 0$ to $T = 5$d) showing the UV traces of both substrate **1** and product **2** during the direct HPLC monitoring of the reaction progress of Novozyme525-catalyzed enantioselective esterification of **1** using 1-butanol in MtBE as nonstandard HPLC solvent.

different ratios for the simultaneous separation of **1** and **2** in one run on Chiralpak IB. A simultaneous baseline separation of both racemic flurbiprofen (**1**) and its corresponding butyl ester (**2**) was achieved in one run using *n*-hexane associated with MtBE as a nonstandard HPLC organic solvent, 2-propanol and trifluoroacetic acid in mobile phase composition (*n*-Hex/MtBE/2-PrOH/trifluoroacetic acid (85:15:0.4:0.1 v/v/v/v) (Fig. 2).

Upon the fulfillment of the enantioselective baseline separation of the enzyme substrate (**1**) and the expected product (**2**), yet, the enzymatic reaction can be easily and directly HPLC monitored in either standard or nonstandard HPLC organic solvents. Thus, the screening of different lipases for the enantioselective esterification of flurbiprofen (Fig. 1) was first performed in acetonitrile (log *P* −0.34) as standard organic solvent. Of all 10 tested

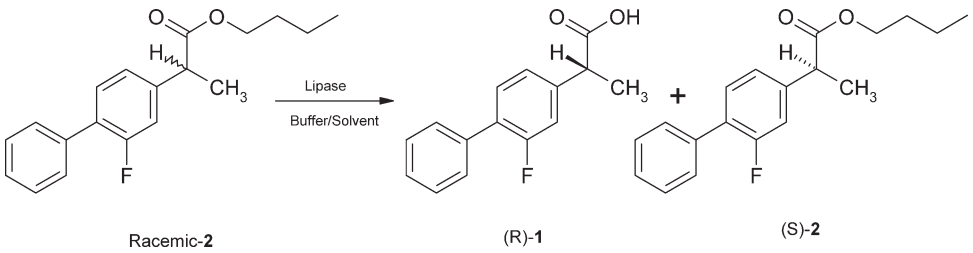

Fig. 4. Lipase-catalyzed enantioselective hydrolysis of butyl ester (**2**) in a biphasic system consisting of nonstandard solvent and phosphate buffer pH 7.

lipases, only lipase from *Candida antarctica* lipase B CAL-B (Novozym435 and Novozym525) showed better activity and enantioselectivity (also called enantiomeric ratio) *E*. The rest did not show any promising results even after 168 hrs. Results are shown in Table 1. Due to the R-stereopreference of *Candida antarctica* lipase B,[15,18] the enantioselective esterification of racemic **1** [(*RS*)-**1**], (*R*)-**1** was the faster reacting enantiomer which was selectively esterified to afford its corresponding butyl ester (*R*)-**2** leaving the second enantiomer of the substrate (*S*)-**1** in enantiomerically enriched form. This was achieved when using different lipases in presence of *n*-butanol in acetonitrile as standard organic solvent (cf. Table 1).

Based on the aforementioned results, two lipases were selected for further investigation in nonstandard organic solvents. Thus, lipase from *Candida Antarctica* CAL-B (Novozym435) and its free form (Novozym525) were used for the enantioselective esterification of racemic flurbiprofen **1** in nonstandard HPLC solvents such as toluene, methyl *tert*-butyl ether (MtBE), ethyl acetate (EtOAc), THF and DCM, results are summarized in Table 2. The reactions were directly monitored by enantioselective HPLC using Chiralpak IB without further workup or removal of the nonstandard solvent. In comparison with the lipase-catalyzed kinetic resolution of (**1**) in acetonitrile as standard organic solvent, excellent enantioselectivities E, enantiomeric excesses of both substrate (**1**) and product (**2**) and faster reaction rates were observed when using nonstandard HPLC organic solvents.

TABLE 3. Enzymatic parameters including enantiomeric excesses of both substrate 2 and product 1 ee_s and ee_p, respectively, conversion and enantioselectivity (*E*) of different lipases screened for the enantioselective hydrolysis of racemic flurbiprofen butyl ester (2) in a biphasic system consisting of MtBE as organic solvent and phosphate buffer pH 7

Lipase	Time (h)	ee_s (%)	ee_p (%)	Conv. %	*E*
Novozym435	72	24.9	22.5	52.4	1.9
Novozym525	96	25.6	19.9	56.3	1.8
Lipozyme	168	9.0	14.2	0.3	1.4
Lipase AS	168	12.8	50.0	0.2	3.4
Lipase PS	168	12.3	77.7	0.1	9.0
Lipase AYS	168	13.1	41.1	0.2	2.7
CRL	168	11.1	93.7	0.1	34.8
Lipase PS-C	168	11.7	62.5	0.1	4.8
CCL	168	11.7	13.2	0.4	1.4
MML	168	9.3	17.0	0.3	1.5

In terms of enantioselectivity (*E*), enantiomeric excess of the resulting product (ee_p) (*R*-ester) at maximum conversion (43.5%), lipase from *Candida antarctica* B (CAL-B) known as Novozym525 showed the best performance in MtBE having log *P* 1.30 (ee_s = 75.7 %, ee_p = 98.2 %, *E* = 263.5 after 120 hrs) (cf. Fig. 3). While in toluene (log *P* 2.50) Novozym525 showed very good enantioselectivity and enantiomeric excess of unreacted substrate (ee_s) (S-acid) at maximum conversion (54.0%), in shorter time (ee_s = 96.1 %, ee_p = 81.8 %, *E* = 39.0 after 16 hrs). Log *P* in Table 2 is defined as the ratio of concentration of a substance in two immiscible phases at equilibrium namely octanol

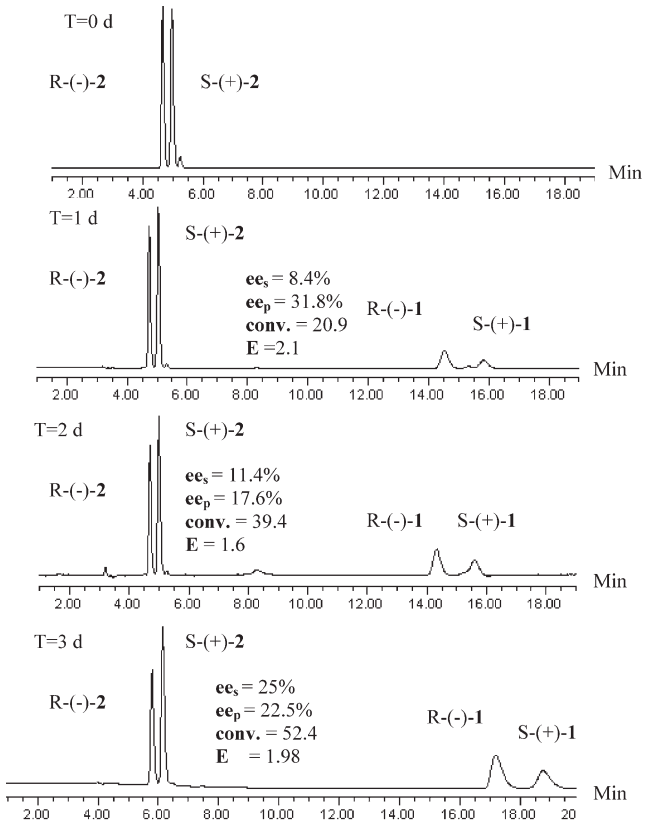

Fig. 5. Selected HPLC chromatograms (from $t = 0$ to $t = 3$ d) showing UV traces of both substrate **2** and product **1** during the direct HPLC monitoring of the reaction progress of Novozym435-catalyzed enantioselective hydrolysis of flurbiprofen butyl ester (**2**) in a biphasic system consisting of MtBE and phosphate buffer pH 7.

TABLE 4. Enzymatic parameters including enantiomeric excesses of both substrate 2 and product 1 ee_s and ee_p, respectively, conversion and enantioselectivity (*E*) of selected lipases screened for the enantioselective hydrolysis of racemic flurbiprofen butyl ester (2) in a biphasic system consisting of different organic solvents and phosphate buffer pH 7

Lipase	Solvent	Time (h)	ee_s (%)	ee_p (%)	Conv. %	*E*
Novozym435	Toluene	144	17.8	4.7	12.6	1.2
	Ethyl acetate	144	8.9	89.9	9.0	20.6
	DCM	144	9.0	33.5	6.4	2.1
Novozym525	Toluene	144	6.6	39.1	2.3	2.4
	Ethyl acetate	144	8.1	88.8	8.4	18.4
	DCM	144	8.2	13.3	0.6	1.4

and water and used to describe the correlation of hydrophobicity and the enantioselectivity *E* of lipases.[19]

Compared to the esterification experiments in the nonaqueous medium consisting of standard and nonstandard HPLC organic solvents described above, the enzymatic hydrolysis of the butyl ester (*RS*)-**2** in aqueous medium proceeded slowly (cf. Fig. 4). Only two lipases namely Novozym435 and 525 were able to conduct poor enantioselective resolution when MtBE was used in combination with sodium phosphate buffer pH 7 in a biphasic system (ee_s 24.9 %, ee_p 22.5, Conv. 52.4 %, $E = 1.9$) for Novozym435 and (ee_s 25.6 %, ee_p 19.9, Conv. 56.3 %, $E = 1.8$ for Novozym525) Table 3. These results promote us to study the screening of both enzymes for the enantioselective hydrolysis of racemic (**2**) in other nonstandard HPLC organic solvents namely toluene, EtOAc and DCM. Results are summarized in Table 4. In terms of the enantioselectivity (*E*), enantiomeric excesses of unreacted substrate (ee_s of the ester (*S*)-**2**), enantiomeric excess of the resulting product (ee_p of the acid (*R*)-**1**) and the reaction time at maximum conversion (52.4%), lipase from *Candida antarctica* B (CAL-B) known as Novozym435 showed the best performance when used in a biphasic solvents consisting of MtBE and buffer pH 7 (ee_s 24.9 %, ee_p 22.5, Conv. 52.4 %, $E = 1.9$ after 72 hours) (cf. Fig. 5).

CONCLUSION

Nonstandard organic solvent is a mandatory mobile phase component to achieve the enantioselective baseline separation of flurbiprofen and its corresponding *n*-butyl ester in on run. Such baseline enantioselective HPLC separation would not be achieved without the immobilization of chiral selector as in Chiralpak IB. Moreover, the lipase-catalyzed esterification in nonstandard solvents showed very high activity and enantioselectivity *E* (263 within 120 hrs and 39 within 16 hrs for MtBE and toluene, respectively). These biotransformation reactions were easily monitored by direct enantioselective HPLC equipped with immobilized chiral stationary phase, namely, Chiralpak IB and a UV-detector. This direct enantioselective HPLC monitoring in nonstandard organic solvents could offer unique capabilities to allow the exploration of the enantioselectivity of lipases toward "difficult to dissolve" substrates in harsh organic solvents.

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