Journal of Chromatography A, 1217 (2010) 1094–1100

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Retention of fluorinated chiral selectors in biphasic fluorinated solvent systems and its application to the separation of enantiomers by countercurrent chromatography

Anna Maria Pérez[a,b], Cristina Minguillón[a,b,*]

[a] Institute for Research in Biomedicine (IRB Barcelona), Parc Cientific de Barcelona (PCB), Baldiri Reixac 10-12, E-08028 Barcelona, Spain
[b] Laboratori de Química Farmacèutica, Faculty of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain

ARTICLE INFO

Article history:
Available online 2 October 2009

Keywords:
Countercurrent chromatography (CCC)
Enantiomer separation
Chiral selector
Ethoxynonafluorobutane
HFE-7200
ENFB
Fluorinated chiral selector

ABSTRACT

Ethoxynonafluorobutane (ENFB) has been used as a component of new biphasic solvent mixtures. The suitability of several mixtures as solvent systems in countercurrent chromatography was tested. The applicability of the ENFB/2-PrOH/H_2O mixture to the separation of enantiomers, in combination with a fluorinated chiral selector (CS), was evaluated. *N*-Perfluoroundecanoyl-L-proline-3,5-dimethylanilide (**2**), analogous to the previously used *N*-dodecyl-L-proline-3,5-dimethylanilide (**1**), was synthesized for this purpose. The capacity of the new solvent system to retain the fluorinated CS in the fluorinated phase used as stationary was examined. Chiral selector **1** was applied in analogous conditions for comparative purposes. Additionally, MTBE/phosphate buffer solvent system was also used with the two CSs. The ENFB/2-PrOH/H_2O (25:35:40) mixture was found to be adequate in the enantioseparation of DNB-Leu and DNB-Leu-*t*Bu. Enantioselectivity was maintained in the fluorinated solvent system without compromising eluting time. The complete separation of DNB-Leu-*t*Bu was achieved and no leaks of CS to the mobile phase were detected.

1. Introduction

Countercurrent chromatography (CCC) is a liquid–liquid separation technique based on the partition of samples between the two non-miscible liquids acting as stationary and mobile phase, respectively [1–3]. It is a highly versatile chromatographic technique. A variety of combinations of non-miscible solvents can be used as solvent systems and diverse elution modes are permitted thanks to the absence of solid support [4]. Moreover, CCC is specially adapted to preparative purposes. The lower solvent consumption and higher sample loading capacity than conventional solid–liquid chromatography are the main features that make it an alternative to HPLC for preparative applications [5,6]. Regarding the separation of enantiomers, the preparative nature of CCC can be of great interest. This technique offers the possibility to produce enantiomerically pure compounds at a lower cost than conventional liquid chromatography. As for other enantioselective separation techniques, a chiral selector (CS) has to be added to one of the phases to produce enantioseparation [7]. The two-phase solvent systems most commonly used in CCC comprise an organic solvent or mixture, the more lipophilic phase, and a strongly polar solution, often aqueous. Enantiorecognition occurs in the liquid phase, either organic or aqueous, which contains the CS. This phase is generally used as stationary phase.

In CCC sample loading capacity and also enantioselectivity are dependent on the amount of CS involved in the separation [7,8]. Therefore, the CS has to be fairly soluble in the solvent that constitutes the stationary phase. Moreover, it should not partition to the mobile phase. Simultaneously, the racemate to be resolved has to distribute between the two phases. This will allow it to interact with the CS in the stationary phase and to be properly eluted with the mobile phase. Unfortunately, these conditions make the search for an adequate solvent system a difficult task when CS and analytes have similar polarities.

When the CS is designed to be confined in the more lipophilic phase, the introduction of a long hydrophobic chain in its structure permits to increase solubility and reduce affinity for the polar phase, while simultaneously increasing the difference in polarity between CS and analytes [9–11]. Additionally, the presence of ionisable groups in analytes can be used to control their partition and elution by properly setting the pH of the mobile phase [12]. Nevertheless, sometimes leaks of the CS to the mobile phase cannot be avoided [9]. In this context, we have considered the particular affinity properties of fluorinated compounds. Thus, the use of

* Corresponding author at: Institute for Research in Biomedicine (IRB Barcelona), Parc Cientific de Barcelona (PCB), Baldiri Reixac 10, E-08028 Barcelona, Spain. Tel.: +34 93 403 71 07; fax: +34 93 403 71 04.
E-mail address: cminguillon@pcb.ub.es (C. Minguillón).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.09.071

a fluorinated CS in combination with a fluorinated solvent system is proposed for the selective retention of the CS in the fluorinated stationary phase of the solvent system.

Fluorous technologies applied to organic synthesis (fluorous reagents, scavengers, catalysts or protecting groups, among others) have been developed since the mid-1990s [13]. The affinity properties of fluorinated compounds have also been applied to separation techniques such as liquid–liquid extraction [14], solid phase extraction and HPLC [15]. However, to the best of our knowledge, the concept has not been applied to CCC, neither for the conventional separation of fluorinated compounds nor for enantioselective purposes.

Recently, ethoxynonafluorobutane (ENFB), a new environment friendly fluorinated solvent designed to be used as a cold cleaner in diverse applications, has been introduced in chromatography [16]. The high lipophilicity of this solvent makes it a suitable substitute of hexane. Moreover, its lack of flammability permits the use of normal phase conditions in LC–MS [17–19]. With the aim to apply the affinity properties of fluorinated compounds to CCC enantioseparation, ENFB has been chosen in this study as a basic fluorinated component in the construction of biphasic solvent systems.

In order to be applicable to enantioseparation, the newly constructed fluorinated biphasic solvent systems have to produce the retention of the fluorinated CS in the fluorinated stationary phase. In addition, enantioselectivity has to be possible in such conditions. Therefore, to test this property a CS-racemic analyte pair with demonstrated enantioseparation in diverse media is required.

The successful separation of DNB-Leu enantiomers by CCC using *N*-dodecyl-L-proline-3,5-dimethylanilide (**1**, Fig. 1) as CS in diverse conditions has been described [8,12]. The same CS was also enantioselective for the non-ionisable DNB-Leu-*t*Bu using heptane/EtOAc/MeOH/H_2O (3:1:3:1) as a solvent system [9]. Unfortunately, in spite of the optimization of the conditions performed by the authors, a continuous leak of CS was detected. These separations have been considered an adequate model to test the applicability of fluorous affinity to CCC enantioseparations. Thus, here the preparation of a CS, analogous to *N*-dodecyl-L-proline-3,5-dimethylanilide **1** is described. The new CS **2** contains a perfluorinated alkyl chain instead of the hydrocarbon chain of **1**. After determining the solubility and partition properties of **2**, this CS is studied comparatively to **1** in the separation of DNB-Leu and DNB-Leu-*t*Bu racemates using conventional and fluorinated solvent systems. In addition CS **3**, a commercially available L-proline-derived chiral compound was also included in the study.

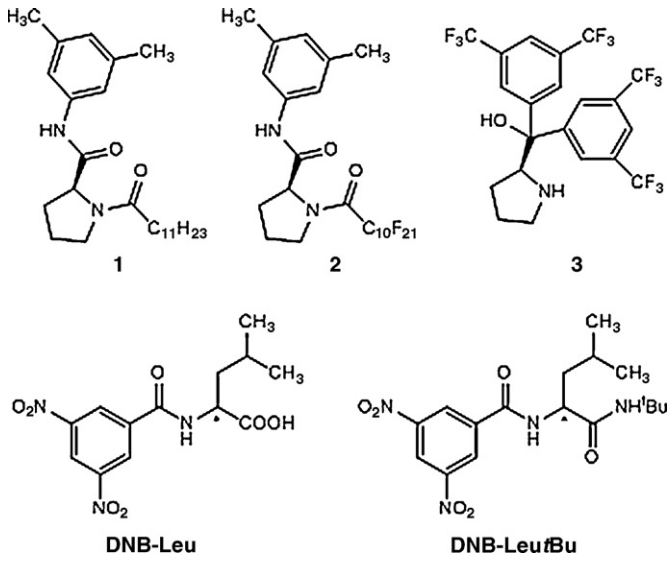

Fig. 1. Chemical structures of the chiral selectors and the racemic compounds used in the study. (CS **1**: *N*-dodecyl-L-proline-3,5-dimethylanilide; CS **2**: *N*-perfluoroundecanoyl-L-proline-3,5-dimethylanilide; CS **3**: (*S*)-α,α-bis[3,5-bis(trifluoromethyl)phenyl]-2-pyrrolidinemethanol).

2. Experimental

2.1. Reagents and apparatus

N-(3,5-Dinitrobenzoyl)-(±)-leucine (DNB-Leu), L-proline, di-*tert*-butyl dicarbonate (Boc_2O), 3,5-dimethylaniline, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), trifluoroacetic acid (TFA), perfluoroundecanoic acid, diisopropylethylamine (DIPEA), chloro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate (TCFH) and (*S*)-α,α-bis[3,5-bis(trifluoromethyl)phenyl]-2-pyrrolidinemethanol (**3**) were purchased from Sigma–Aldrich (Steinheim, Germany). Ethoxynonafluorobutane (ENFB) (Novec HFE-7200, 3 M) was purchased from Twintec S. L. (Manresa, Barcelona, Spain). *N*-(3,5-Dinitrobenzoyl)-*tert*-butyl-(±)-leucinamide and **1** were synthesized as described previously [9]. Liquid phases for CCC and HPLC were prepared from analytical reagent-grade sodium monohydrogenphosphate and sodium dihydrogenphosphate, Milli-Q water and the required HPLC-grade solvents.

NMR spectra (^1H, ^{13}C and ^{19}F) were recorded on a Varian Mercury 400. Chemical shifts are quoted in δ values downfield from TMS. MS MALDI-TOF spectra were obtained from a 4700 Proteomics analyzer (Applied Biosystems). Optical rotation values were determined in a Perkin-Elmer 241 polarimeter.

The CCC experiments were performed in a high performance countercurrent chromatograph (HPCCC) Mini-DE (Dynamic Extractions, Slough, UK). The apparatus was connected to an external cooling system which allowed the device to accurately control its operating temperature at 25 °C. The rotational speed was set at 2100 rpm. The HPCCC device is provided with a coil of 17.8 mL capacity made of a 0.8 mm bore Teflon tubing wound on a spool with an external diameter of 7 cm. The distance between the central rotor axis and the coil axis was 5.0 cm. The β-value ranges from 0.5 to 0.76. This instrument was connected to a conventional HPLC system (pump, autosampler, UV detector, and chromatography data station software) model HP 1100 (Agilent Technologies, Palo Alto, CA, USA). A manual Rheodyne injector equipped with a 0.34 mL loop was used. The elution of the analytes was directly monitored by UV detection (254 nm). The fractions of the eluate (1 fraction/min) were collected throughout the experiment (Gilson FC 203B fraction collector) and analysed by enantioselective HPLC to determine their enantiomeric composition. Elution profiles were constructed from the obtained data. The same HPLC system, using the autosampler and replacing the CCC device by the appropriate HPLC chiral column, was used for this purpose.

2.2. Preparation of CS 2 (Fig. 2)

Anilide **4** was obtained as described [20]. Thus, 3,5-dimethylaniline (150 mg, 1.2 mmol) was added to a solution of *N*-Boc-L-proline (250 mg, 1.1 mmol) and EEDQ (299 mg, 1.2 mmol) in dichloromethane (20 mL). The mixture was stirred at room temperature for 24 h. The resulting solution was washed successively with 2N HCl, 5% $NaHCO_3$ and H_2O. The crude product obtained from the evaporation of the organic extracts was treated with a solution of TFA in CH_2Cl_2 (30:70, v/v) without any previous purification. The solution was stirred at r.t. for 3 h. The resulting mixture was neutralized with NH_4OH and extracted with CH_2Cl_2. The oily material obtained from the evaporation of solvent was purified by crystallization of the hydrochloride form using ethanol/water (80% yield).

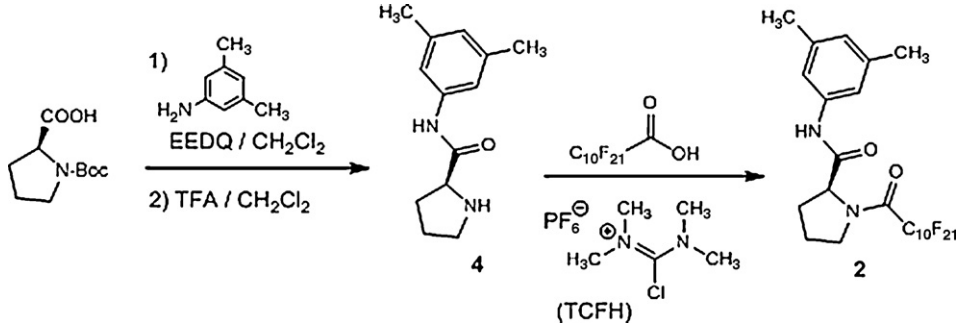

Fig. 2. Synthetic pathway followed in the preparation of CS **2**.

TCFH [21] (320 mg, 0.8 mmol) was added to a solution of perfluoroundecanoic acid (320 mg, 0.6 mmol) in CH_2Cl_2 (5 mL). The mixture was stirred 1 h at r.t. and a mixture of **4** (100 mg, 0.5 mmol) and diisopropylethylamine (105 μL, 0.8 mmol) in CH_2Cl_2 (2 mL) was added drop-wise. After 18 h at r.t., the resulting solution was washed with 5% $NaHCO_3$ and H_2O. The crude product obtained after removal of solvent was purified by chromatography using CH_2Cl_2/MeOH (9:1). Compound **2** was obtained as a yellowish solid (244 mg, yield 70%). $[\alpha]^D$: −38 (c:1, $CHCl_3$). MALDI calcd. [M+H] 765.12; found [M+K] 803.07; [M+Na] 787.10; [M+H] 765.10.

^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.02 (m, 2H, C^4H_2); 2.20 (s, 6H, CH_3Ar); 2.34 (m, 2H C^3H_2); 3.83 + 3.97 (m + m, 2H, C^5H_2); 4.78 (dd, 1H, C^2H_2); 6.64 (s, 1H, $C^{4'}H$); 6.99 (s, 2H, $C^{2',6'}H$); 8.50 (ba, 1H, NHCO).

^{13}C NMR (100.6 MHz, $CDCl_3$), δ: 21.4 (2 CH_3Ar); 25.7 (C^4H_2); 27.1 (C^3H_2); 48.3 (C^5H_2); 63.8 (C^2H); 117.6 ($C^{2',6'}H$); 126.1 ($C^{4'}H$); 137.6 ($C^{1'}H$); 138.6 ($C^{3',5'}H$); 157,9 (CON); 167,8 (CONH).

^{19}F NMR (376.2 MHz, $CDCl_3$), δ: −74.1 (12F); −80.2 (3F); −115.7 (2F); −126.3 (2F); −179.8 (2F).

2.3. Delimiting binary phase composition of ternary mixtures

The range of ternary compositions that produce biphasic mixtures was delimited following the procedure described [22] using individual solvent volumes. Thus, 15 mixtures of either 2-propanol, ACN or MeOH (volumes ranging from 0.25 to 15 mL) and water (from 5 to 0.25 mL) were prepared in test tubs with screw caps. In each test tube known volumes of ENFB were added until turbidity (two phases) was observed. The graphical representation of the resulting compositions delimits the domain in which a biphasic mixture was obtained at room temperature (25 °C) from the three solvents involved (Fig. 3).

2.4. Selection and preparation of the solvent system

The suitability of several compositions as solvent system for the CCC enantioseparation of the racemates using the CSs of interest, was determined as follows. Several biphasic ternary mixtures were prepared by mixing measured volumes of individual solvents in separatory funnels. After shaking, the mixtures were allowed to separate and equilibrate overnight. The considered CS (5 mg, in the order 6–12 μmol, depending on the CS considered) and the racemate (1 mg, in the order of 3 μmol) were added to test tubes containing 2 mL of the lower phase and 2 mL of the upper phase. After shaking and equilibration, the distribution of solutes was qualitatively controlled by TLC (chloroform/MeOH; 9:1). The mixtures that retained the CS in the lipophilic phase and permitted the partition of the racemate are indicated in Table 1. When a suitable composition was found, the maximum amount of CS soluble in these conditions was determined by adding increasing amounts of CSs to 5 mL of the organic phase mixture. An exactly measured aliquot of the resulting saturated solution was exhaustively evaporated and the weight of the residue obtained was determined.

2.5. CCC experimental conditions

To establish working conditions with the new solvent systems, their stability inside the CCC device was tested first. Thus, after filling the centrifuge with the stationary lipophilic phase, the rotor speed was set a 2100 rpm. The elution mode was set tail-to-head when fluorinated solvent systems were used. This mode leaves the denser fluorinated phase as stationary. For MTBE/buffer mixtures the system was connected head-to-tail (less dense organic phase stationary). The flow of the mobile phase was modified to optimize stationary phase retention and analyte retention. Flow rate was set at 1 mL/min when the MTBE/phosphate buffer solvent system was used. When the fluorinated solvent system was used flow rate was set at 0.5 mL/min in the separation of DNB-Leu enantiomers and at 1 mL/min for DNB-Leu-*t*Bu. The volume of stationary phase displaced (V_M) during equilibration was measured to calculate the amount of CS involved in the separation. The analytes (0.023 mmol) were injected in 0.34 mL (2% of the column volume) of a mixture of upper and lower phase. The elution was directly monitored by

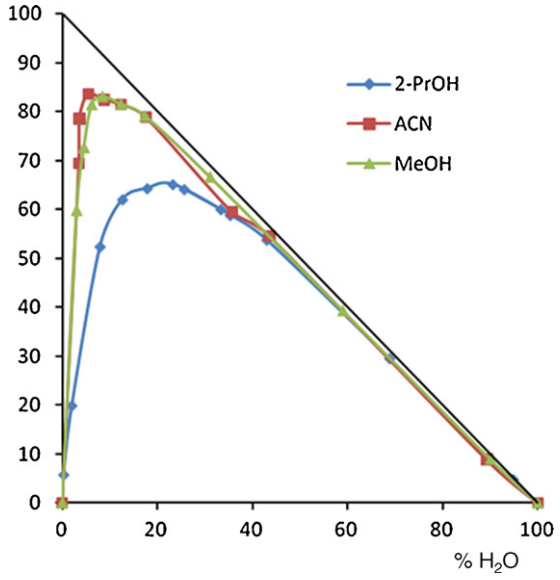

Fig. 3. Ternary diagrams. The compositions under the bimodal line correspond to biphasic mixtures (horizontal axis: %H_2O; vertical axis: % of either 2-PrOH, ACN or MeOH). The content of H_2O and one of the solvents indicated (2-PrOH, ACN or MeOH) can be directly determined in the horizontal and vertical axis, respectively. The ENFB content can be calculated by difference (volume percentages for individual solvents).

Table 1
Ternary mixtures tested and their properties respect CSs and DNB-Leu.

Constituents of the solvent system	Volume ratios (v:v:v)	CS **1**	CS **2**	CS **3**	DNB-Leu	Solubility in the lipophilic phase		
						CS **1**	CS **2**	CS **3**
ENFB/ACN/H_2O	17:43:40	P[a]	NP	P	P	Insoluble	11 mg/mL (14 mM)	Not det.
ENFB/MeOH/H_2O	21:33:40	NP	NP	NP	NP	Slightly sol.[b]	Slightly sol.	Slightly sol.
ENFB/MeOH/H_2O	46:295:40	NP	NP	NP	NP	Insoluble	Slightly sol.	Slightly sol.
ENFB/2-PrOH/H_2O	48:12:40	P	NP	P	P	Slightly sol.	48 mg/mL (60 mM)	Not det.
ENFB/2-PrOH/H_2O	90:35:40	P	NP	P	P	Slightly sol.	17 mg/mL (20 mM)	Not det.
ENFB/2-PrOH/H_2O	25:35:40	NP	NP	NP	P	31 mg/mL (70 mM)	62 mg/mL (80 mM)	23 mg/mL (40 mM)
MTBE/sodium phosphate buffer 50 mM pH 6.0	–	NP	NP	NP	P	42 mg/mL (100 mM)	57 mg/mL (70 mM)	202 mg/mL (380 mM)

[a] Determined qualitatively by TLC. P: partitioned into the two non-miscible liquid phases of the indicated solvent mixture; NP: no partitioned.
[b] Slightly sol.: <1 mg/mL.

UV detection at 254 nm. The eluate was collected in 1 min fractions which were further analysed.

2.6. Analysis of fractions

The fractions collected during the experiment were individually analysed by enantioselective HPLC to determine the enantiomeric content. Thus, for DNB-Leu containing fractions 1 mL of $CHCl_3$ and 3 drops of HCl 2N were added to each aqueous fraction. 750 μL of the organic lower phase was placed in vials to be analysed. Only 1 mL of $CHCl_3$ was added to DNB-Leu-*t*Bu containing fractions and the resulting organic phase was analysed. The determination of the enantiomeric content in DNB-Leu fractions was performed on a column containing *N*-(3,5-dimethylphenyl)-(4*R*)-(3,5-dimethylphenylaminocarbonyloxy)-L-prolinamide as CS [23] using heptane/2-PrOH/TFA (80:20:0.5) as mobile phase (k'_1, 3.3; α, 1.6; Rs, 1.7). A (*S*)-naproxen derivatized chiral stationary phase [24] was used in the analysis of DNB-Leu-*t*Bu (mobile phase: heptane/2-PrOH, 95:5; k'_1, 0.6; α, 3.4; Rs, 3.4).

3. Results and discussion

Fluorinated solvents have been developed in an effort to reduce environmental problems caused by the extensive use of certain organic solvents. Due to its low toxicity, ENFB constitutes an alternative to chlorinated or hydrocarbon solvents. The successful results recently obtained in the replacement of *n*-hexane by ENFB in HPLC enantioseparation [16,18] encouraged us to introduce its use as a component in a CCC solvent system.

Several factors should be taken into consideration when the design of a new solvent system to be used in CCC is undertaken. The two phases have to separate readily. Moreover, the solvent system should be stable and should not emulsify in the CCC device under working conditions. In addition, the solvent system should be adapted to the sample to be separated. In conventional separations, the ideal solvent system generates a differential partition for the components in the sample between the two phases. The high diversity of biphasic solvent systems that can be used in CCC permits selectivity to be modulated in any particular separation.

When a chiral separation is the objective, two solutes (the CS and the racemate) are involved in the process. In this case, the solvent system must produce the retention of one of them, the CS, in the stationary phase while the racemate is partitioned. When the CS and the racemate have similar polarity characteristics, the search for a solvent system that fulfils all the requirements may be complicated. Moreover, the solvent system should maintain enantiorecognition. The solvation of the CS and enantiomers in the stationary phase may prevent association between these two species and therefore compromise enantiomeric recognition. The possibility to apply the affinity properties of fluorinated compounds in this context has been considered in the present study. To assess the feasibility of this approach, a solvent system containing a fluorinated component and a fluorinated CS are required.

3.1. Synthesis of N-perfluoroundecanoyl-L-proline-3,5-dimethylanilide (CS **2**)

The synthesis of CS **2** was first undertaken following the same strategy described for *N*-dodecyl-L-proline-3,5-dimethylanilide **1** [9]. Thus, L-proline was treated in acylating conditions using either perfluoroundecanoyl chloride or, alternatively, the corresponding acid and a coupling agent such as 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) or *N*,*N*′-diisopropylcarbodiimide (DIPCDI). Given the very low yields obtained (in the order of 7%, depending on the particular conditions applied), direct acylation of **4** was attempted (Fig. 2). CS **2** was obtained in good yield (70%) using perfluoroundecanoic acid and chloro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate (TCFH) as a coupling agent in CH_2Cl_2.

3.2. Design and selection of solvent systems

Most fluorous solvents, including ENFB, are highly hydrophobic. Therefore, the mixture ENFB/water shows extreme polarities in the two phases. However, a certain polarity is needed in the more lipophilic phase to dissolve the CS and to allow partition of analytes. A third solvent of intermediate polarity, miscible with ENFB and water, can provide the required properties. Therefore, phase diagrams for several ternary mixtures were constructed. Three solvents MeOH, ACN and 2-PrOH, all accomplishing the requirements of miscibility and polarity, were chosen. The compositions at which the ternary mixtures ENFB/MeOH/H_2O, ENFB/ACN/H_2O, and ENBF/2-PrOH/H_2O produce biphasic solvent systems were examined (Fig. 3). All three mixtures behaved like type I solvent systems [2].

In spite of the complete miscibility of MeOH and ACN in ENFB [25], almost the entire amount of these solvents added to the biphasic ENFB/water mixture remained in the polar phase of the ternary mixtures. This effect was less pronounced for 2-PrOH. Mixtures ENFB/MeOH/H_2O were discarded for further CCC assays as a result of the low solubility of CSs in the more lipophilic phase and the lack of partition of DNB-Leu (Table 1).

In ENFB/2-PrOH/H_2O mixtures, the retention of the three CSs in the fluorinated phase was attained only when a considerable relative amount of 2-PrOH was used. For mixtures containing a high relative amount of ENFB, only **2** was retained in the fluorinated phase. Nevertheless, DNB-Leu was partitioned between the two phases of the three biphasic ENFB/2-PrOH/H_2O mixtures tested. Therefore, although the polarity of the solvent system can be detrimental for enantioseparation [8,12], ENFB/2-PrOH/H_2O (25:35:40)

Table 2
CCC runs.

CS	CS_{st} (mmol)	Racemate	$r_{CS/rac}$	Flow rate (mL/min)	t_1 (min)	t_2 (min)	α_{CCC}[a]	R_s[a]	eo
Solvent system: MTBE/50 mM sodium phosphate buffer (pH: 6.0) (Fig. 4a)									
1	0.49	DNB-Leu	21.50	1	43	77	1.89	1.54	*R*
2	0.42	DNB-Leu	18.26	1	29	37	1.40	0.42	*R*
3	0.57	DNB-Leu	22.04	1	54	62	1.15	0.60	*S*
Solvent system: ENFB/2-PrOH/H_2O (25:35:40) (Fig. 4b)									
Blank	–	DNB-Leu	–	0.5	28	–	–	–	–
1	0.40	DNB-Leu	17.60	0.5	18	22	1.80	0.60	*R*
2	0.42	DNB-Leu	18.26	0.5	18	23	1.83	1.19	*R*
3	0.44	DNB-Leu	19.17	0.5	23	25	1.21	0.28	*S*
Solvent system: ENFB/2-PrOH/H_2O (25:35:40) (Fig. 4c)									
Blank	–	DNB-Leu-*t*Bu	–	1	24	–	–	–	–
1	0.48	DNB-Leu-*t*Bu	20.86	1	29	33	1.16	0.36	*R*
2	0.42	DNB-Leu-*t*Bu	18.26	1	30	48	1.75	1.50	*R*
3	0.44	DNB-Leu-*t*Bu	19.17	1	62	–	–	–	–

CS_{st}, mmol of CS retained in the stationary phase; $r_{CS/rac}$, molar ratio CS/racemate; t_1 and t_2, retention time for each enantiomer; α_{CCC}, selectivity factor; R_s, resolution; eo, elution order, configuration of the first eluted enantiomer; Conditions: ω = 2100 rpm; amount of racemate injected: 0.023 mmol.

[a] Calculated on elution profiles.

was considered a suitable composition to perform comparative studies for the three CSs of interest.

3.3. CCC runs

Firstly, blank experiments without CS were performed to assess the stability of the solvent system in the CCC working conditions and the retention of the analytes using a flow rate of 0.5 mL/min. ENFB/ACN/H_2O and ENFB/2-PrOH/H_2O mixtures were stable inside the CCC device. Retentions of stationary phase (S_f) in the order of 80–89% were obtained at 2100 rpm. At this point, given that DNB-Leu is an ionisable compound, the use of a buffer of controlled pH instead of water was considered [10,12]. However, when 50 mM sodium phosphate buffer was used DNB-Leu eluted at the void volume, even at acidic pH (pH 6.0). Therefore, water was used in the fluorinated solvent systems to perform the subsequent experiments (Table 2).

DNB-Leu-*t*Bu was included in the study as a non-ionisable analyte whose elution cannot be controlled by setting the pH of the mobile phase. The preliminary TLC tests showed the almost complete retention of this analyte in the organic lipophilic phase of the solvent system. Nevertheless, a blank CCC run was performed in the same conditions previously applied to DNB-Leu. As expected, the analyte showed a high retention time (>60 min), although, lower retention times were obtained by increasing the flow rate to 1 mL/min.

The separation of DNB-Leu enantiomers in a ENFB/2-PrOH/H_2O (25:35:40) solvent system was performed at a fixed 30 mM CS concentration. Although still far from saturation, this concentration produced an equivalent enantioselectivity value equivalent for **1** and **2** of 1.8. This value was comparable to that obtained for **1** in MTBE/phosphate buffer system at the same CS concentration. Therefore, it can be assumed that enantioselectivity is maintained in the fluorinated solvent system.

The solvent systems ENFB/ACN/H_2O (17:43:40) and ENFB/2-PrOH/H_2O (48:12:40) were also tested in the enantioseparation of DNB-Leu with CS **2** at 14 and 30 mM concentrations, respectively. CS **2** did not show any enantioselectivity in the ACN containing system. Nevertheless, the use of ENFB/2-PrOH/H_2O (48:12:40), with a lower content in 2-PrOH, produced similar results to those obtained formerly using a more polar mixture (ENFB/2-PrOH/H_2O (25:35:40)).

When the results obtained with ENFB/2-PrOH/H_2O (25:35:40) and MTBE/phosphate buffer systems were compared, the most remarkable differences were the lower retention time and the lower resolution observed in the fluorinated solvent system (Fig. 4). Thus, run times of less than 30 min were adequate in this latter while a run time of almost 100 min was necessary to perform the same separation in MTBE/buffer. Nevertheless, resolution was clearly lower in ENFB/2-PrOH/H_2O. Although this can be considered a major drawback for analytical purposes, low resolution in CCC can be improved by applying modified multiple dual mode [26], which has the same effect as increasing the length of the column. Moreover, given the preparative applicability of CCC, techniques such as peak shaving and recycling, which are common practice in preparative applications, can be used to improve the obtained separation. For preparative purposes, the reduced flow rate makes ENFB/2-PrOH/H_2O advantageous over the MTBE/buffer solvent system.

Regarding the retention of the CSs in the ENFB/2-PrOH/H_2O solvent system, while CS **2** remained confined in the fluorinated stationary phase, continuous leaking of CS **1** into the mobile phase was detected. This finding was attributed to the absence of fluorine atoms on CS **1**. CS **3**, containing four trifluoromethyl groups, was also retained in the fluorinated phase of the solvent system.

CS **2** was less enantioselective in the MTBE/buffer solvent system, in spite of having the same chiral moiety as **1**. Moreover, in spite of the long retention produced when **3** was used, this CS was less enantioselective for DNB-Leu than **1** and **2** in the two solvents systems. It is also worth noting that **3** produced the inverse elution order of enantiomers than **1** and **2**, which is an indication of a different recognition mechanism.

Nevertheless, it is in the separation of non-ionisable compounds where the fluorinated solvent system demonstrates effectiveness and usefulness. For these analytes it is not possible to control retention by changing the pH of the mobile phase and only differences in polarity can be applied in conventional conditions. For this reason, the enantioseparation of DNB-Leu-*t*Bu was undertaken using the fluorinated solvent system. In spite of the complete separation obtained for this compound when CS **1** (10 mM) was used in heptane/EtOAc/MeOH/H_2O (3:1:3:1) [9], only a partial separation was obtained with the same CS (30 mM) in ENFB/2-PrOH/H_2O (25:35:40). In both cases a continuous leak of the CS to the eluate was detected. In contrast, when CS **2** (30 mM) was applied the complete separation of DNB-Leu-*t*Bu enantiomers was attained in 64 min. This observation demonstrates the effectiveness of the pair fluorinated solvent system/fluorinated CS in the enantioseparation of the non-ionisable DNB-Leu-*t*Bu.

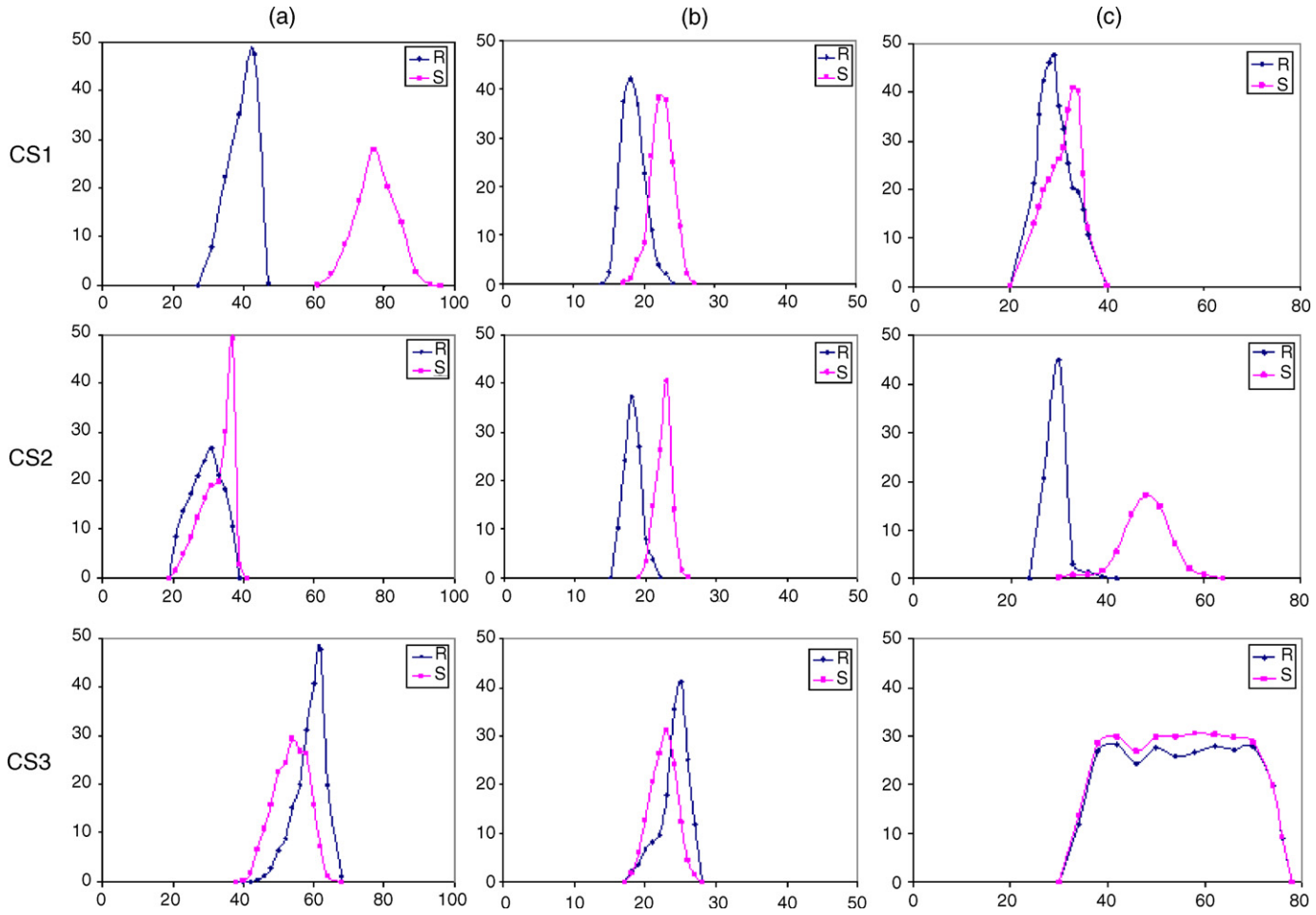

Fig. 4. Elution profiles obtained for the enantioseparation of (a) DNB-Leu (MTBE/sodium phosphate buffer, 1 mL/min); (b) DNB-Leu (ENFB/2-PrOH/H_2O (25:35:40), 0.5 mL/min) and (c) DNB-Leu-*t*Bu (ENFB/2-PrOH/H_2O (25:35:40), 1 mL/min) using CS **1**, **2** and **3**. See Table 2 for detailed experimental conditions.

4. Conclusion

The use of fluorinated solvent systems in CCC opens up a new field of applications because of the particular selectivity shown by these solvents. In this study we describe new biphasic solvent systems containing a fluorinated solvent (ENFB). The new solvent systems were stable at room temperature and performed well in CCC. Regarding their use in enantioseparation, the inclusion of a fluorinated chain in the CS provides it with the required properties of solubility and retention in the fluorinated stationary phase. Moreover, the CS maintains its enantioselectivity in these conditions without leaking to the eluate. The study shows the potential of this approach to the successful separation of non-ionisable chiral compounds that have similar polarity properties to the CS used in their enantioseparation by CCC.

Acknowledgements

Financial support from the *Ministerio de Ciencia e Innovacion* and from the European Regional Development Fund (ERDF) (CTQ 2006-03378/PPQ) is acknowledged. Partial support from the *Comissionat per a Universitats i Recerca* of the *Generalitat de Catalunya* (project 2005-SGR00180) is also acknowledged. The authors are indebted to Professor I. A. Sutherland and Dr. S. Ignatova for the loan of the CCC device and also for fruitful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.09.071.

References

[1] A. Berthod (Ed.), Countercurrent Chromatography, the Support-free Liquid Stationary Phase (Comprehensive Analytical Chemistry), vol. XXXVIII, Elsevier, Amsterdam, 2002.
[2] A.P. Foucault (Ed.), Centrifugal Partition Chromatography (Chromatographic Science Series), 68, Marcel Dekker, New York, 1995.
[3] W.D. Conway, Countercurrent Chromatography, Apparatus. Theory and Applications, VCH Publishers, New York, 1990.
[4] A. Berthod, T. Maryutina, B. Spivarov, O. Shpigun, I.A. Sutherland, Pure Appl. Chem. 81 (2009) 355.
[5] I.A. Sutherland, P. Hewitson, S. Ignatova, J. Chromatogr. A, in press, doi:10.1016/j.chroma.2009.03.040 (available on line: 19 March 2009).
[6] I.A. Sutherland, P. Hewitson, S. Ignatova, J. Chromatogr. A 1216 (2009) 4201.
[7] E. Pérez, C. Minguillón, in: G. Subramanian (Ed.), "Counter-Current Chromatography in the Separation of Enantiomers". Chiral Separation Techniques. A Practical Approach, Wiley–VCH, Germany, 2006.
[8] Y. Ma, Y. Ito, Anal. Chem. 67 (1995) 3069.
[9] L. Oliveros, P. Franco, C. Minguillón, E. Camacho-Frias, A.P. Foucault, F. Le Goffic, J. Liq. Chromatogr. 17 (1994) 2301.
[10] P. Franco, J. Blanc, W.R. Oberleitner, N.M. Maier, W. Lindner, C. Minguillón, Anal. Chem. 74 (2002) 4175.
[11] E. Gavioli, N.M. Maier, C. Minguillón, W. Lindner, Anal. Chem. 76 (2004) 5837.
[12] B. Delgado, E. Pérez, M.C. Santano, C. Minguillón, J. Chromatogr. A 1092 (2005) 36.

[13] W. Zhang, Tetrahedron 59 (2003) 4475.
[14] D.P. Curran, Angew. Chem. Int. Ed. 37 (1998) 1174.
[15] A.B. Daley, R.D. Oleschuk, J. Chromatogr. A 1216 (2009) 772.
[16] M.Z. Kagan, J. Chromatogr. A 918 (2001) 293.
[17] M.Z. Kagan, M. Chlenov, C.M. Kraml, J. Chromatogr. A 1033 (2004) 321.
[18] J. Ding, M. Desai, D.W. Armstrong, J. Chromatogr. A 1076 (2005) 34.
[19] M. Kagan, M. Chlenov, S. Melnikov, O. McConnell, A.C. Bach, G. Carter II., A. Failli, T.J. Caggiano, J.S. Shumsky, D. Lubda, J. Comb. Chem. 11 (2009) 704.
[20] W.H. Pirkle, P.G. Murray, J. Chromatogr. 641 (1993) 11.
[21] A. El-Faham, F. Albericio, J. Org. Chem. 73 (2008) 2731.
[22] M.J. Ruiz-Angel, V. Pino, S. Carda-Broch, A. Berthod, J. Chromatogr. A 1151 (2007) 65.
[23] R. Sancho, C. Minguillón, J. Chromatogr. B 875 (2008) 93.
[24] L. Oliveros, C. Minguillón, B. Desmazières, P.L. Desbène, J. Chromatogr. 589 (1992) 53.
[25] Data provided by the manufacturer.
[26] N. Rubio, S. Ignatova, C. Minguillón, I.A. Sutherland, J. Chromatogr. A, in press, doi:10.1016/j.chroma.2009.10.006.