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Screening approach for chiral separation of pharmaceuticals Part III. Supercritical fluid chromatography for analysis and purification in drug discovery

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Abstract

High-throughput and performance analysis and purification of enantiomers are important parts of drug discovery and provide high-quality compounds for pharmacological testing. We have previously reported two parts describing chiral chromatographic screens using normal-phase (NPLC) and reversed-phase (RPLC) liquid chromatography, in order to cope with increasing numbers of new compounds generated by chemistry programs. We present in this part the development and implementation of a third faster screen using supercritical fluid chromatography (SFC) to maximize chance in achieving rapid enantiomer resolution of large numbers of compounds in a minimum of time. The SFC screen utilizes a narrow combination of only four columns (Chirlapak AD and AS, and Chiralcel OD and OJ) and two solvent modifiers (methanol and isopropanol). A modifier and column-switching setup was employed to allow the entire screening process to be serially run in the order AD > OD > OJ > AS and methanol > isopropanol, so that the screening for a given molecule can be stopped when separation is achieved. The switching system was fully automated for unattended operation of multiple compounds. An optimization procedure was also defined, which can be performed if needed for unsuccessful separations in the screening step. The chiral SFC strategy proved its performance and robustness in resolution of hundreds proprietary chiral molecules generated by drug discovery programs, with a success rate exceeding 95%. In addition, the generic capability of the strategy was evaluated by applying the screen and optimization methodology to a test set comprising 40 marketed drugs differing from proprietary compounds in terms of chemical diversity, revealing a similar high success rate of 98%. Chiral separations developed at the analytical scale work easily and equally well at the semi-preparative level, as illustrated with an example. The SFC screen allows resolution of compounds that were partially separated by NPLC or not separated at all by RPLC, demonstrating the utility of implementing complementary chromatographic techniques. The SFC screen is currently an integral part of our analytical support to discovery chemical programs and is considered the first try for chiral separations of new compounds, because it offers a higher success rate, performance and throughput.
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1. Introduction

The pharmaceutical industry strives to produce effective, safe and high quality medicines. However, the research-based drug industry still faces today the major challenge of shortening discovery time to push truly new drug candidates into the development pipeline [1]. High-throughput technologies that speed drug discovery for identifying promising leads became, in the 1990s, mainstays in drug discovery programs. But vast compound libraries are useless without the analytical means to control their quality (purity and identity). As a consequence, the number of samples submitted by medicinal chemists for analysis has significantly increased over recent years. On the other hand, in the 2000s, the goal of discovery

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has shifted from a sole pursuit of high-throughput towards performing high quality and innovative compounds. Under this new view, the biggest challenges to analysts are to minimize assay time and maximize analytical information by utilizing newer technologies and approaches in order to perform rapid analytical method development. The goals from these approaches are to provide chemists with the appropriate answers to their synthesis in a short time frame.

As part of the continuing effort to improve safety and efficacy of drugs, special attention of both pharmaceutical companies and regulatory agencies has been focused on chiral drugs. Because, each enantiomer can produce different therapeutic or adverse effects, and may even be metabolized differently [2], the analyst plays a critical role in the chiral drug discovery process. If a drug candidate is developed as a single enantiomer, analytical support is needed to assess the viability of enantioselective syntheses and to verify the chiral purity of single isomers. In addition, the toxicological and pharmacological effects of the pure enantiomers must be established. Thus, separation of enantiomers has also been addressed very early, for purification of modest amounts (milligrams to grams) for pharmacological testing. The separation of a pair of enantiomers bring into play subtle stereo selective interactions with a chromatographic chiral stationary phase (CSP), like a receptor-ligand. Thus, it is not evident to predict separation and elution order from their chemical structure. Fortunately, a large number of commercial CSPs (more than a hundred) are available. But, appropriate CSP and elution solvent are compound-specific and difficult to select. It is not unusual that similar racemic compounds from the same synthetic route with a slight change in just one functional moiety may require totally different CSP/solvent combinations in order to achieve the desired enantiomeric resolution. Separation of enantiomers can be performed by various separation techniques, including gas chromatography (GC), liquid chromatography (HPLC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE) and capillary electrochromatography (CEC). However, the already widespread use of HPLC for pharmaceutical analysis has favored this technique for chiral drug separations. But, several limitations have been encountered during development of chiral HPLC methods. First, choosing the best CSP and eluent, mainly a trial and error process, can require a significant investment in time and cost for each racemate due to long equilibration after changes in columns and mobile phases. Second, the low efficiency of HPLC results in long analysis times that limit the throughput and broad peaks that may preclude reliable determination of enantiomeric purity.

Because of such difficulties, in the last few years SFC has gained ground against HPLC for separating drug enantiomers. The application of SFC to enantiomeric separations, using chiral columns originally designed for HPLC, was first reported in 1985 [3]. Since that report, the separation of enantiomers has increasingly been identified as an area in which SFC offers distinct advantages over HPLC and has been the subject of various articles, reviews and books [4–15]. The major interest is that higher flow rates can be used in SFC to take advantage of the high diffusivity of supercritical fluids reducing analysis time without compromising efficiency. Thus, column equilibration occurs within a few minutes, speeding the optimization of chromatographic parameters. In addition, during early stages of drug discovery, small quantities (milligrams to grams) of each enantiomer may be required to assess activity and toxicity. Chromatographic purification is now recognized as a much faster approach to obtaining pure enantiomers than asymmetric synthesis, recrystallizations or other purification routes. In these cases, SFC offers by far the easiest path to early testing. Preparative scale SFC offers dramatically high resolution that improves throughput and pure product recovery, reduces solvent consumption and replaces toxic and flammable solvents used in HPLC [16–18].

When exploring new synthetic routes, generic and high-throughput and -performance chiral separations are needed to provide chemists with quick and suitable answers to their synthesis. In order to maximize the chance to achieve rapidly a separation, we developed screens with most separating techniques commonly used in pharmaceutical industry, including CE [19], NPLC [20] and RPLC [21]. Chiral SFC with a screening approach has been reported, using a set of four columns with several mobile phases [22,23]. In the present article, we report a faster chiral SFC screen, using a narrow combination of only four columns and two modifiers at a single concentration. A modifier- and column-switching system was employed to allow the entire screening process to be used in a sequential mode when dealing with few compounds, or fully automated for unattended operation of multiple compounds. We also defined an optimization procedure, which can be performed if needed for unsuccessful separations in the screening step. The experimental conditions selected for screening and optimization were statistically obtained from 10 years experience in separation of hundreds proprietary chiral molecules generated during early drug discovery programs. The strategy was tested with a set of 40 marketed chiral drugs, in order to evaluate the generic capability of the screening and optimization procedures. The initial part of the article reviews knowledge from literature and our practice on chiral SFC, with emphasis on the unique characteristics of SFC and parameters impacting the enantioselectivity. The latter part of the article demonstrates through an example, how the overall screening and optimization process works and how the best separation is then transferred to preparative separation for isolating pure enantiomers.

2. Experimental

2.1. Chemicals

Carbon dioxide (5.5 SFC/SFE grade) was obtained from Messer (Poole, Germany), methanol, isopropanol and ethanol, both HPLC grade, were obtained from Merck (Darmstadt, Germany). Diethylamine, triethylamine, iso-

propylamine, *N*-dimethylethylamine, trifluoroacetic acid and heptafluorobutyric acid were obtained from Fluka (Buchs, Switzerland).

2.2. Chiral test compounds

Acebutolol hydrochloride alprenolol hydrochloride, atropine, (±) bupropion hydrochloride, clenbuterol hydrochloride, cyclothiazide, ephedrine hydrochloride, (±) epinephrine hydrochloride, (±) flurbiprofen, (±) fenoprofen calcium salt hydrate, ibuprofen, ketamine hydrochloride, ketoprofen, (±) metroprolol tartrate salt, morphine sulfate pentahydrate, nadolol, (+) naproxen, (−) naproxen sodium salt, oxprenolol hydrochloride, pindolol, praziquantel, promethazine hydrochloride, DL-propranolol hydrochloride, (±) sotalol, (±) sulpiride, suprofen, (±) tetramisol hydrochloride, (±) thiopental, *trans*-stilbene oxide, (±) verapamil hydrochloride, warfarin, and mandelic acid were obtained from Sigma–Aldrich (Steinheim, Germany). (±) 3,5-Difluoro mandelic acid was obtained from Lancaster Synthesis (Strasbourg, France). Acenocoumarol, fluoxetine hydrochloride, hexobarbital, methadone hydrochloride, mianserin, oxazepam, and propiomazine, all were gifts from diverse sources. (±) Amisulpride (SL91.1077-10), PCR 4099 and (±) diltiazem hydrochloride (SL85.0294-10) were from Sanofi-Synthelabo Recherche.

2.3. Chiral columns

Chiralcel OD: cellulose tris(3,5-dimethylphenyl carbamate), Chiralpak AD: amylose tris(3,5-dimethylphenyl carbamate), Chiralcel OJ: cellulose tris(4-methylbenzoate), and Chiralpak AS: amylose tris [(*S*)alpha-methylbenzyl carbamate] columns, were purchased from Chiral Technologies (Illkirch, France). Dimensions were 250 mm × 4.6 mm i.d., 10 μm-particle size and 250 mm × 21 mm i.d., 10 μm for analytical and preparative columns, respectively. The 5 μm-particle size versions AD-H, OD-H, OJ-H and AS-H were also used.

2.4. SFC instrumentation

Three SFC systems were used in this study.

Analytical BI-SFC system from Berger Instruments (Newark, DE, USA) comprises a FCM-1200 dual-pump fluid control module for delivering carbon dioxide (CO_2) and modifier, an ALS-719 automatic liquid sampler, a TCM-2000 thermal control module for column heating and cryogenic cooling (using liquid CO_2) in the sub-0 to 150 °C range. An Agilent 1100 UV photodiode array detector (Agilent Technologies, Waldbronn, Germany) equipped with a high-pressure flow cell standing up to 400 bar was used. UV signal was recorded at 220 nm. Instrument control, data acquisition and data processing, were performed by a ChemStation or ProNTo software. Two Valco valves (VICI, Houston, TX, USA), controlled via remote logic level signal, allow switching between different columns and modifiers (Fig. 1).

Analytical/semi-preparative SF3 system from Gilson (Villiers-le-Bel, France). CO_2 was pumped with a model 306 pump. Cooling of the pump head and CO_2 line was achieved with a coil alimented by a Lauda chiller (Brinkman Instruments). Modifier was pumped with a model 306 pump. Mixing of CO_2 and modifier took place in a model 811C dynamic mixer with a 1.5-ml mixing chamber. Sample injections were made using a model 233XL injector. Detection was accomplished at 210 nm using a model 155 variable-wavelength UV detector with a 7 μl high-pressure flow cell. Outlet column pressure was controlled by a model 821 pressure regulator.

Preparative APS 1010 system with AutoPrep option from Berger Instruments, consisted of two Varian SD-1 pumps (Walnut Creek, CA, USA), one of which was extensively modified to pump CO_2, a special pump head heat exchanger, a Julabo FT401 chiller (Labortechnik GmbH, Seelback, Germany), a model Knauer 2600 UV detector with high-pressure flow cell (Berlin, Germany), a model SCM 2500 phase separator (Berger Instruments) with selection valve, and a set of collection vessels in a Bodan robot. Samples were applied using a six-port injection valve (Valco, Houston, TX) with a 2-ml sample loop and a model YP-3000 syringe pump (Cavro, San Jose, CA).

3. Results and discussion

3.1. Definition of a screening and optimization strategy

In this part, we describe how we select the experimental conditions for the screening and optimization strategy. Selection was based on knowledge in chiral SFC from literature and from our practice in separation of hundreds proprietary chiral molecules generated during drug discovery programs.

3.1.1. Selection of chiral columns

A wide range of chiral stationary phases (CSP) can be used in SFC, nearly all commercially available CSPs designed for use in NPLC [8,9,12–14]. Among these, polysaccharide phases developed by Okamoto and co-workers [24,25] have been extensively employed in HPLC [26–28] and found to be also very versatile for SFC [29–32]. Several authors performed detailed comparison of HPLC and SFC on polysaccharides [11,31,33,34]. Column equilibration and parameter optimization were generally accomplished more rapidly in SFC than in HPLC. SFC provided often, but not always, improved resolution of the racemic compounds in a shorter period of time than HPLC. In some instances, SFC provided separation capabilities not readily accessible in HPLC. After a trial of several CSP types (including Pirkle-type, macrocyclic antibiotics and polysaccharide derivatives), we selected four polysaccharide columns (Chiralpak AD, Chiralpak AS, Chiralcel OD and Chiralcel OJ), because their wide enantiose-

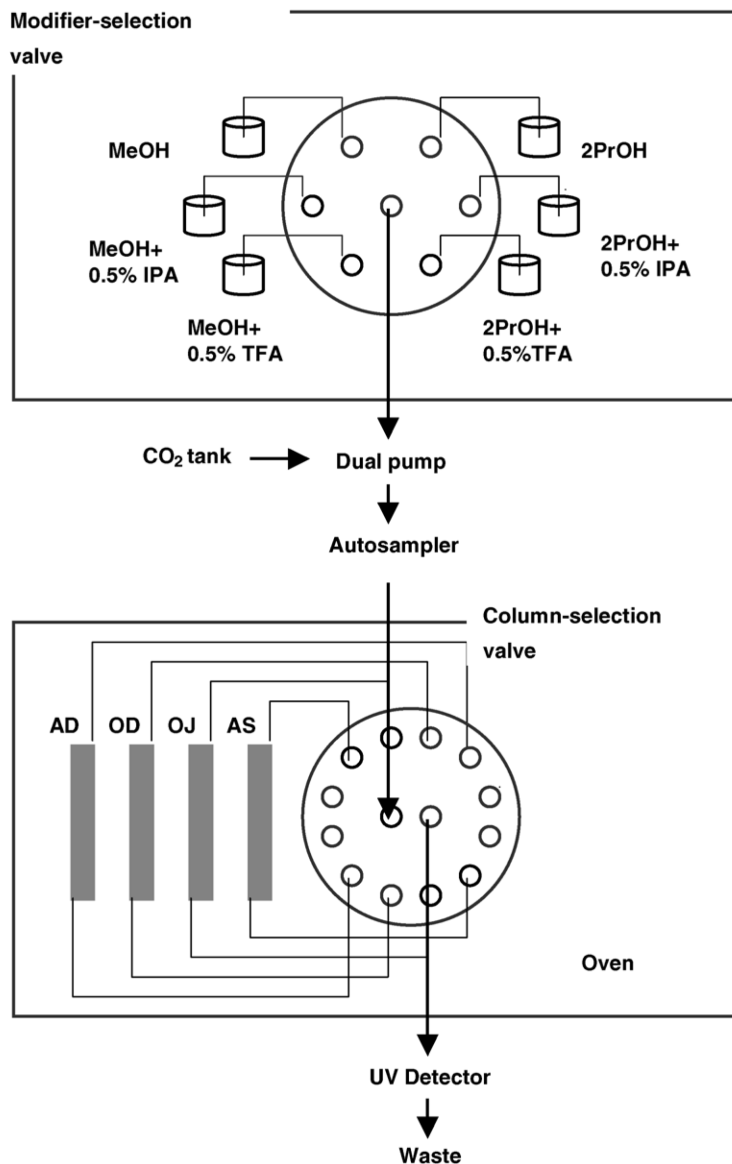

Fig. 1. Chiral column- and modifier-switching system used in the SFC screen.

lective applicability. Enantioseparations obtained with more than 500 proprietary chiral compounds, gave a success rate rank AD (60%) > OD (31%) > OJ (8%) > AS (2%) as shown in Fig. 2. This result is not in agreement with a recent observation in chiral SFC on less compounds, reporting a success rate in the order AD > AS > OJ > OD [23]. The enantioselectivity of the modified polysaccharides depends not only on their helical conformation, but also upon the nature of substituents introduced during their derivatization process [24,25]. Dramatic difference in stereo selectivity of polysaccharide CSPs toward PCR 4099 is shown in Fig. 3. As can be seen, PCR 4099 enantiomers were resolved on an amylose (AD) and a cellulose (OJ) columns, whereas no separation was obtained on the other cellulose (OD) and amylose (AS) columns. This example shows how the selection of an appropriate column among the four CSPs to resolve a pair of enantiomers is

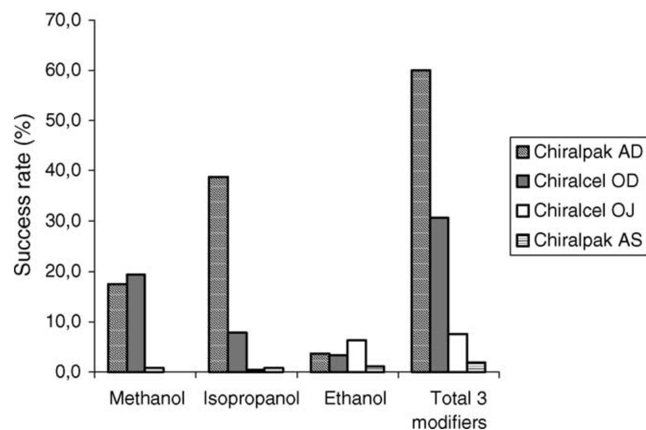

Fig. 2. Success rate obtained with chiral polysaccharide-columns and modifiers for SFC enantioresolution of proprietary compounds.

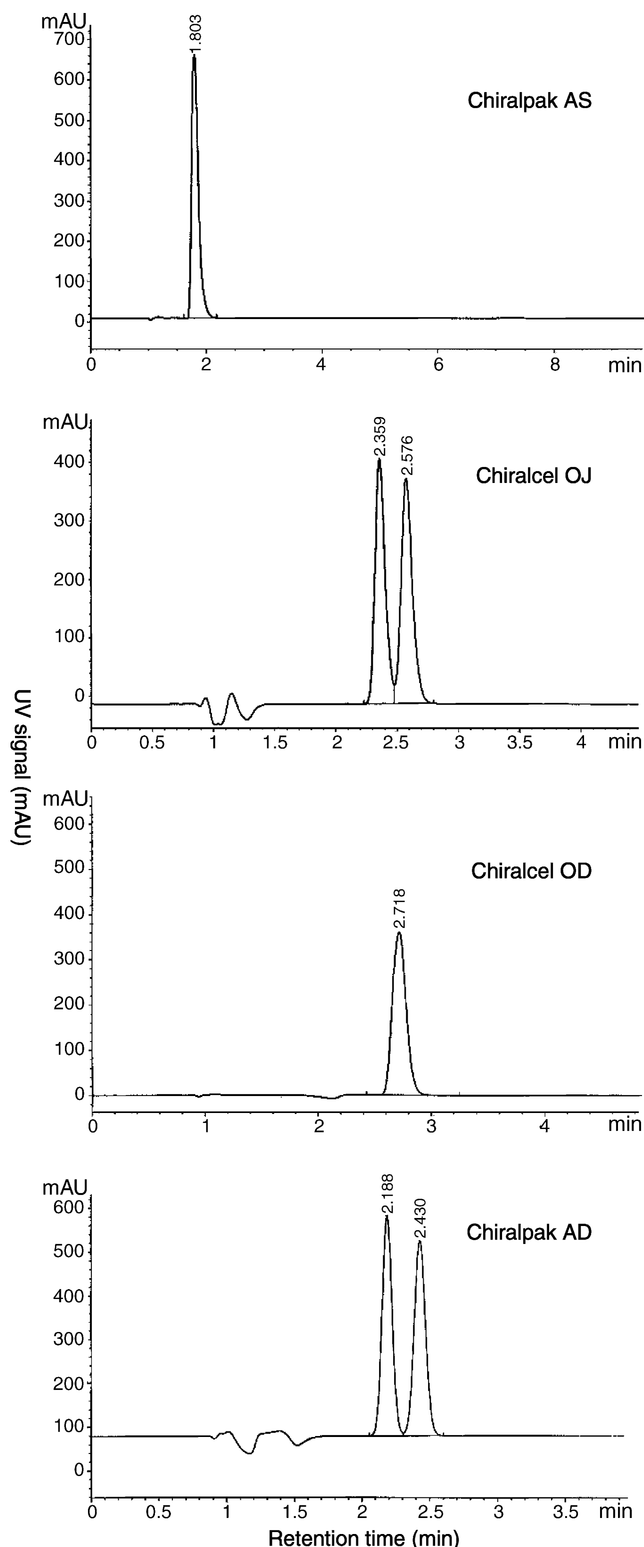

Fig. 3. Selectivity of polysaccharide-columns for PCR4099 enantiomers. CO_2–isopropanol (80:20) containing 0.5% IPA, 200 bar, 30 °C, 3 ml/min.

not easy to predict from their chemical structure. Therefore we selected the four columns for the screening. The change from one column to another was facilitated by the use of a switching-valve (see Fig. 1).

3.1.2. Selection of mobile phases

Pure CO_2 is a non-polar solvent, not adequate to elute organic drug-like compounds. Almost all the compounds of pharmaceutical interest have hydrophobic and hydrogen-bonding donor and acceptor sites to interact with the CSP. They were highly retained and the use of a polar organic modifier was necessary in order to obtain acceptable analysis times. Fortunately, CO_2 is completely miscible with nearly all commonly used organic solvents, including the most polar methanol (MeOH) and acetonitrile (ACN). In contrast, MeOH and ACN are rarely used as modifiers in NPLC because they are immiscible with hexane and pentane. When using CO_2-modifier mixtures, subcritical conditions are applied, but as there is continuity in the fluid properties, the separative advantages of supercritical fluids remain. The modifier has a large effect on chiral SFC. The retention of enantiomers is very much influenced by the amount of modifier, while the enantioselectivity remains modifier type-dependent [5]. In general, retention decreases as the modifier concentration increases because the solvent molecules compete with the enantiomer molecules for the specific adsorption sites on the CSP. Retention appears to be a steeper function of concentration than in non-chiral separations, more than doubling with a two-fold decrease [5]. At low modifier concentration, enantiomers often exhibit long retention times and peak shapes tend to degrade on CSPs more rapidly than those in non-chiral columns. Unusual stereo selective effects, like elution order, which may be inversed when changing from one modifier to another one, are reviewed for various racemic compounds with emphasis on polysaccharides [35]. Most often, an alcohol is used as the polar modifier. Enantioseparations obtained with more than 500 proprietary chiral compounds in our laboratory, gave a success rate rank isopropanol (48%) > methanol (38%) > ethanol (14%) as shown in Fig. 2. Despite the superiority of isopropanol (2PrOH) over MeOH and ethanol (EtOH), we consider MeOH the first modifier try in SFC because it produces highly efficient separations. Another reason, MeOH combines low viscosity and high polarity with a low boiling point, which is favorable when the method is transferred to preparative purification. Although MeOH and 2PrOH proved to be the most suitable modifiers for efficient separation of a wide variety of racemates, EtOH has been revealed to be effective for enantioseparation of some compounds. For example, EtOH allows resolution of ephedrine enantiomers for which MeOH and 2PrOH give no separation (Fig. 4). On the other hand, we found acetonitrile a poor modifier, giving rarely an enantioseparation. Yet, interesting enantioselectivity can be obtained for particular compounds, like clenbuterol enantiomers which are separated with any of the modifiers tested (Fig. 5). It should be noted that the highest resolution was obtained with acetonitrile ($R_s = 23.17$) compared to MeOH (3.38), 2PrOH (2.98) and EtOH (2.97). However, as far as enantioselectivity is concerned, it is difficult to predict which solvent will be the most favorable modifier, as demonstrated by the above examples. In the perspective of a fast screen, we selected only two mod-

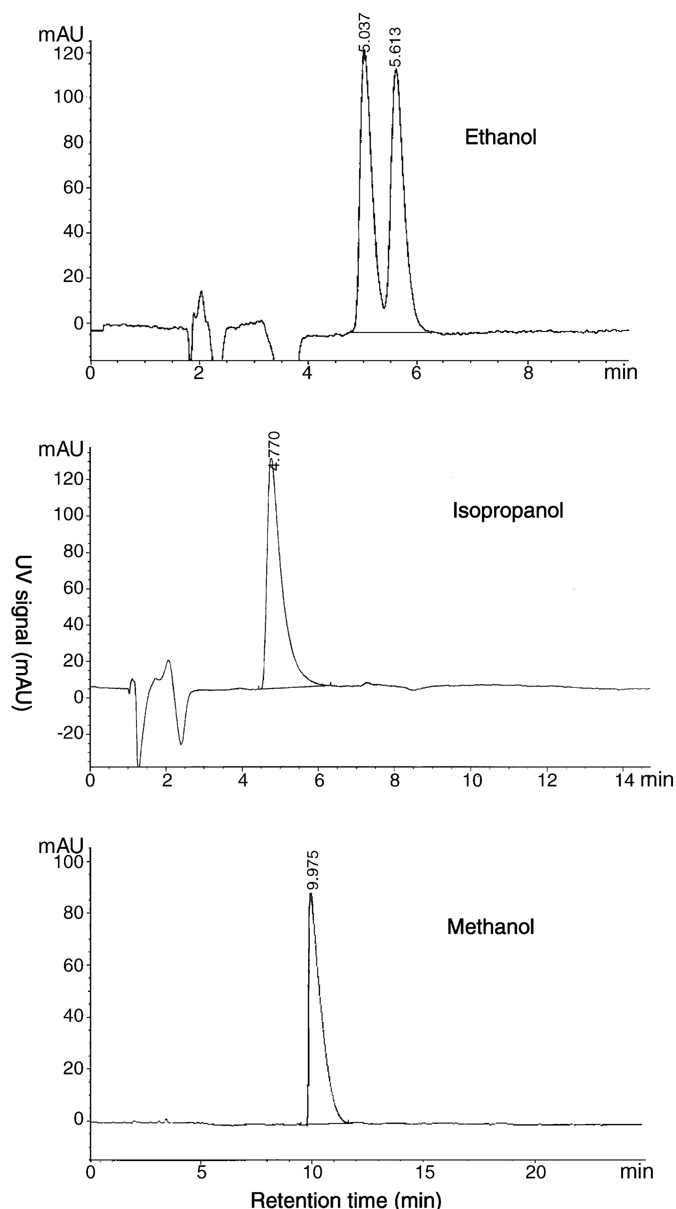

Fig. 4. Selectivity of modifiers for ephedrine enantiomers. Chiralpak AS, CO_2–methanol (90:10) containing 0.5% IPA, 30 °C, 200 bar, 3 ml/min; CO_2–isopropanol (80:20) containing 0.5% IPA, 30 °C, 200 bar, 3 ml/min; CO_2–ethanol (85:15) containing 0.5% IPA, 30 °C, 100 bar, 1.5 ml/min.

ifiers (MeOH and 2PrOH) at a single concentration (10 and 20%, respectively), because they produce more than 80% success.

For most small drug-like molecules, either the enantiomer peaks does not elute or they elutes with severe broadening and distortion, when the modifier was used alone. To improve the peak shapes of strong bases, a strong basic additivee is added in mobile phase. Similarly, to elute a strong acid, a strong acidic additive is added [36], as shown for clenbuterol (Fig. 6a) and mandelic acid (Fig. 6b). Whereas, we observed no impact on neutral compounds. Additives can provide increased efficiency by minimizing undesirable interactions between the enantiomers and the residual silanol

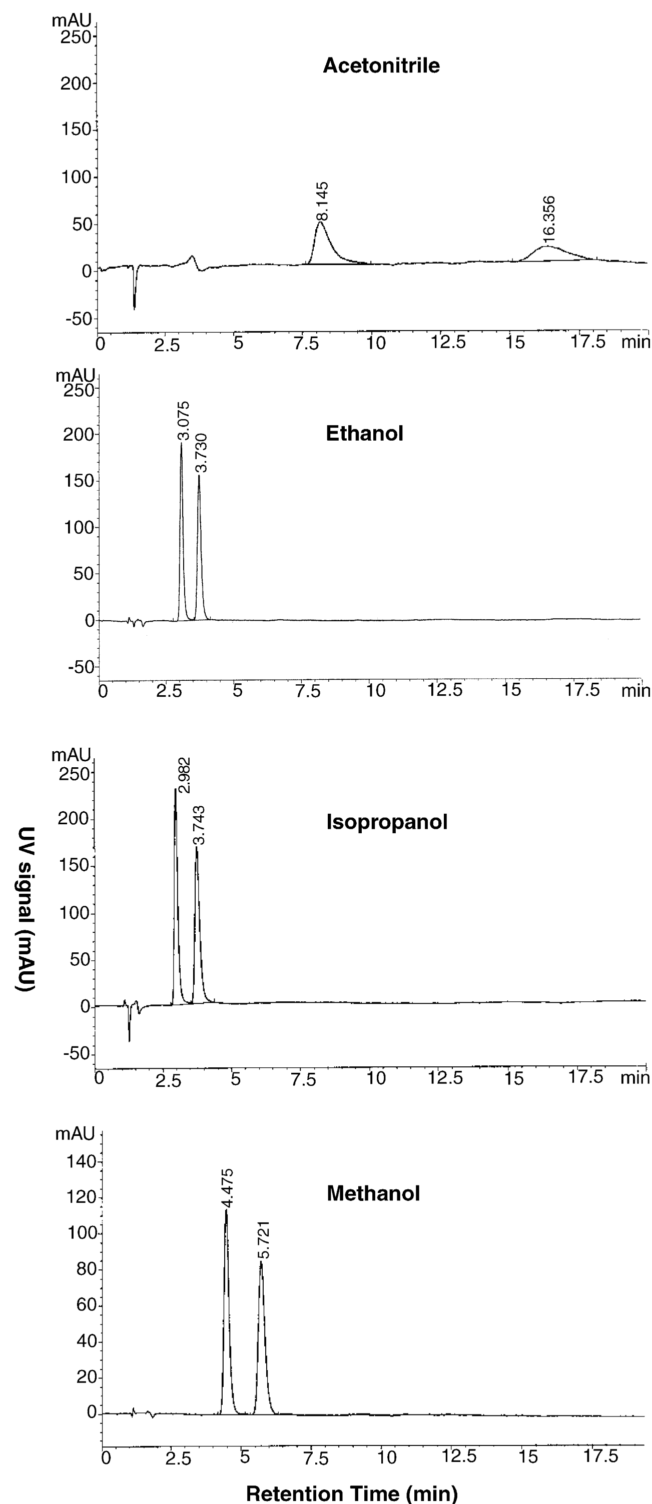

Fig. 5. Selectivity of modifiers for clenbuterol enantiomers. Chiralpak AD, 200 bar, 30 °C and 3 ml/min, CO_2–methanol (90:10), CO_2–isopropanol (80:20), CO_2–ethanol (85:15) and CO_2–acetonitrile (70:30), 0.5% IPA as additive.

groups on the CSP. Ionization suppression seems to be a major mechanism in SFC. The type of additive (acidic or basic) that gives the best resolution was found to be dependent upon the functionality and charge as the chiral com-

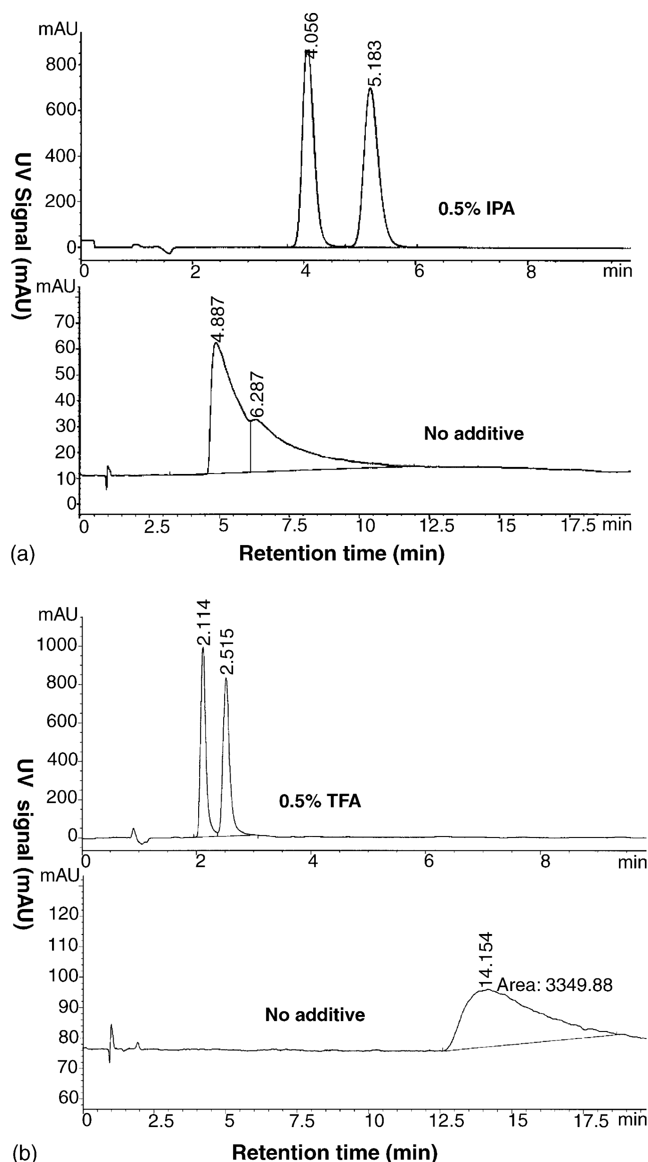

Fig. 6. Effect of additive on separation of (a) clenbuterol and (b) mandelic acid enantiomers. Chiralpak AD, CO_2–methanol (90:10), 200 bar, 30 °C and 3 ml/min.

pound. Charged chiral compounds are affected to a much greater extent. Additives that act as competing ions of the same charge as the chiral compound dramatically improve efficiency and resolution [37]. We investigated various additives in our laboratory and found that isopropylamine (IPA) performs best than diethylamine (DEA), triethylamine (TEA) and *N*-dimethylethylamine (NDMEA) for basic compounds, and trifluoroacetic acid (TFA) best than heptafluorobutyric acid (HFBA) for acidic substances. In addition, IPA has a lower boiling point (34 °C) than DEA (55 °C) and TEA (89 °C), which is considered important when transferring the method to preparative purification where all traces of additives and modifier must be removed under mild temperatures to avoid compounds degradation. Since, additives are in general strongly retained on the CSPs, and their effect may persist even after they are removed from the eluent [38,39], we selected for screening a concentration of 0.5% IPA for basics and 0.5% TFA for acids, as we did not observe substantial change in separation efficiency at higher concentrations (results not shown). Bifunctional compounds can be analyzed with any of these additives, whereas neutral compounds need no additive. However, in the perspective of a screening strategy, a reduced set of mobile phases would be more suitable. Thus, for simplicity, the samples are segregated into two pools, the basics/bifunctionals/neutrals are screened with two mobile phases (MeOH and 2PrOH, each containing 0.5% IPA) and the acids are screened with two other mobile phases (MeOH and 2PrOH, each containing 0.5% TFA). The change from one modifier to another was achieved by a switching-valve (see Fig. 1).

We also defined an optimization procedure in order to improve resolution for unsuccessful separations in the screening run. At first, we change modifier concentration for adjusting enantioselectivity. The percentages ranged from 2 to 20% for MeOH and EtOH and 5 to 30% for the less stronger solvent 2PrOH. Second, we replace MeOH and 2PrOH by EtOH, as will be illustrated further.

3.1.3. Selection of operating parameters: temperature, pressure and flow rate

It has been observed that subtle changes in temperature can dramatically affect selectivity or relative retention of enantiomers with polysaccharide phases [35]. Increased temperature commonly gives decreased retention, in accordance with non chiral chromatography, but an opposite effect can be observed. In most cases, the enantioselectivity decreased when the temperature increase. For this reason, most chiral separations in SFC are performed at ambient or sub ambient temperatures [40,41]. Sub ambient operation is not useful in HPLC, because the high viscosity of liquids gives slow analyses with degraded efficiency. In SFC, the viscosity of CO_2 is much lower, and diffusivity much higher than in liquids at room and sub ambient temperatures. A sub ambient and especially cryogenic temperature is particularly useful for enantiomers having low configurational stability at ambient temperature. The enantiomers of several compounds that show low enantioselectivity at room temperature have been fully separated at cryogenic temperatures as low as −47 °C on Pirkle phases [42]. Smith et al. found on the Chiralcel OD [43] that two potassium channel activator analogues, which differed only by replacement of a benzoyl by *n*-pentanoyl group, showed quite strikingly different temperature dependencies in SFC. This indicates that one compound is above, and the other below, its enantioselective temperature, at which separation of enantiomers is not possible. Recently, we succeeded to separate eight metabolite isomers (bearing two chiral and one ethylenic bond geometric centres) of the antithrombotic drug clopidogrel by SFC on Chiralpak AD in less than 10 min by operating the column at 5 °C [44]. We select a temperature setting of 30 °C for screening to avoid modifying too many variables. However, as a last resort when separation fails, op-

timization of enantioselectivity should always include investigation of temperature dependence, since this can enhance the separation factor needed for complete resolution.

Most studies have indicated little effect of pressure on stereo selectivity in chiral SFC [3,45]. An increase of pressure decreased the retention, but the resolution was only slightly affected. At low pressure, peak shapes tend to degrade. Higher optimum flow rates can be achieved in SFC with lower pressure drops than typical in HPLC. Because the van Deemter curves for supercritical fluids are flatter and the viscosity is lower, operation at much higher velocities is both desirable and practical. Thus, SFC places far less stress on expensive chiral columns [5]. A pressure of 200 bar was utilized to carry out the screening due to its small impact on retention and enantioselectivity.

Diffusion coefficients are roughly three to five times higher in CO_2-modifier mixtures than they are in a liquid. This higher diffusion translates into higher optimum linear velocity. An important aspect of the high diffusivity, the fluids equilibrate extremely rapidly and retention times stabilize in as few as three to five column volumes, which is unusually fast compared to HPLC. The minimum equilibration when changing from one column to another or from one modifier to another during method development, requires a pumping of three to five column volumes, which correspond to 2.4–4.0 min for a standard chiral column [5]. An increase of flow rate markedly decreased the retention, but the resolution was only slightly changed. Consequently, the same work can be done three times faster, or three times more work can be done in the same time. This is an important advantage for high-throughput. A flow rate of 3 ml/min was utilized to carry out the screening due to its smaller impact on enantioselectivity. However, the flow rate can also be lowered for further optimization if needed as will be illustrated further.

3.2. Evaluation of the screening and optimization strategy

The experimental conditions selected above for a screening and opimization strategy have proved to be of a large applicability for proprietary compounds with a high success rate exceeding 95%. In the aim to test the generic ability of the methodology, a set of 40 marketed chiral drugs and analogs differing in chemical diversity from proprietary compounds, was submitted to SFC screen. The screen was carried out on the analytical Berger BI-SFC system, which is equipped with the column- and modifier-switching valves. Samples were dissolved to 1 mg/ml in methanol and 10 μl were injected. Poorly soluble compounds were primarily dissolved in DMSO and subsequently diluted in methanol. The 30 basic/bifunctional/neutral and 10 acidic compounds were analyzed under the defined screening conditions (i.e. four AD/OD/OJ/AS columns, two eluents: 10% MeOH and 20% 2PrOH each containing 0.5% IPA or TFA as modifiers in CO_2, 200 bar as pressure, 30 °C as temperature and 3 ml/min as flow rate), generating 320 chromatograms. Further optimization was performed for non- or partially separated ($R_s < 1.5$) compounds. The best results for each chiral column are given (printed in bold) in Table 1. Under the screening conditions, 28 out of 40 compounds are completely resolved ($R_s \geq 1.5$) on at least one column, with good peak shape and mostly very short analysis times (less than 10 min). It can be seen that nine compounds are almost separated ($R_s > 1.0$): acebutolol, bupropion, fluoxetine, methadone, sulphide, cyclothiazide, thiopental, ibuprofen and ketoprofen, requiring further optimization. One compound shows a beginning of separation: nadolol, while only two compounds show no separation at all: ephedrine and naproxen.

For non- or insufficiently-resolved compounds during the screening (12 out of 40), we applied an optimization procedure. The first step in optimization consists in fine-tuning the concentration of the modifier. Changing the %MeOH or 2PrOH gave an improved resolution for all nine compounds incompletely resolved during the screening run. However, changing the % modifier gave no result for the three compounds ephedrine, nadolol and naproxen on any of the four columns. The second step in optimization is a shift to EtOH as modifier. On changing to EtOH at 10%, a good resolution ($R_s = 1.30$, 2.88 and 2.28) occurred for the four nadolol stereoisomers. For ephedrine, an insufficient separation occurred with 15% EtOH only on AS column. But, lowering pressure to 100 bar and flow rate to 1.5 ml/min resulted in an acceptable separation ($R_s = 1.37$). Executing the optimization procedure gave no result for naproxen on any of the four columns. For that particular compound, it is advisable to use another separation technique. It is interesting to note that naproxen was previously resolved on AD column ($R_s = 1.68$) under RPLC [21] and not separated under NPLC screen [20]. In contrast, three compounds (ephedrine, nadolol and sulphide), which were not separated by RPLC and showed some degree of resolution in NPLC, are completely resolved by SFC. This demonstrates the complementarity of separation techniques working in aqueous (RPLC) and non-aqueous (NPLC and SFC) environment. However, sufficient separations obtained in the screening can be optimized further, in order to improve separation or reduce analysis time. As an example, PCR 4099 which showed good separation during screening with 20% 2PrOH on AD ($t_R = 2.19$ and 2.43 min, $R_s = 1.65$) gave better resolution when adjusting the modifier content to 10% 2PrOH and replacing AD column (10 μm pore size) by the more efficient AD-H (5 μm pore size) ($t_R = 4.68$ and 5.57 min, $R_s = 4.27$).

In the end, evaluation of the proposed strategy pointed out that it allows achieving baseline-resolution ($R_s \geq 1.5$) of 28 out of 40 compounds (70%) after the screening step and 39 out of 40 (98%) after the optimization procedure. Following screening, it was seen that AD column showed enantioselectivity for 24 out of 40 compounds (60%), followed by OD, OJ and AS which resolve 10 (25%), 9 (23%) and 5 compounds (13%), respectively, as indicated in Fig. 7. Further optimization improves the success rate for both columns: 32 compounds (80%) for AD, 19 (48%) for OD, 17 (43%) for

Table 1
SFC screening and optimization results obtained with 40 marketed chiral drugs

Compounds	Column	Modifier[a]	t_R (min)	R_s
Basics/bifunctionals/neutrals				
Acebutolol	AD	15% 2PrOH	11.85–13.46	1.19
	OD	**5% MeOH**	**17.97–22.94**	**1.48**
	OJ			ns
	AS	20% 2PrOH	18.27–19.14	0.31
Alprenolol	**AD**	**10% MeOH**	**2.49–2.73**	**1.61**
	OD	**10% MeOH**	**3.24–4.53**	**4.27**
	OJ			ns
	AS			ns
Amisulpride	**AD**	**10% MeOH**	**16.40–18.80**	**1.51**
	OD	20% 2PrOH	11.00–11.92	0.65
	OJ			ns
	AS	**20% 2PrOH**	**28.01–35.59**	**1.49**
Atropine	AD	10% MeOH	6.44–6.97	1.13
	OD	**10% MeOH**	**6.76–7.66**	**1.54**
	OJ			ns
	AS	20% 2PrOH	11.36–12.26	0.45
Bupropion	**AD**	**5% MeOH**	**3.28–4.14**	**1.67**
	OD			ns
	OJ			ns
	AS			ns
Clenbuterol	**AD**	**10% MeOH**	**3.71–4.66**	**3.59**
	OD	**5% MeOH**	**17.38–19.62**	**1.47**
	OJ			ns
	AS	**10% MeOH**	**10.37–13.74**	**2.76**
Diltiazem	**AD**	**10% MeOH**	**3.83–4.68**	**2.26**
	OD	**10% 2PrOH**	**3.73–4.65**	**2.77**
	OJ			ns
	AS			ns
Ephedrine	AD			ns
	OD			ns
	OJ			ns
	AS	**15% EtOH**[b]	**5.04–5.61**	**1.37**
Epinephrine	**AD**	**10% MeOH**	**7.09–7.99**	**1.58**
	OD	10% MeOH	23.45–24.13	0.59
	OJ			ns
	AS		11.67–12.58	0.64
Fluoxetine	**AD**	**7% MeOH**	**3.52–3.90**	**1.56**
	OD	5% MeOH	7.53–7.89	0.55
	OJ			ns
	AS			ns
Ketamine	**AD**	**10% MeOH**	**2.66–2.85**	**1.49**
	OD			ns
	OJ	**10% MeOH**	**2.37–2.83**	**2.06**
	AS	**20% 2PrOH**	**2.09–2.62**	**1.96**
Methadone	AD	20% 2PrOH	1.73–1.81	0.66
	OD	10% MeOH	5.42–5.94	1.16
	OJ	**5% MeOH**	**3.02–3.46**	**1.94**
	AS			ns
Metoprolol	**AD**	**20% 2PrOH**	**2.51–2.91**	**1.81**
	OD	**10% MeOH**	**3.71–9.40**	**10.23**
	OJ			ns
	AS	20% 2PrOH	3.23–3.76	1.13
Mianserin	**AD**	**10% MeOH**	**2.71–3.95**	**6.05**
	OD	**20% 2PrOH**	**2.79–3.20**	**1.69**
	OJ	**10% MeOH**	**2.46–3.41**	**3.64**
	AS			ns

Table 1 (*Continued*)

Compounds	Column	Modifier[a]	t_R (min)	R_s
Nadolol	AD	10% MeOH	8.91–11.68–15.29	2.92–3.15
	OD	**10% EtOH[c]**	**10.85–12.45–17.30–21.76**	**1.30–2.88.2.28**
	OJ			ns
	AS	20% 2PrOH	15.01–20.71	1.48
Oxprenolol	**AD**	**10% MeOH**	**2.94–3.29**	**1.90**
	OD	**20% MeOH**	**2.00–3.30**	**5.94**
	OJ			ns
	AS	10% 2PrOH	3.27–3.63	0.79
PCR 4099	**AD**	**20% 2PrOH**	**2.19–2.43**	**1.65**
	OD			ns
	OJ	**20% 2PrOH**	**2.36–2.58**	**1.49**
	AS			ns
Pindolol	**AD**	**10% MeOH**	**9.68–10.99**	**1.78**
	OD	**20% MeOH**	**6.08–23.82**	**11.93**
	OJ	**10% MeOH**	**29.77–35.81**	**1.51**
	AS	**20% MeOH**	**7.19–8.57**	**1.67**
Promethazine	**AD**	**20% 2PrOH**	**2.66–2.88**	**1.50**
	OD			ns
	OJ	**5% 2PrOH**	**10.97–13.41**	**1.50**
	AS			ns
Propiomazine	**AD**	**5% 2PrOH**	**36.72–42.58**	**2.48**
	OD			ns
	OJ	**20% 2PrOH**	**2.04–2.39**	**1.49**
	AS	20% 2PrOH	3.24–3.80	1.11
Propranolol	**AD**	**10% MeOH**	**4.73–5.82**	**3.26**
	OD	**10% MeOH**	**12.77–21.11**	**5.27**
	OJ			ns
	AS	20% MeOH	3.18–3.33	0.47
Sotalol	**AD**	**10% MeOH**	**7.19–8.61**	**1.67**
	OD	20% 2PrOH	9.66–10.40	0.54
	OJ			ns
	AS	20% MeOH	6.60–7.45	0.74
Sulpiride	**AD**	**25% MeOH**	**9.08–10.82**	**2.36**
	OD	10% MeOH	31.92–34.74	1.09
	OJ			ns
	AS	**25% MeOH**	**6.26–7.41**	**1.54**
Tetramisol	**AD**	**10% MeOH**	**5.06–5.51**	**1.64**
	OD			ns
	OJ	**10% MeOH**	**6.94–8.55**	**2.73**
	AS	20% 2PrOH	5.72–6.18	0.74
Verapamil	**AD**	**20% 2PrOH**	**2.32–2.56**	**1.69**
	OD	10% MeOH	5.43–5.84	0.81
	OJ	**5% 2PrOH**	**8.83–10.61**	**1.61**
	AS			ns
Cyclothiazide	**AD**	**25% MeOH**	**8.29–11.48–15.16–16.42**	**3.88–3.12–0.92**
	OD	**20% 2PrOH**	**17.69–19.48–25.59–27.13**	**0.69–2.11–0.96**
	OJ	**20% MeOH**	**10.59–12.79–14.98–16.05**	**2.10–1.67–0.69**
	AS	**30% 2PrOH**	**15.14–17.62–27.64–33.31**	**1.11–3.51–1.09**
Oxazepam	**AD**	**20% 2PrOH**	**6.38–15.99**	**12.93**
	OD	**10% MeOH**	**15.79–24.56**	**6.44**
	OJ	**10% 2PrOH**	**9.38–11.24**	**1.71**
	AS	**20% MeOH**	**8.14–14.14**	**5.68**
Praziquantel	**AD**	**30%2PrOH**	**3.01–3.71**	**2.78**
	OD	**20% 2PrOH**	**4.55–5.37**	**1.79**
	OJ			ns
	AS	20% MeOH	5.32–5.87	0.95
Thiopental	AD	10% MeOH	3.48–3.58	0.55
	OD	5% MeOH	6.27–6.54	0.57

Table 1 (*Continued*)

Compounds	Column	Modifier[a]	t_R (min)	R_s
	OJ	**5% MeOH**	**3.68–4.17**	**2.17**
	AS			ns
Trans-stilbene oxide	**AD**	**10% MeOH**	**2.85–3.91**	**5.38**
	OD			ns
	OJ	**10% MeOH**	**3.03–4.03**	**4.22**
	AS	**10% MeOH**	**3.02–4.43**	**3.41**
Acidics				
Acenocoumarol	**AD**	**25% MeOH**	**5.53–11.64**	**6.02**
	OD	**20% 2PrOH**	**6.54–9.70**	**4.39**
	OJ	**10% MeOH**	**16.67–22.81**	**4.36**
	AS	**20% 2PrOH**	**11.35–15.93**	**2.23**
Fenoprofen	**AD**	**20% 2PrOH**	**2.13–2.30**	**1.45**
	OD			ns
	OJ	10% 2PrOH	3.37–3.36	1.12
	AS			ns
Flurbiprofen	**AD**	**10% MeOH**	**3.23–4.43**	**5.72**
	OD			ns
	OJ			ns
	AS			ns
Hexobarbital	**AD**	**10% MeOH**	**2.59–7.84**	**16.39**
	OD	**7% MeOH**	**16.46–18.67**	**1.62**
	OJ	10% 2PrOH	3.42–3.63	0.74
	AS	**5% MeOH**	**20.12–26.58**	**2.54**
Ibuprofen	**AD**	**5% MeOH**	**5.88–8.15**	**4.82**
	OD			ns
	OJ	10% MeOH	2.32–2.58	1.32
	AS			ns
Ketoprofen	AD	10% 2PrOH	7.00–7.48	1.16
	AD	20% 2PrOH	2.65–2.83	1.08
	OD			ns
	OJ	**5% 2PrOH**	**5.45–6.37**	**1.72**
	AS			ns
Mandelic acid	**AD**	**20% 2PrOH**	**2.19–2.50**	**1.97**
	OD	**10% MeOH**	**2.83–4.37**	**6.32**
	OJ			ns
	AS			ns
Naproxen	AD			ns
	OD			ns
	OJ			ns
	AS			ns
Suprofen	**AD**	**10% MeOH**	**6.17–8.03**	**4.24**
	OD	**12% 2PrOH**	**22.91–25.47**	**1.60**
	OJ	**20% MeOH**	**5.13–5.76**	**1.69**
	AS	**20% 2PrOH**	**5.38–6.21**	**1.69**
Warfarin	**AD**	**10% MeOH**	**10.44–20.66**	**7.36**
	OD	**10% MeOH**	**8.15–18.72**	**11.15**
	OJ	**10% MeOH**	**13.13–16.59**	**3.01**
	AS	**7% MeOH**	**8.36–10.52**	**1.88**

ns: no separation.
[a] % Modifier containing 0.5% IPA for basic/bifunctional/neutral or 0.5% TFA for acidic compounds.
[b] 100 bar and 1.5 ml/min.

OJ and 12 (30%) for AS, as shown in Fig. 7. Although the AS column performed less, it exhibit complementary selectivity to AD, OD and OJ. Ephedrine was separated only on AS column. It is therefore recommended to perform the screening with both columns in the order AD > OD > OJ > AS. The score obtained for the four chiral columns with marketed drugs (Fig. 7) was similar to that observed for proprietary compounds (Fig. 2).

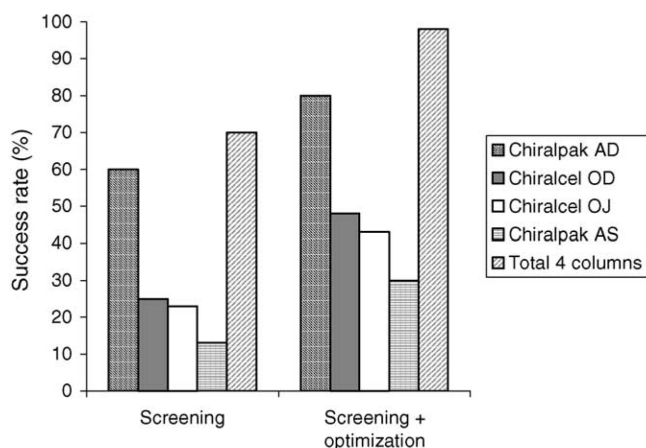

Fig. 7. Success rate obtained with chiral polysaccharide-columns for SFC enantioresolution of 40 marketed chiral drugs.

3.3. Scaling up analytical to preparative purification of enantiomers

The flow rate 3.0 ml/min used with analytical columns (4.6 mm i.d.) can be proportionally scaled up to 50 ml/min for semi-preparative columns (21 mm i.d.) without much change in the peak's retention times, capacity factors or resolution. In order to transfer developed methods to a purification process, the highest resolution is needed to allow large sample loading onto a preparative column. As an example, we describe here the different steps followed in a purification study. The *R*-enantiomer of 3,5-difluoro mandelic acid, which is known to be levogyre, is expected to give the active diastereoisomer. Purification by preparative SFC from the commercial racemic starting reagent permits the synthesis of a chiral proprietary compound directly with the appropriate enantiomer (Fig. 8).

The purification study started with an analytical method development, which was carried out on the Gilson SF3 SFC, prior to preparative separation on the Berger APS 1010 SFC system. The SF3 system can work both at analytical and semi-preparative scales. Although very handy for method optimization, it is quite limited for higher scale use. It can provide flow rates of no more than 10 ml/min with a rather low loading capacity (typically 10 mg of racemate per purification run). At collection, the fluid forms a spray in a non-contained environment, leading to product loss, therefore low recovery and possible workspace contamination. It is necessary to keep the entire system in a ventilated self-contained environment. The APS 1010 system is very suitable for preparative scale separation, with flow rates up to 50 ml/min and loading capacities up to 100 mg, the sample collection (up to 144 fractions) being automatically run by a Bohdan robot. As opposed to the SF3 system, it is provided with a phase-separator that prevents aerosol formation at collection. The fractions are collected into a self-contained rack, which avoids sample loss and environment contamination.

Analytical method development started with selection of the best chiral column. A beginning of separation was observed on AD column, which was then selected for further work (Fig. 9a). EtOH showed the best separation ($R_S = 1.6$) compared to MeOH and 2PrOH (Fig. 9b). Fluid composition was studied, and EtOH at 3% showed significantly improved separation compared to higher percentages, giving retention times of 15.9 and 20.2 min with a resolution factor of 2.7. The obtained chromatographic profile was considered suitable for preparative scale-up (Fig. 9c). Although the lower the alcohol content, the better the separation, it was decided to work under 3% EtOH because of the limited solubility of 3,5-difluoro mandelic acid in the fluid. Sample overloading was studied in order to determine the maximum amount of compound to be injected per purification run. The amounts loaded are directly transferable to preparative scale (Fig. 9d). The separations shown in Fig. 9d remain acceptable up to 30 mg injected.

Purification was achieved using a preparative Chiralpak AD-H column (21 mm i.d.) at a flow rate of 50 ml/min.

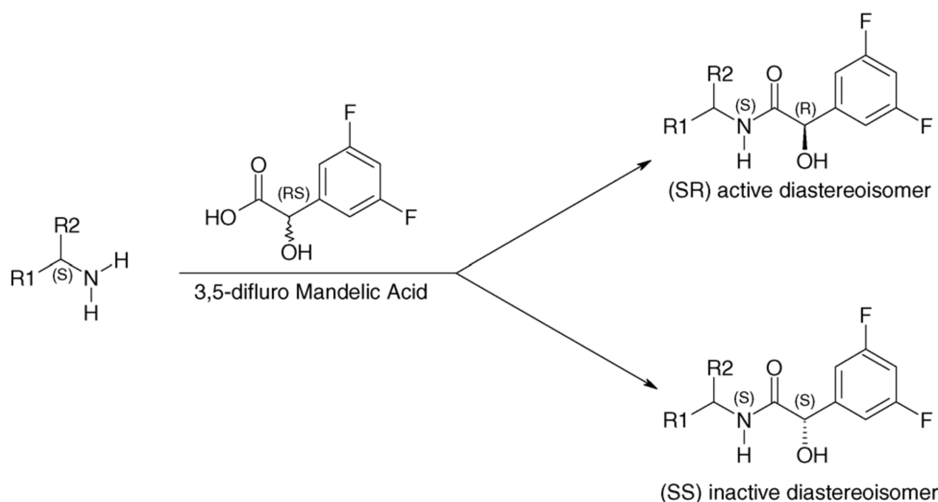

Fig. 8. Synthesis scheme of (*R*)- and (*S*)-enantiomers from racemic 3,5-difluoro mandelic acid.

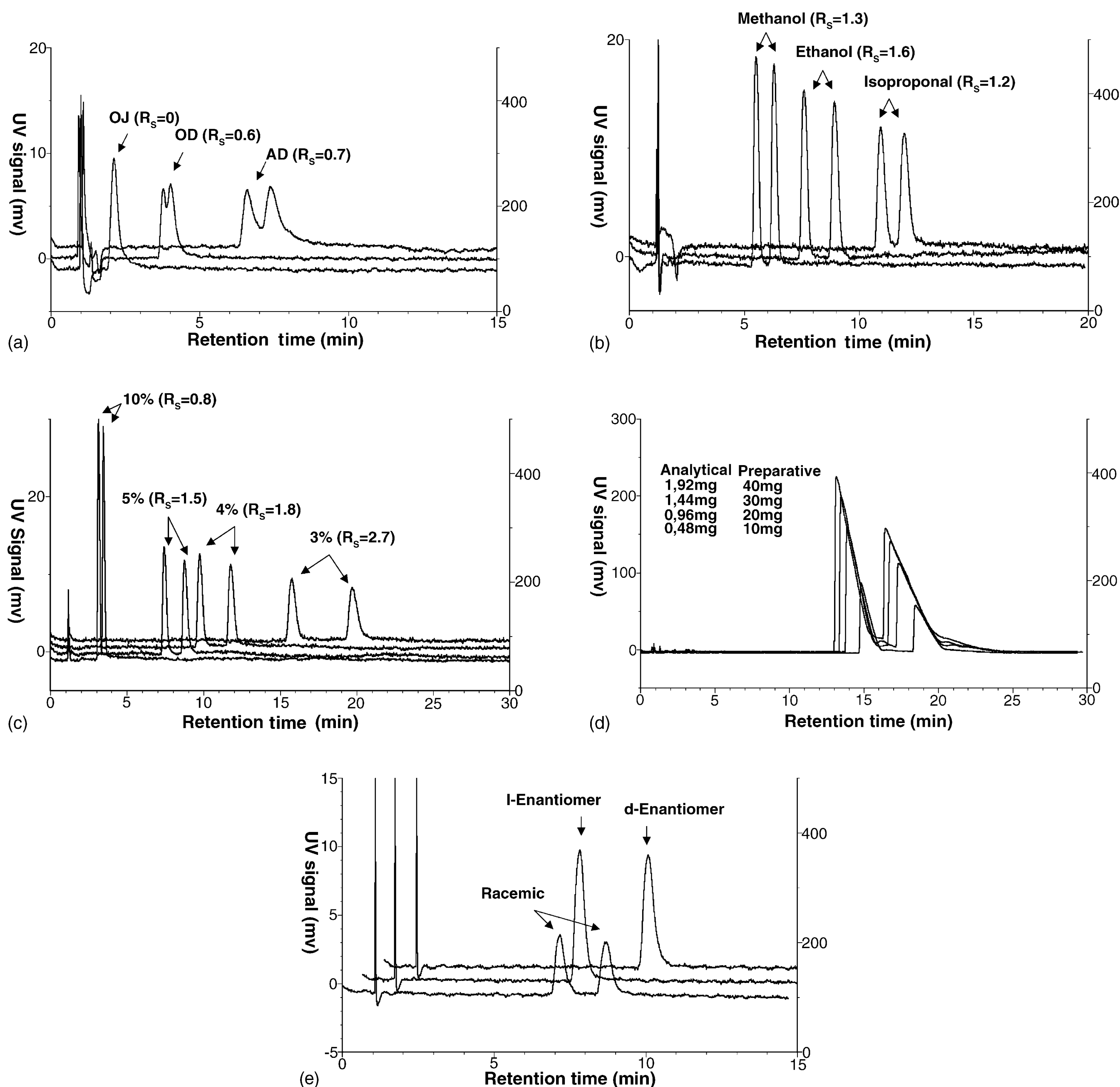

Fig. 9. Chiral SFC purification of 3,5-difluoro mandelic acid enantiomers: (a) selection of chiral column: CO_2–isopropanol (95:5), 3 ml/min, 200 bar; (b) selection of modifier: analytical Chiralpak AD-H (4.6 mm i.d.), CO_2-modifier (95:5) containing 0.5% TFA, 2.4 ml/min, 100 bar; (c) selection of % ethanol, 2.4 ml/min, 100 bar; (d) sample overloading effect: CO_2–ethanol (97:3) containing 0.5% TFA, 100 bar, 2.4 ml/min; (e) control of enantiomeric purity of isolated enantiomers: analytical Chiralpak AD-H, CO_2–ethanol (94:6) containing 0.5% TFA, 3 ml/min, 200 bar.

Good correlation between analytical and preparative chromatograms was observed. An amount of 450 mg of (±) 3,5-difluoro mandelic acid was separated in 15 purification runs of 30 mg each, requiring 6 h. Collected fractions for each enantiomer were pooled, evaporated and lyophilized. Amounts of 170 mg of L-enantiomer and 153 mg of D-enantiomer (optical rotation signals were monitored on-line by a Jasco chiral detector) were obtained. The mass recovery rate was 72%. Both D- and L-enantiomers were obtained with an enantiomeric purity of 100% (Fig. 9e).

4. Conclusion

Toward the goal of accelerating the overall chiral drug discovery process, we developed and implemented generic high-throughput chiral separation screens in order to speed method development for providing chemists with quick and suitable answers to their synthesis. We previously suggested that the most effective way to develop rapidly chiral separations turned out to be the screening of a small combination of the most successful chiral selectors and eluents [19–21].

The drug discovery phase requires a generic and fast screening for running as many structurally diversified compounds as possible with few assays to achieve an "acceptable separation" for most of them. The separation obtained in the screening step gives the chemist a rapid estimation of enantiomeric purity, and also serves as a good starting point for resolution and loadability optimization for preparative purification. Advanced optimization using experimental design can be employed to achieve "optimal separation" necessary for method robustness and quantification ability, needed in pre-clinical and clinical drug development stages. In order to handle the chemical diversity of drug discovery molecules (building blocks, intermediates and drug candidates), and to maximize the chance to achieve rapidly a separation, analysts need to use different techniques in unison. Because different chromatographic and electrophoretic techniques have different separation mechanisms which can lead to different selectivities. So if a separation fails under aqueous media (i.e. RPLC or CE), it is likely that the same compound will succeed under non-aqueous environment (i.e. NPLC or SFC). For this reason, we developed several chiral screens using CE [19], NPLC [20] and RPLC [21]. Knowledge about these screens was formalized in a knowledge-based system (chiral KBS) software to assist the analyst during method development [46,47]. Additional chiral screens utilizing CEC [48] and polar organic solvent chromatography (POSC) [49] are in current progress.

Compared to NPLC and RPLC, the SFC screen (using the same chiral columns) achieves resolution of compounds that were only partially separated by NPLC or not separated at all by RPLC. Thus, we consider SFC the first try for chiral separations of new compounds in the support to chemical synthesis, where high-throughput and -performance are greatly appreciated. The inherent advantages of supercritical fluids over liquids endow chiral SFC with numerous advantages in comparison to chiral HPLC-based techniques. On the analytical scale, the higher speed chromatography and faster re-equilibration makes method development dramatically faster. These advantages become more compelling as the scale of the chromatography increases, due to the lower operating costs.

Despite the former advantages, the following technological and commercial problems have combined to limit the penetration of SFC in analytical laboratories: (i) unfamiliarity with SFC technique; (ii) hardware complexity; (iv) high capital cost; and (v) disinterest of the major instrumentation manufacturers. Nowadays, despite these limitations, enantiomeric separation is the most successful field of application of SFC. SFC is becoming the technique of reference for analysis and purification of enantiomers.

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