Expanding the potential of chiral chromatography for high-throughput screening of large compound libraries by means of sub–2μm Whelk-O 1 stationary phase in supercritical fluid conditions

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Abstract

With the aim of exploring the potential of ultrafast chiral chromatography for high-throughput analysis, the new sub–2 micron Whelk-O 1 chiral stationary phase (CSP) has been employed in supercritical fluid conditions to screen 129 racemates, mainly of pharmaceutical interest.

By using a 5-cm long column (0.46 cm internal diameter), a single co-solvent (MeOH) and a 7-min gradient elution, 85% of acidic and neutral analytes considered in this work have been successfully resolved, with resolution (Rs) larger than 2 in more than 65% of cases. Moreover, almost a half of basic samples that, for their own characteristics, are known to be difficult to separate on Whelk–O 1 CSP, have shown Rs greater than 0.3. The screening of the entire library could be accomplished in less than 24 h (single run) with 63% of positive score.

For well-resolved enantiomers (Rs roughly included between 1 and 3), we show that method transfer from gradient to isocratic conditions is straightforward. In many cases, isocratic ultrafast separations (with analysis time smaller than 60 sec) have been achieved by simply employing, as isocratic mobile phase, the eluent composition at which the second enantiomer was eluted in gradient mode.

By considering the extension and variety of the library in terms of chemico-physical and structural properties of compounds and numerousness, we believe that this work demonstrates the real potential of the technique for high-throughput enantioselective screening.

1. Introduction

The production of new chiral molecules to be evaluated as potential drug candidates has experienced, over the last 15 years, an impressive growth in medicinal and pharmaceutical chemistry. To assess the true potency of each single enantiomer, highly-efficient and fast separation methods are needed. Among them, direct enantioselective chromatography, thanks to the broad availability of Chiral Stationary Phases (CSPs) and modes of operation, predated the field becoming *de facto* an essential tool in the area of drug discovery [1–6]. Depending on the stage in chiral drug discovery, goals and needs are different. At the initial stages of the process, indeed, ~~the possibility of~~ a quickly screening of large libraries of chiral molecules ~~which~~ is fundamental [7]. High throughput methods need enantioselective systems able to separate the largest number of structurally and chemically different molecules with minimum changes of experimental conditions. On the other hand, once a racemic candidate has been selected for successive steps of drug development, the optimization of the separation (possibly under isocratic preparative conditions) is strongly facilitated by the possibility of transferring all or part of the information gathered in the screening phase with great practical and economic advantages.

Enantioselective Supercritical Fluid Chromatography (eSFC) is nowadays a preferred solution for the development of fast-screening methods [8–15]. Supercritical or subcritical CO_2-based mobile phases are indeed characterized by lower viscosity and faster mass transfer properties than typical liquid chromatography eluents, which allows for the reduction of analysis time without losing efficiency. In addition, as they permit significant reduction of organic solvent consumption (estimable up to 20–30% in volume), SFC methods are considered to be greener than both normal- and reversed-phase chromatography, ~~which is significant~~ especially from the industrial point of view. The fast eSFC screening of chiral molecules has been most of times

realized by using polysaccharide-based CSPs with gradient elution [16-17]. Analysis time in the order of 15-20 min ~~or longer~~ were considered very satisfactory in chiral separations until 5-10 years ago, when a barrier to the development of very fast separations is essentially represented by the 5 µm particle size of the packing bed [16]. Reduced analysis time can be obtained by using the chiral stationary phases developed on 3 µm particle size [18-21]. However, the recent development of CSPs in sub-2 µm format has however opened new frontiers in the field of enantioselective ultra-high performance SFC (eUHPSFC) [22]. As a matter of fact, however, this transition has so far effectively affected only brush-type CSPs, in part because of practical difficulties to adapt the surface modification chemistry of classical chiral stationary phases to smaller particles (a very common problem encountered during the preparation of sub-2µm CSPs is their tendency to aggregate during synthesis, which provokes the attainment of broad particle size distribution unsuitable for efficient packing) and, in part, due to the scarcity of fundamental studies in mass-transfer mechanisms in CSPs [22, 23]. On the other hand, brush type CSPs and, in particular, Whelk-O 1 type have been prepared in sub-2 µm format with excellent results [24-26]. Indeed, phenomena such as particle aggregation and clogging or the non-uniformity/excess of selector coating are not frequently encountered during the preparation of these phases, which on the other hand exhibit fast adsorption/desorption kinetics possibly due to the monomeric nature of selectors.

In this work, we report about the use of a Whelk-O 1 CSP, prepared by employing 1.7 micron high-surface totally porous spherical particles, to screen a very large library made of 129 racemic compounds in eUHPSFC. Very promising results have been obtained under fast-gradient conditions (7 min total analysis time) for compounds with significantly different chemico-physical properties, including acidic, neutral and even basic ones, which are not ideal molecules

to be separated on these stationary phases [27]. In the light of the numerousness of the compound library and its variety, this study represents, to the best of our knowledge, the first example demonstrating the real feasibility of high-throughput screening of enantiomers in eUHPSFC. We also show that, when the separation of enantiomers is reasonably satisfactory ($1 < Rs < 3$), a simple criterion to achieve very fast and efficient chiral separations under isocratic conditions is that of operating the column at a mobile phase composition close to that in which the second enantiomer has been eluted under gradient mode. In cases of smaller Rs, this proof-of-concept finding has been in any case particularly important, as it has provided reliable initial conditions for optimization of the isocratic separation of enantiomers.

2. Experimental

2.1 Materials and chemicals

All reagents and solvents were purchased from Sigma Aldrich (Milano, Italy) and used without further purification. Grade 5.5 carbon dioxide was from Gruppo SAPIO (Milano, Italy). HPLC gradient grade methanol was further filtered on 0.2 µm Omnipore filters (Merck Millipore, Darmstadt, Germany), prior to use in the UHPSFC system. Syncronis silica 1.7 µm (pore size 120 Å, particle size 1.7 µm and specific surface area 320 m^2 g^{-1}) was a gift from Thermo Scientific (Waltham, MA, USA). The (*S*,*S*) Whelk-O 1 selector and the HPLC (*S*,*S*) Whelk-O 1 analytical column (particle size 5µm, 150 mm x 4.6 mm ID) were donated by Regis Technologies Inc.® (Morton Grove, IL, USA). Chiral samples were available from previous studies or were provided by Regis Technologies Inc. ® (Morton Grove, IL, USA). The complete list of analyzed samples is reported in Table 1. Empty stainless steel column was from IsoBar Systems by Idex (Wertheim-Mondfeld, Germany).

2.2 Instrumentations

UHPLC Instrumentation. The UHPLC chromatographic system used was an UltiMate 3000 RS system from Thermo Fisher Dionex (Sunnyvale, California), consisting of a dual gradient RS pump (pressure up to 1034 bar under reversed phase conditions, up to 800 bar under normal phase conditions; flow rates up to 8.0 mL/min), an in-line split loop Well Plate Sampler, a thermostatted RS Column Compartment (temperature range 5-110°C) and DAD detector with a 2.5 µL flow cell. The DAD was set at a filter time constant of 0.002 s, a data collection rate of 100 Hz and a response time of 0.025 s. Viper capillaries and fittings were used, with the two capillary Viper tubes (350 mm x 0.13 mm I.D.) producing an extra-column volume of 9.3 µL. Data acquisition and processing was performed with Chromeleon 6.8 software from Thermo Fisher. Detection of all tested analytes was carried out at two different wavelengths (214 nm and 254 nm). The injection volume ranged between 1 – 2 µL on the eUHPLC 1.7 µm columns and between 5 - 10 µL on the eHPLC columns. The UHPLC system was characterized prior to the analysis, yielding a total extra-column volume of 19 µL (variance, $\mu_{2,extra,f0.5}$= 12.2 μL^2), obtained by injecting toluene and using a zero dead volume connector.

UHPSFC Instrumentation. A Waters Acquity UPC^2 (Ultra Performance Convergence Chromatography) was used to perform SFC analyses. The system was equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 4 mL/min and maximum system pressure of 414 bar. A 250 µL mixing chamber is present in the delivery system. The system also comprised an autosampler with a 10 µL loop, a column oven compatible with temperatures up to 90 °C, an UV detector equipped with an 8 µL flow–cell and a backpressure regulator (BPR). The injector/column inlet and column/detector connection tubes were 600 mm long and had an I.D. of 0.175 mm. The extra-column and dwell volumes of this instrument were

estimated to be 60 µL and 440 µL respectively [28]. Data acquisition and control of the UHPSFC system was performed with the Empower 3.

2.3 Chiral Whelk–O 1 stationary phase and columns

The (*S*,*S*) Whelk-O 1, 1.7-µm chiral stationary phase (CSP) was prepared as described in reference [29, 30]. A 50 x 4.6 mm L x I.D. column was used in this study. The unoptimized packing procedure consists of acetone slurry (composition 10% w/v), 900 bar packing pressure and hexane as flushing solvent. End frits of 0.5 µm were used. To comparative purpose, a Regis (*S*,*S*) Whelk-O 1, 5 µm analytical column (150 mm x 4.6 mm ID) was employed.

2.4 Methodology

The van Deemter equation ($H = A + B/\varphi + C*\varphi$) was used to fit the experimental data, allowing to compare the efficiency of the new (*S*,*S*) Whelk-O 1, 1.7 µm column with the commercially available (*S*,*S*) Whelk-O 1 columns packed with 5 µm (eHPLC column). Data fitting of the van Deemter curves was performed using Origin 6.0 software, while further calculations were performed with MS Excel 2010 software. Van Deemter plots were produced by inspection of column efficiencies using *trans*-stilbene oxide (TSO) as chiral probe. The data obtained were not corrected for the extra-column peak broadening. For both 5 µm and 1.7 µm columns, the mobile phase consisted of 90/10 *n*-hexane/EtOH +1% MeOH in Normal Phase Liquid Chromatography, and of CO_2/MeOH, 80/20 in SFC conditions. A backpressure of 1800 psi was set in the UHPSFC system. The temperature of the column was set at 25 °C in all experiments, both in LC and SFC. UV detection was performed at 240 nm. The number of theoretical plates N was calculated for

every sample according to the European Pharmacopeia using the peak width at half height as implemented in the Chromeleon 6.8 software. An average of two measurements was used for each determination. For the present work, the HETP (H) values were not corrected for the extra-column volume as, although theoretically correct, it would, nevertheless represent a different situation from the one experimentally observed in the lab.

The chiral samples were resolved by using methanol as organic solvent in linear gradient elution (9 min total cycle time): starting from 2% to 30% of MeOH in 5 min, maintaining 30% MeOH for 2 min. The re-equilibration step required 2 min. Mobile phase composition in isocratic elution for each sample was optimized starting from resolution *versus* retention time scatter plot as explained in result and discussion section. Trifluoroacetic acid (TFA), for neutral and acidic compounds, and ammonia (NH_3) 7N ca. solution in methanol, for basic samples, were used as additives in the organic solvent (0.1 % v/v). For SFC measurements, the following conditions were employed: 124 bar backpressure, 35°C temperature, 3.5 ml/min flow rate and 210–300 nm UV detection. The system pressure ranged from 263 bar at initial condition of gradient to 298 bar at the end. Measurements were repeated twice and average values were used for calculations. Hold-up time was simply estimated from the first negative deviation of the signal. For each enantioseparation, the resolution (Rs) and plate number (N) were calculated according to the European Pharmacopeia using peak width at half height ($w_{0.5}$). In particular, resolution was calculated as:

$$Rs = 2[(t_r)_2-(t_r)_1]/(w_{0.5})_2+(w_{0.5})_1$$

where t_r is the retention time. Subscripts 1 and 2 refer to the firstly and secondly eluted peak of racemic sample. The retention time of eluted peaks was not corrected for the dwell volume. The

percentage of methanol in the gradient ramp was estimated by proportion considering the maximum value of methanol content as 28% and adding the 2% of initial conditions. That's means: $\%MeOH_{2nd\ peak} = (28 \times tr_2/5) + 2$. In the result evaluation, peaks with Rs > 2.0 are considered to be "largely separated", those with 1.0 < Rs < 2.0 are "baseline separated" and, finally, peaks with 0.3 < Rs ≤ 1.0 are "partially resolved". Rs = 0 means no separation.

3. Results and Discussion

3.1 Kinetic performance evaluation.

The (*S*,*S*) Whelk-O 1, 1.7 µm CSP has been previously fully characterized in terms of kinetic and thermodynamic properties in normal phase and reversed phase eUHPLC [26]. Before developing the fast gradient screening, an evaluation of the kinetic performance of this column was performed in SFC conditions. Furthermore, by including the 5-µm column in this evaluation, a direct comparison between eHPLC, eUHPLC, eSFC and eUHPFC was obtained and is summarized in Figure 1. Mobile phases were chosen in order to have as similar as possible retention factors between the two columns on the two different systems. Van Deemter curves were then plotted using the first eluting enantiomer of TSO. The H_{min} in eUHPLC is reached at a flow rate twice higher than in eHPLC: 2.04 mL/min and 0.84 mL/min respectively. A H_{min} of 4.44 µm and 13.9 µm were respectively obtained for the eUHPLC and eHPLC columns. In 5 µm particle domain, the optimal flow rate was as expected higher in SFC compared to HPLC, corresponding to a two-fold increase in supercritical conditions. Interestingly, the minimum of the van Deemter curve could not be obtained for the 1.7 µm column in eUHPSFC: at the maximum flow rate permitted (4 mL/min) the column efficiency continued increasing, as can be also seen by the descending curve in Figure 1. The number of theoretical plates per meter was in

this case 235000 plates at 4 mL/min, but a prediction of the minimum of the van Deemter curve obtained using data fitting as incorporated in Origin 6.0 yielded a flow rate of approximately 5.00 mL/min (improved speed factor of 2.4). This would be in agreement with the generally observed that an increase of the optimal flow rate of a factor of 2-2.5 times is to be expected when transitioning from LC to SFC. As a consequence higher linear velocities can be used in UHPSFC without loss in efficiency, leading to a reduced analysis time, which is a basic principle in the transfer from ultra-fast to ultra-high performance conditions. In addition to this, the extra-benefit of the use of sub-2-µm particles, advantageous in kinetic terms, allows to further decrease analysis time while gaining in efficiency and thus in resolution achieving the ultra-high performance separation. The chromatographic traces, reported in figure S1, showed that in a quasi-optimal UHPSFC conditions (flow 4.0 mL/min), due to the instrumental limitation, the Rs values were greater than those recorded in UHPLC. The enantiomers of TSO and haloxyfop-2-ethoxyethyl (a and c in figure S1) were separated with a gain in Rs of 1.31 and 1.34 factors respectively. For the enantiomers of benzoin (b, figure S1) the slight Rs increase is offset by a 3-fold reduced analysis time.

3.2 Fast gradient screening

Chiral compounds considered in this work have been selected as to cover a broad range of chemically different samples. They include drugs and chiral intermediates of pharmaceutical, biological and medicinal interest (see Table 1 for the complete list of compounds). The chemical structure of all samples has been reported in the Supporting Information (Table S1). As it can be seen the ensemble of compounds considered in this study is extremely heterogeneous both as physico-chemical and structural properties. In the histogram chart of Figure 2, all compounds have been classified according to their principal employment on human and plant diseases. The

most represented classes are beta–blockers (18 compounds), agrochemicals (10), antidepressants (10) and antihypertensives (9), covering in total 36% of cases. Samples with unknown activity have been generically classified as "organic compounds" and are 32% of racemates. Of the 129 samples, a useful classification of racemates was done based on a primary chemical property: 33 were acidic (bearing a carboxylic group), 38 neutral and 58 basic (with at least an amino group).

The geometry of the column employed for the fast screening of these compounds, 50 × 4.6 mm I.D., has been chosen to reduce the effect of extra-column band broadening and simultaneously to allow for short analysis time [25, 31].

Figure 2

The organic modifier employed for the fast gradient screening of compound library was methanol as it combines low viscosity and high polarity and is characterized by a low boiling point (useful behavior for scaling-up to preparative level) if compared to other modifiers often employed in SFC, such as ethanol and isopropanol. In all cases, the gradient program was performed by linearly increasing the amount of methanol in mobile phase from 2 to 30% v/v in 5 min and therefore the sub-critical conditions are reached. However, since numerous studies have reported continuity of all chromatographic properties between sub- and super-critical domains [32, 33], in this work we will only speak in terms of super-critical conditions. Depending of compound's nature and to improve peak shape, trifluoroacetic acid (0.1% v/v) was added to the mobile phase for the separation of acidic samples and ammonia (0.1% v/v) was employed for the separation of basic ones. As the presence of additives does not affect the shape of neutral compounds, it was decided to use the same mobile phase to elute both acidic and neutral analytes. This choice is essentially practical and reflects the attempt of performing the entire

screening procedure with minimal changes of experimental conditions. The screening with combined acid and basic additives in a single mobile phase has been reported in literature, but its use is still not widespread [12, 13]. Therefore, the screening of all 129 compounds was achieved with a same gradient program and only two mobile phases (one with methanol/trifluoroacetic acid and the other with methanol/ammonia).

Table 1

A flow rate of 3.5 ml/min allowed for ultra-high performance SFC condition, meaning a combination of very fast and highly efficient separations. In fact, most of samples ~~being~~ were eluted and successfully separated in less than 4 min, as shown in Table 1 where the retention time of the latter eluted enantiomer (t_{r2}), the percentage of methanol when the more retained enantiomer is eluted (%B) and the observed resolution (Rs) are reported. As expected, best results were obtained with acidic and neutral compounds. As an example, ~~two~~ chromatograms showing the enantioseparation of neutral compounds Praziquantel (entry 41 of Table 1, resolved in 3 min with Rs = 5.08), ~~and~~ of Kavain (entry 36, less than 2.5 min analysis time and Rs =1) and the basic compound Mianserine (entry 77 of Table 1, resolved in 3.5 min with Rs = 1.81) are reported on the right side of Figure 3.

However, it is remarkable to notice that the (*S*,*S*) Whelk-O 1, 1.7 µm CSP performed well also with basic enantiomers. Indeed, 47% of basic compounds was separated with Rs about 0.3. These findings not only confirm the ability of Whelk-O 1 selectors to separate acidic species but somehow show that this CSP can be successfully employed also with basic enantiomers. In terms of efficiency of sample screening, 63% of the analytes (81 of 129 enantiomeric pairs) were

284 resolved and 47 compounds, corresponding to 58% of positive score, have been separated with
285 Rs > 2.0 (Figure 3 left).

286 **Figure 3**

287 For the sake of clarity, the information of the screening has been condensed in Figure 4. The plot
288 on the left refers to the separation of acidic and neutral compounds (mobile phase modifier:
289 methanol plus 0.1% v/v trifluoroaceitc acid), that on the right is for the basic ones (mobile phase
290 modifier: methanol plus 0.1% v/v ammonia). Each point in a plot corresponds to an entry of
291 Table 1 and essentially contains three information: retention time of secondly eluted enantiomer
292 (bottom x–axis), resolution of separation (y–axis) and percentage of mobile phase modifier
293 required for the elution of the more retained enantiomer (top x-axis). Very high values of
294 resolution (Rs >13) were obtained for *trans*–stilbenoxide (entry 50), Benzoin (entry 51) and
295 Naproxen (entry 11). Most separations were characterized by 1.00 < Rs < 5.00. The zoomed area
296 in Figure 4 (bottom left) refers to the zone characterized by Rs < 4.00 and retention time < 2.5
297 min, where a large number of acidic compounds was eluted.

298 **Figure 4**

299 **3.2 Method transfer from gradient to isocratic conditions**

300 Isocratic conditions are usually preferred for purity control (i.e., determination of enantiomeric
301 excess) and purification of chiral samples (i.e., under nonlinear conditions). Going from gradient
302 to isocratic conditions however is not always straightforward, especially if the resolution is low.
303 For samples included in the zoomed area of Figure 4 and with a 1.5 < Rs < 5, we show that a
304 simple empirical criterion can be employed to find suitable conditions for ultrafast separation in

isocratic mode. This proof of concept finding is that one should use, in isocratic mode, a mobile phase composition that corresponds to that of the elution of the more retained enantiomer under gradient mode. When Rs becomes larger than roughly 5, this concept helps to get reliable initial conditions from which to start to achieve an ultrafast enantioseparation. For example, the enantiomers of Abscisic acid (entry 4 in Table 1) have been baseline resolved (Rs = 2.21) in less than 60 sec by using a mobile phase containing 15% v/v organic modifier, that is the composition at which the second enantiomer of 4 is eluted in gradient mode (see Table 1). Figure 5 (top) reports the corresponding chromatogram. The other chromatograms shown in Figure 5 refer to the ultrafast separation of other two racemates, Acenaphthenol (entry 57, middle chromatogram) and Bendroflumethiazide (entry 105, bottom chromatogram). In particular, Acenaphtenol exhibited a very large resolution under gradient conditions (Rs 4.5). To perform an ultrafast separation (less than 1 min) of its enantiomers, we had to use twice the amount of modifier at which the more retained enantiomer was eluted in gradient mode. In this case the information coming from gradient elution was important to set up reliable initial conditions. In cases where resolution is low (Rs < 1.5), separations took a longer time which is in any case very short if compared to that of typical enantioseparations. As an example, the enantiomers of Ketoprofen (entry 6) were *quasi*-baseline separated in less than 2.5 min (Rs =1.25, see Figure S2-a of Supporting Information) while those of Kavain (entry 36) needed less than 4 min (Rs =0.9, Figure S2-b of Supporting Information).

Figure 5

As a final comment, we want to point out that in cases of low Rs, the use of longer column can help to improve separation. As an example of the very efficient separations that can be achieved in eUHPSFC, the chromatograms for the elution of the enantiomers of two pesticides

(Quizalofop-methyl, entry 49, and Flamprop-methyl, entry 48) have been presented in Figure S3 of Supporting Information. In these cases, efficiency larger than 193,000 theoretical plates per meter were reached by using a 10 cm long column packed with the Whelk-O 1 CSP.

4. Conclusions

In this proof of concept study we have shown that the high-throughput screening of large compound library by enantioselective SFC is reality. In less than 24 h, by using a 5 cm long column packed with 1.7 µm totally porous Whelk-O 1 particles and fast gradient elution (total analysis time 9 min, including column re-equilibration), 129 racemates with significantly different chemico-physical properties could be screened with a positive result of 63%. In particular, 85% of acidic and neutral analytes have been effectively resolved. Of these 66% have been separated with Rs > 2.0. Moreover, the CSP performed very well also towards the separation of basic compounds, which have been considered not to be good candidates for separation on this chiral selector.

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Conflict of interest

Authors declare no conflict of interest.

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Figure captions.

Figure 1. Comparison of van Deemter plots in LC and SFC at 25 °C for the first eluting enantiomer of TSO. Columns: A) Whelk-O 1, 150 mm x 4.6 mm I.D., 5 µm, red and orange curves; B) Whelk-O 1, 50 mm x 4.6 mm I.D., 1.7 µm, blue and green curves. Data not corrected for extra-column band broadening effect. (For interpretation of the colors in this legend, please refer to the web version of the article).

Figure 2. Classification of investigated samples based on their chemical properties (acidic, neutral, basic) and pharmacological activity. For those compounds whose pharmaceutical activity is unknown/unspecified the generic expression "organic compounds" has been employed.

Figure 3. Left: pie chart representing numerical proportion of resolved enantiomers with Rs>2, 1<Rs<2 and 0.3<Rs<1.0. Right: Examples of experimental chromatograms in gradient elution. Top: Praziquantel, entry 41 of Table 1; middle: Kavain, entry 36; bottom: Mianserine, entry 77. (For interpretation of the colors, please refer to the web version of the article).

Figure 4. Scatter plots showing the results of the screening. Left: acidic and neutral samples. Zoomed area (bottom left) corresponds to the zones where the largest part of acidic compounds has been eluted. Right: basic samples. Limit values of Rs (0.9 for acidic and neutral compounds and 0.3 for basic ones) have been represented in the plots by dotted lines. See text for details.

Figure 5. Examples of ultrafast enantioseparations in isocratic conditions. Experimental details in the text.