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Ultrafast chiral separations for high throughput enantiopurity analysis

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Abstract: Recent developments in fast chromatographic enantioseparations now make high throughput analysis of enantiopurity on the order of a few seconds achievable. Nevertheless, routine chromatographic determinations of enantiopurity to support stereochemical investigations in pharmaceutical research and development, synthetic chemistry and bioanalysis are still typically performed on the 5-20 min timescale, with many practitioners believing that sub-minute enantioseparations are not representative of the molecules encountered in day to day research. In this study we develop ultrafast chromatographic enantioseparations for a variety of pharmaceutically-related drugs and intermediates, showing that sub-minute resolutions are now possible in the vast majority of cases by both supercritical fluid chromatography (SFC) and reversed phase liquid chromatography (RP-LC). Examples are provided illustrating how such methods can be routinely developed and used for ultrafast high throughput analysis to support enantioselective synthesis investigations.

The past few years have seen dramatic improvements in the speed of chromatographic enantioseparations.[1-6] Long a preferred technique for analysis of enantiopurity to support enantioselective synthesis or bioanalytical investigations,[7,8] chiral chromatography has evolved from typical run times of 20-40 minutes in the 1980s and 1990s to 5-10 minutes in the 2000s, to recent examples of ultrafast sub-minute separations, some taking only a few seconds.[8,9] A variety of factors have contributed to this speed revolution, including improved chiral stationary phases (CSPs), instrumentation and chromatographic particle technology.[1,6,9,10] Equally important has been a growing dissatisfaction with legacy methods that are poorly suited to high throughput experimentation,[11] and an emerging understanding of the theory and practice underlying ultrafast chromatographic separations. At this point in time "world speed records" for chromatographic enantioseparations of particular molecules are broken on a routine basis,[1-3,12] and the whole movement toward fast chromatographic separations promises to significantly disrupt conventional workflows in enantioselective synthesis and pharmaceutical chemistry.

Ultrafast chiral chromatography offers a tremendous potential for high-throughput enantiopurity assays, with analysis time that is competitive with sensor-based analytical approaches.[13] Nevertheless, most researchers currently utilizing chiral chromatography as an analytical tool are still using analysis times of 5-10 min per sample. While these longer assays may be fine for the analysis of a few samples, they are poorly suited for research investigations involving screening and high throughput experimentation. In this study we investigate the ability to develop fast chromatographic enantioseparations for a variety of pharmaceutical-related drugs and intermediates, showing that sub minute separations are now possible in most cases. We illustrate how such methods can be routinely developed, and how ultrafast chromatographic enantioseparations can be used for high throughput analysis to support enantioselective synthesis investigations.

The chromatographic enantioseparation of a group of 50 different racemates (Figure S1, ESI) was investigated in order to gauge the generality of sub-minute chromatographic resolutions. Many of the compounds in the group come from a diverse standard set of chiral drugs and synthetic intermediates developed in our labs to assess performance and generality of new CSPs,[14] a sample set that intentionally includes some difficult to resolve analytes such as compounds **11**, **18**, **24**, **30**, **38** and **39**. Additional challenging racemates such as ibuprofen (**6**), 1-tetralol (**10**) and compounds **3** and **19** were added to increase the range of functional group diversity within the set. In addition, the family of warfarin (**41**) and related hydroxylated metabolites (**42-46**) was included. Each of the 50 compounds was then subjected to method development screening using both chiral RP-LC and SFC. Our method development screening

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involves the use of a standard gradient elution (1 to 40% organic modifier over 5 min for SFC[15] and 5 to 80% over 10 min for HPLC) on a series of columns containing different chiral stationary phases (CSPs).

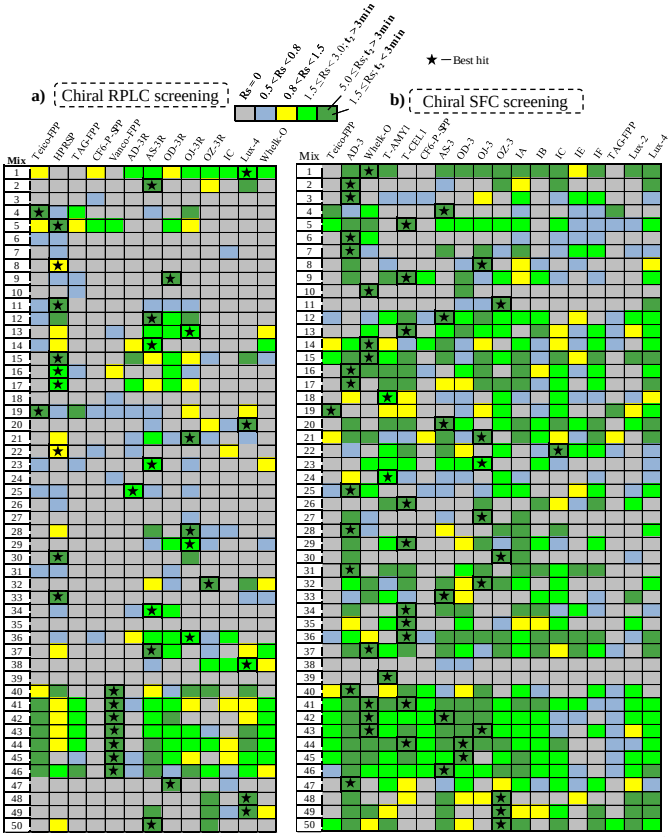

Figure 1. Scoring system. **a)** chiral RP-LC screening of 50 enantiomeric mixtures. **b)** Chiral SFC screening of 50 enantiomeric mixtures.

The complete list of columns and conditions evaluated in the study is shown in table S1 (ESI). A total of 13 CSPs in RP-LC and 18 CSPs in SFC from different manufacturers (Waters Co., Chiral Technologies, Regis Technologies, Phenomenex and AZYP LLC) were selected for this evaluation. Most of these columns are conventional 2.5 and 3 µm coated and immobilized polysaccharide-based CSPs. Some other relatively new chiral selectors based on macrocyclic glycopeptide bonded to sub-2 µm fully porous particles (Teico, TAG and Vanco)[5,16] or 2.7 µm fused-core particles (HPRSP)[5,17] were included. As shown in table 1 some of these CSPs can be used in either the RP-LC or SFC applications mode without any performance degradation.

The initial chiral RP-LC and SFC screenings, summarized in Figure 1 (and chromatograms shown in Figure S2 and S3), help to identify those CSPs and conditions that hold the most promise for developing an ultrafast separation method for each racemate. Some mixtures are easily separated, showing good resolution with a number of different CSPs and conditions, *e.g. trans*-stilbene oxide (TSO, **1**), synthetic intermediates **5**, **12**, warfarin and hydroxylated warfarin metabolites (**41-46**). Others are more challenging, showing only partial resolution on a single CSP or just a few hits, *e.g.* 1-tetralol (**10**) and compounds **24**, **38** and **39**. A simple scoring system helps to visualize the best outcome for each mixture across both SFC and RP-LC experiments. We chose to focus on resolution (*Rs*) and speed, but different scoring systems focusing on other aspects of performance could be imagined.[18] Baseline separations ($Rs \geq 1.5$) are denoted with a bright green color, while separations achieved in less than 3 min with a $Rs \geq 1.5$ or separations above 3 min with $Rs \geq 5.0$ are denoted by a dark green color. The best CSP for separation of each mixture is highlighted with a star.

Overall, the SFC screens (Figure 1b) show greater generality for enantioseparation than the RP-LC screens where the separations were dominated by only a few CSPs (Figure 1a).

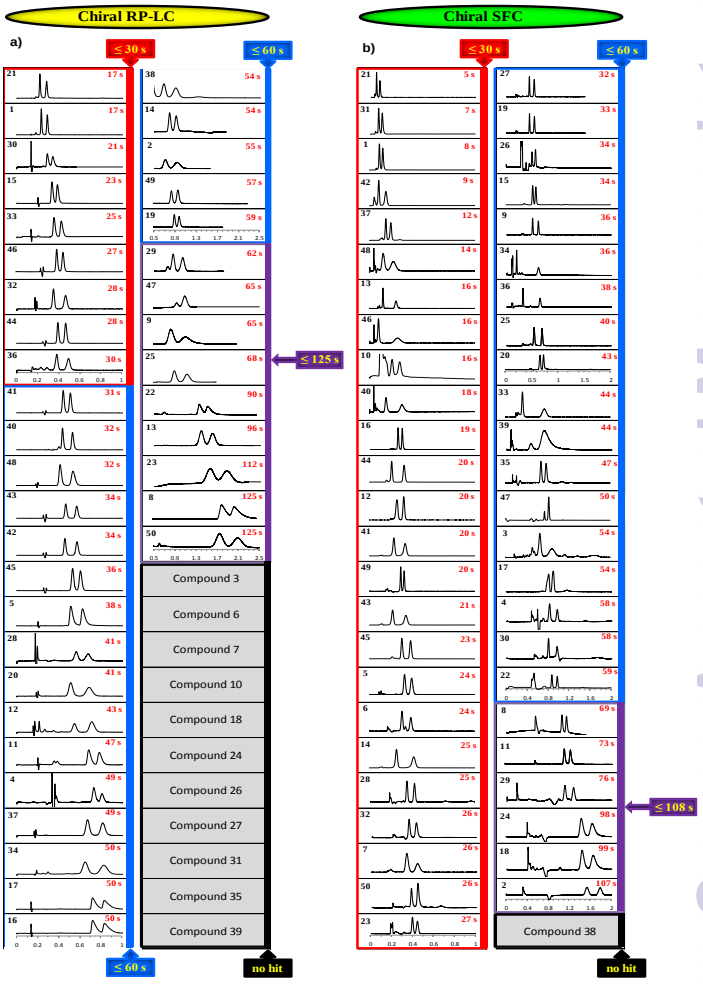

Figure 2. Ultrafast chiral separations of all mixtures by RP-LC (**a**) and SFC (**b**). Method conditions are detailed in table 3 and 4. Detection was performed at sampling frequency of 80 Hz and the lowest available response time.

These results clearly illustrate why in recent years SFC has become the workhorse technique for separation and purification of enantiomers in the pharmaceutical industry and benefits from a broad screening approach.[19] However, the two techniques are in some ways complimentary, with compounds that are poorly resolved across almost all SFC columns and conditions (*e.g.* intermediates **11** and **38**) sometimes showing improved resolution by RP-HPLC, affording additional options for method development. Interestingly, all warfarin and hydroxylated metabolites can be baseline resolved in less than 2 min using a single screening method on the Vancomycin FPP column with 150 mM $NaClO_4$ in 0.02 % $HClO_4$:CH_3CN mobile

phase (Figure 2 and S2). It is noteworthy that all compounds in the study showed at least some resolution on at least one of the CSPs, a testimony to both the power of contemporary enantioselective chromatography and the value of the combined CSP screening approach. In some cases it can be challenging to determine which of the different enantioseparations offers the greatest potential for developing an ultrafast method, for example, when comparing a 2 minute method with baseline resolution (Rs = 1.5) and a 4 min method where Rs = 4. In such cases, calculation of the previously described tmin cc term[4] not only allows for easy selection of the best method, but also provides an estimate of the optimal time for ultrafast separation (Table S2).

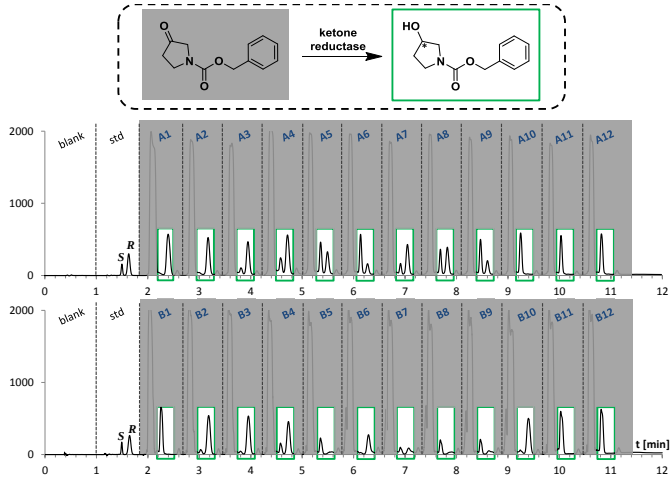

Figure 3. MISER chiral SFC for high-throughput enantiopurity analysis of an alcohol obtained from a ketone *via* enzymatic catalysis. Conditions for reactions and MISER SFC experiments are described in the experimental section.

With the selection of the best CSP for each of the 50 racemates in hand, we next focused on the development of ultrafast chiral methods for each compound. This generally involves changing the gradient elution used in the screening methods to an isocratic elution profile where the solvent composition remains constant throughout the separation. Using isocratic mode rather than gradient elution for ee analysis offers a significant advantage from a speed and simplicity perspective, as column equilibration is no longer required between sample runs. In addition, shorter columns are often used, typically operating at significantly higher flow rates than those used in the CSP screening methods. The detector sampling frequency and the detector response time settings become critically important for rapidly eluting analytes and highly efficient separations, as highlighted in recent studies.[1,5,20,21] Using a detector setting of 80 Hz sampling frequency and the lowest available response time, combined with the use of high flow isocratic elution on short columns packed with the optimal CSP identified in screening, ultrafastfast enantioseparations were developed for 38 out 50 analytes by RP-LC and 49 out of 50 by SFC (Figure 2 and S4). Table S4 and S5 summarize the chromatographic conditions for the optimized separations, as well as the respective retention time of the more retained enantiomer (t_2) and separation factors (α). In general, SFC provides better peak shape and faster analysis, but also much better overall selectivity than RP-LC. Figure 4a also shows that all 38 of the RP-LC enantioseparations can be performed in less than 2.1 min, with nine of them under 30s (highlighted in red), 21 separations between 30 and 60s (highlighted in blue), and nine between 60 and 125s (highlighted in violet). On the other hand, all 49 baseline SFC enantioseparations illustrated in figure 4b were achieved in less than 2.0 min, with 25 of them under 30s, 18 separations between 30 and 60s, and six between 60 and 108s.It is important to point out that all of the 11 racemic mixtures that were not be resolved by RP-LC (3, 6, 7, 10, 18, 24, 26, 27, 31, 35 and 39), were easily separated using SFC.

Only a single racemate (38) was not baseline resolved by SFC, however this compound was nicely resolved by several RPLC methods, highlighting the complementary nature of the two techniques. Separations factors (α) ranged from 1.14 to 2.02 by RP-LC and 1.20 to 5.10 by SFC. These results clearly show that ultrafast chromatographic enantioseparation methods can be developed for many pharmaceutical drugs and synthetic intermediates, with half of all separations in this study being achieved in less than 30s and 86% in less than 1 min. Even more exciting is the fact that the slowest enantioseparations (violet bracket) ranged from only 1 to 2.1 min (18% by RP-LC and 12% by SFC), which are much faster than the standard enantioseparation methods generally practiced by researchers in enantioselective synthesis. Additional gains in speed can be obtained for some of these separations with the use of shorter 1-2 cm columns (e.g. compound 34 and 36 by SFC). It is expected that continuing development of column, CSP and instrument technologies over the coming years will lead to even further improvements in chiral chromatographic performance, with many, or even most, separations becoming achievable in just a few seconds.

While this study employs relatively state of the art HPLC and SFC instrumentation, for most laboratories, significant gains in speed do not require a wholesale replacement of existing analytical equipment. However, modification of older instrument to minimize extracolumn volumes by replacing high volume mixers and connecting fittings with low volume alternatives is recommended[3,21], as is the aforementioned switch to fast sampling rates and detector response times. It should also be noted that this study focuses on simple two component enantiomer separations, while some real world stereochemical problems require an enantioseparation to be performed in the presence of a variety of additional peaks and components, thereby increasing the difficulty of developing ultrafast analysis methods. Figure 5 showcases an example of how ultrafast enantiopurity analysis can be swiftly integrated into standard workflows for catalyst identification and process optimization. In this instance, a high throughput analysis method was required to enable screening of the enzymatic ketoreductase-catalyzed reduction of a prochiral ketone to afford the corresponding alcohol (compound 47) in high enantiopurity. CSP screening (Figure 1) followed by method development optimization afforded the 46s ultrafast chiral SFC assay shown in Figure 2, with co-injection of starting ketone showing early elution well away from the desired enantiomer pair (Figure 3). While this method would be well suited for the

direct study of a few samples, larger scale screening can benefit from MISER analysis (multiple injections within a single experimental run),[22] where injections from a number of different samples within a single chromatogram facilitates visual comparison and the rapid selection of the best performing reaction conditions. Rapid MISER SFC[23] analysis using a sample injection interval of 50 s afforded a convenient high throughput analysis method with a plate time (time for analysis of a 96 well microplate) of only 80 min. Two rows of 12 samples each are shown in Figure 4, with a number of enzymes identified that afford not only good conversion but also high enantioselectivity for the formation of either the (*R*) and (*S*) product enantiomers (Table S3). Ultrafast chiral chromatographic analysis is well suited to such first round in a high-throughput mode, with conventional chromatographic analysis often being used as a confirmatory assay.

In conclusion, chromatographic enantioseparations taking less than 1 minute can now be achieved for most racemic mixtures using state of the art stationary phases, columns and chromatographic equipment. Fast enantioseparations are also possible with older instrumentation, with the use of relatively inexpensive stationary phases packed into high efficiency short columns. A simple and straightforward approach to method development involves initial screening of a variety of stationary phases to identify a leading candidate, followed by optimization of column length, flow rate and eluent composition. The resulting ultrafast method can be used for routine stereochemical analysis, or can form the basis for a MISER method for high throughput analysis. While 5-30 minute methods for the chromatographic analysis of enantiopurity are still used to support research investigations in many synthetic chemistry, bioanalysis and pharmaceutical research laboratories, in many cases these assays can be easily replaced by much faster methods enabling ee analysis of over one thousand samples in an 8 h workday. Consequently, ultrafast chromatographic enantioseparations are expected to greatly enable faster and more efficient research investigations and the broader adoption of high throughput experimentation approaches in stereochemical research.

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