Orthogonality of SFC versus HPLC for Small Molecule Library Separation

Harold N. Weller,*,† Katalin Ebinger,‡ William Bullock,† Kurt J. Edinger,‡ Mark A. Hermsmeier,† Steven L. Hoffman,† David S. Nirschl,† Thomas Swann,‡ Jiang Zhao,‡ Jeffrey Kiplinger,§ and Paul Lefebvre§

Bristol-Myers Squibb Company, Applied Biotechnology Division, PO Box 4000, Princeton, New Jersey 08543, Bristol-Myers Squibb Company, Applied Biotechnology Division, 5 Research Parkway, Wallingford, Connecticut 06492, and Averica Discovery Services Inc., One Innovation Drive, Three Biotech, Worcester, Massachusetts 01605

Received June 29, 2010

Preparative HPLC and HPLC-MS are well established as the methods of choice for purification of pharmaceutical library compounds. Recent advances in supercritical fluid chromatography (SFC) have now made SFC a viable alternative to HPLC for this application. One of the potential arguments for using SFC in place of, or in addition to, HPLC is that it may offer different selectivity and thus has the potential for improved separation success rates. In this paper, we examine relative success rates for SFC and HPLC in obtaining adequate selectivity for successful separation. Our results suggest that use of SFC in addition to HPLC may result in a slight (1−2%) improvement in success rate compared to use of HPLC alone.

Introduction

Small molecule drug discovery relies upon a continuing supply of new compounds for primary biochemical testing and structural optimization. Once a lead compound has been discovered, lead optimization proceeds via iterative synthesis of individual compounds or small libraries with the goal of optimizing the structure with respect to desired activities and undesired liabilities. High purity is required at this stage to ensure accuracy of test results, and preparative gradient reverse phase HPLC has become the method of choice for purification of new drug discovery molecules.[1] The use of near universal gradient methods minimizes method development time, and typically the entire sample can be purified in a single preparative HPLC injection, thus the overall purification cycle time can be short. Supercritical Fluid Chromatography (SFC) has become well established for bulk purification of pharmaceutical intermediates[2] but, until recently, technical challenges associated with library compound purification have limited its application in this arena. New advances in SFC technology, however, now make SFC a viable alternative to HPLC for purification of diverse compounds.[3−6] The choice of whether to use HPLC, SFC, or a combination of the two for a specific purification application now depends on the relative costs and benefits of the techniques. Processing delays can add significantly to the both cost and cycle time. Lean Sigma analysis has highlighted batch size and rework, which results when the first attempt to purify a compound fails, as significant sources of delay.[7] Various strategies have been described to minimize HPLC rework, including use of shallow compound specific focused gradients instead of broad, universal gradients,[8−10] use of longer columns and gradient run times,[11] and more extensive method scouting prior to purification. Since retention behavior is quite different for SFC than for HPLC,[12,13] one option for improving selectivity, and thus first attempt success rates, may be to include SFC in addition to (or in place of) HPLC during the method scouting and development phase. This, in turn, has the potential to reduce rework resulting from failed purification attempts. As part of our ongoing evaluation of the two techniques, we report here an analysis of separation success rates by both HPLC and SFC for a large collection of crude reaction mixtures containing drug discovery compounds.

Several studies have been reported demonstrating that typical drug like compounds can be chromatographed and detected by SFC as well as by HPLC.[14−21] In particular, a landmark study by Pinkston[22] showed that HPLC and SFC were about equally successful in providing useful chromatography retention times for a large sample (>2000 compounds) of diverse drug-like compounds. The Pinkston study did not address, however, separation of the target drugs from associated impurities. Other studies have described a variety of methods for estimating relative orthogonality of various stationary and mobile phases,[23−26] but have not shown the impact of orthogonality (*different* selectivity) on the ability to quickly identify a successful purification method (*improved* selectivity) in our application. We therefore sought to study the relative success of HPLC and/or SFC, using commonly used stationary and mobile phases, for identifying an analytical method that would separate a drug target from its associated impurities in crude reaction mixtures of the

* To whom correspondence should be addressed. E-mail: Harold.Weller@bms.com.
† Bristol-Myers Squibb Company, Princeton, New Jersey.
‡ Bristol-Myers Squibb Company, Wallingford, Connecticut.
§ Averica Discovery Services Inc., Worcester, Massachusetts.

10.1021/cc100118y
Published on Web 10/05/2010

Table 1. Chromatography Methods

name	column[27]	organic modifier	buffer	flow rate (mL/min)	gradient time (min)	total volume (mL)	gradient range (% organic)	ions	peak capacity
HPLC-1	XbridgeC$_{18}$	CH_3CN	NH_4OAc	2.0	8	16	5−95	ESI^+	72
HPLC-2	XbridgeC$_{18}$	CH_3OH	NH_4OAc	2.0	7	14	15−95	ESI^+	61
HPLC-3	ShieldRPC$_{18}$	CH_3CN	TFA	2.0	8	16	5−95	ESI^+	78
HPLC-4	ShieldRPC$_{18}$	CH_3CN	NH_4OH	2.0	8	16	5−95	ESI^+	77
SFC-1	2-ethylpyridine	CH_3OH	NH_4OAc	5.0	5	25	5−60	$APCI^+$	98
SFC-2	cyano-diol	CH_3OH	NH_4OAc	5.0	5	25	5−60	$APCI^+$	80
SFC-3	PolarRP	CH_3OH	NH_4OAc	5.0	5	25	5−50	$APCI^+$	90
SFC-4	PVA-SIL	CH_3OH	NH_4OAc	5.0	5	25	5−60	$APCI^+$	106

type encountered in the drug discovery synthesis lab. Our assumption was that the successful analytical method could be scaled up to preparative scale to provide pure product. Preparative scale separation, however, was not performed as part of this study.

Experimental Section

Samples. Samples for our study were crude synthesis reaction mixtures from ongoing drug discovery synthesis at BMS. We evaluated a total of 1258 reaction mixtures from 36 different small molecule compound libraries representing typical reaction types (16-acylations, 10-Suzuki couplings, 5-displacements, 3-reductive aminations, and 2-miscellaneous reactions). Reactions were performed at a scale of 25−100 micromoles. Since these were crude synthesis reactions and synthesis reactions sometimes fail, we did not know at the outset how many of these mixtures actually contained the intended products.

Sample Preparation. Crude synthesis products were dried by centrifugal concentration, dissolved in dimethylformamide (1.8 mL), and transferred into a 96-well deep block. Aliquots were removed and transferred to two identical 96-well deep blocks (one for HPLC analysis and one for SFC analysis). The aliquots were subsequently diluted to a target concentration of 4 mM for analysis. Aliquot size and dilution volume were calculated based on reaction scale and estimated reaction efficiency to achieve approximate target concentration. Analysis plates were press sealed with aluminum foil (5″ × 6″ × 0.001″, All-Foils Inc., Cleveland OH) and, if necessary, stored at 4 °C until analysis. For HPLC and SFC analysis, ten microliters of the above solution (approximately 40 nanomoles) was injected for analysis.

Chromatography Methods. We analyzed each sample by four different gradient HPLC-MS methods and four different gradient SFC-MS methods, shown in Table 1. The HPLC methods were chosen to mimic methods commonly used in our laboratory for this application, guided by results from a prior unpublished study in our laboratories that suggested that these methods offer significant orthogonality. All HPLC columns were 4.6 × 50 mm dimension packed with 5-μm particles. Analytical HPLC-MS analysis was performed on a Waters MassLynx 4.0 driven analytical system equipped with a Waters ZQ mass spectrometer (using an ESI+ probe with a cone voltage of 20 V, a desolvation temperature of 350 °C and a source temperature of 125 °C), a CTC-Leap HTS-PAL autosampler, an Agilent 1100 quaternary pump, and an Agilent 1100 photodiode array (PDA) detector. All SFC columns were 4.6 × 150 mm dimension packed with 5-μm particles of different phases,[27] including 2-ethyl pyridine, cyano-diol, PolarRP, and PVA-SIL. The SFC methods were chosen to be representative of typical methods that have been reported for this application.[28] Column orthogonality scores as reported by West and Lesellier were also taken into account in SFC column selection to maximize orthogonality.[25,26] Analytical SFC-MS analysis was performed on a TharSFC analytical SFC system equipped with a Waters ZQ mass spectrometer, Waters 2998 photodiode array (PDA) detector, TharSFC Alias auto sampler, TharSFC high pressure CO_2 and solvent pump, Thar SFC 10-port column switching column oven, TharSFC analytical automated back pressure regulator, and controlled by Waters MassLynx 4.1 software. SFC-MS data were acquired using the $APCI^+$ ionization mode, with cone voltage of 40 V and probe temperature set at 550 °C. Gradient times, column lengths, and flow rates for HPLC and SFC were chosen in an attempt to approximately match peak capacities,[29] as measured using a standard test mix, across all eight methods while still being representative of typical high throughput analysis methods. As shown in Table 1, the SFC methods in practice had slightly larger peak capacity than the HPLC methods, thus presumably leading to slightly higher resolution values in the SFC methods than for HPLC methods having similar selectivity.

Data Interpretation. Data files were analyzed using an analysis tool called "ChromRez". Overall, the software identifies three peaks: the target product, the first significant peak eluting immediately prior to the target, and the first significant peak eluting immediately after the target. First, the software locates peaks for target product by searching the MS data for the specified molecular ion. Once the target product is found, the nearest significant (UV peak area >5% of target product peak area) chromatographic neighbors are also located to the right and left of the main peak in the UV chromatogram. Next, peak widths at half height are calculated for target and nearest neighbor peaks. Finally resolution values (R) between the target and its two nearest neighbors are calculated using eq 1.[30] Resolution data and chromatograms (UV, total ion current, extracted ion current) are displayed on screen alongside mass spectra of individual peaks. All data were manually reviewed after processing by ChromRez in an attempt to eliminate errors caused by hidden peaks, unresolved, or partially resolved peaks, and incorrect peak assignments; resolution data were recalculated when necessary based on outcome of the manual review. Once the processing was complete, all associated data, along with any messages (missing peaks, poor peak shape, etc.) were delivered for further processing and tabulation in Microsoft Excel. Resolution between the target and the two nearest

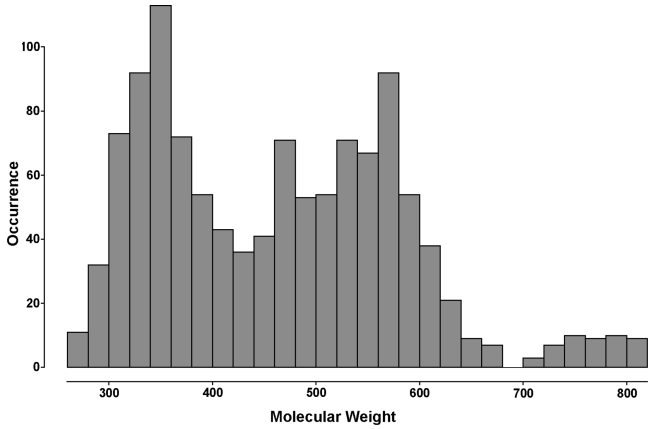

Figure 1. Molecular weight distribution.

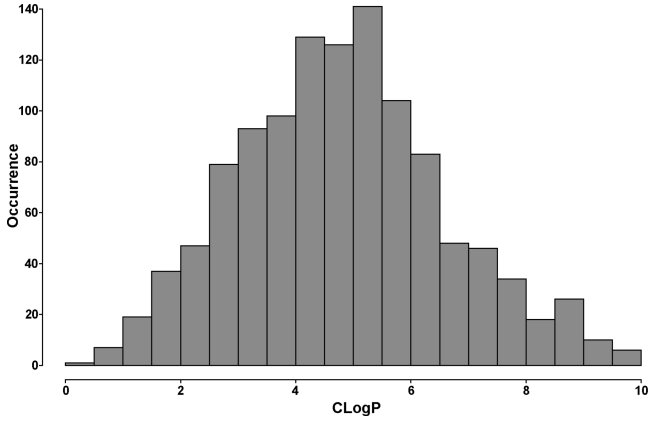

Figure 2. cLogP distribution.

neighbors were compared and only the lower of the two nearest neighbor resolutions was considered. When the target was found and lowest neighbor resolution was >1.5 the separation was scored as a success as described in the results section below.

$$R = \frac{(t_{R,p2} - t_{R,p1})}{(w_{1/2h,p1} + w_{1/2h,p2})/1.18} \quad (1)$$

Results

All 1258 reaction mixtures were analyzed by the eight chromatography methods (4 HPLC and 4 SFC) described in Table 1. Of the 1258 reaction mixtures, the target product was detected ($M + H^+$ present in the LC-MS or SFC-MS trace) in 1158 mixtures (92% of mixtures) by at least one of the eight methods. This is consistent with historical chemical synthesis success rates in our laboratory. Molecular weight and cLogP[31] distributions for the 1158 found target products are shown in Figures 1 and 2. Both molecular weight (median 462) and cLogP (median 4.8) distributions for the compounds in this study exhibit a small shift toward slightly higher values than those reported for common drug databases.[32]

Of the 1158 mixtures where the product was successfully detected, 1155 (99.7% of those detected) were detected by at least one LC-MS method, while only 1072 (92.6% of those detected) were detected by at least one SFC-MS method. Of the 1258 reaction mixtures, product was detected in all eight methods for 1021 mixtures (81% of the total).

Table 2. Separation Success Rates ($R > 1.5$) as Percent of All 1158 Mixtures

method	method short description	success rate
HPLC-3	Shield RP/CH_3CN/TFA	81%
HPLC-1	XBridge/CH_3CN/NH_4OAc	80%
HPLC-2	XBridge/CH_3OH/NH_4OAc	74%
HPLC-4	Shield RP/CH_3CN/NH_4OH	74%
SFC-3	polar RP	76%
SFC-4	PVA Sil	69%
SFC-2	cyano-diol	68%
SFC-1	2-ethyl pyridine	66%

Table 3. Separation Success Rates ($R > 1.5$) as Percent of 1021 Mixtures

method	method short description	success rate
HPLC-3	Shield RP/ACN/TFA	85%
HPLC-1	XBridge/ACN/NH_4OAc	84%
HPLC-2	XBridge/MeOH/NH_4OAc	77%
HPLC-4	Shield RP/ACN/NH4OH	77%
SFC-3	Polar RP	85%
SFC-4	PVA Sil	77%
SFC-2	cyano-diol	77%
SFC-1	2-ethyl pyridine	75%

For all chromatography traces where the target product was detected, ChromRez was used to estimate resolution between the target peak and its nearest neighbor in the UV chromatogram. For this purpose, adjacent peaks with areas less than 5% of the target peak area were disregarded. Every chromatogram was inspected manually to look for hidden or overlapping peaks that may have been missed by ChromRez. This was facilitated by the ability to compare mass spectra of UV peak tops for all eight methods in sequence, thus aiding identification of spurious peaks in the mass spectrum when impurities overlapped. When overlapping peaks were apparent a judgment call was made, ChromRez was overruled, and the resolution was manually recalculated or simply entered as 0 or 0.5.

Our experience with preparative HPLC has suggested that an analytical separation with resolution of 1.5 or greater can generally be scaled to preparative scale, whereas purification is far more challenging when analytical resolution is less than 1.5. We used that definition to score each of the eight methods for the number of target compounds that could be successfully separated ($R > 1.5$) from impurities, resulting in a Boolean result (successful or not successful) for each separation. To eliminate potential bias because of the different MS ionization modes used in the SFC and HPLC assays, we performed this analysis using all 1158 samples where the target was found at least once as well as using only the 1021 mixtures where the target was found in all eight methods. Results of this analysis are shown in Tables 2 and 3.

Discussion

Our results are consistent with previously published reports that the majority of drug-like compounds chromatograph well by both HPLC and SFC. Analysis of the data for all compounds (Table 2) suggests that the HPLC methods (74−81% success) provide a successful separation slightly more frequently than the SFC methods (66−76% success). Our definition of a successful separation requires both that

Table 4. Separation Success Rates ($R > 1.5$) for 147 Mixtures That Failed First Pass HPLC Separation

method type	method short description	success rate
HPLC-3	Shield RP/CH_3CN/TFA	N/A
HPLC-1	XBridge/CH_3CN/NH_4OAc	65%
HPLC-2	XBridge/CH_3OH/NH_4OAc	48%
HPLC-4	Shield RP/CH_3CN/NH_4OH	53%
SFC-3	Polar RP	81%
SFC-4	PVA Sil	73%
SFC-2	cyano-diol	76%
SFC-1	2-ethyl pyridine	71%

the target compound be detected ($M + H^+$) by mass spectrometry and that it be resolved from its nearest neighbor by at least $R = 1.5$. The target was detected less frequently in the SFC methods (92.6%) than in the HPLC methods (99.7%). A compound may fail to be detected for one of two reasons: either it fails to chromatograph well (for example, it elutes at the solvent front or never elutes at all), or the mass spectrometer does not provide a molecular ion $(M + H)^+$ because of either poor ionization or extensive fragmentation. In our study, the HPLC system was equipped with electrospray ionization (ESI), while the SFC system was equipped with atmospheric pressure chemical ionization (APCI). APCI was chosen for our SFC analysis because of its relative independence of fluid flow variations that occur during gradient elution,[33] particularly since our system was set up without a splitter or make up flow. We generally saw much more extensive fragmentation on the SFC methods running APCI, so our results may be biased by the mass spectrometer detection method rather than the chromatography. To eliminate this bias, we also examined the data using only those compounds that were identified by mass spectrometry in all eight methods (at the risk of ignoring chromatography failures because of unretained and completely retained compounds in SFC). In that analysis (Table 3), the HPLC and SFC methods provided essentially equivalent overall separation success rates (77−85% for HPLC, 75−85% for SFC).

Ideally, the minimum number of analyses required to achieve successful separation should be generated for each crude sample. If a single analysis generates a successful separation, that is defined as a "first-pass" separation. Any further analyses generated after a first-pass failure would be termed second-pass, third-pass, etc. Based on the body of previously published work, we were not surprised to find that HPLC and SFC have about equivalent success rates at providing first-pass separation. The question of most interest to us, however, was whether introducing an orthogonal technique for the second attempt would significantly increase second-pass success rates. For example, target product was detected but failed to separate 15% of the time by the best HPLC method. Would we have greater success in the second attempt to find a successful separation method for those compounds by using an SFC method instead of an orthogonal HPLC method? The most successful method for resolution of compounds detected in all methods was HPLC-3 (Table 3). We examined mixtures (147 samples) that failed under those conditions to determine which of the remaining seven methods provided the greatest success rate for that subset of compounds. Results are shown in Table 4. The four SFC methods all appear to offer greater second pass success (71−81%) than any of the HPLC methods (53−65%). The difference between the best SFC method (SFC-3, 119 compounds salvaged in second pass) versus the best HPLC method (HPLC-1, 95 compounds salvaged) is 24 compounds, or about 2.4% of the total.

It is generally assumed that changes to separation chemistry that lead to large retention differences between methods will have the greatest chance of also leading to changes to (e.g., improved) selectivity. To assess this assumption, we created retention time correlation plots for all 1021 compounds on each possible pair of columns. In particular, we created separate plots showing retention time for each compound on each column/method versus retention time for the same compounds on the most successful first pass method (HPLC-3); two examples of this are shown in Figure 3. We then calculated linear correlation coefficients (represented by R^2)[34] for each plot using the built-in Excel tool. Figure 4 shows retention time correlation coefficients (R^2) (on the X axis) versus percent of samples that failed on first pass analysis (using method HPLC-3) but succeeded during second pass analysis using the indicated method (on the Y axis). As seen from the graph, with the exception of method HPLC-1, methods with the poorest retention time correlation with the reference method gave the highest second pass success rate. Since all correlation coefficients are low, however, and the result for method HPLC-1 is inconsistent, it is difficult to conclude from these data that orthogonality of retention times will necessarily lead to orthogonality of separation success.

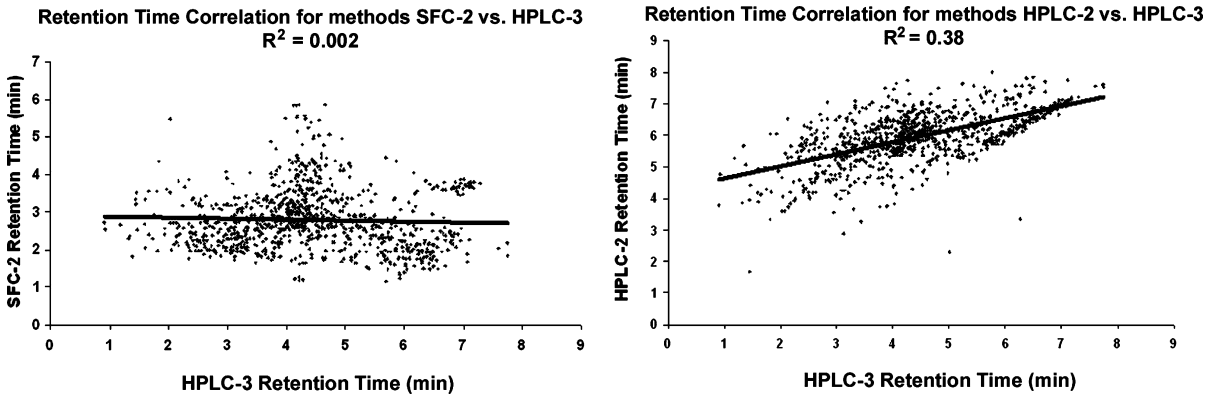

Figure 3. Poor column to column retention time correlation (left) and better correlation (right).

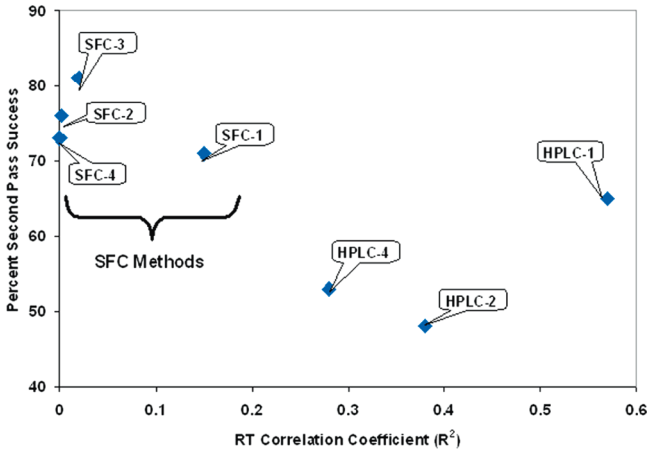

Figure 4. Retention time correlation vs second pass success.

Table 5. Cumulative Success Rate for 1021 Samples Through Four Analysis Methods

number of methods	HPLC only (method/cmpds/percent)	SFC only (method/cmpds/percent)	best of HPLC + SFC (method/cmpds/percent)
1	HPLC-3/812/85%	SFC-3/815/85%	HPLC-3/815/85%
2	HPLC-1/908/95%	SFC-2/912/95%	SFC-3/931/97%
3	HPLC-2/923/96%	SFC-1/941/98%	SFC-2/947/99%
4	HPLC-4/934/97%	SFC-4/949/99%	SFC-1/954/99%

The success rate analysis can be repeated iteratively by continuing to retrospectively examine those samples for which a successful analysis has not yet been obtained after a given number of methods. This is shown in Table 5. Three scenarios are shown: HPLC only, SFC only, and HPLC + SFC. For the HPLC only scenario, only the four HPLC methods were considered. As shown in Table 3, the most successful HPLC method (HPLC-3) had 85% success. Examination of the 15% of samples that failed to provide successful separation shows that the HPLC method that provided greatest separation success for that subset of samples was HPLC-1 at 65% success (Table 4). The combined total successful separations using those two methods are 95%. Continuing retrospective analysis for the 5% of compounds for which successful separation has still not been obtained eventually leads to finding a successful method 97% of the time after four HPLC analyses (Table 5). Similar analysis starting with the best SFC method and continuing through the SFC methods leads to a total success rate of 99%. Considering all eight methods, including both HPLC and SFC, also leads to 99% overall success after including the best four out of eight methods. Note that we do not have a baseline standard including eight HPLC or eight SFC methods, so all we can do is estimate a maximum value for adding SFC to HPLC (versus having eight HPLC or eight SFC methods) in this case. The difference between using four HPLC methods or using a combination of HPLC and SFC methods is 20 compounds, or about 2% of the total. This results from use of one HPLC method and three SFC methods. The data in Table 5 are also summarized graphically in Figure 5.

The data in Table 5 and Figure 5 are derived from only those samples where the molecular ion was found in all eight methods. This removes potential detection bias because of the difference in mass spectrometer ionization but may ignore

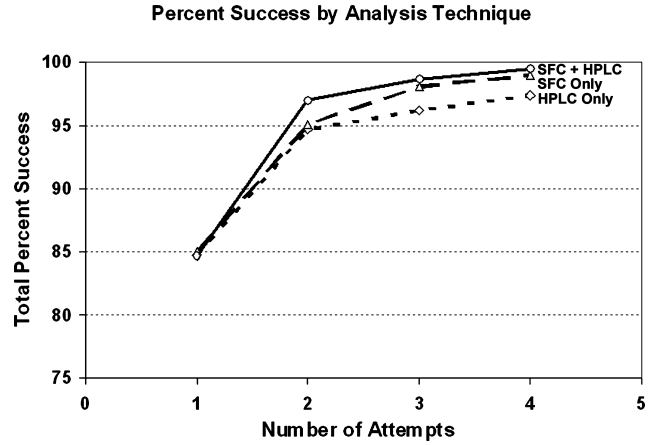

Figure 5. Cumulative success rate for 1021 samples through four analysis methods.

Table 6. Cumulative Success Rate for 1158 Samples Through Four Analysis Methods

no. of methods	HPLC only. (method/cmpds/percent)	SFC only (method/cmpds/percent)	best of HPLC + SFC (method/cmpds/percent)
1	HPLC-3/946/82%	SFC-3/891/77%	HPLC-3/946/82%
2	HPLC-1/1071/92%	SFC-4/1003/87%	SFC-3/1089/94%
3	HPLC-2/1096/95%	SFC-2/1032/89%	HPLC-1/1114/96%
4	HPLC-4/1114/96%	SFC-1/1049/91%	HPLC-4/1125/97%

samples that simply to not chromatograph well by SFC and may not be representative of real world experience. Repeating the analysis but including all mixtures where the target was found by at least one method, leads to the result summarized in Table 6 and Figure 6. In this case, SFC does not perform as well as HPLC because of failure to identify a molecular ion in a significant number of samples. After choosing the best four methods (a combination of three HPLC and one SFC method), the number of successful samples (1125) is only 11 samples (1%) greater than when using HPLC alone.

Conclusions

The purification method of choice in most drug discovery laboratories is currently preparative HPLC. The method is both robust and universal, and the costs associated with operation of an HPLC based laboratory are well understood. When considering introduction of a new technology into an

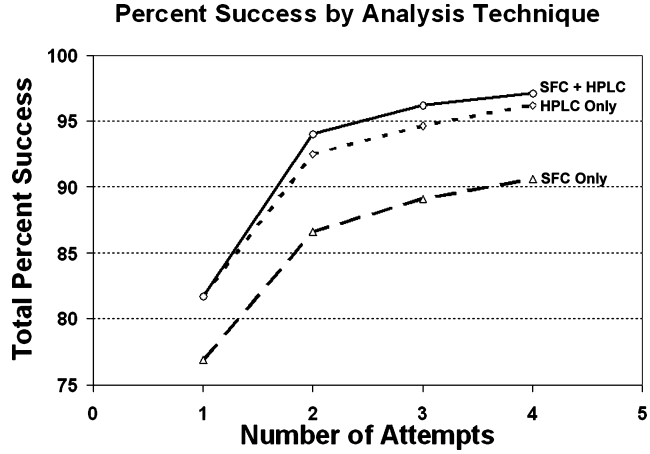

Figure 6. Cumulative success rate for 1158 samples through four analysis methods.

existing laboratory, both the costs and the benefits of that new technology will be taken into account. One of the potential benefits of introducing SFC technology into an HPLC-based laboratory supporting drug discovery is the potential for separation orthogonality. If a target separation method can be developed in less time by using SFC or by using a mixture of HPLC and SFC, then the time savings represents part of the "return" on investment in SFC technology. Our results suggest that our high throughput drug discovery purification laboratory using a standard screening battery of up to four typical separation methods will improve its screening success rate by 1−2% by incorporation of SFC in addition to reverse phase HPLC. These results are, of course, highly specific to the methods and the specific synthesis reaction mixtures we used in our study and do not take into account the potential benefits of other HPLC methods including normal phase and hydrophilic interaction chromatography (HILIC). The potential success rate benefits of SFC must be weighed against any other potential costs or benefits of SFC versus HPLC in making the decision of whether or not to use SFC, HPLC, or a combination of the two in a high throughput purification laboratory. Since the methods we used were typical of common practice in use today, further research may be required to identify a suite of SFC methods (columns, buffers, etc.) that provides greater complementarity to reverse phase HPLC than those we used. In addition, improvements to match MS ionization success rates among HPLC-MS, analytical SFC-MS, and preparative SFC-MS would make comparisons more meaningful and may improve the opportunity for overall success when using SFC-MS.

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(27) Columns were obtained from the following sources: **Xbridge C18:** XBridge C_{18} 5 μ, 4.6 mm × 50 mm column from Waters Corporation (Milford, MA). XBridge is a trademark of Waters Corporation. **ShieldRP:** XBridge Shield RP- C_{18} 5 μ, 4.6 mm × 50 mm column from Waters Corporation (Milford, MA). XBridge is a trademark of Waters Corporation. **2-Ethyl Pyridine:** 2-Ethyl pyridine 60A 5 μ, 4.6 mm × 150 mm column from Princeton Chromatography (Princeton, NJ). **Cyano-Diol:** 2-CN:Diol 60A, 5 μ, 4.6 mm × 150 mm column from Princeton Chromatography (Princeton, NJ). **PolarRP:** Synergi Polar-RP, 5 μ, 80A, 4.6 mm × 150mm column from Phenomenex (Torrance, CA). Synergi is a trademark of Phenomenex Corporation. **PVA-SIL:** YMC-Pack PVA-Sil-NP S-5 μ, 4.6 mm × 150 mm column from YMC America (Allentown, PA).

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CC100118Y