Modern Chiral Separations using HPLC and SFC for Method Development and Prep Purification

This article discusses factors important to operating a fast (high throughput) and efficient HPLC/SFC method development and prep purification laboratory. Discussion will include procedures for quickly developing separation methods and purifying pharmaceutical candidates as well as the systems, instruments, columns, and solvents we use to develop methods and purify samples in one week. Select examples will be presented.

Key words: Chiral, Chiral Separations, HPLC, SFC, Method Development, Prep Purification

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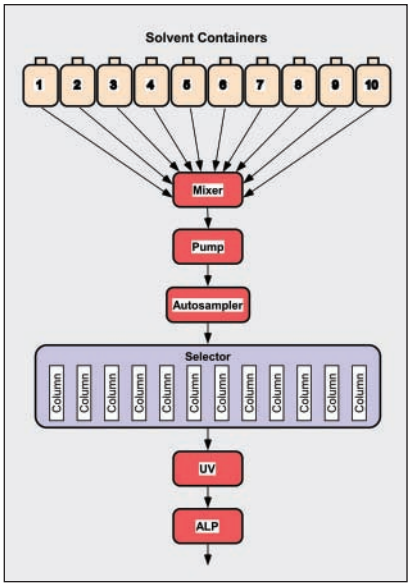

Figure 1: Method Screening Flow Diagram

INTRODUCTION

Competitive and economic factors are driving pharmaceutical companies towards increased efficiency and cost reduction. Most new pharmaceutical candidates produced by medicinal chemistry need to be analysed and purified by HPLC/SFC quickly in order to decide which compounds are worth further development – often creating a throughput bottleneck.

In order to develop a good method you must start with a well chosen set of columns and solvents and screen a productive variety of conditions to determine the best method for a particular compound. An example of useful columns and solvents are listed in *Table 1* below. Other useful columns are manufactured by companies including Phenomenex, Regis, Kromasil, and ASTEC (now Supelco). Similarly other solvents can prove useful, but if too many conditions are screened the time to screen becomes unrealistically long. This realistic example is intended to illustrate bounding the number of methods to be screened and should not be interpreted as the correct answer for all applications.

EXPERIMENTAL

A description of our experimental conditions and results are presented below and divided into 3 sections: Method Screening & OptimiSation, Preparative Purification, and Chiral Detection. All experimental work was done in our labs over the last few years.

METHOD SCREENING & OPTIMISATION

A modern method screening system flow diagram is illustrated in *Figure 1* and an example is pictured below in *Figure 2*. The system pictured in *Figure 2* is controlled by AutoMDS software (PDR-Chiral, Lake Park,

Table 1: Example of useful columns and solvents for chiral HPLC and SFC screening

Column	Manufacturer		HPLC Solvents	SFC Solvents
OD-H	Daicel	1	Hexane	CO2
AD-H	Daicel	2	Isopropanol	Isopropanol
AS-H	Daicel	3	Ethanol	Ethanol
OJ-H	Daicel	4	Methanol	Methanol
IA	Daicel	5	Acetonitrile	Acetonitrile
IB	Daicel	6	Methyl tert Butyl Ether	Methyl tert Butyl Ether
IC	Daicel	7	Ethyl Acetate	Ethyl Acetate
		8	Tetrahydrofuran	Tetrahydrofuran
		9	Methylene Chloride	Methylene Chloride

FL USA) and includes a 20 bottle low pressure gradient mixer feeding an Agilent 1100, a 24 position heated/cooled column selector fitted with up to 22 columns and 2 bypass lines, and an Advanced Laser Polarimeter (ALP) for uniquely identifying enantiomers and tracking elution order.

Figure 2: AutoMDS on Agilent 1100 with 22 Columns & 20 Bottles

In order to make method development fast and automated we build relatively large screening sequences that differ based on column type (coated vs bonded) and eluent phase (normal, polar, and reverse). The reason we separate based on column type is that coated phases can be easily ruined by strong solvents, as opposed to bonded phases which can handle almost any solvent. Depending on analyte characteristics, acid or base additives are included in eluents.

We suggest developing the master method libraries listed in *Table 2* below. Sequences are written by selecting an appropriate master method, deciding which columns and solvents from the master set are to be screened, and then building a sequence of only those methods. We consider miscibility of solvents when arranging methods in a sequence and we include equilibration of about 1 column volume before each injection.

Table 2: Suggested master method libraries for chiral screening

Column	Eluent	System
Coated	Normal	HPLC
Bonded	Normal	HPLC
Coated	Polar	HPLC
Bonded	Polar	HPLC
Coated	Reverse	HPLC
Bonded	Reverse	HPLC
Coated	Normal	SFC
Bonded	Normal	SFC

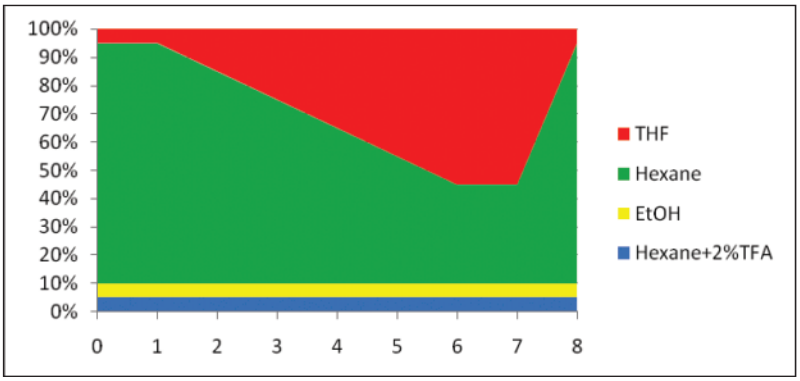

Figure 3: Gradient Profile using Additive in only 1 Bottle

Gradient methods prevail in our screening method libraries because they save time and sample by exposing a single injection to varying eluent composition quickly. Multi solvent bottle gradients (more than binary) are often used to maintain constant eluent additive concentration without requiring additive to be in every solvent bottle. *Figure 3* below shows an example where the eluent has a constant amount of TFA (5% of 2% = 0.1%) and a constant amount of Ethanol (5%), even though the percent contributions of Hexane and THF vary in a linear gradient from 90/5 to 40/55 between 1 and 6 minutes. Using this scheme, we can run acid, neutral, or base gradients against Hexane, for example, by adding only 1 bottle of Hexane with acid additive and one bottle of Hexane with base additive, rather than needing an acid, neutral, and base set of all solvents. These techniques allow us to screen a wide variety of normal, reverse, and polar organic phase eluents with acid, neutral, or base additives using only 20 bottles total. To avoid column + additive memory effects we load column selectors with 3 of each type of column, one for acid, neutral, and base eluents. Thus we do not need to spend time equilibrating our columns because of eluent additive changes.

Methods should be optimiSed based on application requirements and scale, e.g. analytical, semi-prep, prep, or process. Important considerations may include separation, elution order, solubility, stability, loading capacity, impurities, speed, and cost of eluents.

PREPARATIVE PURIFICATION

After method optimisation, scale-up to prep is usually straight forward if the method was developed and tested against appropriate requirements. Loading, cycle time, and other parameters can be determined using an analytical column and relatively simple mathematics can be used to scale performance up to prep. We developed a calculation worksheet called "Prep Predictor" (free on our web site) for pragmatically comparing methods for prep purification. Prediction accuracy is typically within 10% on a complete job. The Prep Predictor quickly summarises important bounding parameters like total run time, total solvent consumed, and total solvent collected (for evaporation). This allows us to do prep chromatography in a very deterministic way with minimal method optimisation at prep scale and minimal confusion about how much time and solvent we need, how big collection vessels should be, and how long rotovapping will take.

A modern HPLC/SFC prep purification system with 2 pumps configured for high pressure mixing, injector, column, detectors, and a collection valve is flow diagrammed below in *Figure 4*. The

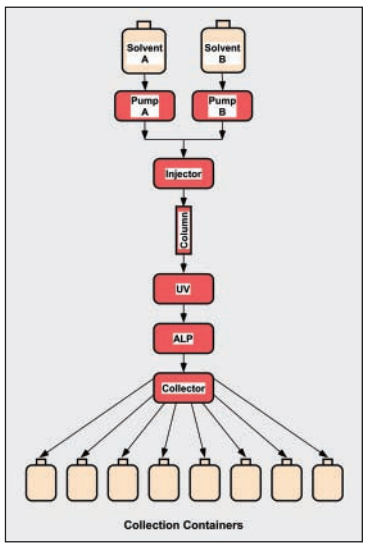

Figure 4: Prep Purification System

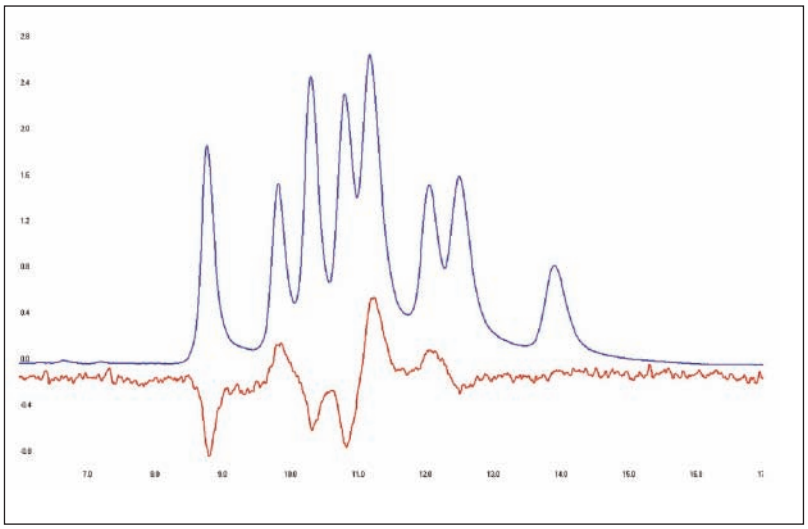

Figure 5: SFC separation of Cypermethrin with 3 chiral centers, SFC

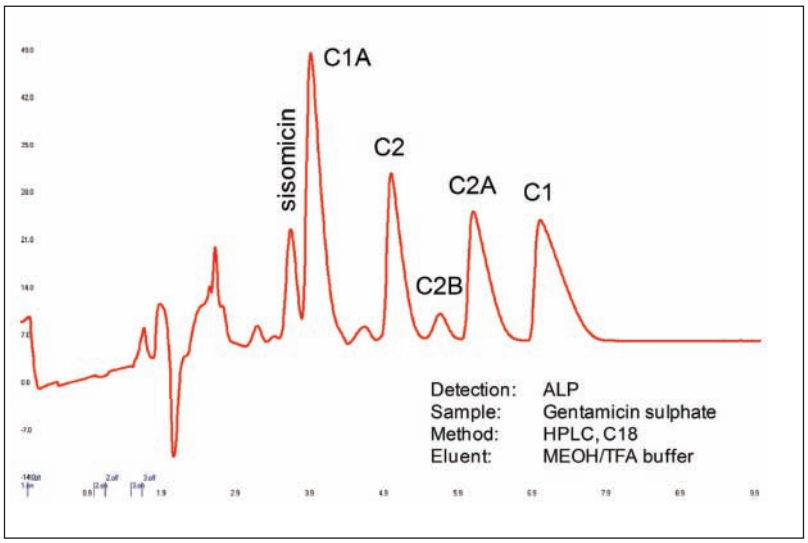

Figure 6: Gentamicin Semi-Prep Separation with ALP Detection

collection valve diverts eluent flow from waste to collection vessels and back to waste under command of AutoPrep software (PDR-Chiral, Lake Park, FL USA). AutoPrep software controls peak collection based on a combination of parameters including: time, detector amplitude and sign, detector derivative and sign, and enantiomeric excess. Collection modes are very robust allowing us to routinely make multi-day continuous runs with no one on-site during evening and night hours. Most of our collection modes dynamically adapt to real time conditions and perform well even during changing conditions.

CHIRAL DETECTION

We find chiral (optical activity) detectors necessary in chiral separations. Both polarimeter-based and circular dichroism-based chiral detectors can be useful to positively identify enantiomers in chromatograms with many peaks, monitor elution order during method development, and collect enantiomeric peaks during prep purification. Circular Dichroism detectors are absorbance-based, require a chromophore, and exhibit limited dynamic range. Polarimeter detectors are not absorbance based so do not require a chromophore and can exhibit very large dynamic range depending on design. Chiral detectors are generally less sensitive that absorbance detectors (UV) and much less sensitive than mass spectrometers (MS). However UV and MS do not offer insight into optical activity.

We use Advanced Laser Polarimeters (ALPs) to track enantiomer elution order, differentiate enantiomers from impurities, identify components in multiple chiral centre compounds, detect compounds without chromophores, and control peak collection in HPLC/SFC purification.

See *Figure 5* where an ALP was used to purify cypermethrin enantiomers using SFC. Cypermethrin has three chiral centres and eight enantiomers. There are no commercially available cypermethrin single enantiomer standards. The ability of ALP to identify enantiomers by peak polarity and area was a big help in interpretation of UV chromatograms and peak identification for prep scale separations. The last peak in this chromatogram is an impurity, not a cypermethrin enantiomer. This was easy to see with a supplemental ALP chromatographic trace.

Figure 6 illustrates where ALP was used to purify Gentamicin analogues using HPLC. Many antibiotics are relatively small molecules with a molecular weight less than 2000 Da. UV detection is challenging because this compounds lack a UV absorbing chromophore. All analogues (C1A, C2, C2A, C2B, C2A, and C1) are separated along with impurities and sisomicin. Method details are Column - Phenomenex Gemini, 4.6 x 250 mm, 5 µm; Eluent - water + TFA 0.04M buffer / methanol, 97/3; Flow - 1 mL/min; Injection - 100µL; Sample – Gentamicin 5mg/mL in water; Temperature - 35°.

RESULTS AND CONCLUSIONS

Using the procedures and equipment mentioned above we are usually able to go from racemic samples we have never seen before to purified enantiomers in one week. This performance is partially due to efficient system configurations and procedures but is also partially due to intelligently limiting the methods we consider to a reasonable set. The systems and equipment described herein are not particularly expensive when compare to the results they deliver. We use ALP detectors on all systems to provide complementary information to UV detection.

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