Super-critical fluid chromatography (SFC) with tandem mass spectrometry (MS/MS) to evaluate the absorption and delivery of individual stereoisomers

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Why consider stereoisomers?

- **Historically, pharma has ignored stereochemistry.**
  - This eventually led to the formation of companies whose mission was to capitalize on (patent/sell) the individual stereoisomers of other company's existing big selling drugs.
  - Success at this new mission caught the attention of mainstream pharma and it seems a lot less likely that it will remain unaddressed.
- **Now that it's on the radar screen, how is this addressed?**
  - Do all the usual pharma R&D on the individual stereoisomers.
  - In our organization that doesn't mean doing everything twice (or more for added chiral centers). It means that enough research is done to find and fully understand the most desirable isomer to put forward into development and then enough follow-up are done to achieve POC.
  - The additional research involves understanding biology (potency, side effects) and chemistry (inter-conversion, deliverability).
- **What additional technologies are needed?**
  - Chiral synthesis will meet some needs, particularly for chiral intermediates.
  - **Chiral separations have a crucial role in both analysis and purification.**

Why use chiral separations?

More specifically, why & how did we use them?

- **Of course, there's the usual enantiomeric excess (%ee) and compound purification needs.**
  - This is what led us to SFC/DAD/ELSD/MS in the first place and then later to SFC/MS/MS for the applications described below.
- **Are there reactivity reasons that the *in vivo* and *in vitro* assays don't agree on which isomer is most potent (i.e. did isomer inter-conversion occur)?**
  - Dose individual stereoisomers (ee = 99+%) and measure ee in plasma and target organs.
  - If the ee changes (beyond statistical variation, i.e. other isomers appear) then inter-conversion has occurred.
- **Is the stereo-specific potency target related or absorption related?**
  - Dose known mixture and measure ee in plasma and target organs.
  - If the preferentially absorbed stereo isomer is the less potent isomer, then suspect target protein as possible source of stereo-specific behavior. This would have to be validated with kinetics experiments.
  - If the preferentially absorbed stereo-isomer also is the more potent isomer, then absorption is the likely source of stereo-specific behavior (commonly observed).

Why use SFC?

- **Chiral Separations**
  - Ø **Reverse phase (RP) LC is possible:**
    - Using gradients is nice for spanning a range of polarities in one chromatogram (different polarities doesn't address stereo isomers).
    - RP chiral separations often are very, very pH dependent and this makes method development difficult.
  - **Normal phase (NP) LC works and is the well proven approach:**
    - Difficult stereoisomers can usually be well resolved.
    - Nevertheless, method development is easier / faster than RP where pH sometimes must be stepped 0.1 pH units. NP usually only requires trying 3 or fewer different buffers.
    - Gradients don't really work because all the action occurs within a very small span of change in mobile phase composition. Methods are isocratic and thus are rarely fast.
    - NP (hexane/heptane) isn't compatible with MS detection.
  - ☺ **SFC**
    - **SFC is NP. Think of it as CO_2 substituted for hexane.**
    - **Gradients work well in SFC and usually span 5 to 50% B (alcohol).**
    - **Combination of gradient and low viscosity often facilitates fast separations.**
    - **SFC works when combined with MS detection, but it's not as easy and doesn't work as well as RP-LC/MS.**

Outline of SFC presentation

- **Chiral method development with SFC**
  - **Columns, solvents, & buffers.**
  - **Pressure / Temperature effects.**
- **Challenges of interfacing SFC to MS**
  - **Splitting flow & using make up solvent.**
  - **CO_2 is different (not inert gas?).**
  - **Other techniques.**
- **Examples of measuring ee in plasma & brains**

Chiral method development with SFC

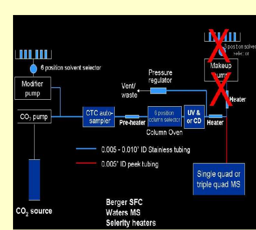
- Columns, solvents, & buffers
  - Normal phase requires a bit more work.
    - Column screening is required (4-6 for chiral).
    - Buffer screening sometimes required (DEA, IPA, FA).
    - Not unusual to need modifier switch (MeOH/EtOH/IPA).
  - Expect to make 10-20 injections to have enough data to be able to choose column, modifier and buffer.
- Pressure / Temperature
  - Pressure at column exit must be regulated upward (100 bar).
  - Pressure drop across column results in significant expansion and thus significant adiabatic cooling (example: leak = snow).
  - Mobile phase should be actively heated before and after column to avoid mixed phases.
  - Temperature regulation offers more limited benefit (but still observable) for the traditional chromatography expectations for reduced viscosity and increased adsorption (mass transfer)

Challenges of interfacing SFC to MS

- Splitting flow & using make up solvent
  - Despite eluent being mostly gas at AP, full flow (1-5 mL/min) into the source (ESI, APCI) doesn't work well (especially ESI: high background, low response).
    - Sample blown away? Too much phase changing?
    - Lower flow, 5-50 µL/min alcohol from column seems to provide better sensitivity.
  - Conventional Wisdom: Make up flow (200-400 µL/min) of alcohol improves signal stability and sensitivity (via dilution of amine buffer?).
- CO_2 is different (not completely an inert gas?)
  - The use of flow injection (FI) on a LC/MS is not a viable approach toward tuning / optimizing make up solvent composition.
  - FI/MS under LC/MS conditions (identical to SFC except no CO_2) often suggests acetonitrile as most sensitive make up solvent.
  - In the presence of CO_2, alcohols for make up flow usually provide better sensitivity.
- Other considerations
  - As the pressure goes below super critical (~75 bar) for ionization, distinct phase layers (large droplets) form raising the background noise.
    - Heating the eluent stream is among the most effective ways to re-homogenize it and stabilize the MS signal.
  - A multi-step approach to temperature control allows full flow (3 mL/min, 20% IPA) into source while achieving best sensitivity (ng/mL).
  - While most use APCI with SFC, we find ESI (heated) is most sensitive.

Instrument photo

SFC/MS or SFC/MS/MS schematic diagram



CO_2 source photo

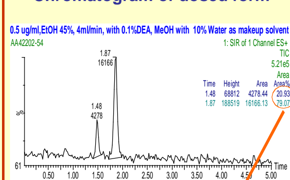
Example use of SFC/MS

- **Question to be addressed: Does absorption play a role in the observed stereo selective *in vivo* efficacy for a compound of interest?**
- Sample preparation

Rat brain extracts were submitted in acetonitrile solution (4/1 ACN ppt). Upon solvent evaporation using nitrogen gas, the brain extracts were reconstituted into 100 µL methanol.

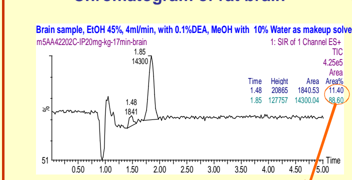
- SFC condition
  - Column: Chiralpak AD, 4.6 x 250 mm, 5µm
  - Modifier: Ethanol with 0.1% DEA (diethylamine)
  - Flow rate: 4 mL/min
  - Nozzle pressure: 150 bar
  - Nozzle temperature : 60 ℃
  - Column temperature: 35 ℃
- MS condition
  - Makeup solvent : Methanol with 10 % Water
  - Flow rate: 0.3 mL/min
  - Positive ESI mode, SIR

Chromatogram of dosed form



Dosed compound ee = 60%

Chromatogram of rat brain



Compound detected from rat brain extract after dosing ee = 77%

Ratio of the two enantiomers has changed!

Absorption is at least partially related to observed stereo-selective activity.

Example use of SFC/MS/MS

- **Question to be addressed: Does inter-conversion of isomers play a role in the observed stereo-selective *in vivo* efficacy for a compound of interest (opposite of that seen *in vitro*).**
- Sample preparation

Rat plasma and brain extracts were submitted in acetonitrile solution (4/1 ACN ppt). Upon solvent evaporation using nitrogen gas, the brain extracts were reconstituted into 100 µL methanol.

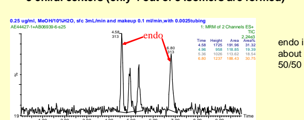
- SFC condition
  - Column: Chiralpak AD, 4.6 x 250 mm, 5 µm
  - Modifier: Isopropylalcohol (IPA) with 0.1% DEA (diethylamine)
  - Flow rate: 3 mL/min
  - Nozzle pressure: 150 bar
  - Nozzle temperature : 60 ℃
  - Column temperature: 40 ℃
- MS/MS conditions

Makeup solvent : Methanol, flow rate: 0.1 mL/min

Positive ESI mode and Double MRM at 313.2/95.1and 313.2/191.2

Chromatogram of standard

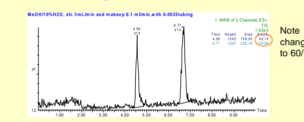
3 chiral centers (only 4 out of 8 isomers are formed)



endo is about 50/50

Chromatogram of 60 ng/mL mixture of the exo and endo isomers.

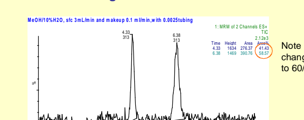
Chromatogram of plasma sample



Note change to 60/40

Only endo dosed and only endo observed

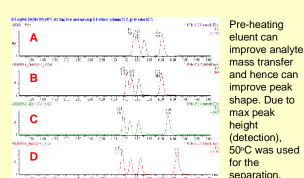
Chromatogram of brain extracts



Note change to 60/40

Only endo dosed and only endo observed. Thus, no interconversion observed.

Temperature tuning the separation



Pre-heating eluent can improve analyte mass transfer and hence can improve peak shape. Due to max peak height (detection), 50°C was used for the separation.

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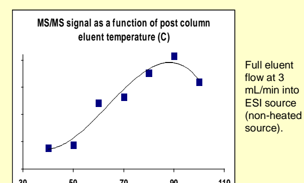
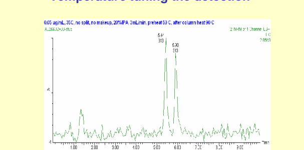
Temperature tuning the detection



Full eluent flow at 3 mL/min into ESI source (non-heated source).

Post column heating homogenizes the eluent and results in more efficient (sensitive) ESI ionization and a more stable MS signal (like observed previously for APCI).

Temperature tuning the detection



Full eluent flow at 3 mL/min and post column heating to 90℃ into ESI source gives at least 5 fold better s ensitivity than any split / make up flow / pre-column heating combination we have found thus far.

[10 µL injection at ~25 ng/mL each isomer]

Summary

- **SFC is a proven technique for chiral analysis and purification for samples that are synthetic in origin.**
- **SFC/MS and SFC/MS/MS are beginning to show promise for study and understanding of stereo specific effects observed *in vivo*.**
- **For a limited number of compounds examined thus far, stereo specific absorption has been observed in all cases (with good precision [ca. 1-2%] even animal to animal).**
- **Thus far (for a limited number of compounds examined), observed *in vivo* biological effects have been consistent with measured differences in absorption (using SFC/MS[n]).**
- **Active control of temperature before and after column can be used to achieve ng/mL sensitivities with full SFC flow (3 mL/min) into a standard (non-heated) ESI source (instead of APCI). While still not quite as sensitive as LC/MS/MS, it seems sufficient for priority compounds which are likely to be well absorbed (SFC/MS/MS not yet needed elsewhere).**