

**Super-Critical Fluid Chromatography (SFC)
with Tandem Mass Spectrometry (MS/MS)
to Evaluate the *in vivo* Disposition of
Individual Stereoisomers of Drugs**

QingPing Han and Mark J. Hayward

**Lundbeck Research USA
Paramus, NJ**



Made it happen:

QingPing Han



Also:

Analytical
@ Lundbeck US
David P. Budac
Xu Zhang

DMPK
@ Lundbeck US
Manual Cujina
Rui Li
A.S. Pieris-Gunatilaka
Dahai Dong
Ron Kong
Gamini Chandrasena
@Lundbeck DK
Mette Nøhr Poulsen
Frank Larsen

Neuro Science
@Lundbeck US
Silke Miller

Med Chem
@ Lundbeck US
Arifa Husain
H.N. Jimenez
Jesse M. Weiss
Chien-An Chen

Why consider stereoisomers?

- **Historically, pharma mostly ignored stereochemistry (prior to 1980s).**
 - Eventually in the 1980s, it became clear that individual stereoisomers can have unique pharmacological profiles and hence medical benefits.
 - Success at meeting previously unmet medical needs with individual stereoisomers has resulted in pharma integrating the study of drug candidate stereo chemistry into R&D.*
- **Now that we are studying stereo chemistry, 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 done to achieve stereo-specific POC.
 - The additional research involves understanding biology (potency, side effects) and chemistry (inter-conversion, availability).
- **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.**

***Reviews on the benefits of chirality in pharmaceuticals:**

I. Agranat, H. Caner, J. Caldwell, *Nat. Rev. Drug Discov.*, 1, 2002, 753.

H. Caner, E. Groner, L. Levy, I. Agranat, *Drug Discov. Technol.*, 9(3), 2004, 105.

Why use chiral separations?

More specifically, why & how did we use them?

- **Of course in drug discovery, 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 bioanalysis 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 [ex. Thalidomide,* profens: R pro-drug → S active**]) ?**
 - 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.
 - Don't forget the other potential answer: metabolism.
- **Is the stereo-specific potency target related*** or ADME related?**
 - Dose known mixture and measure ee in plasma and target organs.
 - If the preferentially available stereo isomer is the less potent isomer, then the drug / target protein combination may be the possible source of stereo-specific behavior. This would have to be validated with further experiments.
 - If the preferentially available stereo-isomer also is the more potent isomer, then drug availability may be the source of stereo-specific behavior (commonly observed).

Recent SFC/MS review with emphasis on drug bioanalysis:
F. Li, Y. Shieh, *J. Sep. Sci.* 31, 2008, 1231.

Recent review on stereo-selective pharmacokinetics:
D.R. Brock, *Biopharm. Drug Dispos.*, 27, 2006, 387.

Drug enantiomer interconversion:

*T. Eriksson, S. Bjorkman, B. Roth, A. Fyge, P. Hoglund,
Chirality, 7, 1995, 44.

**A.J. Hutt, J. Caldwell, *J. Pharm. Pharmacol.* 35, 1983, 693.

Highly stereo-specific potency at target:

***P. Baumann, D.F. Zullino, C.B. Eap,
Europ. Neuro-psychopharmacol. 12, 2002, 433.

Why use SFC?

● Chiral Separations

⊘ 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 CO₂ 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 (usually at least 3x faster than NP-LC).
- SFC works when combined with MS detection, but historically it has not been as easy and hasn't worked as well as RP-LC/MS.

Outline of SFC presentation

- Overview: Chiral method development with SFC
 - Columns, solvents, & buffers.
 - Pressure / Temperature effects.
 - Instrument photos.
- Early examples of measuring ee in plasma & brains before focusing on the MS interface.
 - Success, but it's a journey and a learning process!
 - ee: constantly different but precise?
- Challenges of interfacing SFC to MS
 - Splitting flow & using make up solvent.
 - CO₂ is different (not inert gas?).
 - Other techniques – all in - no BPR – use temperature.
- More detailed example of measuring ee in plasma & brains after focusing on the MS interface and a head to head comparison of SFC/MS/MS with LC/MS/MS.
 - More lessons learned, but finally truly comparable results achieved SFC with LC using MS detection!

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 mass transfer benefit (but still observable) for the traditional chromatography expectations for reduced viscosity, but the selectivity changes can be very significant.

Instrument Photo



CO₂ Source Photos



Example 1: Use of SFC/MS

(Discovery – small study, can't show compound)

- **Question to be addressed: Does enantiomer availability 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 μ L methanol.

- **SFC condition**

- Column: Chiralpak AD, 4.6 x 250 mm, 5 μ m
- Modifier: Ethanol with 0.1% DEA (diethylamine)
- Flow rate: 4 mL/min
- Nozzle pressure: 150 bar
- Nozzle temperature : 60 °C
- Column temperature: 35 °C

- **MS conditions**

Makeup solvent : Methanol with 10 % Water, flow rate: 0.3 mL/min

Positive ESI mode, SIR

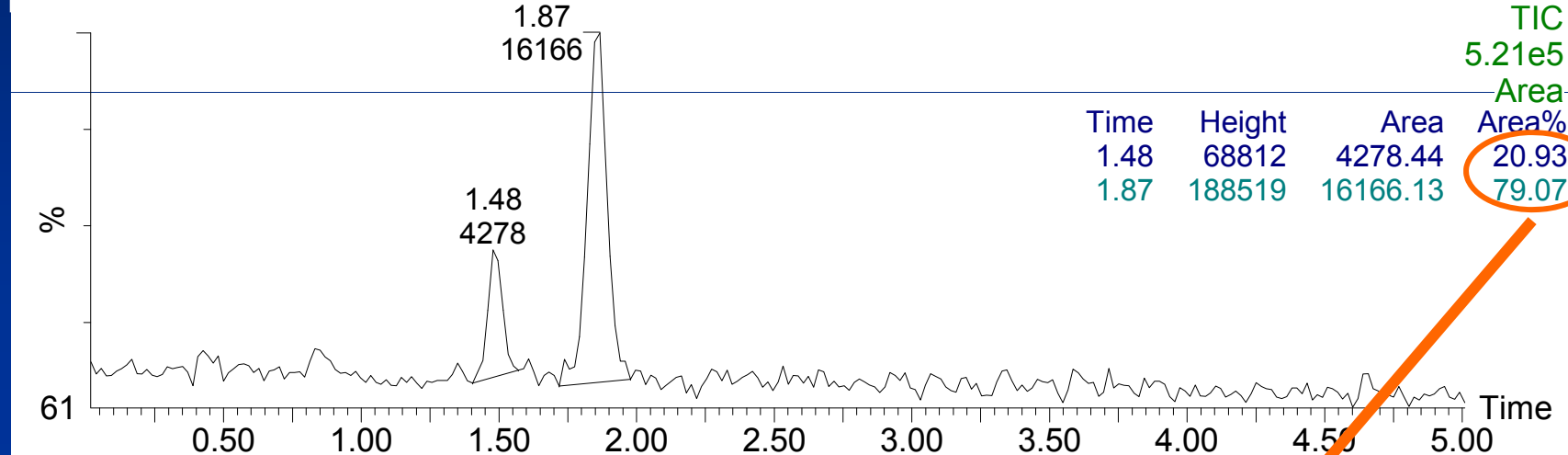


Chromatogram of dosed form

0.5 ug/ml, EtOH 45%, 4ml/min, with 0.1% DEA, MeOH with 10% Water as makeup solvent

1: SIR of 1 Channel ES+

TIC
5.21e5

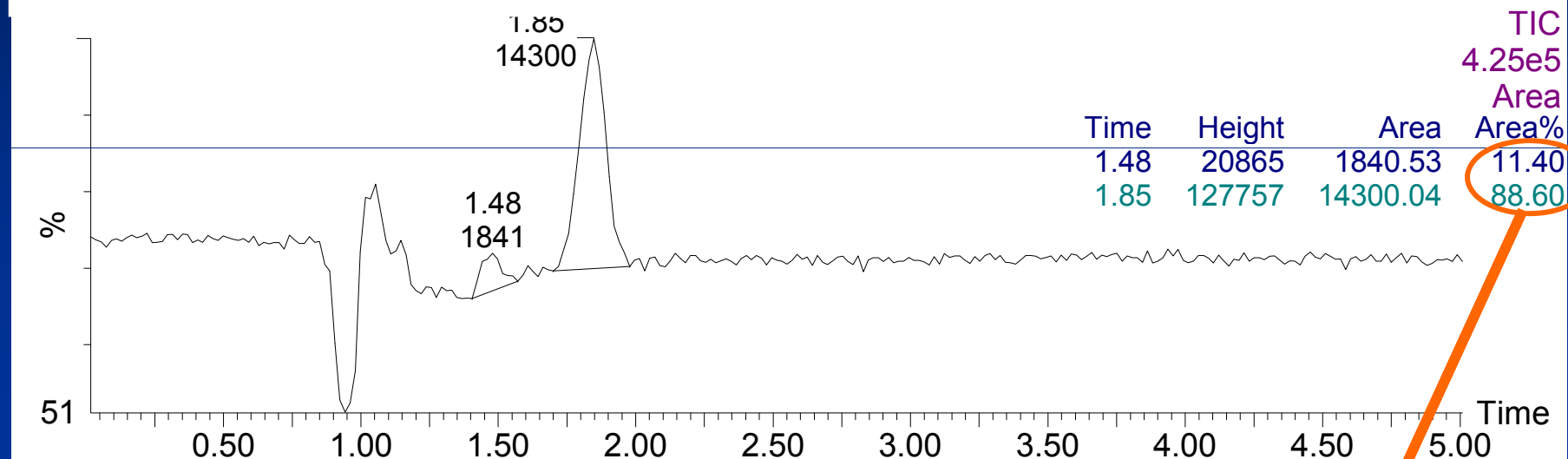


Dosed Compound ee = 60%

Chromatogram of Rat Brain

Brain sample, EtOH 45%, 4ml/min, with 0.1%DEA, MeOH with 10% Water as makeup sol

1: SIR of 1 Channel ES+



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

Ratio of the two enantiomers has changed!

New ratio differs by <2% between 2 animals

Availability seems to be at least partially related to observed stereo-selective activity (more active enantiomer also more available).

Example 2: Use of SFC/MS/MS

(Discovery – small study, can't show compound)

• **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 μ L methanol.

• **SFC condition**

- Column: Chiralpak AD, 4.6 x 250 mm, 5 μ m
- Modifier: Isopropylalcohol (IPA) with 0.1% DEA (diethylamine)
- Flow rate: 3 mL/min
- Nozzle pressure: 150 bar
- Nozzle temperature : 60 °C
- Column temperature: 40 °C

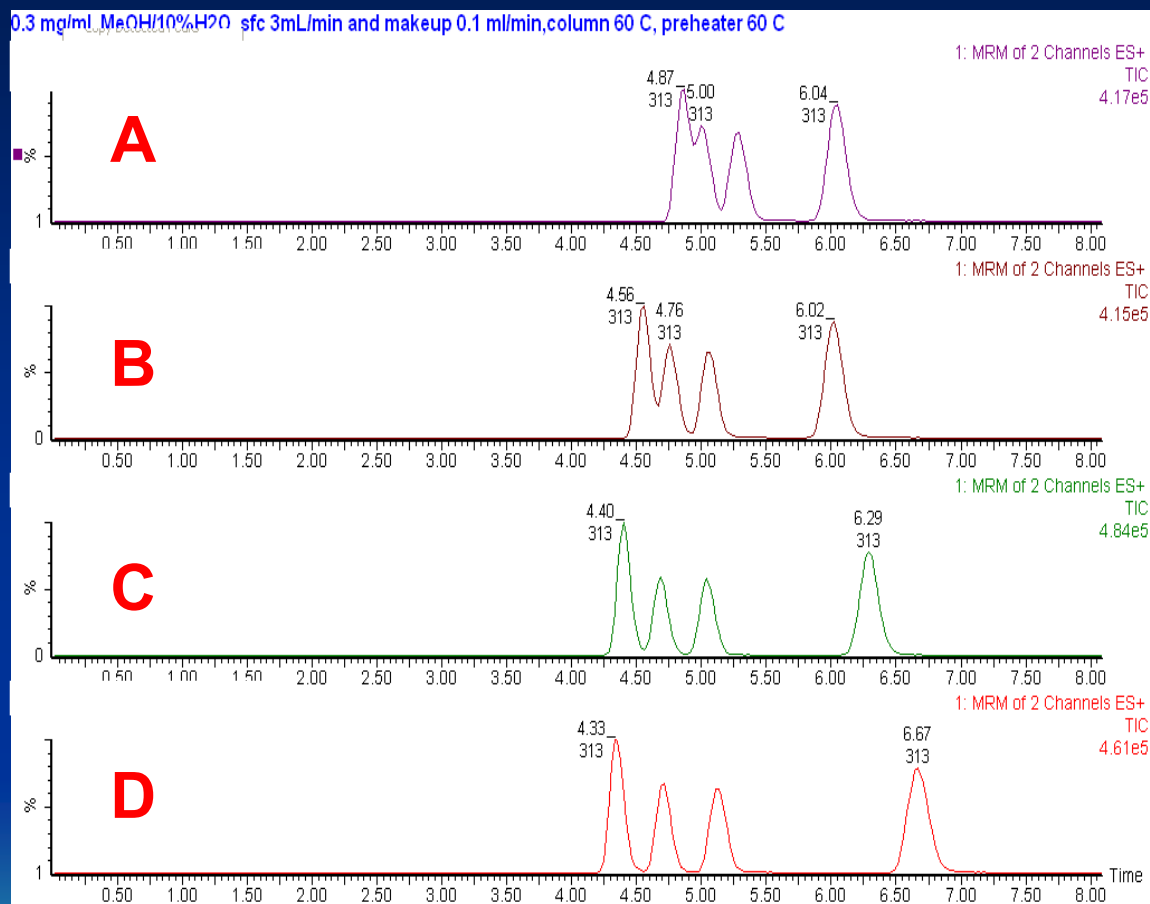
• **MS/MS conditions**

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

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

Temperature tuning the separation

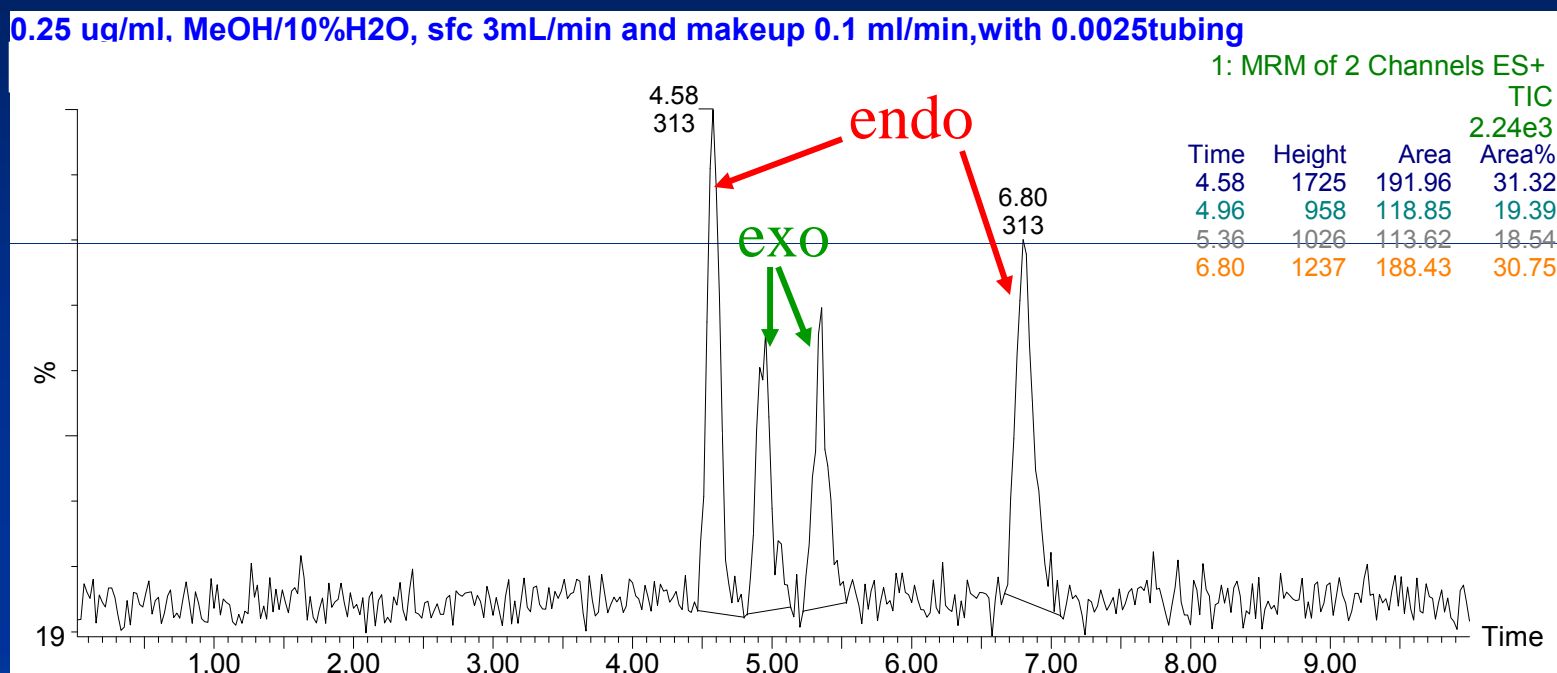
Pre-heating eluent can improve analyte mass transfer and hence can improve peak shape. Due to max peak height (detection) and sufficient separation, 50°C was used for the separation.



Mixture of endo/exo isomers at (A) 30, (B) 40, (C) 50, and (D) 60 °C

Chromatogram of standard

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

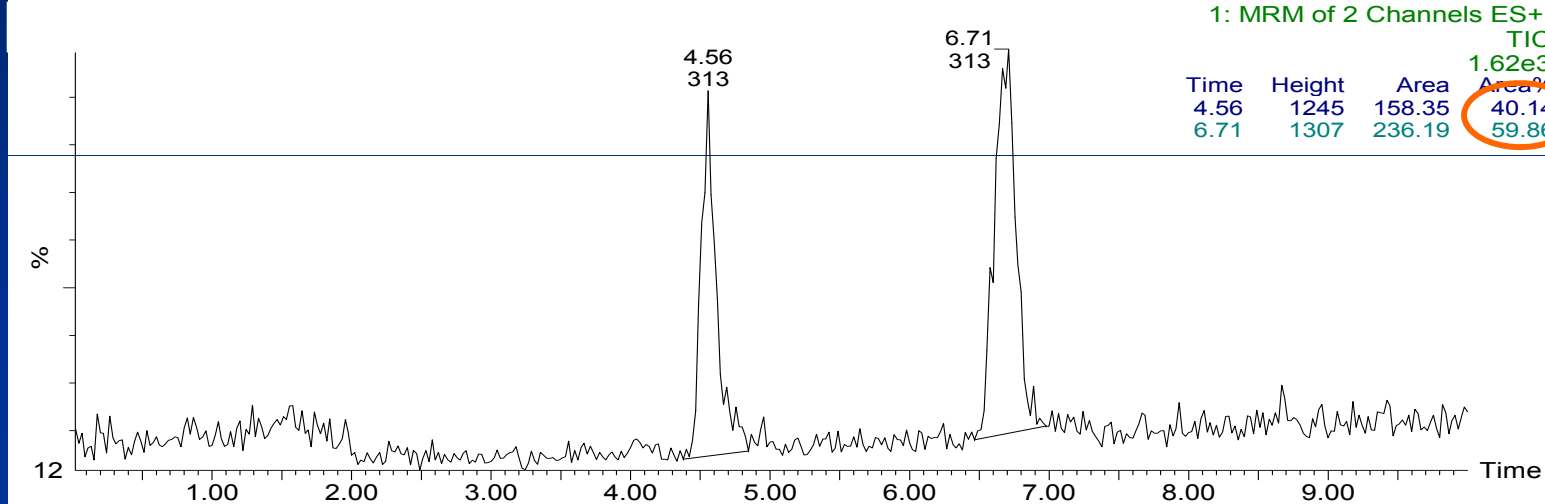


endo is
about
50/50

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

Chromatogram of Plasma Sample

MeOH/10%H₂O, sfc 3mL/min and makeup 0.1 ml/min, with 0.0025tubing

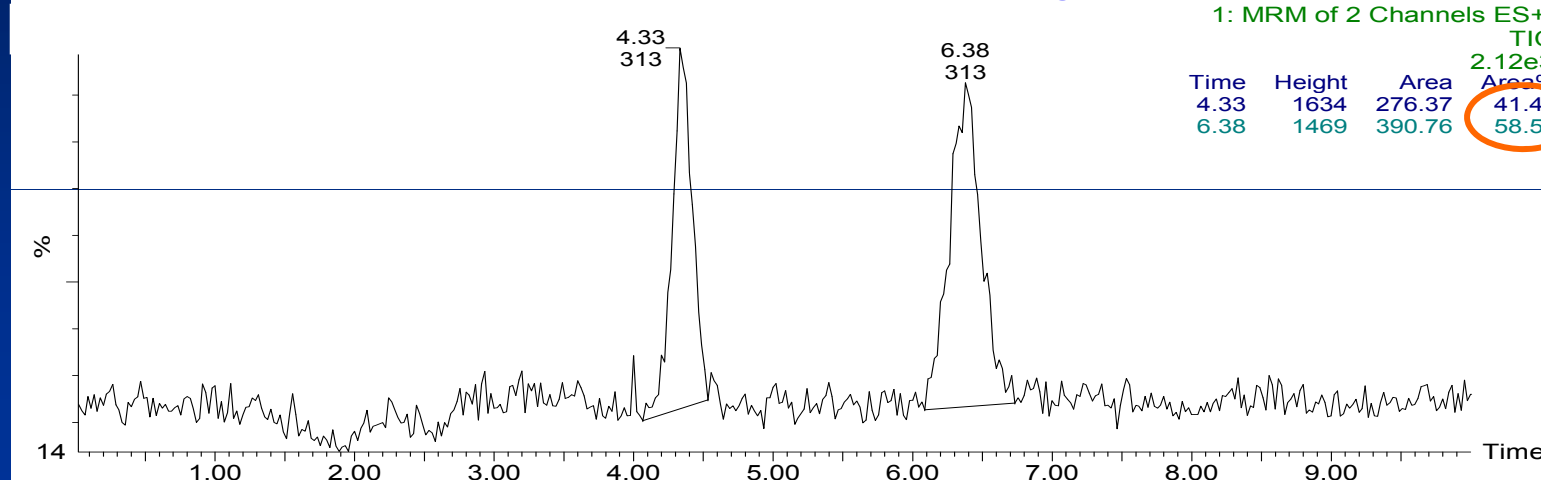


Note
change
to 60/40.
Difference
between
values for
2 animals
<2%.

Only endo dosed and only endo observed.

Chromatogram of Brain Extracts

MeOH/10%H₂O, sfc 3mL/min and makeup 0.1 ml/min, with 0.0025tubing



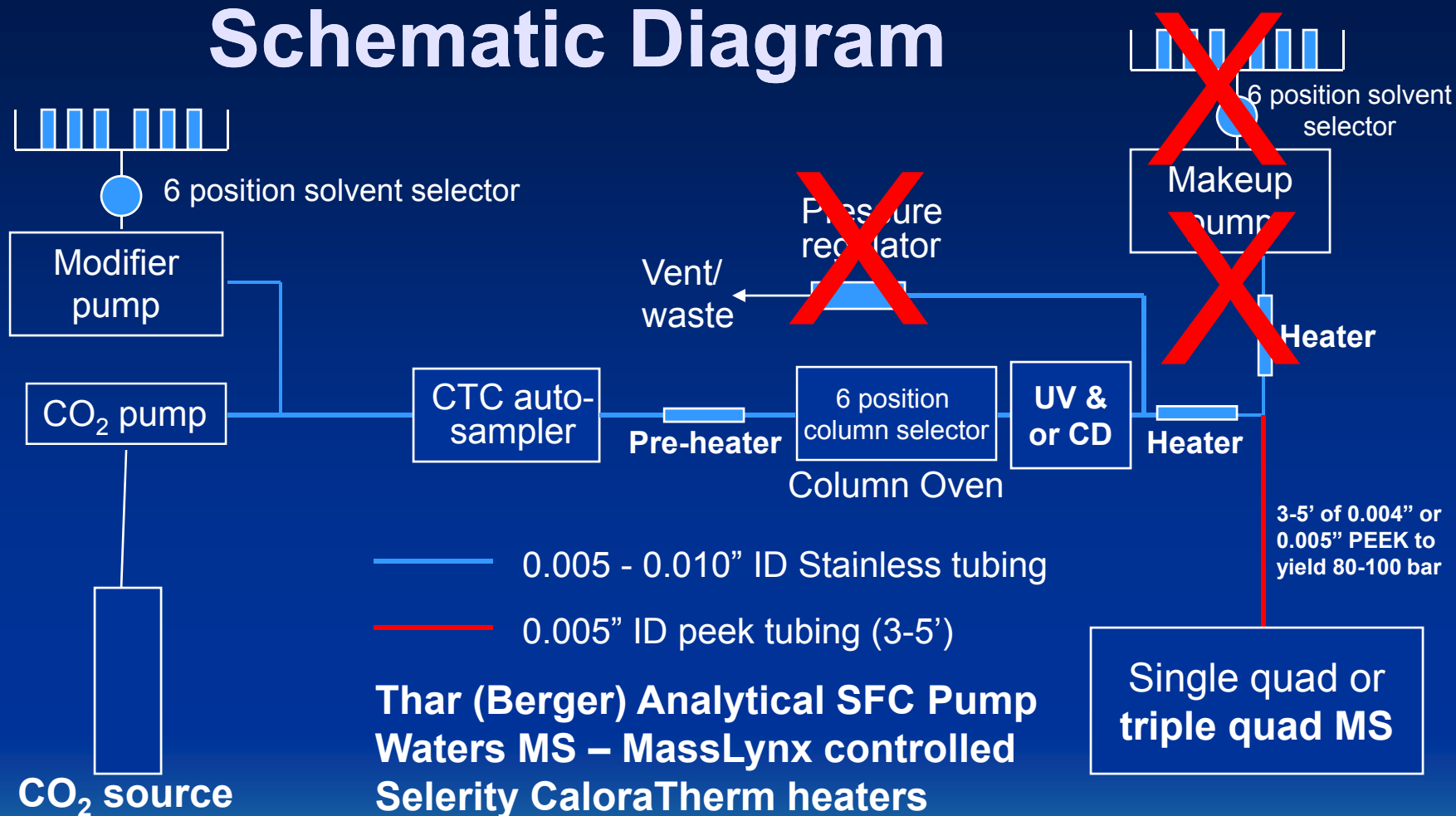
Note change to 59/41. Difference between values for brains vs. plasma also <2%.

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

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) hasn't worked well (especially ESI: high background, low response).
 - Sample blown away? Too much phase changing?
 - Lower flow, 5-50 $\mu\text{L}/\text{min}$ alcohol from column seems to provide better sensitivity.
 - Conventional Wisdom: Make up flow (200-400 $\mu\text{L}/\text{min}$) of alcohol improves signal stability and sensitivity (via dilution of amine buffer?).
- **CO₂ 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 CO₂) often suggests acetonitrile as most sensitive make up solvent.
 - In the presence of CO₂, 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 while under pressure (80-100 bar) is among the most effective ways to maintain a homogeneous fluid @AP and stabilize the MS signal.
 - A multi-step approach to temperature control allows full flow (3-4 mL/min, 20% IPA) into source while achieving good sensitivity (ng/mL).
 - While most use APCI with SFC, we find ESI (heated) is most sensitive.

SFC/MS or SFC/MS/MS Schematic Diagram



Thorough review of the early days of SFC/MS with emphasis on interface:
M.T. Combs, M. Ashraf-Khorassani, L.T. Taylor *J. Chromatogr. A* 785, 1997, 85.

Suggested no BPR needed:
J.D. Pinkston, *Eur.J. Mass Spectrom.* 11, 2005, 189.

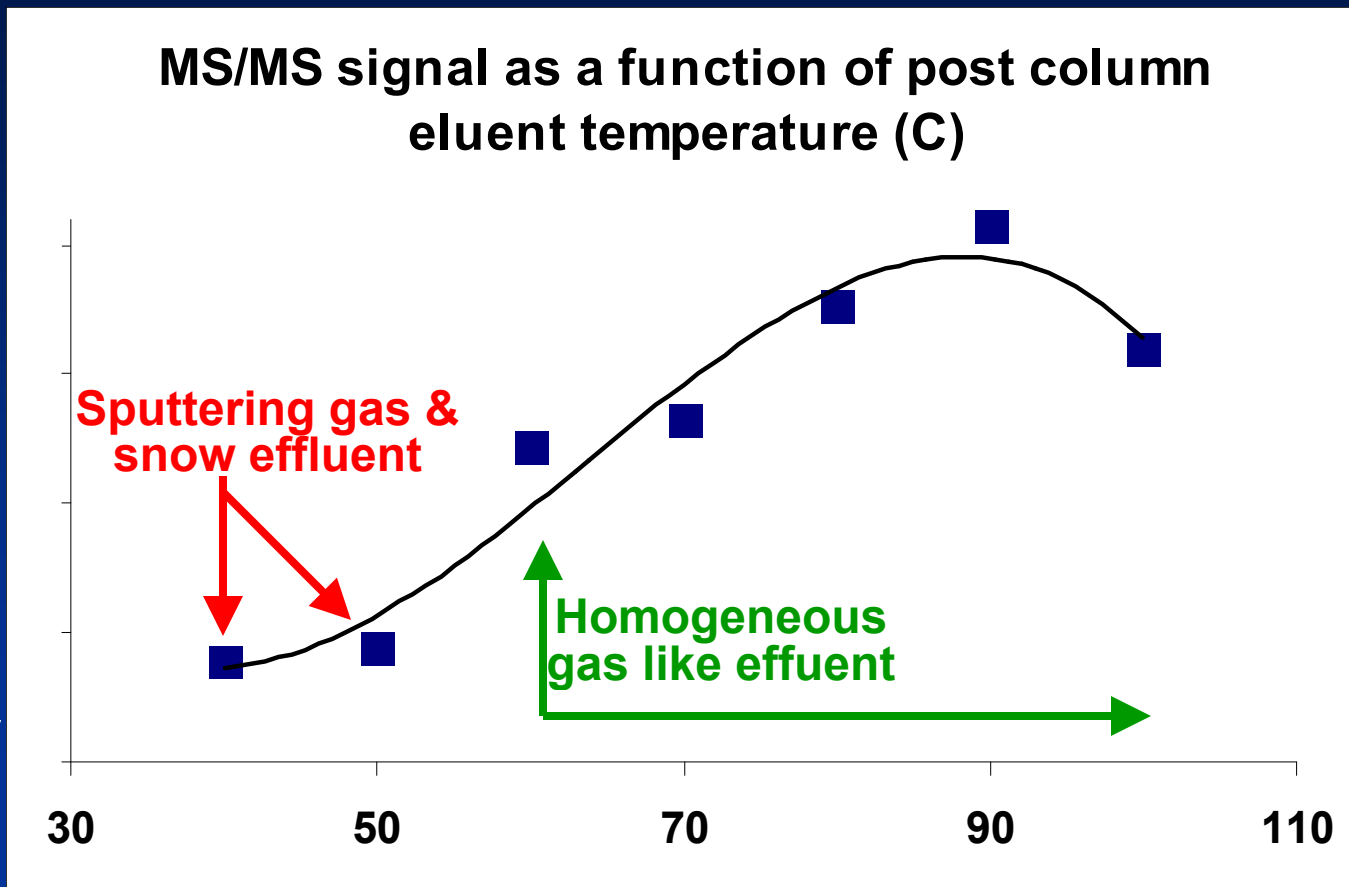
Traditional split interface SFC/MS set up:
P.J.R. Sjoberg, K.E. Markides *J. Chromatogr. A* 785, 1997, 101.
T. Baker, J.D. Pinkston *J. Am. Soc. Mass Spectrom.* 9, 1998, 498.
D.G. Morgan, K.L. Harbol, N.K. Kitrinis *J. Chromatogr. A* 800, 1998, 39.
M. Garzotti, M. Hamdan, *J. Chromatogr. B* 770, 2002, 53.
B. Bolanos, et.al. *Int. J. Mass Spectrom.* 238, 2004, 85.

Full flow into APPI, ESI, APCI MS sources with good results:
R.A. Coe, J.O. Rathe, J.W. Lee *J. Pharm. Biomed. Anal.* 42, 2006, 573.

Noted importance of temperature:
F Sadoun, H. Virilizer, P.J. Arpino *J. Chromatogr.* 647, 1997, 351.

Temperature tuning the detection

Post column heating (prior to expansion to AP) homogenizes the eluent and results in more efficient (sensitive) ESI ionization and a more stable MS signal (like observed with HPLC & previously for hot APCI).



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

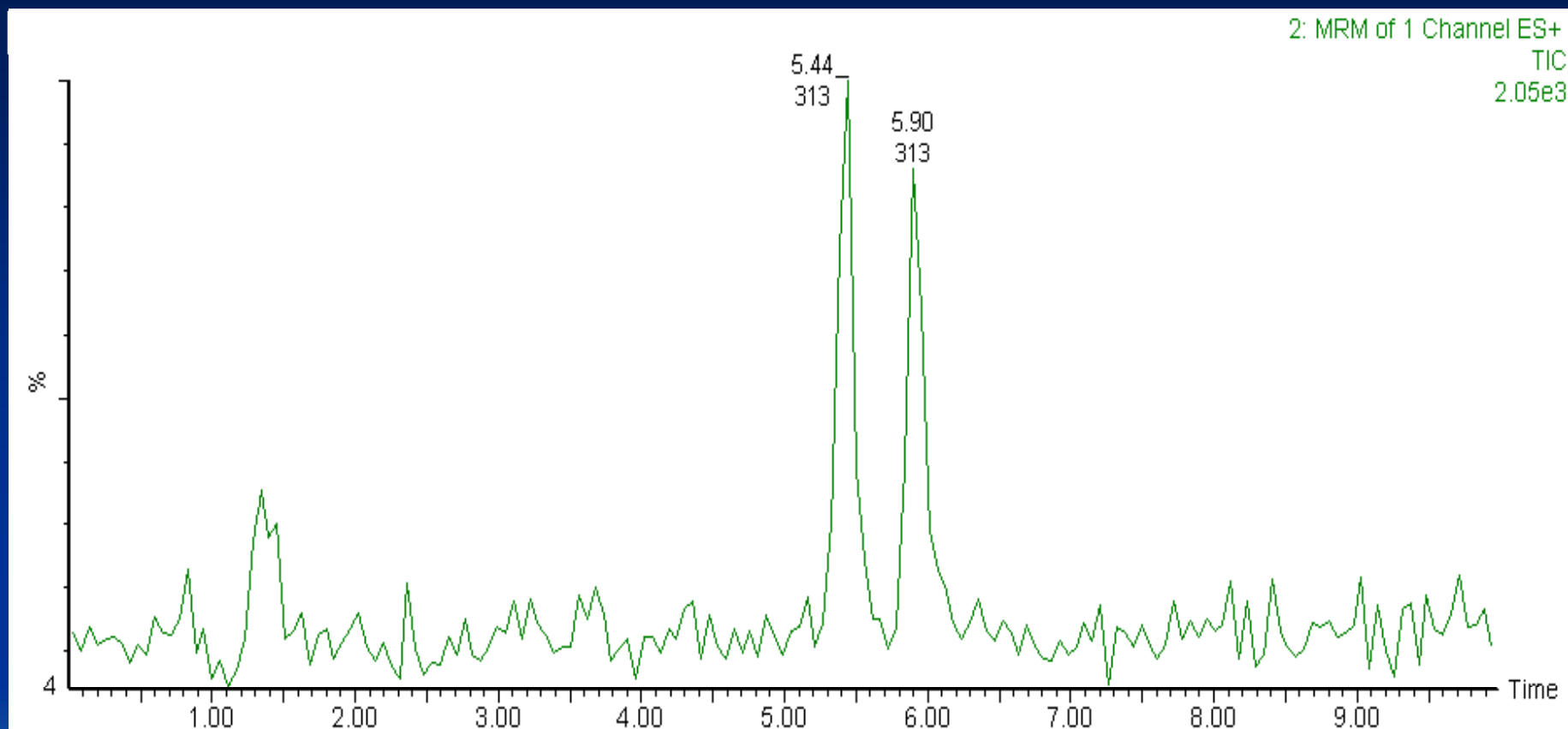
Post column heat source: Selerity CaloraTherm MS: Waters Quattro Premier XE Z-Spray

Now it makes sense that so many with SFC gravitated toward well heated APCI!

Expected 40°C drop in temperature on expansion to AP is consistent with:
F Sadoun, H. Virlizier, P.J. Arpino *J. Chromatogr.* 647, 1997, 351,
where 50°C at AP was found optimal for the SFC input into ESI (custom heated sprayer).

Temperature tuning the detection

makes ng/mL measurements routine (helps get answer quicker)



Full eluent flow at 3 mL/min into ESI source with post column heating to 90°C gives at least 5 fold better sensitivity than any split / make up flow / pre-column heating combination we have found thus far. Also, nice linear dynamic range (10^3).

[10 μ L plasma injection at ~25 ng/mL total - after 4/1 ACN ppt]

Example 3: Better Use of SFC/MS/MS

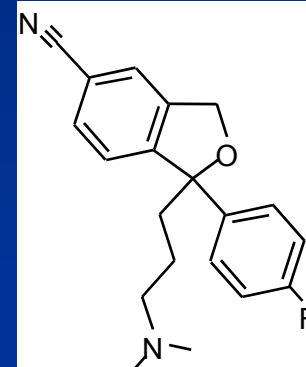
(Development –larger study, can show compound, now using heated interface)

Overall study objective (met):

- To investigate the inhibitory effect of R-citalopram on S-citalopram (escitalopram, Lexapro[®]) produced increases of brain serotonin levels in rats. Rats were dosed chronically via an osmotic minipump placed in under the skin in the scapula area. The R-citalopram and S-citalopram doses were chosen to target the clinically relevant plasma concentrations measured in patients treated with racemic citalopram (Celexa[®]).

Addition analytical learning objectives:

- Large number of animals (44) and samples in study allows testing of enantiomer ratio variation → can 2% precision for different enantiomer availabilities be achieved in larger study and what does it mean?
- Thoroughly test the full flow, heated eluent approach for SFC connected to cold ESI → is it robust?
- Thoroughly compare SFC/MS/MS with LC/MS/MS → are they truly comparable yet?

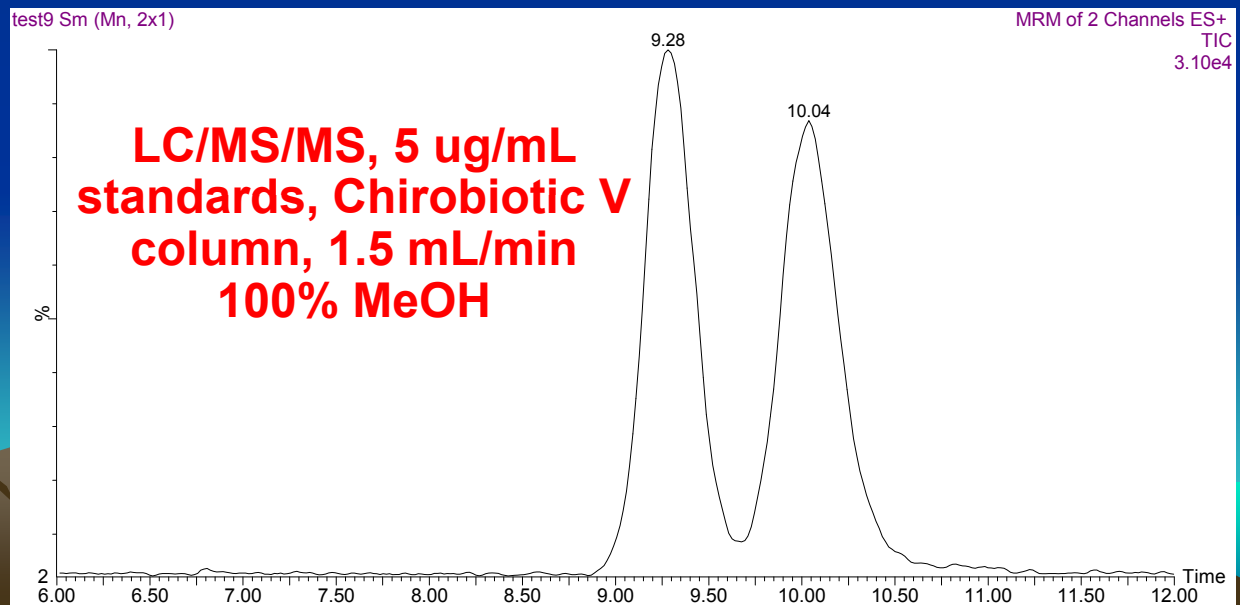
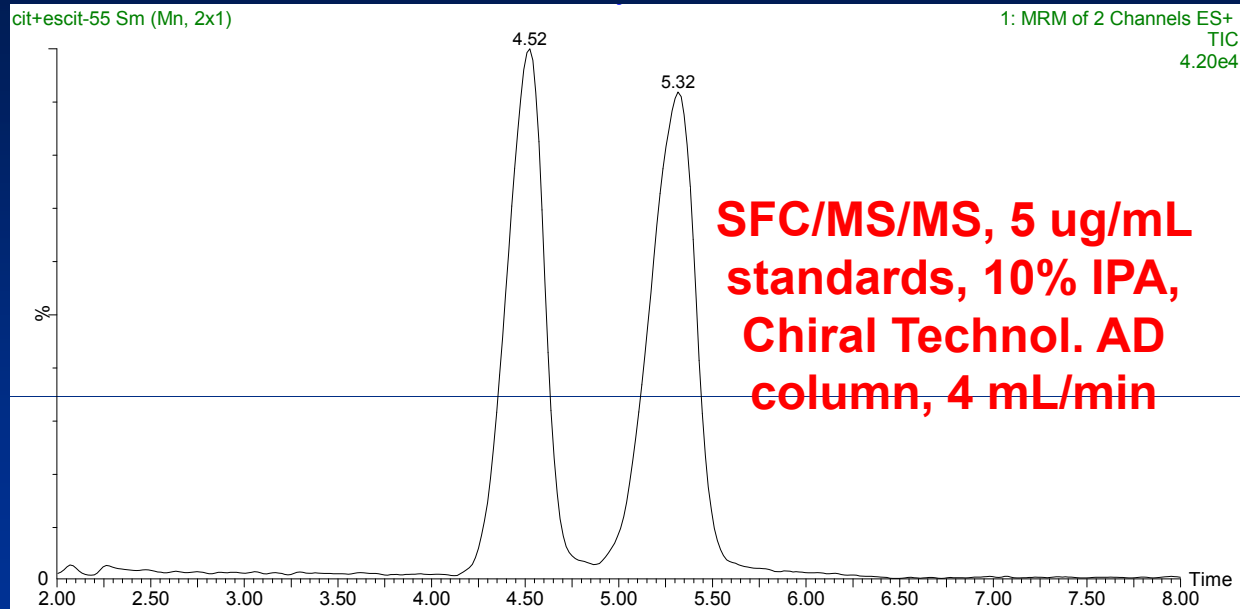


In the end, we still had lessons to learn!

S-Citalopram is the most selective SSRI:

K. Bezchlibnyk-Butler, L. Aleksic, S.H. Kennedy, *J. Psychiatry Neurosci.*, 25, 2000, 241.

First step: SFC and LC methods

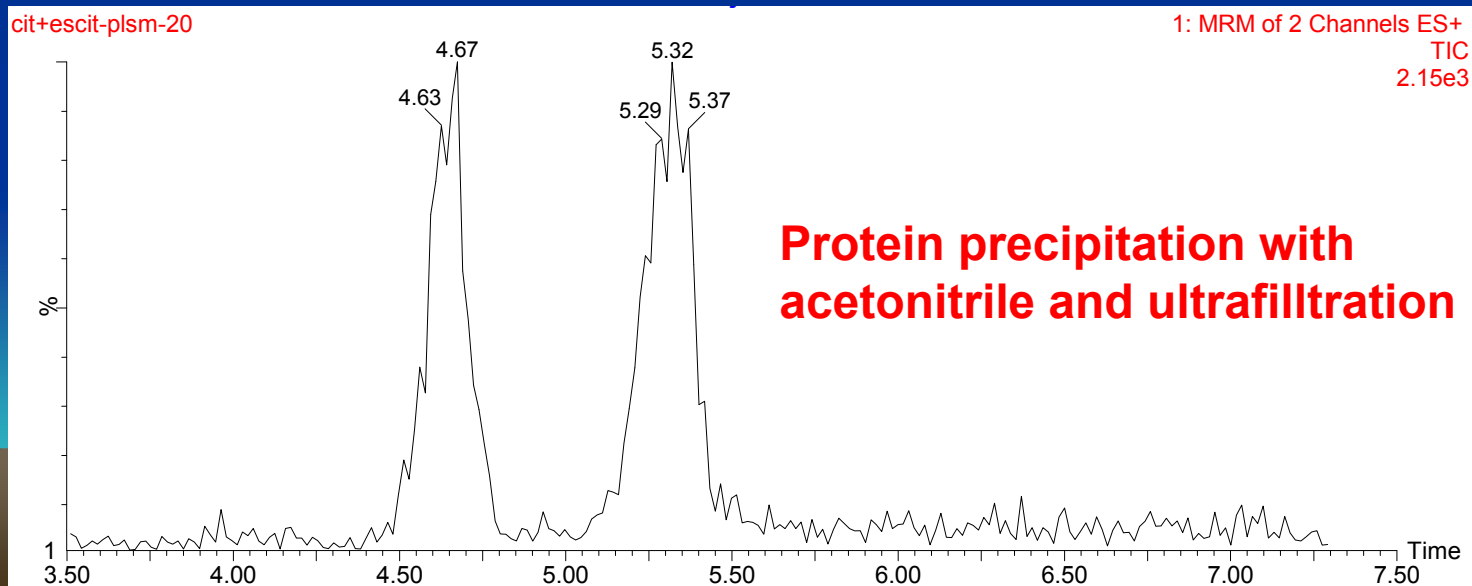
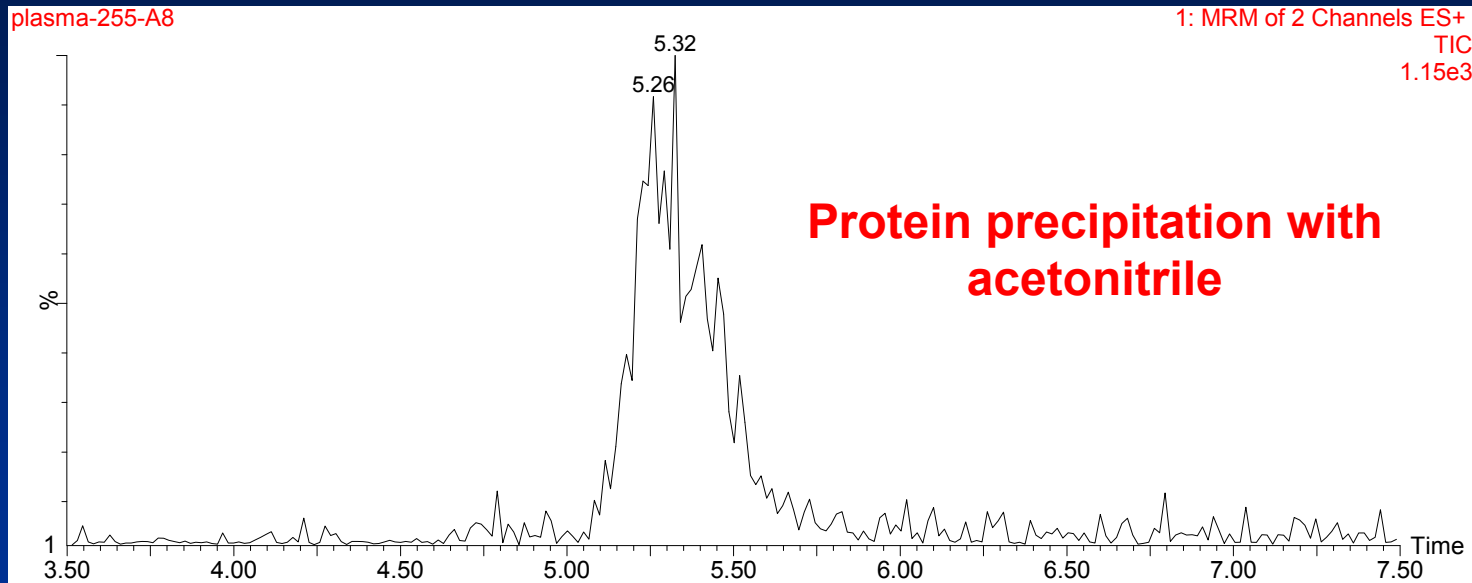


First step: SFC and LC methods

- SFC conditions (inst: Thar/Berger analytical FCM + CTC):
 - Column: Chiralpak AD, 4.6 x 250 mm, 5 μ m
 - Modifier: 10% Isopropylalcohol (IPA) with 0.1% DEA (diethylamine)
 - Flow rate: 4 mL/min
 - Column temperature: 35 °C
- LC Conditions (inst: Waters Acquity UPLC pump + AS):
 - Column: Chirobiotic V (5 μ m, 250 \times 4,6 mm ID), Astec # 11024 with precolumn: Ultron ES-OVM-G 10 \times 4 mm, Part No. 712111630 from Agilent.
 - Mobile phase: Ammonium trifluoroacetate 0.1% in methanol
 - Flow: 1.5 mL/minute (split to MS 1:5)
 - Column temperature: 20°C
- MS/MS conditions (Waters Quattro Premier XE)
 - Positive ESI mode and Double MRM at 325/109 and 325/262

Next step: Try a sample with matrix

Lesson time – need to further consider matrix effects!

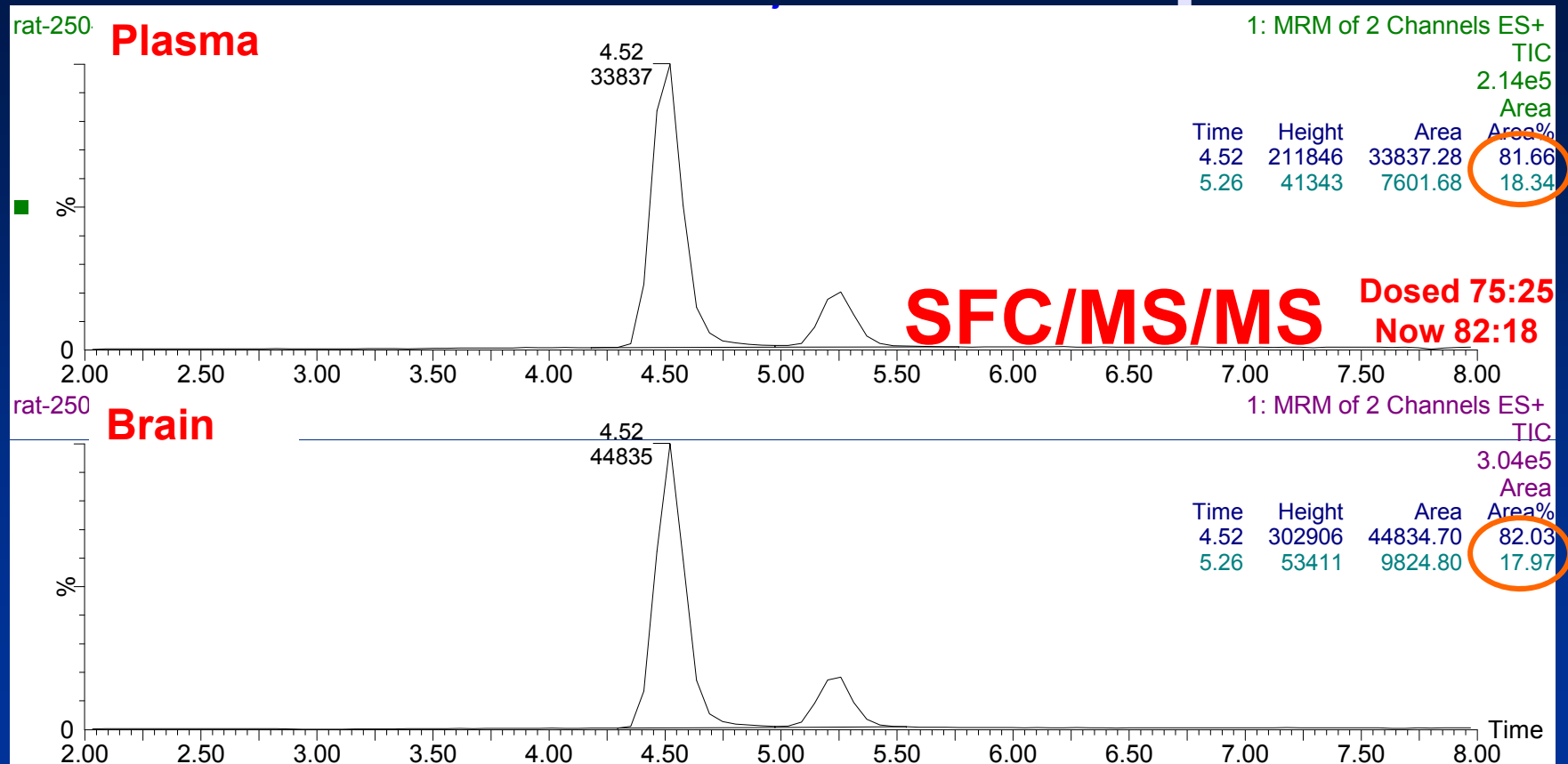


Revised sample clean up: Liquid-liquid extraction with pH help

Sample preparation

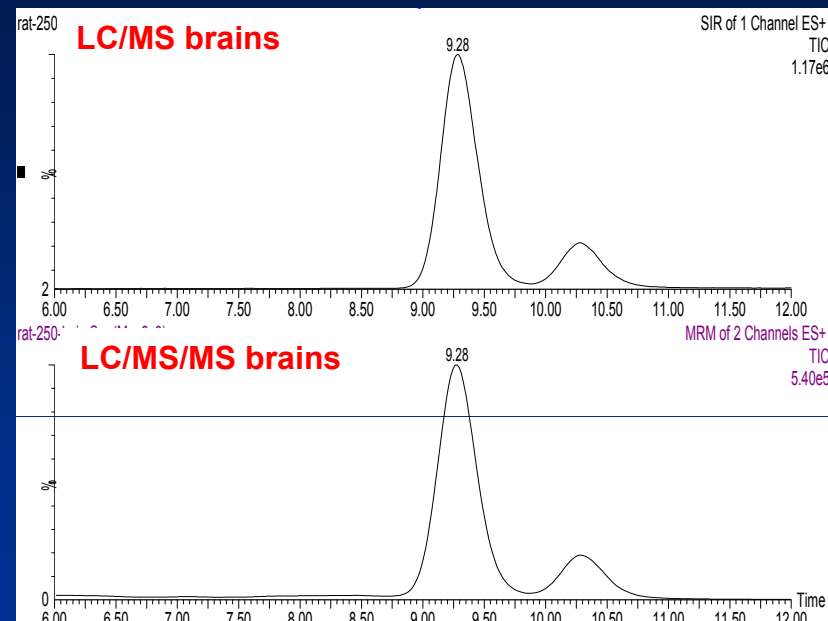
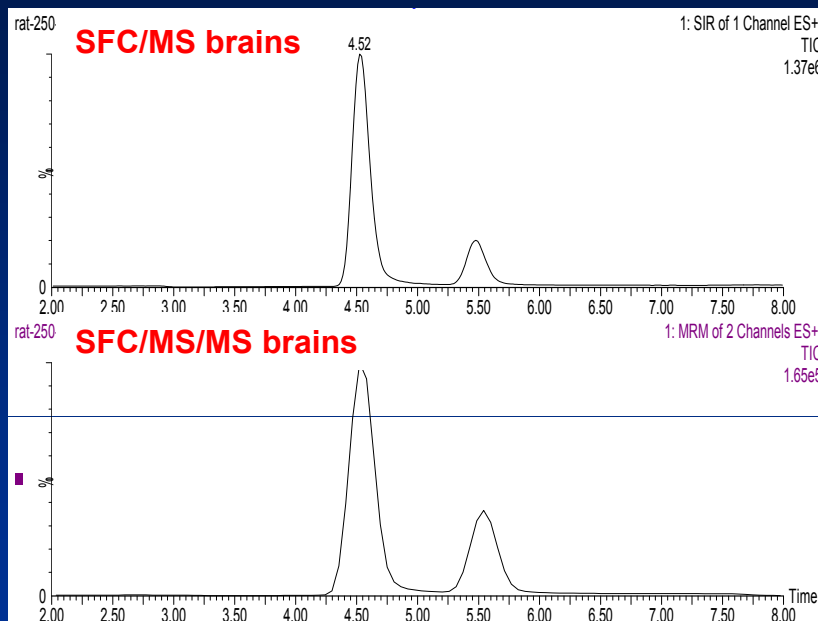
- Addition of 50 μl 1 N Sodium Hydroxide, the samples (0.2 to 1 mL) are extracted with 6.0 ml Heptane:Isoamylalcohol (98.5:1.5) by shaking or vortexing for 15 minutes. After centrifugation for 5 minutes at 3000 rpm (2000G) the aqueous phase is frozen in an ice bath made of dry ice in ethanol. The organic phase is transferred to new tubes containing 0.1 ml Hydrochloric Acid 0.1N. These tubes are shaken or vortexed for 15 minutes and centrifuged for 5 minutes at 3000 rpm. The samples are again frozen in an ice bath and the organic layer discarded.
- The aqueous phase is thawed and approximately 75 μL vaporized to dryness and the residue dissolved in 500 μL methanol and transferred to auto-sampler vials.

Now with cleaner samples!



- At least 2 orders of magnitude more signal. $S/N > 1000$ for 10 μ L injection of citalopram @ ≈ 100 ng/mL in plasma.
- More signal to noise yields even better precision.
- In general, chiral separations are nowhere close to ordinary reverse phase gradients (often easily measured with just ACN crash for prep) in their ability to avoid co-elution of interfering biological matrix components (SFC or LC).
- Also very interesting: dosed 75/25 R/S, observed average 82/18 R/S, and *S* is the potent enantiomer!

SFC/MS/MS vs. LC/MS/MS and MS/MS vs. MS (for both SFC & LC)



- 2 samples from 44 animals + half of all samples were serially diluted 3 times to ensure no detector saturation for a total of 210 samples each run 4 times. LC and SFC used the same MS/MS. Peak shape and response were steady throughout the 840 analyses.
- SFC → S/N 2-3k with MS > MS/MS.
- LC → S/N 6-9k with MS/MS > MS (LC vs. SFC = MeOH vs. IPA?).
- Animal to animal variation in enantiomer ratio < 2%.
- Brain to plasma (same animal) variation in enantiomer ratio ≤ 1%.

What did we learn about SFC and its use in chiral bioanalysis?

- Sample preparation / clean up is more crucial for chiral bioanalysis (LC or SFC). However, MS/MS may not be needed with good clean up (MS suffices).
- Simple heated SFC interface (prior to expansion to AP) with full flow into ESI-MS has shown to be robust for many batches of 100s of bioanalysis samples.
- After learning the lessons described here, we find that SFC and LC can work equally well when using “off the shelf” cold ESI-MS detection. Previously observed primarily for well heated APCI-MS.
- Now, using ESI, functionally equivalent bioanalysis performance levels (precision, sensitivity, dynamic range) as well as robustness can be achieved with LC/MS/MS or SFC/MS/MS.
- Chiral method development is still significantly easier by SFC (routine generic gradients work).

What did we learn about chiral bioanalysis?

- Observation of enantiomer ratios that are different from dosed ratio appears to be a general feature of chiral bioanalysis.
- Even though the total fraction absorbed typically varies by a factor of 2 animal to animal, these different enantiomer ratios tend to have far less variation than the total fraction absorbed.
- We hypothesize that all ADME aspects of enantiomers are identical (i.e. not stereo specific) except for the mechanism(s) and hence rates of metabolism. This hypothesis (and our observed basis data) is entirely consistent with what is known in general* and for the well studied drug citalopram.**
- The rates of metabolism appear to be different in all cases we have examined thus far and this leads to the ee always being different than dosed but essentially the same ($\sigma \leq 2\%$) for all samples taken at the same time (even those from different animals).

*Recent review on stereo-selective drug metabolism:
H. Lu, *Expert Opin. Drg Metab. Toxicol.*, 3(2), 2007, 149.

*Recent review on stereo-selective pharmacokinetics:
D.R. Brock, *Biopharm. Drug Dispos.*, 27, 2006, 387.

**Studies showing S-citalopram metabolizes faster than R-citalopram:

J. Sidhu, M. Priskorn, M. Poulsen, A. Segonzac, G. Grollier, F. Larsen, *Chirality*, 9, 1997, 686.

L.L. von Moltke, D.J. Greenblatt, G.M. Giancarlo, B.W. Granda, J.S. Hartmatz, R.I. Shader, *Drug Metab. Disp.*, 29(8), 2001, 1102.

F.C. Kugelberg, B. Carlsson, J. Ahlner, F. Bengtsson, *Chirality*, 15, 2003, 622.

Summary

- SFC/MS and SFC/MS/MS are showing truly promising results for the study and understanding of stereo specific effects observed *in vivo*.
- Active control of SFC eluent temperature before the column can significantly enhance selectivity of the chiral separation.
- Active control of SFC eluent temperature after column (heated interface to MS) can be used to readily achieve low ng/mL MS sensitivities with full flow (3-4 mL/min) into a standard (non-heated) ESI source (instead of split flow and the usual heated APCI).
- The precision, sensitivity, and dynamic range of LC and SFC with MS detection are functionally equivalent and routinely provide truly comparable quantitative results. *Chiral method development easier by SFC.*
- For the limited number of compounds examined thus far, stereo specific availability has been observed in all cases with good precision [ca. 1-2%] even animal to animal. *This is a real effect* and is presumed to be exclusively the result of different stereo-specific rates of metabolism.
- Thus far, the observed *in vivo* biological effects (efficacy) have generally (but not always) been consistent with measured differences in availability (more available = more active). Despite our energetic search for it, we have yet to see *in vivo* enantiomer inter-conversion.
- In one case we have examined, citalopram, the efficacious enantiomer (S) is not the one with the greater *in vivo* availability (R).