

"Methods Development Process for Bioanalysis"

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What is Bioanalysis?

- In “Bioanalysis”, we are “measuring the quantity of molecules of interest (analytes) in a complex biological matrix.” This matrix is can be plasma, serum, whole blood, bone marrow, CSF, microsomes, hepatocytes, hair, brain, bile, or any other type of biological tissue, fluid, waste product, or substance.

To measure, we must separate.

- Biological samples contain thousands of small and large molecule compounds. We must purify our compounds of interest if we are going to see them above the background.
- Our method must be “specific” for our analyte.

Paths to molecular specificity

- Molecular size – size exclusion chromatography
- Iso-electric point – electrophoresis
- Partition coefficient relative to stationary phase and mobile phases – normal and reverse phase chromatography, Solid Phase Extraction (SPE)
- Partition coefficient relative to two immiscible solvents – liquid/liquid extraction
- Ion pairing – Ion Exchange Chromatography, and ion exchange SPE

Further specificity using Mass Spec²

- Molecule must be ionized
- Ion must be same nominal mass of correct analyte ion species
- Ion must form same fragment ion, as analyte ion species

Terminology

- LLOD – Lower limit of Detection
 - The smallest amount your system can distinguish reliably from background, (but not necessarily be able to quantitate)
 - Typically about 3x signal to noise
- LLOQ – Lower limit of Quantitation
 - The lowest passing standard on your system's linear standard curve, and smallest amount of analyte your system can measure with a reasonable degree of confidence
 - Typically about 10x signal to noise
- ULOQ – Upper limit of Quantitation
 - The highest passing standard on your system's linear standard curve and the largest amount of analyte your system can measure with a reasonable degree of confidence

Terminology Continued

- BLQ – Below limit of Quantitation
 - Any number that comes in less than your LLOQ
- ALQ – Above limit of Quantitation
 - Any number that comes in greater than your LLOQ
- QC – Quality control sample
 - An artificial sample made to mimic a real-life sample, but one for which you know the analyte concentration with reasonable confidence. QC samples should ideally be made by a different individual and using a different weighing than for the standards.
- Standards – Artificial samples used to produce you standard curve

Steps in Method Development

1. Look at your molecule and identify potential method development issues.
 - a. Potential ionization site. Positive ionization? (amine or other basic site) Negative Ionization? (carboxylic acid, alcohol, or other sit that will stabilize a negative charge)
 - b. How greasy/hydrophobic, or hydrophillic is your molecule?
 - c. What is the pKA? – key information

Develop the Mass Spec method

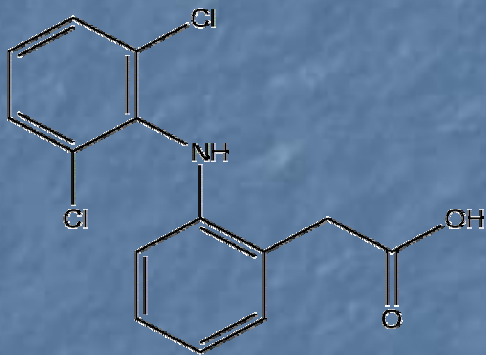
- Weigh out about 5 mg of your pure standard into a vial. Measure weight on a 0.1 or 0.01 mg analytical balance and record. Dilute to exactly 1.0 mg/mL in a solvent that will easily dissolve your compound. (Methanol or Acetonitrile usually – but DMSO is O.K. too.)
- Prepare two standards for your analytes in 50/50 Methanol/Water with 0.1% Formic Acid
 - 1 $\mu\text{g}/\text{mL}$
 - 100 ng/mL
- Set up the mass spectrometer to accept an infusion of 1 $\mu\text{g}/\text{mL}$ at 10 μL per minute.

Mass Spec method cont.

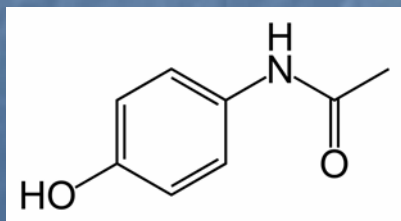
- Infuse 1 $\mu\text{g/mL}$ into Mass Spec and look at the total scan in Q1 across a range from 100 to 1000 amu (small molecules) in positive then negative ionization mode.
- Notice the following:
 - What is the most prominent peak?
 - Does the M+1 or M-1 mass show up clearly in the spectrum?
 - In which spectrum, positive or negative does the analyte show up most prominently?

Some structures to talk about.

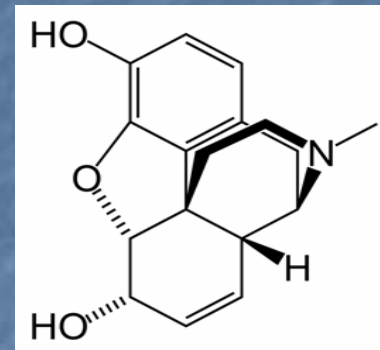
Diclofenac



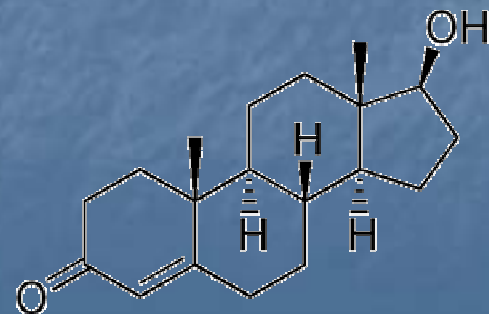
Acetaminophen



Morphine



Testosterone



MS method cont.

- Narrow scan range to 3 amu with M+ or M- focused in the center of the scan. Switch infusion solution to 100 ng/mL, (assuming a steady signal from the parent ion peak).
- Some instruments will auto tune at this point or even earlier, in which case perform the autotune function.
- After autotune, tweak parameters such as gas flow and pressure not optimized in autotune.

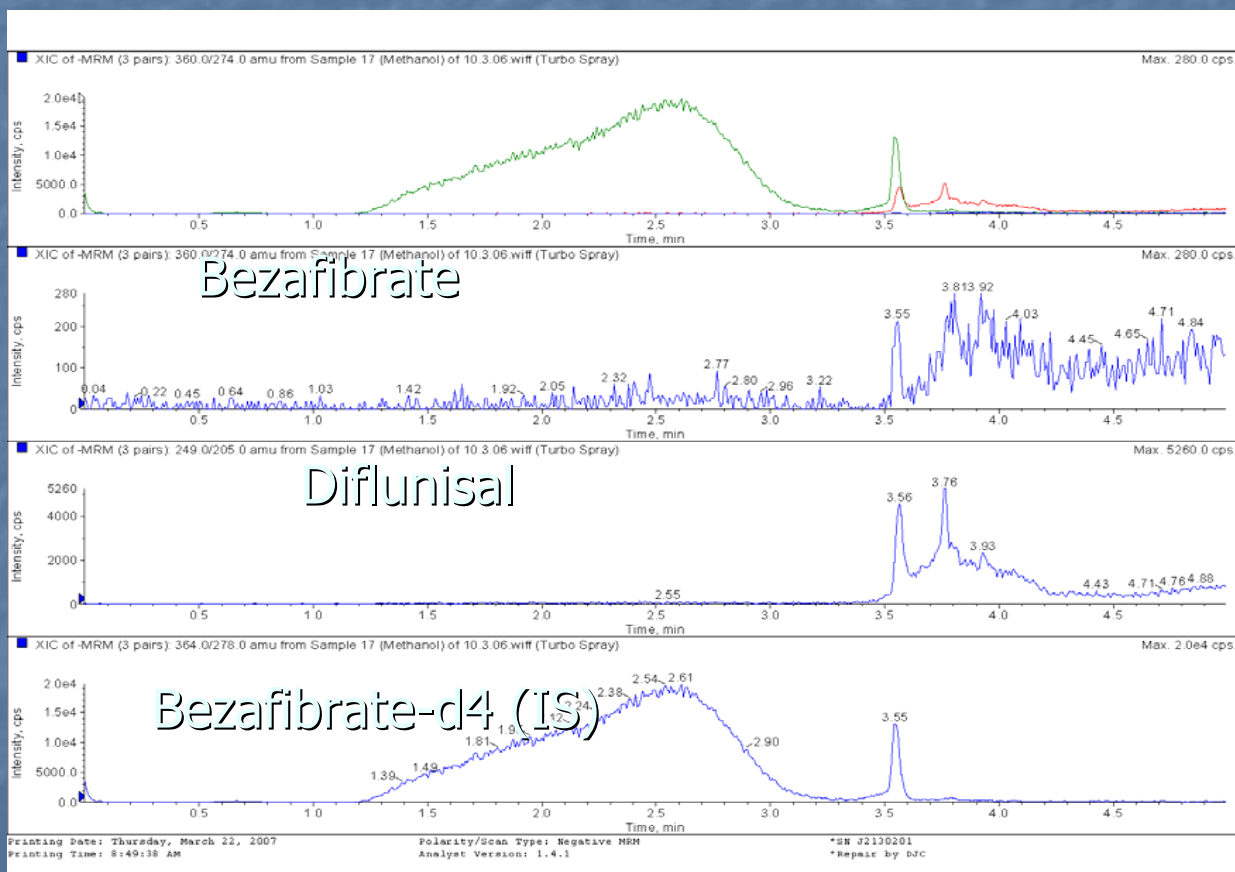
MS method cont.

- Set up LC to flow into Mass Spec and T in the analyte, (keep flow at 10 to 100 $\mu\text{L}/\text{minute}$).
- Set LC pumps to expected organic elution % for analyte, and set desired flow.
- Re-optimize gasses and spray needle position to obtain highest sensitivity

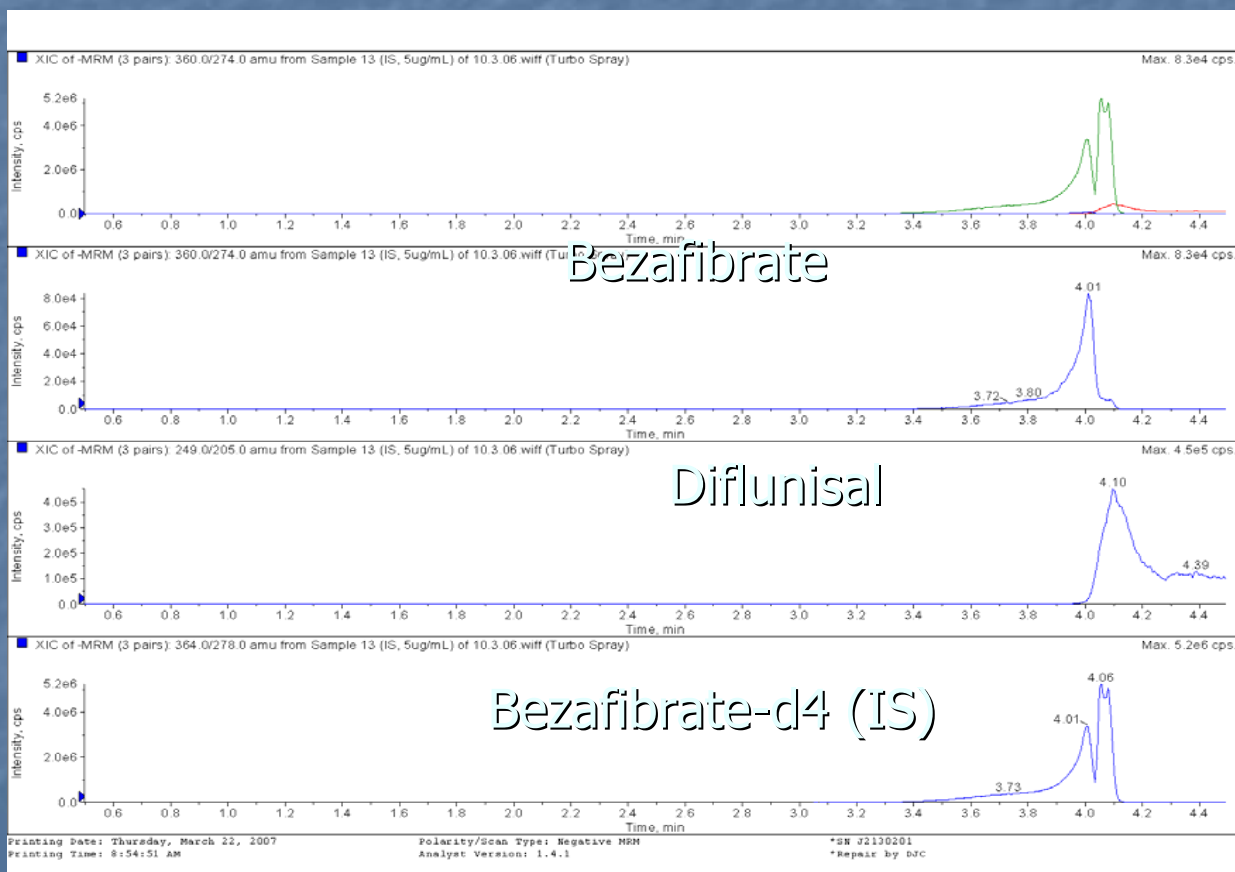
Ready to test neat standard.

- Set up preliminary LC system.
- Starting point:
 - Guess the pK_A of your compound. If it's an acid, start with 5 mM Ammonium Acetate (almost neutral pH). If it's a base, 0.3 % Formic Acid is a good starting point.
- Set up LC Gradient.
 - Good starting gradient is: begin at 10% Acetonitrile in 90% Water

Ghost Peaks after MeOH injection

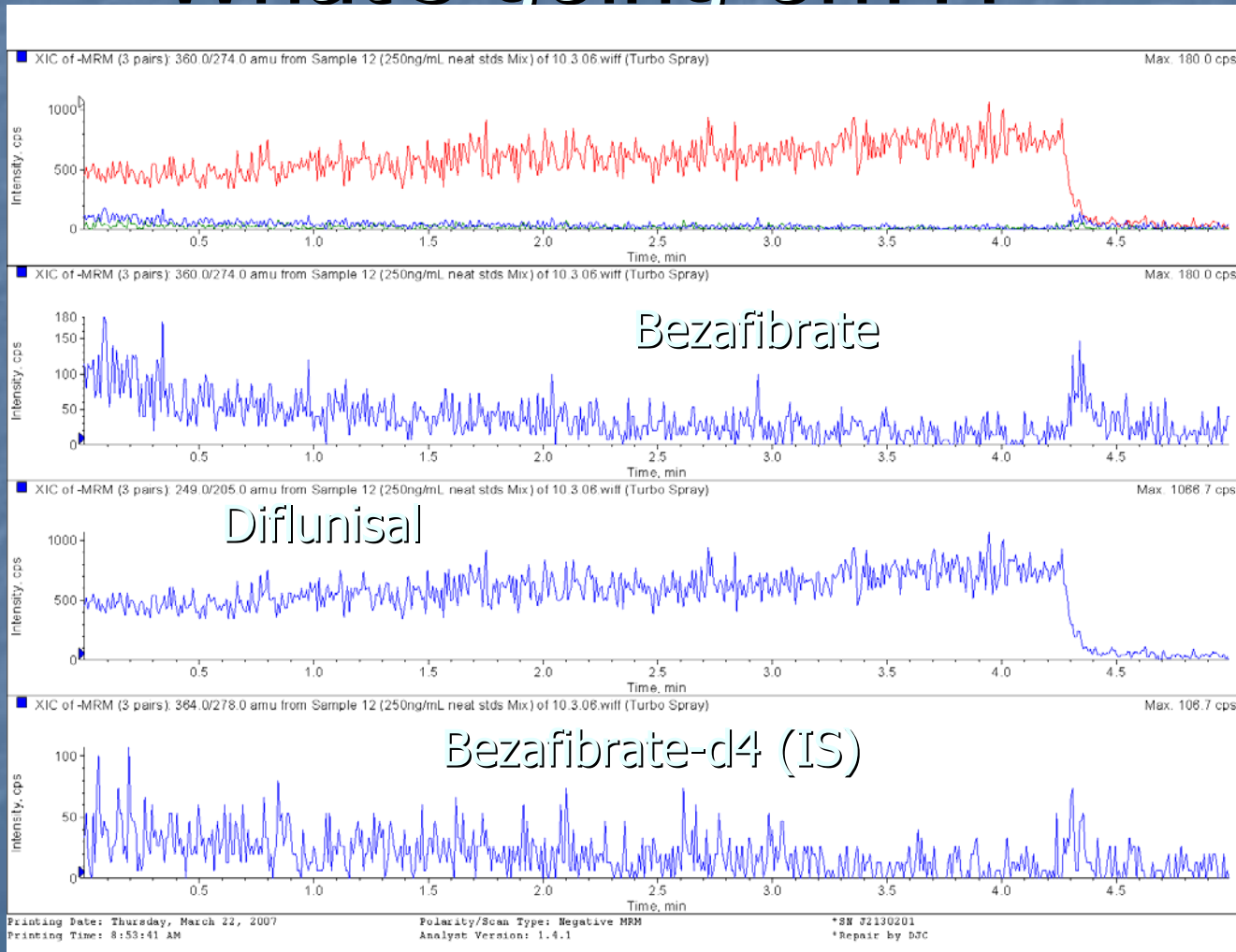


5 $\mu\text{g}/\text{mL}$, late retention poor peak shape... What's going on?

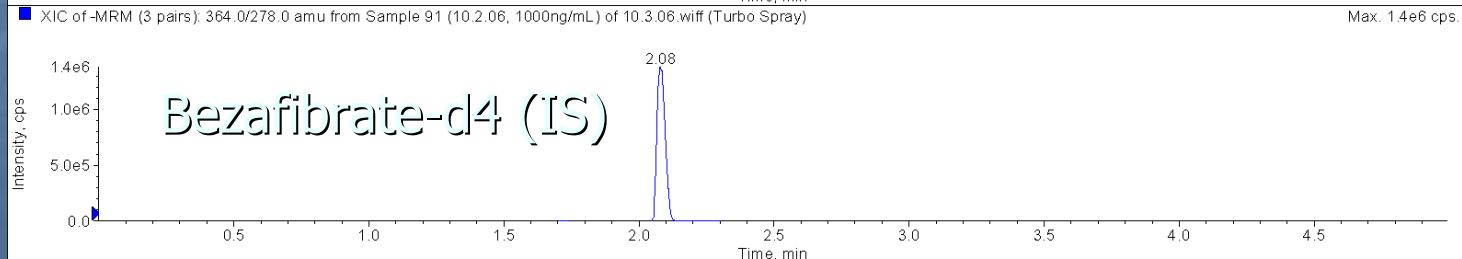
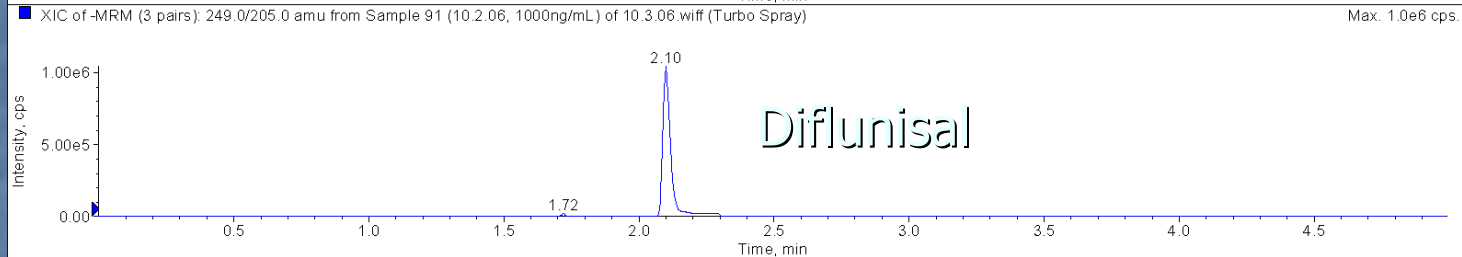
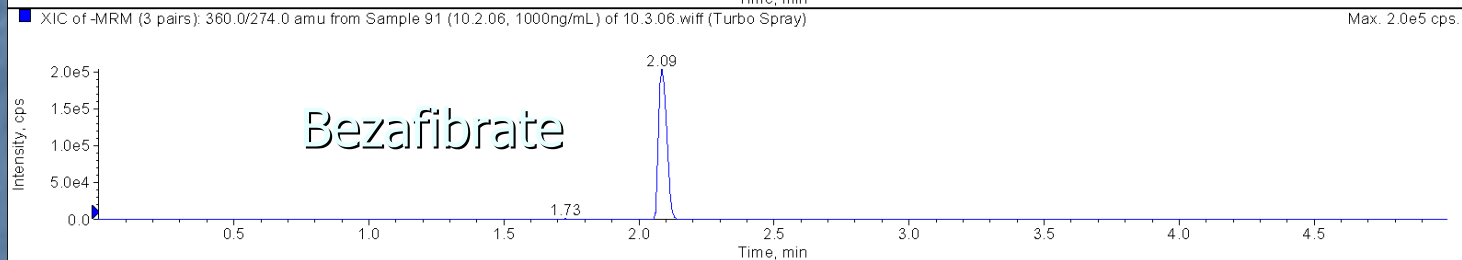
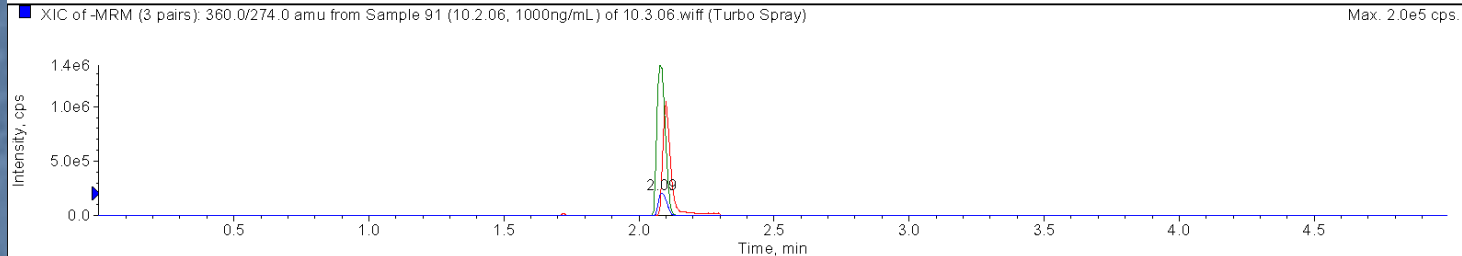


250 ng/mL injection

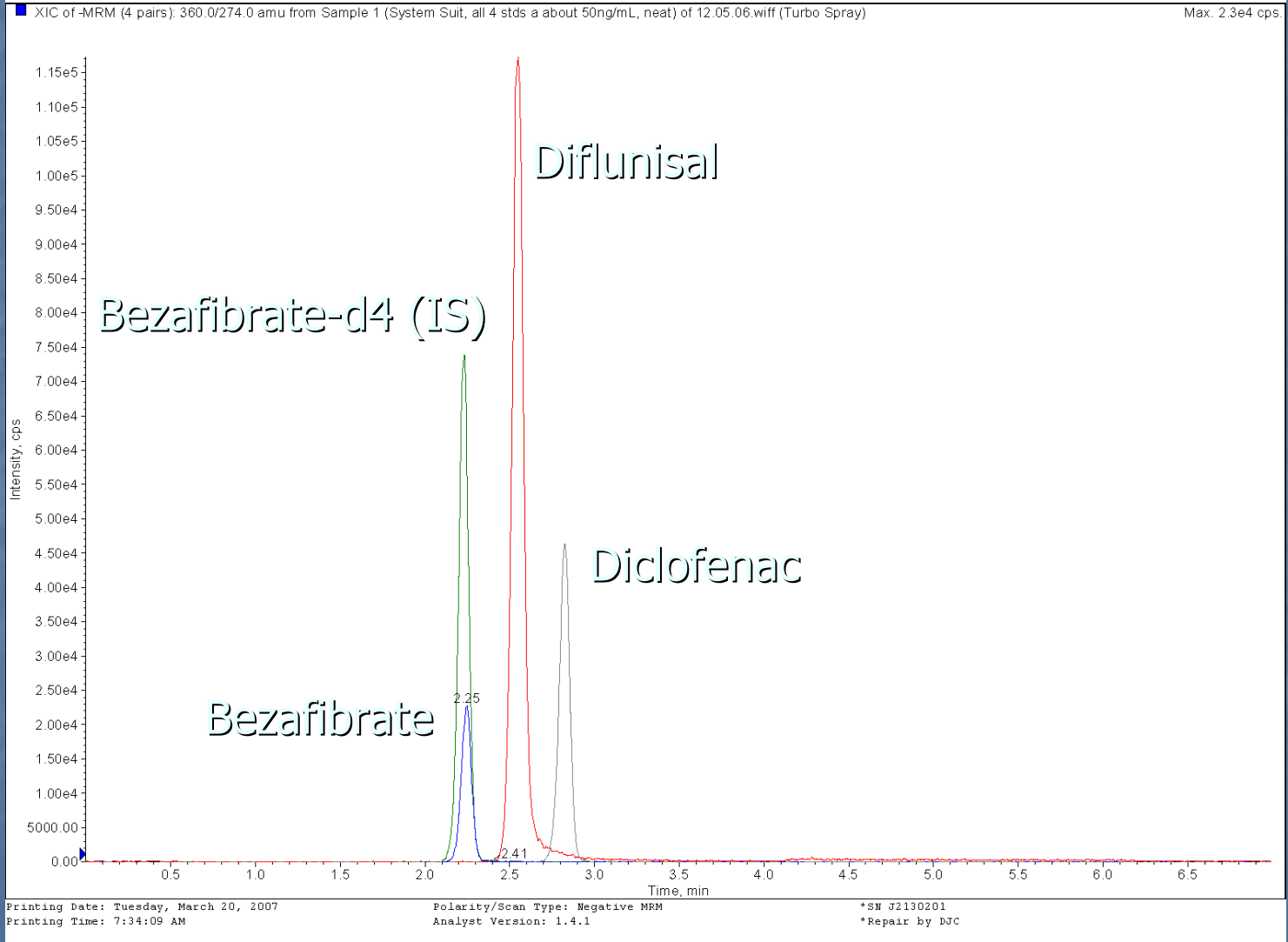
What's going on???



Higher flow now, using ballistic gradient.
Peaks are showing up nicely with good peak shape,
and good retention... But poor selectivity!



Modify gradient with shallow slope in working region. Now we have good Retention and Selectivity!!!



Ready to test neat standard, cont.

- Set up LC Gradient.
 - Good starting gradient is:
 - begin at 10% Acetonitrile in 90% Water, hold 30s
 - Increase to 25% organic in 30s
 - Increase to 95% organic in 120s
 - Hold at 95% organic for 120s
 - Decrease to 10% organic over 5s
 - Hold for 60s at 10% organic
- Inject 1 μL of 1 $\mu\text{g}/\text{mL}$ neat standard.

Evaluate situation and adjust.

- If peak is split, pKA of compound is probably close to pH of Aqueous mobile phase. Your compound should be ionized - so get the Aqueous MP away from your analyte in the direction of ionization.
- Acids – make MP higher pH using Ammonium Hydroxide.
- Bases - make MP lower pH (Formic Acid alone or even TFA).

Evaluate situation and adjust.

- Broad but gaussian peaks – try a less greasy stationary phase, C8 instead of C18. Increase flow rate. Increase steepness of gradient.
- Tailing – change pH, use less retentive column – confirm “fitness” of column with known standards.
- Overlapping analytes – make gradient more shallow and more in the “working zone”

Reinject and re-optimize...

- Change conditions and re-inject. When you get a good method with neat standards, re-optimize the mass spec for sensitivity.
- Set % organic and solvent flow to analyte peak elution %.
- T in 100 ng infusion standard and optimize MS/MS.
- Re-inject a neat standard curve between desired LLOQ/10 and ULOQ.
- Evaluate overall sensitivity and linearity.
- Neat standard should be visible at 1/2 desired LLOQ.

Decide on Internal Standard

- Ideal internal standard: Deuterium or other stable isotope labeled standard that is at least $-d_4$. (C13 is fine too – but Deuterium is used in 99% of “Stable Labeled” cases.)
- Also O.K. – but usually with less precision and accuracy – another compound that is as close as possible in physicochemical nature to your analyte. For example, the compound with a one carbon longer or shorter chain. An isomer that separates by HPLC and fragments differently may also be used, but this is dangerous. Substituting a hydrogen with a fluorine is also good. A completely different compound may also be used in the case of protein precipitation methods, but as extraction becomes more specific to the analyte (LLE or SPE) the IS may not perform well at all.

Extraction Method Development

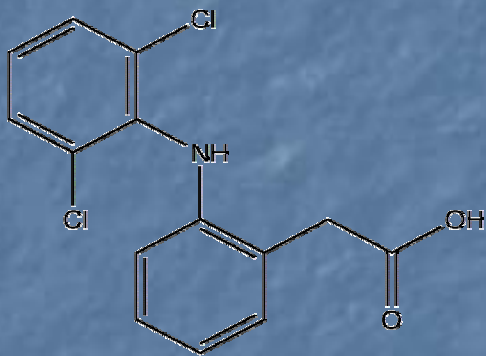
- Get your sample as clean as possible.
- Mass Spec is about signal to noise, not just signal.

Types of Extraction for Bioanalysis

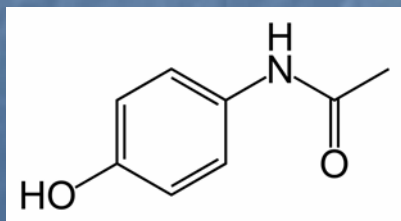
- Protein Precipitation
 - Easy and Fast
- Liquid/Liquid Extraction
 - Usually Cleaner – but more work.
- Solid Phase Extraction
 - Can be very specific and clean – good if you have a large sample to concentrate. Mixed mode Ion Exchange/C18 or HLB is usually best

Some structures to talk about.

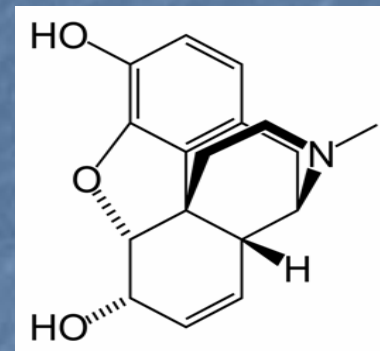
Diclofenac



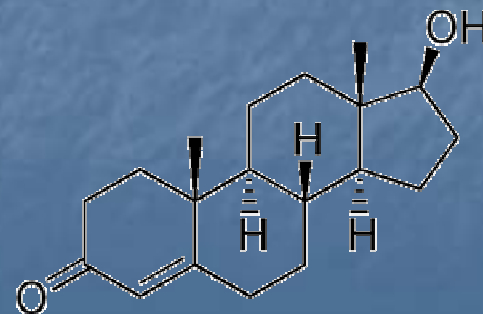
Acetaminophen



Morphine



Testosterone



Protein Precipitation

- For groups of samples < 500, this is usually the method of choice.
- Straightforward, easy, usually effective
- Fine for most drugs
- Tends to be less clean than other methods, but if a small amount is injected (1 or 2 μL), it's usually rugged enough.

Protein Precipitation Method

- Prepare a 50 to 500 ng/mL solution of your internal standard in either MeOH or AcN.
- Pipette 25 to 100 μ L of plasma, serum, brain or other matrix into a tube or plate well.
- Add 50 to 1000 μ L of IS/organic solution.
- Cap, and vortex vigorously for exactly X minutes (treat all samples exactly the same) X = 5 to 30. VWR multitube vortexer is the best for this. (Make sure caps don't leak during vortexing.)
- Centrifuge for ≥ 10 minutes at ≥ 3000 g.
- Transfer 96 well plate (or tubes) to autosampler.
- Make sure that autosampler needle stays well away from protein pellet in the bottom of tube/well.

Protein Crash cont.

- You can add the following step:
 - Transfer supernatant after centrifugation step to a clean 96 well plate or tubes. (Then centrifuge again.)
 - You may add the IS in a separate step, but this is usually unnecessary.

Notes:

- Try to inject as little as possible of the final solution to achieve desired LLOQ. Sometimes a more diluted sample (higher proportion of crash solution) will achieve better signal to noise).
- Traditionally, a 1:1 or 2:1 Organic:Plasma ration has been used. We believe that a 3:1 to 10:1 Organic to Plasma ratio is better. Samples are cleaner this way.

Liquid/Liquid Extraction

- Based on partitioning of analyte into an organic solvent that is immiscible with water (biological matrices are usually water based)
- Analyte should have a higher affinity for the organic solvent used than for water.
- Very good results with the more hydrophobic analytes such as steroids or sphingolipids.

LLE continued

- Choose a solvent that is close to polarity of analyte. However, it is always a good idea to run a systematic experiment using a wide variety of solvents.
- Extraction experiment: $n=3$, aliquot 50uL of a 200 ng/mL plasma standard. Extract with 500uL of each of the following solvents (18 samples)
- Solvents most often used for LLE:
 - Ethyl Acetate
 - MTBE
 - Hexane
 - Toluene
 - *Methylene Chloride (heavier than water)*
 - *Chloroform (heavier than water)*

Try these too

- Worth a try for LLE:*
- Bromo Benzene (*heavier than water, needs teflon liner or glass*)
- 2,2,2-tri-Chloroethanol (*heavier than water, needs teflon liner or glass*)
- Simply add the solvent to the sample, let sit for 30 minutes, sample close to bottom, drawing up 1 or 2 uL of sample and inject. These are very aggressive solvents – be careful.

* Personal communication from Mark Hayward

LLC Method

- Add pH buffer to neutralize sample, (sample:buffer, 50:50). For best extraction, analyte should not be ionized.
- Cap and Vortex
- Add extraction solvent. Solvent to sample ratio should be at least 2:1. 5 or 10:1 is preferable.
- Cap and Vortex
- Transfer organic supernatant, aqueous layer is often frozen first
 - If organic is heavier than water, transfer organic zone with pipette.
- Dry down sample under nitrogen with gentle heat (typically 40°C).
- Reconstitute sample in initial mobile phase mixture for gradient run (typically 10% organic, 90% A).
- Vortex
- Centrifuge
- Analyze by LC/MS/MS

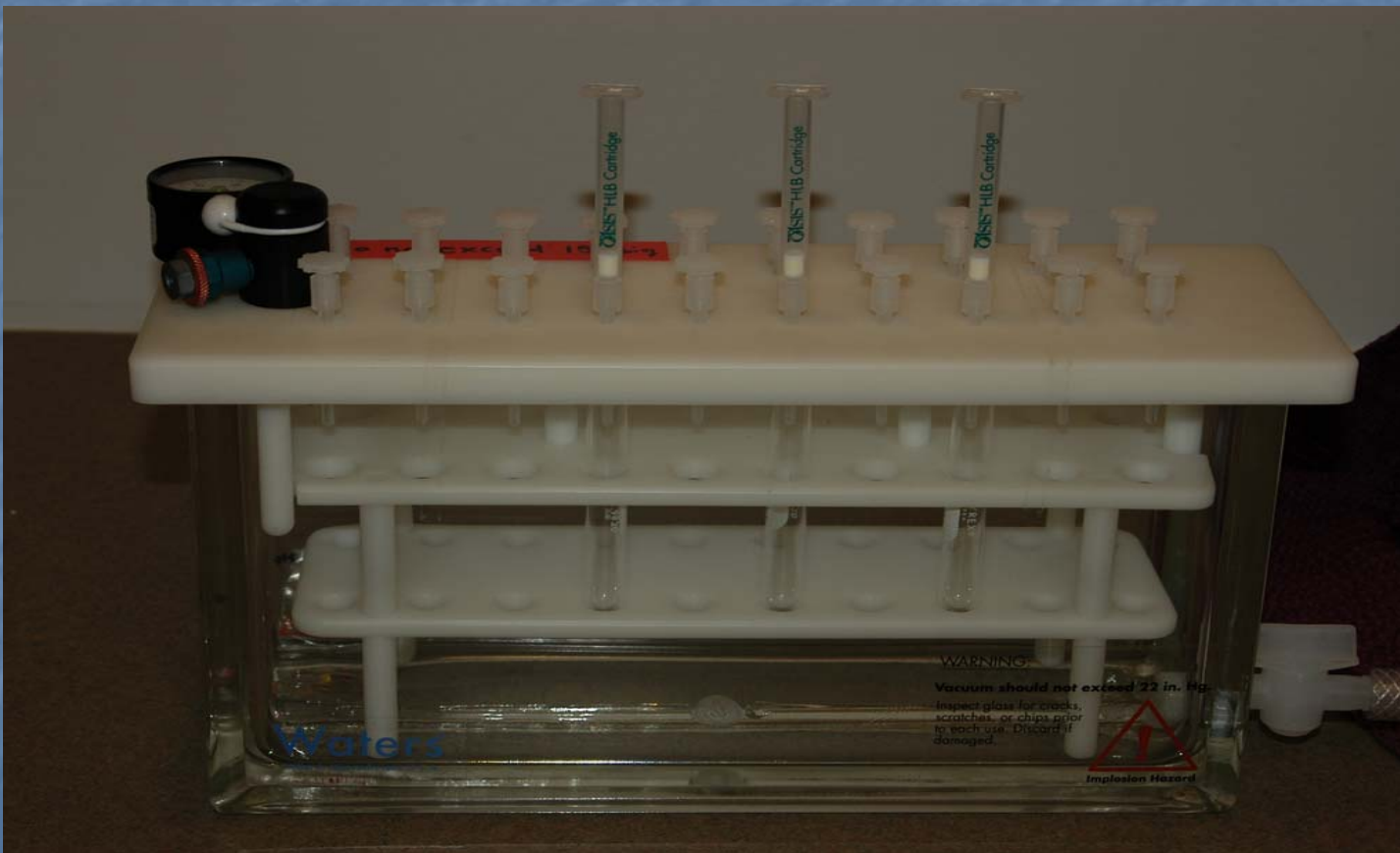
Solid Phase Extraction (SPE)

- Acts like a preliminary chromatography system.
- Works best in combination methods such as mixed ion-exchange/C18 or HLB
- Can be used to pre-concentrate a large volume of sample when very sensitive (low pg/mL) methods are required.

Discrete tube Solid Phase Extraction setup



Discrete tube Solid Phase Extraction setup, closeup



96 well plate format Solid Phase Extraction setup



Automated, 96 well plate evaporator with heat control



SPE continued

C18 or Waters HLB

- Pre-condition SPE cartridge with Methanol, then Aqueous
- Add sample
- Wash sample with aqueous and/or low strength organic (20 to 40% MeOH)
- Elute with high strength organic
- Dry under Nitrogen stream w/ gentle heat.
- Reconstitute, and centrifuge as in LLE method.

SPE Continued

Mixed Cation or Anion Exchange/HLB or C18

- Ionized Analyte binds strongly to counterion in SPE cartridge while aggressively washed with strong organic solution.
- Better for concentrating and cleaning samples for bioanalysis than straight C18 or HLB.
- More steps than other sample prep methods.

SPE Continued

Mixed Cation or Anion Exchange/HLB or C18

- Condition column with acidified methanol (2% formic acid in methanol)
- Wash with 2% FA in water
- Load sample onto column
- Wash with 2% FA in water
- Wash with 2% FA in pure methanol
- Elute with 5% ammonium hydroxide in methanol
- Dry and process as in previous C18/HLB SPE

Standard Curve

- This is your measuring stick with which you measure your samples and QCs.
- Standards must have $\leq 2\%$ organic solvent contribution.
- Lowest and highest standards must bracket your sample concentrations.
- Must have sufficient points to provide accurate measurements, (typically 8 or more points over 3 orders of magnitude).

Quality Control Samples

- Make up with a separate weighing.
- One person makes the standards, another makes the QCs.
- Different dilution scheme and different concentration than the standards.
- Organic solvent composition $\leq 1\%$
- Highest QC 70 to 90% concentration of ULOQ standard, Lowest QC may be 1.5 x conc. of LLOQ standard.
- QC samples mimic samples. They are the final judge of system fitness (not the standards).

Summary

- Make up Standards and QCs carefully. They are your measuring stick.
- Bracket real samples
- Clean samples and good chromatography make your method more sensitive and specific to your analyte
- Auto tune mass spec then tweak parameters (especially gas flow and temperatures) manually
- Keep Mass Spec clean – less is often more. Strive for good signal to noise, not high signal.

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