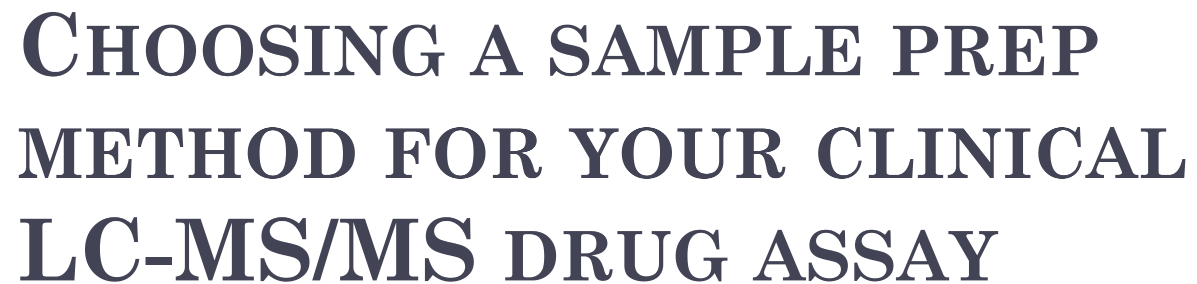

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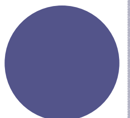
AIT Laboratories
IATDMCT Workshop, #W111
October 4, 2009

Why perform extractions?

- To detect all or specific analytes we must remove them from the matrix interferants
- Type of extraction varies with compound and willingness to sacrifice…
  - Chromatography
  - Column life
  - Speed of extraction
  - Instrument maintenance



Components of whole blood

Sugars
400 Da

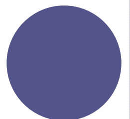
Vitamins
1000 Da

Proteins
10000 Da

Antibodies
150,000 Da

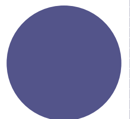
Blood clotting factors
50 – 300,000 Da

Small molecule drugs are <400 Da – needle in a haystack



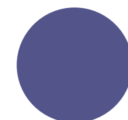
History of Extractions

- LLE, SPE, LLE with back extraction
- All of above, plus derivitization for GC methods
- Labor and supply intensive
- Shift to LC/MS then to LC-MS/MS
  - More selectivity
  - Reduced background
  - No requirement for derivitization
- Ideal techniques
  - Crash and shoot for Blood, Serum, Plasma, Tissue, Vitreous methods
  - Dilute and shoot for Urine methods
  - Not always this easy!



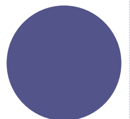
Protein Precipitation

- Due to the high protein content of whole blood a protein precipitation step is commonly performed prior to chromatographic analysis
- The removal of protein, salts and lipids from blood samples prior to LC-MS/MS is essential for obtaining good analytical results and maintaining instrument performance
- Protein precipitations result in the removal of such interferants

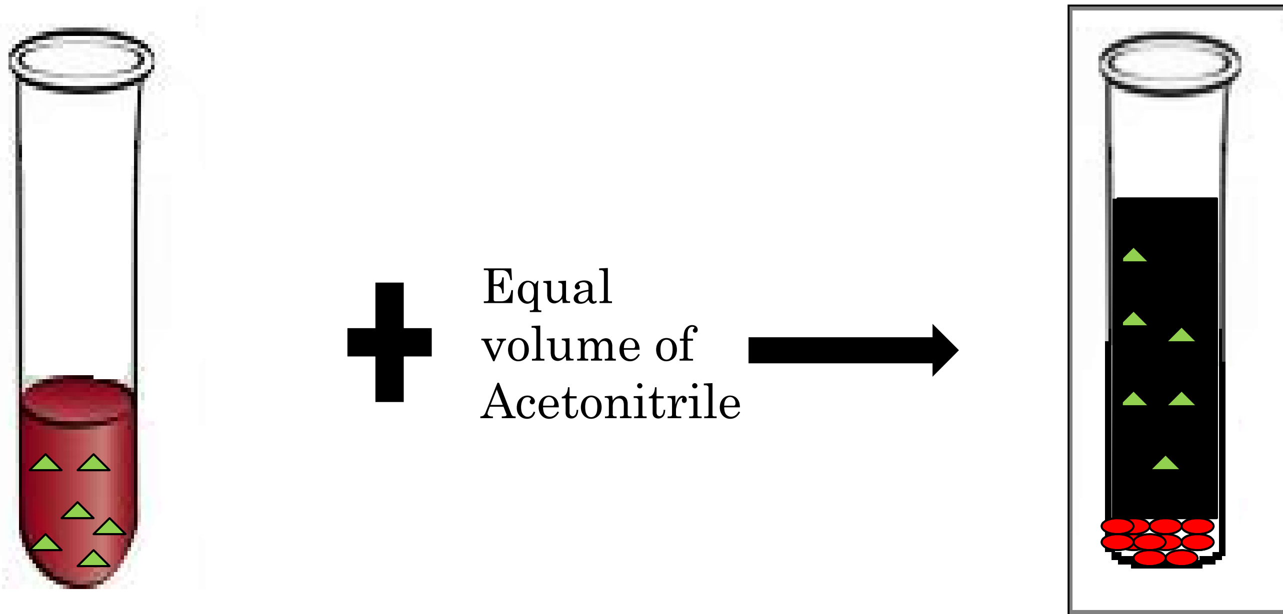


PROTEIN 'CRASHING'

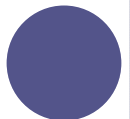
- Addition of organic solvents such as acetonitrile will cause proteins in the solution to denature and precipitate out
- The solid is called the precipitate and in most cases it will 'fall' or 'crash' out of the solute phase, then the sample is centrifuged to pellet the solids
- Once the proteins have been precipitated out of solution the remaining aqueous layer will contain drugs of interest



'Crash and Shoot' method



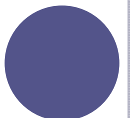
- Addition of an equal volume of ACN causes all of the proteins to fall out of solution
- Any drug present now exists in the acetonitrile layer and can be directly injected into the LC-MS/MS



4 METHODS TO COMPARE

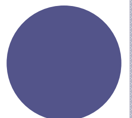
- Urine
  - Methadone & EDDP
    - Pain management, several hundred per day
  - Ethyl Glucuronide
    - Pain management, "no alcohol" contracts between patients and physicians

- Blood
  - THC & THC-COOH
    - Postmortem
  - General Unknown Screen
    - Postmortem



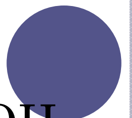
Urine Methadone & EDDP

- 500 µL sample, calibrator, controls
  - Single point calibrator at 300 ng/mL
  - Controls at 225 ng/mL, 375 ng/mL, negative
  - El Sohly calibrators and controls
- 2 mL internal standard
  - Methadone d^3, EDDP d^3 at 75 ng/mL each, prepared in ultrapure water
- Vortex 20 seconds
- Centrifuge 5 min at 3000 x g
- Transfer to vial or microplate
- Run on UPLC-MS/MS
  - Column Waters HSS T3 2.1 x 50mm, 1.8 µm particle size
  - Sciex 3200 MS/MS
  - Mobile phases: 0.1% formic acid in water, 0.1% formic acid in MeOH



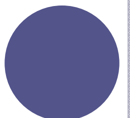
Urine Ethyl Glucuronide (EtG)

- 1000 µL sample, calibrator, controls
  - 9 point calibration curve, 0 to 10000 ng/mL
  - Controls at 250 ng/mL, 1000 ng/mL
  - All calibrators and controls prepared in house with synthetic urine
- 50 µL internal standard
  - EtG d^5 10 µg/mL in MeOH
- Vortex 20 seconds
- Centrifuge 10 min at 3000 x g
- SPE
  - UCT Clean Screen EtG Carbon
  - Elute with 1% formic acid in MeOH – Make fresh daily
  - Dry down under nitrogen, reconstitute with water
- Transfer to vial or microplate
- Run on UPLC-MS/MS
  - Column Waters HSS C_{18} 2.1 x 150mm, 1.8 µm particle size
    - Column held at 50°C for enhanced resolution
  - Waters TQD MS/MS
  - Mobile phases: 0.1% formic acid in water, 0.1% formic acid in MeOH



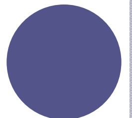
Methadone vs. EtG

- Methadone – simple dilute & shoot
- EtG – SPE method
- Questions
  - Why can we use a dilute & shoot with Methadone, but have to use SPE for EtG?
  - Not a typical reverse phase SPE column for EtG?
  - Why can we use a single point curve with Methadone vs. a multi-point curve for EtG?
  - Are there tradeoffs for the simple Methadone extraction



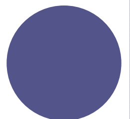
Methadone vs. EtG

- Why dilute & shoot for Methadone vs. SPE for EtG
  - Ion suppression from co-eluting matrix interferents can be a major problem in LC-MS/MS
  - Methadone different enough from interferents that co-eluting compounds are low
  - EtG, an ethyl group attached to glucuronic acid, is more similar to the interfering substances
  - Methadone tends to be easier to ionize via ESI than EtG, so there is less of an impact by interferents
- Why the atypical column for EtG? (Graphite carbon column)
  - When using RP chromatography, extraction method should not be based solely on RP, or you will concentrate co-eluting interferents
  - Need to selectively separate the analyte from interferents
    - Different mobile phase pH
    - Alternative SPE chemistries - mixed-mode, ion-exchange, amine, or graphite



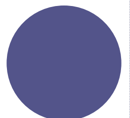
Methadone vs. EtG

- Single point calibrator for Methadone, but a multi point curve for EtG?
  - On any instrument the total response curve for an analyte tends to be sigmoidal in shape
  - Generally dilute/concentrate analytes so that the AMR falls within the linear portion of this curve
  - Response curve for Methadone is linear in our target range, with just a dilution
  - Because EtG is a poor ionizer, we had to concentrate the samples to get adequate signal
    - This results in more species in the source, possibly a saturation effect at the top end of our range causing the curve to flatten out
    - Need a quadratic fit model, thus more curve points



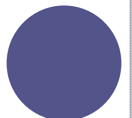
Methadone vs. EtG

- Don't appear to be any tradeoffs for the simple Methadone extraction
  - One of our cleanest methods on the instrument
  - Chromatography universally good
  - Column life is very good
    - We run Methadones on same column as Buprenorphine/Nor (one of our dirtiest assays) get roughly 1000 injections per column
    - Do use precolumns
    - On HPLC-MS/MS, didn't run with anything else, 3000-5000 injections.
  - Small gains in column life don't outweigh costs of more robust Methadone extraction
  - EtG column life of ~1200 injections
- They can't all be Methadones!



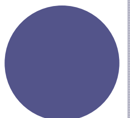
Blood General Unknown Screen

- 250 µL sample and controls
  - 7 controls, containing ~110 analytes in all, prepared in NaF treated negative blood
- 1 mL crash solution
  - Cold Acetonitrile/Proadifen IS at 10 ng/mL
- Vortex 5 minutes
- Centrifuge 5 min at 3000 x g
- Transfer to glass vial or microplate
- Run on UPLC-ToF MS
  - Column Waters HSS T3 2.1 x 100mm, 1.8 µm particle size
  - Waters LCT Premier ToF MS
  - Mobile phases: 0.1% formic acid in water
  - Optima grade MeOH – very important



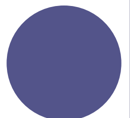
Blood THC & THC-COOH (part 1)

- 500 µL sample, calibrator, controls
  - 8 point calibration curve, 0 to 100 ng/mL
  - Controls at 10 ng/mL, 40 ng/mL
  - All calibrators and controls prepared in house with NaF treated blood
- 1 mL crash solution
  - Cold Acetonitrile containing THC d^3 & THC-COOH d^3 at 25 ng/mL each
- Vortex 2 minutes
- Let stand 5 minutes
- Vortex 10 seconds
- Centrifuge 10 min at 3000 x g
- Transfer to glass culture tube
- 1 mL water
- Vortex 10 seconds



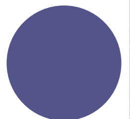
Blood THC & THC-COOH (part 2)

- SPE
  - Cerex Polychrom THC, SPEWare
  - Wash (Water/ACN/Ammonium Hydroxide) & elution (hexane/ethyl acetate/glacial acetic acid) solutions - make fresh daily
  - Dry down under nitrogen
  - Reconstitute with 100 µL ACN
  - Vortex 5 minutes
  - Add 150 µL ACN/Water (50/50)
  - Vortex 5 minutes
- Transfer to vial or microplate
- Run on UPLC-MS/MS
  - Column Waters BEH C_{18} 2.1 x 50mm, 1.7 µm particle size
  - Waters Quattro Premier XE MS/MS
  - Mobile phases: 0.1% formic acid in water, 0.1% formic acid in ACN



GUS VS. THC

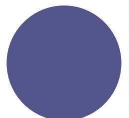
- Blood screen – simple crash & shoot
- THC – crash & shoot, followed by SPE, followed by dry down and recon in ACN first, then addition of ACN/water
- Questions
  - Why does the crash & shoot not work for THC?
  - Are there any tradeoffs for using the crash & shoot for blood screens?
  - Why the two step reconstitution for THC?



GUS vs. THC

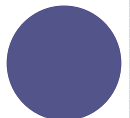
- Crash & shoot for GUS method
  - Benefits
    - Speed & High throughput (~2000 samples per month)
    - Amenable to a wide range of analytes in a wide range of matrices
      - ~330 compounds in library
  - Drawbacks
    - More matrix suppression, higher background
    - No concentration of low level analytes, smaller on column values
    - No dilution of high level analytes, possible detector saturation and false negative
    - Must supplement ToF with Immunoassay
      - Opiates, Oxycodone
      - Cannabinoids

- SPE method THC & THC-COOH
  - Need additional sensitivity, thus must concentrate sample
    - LLOQ is 1 ng/mL for THC & THC-COOH
  - Less matrix suppression with SPE, important with low concentration drugs



GUS vs. THC

- Blood THC column life
  - We change column at ~1000 injections, but probably not necessary
  - 1700 injections on one column, still looked good
- GUS column life
  - Approximately 1200 to 1800 injections
  - Had one column last until 2300 injections
  - Majority of our samples for GUS are postmortem matrices
- "Double" reconstitution for THC
  - THC is very 'sticky'
  - If acetonitrile:water blend used by itself, THC tends to stick to the tube
  - By using the acetonitrile first, THC fully dissolves in organic, then dilute with the 50:50 acetonitrile:water blend to make it more compatible with the UPLC method & to fully solubilize the THC-COOH



Thank You!

- Questions??

- Please feel free to e-mail us with any questions:
  - aterrell@aitlabs.com or
  - toxicologist@aitlabs.com