Antifungals  TDM and DAU Chiral Analysis by LCMSMS

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AACC Past President

Mass Spectrometry and Separation Sciences for Laboratory Medicine
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Disclosure

• LCMSMS Grant – ThermoFisher (2012-14)

• Speaker honorarium – Beckman

• Consultations – Preferred Pain Management/Heag/PCLS
Acknowledgement

• Dr. Elizabeth Palavecino
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• Lilly Yee (UWI –Mil.)
• Mark E Hindsdale
• William Nell
• Andrew Vennis
• ThermoFisher scientists
• Sciex scientists
Objectives

1. Antifungals TDM

2. Chiral Analysis:
   - Basic
   - Amphetamines
   - Fluoxetine (LC & CE)
   - Methadone
Voriconazole references


Voriconazole

- Invasive fungal diseases - significant morbidity and mortality in immunocompromised patients
- Voriconazole - a triazole antifungal, the first line treatment of invasive fungal infections
- Voriconazole – high inter-individual variations, non-linear saturation pharmacokinetics, and a narrow therapeutic range
- PO – 200 mg to 300 mg Q12 h 2.5-fold AUC
- Bioavailability - 96%
Voriconazole PK

- $T_{\text{max}}$ – 1 – 2 hr, $T_{1/2}$ - 6 h, SS – 5 d, $V_d$ – 4.6 L/Kg
- Metabolism – CYP 2C19 (major determinants of toxicity, Asian with higher frequency), 2C9 and 3A4
- Inhibitory effect on CYP 2C19, 2C9 and 3A4
- May increase calcineurin inhibitors such as CsA by 2 to 3 times
- Excretion – extensive hepatic metabolism with <2% unchanged

http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021630s023lbl.pdf
Voriconazole TDM

TDM - maximize the efficacy, decrease the risk of toxicity and improve the treatment response in invasive fungal infection

• Trough conc.
• Treatment for > 1 week and more than additional 7 days
• Toxicity
• Drug interaction
• Therapeutic range: 1- 5.5 mg/L

Voriconazole
3/10-9/21/15, n=146

<table>
<thead>
<tr>
<th>Conc. mg/L</th>
<th>n</th>
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<tr>
<td>&lt;1.0</td>
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<td>1.0-5.5</td>
<td>79</td>
<td>54</td>
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<tr>
<td>&gt;5.5</td>
<td>30</td>
<td>21</td>
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</table>
A voriconazole patient

• 53 yr old male, haploidentical peripheral blood stem cell transplant for his AML in remission with cells from his son

• Post-transplant complications- intractable hiccups with management leading to serotonin syndrome, chemotherapy induced nausea and vomiting, mucositis, neutropenic methicillin-sensitive staph aureus bacteremia with sepsis, multifocal pneumonia with positive Cytomegalovirus and Aspergillus platelia from Broncho alveolar lavage

• Improved clinically with Ganciclovir and Voriconazole oral doses 500 mg two times daily

• Serum Voriconazole conc. measured by mass spectrometry, showed with corresponding oral doses in next table.
A Voriconazole patient (cont.)

Comments

- Even with critical values, physician continues on given patient a high dose Voriconazole because of invasive aspergillosis.

- After improvement, oral dose lowered to 300 mg twice daily as a prophylactic dose.

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<tr>
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<td>500 mg two times daily</td>
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<td>10.7</td>
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<td>500 mg two times daily</td>
<td>14.9</td>
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<td>5-4-15</td>
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<td>5-7-15</td>
<td>500 mg two times daily</td>
<td>8.2</td>
<td>critical</td>
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<tr>
<td>5-11-15</td>
<td>500 mg two times daily</td>
<td>5.9</td>
<td>critical</td>
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<tr>
<td>5-21-15</td>
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<td>6-23-15</td>
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<td>6-29-15</td>
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<tr>
<td>9-14-15</td>
<td>300 mg two times daily</td>
<td>1</td>
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Posaconazole

- Posaconazole - a novel lipophilic antifungal triazole that inhibits cytochrome P450-dependent 14-α demethylase in the biosynthetic pathway of ergosterol.

- Broad-spectrum against opportunistic, endemic and dermatophytic fungi - organisms such as *Candida glabrata*, *Candida krusei*, *Aspergillus terreus*, *Fusarium* spp. and the Zygomycetes.

- Oral suspension

- $V_d$ - 5 l/kg, $t_{1/2}$ - 20 h.

- Not metabolized significantly, primarily excreted in an unchanged

- Although it is inhibitory, cytochrome P3A4 has no effect on 1A2, 2C8, 2C9, 2D6 and 2E1 isoenzymes, and therefore, a limited spectrum of drug–drug interactions can be expected.

- Pharmacokinetic studies in special populations revealed no necessity for dosage adjustment based on differences in age, gender, race, renal or hepatic function.

- Wake Forest patients (n=3 from 8/27-9/11/15) – (0.10 & 0.12) <0.19 and 0.20 mg/L
Posaconazole references


Distribution of serum posaconazole levels obtained by the Fungus Testing Laboratory, San Antonio, TX, from 26 December 2007 through 30 December 2008.

Voriconazole and Posaconazole LCMSMS assays

- Mix 100 µL aliquot patient samples or calibrator + 300 µL precipitating reagent (Methanolic D3 Voriconazole or D3 Posaconazole)
- Vortex for 1 min for deproteinization
- Centrifuge at 4000 RPM for 10min
- Mix 10 µl of the supernatant and pipette into 2ml vials and add 990 µl water.
- LCMSMS: C-18 column, and quantitation fragments: Voriconazole - 281 m/z; Posaconazole - 614 m/z
- Reference range: Vor. - 1.0 to 5.5 mg/L; Pos – 0.7 to 3.65 mg/L
- Calibration Ranges: Vor. - 0.1 to 6.2 mg/L; Pos - 0.19 to 5.54 mg/L
- CVs: Vor. - 0.7 to 5.5%; Pos. - 3.5 to 5.5%
- Recent published voriconazole protocols: Ref. 7, 8 (LCMSMS) and 9 (GC/MS)
Voriconazole monitoring (6 standard and patient)
Posaconazole

0.46 mg/L standard

0.10 mg/L patient
Objectives

1. Antifungals TDM

2. Chiral Analysis :
   - Basic
   - Amphetamines
   - Fluoxetine (LC & CE)
   - Methadone
Two enantiomers of a generic amino acid that is chiral
Chiral references


Chiral Recognition in Separation Science: Overview  G. Scriba

\[ (R)-A + (R)-S \xleftrightarrow{K_r} [(R)-A---(R)-S] \]

\[ (S)-A + (R)-S \xleftrightarrow{K_s} [(S)-A---(R)-S] \]

32 Chapters

1. Chiral Recognition in Separation Science: An Overview
2. Enantioseparations by Thin-Layer Chromatography
3. Gas-Chromatographic Enantioseparation of Unfunctionalized Chiral Hydrocarbons: An Overview
4. HPLC Enantioseparation on Cyclodextrin-Based Chiral Stationary Phases
5. Enantioseparations by High-Performance Liquid Chromatography Using Polysaccharide-Based Chiral Stationary Phases: An Overview
6. Common Screening Approaches for Efficient Analytical Method Development in LC and SFC on Columns Packed with Immobilized Polysaccharide-Derived Chiral Stationary Phases
7. Chiral Separations by HPLC on Immobilized Polysaccharide Chiral Stationary Phases
8. Enantioseparations by High-Performance Liquid Chromatography Using Macrocyclic Glycopeptide-Based Chiral Stationary Phases: An Overview
9. Enantioseparations of Primary Amino Compounds by High-Performance Liquid Chromatography Using Chiral Crown Ether-Based Chiral Stationary Phase
10. Screening of Pirke-Type Chiral Stationary Phases for HPLC Enantioseparations
11. Enantioseparations by High-Performance Liquid Chromatography Based on Chiral Ligand-Exchange
12. Enantioseparations by High-Performance Liquid Chromatography Using Molecularly Imprinted Polymers
13. Chiral Mobile Phase Additives in HPLC Enantioseparations
14. Chiral Benzofurazan-Derived Derivatization Reagents for Indirect Enantioseparations by HPLC
15. Separation of Enantiomeric 1-(9-Anthryl)-2,2,2-trifluoroethanol by Sub-/Supercritical Fluid Chromatography
16. Chiral Separations by Simulated Moving Bed Method Using Polysaccharide-Based Chiral Stationary Phases
17. Enantioseparations by Capillary Electrophoresis Using Cyclodextrins as Chiral Selectors
18. Application of Dual Cyclodextrin Systems in Capillary Electrophoresis Enantioseparations
19. Enantioseparations in Nonaqueous Capillary Electrophoresis Using Charged Cyclodextrins
20. Use of Macro cyclic Antibiotics as the Chiral Selectors in Capillary Electrophoresis
21. Application of Polymeric Surfactants in Chiral Micellar Electrokinetic Chromatography (CMEKC) and CMEKC Coupled to Mass Spectrometry
22. Cyclodextrin-modified Micellar Electrokinetic Chromatography for Enantioseparations
23. Cyclodextrin-Mediated Enantioseparation in Microemulsion Electrokinetic Chromatography
24. Chiral Separations by Capillary Electrophoresis Using Proteins as Chiral Selectors
25. Enantioseparation by Chiral Ligand-Exchange Capillary Electrophoresis
26. Experimental Design Methodologies in the Optimization of Chiral CE or CEC Separations: An Overview
27. Chiral Capillary Electrophoresis–Mass Spectrometry
28. Application of Chiral Ligand-Exchange Stationary Phases in Capillary Electrochromatography
29. Polysaccharide-Derived Chiral Stationary Phases in Capillary Electrochromatography Enantioseparations
30. Open Tubular Molecular Imprinted Phases in Chiral Capillary Electrochromatography
31. Enantioseparations in Capillary Electrochromatography Using Sulfated Poly β-Cyclodextrin-Modified Silica-Based Monolith as Stationary Phase
32. Cyclodextrin-Mediated Enantioseparations by Capillary Electrochromatography
13 of 32 Chapters
Chiral Recognition in Separation Science: An Overview
Polysaccharide Chiral Stationary phases, Scriba (Ed.)

1. HPLC Enantioseparation on Cyclodextrin-Based Chiral Stationary Phases
2. Enantioseparations by High-Performance Liquid Chromatography Using Polysaccharide-Based Chiral Stationary Phases: An Overview
3. Common Screening Approaches for Efficient Analytical Method Development in LC and SFC on Columns Packed with Immobilized Polysaccharide-Derived Chiral Stationary Phases
4. Chiral Separations by HPLC on Immobilized Polysaccharide Chiral Stationary Phases

1. Enantioseparations by High-Performance Liquid Chromatography Using Macrocyclic Glycopeptide-Based Chiral Stationary Phases: An Overview

2. Screening of Pirkle-Type Chiral Stationary Phases for HPLC Enantioseparations

3. Enantioseparations by High-Performance Liquid Chromatography Based on Chiral Ligand-Exchange Capillary Electrophoresis

1. Enantioseparations by Capillary Electrophoresis Using Cyclodextrins as Chiral Selectors

2. Use of Macrocyclic Antibiotics as the Chiral Selectors in Capillary Electrophoresis

3. Chiral Separations by Capillary Electrophoresis Using Proteins as Chiral Selectors

4. Chiral Capillary Electrophoresis–Mass Spectrometry

5. Cyclodextrin-Mediated Enantioseparations by Capillary Electrochromatography
Chiral separation

- TLC
- GC
- HPLC
- Supercritical fluid chromatography
- Capillary electrophoresis
- Capillary electrokinetic chromatography (EKC)
- Micellar EKC
- Microemulsion EKC
- Capillary electrochromatography

- Indirect methods – enantiomers reacting with an enantiopure reagent, pair of diastereomers forms, separation under achiral condition, e.g. GC

- **Direct methods** - *enantiomeric separation in a chiral environment, e.g. HPLC Chirobiotics*
Validation of LC-TOF-MS screening for drugs, metabolites, and collateral compounds in forensic toxicology specimens


• LC-TOF-MS – gentle electrospray ionization, accurate mass, and retention data for ID
• Automated SPE, 13 m gradient, resolve isobaric compounds within 15 ppm, and <10 S separation
• Blood, urine postmortem, DUID, DFSA
• Stimulants, benzo., opiates, muscle relaxants, hypnotics, antihistaines, antidepressants, newer synthetic drugs - “designer” “loop holes” “Spice/K2 “cannabinoids, and cathinone” bath salt”
## WF Pain Management Drug Panel

<table>
<thead>
<tr>
<th>Substance</th>
<th>Compound</th>
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</thead>
<tbody>
<tr>
<td>11-nor-9-Carboxy-Δ9-THC</td>
<td>Codeine</td>
</tr>
<tr>
<td>6-Acetylmorphine</td>
<td>Desalkylflurazepam</td>
</tr>
<tr>
<td>7-Aminoclonazepam</td>
<td>Diazepam</td>
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<tr>
<td>7-Aminoflunitrazepam</td>
<td>EDDP</td>
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<tr>
<td>alpha-Hydroxyalprazolam</td>
<td>Estazolam</td>
</tr>
<tr>
<td>alpha-Hydroxymidazolam</td>
<td>Fentanyl</td>
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<tr>
<td>alpha-Hydroxytriazolam</td>
<td>Hydrocodone</td>
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<tr>
<td>Alprazolam</td>
<td>Hydromorphone</td>
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<tr>
<td>Amphetamine</td>
<td>Lorazepam</td>
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<tr>
<td>Benzoylecgonine</td>
<td>MDA</td>
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<tr>
<td>Buprenorphine</td>
<td>MDEA</td>
</tr>
<tr>
<td>Butalbital</td>
<td>MDMA</td>
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<tr>
<td>Carisoprodol</td>
<td>Meperidineden</td>
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<tr>
<td>Clonazepam</td>
<td>Meprobamate</td>
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WF Pain Management Drug Panel

<table>
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<tr>
<th>Drug</th>
<th>Drug</th>
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<tr>
<td>Methadone</td>
<td>Oxycodone</td>
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<td>Methamphetamine</td>
<td>Oxymorphone</td>
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<tr>
<td>Morphine</td>
<td>PCP</td>
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<tr>
<td>N-Desmethyl-cis-tapentadol</td>
<td>Pentobarbital</td>
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<tr>
<td>N-Desmethyl-cis-tramadol</td>
<td>Phenobarbital</td>
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<td>Norbuprenorphine</td>
<td>Pregabalin</td>
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<td>Nordiazepam</td>
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<td>Secobarbital</td>
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<td>Tapentadol</td>
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<td>Temazepam</td>
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<td>Tramadol</td>
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<td>Noroxymorphone</td>
<td>Zolpidem</td>
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<tr>
<td>Norpropoxyphene</td>
<td>Zolpidem phenyl-4-carboxylic acid</td>
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<tr>
<td>Oxazepam</td>
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</tr>
</tbody>
</table>
Differentiation of Illicit D-Methamphetamine from Over-the-Counter L-Methamphetamine by LC-MS

- Astec® CHIROBIOTIC® V2 column (25 cm x 4.6 mm, 5 µm)
- Mobile phase consisted of 0.04% w/v ammonium trifluoroacetic acid in water:methanol (5:95, v/v)
- Flow rate was set at 1 mL/min. run time was 13.00 min
- Retention times - 10.75 and 11.62 min for D- and L methamphetamine
- Mass spectrometer in ESI+ and MRM modes, 2 transitions 150.0..91.0 and 150.0..119.0.
- Other publications: Ref. 2, 13 and 14.

Marc D. Julliard, Jason E. Strull, Dylan M. Stone, David S. Bell, Reporter US Volume 31.2
Differentiation of Illicit D-Methamphetamine from Over-the-Counter L-Methamphetamine by LC-MS
Lilly Yee, M.Sc.
Chiral liquid chromatographic analysis and chiral pharmacology of fluoxetine.
University of Wisconsin-Milwaukee

$S$-Fluoxetine

$R$-Fluoxetine
Interaction between enantiomers and a Chiral Receptor
Figure 4. Chemical Structure of Acetylated β-Cyclodextrin (Ac = COCH$_3$).
Rabbit plasma 6 hr post-injection

Conditions: β-cyclodextrin, MP-MeOH/0.3% triethylamine buffer, FR 1mL/min, 40°C and 214 nm
Chromatogram of Plasma sample extract

Conditions: Chirobiotic V, 100 µg/L Fluoxetine and norfluoxetine std., MP – EtOH/1% triethylamine(9:1), FR-1 mL/min, 265 nm
Enantiomeric separation of fluoxetine and norfluoxetine in plasma and serum samples with higher detection sensitivity capillary electrophoresis, *Electrophoresis* 1999;20:3432-8

Figure 7. Analysis of an extracted plasma sample from a patient under treatment of depression with Flx, 8 h after the last intake. Experimental conditions as in Fig. 4.

**Findings**
- \( R \text{Flu} < S \text{Flu} \) due to increased metabolism
- \( R \text{Nor-Flu} \) and \( S \text{NorFlu} \) - variable

**Conditions**
- 0.075 mm ID fused-silica
- Zeta-shaped detection cell
- Voltage 25 kV
- Cyclodextrin buffer – 0.5 mg/L of DM-\( \beta \)-CD and 0.6 mg/mL of PH-\( \alpha \)-CD
Increased use of Heroin and Treatments

Higher purity ~ the user
  - Ability to snort
    • No risk of AIDS
    • 4 hour “High” @ $10 - $20 for each dose
    • Not an Aggressive “High”
  - Ability to smoke

Treatment – Methadone, Buprenorphine, (Prescribed Heroin in Switzerland!!)
Methadone – Chiral pharmacology

- Methadone activity is almost solely to the drug itself rather than the metabolites.
- Half life is variable 15-55 hrs.
- R methadone – longer $t_{1/2}$ & larger $V_d$, affected by CYP450.
- R Methadone active form is 25-50 times more active than S.
- However – CYP 2B6 poor metabolizer and S-methadone → cardiotoxicity.
- R methadone – longer $t_{1/2}$ & larger $V_d$, affected by CYP450.
- R/S ratios in AM settings – inter-individual variations, 0.5 to 2.5, average <1.0.
Methadone Therapeutic and Toxic levels

Chronic administration:
100-200 mg daily 0.83 mg/L (0.57-1.06), 24hrs 0.46 mg/L
t_{1/2} = 25 hrs

Lethal Concentrations:

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<tr>
<th></th>
<th>Blood</th>
<th>Brain</th>
<th>Liver</th>
<th>Bile</th>
<th>Kidney</th>
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<td>avg</td>
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<td>1.0</td>
<td>3.8</td>
<td>7.5</td>
<td>2.9</td>
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<tr>
<td>Range</td>
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<td>0.5-1.4</td>
<td>1.8-7.5</td>
<td>2.9-18.0</td>
<td>1.1-6.0</td>
</tr>
</tbody>
</table>
Methadone Metabolism (2003-4)

*Asymmetric carbon
Moody.SOFT WS 2003
Winecker, Clin For Tox News(AACC), June 2003
Chiral Analysis of Methadone and its Main Metabolite EDDP in Postmortem Blood by Liquid Chromatography–Mass Spectrometry

Sys Stybe Johansen* and Kristian Linnet
Section of Forensic Chemistry, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
Protocol

• Mix blood with water and IS solution
• Add NaOH, then extract with butyl acetate, spin, remove organic and dry down
• Mix with MP
• LCMSMS
  – Agilent LC 1100 with chiral AGP column (100 mmX 4.0 mm)
  – Quattro micro from Waters
  – Positive ESI
  – Isocratic, 0.3 mL/min, 25°C, 34 min run
  – Calibration from 0.003 to 2.5 mg/kg blood
<table>
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<th></th>
<th>Retention Time (min)</th>
<th>Collision 1 (V)</th>
<th>Transition 1 MRM</th>
<th>Collision 2 (V)</th>
<th>Transition 2 MRM</th>
<th>Ion ratio MRM2/MRM1</th>
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</thead>
<tbody>
<tr>
<td>R-Meth*</td>
<td>22.00</td>
<td>26</td>
<td>310 → 265</td>
<td>15</td>
<td>310 → 105</td>
<td>2.46</td>
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<tr>
<td>R-Meth-IS</td>
<td>26</td>
<td>313 → 268</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>S-Meth</td>
<td>26.50</td>
<td>26</td>
<td>310 → 265</td>
<td>15</td>
<td>310 → 105</td>
<td>2.47</td>
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<td>313 → 268</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>R-EDDP</td>
<td>18.50</td>
<td>30</td>
<td>278 → 249</td>
<td>23</td>
<td>278 → 234</td>
<td>1.90</td>
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<tr>
<td>R-EDDP-IS</td>
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<td>281 → 249</td>
<td>–</td>
<td>–</td>
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<tr>
<td>S-EDDP</td>
<td>20.05</td>
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<td>278 → 249</td>
<td>23</td>
<td>278 → 234</td>
<td>1.91</td>
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<tr>
<td>S-EDDP-IS</td>
<td>30</td>
<td>281 → 249</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

* Meth: methadone. The cone energy was 25 V for methadone and 40 V for EDDP, and the source and desolvation temperatures were 130°C and 375°C, respectively.
Figure 1. Ion chromatograms of extracted blank whole blood (A) and a standard sample at 0.003 mg/kg (B). The first trace is EDDP-IS, then EDDP's quantitative trace, methadone-IS, and the last trace is methadone. The elution order is R-EDDP at 18.5 min, S-EDDP at 20.0 min, R-methadone at 22.0 min, and S-methadone at 26.5 min.
Forensic Toxicology Methadone

Preliminary study – n = 10

Methadone deaths due to overdose, higher parent drug, thus R/S ratio higher? “rough estimation”

Blood/plasma ratio – average 0.75

Methadone – postmortem redistribution, heart/femoral blood 0.8 to 1.4 with an average of 1.1
Subclavian/heart blood 0.3 to 2.03
R methadone $V_d$ – higher influx postmortem

EDDP – Blood 1/5 of methadone, Urine, same conc. EDDP/methadone higher urine conc. in maintenance than acute overdose
R<S, R-methadone to R-EDDP lower clearance

Findings – Methadone median R/S = 1.46
EDDP median R/S = 0.80
Chiral Pharmacogenomics of Methadone Therapy for Drug Addiction

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- N = 35 patients, SS > 4 wk, 10-30 mg
- IRB approval and trough blood draw
- Patient samples genotyped for CYP 2D6, 2C9, 2C19, 3A4 and 3A5, using Pyrosequencing
- Methadone therapy, effectiveness and toxicities evaluated by using opioid dose adequacy scale (ODAS) and a checklist of medication side-effects, supplemented by Beck Depression scale
Chiral Methadone Analysis

• SPE with Clean Screen (UCT)

• Separation of R and S enantiomers of free and total methadone and free and total EDDP was by HPLC on a Chiral-AGP 100 × 3.0 mm (0.5 μm) column followed by analysis on an Applied Biosystems API-4000 tandem mass spectrometer (LC/MS/MS)

Total Methadone Plasma Level and Dose

\[ y = 0.15x + 0.17 \]

- \[ R^2 = 0.23 \]
CYP450 Alleles and Adjusted Methadone Enantiomer Concentrations

CYP450 Alleles

- CYP2D6 xN
- All WT
- CYP2D6 *2HM
- CYP2D6 *4HM
- CYP3A5 *3HT

Adjusted Methadone Enantiomer Concentrations

- T/MDN
- R-MDN
- S-MDN
- R-EDDP
- S-EDDP

CYP450 Alleles

- CYP2D6 xN
- All WT
- CYP2D6 *2HM
- CYP2D6 *4HM
- CYP3A5 *3HT
Findings

- Total methadone concentrations not well correlated to dose

- Metabolic ratio indicates that s-methadone is preferentially metabolized, or r-methadone has decreased clearance
Conclusions
Enabling Precision/Personalized Medicine?

**Antifungals TDM** – Voriconazole well accepted, useful for drug-drug interaction., posaconazole – pending., and lack survey -peer comparison needed!

**Chiral DAU** -

- Clinical applications well established for some DAUs such as amphetamine
- Pending for others – fluoxetine and methadone
- May help to differentiate acute vs chronic ingestion of methadone
- Complementary to Pharmacogenomics

*Opportunities for medical laboratory scientists to contribute to R&D*