

Analysis of Benzodiazepines in Blood and Urine with Automated Disposable Pipette Extraction and HPLC/MS/MS

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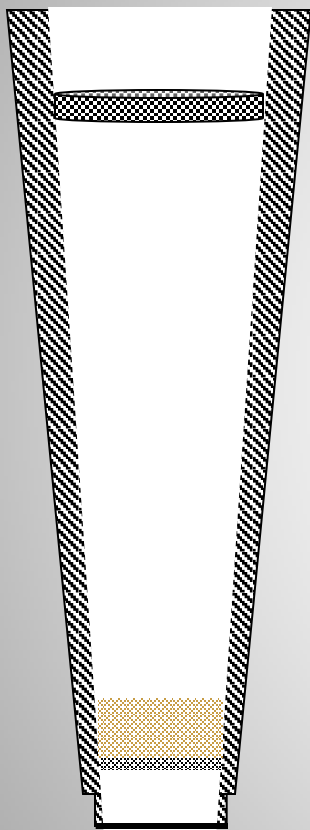
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Outline

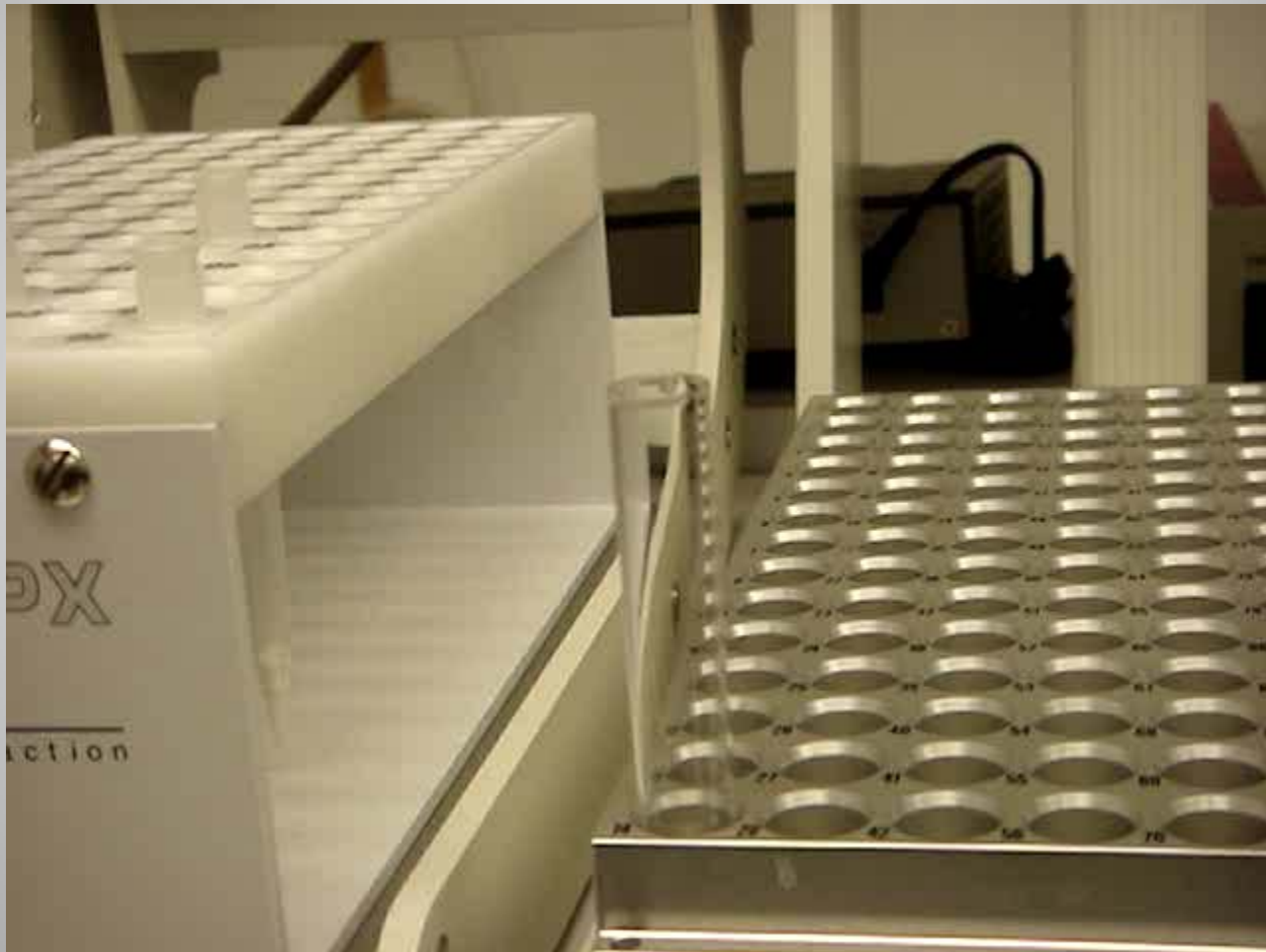
- Disposable Pipette Extraction (DPX)
 - Extraction process
 - Automation
- Benzodiazepines in urine
 - Reverse phase (RP) (GC/MS)
 - Cation exchange (CX)
 - Weak anion exchange (WAX)
- Benzodiazepines in blood
 - Limitation due to protein precipitation
 - Cation exchange
- Sample Collection Tips (SC-Tips)
- Conclusions

Disposable Pipette Extraction (DPX)

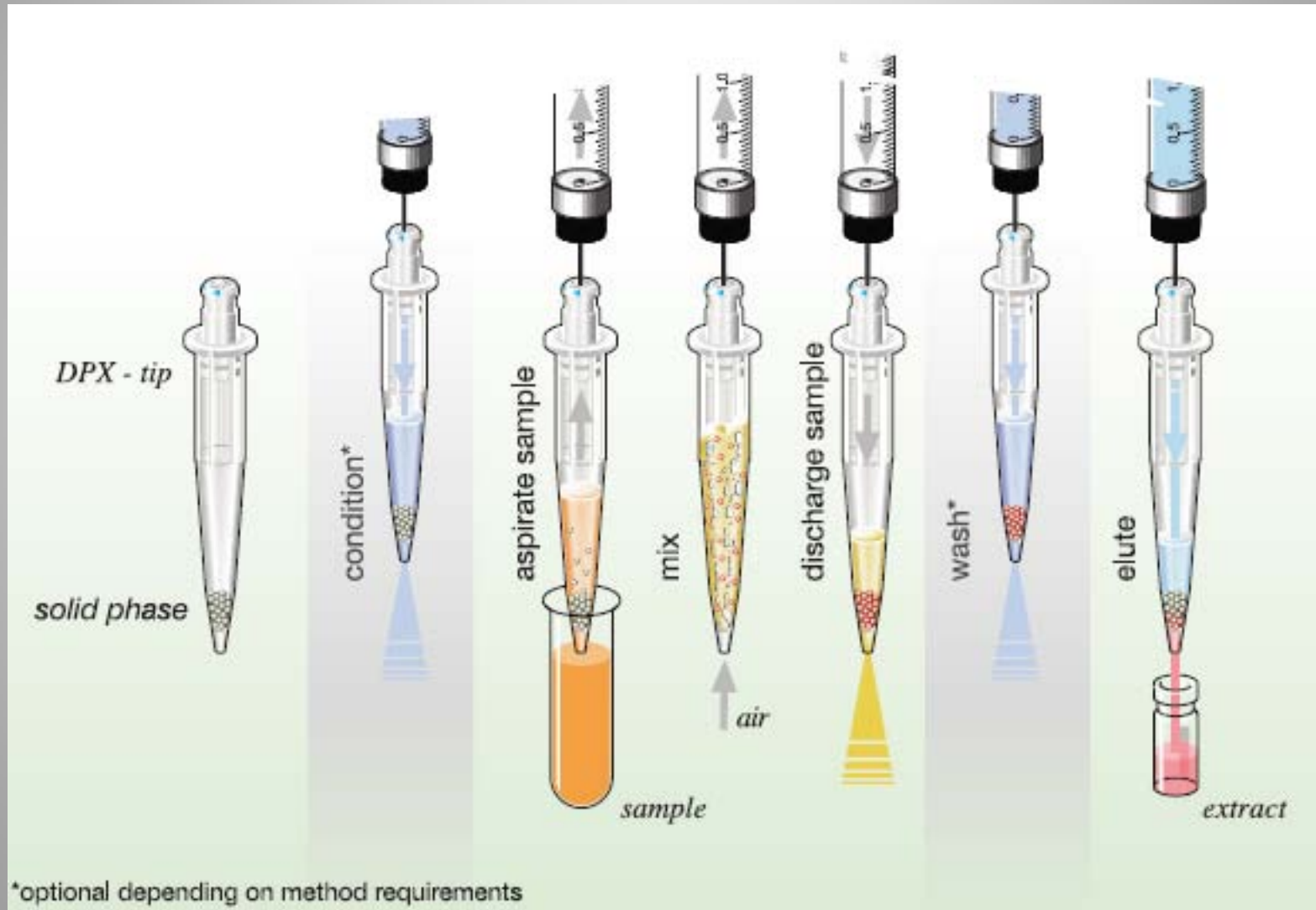


- Extractions are rapid—app. 3 minutes
- Flow is bi-directional
- Loosely contained sorbent—mixing
- No conditioning steps required
- Extraction efficiencies based on equilibration time, not flow rate
- Use less sorbent--less solvent required
- Minimal solvent waste generated
- Readily automated

DPX High Performance SPE (GEL)



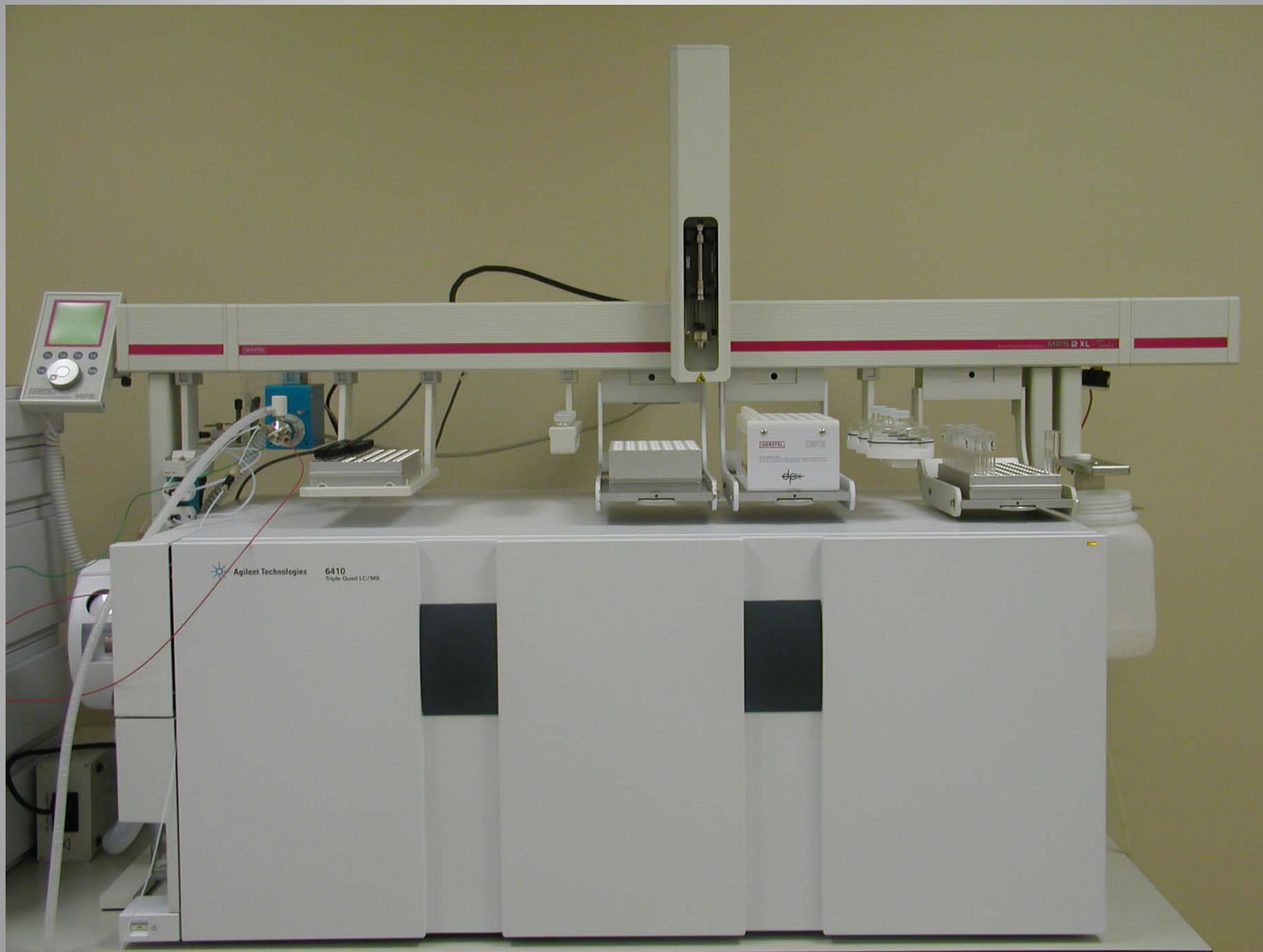
Automated DPX



Automated DPX for GC/MS



Automated DPX for LC/MS/MS



Sample Prep for Automated DPX

➤ Blood specimens:

- 1) Spike sample with internal standard
- 2) Precipitate proteins with acetonitrile
- 3) Centrifuge and transfer to clean labeled tube
- 4) Place on sample tray rack of MPS

➤ Urine specimens:

- 1) Spike samples with internal standard
- 2) Perform hydrolysis if needed
- 3) Transfer set volume to clean labeled tube
- 4) Place on sample tray rack of MPS

Automated DPX Method

➤ DPX-RP and DPX-WAX

- Wet the DPX sorbent using 250µL of 30% acetonitrile (10% provides better recoveries of drugs using reversed phase mechanisms) in water.
- Aspirate the entire sample solution and mix with 1.3mL of air.
- Equilibrate for 20 seconds before dispensing the entire contents to waste.
- Wash the DPX sorbent by dispensing 500µL of 10% acetonitrile in water into the top of the tip.
- Elute benzos by dispensing 700µL of 100% acetonitrile (or 50% methanol in acetonitrile for benzos using DPX-WAX) through the top of the DPX tip into 2mL autosampler vials.

➤ DPX-CX

- Add 100 µL of 1 M HCl to ensure benzos are protonated for ion exchange (low pKa values)
- Wet the DPX sorbent using 250µL of 30% acetonitrile in water.
- Aspirate the entire sample solution and mix with 1.3mL of air.
- Equilibrate for 20 seconds before dispensing the entire contents to waste.
- Wash the DPX sorbent by dispensing 500µL of 10% acetonitrile in water through the top of the tip.
- Wash by dispensing 700µL of 100% acetonitrile through the top of the tip.
- Elute benzos by dispensing 700µL of 4% ammonium hydroxide in acetonitrile/methanol (50/50)

LC Parameters

HPLC: Agilent 1200 HPLC

HPLC Column: Zorbax Eclipse XDB-C18 RRHT column (2.1 x 50mm, 1.8 μ m)

LC/MS: Agilent 6410 Triple Quadrupole Mass Spectrometer with electrospray source

Autosampler: GERSTEL MPS 2XL with Active Washstation

Injection: 6 port (0.40mm) Cheminert C2V injection valve; 20 μ L stainless steel sample loop; 2.5 μ L injection volume

LC Parameters

Mobile Phase A = 5mM ammonium formate with 0.05% formic acid. Mobile Phase B = Methanol with 0.05% formic acid.

Time(min)	Flow(mL/min)	Pressure(bar)	%Solv Ratio B
0	0.5	600	5
0.5	0.5	600	5
1.5	0.5	600	30
3.5	0.5	600	70
4.5	0.5	600	95
6.5	0.5	600	95
7.5	0.5	600	5

LC/MS/MS

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
a-OH-alprazolam	<input type="checkbox"/>	325	Unit	297	Unit	75	120	30
Lorazepam	<input type="checkbox"/>	321	Unit	275	Unit	75	140	20
Clonazepam	<input type="checkbox"/>	316	Unit	270	Unit	75	120	25
Flunitrazepam	<input type="checkbox"/>	314	Unit	268	Unit	75	160	30
Alprazolam	<input type="checkbox"/>	309	Unit	281	Unit	75	160	25
Temazepam	<input type="checkbox"/>	301	Unit	255	Unit	75	120	35
Oxazepam	<input type="checkbox"/>	287	Unit	241	Unit	75	120	20
Diazepam	<input type="checkbox"/>	285	Unit	222	Unit	75	160	25
Nitrazepam	<input type="checkbox"/>	282	Unit	236	Unit	75	160	25
d5-nordiazepam	<input type="checkbox"/>	276	Unit	213	Unit	75	160	30
Nordiazepam	<input type="checkbox"/>	271	Unit	140	Unit	75	160	30

Source Parameters

Parameter	Value
Gas Temp (°C)	350
Gas Flow (l/min)	12
Nebulizer (psi)	35
Capillary (V)	4000

DPX Methods

Urine

- Reversed phase (RP)
 - styrene divinyl benzene
 - pi-pi interactions
- Cation exchange (CX)
 - sulfonated divinyl benzene
 - pi-pi interactions
 - Reverse phase
- Weak anion exchange (WAX)
 - polyamino divinyl benzene
 - pi-pi interactions
 - hydrogen bonding

Blood—acetonitrile ppt.

- Cation exchange (CX)
 - sulfonated divinyl benzene
 - pi-pi interactions
 - Reverse phase
- Weak anion exchange (WAX)
 - polyamino divinyl benzene
 - pi-pi interactions
 - hydrogen bonding
- *Sample Collection Tips (SC-Tips)
 - Rapid clean up method (1 min)
 - Remove matrix
 - Filtration tips

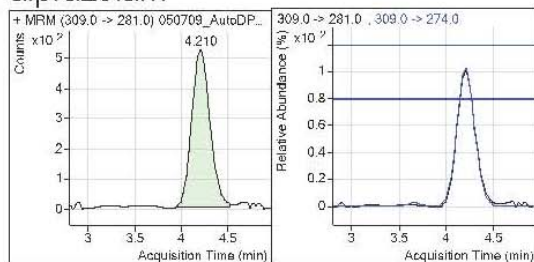
Benzos—urine using DPX-RP

(with GC/MS following MTBSTFA derivatization)

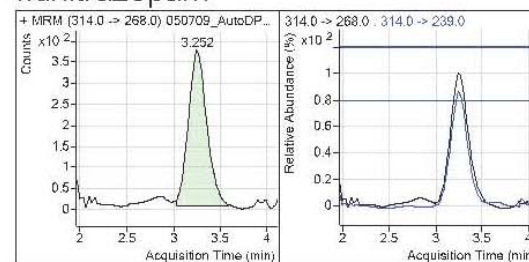
Compound	% Recovery
alprazolam	87.1
a-OH-alprazolam	88.6
clonazepam	100.1
diazepam	73.7
flunitrazepam	95.3
lorazepam	88.7
nitrazepam	98.7
nordiazepam	88.9
oxazepam	87.4
temazepam	78.5

Benzos—urine using DPX-CX MS/MS transitions

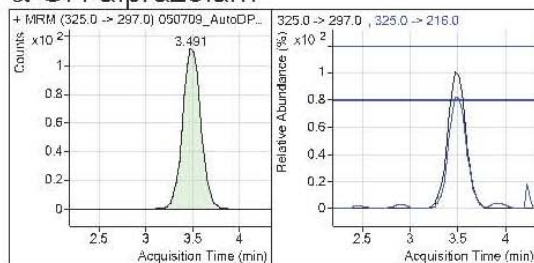
alprazolam



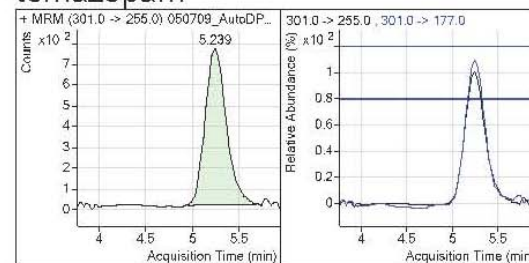
flunitrazepam



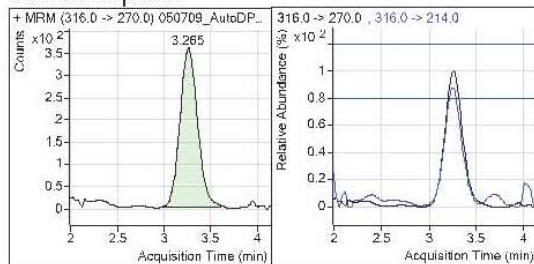
α -OH-alprazolam



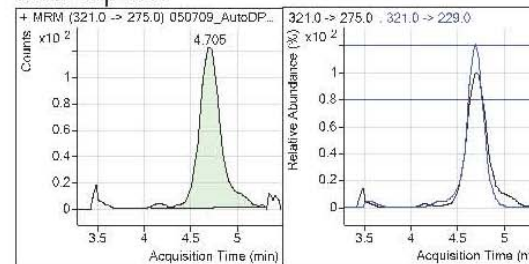
temazepam



clonazepam

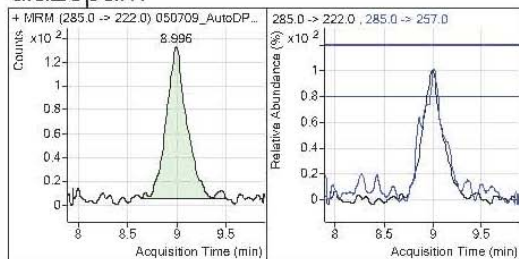


lorazepam

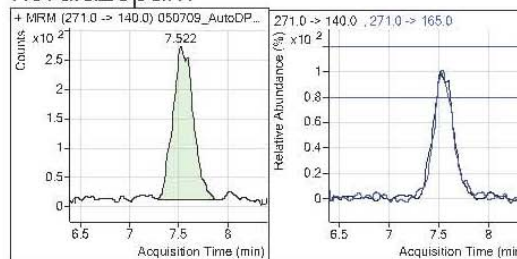


Benzos—urine using DPX-CX MS/MS transitions

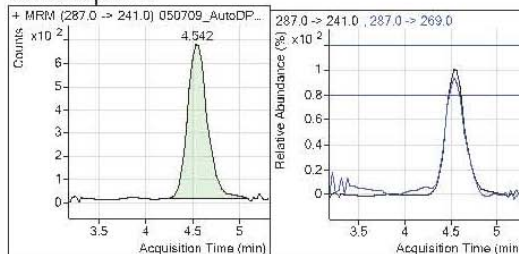
diazepam



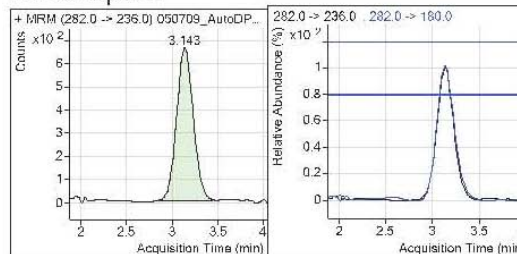
nordiazepam



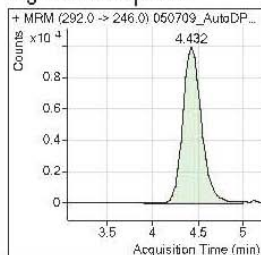
oxazepam



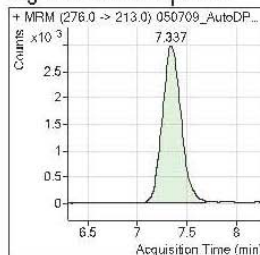
nitrazepam



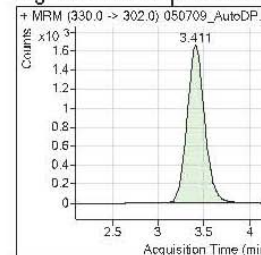
d₅-oxazepam



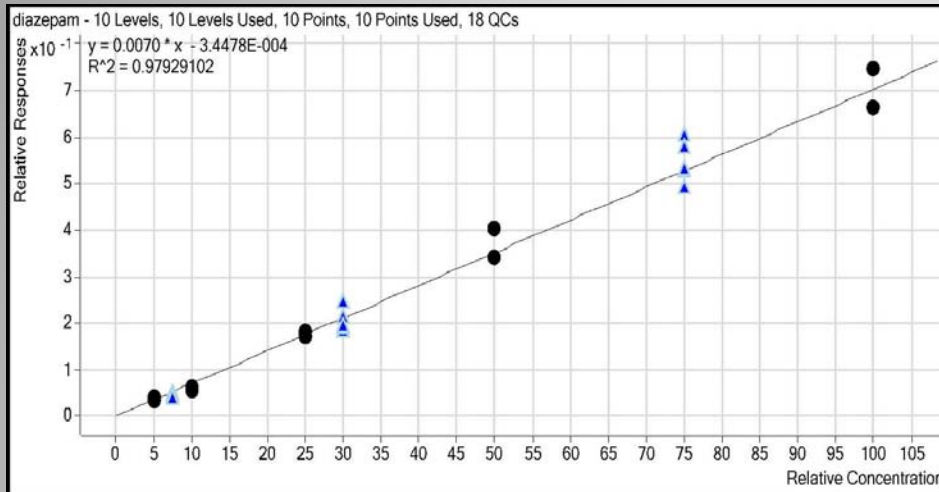
d₅-nordiazepam



d₅-α-OH-alprazolam

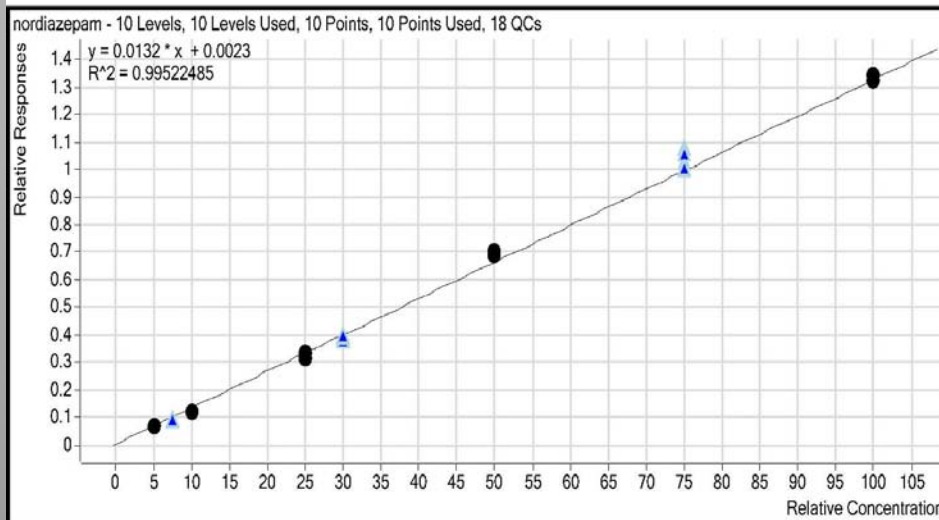


Benzos—urine using DPX-CX



d₅-Nordiazepam was used as internal standard; better reproducibility with matched deuterated internal standard.

Nevertheless, good linearity is achieved with all benzos analyzed.



Benzos—urine using DPX-CX and DPX-WAX

DPX-CX

Compound	% Recovery
alprazolam	121
a-OH-alprazolam	102
clonazepam	181
diazepam	108
flunitrazepam	95
lorazepam	60
nitrazepam	85
nordiazepam	100
oxazepam	98
temazepam	112

DPX-WAX

Compound	% Recovery
alprazolam	86.0
a-OH-alprazolam	75.6
clonazepam	86.1
diazepam	88.0
flunitrazepam	87.1
lorazepam	84.4
nitrazepam	76.1
nordiazepam	93.3
oxazepam	89.9
temazepam	93.9

Benzos—whole blood using DPX-WAX protein precipitation is required to use DPX

DPX-WAX

Analyte	%Recovery
a-OH-alprazolam	28.5
d5-Nordiazepam	-
Nordiazepam	92.2
Lorazepam	44.3
Clonazepam	58.2
Flunitrazepam	66.4
Alprazolam	43.9
Temazepam	87.0
Oxazepam	49.0
Diazepam	109.4
Nitrazepam	85.7

Note: additional elution solvent (w/ methanol) should improve recoveries.

Sample Collection Tips (SC-Tips)

Protein precipitation and “clean up” in one step!

60 second extraction for blood and hydrolyzed urine



Comprehensive screening using LC/MS/MS

Sample Collection Tips (SC-Tips)

- Opiates—6MAM, codeine, hydrocodone, hydromorphone, morphine, oxycodone, oxymorphone
- Opioids—fentanyl, methadone, buprenorphine, EDDP, norbuprenorphine, norfentanyl
- Benzos—hydroxyalprazolam, lorazepam, nordiazepam, oxazepam, temazepam, diazepam, alprazolam, flunitrazepam, 7-aminoflunitrazepam, nitrazepam
- Barbs—amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital, secobarbital
- Analgesics—propoxyphene, norpropoxyphene, tramadol, o-desmethyltramadol

Comprehensive screening using LC/MS/MS

Sample Collection Tips (SC-Tips)

- Anticonvulsants/muscle relaxants—gabapentin, carisoprodol, meprobamate
- Hallucinogens—COOH-THC, PCP
- Stimulants—amphetamine, benzoylecgonine, cocaine, MDA, MDMA, methamphetamine, methylphenidate

Future Research

- More benzodiazepines to extend list to 30 to 40
- More replicates for *reproducibility and validation*
- Optimization of DPX methods for whole blood!
- Determination of ion suppression and matrix effects for SC-Tips for whole blood specimens
- Use of SC-Tips for rapid comprehensive screening of benzos as well as numerous other drugs and drug classes

Acknowledgments

- Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC
- DPX Labs, LLC, Columbia, SC
- GERSTEL
- Agilent Technologies

Visit www.DPXLabs.com for more information (copy of presentations).