

High Throughput Screening of Veterinary Drug Residues:



Challenges and Successes

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Goals of High Throughput

- * Higher numbers of samples analyzed
- * Higher numbers of analytes per sample

Consistent with FDA/ORA Strategic Plan

Approaches to Increasing Sample Throughput

- ⇒ *Streamline sample preparation*
- ⇒ Prescreen samples for known residues
- ⇒ Multi-residue LC-MS/MS screening methods

1. Prescreen samples for known residues

LC-MS/MS is a valuable tool

- * "universal detector"; sensitive and selective
- * able to obtain quant and qual information

BUT it is a limited lab resource!

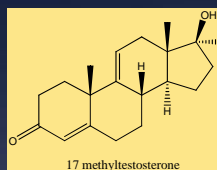
*Can we prescreen samples prior to
LC-MS/MS analysis with rapid test kits?*

1. Prescreen samples for known residues: Immunochemical methods

- * Based on antigen (analyte)/antibody reactions
- * Usually competitive binding assay to give color or physical change
- * Designed to be rapid; analyze many samples quickly
- * Types include ELISA (Enzyme-linked immunosorbant assay) and optical biosensors



Methyltestosterone



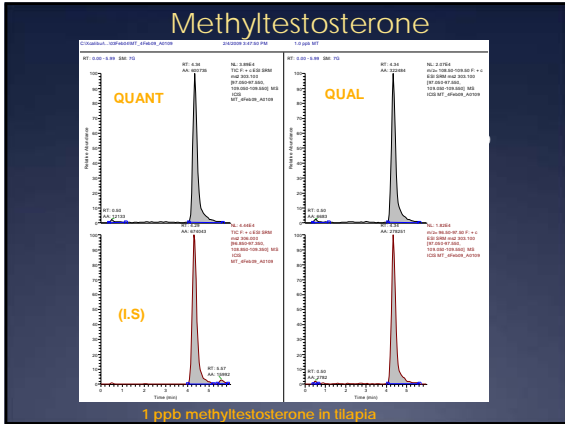
17 methyltestosterone

- * Given to newly hatched fish for sex reversal (all male population)
- * Concerns regarding residues
- * Interested in 0.8 ppb in tilapia



Method

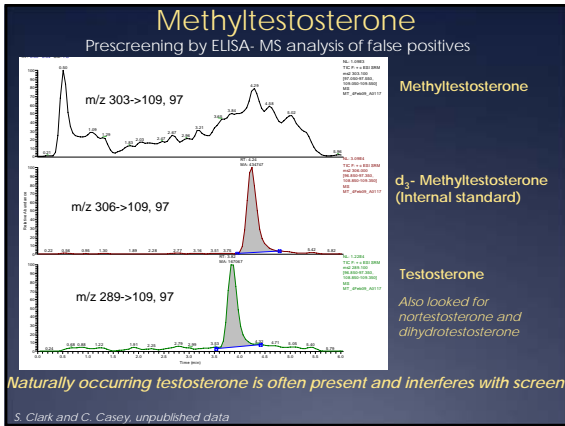
- Extract with acetonitrile
- Liq-liq extraction with ethyl acetate; clean-up with anionic exchange SPE
- Liq-liq extraction into hexane; clean-up with florisil SPE
- LC-MS/MS analysis by APCI or ESI using d₃-17 methyltestosterone as I.S.



Methyltestosterone

Can we prescreen using ELISA?

- * Test kit developed for testosterone that cross-reacts with methyltestosterone
- * Abbreviated extraction procedure
- * Found approximately 25% of samples were presumptive positive
- * These sample did not confirm methyltestosterone by LC-MS/MS

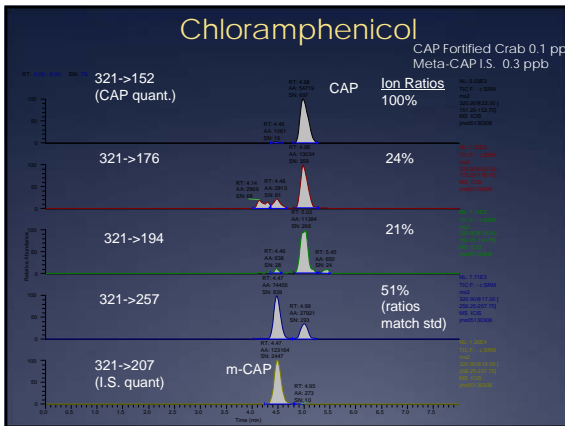


Chloramphenicol

- * Used as antibiotic, hand sterilizer
- * Concerns regarding aplastic anemia
- * Interested in 0.3 ppb in shrimp, crab

Typical Method for Shrimp and Crab:

- Extract w/ ammoniated ethyl acetate; clean-up w/ hexane then SPE
- Negative ion electrospray LC-MS/MS
- Often use an internal standard



Chloramphenicol

Screening by Surface Plasmon Resonance Biosensor Analysis

- * CBP is mixed with crab or shrimp sample extracts and reacts with CAP molecules present in the sample.
- * The sample is then injected into the CAP biosensor flow cell, where free CBP binds to the biosensor causing a shift in the angle of light reflection.
- * This creates a signal that correlates to the quantity of CAP in the sample.

Chloramphenicol

Comparison of Biosensor Results to LC-MS/MS

- * Samples previously analyzed by LC-MS/MS were reanalyzed with biosensor
- * Most samples were negative by both methods
- * Five of the 201 samples had biosensor CAP concentrations >0.3 ng/g, which would necessitate confirmation by LC-MS/MS.
- * CAP was not found by LC-MS/MS in 3 of the 5 samples, resulting in a 1.5 % false positive rate for this study. The false negative rate was 0 %.

Andersen et al. unpublished data

Chloramphenicol

Advantages of Biosensor Screening

- * *Faster Sample Prep (~ 40 subsamples/day/analyst)*
- * *High Throughput Analysis – 8 minutes per sample*
- * *No Interpretation of Results Required*
- * *Low Limit of Detection – 0.073 ng/g for CAP in shellfish*

1. Prescreen samples for known residues

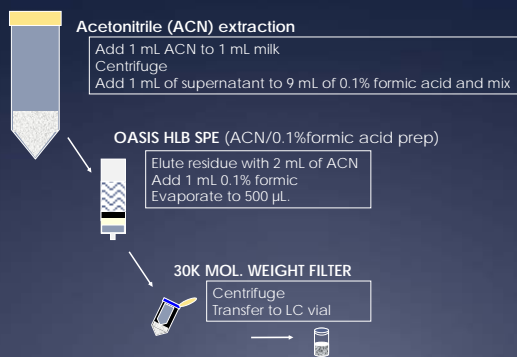
Prescreen samples with immunochemical tests

- + Success: Can reduce burden on LC-MS/MS instruments for initial analysis
- × Challenges: Are kits available for appropriate analytes? Does using the kits save time and money compared to regular analyses?
- × Future work: Develop prescreening methods for sulfonamide residues in fish; also groups working on screening for nitrofurans

2. LC-MS/MS Analysis for Screening Large Numbers of Residues

- * Multi-class residue analysis
- * Generic extraction
- * Primarily qualitative- screening & confirmation; can also perform quantitation
- * Method can be flexible and incorporate new compounds at any time
- * There are many examples for veterinary drug residues including for fish, meat, milk

Milk Multi-residue Screening Method: Extraction



Milk Multi-residue Screening Method

In Original Procedure: Two Types of LC-MS/MS Methods Used:

	Screening Method	Class Specific Methods
# Residues Monitored	All 24	4-8
# SRM transitions/residue	1	3
# Time Segments/run	7+	3-5
# Scan Events /time segment	3-6	2-4

Screening: Used to monitor for the possible presence of any of the residues

Class Specific Methods: Used for confirmation and semi-quantification

Milk Multi-residue Screening Method

A milk sample fortified with drugs at tolerance or safe levels was used as a positive (threshold) control. To be presumptive positive, the response in the unknown sample should be at least 50% of what is observed in the threshold sample.

If presumptive positive, reanalyzed extract using class specific LC-MS/MS program(s) to collect confirmatory ions.

Tolerance or safe levels:

Sulfonamides :	10 ppb
β-lactams:	10 ppb except PEN G (5 ppb), CEPH (20 ppb)
Tetracyclines :	100 ppb (combination <300 ppb)
Macrolides:	50 ppb (ERY, TYL), 100 ppb (TIL)
Fluoroquinolones:	5 ppb (no tolerance set)
Others:	FLU-OH (2 ppb), BAC (500 ppb), THBZ (50 ppb), TRIP (20 ppb)

*Aminoglycosides and avermectins were not recovered with extraction method

Milk Multi-residue Screening Method

Increasing Throughput

- * Decrease chromatographic run-time by using UHPLC – columns with particle size < 2 micron
- * Collect all three SRM transitions for each analyte in one run
- * Use SRM start/stop times instead of time segments

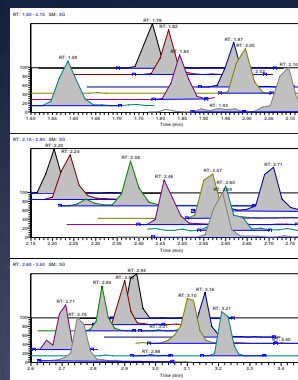
Milk Multi-residue Screening Method

Switch to UHPLC

- * Changed column from 2.1 x 100 mm, 3.5 μm C18-AQ to 2.0 x 50mm, 1.7 μm C18 or Phenyl
- * Flow rate changed from 250 to 400 μL/min
- * Injection volume changed from 10 to 5 μL
- * Gradient changed accordingly

Milk Multi-residue Screening Method

UHPLC Results



- * TIC chromatograms for mixed standard at 1X
- * Adequate separation in <5 min (Phenyl column shown)

Milk Multi-residue Screening Method

Switch from collecting 1 SRM to 3 SRM for all 26 compounds

	Cycle times (sec)	Dwell times (ms)	Typical peak widths (s)	Typical # data points/peak	Calculated # data points/peak
HPLC 1 SRM/cmpd (26 SRM)	NA	200	30-50	Varies 20-50+	30,000/200 = 150; 30,000/6*200 = 25
HPLC 3 SRM/cmpd (78 SRM)	5	-64	30-50	5-8	30/5 = 6
HPLC 3 SRM/cmpd (78 SRM)	2.5	-32	30-50	8-20	30/2.5 = 12
UHPLC 1 SRM/cmpd (26 SRM)	1.2	-46	10-12	6-9	10/1.2 = 8.33
UHPLC 3 SRM/cmpd (78 SRM)	1.2	-15	10-12	6-9	10/1.2 = 8.33

Milk Multi-residue Screening Method

Comparison Results

Signal abundance ($\times 10^4$) and signal to noise (S/N) for several compounds at 1X

CMPD	HPLC 1 SRM (ST = 200 ms)	HPLC 3 SRM (CT = 2.5 s)	UHPLC 1 SRM (CT = 1.2 s)	UHPLC 3 SRM (CT = 1.2 s)
SMZ	25 (S/N=455)	58 (S/N=4119)	6.5 (S/N=151)	23 (S/N=6632)
PEN G	19 (S/N=267)	62 (S/N=273)	6.1 (S/N=120)	5.8 (S/N=162)
FLU-OH	75 (S/N=6840)	83 (S/N=2387)	19 (S/N=4618)	24 (S/N=1345)
OTC	736 (S/N=2088)	861 (S/N=1554)	292 ()	390 (S/N=26,587)
TYL	37 (S/N=444)	172 (S/N=1400)	19 (S/N=1459)	84 (S/N=1321)

St = scan time; CT = cycle time

- * Collecting 3 SRM gives higher abundance than 1 SRM for HPLC and UHPLC
- * HPLC gives higher signal abundance; often higher or similar S/N

Milk Multi-residue Screening Method Comparison - speed

Experiment (for 10 or 50 samples)	# analytical runs	Time/run (min)	Time (min)	Time (hr)
HPLC- Screen (1 SRM) + Four confirmatory (3 SRM) methods	50 250	25	1,250 6,250	20.8 104.2
HPLC 3 SRM screen/confirm	10 50	25	250 1,250	4 20.8
UPLC 3 SRM screen/confirm	10 50	5	50 250	<1 4

*Time benefit: depends on number of samples
and number of presumptive positive*



Milk Multi-residue Screening Method

Currently:

- * Using HPLC with 3 SRM/compound
- * Re-evaluate depending on sample load

Ongoing Projects:

- * Screening imported dairy products
- * Follow-up to tissue residue violations

2. LC-MS/MS Analysis for Screening Large Numbers of Residues

- + Success: Can obtain information for many compounds for single sample collected and processed.
- × Challenges: Need to weigh advantages of speed/data quality for multi-residue methods; data analysis can be bottleneck.
- ◁ Future work: Expand beyond target list of compounds using accurate mass instruments.

Conclusions

- * Prescreening samples and implementing multi-residue methods can potentially increase sample throughput while effectively managing LC-MS/MS instrument time.
- * Need to be aware that other performance aspects (specificity, data quality) may suffer as a result of higher analysis speed.