DEFINITIVE DRUG AND METABOLITE SCREENING IN URINE BY UPLC-MS/MS USING A NOVEL CALIBRATION TECHNIQUE

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INTRODUCTION

- Drug screening is an essential analytical tool in toxicology for detection of therapeutic, illicit and emerging drug use.
- While definitive analytical techniques *e.g.,* LC (or GC) coupled with mass spectrometry, are widely used in forensic casework, other areas of toxicology testing continue to rely heavily on immunoassay (IA) methods for presumptive identification.
- Immunoassays are typically class-specific screens, thus multiple tests are required to screen for the most common drug classes encountered in toxicology.
- Immunoassays employ relatively high cut-off thresholds and are calibrated using a representative analyte from the class, at a single concentration.
- However, cross-reactivity for other agents within the class (and metabolites) can be variable leading to false positives and false negatives. Thus presumptive positive IA screens require further testing (using definitive methods) to identify the specific drug substance (s) and to further verify the screen result.
- LC-MS/MS is a definitive technique and is known to provide improved selectivity and sensitivity for toxicology screening.
- Multi-analyte LC-MS/MS methods however often involve complex sample preparation methods; long analytical run times and rely on inclusion of stable isotope labelled internal standards to compensate for the phenomenon of matrix effect and to ensure accurate quantitation.
- Replacement of multiple IA by a single LC-MS/MS method is attractive but success relies on addressing the 3 key points above.

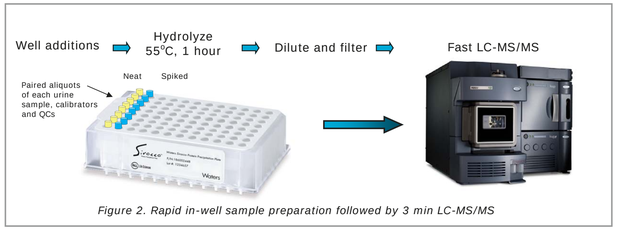

Figure 2. Rapid in-well sample preparation followed by 3 min LC-MS/MS

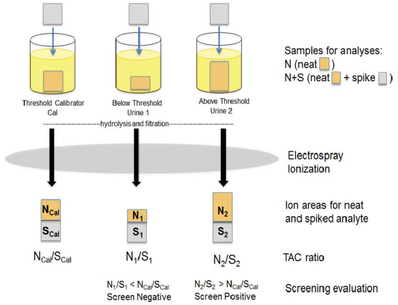

Figure 1. Threshold accurate calibration (TAC) concept.

METHODS

A novel technique Threshold Accurate Calibration (TAC) was developed[1] to achieve both normalization of matrix effects and threshold accurate detection (Figure 1). The method was developed for 33 drugs or metabolites from the following classes: opiates, opioids, benzodiazepines, amphetamines, amphetamine derivatives, cathinones, cocaine metabolite and hallucinogens.
The TAC approach involves testing urine with (spiked) and without (neat) reference-analytes spiked at their cut-off threshold (range 10-100 ng/mL).

Sample preparation

Figure 2 summarizes sample preparation, 200µL of each urine (calibrator, QC or test sample) was added to two paired wells of a 96-well Sirocco™ protein precipitation plate (Waters, MA) along with 50µL of methapyrilene (recovery control at 200ng/mL) and 50µL hydrolysis solution (β-glucuronidase in solution, IMCSzyme, SC).

One of the aliquots was spiked with 50µL of a mixture of the 33 analytes at their respective threshold concentration (range 10-100ng/mL in diluent). The other aliquot (the corresponding 'neat' sample) was spiked with 50µL blank diluent. Following incubation for 1 hour, 600µL of LC-MS/MS mobile phase was added to all wells and the samples filtered using a vacuum manifold and negative pressure. Five microlitres of the filtrate was analysed by LC-MS/MS.

UPLC-MS/MS

Waters® ACQUITY UPLC® I-Class-XEVO® TQD (Figure 2)

Column:	ACQUITY UPLC BEH phenyl column, 45°C
Mobile Phases:	(A) 2mM ammonium formate in water
	(B) 2mM ammonium formate in methanol
Run Time:	3 min gradient
Acquisition mode:	ESI+ multiple reaction monitoring (MRM), 2 transitions per analyte

Data processing

The TAC ratio of 'neat' to 'spiked' peak-area response was determined for each specimen and compared with the ratio obtained for the urine calibrator containing drugs at the cut-off threshold concentration. Analytes with a TAC ratio exceeding the calibrator TAC ratio are POSITIVE. Analytes with a TAC ratio below that of the calibrator are NEGATIVE.

$$\text{TAC ratio} = \frac{\text{'Neat' peak-area response}}{\text{'Spiked' peak-area response} - \text{'Neat' peak-area response}}$$

RESULTS AND DISCUSSION

The utility of the novel approach was demonstrated by an initial evaluation of typical matrix effects obtained with negative urine specimens supplemented with the analytes at 100ng/mL. Within-analyte matrix effect varied significantly between urine specimens (Figure 3) and without correction would impact on the accuracy of the calculated concentration and overall accuracy of the screen.

The TAC approach however normalized the variable matrix effect and allowed consistent, threshold-accurate detection of all 33 analytes investigated (Figure 4). Accuracy was further demonstrated by replicate analysis of QCs at concentrations around the target threshold *i.e.,* at 50%, 75%, 125% and 150% of threshold concentration. Average accuracy averaged 99.0% (90.5-117%) across all analytes.

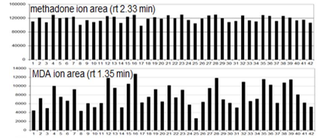

Figure 3. Example of variation in peak-area response in 42 negative urine specimens supplemented with 100 ng/mL concentration of analytes. Of the two analytes shown, MDA shows more significant variation in response owing to matrix effects.

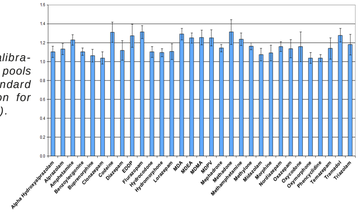

Figure 4. Mean TAC ratio (±SD) for calibrators prepared in seven negative urine pools by supplementing with reference standard material at the threshold concentration for all 33 analytes (expected TAC ratio = 1).

The assay was validated according to laboratory standards provided by New York State's Department of Health standards[2]

Method correlation studies were performed using immunoassay screen-positive specimens resulting in 178 drug and metabolite finding by currently validated definitive confirmation methods. Complete concordance was found with case and proficiency specimen findings by definitive screening and confirmation testing as shown in Figure 5.

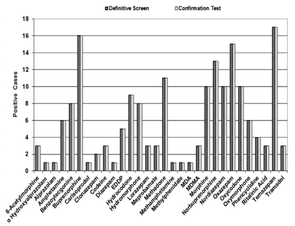

Figure 5. Comparison of positive analyte finding (N=178) by definitive screening method in proficiency and case specimens compared with analyte detection by confirmatory LC-MS/MS testing.

CONCLUSIONS

- **A UPLC-MS/MS assay has been developed and validated for multi-analyte screening in urine and provides an alternative to presumptive immunoassay screening.**
- **The assay employs a novel calibration technique to achieve normalization of matrix effect, without the use of internal standards, and provides threshold accurate screening for drugs and metabolites.**
- **Future activities will focus on automation of the TAC procedure.**

References

1. Rosano TG, Ohouo PY, LeQue JJ, Freeto SM and Wood M. Definitive Drug and Metabolite Screening in Urine by UPLC-MS/MS using a Novel Calibration Technique. *J. Anal. Toxicol.*, (2016) *In press.*
2. Clinical Laboratory Evaluation Program – Survey Standards, New York State Department of Health, http://www.wadsworth.org/labcert/clep/standards.htm.