ThP 203

Automated Targeted Screening of Benzodiazepines in Urine using LDTD-MS/MS at 400 Samples per Hour

Alex Birsan[1], Annie-Claude Bolduc[2], Serge Auger[1], Gregory Blachon[1] Jean Lacoursière[1] and Pierre Picard[1]
1)Phytronix Technologies, Québec, CANADA 2) Université Laval, Québec, QC, Canada

OVERVIEW

Purpose

- High-throughput screening of Benzodiazepines in urine.

Method

- Enzymatic hydrolysis followed by a Liquid-Liquid extraction was used for the Benzodiazepines analysis.
- Quantification: Linearity: $r^2 > 0.995$ over the calibration range (50 to 1000 ng/mL)
- **Samples were analyzed with a run time of 6 seconds using LDTD-MS/MS system**

INTRODUCTION

Toxicology laboratories generally use screening methods to obtain a semi-quantitative response for drug samples. Some screening techniques are fast but lack specificity and generate by far too many false positive results. Confirmation of those additional false positive samples is both time and cost consuming. Using mass spectrometry combined with high-throughput LDTD® Ion Source enhances specificity at equivalent or better speed. Method assessment is achieved by cross validation with LC-MS/MS, the standard gold method, on the same sample extracts. LC runs were adapted to crude sample preparation by using a 30 minute run in order to reduce ionic suppression. Purified beta- glucuronidase enzymes are used to reduce incubation time to 15 minutes (instead of 1 hour in the original method). Comparison with conventional glucuronidase enzyme incubation is performed in order to validate the obtained results. Complete workflow uses TECAN robotic system with 8 channels liquid handler (**Figure 1**).

LDTD® Ionization Source:

The LDTD uses a Laser Diode to produce and control heat on the sample support which is a 96 wells plate**.** The energy is then transferred through the sample holder to the dry sample which vaporizes prior to being carried by a gas in a corona discharge region. High efficiency protonation and strong resistance to ionic suppression characterize this type of ionization, and is the result of the absence of solvent and mobile phase. This allows for very high throughput capabilities of 6 seconds sample-to-sample analysis time, without carry over.

METHOD

Automated Workflow

The workflow uses the Tecan Freedom Evo® liquid handling **(Figure 1)** system to deliver a fully automated screening method. Sample preparation to LazWell spotting is performed automatically using the Evo platform and LDTD-MS/MS (**Figure 2)** achieves a total analysis throughput of 400 samples/hour.

Figure 1 Tecan Freedom Evo® robot system

Figure 2 LDTD system on Sciex 5500 QTrap®

Sample + β-Glucuronidase hydrolysis:
50 µL patient sample (or standard)
15 µL of purified Beta- glucuronidase enzyme
25 µL of rapid hydrolysis buffer containing IS solution (200 ng/mL in MeOH)
Vortex

Incubations:
15 minutes at 55°C

Liquid Liquid extraction (LLE) procedure:
25 µL of Na_2CO_3 0.5M pH 10 buffer
400 µL Ethyl Acetate/Hexanes 1:1
Vortex
Wait for phase separation

LazWell transfert:
Transfer 4 µL of organic upper layer in LazWell™
Dry prior to analysis (Hotel dryer)

LDTD-MS/MS analysis:

Instrumentation
- LDTD model: S-960
- MS: Sciex 5500 QTrap®

LDTD Parameters
- Laser power pattern :
  - ➢ Increase laser power to 65 % in 3.0 sec
  - ➢ Maintain for 1 sec
  - ➢ Decrease laser power to 0 %
- Carrier gas flow (Air) : 3 L/min

MS/MS Parameters
- APCI (+)
- Dwell: 5 msec
- Corona discharge: 3 µA
- DP: 100 V
- MRM mode (see **Table 1**)

Compound	Q1	Q3	CE (V)
Nordiazepam	271.1	140.2	32
7-Aminoflunitrazepam	284.1	236.0	36
Diazepam	285.0	154.1	32
7-Aminoclonazepam	286.1	222.2	30
Oxazepam	287.0	240.5	30
Estazolam	295.0	205.0	48
Temazepam	301.1	254.6	25
Alprazolam	311.0	274.0	40
Lorazepam	321.0	275.0	23
α-OH-Alprazolam	325.1	204.9	54
2-OH-Ethylflurazepam	333.1	211.2	46
α-OH-Midazolam	342.1	203.0	35
α-OH-Triazolam	359.0	331.0	36
Chlordiazepoxide	300.0	227.0	35
Clonazepam	316.0	214.0	50
Flunitrazepam	314.0	240.0	40
D5-Oxazepam	292.0	245.9	32
D5-Temazepam	306.1	259.6	25
D4-α-OH-Triazolam	363.1	335.0	36

Table 1 MRM method transitions

RESULT

Linearity results:

A standard calibration curve (with all drugs) ranging from 50 to 1000 ng/mL has been prepared in blank urine matrix and analyzed in triplicate. All curves have 0.995 coefficients or better. **Figure 3** presents typical calibration curves for Oxazepam with LDTD .

Cross validation:

The most important aspect in a screening method is to provide a Positive flag for all samples that contain targeted drugs. Using both enzyme preparations, no false negative reports were observed using LDTD-MS/MS with the 38 real patient samples tested. During the assay, false positive results were observed on 2 particular samples. A closer look at the integration shows a reduced signal for all internal standards under the acceptance level. Suppression effects are too significant to have an adequate quantitation. Further analysis shows that those 2 samples contain extreme concentrations of opiates (hundreds of µg/mL) causing this effect. The use of deuterated internal standards corrects the quantitation for all the other samples. A threshold level of IS area is used for the identification of overdosed urine samples. **Figure 4** below compares LDTD-MS/MS results against LC-MS/MS results for all benzodiazepine compounds.

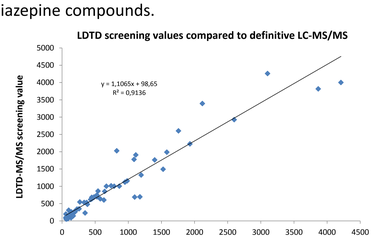

Figure 4 Real samples screening values compared to LC-MS/MS definitive measurement

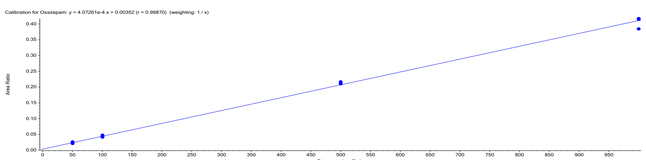

Figure 3 Oxazepam calibration curve with LDTD

Purified vs standard β-glucuronidase:

Two β-glucuronidase have been tested. The purified enzyme allows an incubation time of 15 min compared to 60 min with the standard one. All patient samples were analysed with both of the enzymes. **Figure 5** below compares the amount of several drugs (Nordiazepam, Alprazolam, Temazepam, Oxazepam, OH-Alprazolam and 7-Aminoclonazepam) for the same positive patient sample given the incubation conditions with each of the two enzymes.

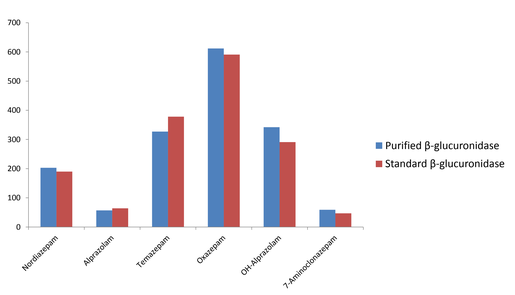

Figure 5 Evaluation of two different types of β-Glucuronidase

CONCLUSION

- **Simultaneous Screening of 16 Benzodiazepines in urine is performed in 6 seconds sample-to-sample by LDTD-MS/MS.**
- **Good precisions and accuracies are obtained. No carryover was observed.**
- **Cross-validation with LC-MS/MS shows no false negative results.**
- **Test of the two β-glucuronidase enzymes gives equivalent results. A four fold decrease of the analysis time is found using the purified one.**
- **Workflow using the Tecan robotic system allows an analysis capability of 400 samples per hour.**