Automated Targeted screening of Benzodiazepines in urine using LDTD-MS/MS at 400 samples per hour rate

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Overview

Toxicology laboratories generally use screening methods to obtain a semi-quantitative response for drug samples. Some screening techniques are fast but less specific 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 enhance specificity at equivalent of better speed. Method assessment is achieved by cross validation with LC-MS/MS, the standard gold method, on same samples extract. LC run were adapted to crude sample preparation by using a 30 minutes run in order to reduce ionic suppression. Purified beta-glucuronidase enzymes are used to reduce incubation time to 15 minutes instead of 1 hour. Comparison with conventional glucuronidase enzyme incubation is performed in order to validate obtained results. Complete workflow use Tecan robotic system with 8 channels liquid handler. Two 96 wells plates are process in parallel to match the analysis capability of LDTD-MS/MS system.

LDTD Ionization Source

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

Sample preparation

Enzymatic hydrolysis

- 25 µL patient sample (or standard)
- 7.5 µL purified Beta-glucuronidase enzyme
- 10 µL rapid hydrolysis buffer
- vortex
- **incubate 15 minutes at 55°C**

Extraction Procedure

- 50 µL Na_2CO_3 0.5M pH 10 buffer
- 400 µL Ethyl Acetate/Hexanes 1:1 containing IS
- vortex
- wait 1.5 minutes for phase separation
- Transfert 2 µL of organic layer in LazWell plate*
- Dry prior to analysis

*LazWell plate coating:
96-wells plates for analysis are pre-coated with 5 µl of an EDTA solution (100 µg/ml in $MeOH/H_2O/NH_4OH$ (75/20/5%)) and dried before sample deposition. This coating improves significantly the Temazepam, Lorazepam and Oxazepam signal.

Instruments setting

1) Mass spectrometer:

Mass spectrometer AB Sciex 5500 QTrap

MRM transitions used with 3 µA corona discharge current, 5 msec dwell time, and a 100 V DP

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
Alpha-OH-Alprazolam	325.1	204.9	54
2-OH-Ethylflurazepam	333.1	211.2	46
Alpha-OH-Midazolam	342.1	203.0	35
Alpha-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-Alpha-OH-Triazolam	363,1	335,0	36

2) LDTD:

LDTD model S-960 operated with a gas flow rate of 3 L/min and a laser pattern ramp from 0 to 65% in 3 seconds, maintaining this power level for 1 second.

3) LC:

Silliachrom SB-C18 (4.6X200mm) column were used with a flow rate of 0.5 ml/min. The following gradiant were used:

Time (min.)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
10	0	100
15	0	100
16	100	0
30	100	0

MPA: Water/Methanol (90/10) + 1% Formic acid

MPB: Water/Methanol (10/90) + 1% Formic acid

Five microliter of samples was injected and Electospray ionization mode was used.

Methodology

The standard curve calibration is made with a stock solutions of each drug spiked in blank urine matrix. The concentrations of the standards are 50, 100, 500 and 1000 ng/mL. The internal standard is prepared with a stock solution of each IS, spiked in methanol for a final concentration of 1000 ng/mL. Each real samples and calibration curve are hydrolyzed using standard β-glucuronidase (1h hydrolysis time) and faster hydrolysis process (15 minutes) for purified enzyme. All digest samples are analyzed using LC-MS/MS and LDTD-MS/MS method.

Results

Linearity from calibration curve is expressed by the correlation coefficient R^2 presented in the following table. All curves have ≥ 0,995 coefficients or better for both LDTD and LC analysis.

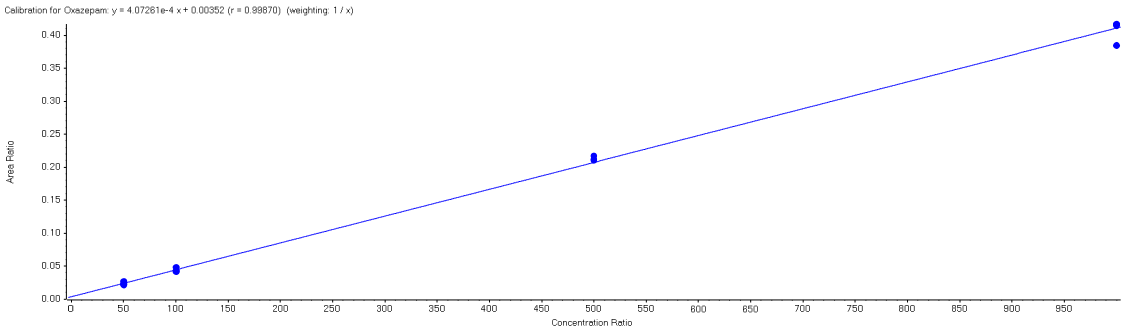

Figure 1: LDTD-MS/MS calibration curve (Oxazepam analysis)

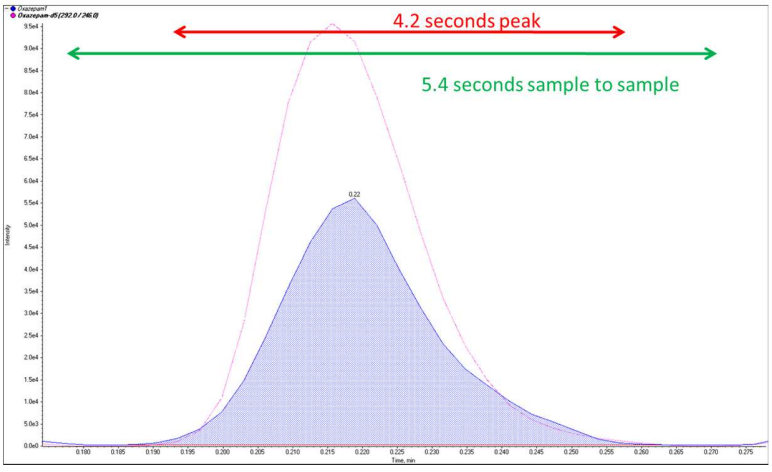

Figure 2: LDTD desorption peak for Oxazepam analysis

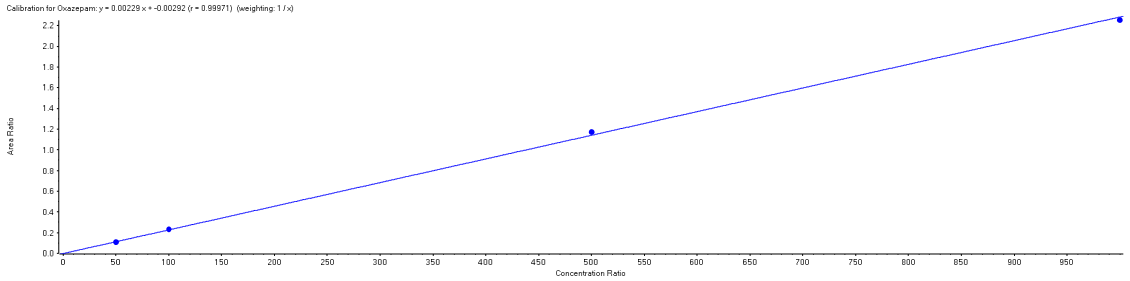

Figure 3: LC-MS/MS calibration curve (Oxazepam analysis)

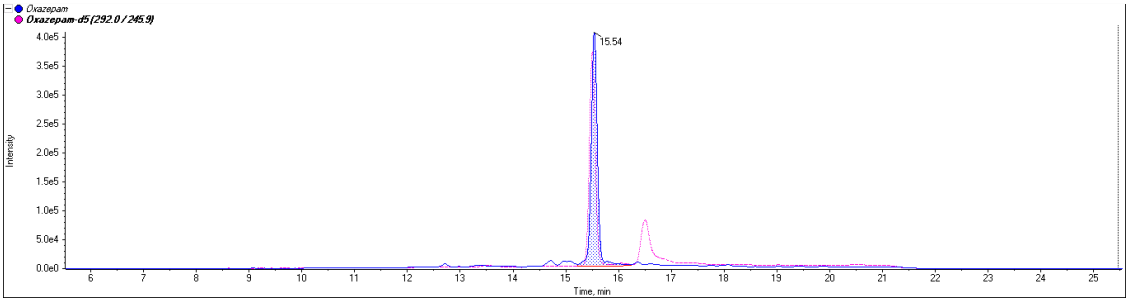

Figure 4: LC-MS/MS chromatogram for Oxazepam analysis

The most important aspect in a screening method is to provide a Positive flag for all samples that contain targeted drugs. Using both enzyme preparation, **no false negative reports were observed** using LDTD-MS/MS with the 38 real tested samples.

During the assay, false positive results were observed on 2 particular samples. Closer look to the integration point out a reduce signal for all internal standard under acceptance level. Suppression effects are significantly high to void adequate quantitation. Further analysis show that those 2 samples contain extreme concentration of opiates (hundreds of µg/ml) causing this effect. Use of deuterated internal standard corrects the quantitation for all the other samples. Threshold level of IS area is used for the identification of overdosed urine sample.

Test of the two β-glucuronidase enzyme preparation give equivalent results. Time is reduced by a factor of 4.

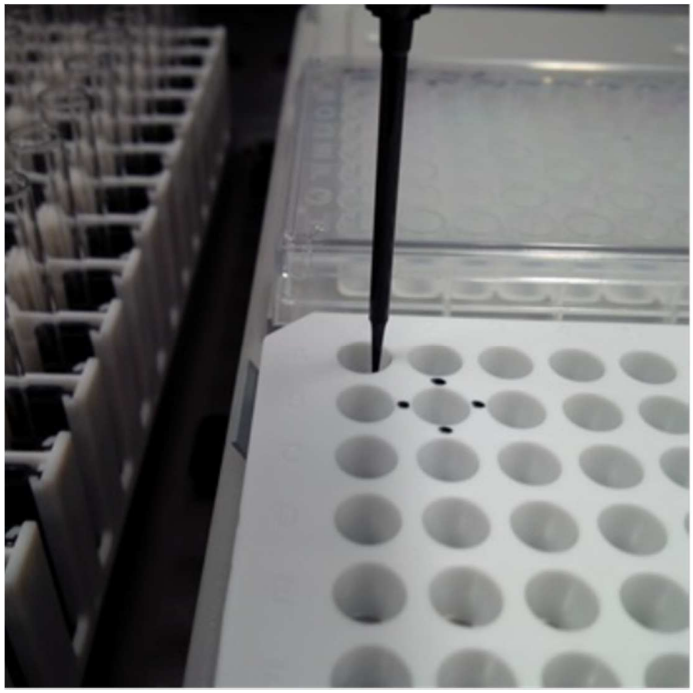

Figure 5: Sample transfer into analysis 96-wells plate

LDTD-MS/MS analysis time of 5.4 seconds sample to sample correspond to 8.7 minutes per plate. Sample preparation was optimized to approach this speed by preparing two plates in parallel. Incubation time of 15 minutes is the most restrictive step. During the action of β-glucuronidase on one plate, extraction and transfer to analyzing plate is processed for the other.

Conclusion:

Purified β-glucuronidase used in the preparation reduces the hydrolysis incubation time to 15 minutes instead of 1 hour. No false negative results are observed using this enzyme preparation. The LDTD technology combined with a mass spectrometer system allows ultra-fast and specific drug screening in urine samples with minimal sample preparation. **One** MRM method and **One** well used to screen 16 drugs in **5.4 seconds per sample.** Complete workflow with robotic system gives a capability of 400 samples per hour cadence with minimal false positive results.