Phytronix Breakaway Technologies

Sciex

Toxicology

Application Note : 2201

Screening of Benzodiazepine Drug Panel in Urine:

Drug Screening in Urine at 8 Seconds per Sample Using LDTD-MS/MS

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Introduction

Toxicological labs need to screen different drugs classes like benzodiazepines, anxiolytics and anticonvulsants using a generic sample preparation and fast analytical technique. Our goal for this application note is to use an automated sample preparation method for a drug panel in urine using a single operation in LDTD-MS/MS.

LDTD-MS/MS offers specificity combined with an ultra-fast analysis for an unrivaled screening method. To develop this application, we focused on performing a quick and simple sample preparation. Twenty-one drugs are analyzed **simultaneously** with **quantitative** screening results obtained in less than 8 seconds per sample. Each drug has been screened based on the industry cut-offs requirement.

Luxon Ionization Source

The Luxon Ion Source® (**Figure 1**) is the second-generation sample introduction and ionization source based on the LDTD® technology for mass spectrometry. Luxon Ion Source uses Fiber-Coupled Laser Diode (Figure 2) to obtain unmatchable thermal uniformity providing more precision, accuracy and speed. The process begins with dry samples which are rapidly evaporated using indirect heat. The thermally desorbed neutral molecules are carried into 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 thermal desorption process yields high-intensity molecular ion signal in less than 1 second sample-to-sample and allows working with very small volumes.

Figure 1 - Luxon Ion Source®

Figure 2 - Schematic of the Luxon Ionization Source

Sample Preparation Method

Sample Collection

Urine samples were collected and transferred into barcoded tubes, readable by the Azeo Liquid Handler.

Automated Sample Extraction

Each barcoded vial was scanned by the Azeo Liquid Handler and an automatic batch file was created. The Azeo extraction system (**Figure 3**) is used to extract the samples using the following conditions:

- 22.5 µL of Internal standard were added to each sample
- 75 µL of urine sample were transferred from the vials to a deep-well plate placed in the Lumo vortexer
  - Mix
- 22.5 µL β-Glucuronidase-RT Enzyme/Hydrolysis buffer were added to each sample.
  - Mix and incubate at room temperature for 15 minutes.
- 225 µL Extraction buffer and 300 µL Acetonitrile were added into the deep-well plate.
  - Mix and centrifuge (5000 rpm) for 5 minutes for phase separation.
- Mix 6.5 µL of desorption buffer with 20 µL of upper layer phase.
- Spot 6 µL of the mixture onto a LazWell™96 plate
  - Dry 5 minutes at 40°C

Figure 3 - Automated extraction system

LDTD®-MS/MS Parameters

LDTD
Model: Luxon S-960, Phytronix
Carrier gas: 6 L/min (air)
Laser pattern:

- 6-second ramp to 55% power
- Hold 2 seconds at 55% power

MS/MS

MS model: Q-Trap System® 5500, Sciex
Scan Time: 5 msec
Total run time: 8 seconds per sample
Ionization: APCI
Analysis Method: Positive MRM mode

Table 1 - MRM transitions for Luxon-MS/MS

	Transition	CE
Meprobamate	219.1 → 158.1	10
Carisoprodol-d_7	269.2 → 183.2	10
Nordiazepam	271.1 → 140.1	27
7-Aminoflunitrazepam	284.0 → 226.0	50
Diazepam	285.1 → 154.1	32
7-Amino Clonazepam	286.1 → 222.2	30
Oxazepam	287.1 → 241.1	30
7-Amino Clonazepam-d_4	290.1 → 226.0	30
7-Aminoflunitrazepam-d_7	291.1 → 230.0	50
Oxazepam-d_5	292.1 → 246.1	30
Temazepam	301.1 → 255.1	25
Temazepam-d_5	306.1 → 260.1	25
Zaleplon	306.1 → 264.1	30
Zolpidem	308.1 → 236.1	35
Alprazolam	309.1 → 274.1	35
Alprazolam-d_5	314.1 → 286.1	35
Clonazepam	316.0 → 214.0	50
Lorazepam	321.0 → 275.0	30
(Alpha) Hydroxyalprazolam	325.1 → 205.1	54
Midazolam	326.1 → 291.1	35
Clozapine	327.1 → 270.1	30
2-Hydroxyethylflurazepam	333.1 → 194.1	30
Alpha-OH-Midazolam	342.1 → 203.1	35
Etizolam	343.1 → 314.0	25
(Alpha) Hydroxytriazolam	359.0 → 331.0	36
Flurazepam	388.1 → 315.0	27

Results and Discussion

Initial Cut-off Test (ng/mL)

A drug list and screening cut-offs required by toxicological labs can be found in **Table 2**.

Table 2 - Analyte cut-offs

Analyte	Cut-off (ng/mL)	Analyte	Cut-off (ng/mL)
(Alpha) Hydroxyalprazolam	50	Alprazolam	50
7-Aminoclonazepam	50	Clozapine	50
Clonazepam	50	Etizolam	50
Diazepam	50	Flurazepam	50
Midazolam	50	Lorazepam	50
(Alpha) OH-Triazolam	50	Meprobamate	100
2-Hydroxyethylflurazepam	50	Nordiazepam	50
Zaleplon	50	Oxazepam	50
7-Aminoflunitrazepam	50	Temazepam	50
Alpha-OH-Midazolam	50	Zolpidem	50

Precision

Spiked samples around the decision point (50% cut-off: QC-L, cut-off: CO and 200% cut-off: QC-H) and blank solutions are used to validate the precision of the method. The peak area against the internal standard (IS) ratio was used to normalize the signal. Replicate extractions are deposited onto a LazWell™ plate and dried before analysis.

The following acceptance criteria were used:

- Each concentration must not exceed 20% CV
- The mean concentration ± 2 times the standard deviation must not overlap with other concentrations at the cut-off.

For the inter-run precision experiment, each fortified sample set is analyzed in triplicate on five different days. **Table 3** shows the inter-run precision results. No overlapping at the cut-off is observed for 2-Hydroxyethylflurazepam and the %CV was below 20%. Similar results are obtained for the other drugs in the panel.

Table 3 - Inter-Run Precision

2-Hydroxyethylflurazepam	QC-L	CO	QC-H
Conc (ng/ml)	25	50	100
N	15	15	15
Mean (ng/mL)	22.9	51.2	103.9
SD	1.6	1.7	3.1
%CV	7.2	3.3	3.0
Mean – 2SD (ng/mL)	19.7	47.8	97.7
Mean + 2SD (ng/mL)	26.3	54.6	110.1

For the intra-run precision experiment, each fortified sample is extracted and analyzed in 8 replicates. Area ratio results are plotted using the ± 2 STD error bars. **Figure 4** shows the intra-run results for 2-Hydroxyethylflurazepam. No overlapping is observed for each concentration and the %CV was below 20%. Similar results are obtained for the other drugs in the panel.

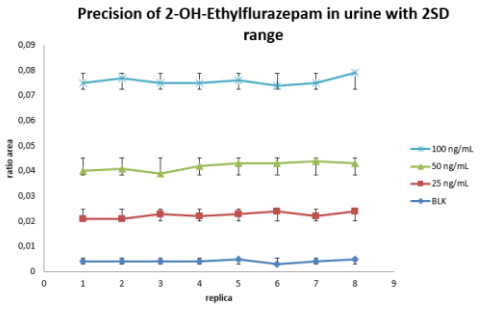

Figure 4 - Intra-Run Precision Curves for 2-Hydroxyethylflurazepam

Multi-matrix validation

Urine samples are collected from 63 patients. Samples are analyzed using LC-MS/MS reference method and LDTD-MS/MS method. The method sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy are verified as followed:

		LC-MS/MS	
		Yes	No
LDTD-MS/MS	Yes	TP (True positive)	FP (False positive)
	No	FN (False negative)	TN (True negative)

Where:

- Sensitivity: (TP / (TP + FN))
- Specificity: (TN / (TN + FP))
- PPV: (TP / (TP + FP))
- NPV: (TN / (TN + FN))
- Accuracy: ((TP+TN) / (TP + FN+TN+FP))

Table 4 shows the analysis result of 63 real samples for 2-Hydroxyethylflurazepam.

Table 4 – 2-Hydroxyethylflurazepam results

2-Hydroxyethylflurazepam		LC-MS/MS	
		Yes	No
LDTD-MS/MS	Yes	TP = 8	FP = 0
	No	FN = 0	TN = 55

Validation results are reported in **Table 5** for 2-Hydroxyethylflurazepam. Similar results are obtained for the other drugs.

Table 5 – Validation results for 2-Hydroxyethylflurazepam

Parameters	2-Hydroxyethylflurazepam
Sensitivity (%)	100
Specificity (%)	100
PPV (%)	100
NPV (%)	100
Accuracy (%)	100

Conclusion

Luxon Ion Source® combined with Sciex Q-Trap 5500 mass spectrometer system allows ultra-fast (**8 seconds per sample**) screening of Benzodiazepine drug panel in urine using a simple and automated sample preparation method.