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Drug Screening and Confirmation Using Two LC Columns and Identical LC-MS/MS Conditions Combined into a Single, Streamlined Forensic Workflow

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Introduction

Methods for detection of drugs and metabolites in forensic toxicology laboratories must meet certain standards to maintain the integrity of lab results in a "screen with confirmation" workflow, where results may be used in legal proceedings. Recently updated standards from The Academy Standards Board (ASB), part of the American Academy of Forensic Sciences (AAFS) define the analytical scope and reportable limits for drug-facilitated crime investigations (ANSI/ASB Standard 121, First Edition 2021).

Forensic toxicology labs have traditionally used immunoassay for drug screening, followed by GC-MS for accurate confirmation and quantitation of drugs and metabolites in biological matrices for drug facilitated crime investigations. Immunoassay only identifies a drug class. Individual drug analytes are identified and quantitated by a more specific technique, like GC-MS. Large sample backlogs and limited resources are driving demand for faster, more efficient forensic toxicology workflows. GC-MS methods are being replaced by LC-MS/MS in forensic toxicology labs because it offers several improvements. LC-MS/MS provides better sensitivity compared to GC-MS for analytes with more diverse properties and does not require derivatization of non-volatile compounds. More recently, methods where both screening and confirmation are performed by LC-MS techniques are increasing in usage because of improved sensitivity and specificity for drug screening compared to immunoassay.

Achieving a More Efficient Drug Screening Workflow

To implement a more streamlined, efficient workflow this technical note describes an LC-MS/MS method for forensic drug screening and confirmation that are integrated to improve efficiency and reduce turnaround time. Two orthogonal LC columns (a Phenomenex Kinetex C18 and a Phenomenex Phenyl-Hexyl) using identical mobile phase and gradient conditions are used for drug screening and confirmation. Harmonizing these method conditions across the two different stationary phases simplifies and creates a more efficient workflow that requires fewer types of instrumentation, one sample preparation procedure, and streamlined data analysis.

LC-MS/MS Analysis

The majority of drugs and metabolites were analyzed using positive mode ESI. Separate LC-MS/MS conditions were required for barbiturates and γ-Hydroxybutyric Acid for optimum ionization, detection and quantitation in negative mode. Standard validation practices followed ANSI/ASB Standard 036, First Edition 2019 Standard Practices for Method Validation in Forensic Toxicology. Method performance indicators included accuracy, precision, measurement uncertainty, calibration models, reportable range, sensitivity, specificity, carryover, interference, ion suppression/enhancement, and analyte stability.

MS/MS Conditions

Ion Source:	ESI	
Polarity:	Positive	Negative
Source Temperature:	350 °C	350 °C
Source Flow Rate:	12 L/min	12 L/min
Nebulizer Gas:	50 psi	50 psi
Capillary Voltage:	+2000 V	-4000 V

LC Conditions – Most Analytes

Column: Kinetex™ 2.6 µm C18, 50 x 2.1 mm (00B-4780-AN)
Kinetex 2.6 µm Phenyl-Hexyl, 50 x 4.6 mm (00B-4495-E0)

Mobile Phase: A: 0.1 % Formic Acid in 25 mM Ammonium Formate
B: Methanol

Gradient:

Time (min)	%B
0	10
1.5	50
4	65
4.5	65
5.5	95
7.5	95
7.75	10
10	10

Flow Rate: 0.4 mL/min
Injection Volume: 10 µL
Temperature: 50 °C
LC System: Agilent® 1260
Detection: MS/MS, positive ionization
Detector: Agilent 6420

LC Conditions - Barbituates

Column: Kinetex 2.6 µm C18, 50 x 2.1 mm (00B-4780-AN)
Kinetex 2.6 µm Phenyl-Hexyl, 50 x 4.6 mm (00B-4495-E0)

Mobile Phase: A: 10 mM Acetic Acid in Water
B: Methanol

Gradient:

Time (min)	%B
0	35
1.25	35
1.26	75
3	75
3.1	35
4	35

Flow Rate: 0.8 mL/min
Injection Volume: 35 µL
Temperature: 50 °C
LC System: Agilent 1260
Detection: MS/MS, negative ionization
Detector: Agilent 6420

LC Conditions - γ-Hydroxybutyric Acid

Column: Kinetex 2.6 µm C18, 100 x 2.1 mm (00D-4780-AN)
Kinetex 2.6 µm Phenyl-Hexyl, 50 x 4.6 mm (00B-4495-E0)

Mobile Phase: A: 10 mM Acetic Acid in Water
B: Methanol

Flow Rate: 0.5 mL/min (Isocratic, 1 % Mobile Phase B for 3 min)
Injection Volume: 1 µL
Temperature: 50 °C
LC System: Agilent 1260
Detection: MS/MS, negative ionization
Detector: Agilent 6420

Sample Preparation

All results were generated from 100 µL urine samples. ToxBox® forensic test kits were manufactured by PinPoint Testing, LLC. These premanufactured kits have calibrators, QCs, and internal standards at precise concentrations in a 96-well plate format for high-throughput testing. The kits have NIST-traceable, certified reference materials (CRMs) for all drug analyte standards, isotopically labeled internal standards and hydrolysis enzyme and buffer incorporated into a suspended-state, "ready-to-go" format. ToxBox kits were stored at −20 °C prior to use. They were removed from storage and used following the PinPoint Testing directions of use.

Calibrators and QC concentrations met sensitivity requirements as outlined in ANSI/ASB Standard 121 (**Table 1**). Three levels of QC were prepared. Four replicates of each QC were analyzed over 5 runs. Separate 96-well plates were prepared and processed for the screening and confirmation analyses. Samples were hydrolyzed in-well using the ToxBox kit prior to extraction and analysis. Analyte extraction was accomplished using a liquid-liquid extraction (LLE) method developed by PinPoint Testing. Briefly, 200 µL or urine was hydrolyzed, then treated with an alkaline pH buffering solution prior to extraction using ethyl acetate. Mixing for LLE was accomplished by aspirating /dispensing each sample 10 times using a multi-channel pipette. The aqueous layer was removed, and the organic portion was evaporated under a gentle stream of Nitrogen and reconstituted for LC-MS/MS analysis.

Results and Discussion

All linear calibration curves had a correlation coefficient (R^2) of >0.99. The 60-drug panel encompassed multiple drug classes, including opioids, sedatives, stimulants, cannabinoids, and other prescribed and illicit drugs (**Table 1**). One standardized LC-MS/MS method was employed for both screening and confirmation, with the mobile phase across the LC columns harmonized to further streamline the workflow. Both columns provided acceptable chromatography for all drug analytes in urine samples processed using the ToxBox kit and protocol (Avram, M. *et al, Chem. Res. Toxicol*., 2023).

Relevant concentrations for all analytes are listed in **Table 1**.

Accuracy was within ±10 % for 99% of the results, except Lorazepam on Phenyl-Hexyl column. Percent Bias was ±10 % for all analytes except for Hydrocodone QC Low (11.7 % on the C18 column and 11.0 % on the Phenyl-Hexyl column), and Lorazepam on the Phenyl-Hexyl column. Within run %CV was within 10 % for all analytes except Lorazepam on the Phenyl-Hexyl column. Between run % CV was within ±10 % for all analytes except γ-Hydroxybutyric Acid QC Low (11.7 % on the C18 column and 11.2 % on the Phenyl-Hexyl column), Norbuprenorphine QC Low (10.7 % on the C18 column and 12.6 % on the Phenyl-Hexyl column), Secobarbital QC High (11.5 % on the Phenyl-Hexyl column) and Lorazepam QC Low and QC Med on the Phenyl-Hexyl column.

Lorazepam showed good results on the C18 column, but variable results on the Phenyl-Hexyl column. Accuracy was 238 %, 198 %, and 117 % for QC Low, QC Med, and QC high respectively. Percent Bias was 135 % for QC Low, 97.6 % QC Med, and 17.7 % QC High on the Phenyl-Hexyl column. Within run %CV was 14.4% for QC Med for lorazepam on the Phenyl-Hexyl column, and between run % CV was Lorazepam with 26.8 % QC Low and 54.9 % QC Med.

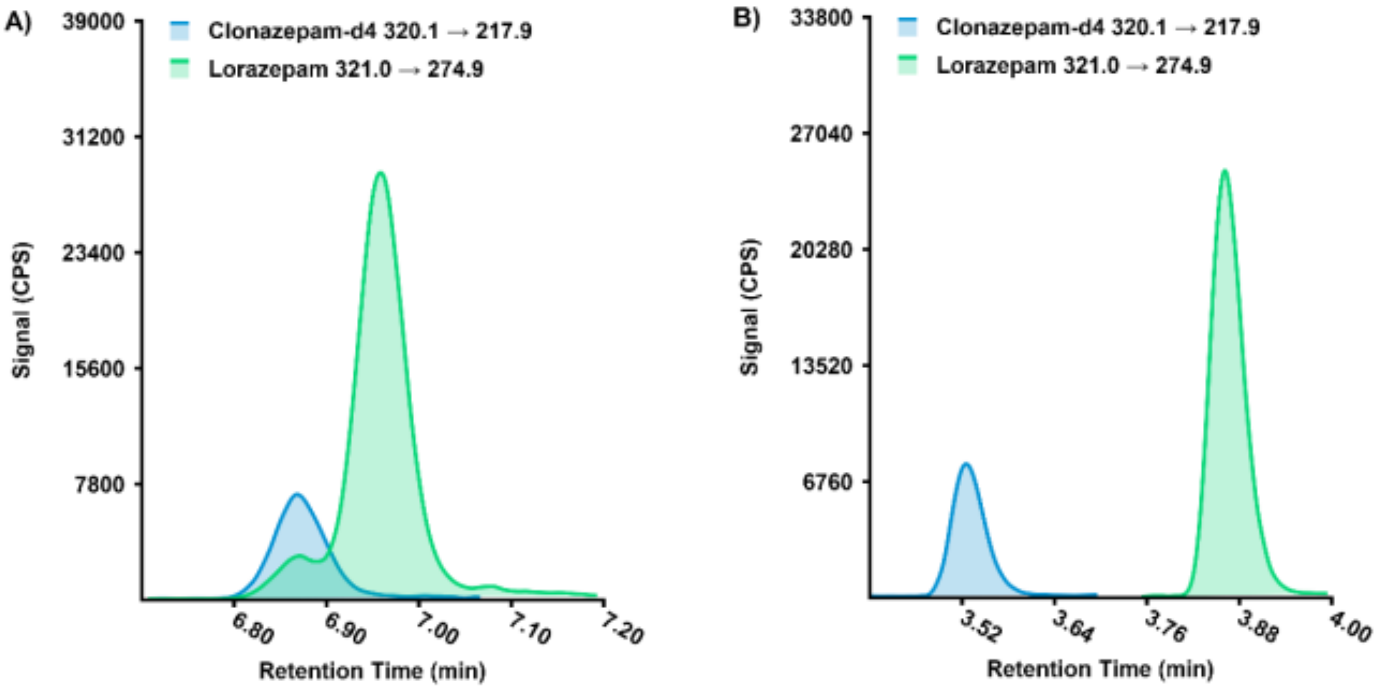
The signal-to-noise ratio of the primary ion used to detect Lorazepam was lower than the other benzodiazepines, so quantitation of lorazepam at the ASB-recommended cutoff of 5 ng/mL was difficult. This study validated a lorazepam cutoff at 10 ng/mL on the Agilent® 6420 LC-MS/MS when the C18 stationary phase is used. Several techniques could be used to improve sensitivity. For example, larger sample volumes could be extracted and concentrated. Utilization of more sensitive LC-MS/MS platforms could also improve Lorazepam detection, but this equipment may not be readily available in state-supported laboratories. Lorazepam confirmation analysis is further complicated with the use of Clonazepam-d4 as an internal standard, which coeluted on the phenyl-hexyl column but not on the C18 column (**Figure 1**).

Interference of clonazepam-d4 prevented accurate low-level quantitation of lorazepam on the Phenyl-Hexyl column. Attempts to modify the mobile phase and gradient conditions did not resolve this interference. Validation results showed that the C18 column was optimal for lorazepam quantitation and the Phenyl-Hexyl column was better suited for screening. In these studies, results for all drug analytes were acceptable for screening and confirmation per the ASB standards.

Table 1. 60 Drug Analytes in the Kinetex™ C18 and Phenyl-Hexyl Column LC-MS/MS Methods. All Concentrations are reported in ng/mL.

Analyte	ASB cutoff	Analytical Meaurement Range	QC Low Target Concentration	QC Med Target Concentration	QC High Target Concentration
6-MAM	-	1 - 1000	3.0	60	900
7-Aminoclonazepam	5	5 - 500	7.5	150	450
α-Hydroxyalprazolam	5	2.5 - 250	7.5	30	150
Alprazolam	-	5 - 5000	15.0	300	4500
Amitriptyline	10	5 - 1000	15.0	300	900
Amphetamine	25	2.5 - 1250	7.5	150	750
Benzoylecgonine	50	25 - 5000	75.0	1500	4500
Brompheniramine	10	1 - 1000	3.0	60	900
Buprenorphine	-	1 - 100	1.5	30	90
Butalbital	100	50 - 10000	150.0	3000	9000
Carbamazepine	-	5 - 500	15.0	60	300
Carisoprodol	100	10 - 10000	30.0	600	9000
Chlorpheniramine	10	1 - 1000	3.0	60	900
Clonazepam	-	25 - 5000	75.0	1500	4500
Cocaethylene	-	2 - 2000	6.0	120	1800
Cocaine	-	10 - 2000	30.0	600	1800
Codeine	10	5 - 1000	15.0	300	900
Cyclobenzaprine	10	5 - 1000	15.0	300	900
Desipramine	10	1 - 1000	3.0	60	900
Dextromethorphan	10	5 - 1000	15.0	300	900
Diazepam	-	1 - 1000	3.0	60	900
Diphenhydramine	10	10 - 1000	15.0	300	900
Doxylamine	10	5 - 1000	15.0	300	900
Fentanyl	1	0.50 - 100	1.5	30	90
Gabapentin	-	100 - 10000	150.0	3000	9000
γ-Hydroxybutyric Acid	10000	1000 - 100000	1500.0	30000	90000
Hydrocodone	10	5 - 1000	15.0	300	900
Hydromorphone	10	5 - 1000	15.0	300	900
Imipramine	10	1 - 1000	3.0	60	900
Ketamine	-	5 - 1000	15.0	300	900
Lorazepam	5	10 - 250	10.0	30	150
mCPP	10	10 - 1000	15.0	300	900
MDA	25	12.5 - 2500	37.5	750	2250
MDMA	25	2.5 - 2500	7.5	150	2250
Meprobamate	100	50 - 10000	150.0	3000	9000
Methadone	-	5 - 5000	15.0	300	4500
Methamphetamine	25	2.5 - 2500	7.5	150	2250
Mitragynine	-	5 - 1000	15.0	300	900
Morphine	10	5 - 1000	15.0	300	900
Norbuprenorphine	-	1 - 100	1.5	30	90
Norchlorcyclizine	10	5 - 1000	15.0	300	900
Nordiazepam	10	5 - 1000	15.0	300	900
Norfentanyl	1	1 - 100	1.5	30	90
Norketamine	10	5 - 1000	15.0	300	900
Nortriptyline	10	5 - 1000	15.0	300	900
o-Desmethyl-tramadol	-	5 - 5000	15.0	300	4500
Oxazepam	10	10 - 1000	15.0	300	900
Oxycodone	1	1 - 500	3.0	60	300
Oxymorphone	10	10 - 500	15.0	60	300
Phencyclidine	-	5 - 500	15.0	60	300
Phenobarbital	100	50 - 10000	150.0	3000	9000
Pregabalin	-	100 - 10000	150.0	3000	9000
Secobarbital	-	50 - 10000	150.0	3000	9000
Tapentadol	-	5 - 1000	15.0	300	900
Temazepam	10	5 - 1000	15.0	300	900
THC-COOH	10	5 - 500	7.5	150	450
Tramadol	10	5 - 1000	15.0	300	900
Zolpidem	-	2 - 2000	6.0	120	1800
Zolpidem-COOH	10	5 - 1000	15.0	300	900
Zopiclone	-	1 - 1000	3.0	60	900

Figure 1 . Extracted Ion Chromatograms for Lorazepam Neat Standard at 50 ng/mL on the Kinetex™ Phenyl-Hexyl Column (A) and the Kinetex C18 Column (B).



Conclusions

This tech note summarizes an elegant, harmonized LC-MS/MS method for screening and confirmation of a large panel of 60 drugs and metabolites in urine that was validated to meet the new ANSI/ASB 121 Standard using Phenomenex columns and the ToxBox® forensic test kits. Employing two complementary LC columns (a Phenyl-Hexyl for drug screening and a C18 for confirmation and quantitation) under identical mobile phase and gradient conditions results in a more efficient workflow that reduces the overall number of reagents required, streamlines processing and data review and harmonizes instrument use. Using LC-MS/MS for screening improves sensitivity and specificity over immunoassay methods that only identify a drug class, and provides a modern solution for labs converting from GC-MS to LC-MS/MS or expanding their existing use of LC-MS/MS. This method should help to increase turn-around-time and reduce sample backlogs compared to traditional immunoassay screening techniques followed by GC-MS drug confirmation methods.

Kinetex™ Ordering Information

2.6 µm Minibore Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡
Phases	**30 x 2.1**	**50 x 2.1**	**75 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
EVO C18	00A-4725-AN	00B-4725-AN	—	00D-4725-AN	00F-4725-AN	AJ0-9298
PS C18	00A-4780-AN	00B-4780-AN	—	00D-4780-AN	00F-4780-AN	AJ0-8951
Polar C18	00A-4759-AN	00B-4759-AN	—	00D-4759-AN	00F-4759-AN	AJ0-9532
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322

for 2.1 mm ID

2.6 µm Analytical Columns (mm)						SecurityGuard ULTRA Cartridges‡	
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
EVO C18	00A-4725-E0	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	00G-4725-E0	AJ0-9296
PS C18	00A-4780-E0	00B-4780-E0	—	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949
Polar C18	00A-4759-E0	00B-4759-E0	—	00D-4759-E0	00F-4759-E0	—	AJ0-9530
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	—	AJ0-9207
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	—	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	—	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	—	AJ0-8770
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	—	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	—	AJ0-8774
F5	00A-4723-E0	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	—	AJ0-9320

for 4.6 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

Need a different column size or sample preparation format?

No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/Chat.

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