Development and Validation of Liquid Chromatography Quadrupole/Time of Flight (LC/QTOF) Drug Screening Method for Over 375 Drugs in Blood using Size Exclusion Chromatography (SEC) at ANSI/ASB Recommended Screening Thresholds

Celia Modell , MSFT, D-ABFT, Brooklynn Molina, MSFT, Jared Castellani, MSFS, D-ABFT-FT, Dustin Smith, F-ABFT
South Carolina Law Enforcement Division, Columbia, SC

Introduction

In forensic toxicology, the drug screening process is a critical component of case examinations. Many widely used screening methodologies exhibit analytical limitations. Advancements in instrumentation have prompted a pursuit of screening techniques that offer an expanded scope, improved sensitivity and specificity, and reduced sample size, all while preserving sample integrity. By employing size exclusion chromatography (SEC), it is possible to enhance sensitivity for low-level analytes by mitigating biological matrix effects. The integration of SEC for sample preparation with liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC/QTOF) for analytical detection facilitates the identification of analytes at the thresholds recommended by ANSI/ASB standards. This validation of over 375 analytes includes the identification of carboxy-THC and barbiturates through the analysis of a single extracted aliquot using both positive and negative modes. This Auto MS/MS LC-QTOF method provides significantly more comprehensive information compared to the current implanted immunoassay technique fundamentally transforming our approach to case work in the future.

Methods

Size Exclusion Chromatography Extraction:

1. 0.5 mL blood sample was aliquoted
2. 10 µL internal standard was added to all samples
3. Protein precipitation dropwise using 750 µL of cold acetonitrile
4. Sample was centrifuged for 10 mins under 4000 RPM then transferred to a clean vial and dried to completion under nitrogen
5. **Size Exclusion Chromatography Extraction (DPX®'s SEC Tips (1500 Da):**
   a) Hydrated resin tip by placing tip base into deionized water
   b) Reconstituted sample vial with 50 µL total volume (10 µL of methanol then vortexed, followed by 40 µL of deionized water)
   c) Sample was vortexed and 40 µL of reconstitution volume was placed on top of resin bed to be filtered gravimetrically to waste
   d) After all liquid has been absorbed into the resin bed the SEC tip was washed with 40 µL of 20:80 methanol: deionized water and gravimetrically filtered through the tip to waste
   e) The tip was transferred over to a new vial and 300 µL of 50:50 methanol: deionized water was eluted and dried under nitrogen to completion
   f) Sample reconstituted in 100 µL of 95:5 mobile phase A and mobile phase B

Instrumentation: Agilent 1260 Infinity II system coupled with an Agilent 6546 QTOF with Auto MS/MS acquisition method

Column: Phenomenex® Kintex 2.6 µm Phenyl Hexyl column (50 x 4.6 mm) paired with Phenomenex® Kintex phenyl guard column.

Positive HPLC Gradient

Time (min)	Pump A %	Pump B%
0.50	95.0	5.0
5.50	5.0	95.0
9.00	5.0	95.0
9.01	95.0	5.0
10.00	95.0	5.0

Negative HPLC Gradient

Time (min)	Pump A %	Pump B%
0.00	60.0	40.0
2.00	2.0	98.0
3.00	2.0	98.0
3.60	60.0	40.0
5.10	60.0	40.0

Mobile Phase A: 0.1% formic acid with 5 mM Ammonium Acetate in Deionized Water
Mobile Phase B: 0.1% formic acid with 5 mM Ammonium Acetate in Methanol

Dual AJS ESI Source Settings

	Positive	Negative
Gas Temp.	250°C	350°C
Drying Gas	10 L/min	12 L/min
Nebulizer	45 psi	15 psi
Sheath Gas Temp	375°C	350°C
Sheath Gas Flow	12 L/min	12 L/min
VCap	3500 V	3500 V
Nozzle Voltage	500 V	500 V

Acquisition Parameters

	Positive	Negative
MS Mass Range	100-950 m/z	75-975 m/z
MS Acquisition Rate/Time	7 spectra/s	7 spectra/s
MS/MS Mass Range	25-950 m/z	25-975 m/z
MS/MS Acquisition Rate/Time	3 spectra/s	3 spectra/s
Collision Energy	10, 20, 40	10, 20, 40
Max Precursor Per Cycle	6	6
Precursor Threshold	800 counts	800 counts

Methods

Using Agilent Qualitative Analysis 10.1 software the following settings were used:

Processing Parameter	Settings
EIC Mass Extraction Window	±10 ppm
EIC RT Extraction Window	±0.7 min
Mass Match Tolerance	±10 ppm
Overall Score Cutoff	40
Absolute Area Cutoff	7000(POS) 2000 (NEG)

Personal Compound Database and Library (PCDL) library subsets were made to process the samples based on the compound's ionization and detection in either the positive or negative method. The overall cutoff score incorporates retention time, mass accuracy, and isotopic fidelity.

Criteria considered for a positive identification include mass accuracy, peak shape, mass spectra, fragmentation spectra consistent with known library spectra and fragmentation, isotopic fidelity and retention time.

Validation Parameters Evaluated

Interference Study

To assess the analytical method for potential interferences, all isobaric compounds from the PCDL containing more than 385 entries were identified. The isobaric analytes were broken down into 43 sets based on their accurate mass. Each set was analyzed on the analytical method as a neat to determine if all analytes could be identified based on retention time and fragmentation patterns. Of the 43 sets, it was determined that 12 contained analytes that could not be resolved chromatographically and therefore required a combined entry within the PCDL. A combined entry was created for the following analytes: ephedrine/pseudoephedrine, chloroethcathinone/chloro N,N-dimethylpentylone, amitriptyline/maprotiline, alprazolam/4-chlorodeschloroalprazoam, quinidine/quinine, crotonyl/cyclopropyl fentanyl and FIBF/p-FBF. In addition, delta8- and delta9-THC and carboxy-THC could not be resolved and therefore a general entry was created.

Ion Suppression/Enhancement Study

Ionization suppression and enhancement was evaluated using a post extraction addition method. Groups of approximately 50 analytes with similar retention times were evaluated. During the assessment, suppression or enhancement was detected for a majority of the analytes. Reflexively, 10 different matrix sources were evaluated during the limit of detection study.

Limit of Detection Study

The lower level of detection was assessed for each drug based on literature and casework. Each of the 10 matrices were extracted in duplicate and evaluated with the qualitative analysis software for consistent detection of the compound and the criteria to properly identify that compound. Per ANSI/ASB Standard 120, during the validation of a non-immunoassay screening technique, confirmation concentrations should be utilized for the recommended screening levels. These confirmation concentrations will apply to non-immunoassay methods unless the screen level indicates N/A. The screening levels illustrated in the figure to the right are developed for immunoassay screening. All validated LOD levels met or exceeded the recommendations established by ANSI/ASB Standard 119 and 120 except for one compound, buprenorphine. Buprenorphine screening/confirmation recommendations for blood DUI cases is 0.5 ng/mL and could not be achieved consistently with this method. Buprenorphine was consistently identified at 1 ng/mL which aligns with our current quantitation method and death investigation recommendations.

Recommended Analytical Scope and Testing

	Death Investigation ANSI/ASB Standard 119		Impaired Driving Investigations ANSI/ASB Standard 120		
	Screen (ng/mL)	Confirmation (ng/mL)	Screen (ng/mL)	Confirmation (ng/mL)	Validated @ LOD*
6-acetylmorphine	N/A	5	N/A	5	1 ng/mL
7-aminoclonazepam	15	15	10	10	10 ng/mL
Alprazolam					
Amphetamine	25	25	20	20	
Benzoylecgonine	50	50	50	50	
Buprenorphine	1	1	1	0.5	1 ng/mL¥
Butalbital	0.1 µg/mL	0.1 µg/mL			0.1 µg/mL
Carisoprodol	1 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	0.1 µg/mL
Clonazepam	15	15	15	10	10 ng/mL
Cocaethylene	N/A	20	N/A	10	
Cocaine					
Codeine	10	10	10	10	
COOH-THC	10	10	10	5	5 ng/mL
Diazepam	50	50	50	20	10 ng/mL
Fentanyl	1	1	1	0.5	0.5 ng/mL
Hydrocodone	10	10	10	10	10 ng/mL
Hydromorphone					1 ng/mL
Lorazepam	15	15	15	10	10 ng/mL
MDA	25	25	25	25	
MDMA					
Meprobamate	1 µg/mL	1 µg/mL	N/A	1µg/mL	0.1 µg/mL
Methadone	50	50	50	20	20 ng /mL
Methamphetamine	25	25	20	20	10 ng/mL
Morphine	10	10	10	10	
Norbuprenorphine			N/A	1	2.5 ng/mL
Nordiazepam	50	50	50	20	10 ng/mL
ODT			N/A	50	
Oxazepam	50	50	50	20	
Oxycodone	10	10	10	10	
Oxymorphone	5	5			1 ng/mL
Pentobarbital	0.1 µg/mL	0.1 µg/mL			0.1 µg/mL
Phenobarbital					
Secobarbital					
Temazepam	50	50	50	20	10 ng/mL
Tramadol			100	50	
Zolpidem	15	15	10	10	
GHB					10 µg/mL

*Established LOD levels exceeded recommendations
¥ Recommendation was not reached.

Results

Stability Study

The stability of processed samples was evaluated over a 96-hour period following extraction. A positive control mix containing 103 analytes was tested at the analytes' established LOD and at 50 times the LOD. Analytes were deemed stable if the response of the analyte from each 24-hour interval did not exceed 25% variation from its original response on the day of extraction. All analytes were considered stable for the 96-hour period, with exception of carboxy-THC and 6-MAM, which showed significant signal reduction within 48-hours.

Case Comparison

A total of 50 case samples were evaluated for the comparison study. All case samples were blindly tested, analyzed and evaluated. The quality controls for this analytical method include a low, mid, and high control followed by a negative control prior to cases with a low control at the middle and end of the run. The positive controls contained 103 analytes and 9 internal standards to encompass a wide range of drug classes and retention times. The cases used for comparison ranged from antemortem and postmortem blood samples. All reported analytes were identified using the new QTOF analytical screening method with one caveat. Due to the larger scope obtained using the QTOF and its increased sensitivity, additional analytes were identified.

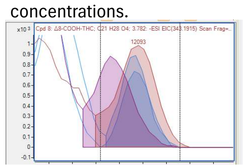
Discussion

Protein Crash v. DPX® SEC Tip

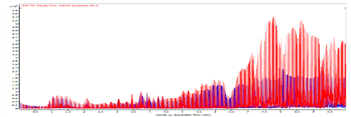
In developing a screening method using QTOF for a wide range of analytes, including acidic, neutral, and basic drugs, significant reductions in background and matrix effects were essential to meet the sensitivity requirements outlined by ANSI/ASB scope recommendations. Various extraction procedures were evaluated to optimize analyte recovery while maintaining instrument cleanliness for enhanced sensitivity. The aim was to minimize sample manipulation to prevent loss of response or identification of low-level analytes.

A comparison of protein precipitation versus the use of DPX® SEC Tips revealed a notable reduction in background noise. DPX® SEC Tips effectively minimized the presence of masses above 1500 Da, and significantly enhanced sensitivity for target compounds. Given the biological matrix's high content of fatty acids and proteins, DPX® SEC Tips filtered out these larger masses, resulting in a cleaner sample for analysis.

When solely using protein precipitation, the instrument struggled to maintain stable tuning and consistent results due to matrix buildup, necessitating frequent maintenance. Moreover, protein precipitation alone was inadequate for detecting the 5 ng/mL COOH-THC screening threshold. However, incorporating DPX® SEC Tips significantly reduced interferences, enabling the detection of COOH-THC at these low concentrations.



Graphic (left): Recovery of COOH-THC with a protein crash (purple and orange) v. recovery with DPX® SEC Tip (blue).Graphic (right): Two extracted samples for 5 ng/mL of COOH-THC using the DPX® SEC Tip.



Chromatogram showing the reduced background from an extracted protein crash (red) compared to using a protein crash in conjunction with the DPX® SEC Tip (blue).

Future

With the completion of this validation in the blood matrix, the next step is to validate the same method for urine samples. The sample preparation approach has already been established, and the drugs have been optimized to align with the lower levels specified in current recommendation standards. Validating the urine method will eliminate the need for immunoassay in our laboratory, allowing us to utilize QTOF as our primary screening technique.

References

ANSI/ASB Standard 036, ANSI/ASB Standard 119, ANSI/ASB Standard 120

VIEW NOW

CONTACT INFORMATION