Development and Validation of LC/QTOF Drug Screening Method for Over 375 Drugs in Blood Using Size Exclusion Chromatography (SEC) Tips

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HIGHLIGHTS: Improved sensitivity, reduced sample volume

SEC

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.

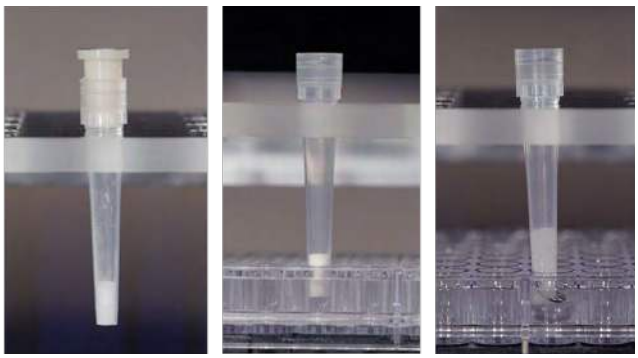

Figure 1. *DPX Technologies' 300 µL SEC Tip as it is received with removable cap (left), without removable cap (center), and swollen (right).*

The integration of INTip™ 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 implemented immunoassay technique, fundamentally transforming our approach to case work in the future.

MATERIALS AND METHODS

Samples were prepared by aliquoting 0.5 mL of blood and adding 10 µL of internal standard to all samples. Protein precipitation was then performed dropwise using 750 µL of cold acetonitrile. The precipitated samples were centrifuged for 10 minutes at 4000 RPM, then transferred to a clean vial and dried to completion under nitrogen. SEC Tips (DPX Technologies, Cat # DPX170471, Columbia, SC) were hydrated by placing the tip into deionized water. The sample was reconstituted by adding 10 µL of methanol, vortexing, then 40 µL of deionized water. The sample was vortexed again and 40 µL of the reconstitution volume was loaded on top of the SEC resin bed. After all liquid was absorbed into the resin bed, the SEC Tip was washed with 40 µL of 20:80 methanol:deionized water and gravity flowed through the tip to waste. The SEC Tip was transferred to a new vial and 300 µL of 50:50 methanol:deionized water was eluted and dried under nitrogen to completion. The sample was reconstituted in 100 µL of 95:5 mobile phase A (0.1% formic acid with 5 mM ammonium acetate in deionized water) and mobile phase B (0.1% formic acid with 5 mM ammonium acetate in methanol). The instrumentation used was an Agilent 1260 Infinity II system coupled with an Agilent 6546 QTOF with Auto MS/MS acquisition method. The column was a Phenomenex® Kintex 2.6 µm Phenyl Hexyl column (50 x 4.6 mm) paired with a Phenomenex® Kintex Phenyl guard column.

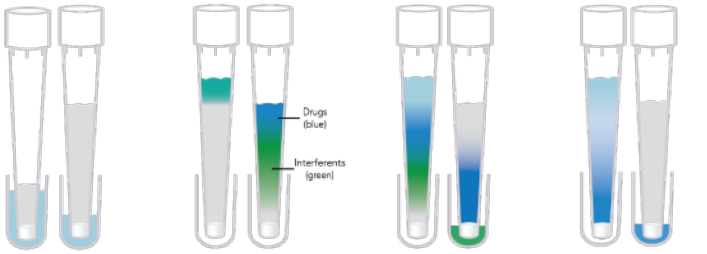

Figure 2. *Sample preparation method using 300 µL SEC Tips.*

VALIDATION PARAMETERS EVALUATED

To assess the analytical method for potential interferences, all isobaric compounds from the Personal Compound Database Library (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.

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 (LOD) 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 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 **Table 1** 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.

RESULTS

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 the exception of carboxy-THC and 6-MAM, which showed significant signal reduction within 48-hours. 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

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 alone versus with the use of SEC Tips revealed a notable reduction in background noise. 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, SEC Tips filtered out these larger masses, resulting in a cleaner sample for analysis.

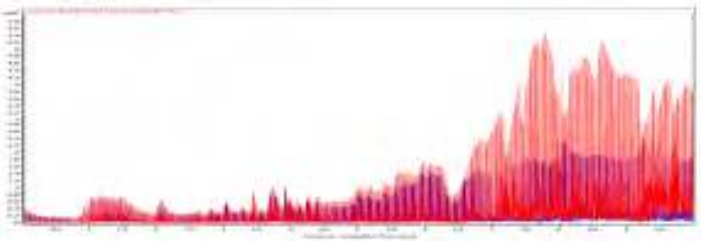

Figure 3. *Chromatogram showing the reduced background from an extracted protein crash (red) compared to using a protein crash in conjunction with DPX Technologies' SEC Tips (blue).*

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 SEC Tips significantly reduced interferences, enabling the detection of COOH-THC at these low concentrations.

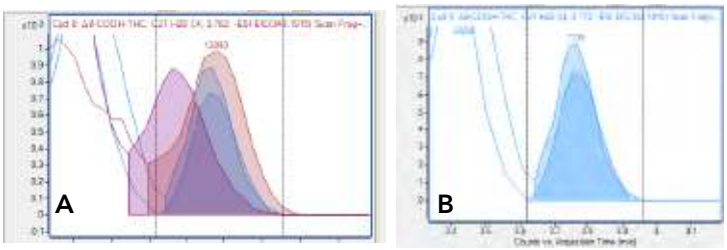

Figure 4. *Detection of COOH-THC at Screening Threshold. **A.** Recovery of COOH-THC with a protein crash (purple and orange) vs. recovery with DPX's SEC Tips (blue). **B.** Two extracted samples for 5 ng/mL of COOH-THC using DPX's SEC Tips.*

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

1. *ANSI/ASB Standard 036*
2. *ANSI/ASB Standard 119*
3. *ANSI/ASB Standard 120*

ACKNOWLEDGMENTS

1. *South Carolina Law Enforcement Division, Columbia, South Carolina*

Table 1. *Recommended Analytical Scope and Testing.*
**Established LOD levels exceeded*
¥ Recommendation was not reached

Analyte	Death Investigation ANSI/ASB Standard 119		Impaired Driving Investigations ANSI/ASB Standard 120		Validated @ LOD
	Screen (ng/mL)	Confirmation (ng/mL)	Screen (ng/mL)	Confirmation (ng/mL)	
6 - acetylmorphine	N/A	5	N/A	5	1 ng/mL
7 - aminoclonazepam	15	15	N/A	10	10 ng/mL
Alprazolam	15	15	10	10	10 ng/mL
Amphetamine	25	25	20	20	10 ng/mL
Benzoylecgonine	50	50	50	50	10 ng/mL
Buprenorphine	1	1	1	0.5	1 ng/mL
Butalbital	0.1 µg/mL	0.1 µg/mL	N/A	N/A	0.1 µg/mL
Carlsoprodol	1 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	0.1 µg/mL
Clonazepam	15	15	15	10	10 ng/mL
Cocaethlene	N/A	20	N/A	10	10 ng/mL
Cocaine	N/A	20	N/A	10	10 ng/mL
Codeine	10	10	10	10	10 ng/mL
C00H-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	10	10	N/A	N/A	1 ng/mL
Lorazepam	15	15	15	10	10 ng/mL
MDA	25	25	25	25	10 ng/mL
MDMA	25	25	25	25	10 ng/mL
Meprobamate	1 µg/mL	1 µg/mL	N/A	1 µg/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	10 ng/mL
Norbuprenorphine	N/A	N/A	N/A	1	2.5 ng/mL
Nordlazepam	50	50	50	20	10 ng/mL
ODT	N/A	N/A	N/A	50	10 ng/mL
Oxazepam	50	50	50	20	10 ng/mL
Oxycodone	10	10	10	10	10 ng/mL
Oxymorphone	5	5	N/A	N/A	1 ng/mL
Pentobarbital	0.1 µg/mL	0.1 µg/mL	N/A	N/A	0.1 µg/mL
Phenobarbital	0.1 µg/mL	0.1 µg/mL	N/A	N/A	0.1 µg/mL
Secobarbital	0.1 µg/mL	0.1 µg/mL	N/A	N/A	0.1 µg/mL
Temazepam	50	50	50	20	10 ng/mL
Tramadol	50	50	100	50	10 ng/mL
Zolpidem	15	15	10	10	10 ng/mL
GHB	N/A	N/A	N/A	N/A	10 µg/mL