Sensitive analysis of therapeutic and abused drugs in whole blood using Tip-on-Tip technology with LC-MS/MS

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INTRODUCTION

LC/MS systems have made a significant impact on sample preparation requirements in forensic toxicology. In particular, highly sensitive LC/MS triple quadrupole instruments allow for low volumes of sample solutions, even when trying to achieve very low detection limits. In addition, the efficiency in HPLC separations minimizes the need for rigorous extraction processes to purify samples for analysis. In this presentation, we demonstrate an improved automated protein precipitation method[1] that provides rapid and sensitive analyses of comprehensive drugs and metabolites in whole blood. The method uses an automated protein precipitation procedure with Tip-on-Tip (ToT) filtration to provide robust sample preparation while minimizing opportunity for human error. By using a very sensitive LC/MS system, we show a quick, low cost sample preparation procedure for accurately and reproducibly quantitating drugs and metabolites in whole blood.

MATERIALS AND METHODS

Blank whole blood samples (from Utak) were spiked at various concentrations (0.5 ng/mL to 64 ng/mL) of 36 common drugs of abuse and their metabolites, including opioids and benzodiazepines (spiked using a mix of single standards ordered from Cerilliant). Using just 100 µL of whole blood, the samples were added directly to vials, and 10 µL of an internal standard (I.S.) mixture was added (at a concentration of 100 ng/mL containing morphine-d_3, 6-mam-d_6, oxycodone-d_6, norfentanyl-d_5, benzoylecgonine-d_8, 7-aminoclonazepam-d_5, fentanyl-d_5, buprenorphine-d_4, and temazepam-d_5). The samples were placed onto a Hamilton Heater Shaker (HHS) and the automated method was started. The solutions were shaken for 5 minutes, then 25 µL of 0.2 g/mL of $ZnSO_4$ was added and shaken for 30 seconds. Subsequently, 250 µL of acetonitrile (ACN) was added and the sample solutions were shaken for 60 seconds to precipitate proteins. After mixing, a wide bore tip aspirated 200 µL of the supernatant. Next, the wide bore tip was positioned into a Low Porosity Filtration Tip (from DPX Technologies), making an air tight seal, then the ToT was moved over a vial rack, and the solution was dispensed into the corresponding vials. The solutions were solvent evaporated using nitrogen and heat, and reconstituted using 100 µL of 5% methanol. See Figure 1 for method schematic.

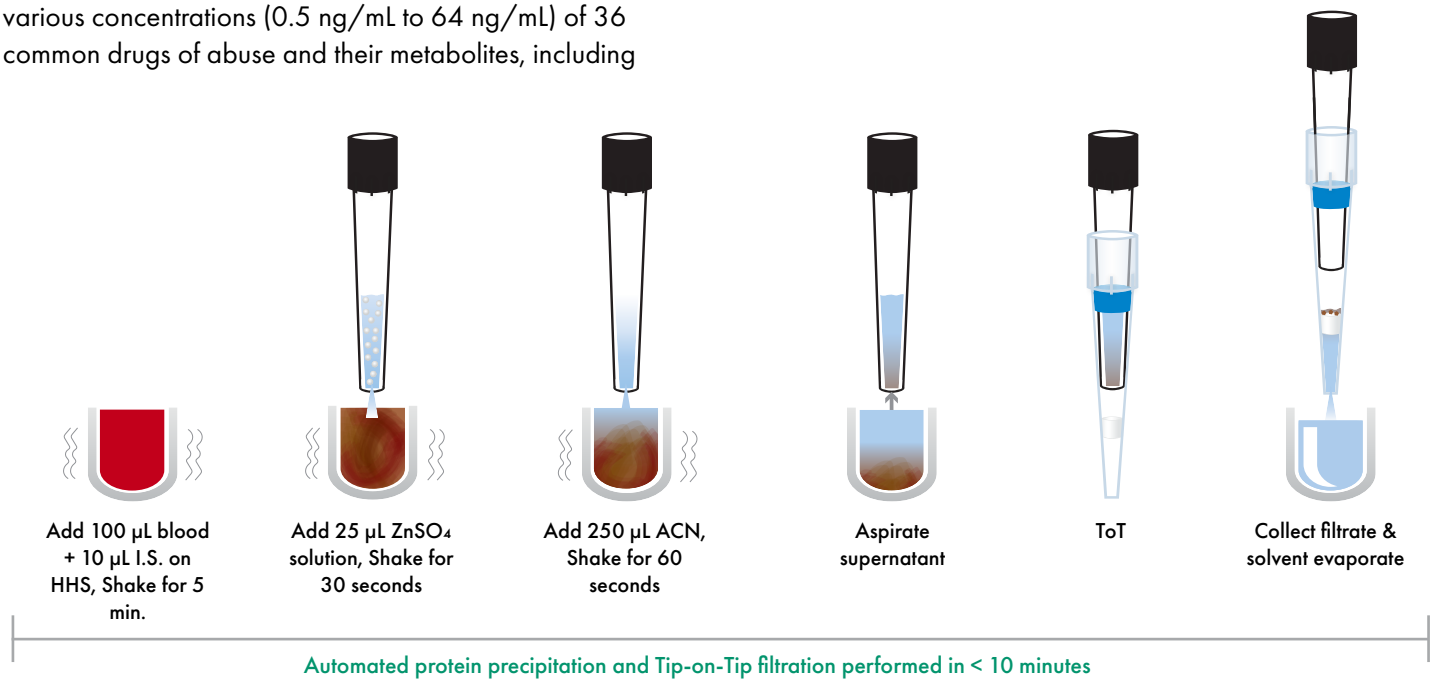
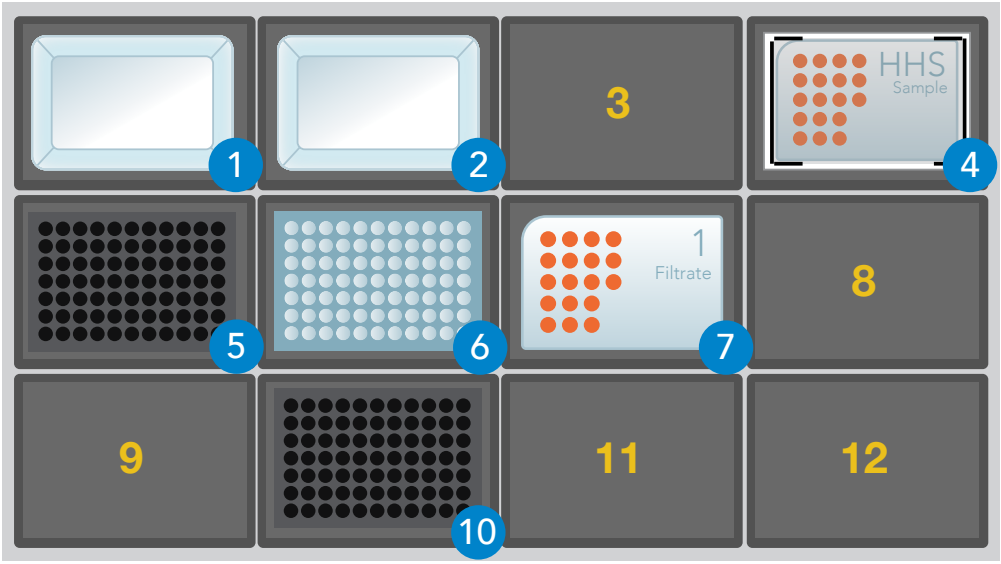


Figure 1. Schematic of INTip Filtration method - powered by Tip-on-Tip technology

A.

B.

C.



1. 0.2 g/mL $ZnSO_4$ Solution
2. ACN
4. Sample *(100 µL blood +10 µL I.S.)* loaded on HHS
5. 300 µL wide bore CO-RE tips
6. LPFT
7. Filtrate Position - 24 vials
10. 300 µL standard CO-RE tips

Figure 2. A. Tip-on-Tip apparatus with Low Porosity Filtration tips

B. Hamilton Heater Shaker

C. The Nimbus96 deck configuration includes reservoirs, well plates and pipette tips to perform the fully automated DPX method.

All analyses were performed using a SCIEX Triple Quad™ 6500+ LC-MS/MS System coupled to an Agilent 1260 LC system (5 µL injection) equipped with a Phenomenex Biphenyl column (Kinetex 2.6 µm, 50 x 3.0 mm). All extractions were performed using a Hamilton Nimbus96 liquid handler. The deck layout is shown in Figure 2.

RESULTS

The automated protein precipitation and Tip-on-Tip filtration was performed in less than 10 minutes, processing up to 24 samples simultaneously using a custom vial rack that holds 24 samples. Alternatively, 96 samples can be processed if using a 96 well plate. Recoveries, which were limited to the efficiency of the protein precipitation, were over 50% for all of the drugs and metabolites. At a concentration of 3 ng/mL, %CVs were less than 10% for almost all of the compounds (meprobamate and carisoprodol were 15% and 11%, respectively). Limits of detection and quantitation were found to be less than 0.5 ng/mL for most of the 36 compounds, suggesting that less sample volume could be utilized for routine analysis. All linear regression values were greater than 0.99 even though most of the compounds did not use matching deuterated internal standards. Table 1 summarizes results for retention time (RT), %CV, R, LOD, and LOQ values using $ZnSO_4$ for all compounds.

Except for benzodiazepines, matrix effects were less than 40% ion suppression. The use of $ZnSO_4$ reduced matrix effects and improved recoveries for basic drugs, in particular. Figure 3 shows recoveries and matrix effects for all compounds comparing the results with and without $ZnSO_4$. Figure 4 shows the chromatogram of all anlytes tested at 1 ng/mL.

CONCLUSIONS

This study demonstrates a rapid, efficient, and sensitive automated method for analyzing common drugs of abuse and their metabolites in whole blood. By using protein precipitation with filtration, costs for sample preparation are greatly reduced. In addition, the reproducibility of this method was very good even though only 9 deuterated internal standards were used, which also reduces costs.

Lower detection limits could be achieved by injecting a larger volume (for example, 10-20 µL) if necessary. However, the LODs achieved in this sensitive method suggest it is feasible to actually reduce the sample volume for routine testing. Further validation studies will be performed through collaboration with a forensic toxicology laboratory.

Table 1. Sample preparation was performed using the DPX method with $ZnSO_4$. The retention time (RT), R, %CV, limit of detection (LOD) and limit of quantitation (LOQ) in ng/mL are listed for all compounds.

	RT	R	%CV	LOD	LOQ
Morphine	1.70	0.9984	6.2%	0.15	0.46
Oxymorphone	1.80	0.9981	7.9%	0.07	0.23
Pregabalin	1.90	0.9964	2.8%	3.10	9.41
Hydromorphone	1.93	0.9981	2.8%	0.03	0.09
Amphetamine	2.11	0.9983	3.7%	0.09	0.28
Gabapentin	2.13	0.9960	4.8%	0.20	0.62
6-MAM	2.28	0.9990	7.0%	0.11	0.33
Methamphetamine	2.28	0.9987	5.0%	0.02	0.07
Codeine	2.28	0.9975	8.4%	0.15	0.45
O-Desmethyltramadol	2.30	0.9986	4.8%	0.01	0.03
Oxycodone	2.34	0.9981	7.5%	0.07	.020
Hydrocodone	2.37	0.9964	4.5%	0.02	0.06
MDMA	2.38	0.9999	2.7%	0.01	0.04
Norfentanyl	2.57	0.9975	5.3%	0.01	0.02
Tramadol	2.61	0.9987	5.6%	0.13	0.41
Benzoylecgonine	2.65	0.9975	5.4%	0.12	0.36
Methylphenidate	2.65	0.9974	3.0%	0.01	0.02
Meprobamate	2.69	0.9978	15.3%	2.67	8.08
Meperidine	2.67	0.9982	4.6%	0.01	0.02
7-Aminoclonazepam	2.72	0.9978	5.1%	0.29	0.86
Zolpidem	2.83	0.9994	6.5%	0.01	0.03
Fentanyl	2.87	0.9994	3.4%	0.01	0.03
Buprenorphine	2.87	0.9973	9.2%	0.32	0.97
Carisoprodol	2.94	0.9981	11.5%	1.05	3.19
Cyclobenzaprene	2.98	0.9964	7.8%	0.13	0.41
Nortriptyline	2.99	0.9980	2.6%	0.07	0.22
Amitriptyline	3.00	0.9983	3.8%	0.02	0.06
Methadone	3.05	0.9978	4.9%	0.03	0.10
Lorazepam	3.08	0.9977	8.6%	0.55	1.66
Clonazepam	3.11	0.9971	8.5%	0.40	1.22
Oxazepam	3.11	0.9982	6.1%	0.10	0.30
α-hydroxyalprazolam	3.16	0.9952	4.0%	0.48	1.44
Nordiazepam	3.18	0.9960	8.4%	0.14	0.42
Temazepam	3.23	0.9994	6.9%	0.06	0.18
Alprazolam	3.24	0.9934	3.5%	0.31	0.95
Diazepam	3.29	0.9995	5.7%	0.33	1.01

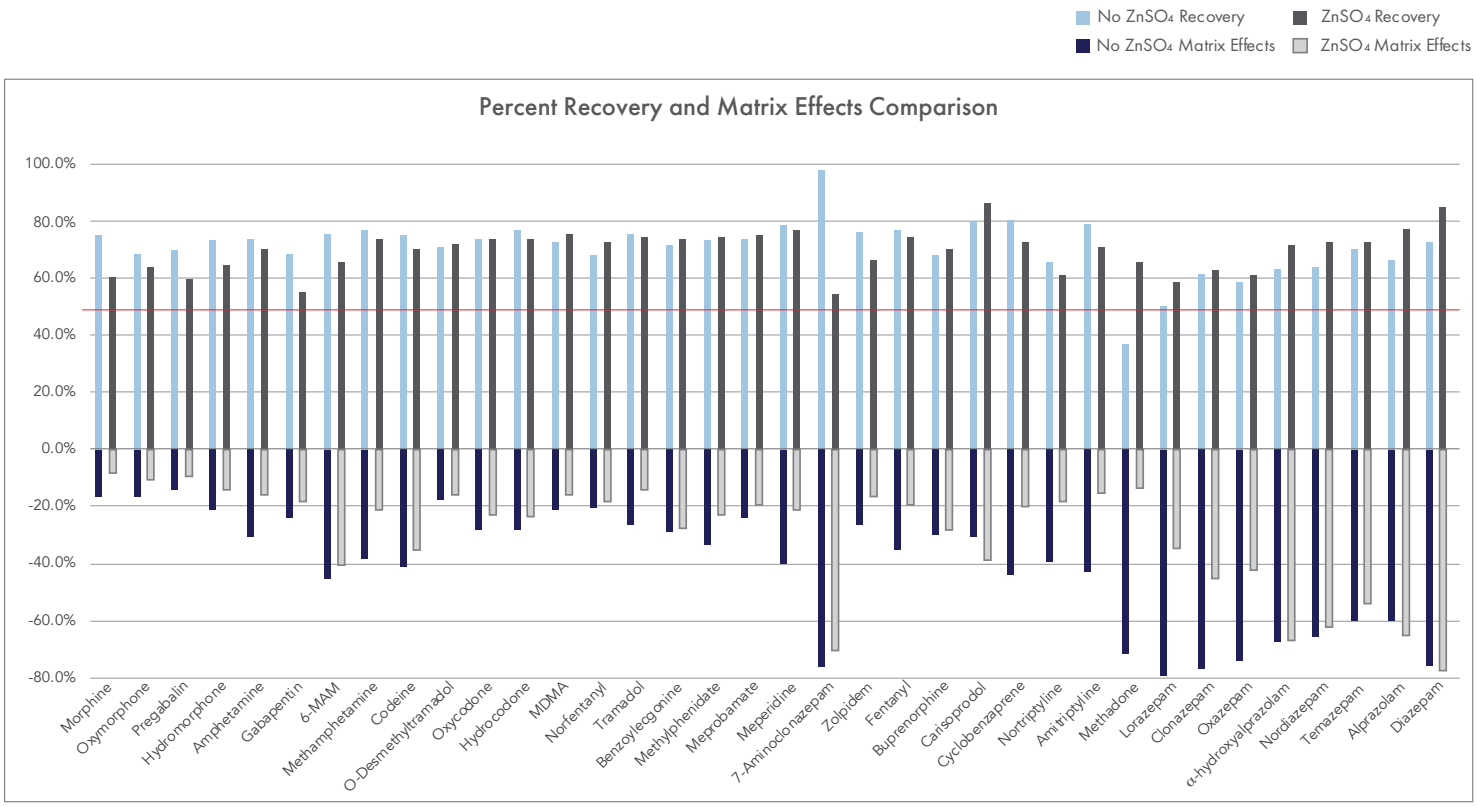


Figure 3. Recovery was 50% or greater for all compounds using the DPX method with $ZnSO_4$. Overall matrix effects were reduced.

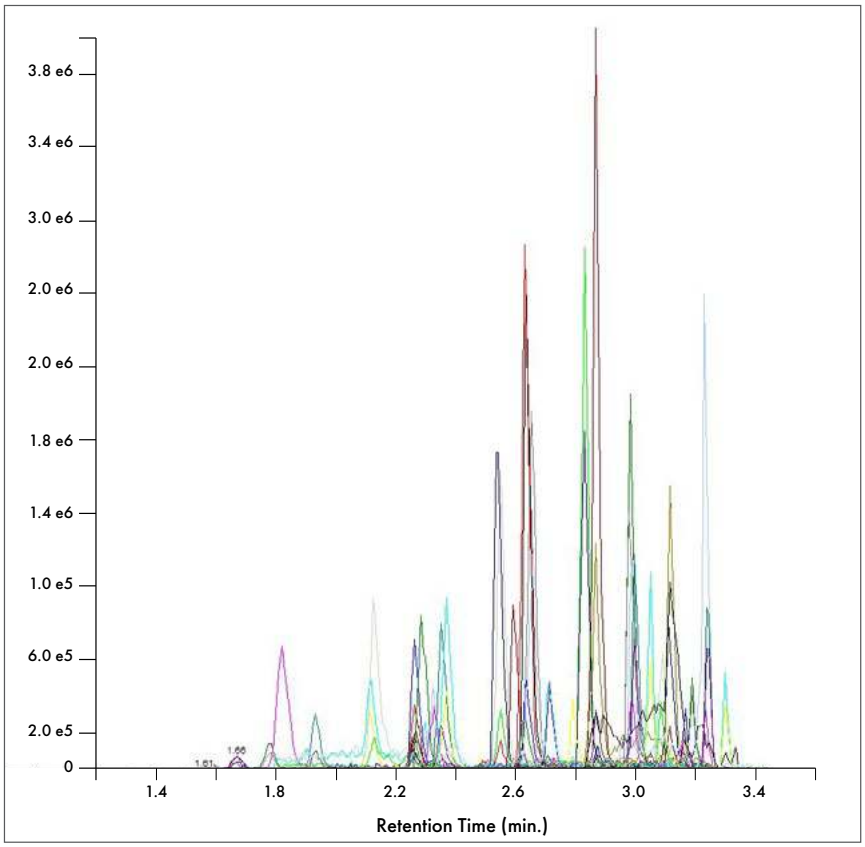


Figure 4. Chromatograms for all analytes tested at 1 ng/mL after sample preparation using the DPX method with $ZnSO_4$.

REFERENCES

1. M. Brusius, "Navigating the Vast Array of Sample Preparation Techniques for Biological Samples—Whole Blood", Chromatography Today, Feb. 2016, 40.