National Institute of Justice, Office of Justice Programs, U.S. Department of Justice

2014-R2-CX-K007

Paper Spray Mass Spectrometry for Rapid Drug and Drug Metabolite Screening Directly from Postmortem Blood Samples

Nicholas E. Manicke
Assistant Professor
Forensic and Investigative Sciences Program
Department of Chemistry and Chemical Biology
Indiana University-Purdue University Indianapolis
nmanicke@iupui.edu
317-274-6819

Spring 2017

Organizational DUNS: 6030079020000
EIN: 35-6001673

Trustees of Indiana University
Internal Account Number: 4394012

Final Summary Overview

I. Purpose

Drug screening is a necessary tool in postmortem investigations. Currently, however, drug screening itself can consume huge amounts of time and money in sample clean-up and analysis, and adding newly emerging compounds can be cumbersome. Paper spray mass spectrometry (PS-MS) provides an alternative method for drug screening that simplifies the screening process while still allowing for rapid and sensitive detection of a wide variety of drugs. The goal of this project was to develop a PS-MS based method to use as a screening procedure in postmortem toxicology. In consultation with AXIS Forensic Toxicology, we compiled a list of some 150 drugs, pharmaceuticals, and metabolites which represents the vast majority (~99%) of the compounds encountered in post-mortem drug screening. Initial screening cutoff objectives for these targets were set in consultation with AXIS and ranged from 1 to 30,000 ng/mL.

II. Project Design and Methods

A. Paper spray

Routine paper spray was performed using Velox sample cartridges on the automated Velox 360 source from Prosolia (Indianapolis, IN, USA). Twelve µL of sample were spotted on the cartridges and allowed to dry at room temperature before analysis. The spray solvent used was 85:10:5:0.01 acetonitrile:acetone:water:acetic acid. In total, 136 µL of solvent were gradually applied to each cartridge.

In addition to the routine method described above, various novel cartridge formats were explored to improve sensitivity.

B. Mass spectrometry

Because no separations are carried out prior to analysis, tandem mass spectrometry (MS/MS) and/or high resolution mass spectrometry are required. Paper spray MS was evaluated both on a triple quadrupole mass spectrometer (Thermo TSQ Vantage) in selected reaction monitoring (SRM) mode and a quadrupole-orbitrap mass spectrometer (Thermo Q-Exactive Focus) operated in MS/MS mode.

III. Data Analysis

A. Triple Quadrupole MS

Two SRM channels were monitored for each target. Positive detection required satisfying two criteria. First, the quantifier SRM had to show signal five times higher than the signal obtained for drug-free blood.

Second, the ratio between the quantifier and qualifier SRM transition had to be within ± 30% of the expected value.

B. Q-Orbitrap MS

All data were automatically processed using TraceFinder v. 3.3 (Thermo Fisher Scientific). Peaks within a 5-ppm window of the target compound's fragment ion were integrated. The analyte peak area was then divided by the area of the corresponding fragment ion of the appropriate internal standard (ISTD). Each calibration point was run in duplicate and the ratios of analyte signal to ISTD signal were plotted against their known concentrations to generate the calibration curve, which was linearly fit using 1/x weighted least squares. Limits of reporting (LORs) were determined by the lowest reliably detected calibrator above noise. The unknown post-mortem (PM) samples were run in triplicate and analyzed by plotting each analyte/ISTD measurement on the corresponding calibration curve to semi-quantitatively determine the amount of drug present.

IV. Findings

A. Fundamental Studies into Ion Suppression and Recovery

Matrix effects are known to occur in paper spray mass spectrometry when analyzing dried biofluids[1-3]. MS signal intensities obtained from the analysis of dried urine, blood, or plasma are almost always lower compared to analyzing an identical quantity of analyte without the presence of biofluid matrices. This phenomenon is common to all ambient or direct ionization methods due to the lack of sample preparation. Despite the presence of matrix effects, surprisingly good detection limits in the single digit or sub-ng/mL range are widely reported in the literature for direct analysis of dried blood spots (DBSs). Nevertheless, minimizing or eliminating matrix effects in paper spray MS would be beneficial because detection limits would be lowered and the need for matrix matched calibrations and stable isotope labeled (SIL) internal standards could be eliminated. To take rational steps to minimize matrix effects, a more rigorous understanding of the causes and the experimental parameters that impact matrix effects is first needed.

We developed a method to measure ion suppression and recovery in paper spray MS. The general method was to spike SIL analogs of each analyte into the spray solvent, while the analyte itself was in the dried biofluid. Intensity of the labeled analog is proportional to ionization efficiency, whereas the ratio of the analyte intensity to the labeled analog in the spray solvent is proportional to recovery. Ion suppression and recovery were found to be compound and matrix dependent. Highest levels of ion suppression were obtained for poor ionizers (e.g.

analytes lacking basic aliphatic amine groups) in urine and approached -90%. Ion suppression was much lower or even absent for good ionizers (analytes with aliphatic amines) in dried blood spots. Recovery was generally highest in urine and lowest in blood. We also examined the effect of two experimental parameters on ion suppression and recovery: the spray solvent and the sample position (how far away from the paper tip the dried sample was spotted). We found that acetonitrile-based solvents show significantly lower levels of ion suppression compared to methanol, although the recovery was somewhat lower as well.

Primary Scholarly Outcome of This Finding:

- Vega C, Spence C, Zhang C, Bills BJ, and Manicke NE: Ionization Suppression and Recovery in Direct Biofluid Analysis Using Paper Spray Mass Spectrometry. *Journal of the American Society of Mass Spectrometry* 27(4), 726-734 (2016). http://dx.doi.org/10.1007/s13361-015-1322-8

B. Drug Screening by Paper Spray MS on a Triple Quad MS

i. Method Development

A significant amount of effort was devoted to determining appropriate detection criteria for paper spray MS/MS on a triple quad MS. The basis for drug detection for paper spray MS on a triple quad is shown in Figure 1; both signal intensity above a certain threshold as well as the ratio between specific fragment ions are used. Detection First, we studied the number of SRM scans required to achieve consistent ion ratios. This is an important parameter because ion ratios between the quantifier and qualifier ions are one of the detection criterion. We identified 26 analytes of our initial test set of 134 drug targets which showed relatively high variation in the intensity ratio between the two selected SRM channels. The relatively high variation may have been due to the large intensity difference between two fragment ions, which ranged from 12:1 to 95:1. The cumulative average of the intensity ratio of the two fragment ions for each target was monitored as a function of time.

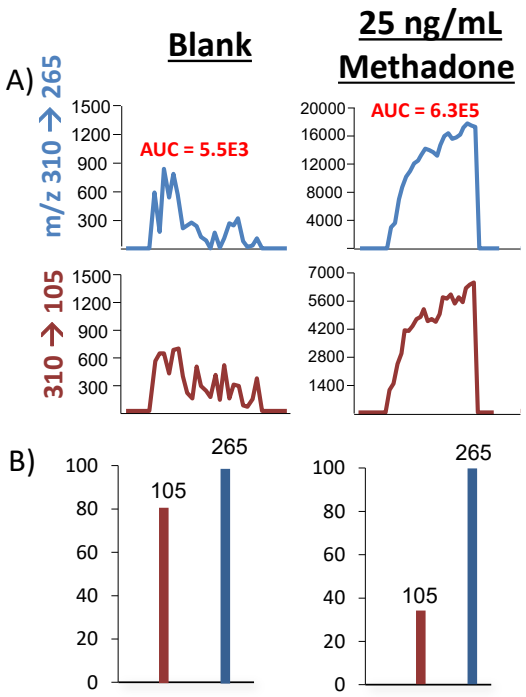

Figure 1. A) SRM Chronograms for two fragment ions of methadone collected in drug free blood and in blood spiked with 25 ng/mL of methadone. B) Relative intensities of SRM channels in drug free and spiked blood samples.

62% of targets showed no significant difference between their "true" fragmentation ratio and the ratio acquired after 15 scans. 92% of targets showed less than ±5% variation between their true ratio and the ratio obtained

after 15 scans. Doubling the number of scans to 30 only increased that percentage to 96%. Fifteen scans of each SRM channel is therefore probably sufficient for most applications. Defining the minimum number of scans needed per SRM channel has direct implications on the analysis time for paper spray. If 15 scans at a dwell time of 0.1s are required for each SRM channel, then 30 analytes can be screened for during a typical 90s paper spray analysis. A larger number of targets can be screened for by decreasing the dwell time or by increasing the paper spray analysis time by replenishing the solvent during analysis.

The second important task was determining an appropriate SRM ratio tolerance, and how that varied as a function of concentration. We measured the deviation of the SRM ratios as a function of the signal-to-blank ratio for about 130 different drug targets. The signal was measured for each target spiked into human blood at their targeted post-mortem toxicological screening cut-off. The blank was determined by measuring the same lot of blood without spiking the target compounds. Not surprisingly, the SRM ratio often deviated significantly from the true value as the signal-to-blank ratio for the lower intensity fragment ion decreased. An appropriate ratio tolerance will therefore depend on what the signal-to-blank ratio is at the screening cutoff. While this number cannot be known for true samples, an average value can be obtained by analyzing drug-free blood samples before and after drug target spiking. If the signal-to-blank ratio is high, ±20% is reasonable while drugs with screening cutoffs closer to the detection limit of the lower abundance SRM channel will require wider tolerance. In this stud, we used an SRM ratio ±30% to minimize the risk of false negatives.

Other aspects of method optimization that were performed were extract solvent selection, sample dilution, and SRM fragment ion selection.

ii. Results for Paper Spray Drug Screening on a Triple Quad

At the beginning of this project, 154 different analytical targets were proposed to investigate the feasibility of paper spray mass spectrometry as an effective means of rapid drug screening. After method development, 16 of the originally proposed targets were concluded to only ionize well in negative mode and will be studied in future experiments optimized for negative ionization. The 16 negatively ionizing targets included all of the barbiturates (amobarbital, butabarbital, butalbital, pentobarbital, phentobarbital, secobarbital) as well as furosemide, hydroxycholoquine, hydrochlorothiazide, ibuprofen, salicylic acid, thiopental, valproic acid, THC, 11-nor-9-Carboxy-THC, and warfarin.

Out of the 134 targets analyzed by paper spray MS/MS on a triple quad, 114 met the detection requirements when spiked into drug-free whole blood at the objective cut-off concentration; the detection

criteria being signal-to-blank ratio of greater than three and SRM ratio deviation less than 30% in their established fragmentation ratio. Of the 20 targets that failed to meet these criteria, half of the time because of low signal-to-blank of the quantifier ion and half of the time due to ratio variability, most likely due to inadequate signal-to-blank on the qualifier ion channel. For these targets, additional method optimization can be performed, such as increasing the SRM dwell time or changing the extraction solvent. In the short term, the limits of reporting can be increased.

iii. Primary Scholarly Outcomes of This Finding:

- Drug Screening using Paper Spray Coupled to a Triple Quadrupole Mass Spectrometer. Rachel Potter, Christine Skaggs, and Nicholas E Manicke. *Analytical Methods*. Submitted
- Potter R and Manicke NE. "Paper Spray Mass Spectrometry for Rapid Drug Screening from Dried Blood Spots." Podium Presentation at the AAFS Annual Scientific Meeting. 2016
- Manicke NE and Potter R. "A New Approach to Drug Screening in Forensic Toxicology: Paper Spray Mass Spectrometry." Poster Presentation at SOFT Annual Meeting, 2015.
- Rachel Potter. Master's Thesis. In preparation.

C. Paper Spray Screening on a Q-Orbitrap MS

i. Method Development

When screening for a relatively larger number of targets using an MS/MS inclusion list, the mass spectrometry settings must be adjusted to ensure an adequate number of scans are obtained for each target within the time the cartridge is spraying. In this assay, we choose 5 scans per target as an adequate number to obtain accurate m/z measurements, accurate ion ratios, and acceptable quantitative performance. An example of the total ion chronogram acquired using the described instrument method is shown in Figure 2a, where each stick is an individual MS/MS scan. A full cycle of these scans completed within ~0.3 min. Five or 6 scans were obtained for each MS/MS scan followed by a zero intensity scan at the end, which was obtained by turning the spray voltage off at 1.6 minutes. A zero intensity scan is required for automatic peak integration through the TraceFinder software. The extracted ion chronogram for cocaine—the filter for which fragmented precursor ions at m/z 304.1543 ±0.5—is shown in Figure 2, demonstrating the number and frequency of MS/MS scans as well as the zero scan. By adjusting the MS resolution to 35,000 and setting the ion injection time to 50 ms, an adequate number of scans for all 138 analytes and 11 internal standards was obtained.

Almost all drugs screened saw the production of more than one fragment ion, in which case the weaker ions could be used as confirmatory ions for positive detection, bolstering selectivity, while the strongest ion would be used for quantitation measurements. In this study, we utilized only a single fragment ion to minimize

the risk of false negatives.

Previous work on the triple quad MS described above determined that 95:5:0.01 methanol:water:acetic acid was the best spray solvent. However, this method had only been used in the generation of analyte calibration curves and had not been tested with any of the PM blood samples. When making the transition from a single living blood donor to multiple deceased blood donors—coupled with differing levels of coagulation—matrix effects started to become more noticeable. This was recognized by the ISTD MS/MS signal from the PM samples being typically weaker than the calibrant samples, showing inconsistency between the unknowns themselves as well as the samples used in generating the calibration curves, which would impede the accuracy of any attempted quantitation. We tested acetonitrile as an alternative organic phase in the spray solvent, which stemmed from our earlier investigations into ion suppression and recovery. This work had demonstrated that acetonitrile-based solvents show lower ion suppression than methanol solvents, presumably because of lower solubility of salts and lipids. However, the use of acetonitrile-water mixtures is problematic as they are less capable than methanol-water mixtures in permeating and fully wetting DBSs. To overcome this, we diluted the blood samples by mixing

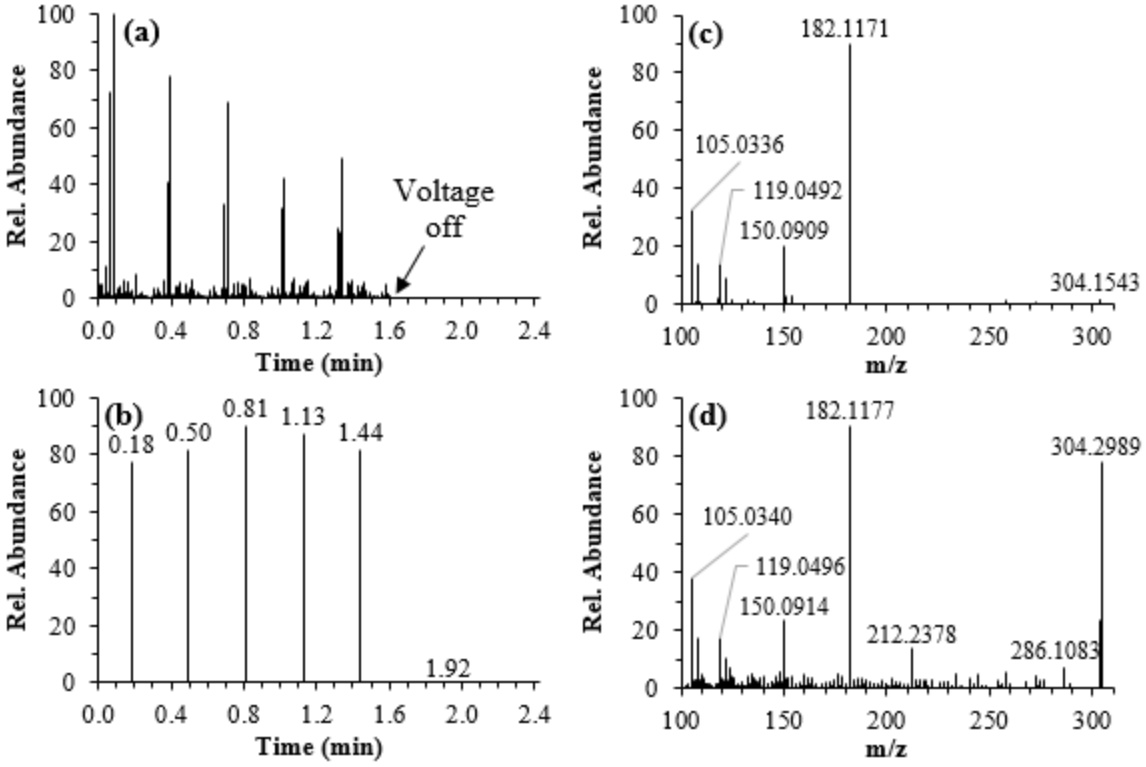

Figure 2. (a) Total ion chronogram (all scans combined). (b) Extracted ion chronogram for cocaine's MS/MS scans. (c) Tandem mass spectrum for a neat standard of cocaine at 200 ng/mL, infused via commercial ESI. (d) Tandem mass spectrum for blood spiked with 16 ng/mL cocaine (0.33x its cutoff), sprayed via paper spray.

1:3 with an aqueous ISTD mixture. We also added acetone to the spray solvent to decrease the surface tension,

further improving the wetting properties of the spray solvent. Ultimately we found that a spray solvent 85:10:5:0.01 acetonitrile:acetone:water:acetic acid at a 5.0 kV onset voltage allowed for proper and consistent spraying across multiple different samples for blood samples that had been mixed 1:3 (v:v) with an aqueous ISTD mixture before spotting. When using the new method, there were no issues with the detection of any ISTDs from any of the PM samples, and almost all of them experienced a notable increase in sensitivity over the original method. Furthermore, the signal achieved using the new method was much more stable and comparable across the different PM and calibrant samples. These facts taken together indicate that the new method of sample preparation as well as the new solvent would allow for more reliable quantitative conclusions to be drawn regarding analyte detection in the PM samples.

ii. Results for Drug Screening by Paper Spray MS on a Q-Orbitrap

For almost all drugs tested, the sensitivity was similar to that obtained on the triple quadrupole, and the lowest concentration calibrant, which corresponded to the desired screening cut-off, could be reliably detected. However, duloxetine, norbuprenorphine, and nortramadol were exceptions to this—their LORs were increased because the raw MS/MS signal provided by lower concentrations was not enough to distinguish the calibrant from the blank signal.

This method was applied to the testing of 30 PM blood samples obtained from AXIS Forensic Toxicology. These 30 samples were taken through drug screening and confirmation by AXIS and then transferred to IUPUI for drug screening by paper spray. The specimens were de-identified and the results were blinded until after the paper spray results were obtained and shared with AXIS. In the 30 PM samples, AXIS reported 86 target compound detections. The paper spray MS screening detected 79 of these instances. Of those 79, 58 instances fell within the paper spray MS reporting range. The average absolute concentration deviation between the PS-MS method and the concentration reported by AXIS was 61%. This is quite good considering that the PS-MS method used here was designed as a rapid screening method intended to give semi-quantitative results only.

The other 21 instances fell below the PS-MS limit of reporting but were still detectable. These 21 target detections were not listed as positive in the initial PS-MS results for these samples, because they fell below the lowest calibrator (the limit of reporting). However, subsequent review of the data showed that these targets were clearly detected. The PS-MS reporting limits were set prior to method development at levels deemed reasonable for PM toxicology. In many cases, PS-MS can detect significantly lower concentrations that the LOR as evidenced here by the 21 samples that were detectable below the LOR.

In 7 cases, PS-MS failed to detect target compounds detected by AXIS. For 5 of those cases, the drug levels reported by AXIS were below the paper spray MS detection limit. For example, clonazepam was reported by AXIS in one sample at a concentration of 8.8 ng/mL. The detection limit of clonazepam in the paper spray method is about 30 ng/mL. In the other two cases of false negatives, AXIS did not collect quantitative data so the concentration is unknown.

There were also a number of false positives in the paper spray MS assays. For example, there were three false positives for opioids (one for oxymorphone and two of hydromorphone). However, other opioids were present in those samples (detected by both IUPUI and AXIS). This result was anticipated due to the large number of opioid isomers among the parent drugs and the drug metabolites, which can result in "off-target" false positive for isomeric opioids. In other cases, the paper spray MS method was adjusted based off what we learned from the analysis of PM blood samples. For example, there were several false positives for amphetamine in the sample set that were eliminated by using a different fragment ion for detection.

iii. Primary Scholarly Outcomes of This Finding:

- McKenna J, Shanks K, and Manicke NE. Toxicological Drug Screening using Paper Spray High-Resolution Tandem Mass Spectrometry (HR-MS/MS). Journal of Analytical Toxicology. 42 (5), 300-310 (2018).
- Josiah McKenna. Master's Thesis. Paper Spray Mass Spectrometry (PS-MS) for Toxicological Drug Screens And Biomonitoring of Chemical Warfare Agent Exposure.
- Manicke NE and Potter R. "Paper Spray Mass Spectrometry for Screening of Illicit Drugs from Blood Samples." Podium Presentation in the New Concepts for Forensic MS section at the Annual ASMS Conference on Mass Spectrometry. 2016.
- Nicholas Manicke. "Post-mortem Drug Screening using Paper Spray MS on a Q-Orbitrap Mass Spectrometer." Poster Presentation at the Annual ASMS Conference on Mass Spectrometry. 2017.

<u>D. Drug Screening in the Negative Ion Mode for Acidic Drugs</u>

Detection of the acidic drugs by paper spray MS is a challenge because of the need to analyze these compounds in the negative ion mode. The difficulty with negative ion detection is the poor stability and irreproducibility of the MS signal. While this problem with the negative ion analysis is known to occur in conventional electrospray[4-7], the effect is even more pronounced in paper spray[3]. Very little work has been done using negative ion paper spray as a result. We explored alternative solvents to improve paper spray stability in the negative ion mode. Most critically, we found that use of chlorinated solvents as the extraction/spray solvent could significantly improve the stability of paper spray ionization in the negative ion mode by suppressing corona discharge formation. By using a 9:1 mixture of methanol:carbon tetrachloride, we significantly improved the

stability and robustness of negative ion mode paper spray to the point that we could perform routine screening and quantitation of acid drugs from blood samples. The barbiturates, which are a significant fraction of the acidic drug targets in this study, could all be detected at the objective screening cut-off set in consultation with AXIS Forensic Toxicology (500 ng/mL in most cases). Moreover, using phenobarbital-d5 as an internal standard, acceptable quantitative performance was achievable, with calibration curve R^2 values of over 0.95 obtained for all of the barbiturates.

Primary Scholarly Outcomes of This Finding:

- Josiah McKenna. Master's Thesis. Paper Spray Mass Spectrometry (PS-MS) for Toxicological Drug Screens and Biomonitoring ff Chemical Warfare Agent Exposure.
- Josiah McKenna and Nicholas Manicke. "Negative Ion Paper Spray for the Detection of Acidic Compounds." Poster Presentation at 65th ASMS Conference on Mass Spectrometry. 2017.
- McKenna J, Dhummakupt ES, Connell T, Demond PS, Miller DB, Nilles JM, Manicke NE, and Glaros T. Detection of Chemical Warfare Agent Simulants and Hydrolysis Products in Biological Samples by Paper Spray Mass Spectrometry. *Analyst*. 142, 1442-1451 (2017).

E. Alternative Cartridge Formats

We developed a prototype paper spray cartridge with the capability to perform extraction and preconcentration automatically. The purpose is to improve detection limits for challenging target compounds. The paper spray cartridge contained an integrated solid phase extraction (SPE) column, and we used it to demonstrate immediate extraction and concentration of analytes from complex samples such as plasma. The cartridge also included the necessary components to perform sample ionization by paper spray. Sample extraction, preconcentration, and ionization from complex samples were therefore all performed on a cartridge that could, in principle, be disposable due to its simple design and low-cost materials. Analysis required the same number of steps as typical paper spray (application of sample, sample drying, and application of extraction/spray solvent). Sample preconcentration and extraction occurred automatically, requiring no human action or secondary device. No pumping was required; both sample and extraction solvent were fed through the device passively by capillary action. After sample application and drying, one step was needed to analyze the sample by MS: a solvent was added to the cartridge, which wicked through the SPE material and the paper spray substrate by capillary action, recovering the analyte and generating gas-phase ions for MS analysis in a single step.

Compared with direct paper spray analysis of dried plasma spots, paper spray analysis employing integrated solid phase extraction improved the detection limits significantly by between 14 to 70 times for the drugs studied thus far. This device shows good potential for improving the capabilities of paper spray for rapid

drug screening.

Primary Scholarly Outcomes of This Finding:

- Zhang C and Manicke NE: Development of a Paper Spray Mass Spectrometry Cartridge with Integrated Solid Phase Extraction for Bioanalysis. *Analytical Chemistry* 87(12), 6212-6219 (2015). http://dx.doi.org/10.1021/acs.analchem.5b00884
- Zhang C and Manicke NE. "Development of a paper spray cartridge with integrated SPE to improve sensitivity for drug detection." Oral Presentation in the Session *Clinical and Forensic Applications of Ambient Ionization Mass Spectrometry*. SciX 2016. Minneapolis MN.
- Zhang C and Manicke NE. "Development of a paper spray cartridge with integrated SPE to improve sensitivity for drug detection." Poster Presentation at the 63rd Annual ASMS Conference on Mass Spectrometry. 2015.

V. Implications for Criminal Justice Policy and Practice in the United States

In an effort to use their resources efficiently, most forensic toxicology laboratories use a two-step process to detect toxicants in biological samples. The first step is screening; ideally, screening tests require little sample manipulation, are fast, inexpensive, sensitive, selective, and cover a broad range of targets. Immunoassays (IA) are the most common method for performing drug screening. Immunoassays have poor sensitivity and higher rates of false positives compared to mass spectrometry. They also have limited multiplexing capability and are slow to adapt to new drug targets. As a result, MS-based drug screening methods have become increasingly popular as screening methods. HPLC-MS-based methods improve significantly on all of the weaknesses of IA, but have an important limitation of their own: they are complex assays and are therefore often limited to large central toxicology labs.

Paper spray MS maintains many of the advantages of HPLC-MS such as its ability to perform multiplex detection of many drug targets and to introduce new drug targets relatively easily. Paper spray MS also has the primary advantage of IA, which is that the test is rapid and simple. The technique is also fairly sensitive; most of the drug targets are screened at 50 ng/mL or lower directly from blood samples without sample preparation. With this type of sensitivity and speed, drug screening can be turned around significantly faster and at low cost. Although the upfront cost of the mass spectrometer required to perform paper spray MS drug screening is significant, the potential savings in time and labor are also substantial.

VI. References

(1) Shi, R.-Z.; El Gierari, E. T. M.; Manicke, N. E.; Faix, J. D. *Clinica Chimica Acta* **2015**, *441*, 99-104.
(2) Zhang, C.; Manicke, N. E. *Anal. Chem.* **2015**, *87*, 6212-6219.
(3) Manicke, N. E.; Yang, Q.; Wang, H.; Oradu, S.; Ouyang, Z.; Cooks, R. G. *Int. J. Mass Spectrom.* **2011**, *300*, 123-129.
(4) Bruins, A. P. In *Electrospray ionization mass spectrometry*, Cole, R. B., Ed.; Wiley: New York, 1997, pp 107-136.
(5) Ikonomou, M. G.; Blades, A. T.; Kebarle, P. *Journal of the American Society for Mass Spectrometry* **1991**, *2*, 497-505.
(6) Van Berkel, G. J. In *Electrospray ionization mass spectrometry*, Cole, R. B., Ed.; Wiley: New York, 1997, pp 65-105.
(7) Yamashita, M.; Fenn, J. B. *The Journal of Physical Chemistry* **1984**, *88*, 4451-4459.