Document Title:	**Evaluating the Effectiveness of Liquid Chromatography-Time of Flight-Mass Spectrometry (LC-TOF-MS) as a Replacement Screening Tool in a Full-Service, State Forensic Toxicology Laboratory**
Author(s):	**Washington State Patrol**
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Evaluating the effectiveness of liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS) as a replacement screening tool in a full-service, state forensic toxicology laboratory

FINAL SUMMARY OVERVIEW

PURPOSE

The purpose of this research was to develop and validate a screening method using LC-TOF-MS that can be replicated in other forensic toxicology laboratories and have a direct impact on their casework accuracy and efficiency. The results of this project would assist other laboratories with their current backlogs and limited resources to decide how best to efficiently process and analyze forensic toxicology evidence.

OBJECTIVES

The objectives for this project were to (1) develop and validate a broad ranging screening method using liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS); (2) compare and evaluate the efficiency, accuracy, sensitivity and breadth of the new LC-TOF-MS screening method with the laboratory's current screening approach; (3) conduct a cost-benefit analysis of the new LC-TOF-MS screening method: and (4) to summarize and disseminate the results of this project to the general forensic toxicology community through publications and presentations.

PROJECT DESIGN

Following the development, optimization and validation of the new LC-TOF-MS method, the new method was used to analyze several hundred actual forensic cases to determine if it identified more compounds than the current screening approach used in the laboratory. A comparison of the two methods (current approach and the new LC-TOF-MS method) was made taking into account the following parameters:

- Sample volume required
- Number and breadth of drugs identified
- Time taken for scientists to prepare and extract the case evidence
- Time taken by the instruments to analyze the evidence
- Time taken for the scientists to subsequently analyze the data
- Time taken for technical and administrative review of all data
- Cost of scientist's time, review time, consumables, and maintaining instrumentation

PROJECT FINDINGS

Development of the LC-TOF-MS test method involved evaluation of extraction techniques to determine the best sample preparation procedure, optimization of mobile phase components and elution gradient, selection of HPLC column and operating temperature, evaluation of positive mode versus negative mode recovery, assignment of internal standard compounds, creation of the in-house target compound database, performance of instrument maintenance and troubleshooting and development of qualitative and semi-quantitative data analysis methods and reporting formats.

As the LC-TOF-MS screening method was intended to be used on all case specimens submitted for analysis, with minimal sample preparation desired, multiple liquid-liquid and

precipitation sample preparation techniques were evaluated. Precipitation with acetonitrile yielded the best overall results, with recovery of the highest number of target compounds, consistent peak area responses and optimal chromatography. Of note is the fact that Δ-9-THC was not detected using this method and the metabolite Δ-9-carboxy-THC was detected only at high concentrations (approximately 100 ng/mL). Subsequently, the laboratory found it necessary to develop a supplemental LC-MSMS screening method for implementation concurrent with the LC-TOF-MS method.

For the method, a volume of 0.8 mL acetonitrile (containing internal standard compounds) was added to 0.2 mL specimen samples, vortex-mixed for approximately 10 sec and centrifuged for 5 min at 3000 rpm. The organic supernatant was then transferred to a conical centrifuge tube and evaporated to dryness under air at 40^0C. Extracts were reconstituted in 50 µL of a 50/50 mixture of HPLC grade methanol and deionized water, vortex-mixed and centrifuged for 2 min at 2000 rpm to collect liquid at bottom of tubes. Extracts were then transferred to polypropylene autosampler vials with integrated inserts and placed on the LC-TOF-MS for analysis. The sample preparation procedure described above proved to be highly efficient, utilizing a small specimen volume and low volumes of organic solvent, and requiring minimal time for extraction.

Mobile phase aqueous components evaluated included 0.1% formic acid, 1mM ammonium fluoride and 5mM ammonium formate, each prepared in deionized water, with acetonitrile and methanol were evaluated for use as organic components. HPLC columns of different diameters, lengths and particle sizes were considered, including C18 and biphenyl phase compositions and columns that employ core shell technology. HPLC column operating temperature and mobile phase flow rate were evaluated to optimize chromatography and elution

time. A mobile phase composition of 5 mM ammonium formate in deionized water and HPLC grade methanol was selected, with column operating temperature of 50°C and flow rate of 0.5 mL/min, utilizing the Agilent Zorbax Eclipse Plus C18, 3.0 x 100 mm HPLC column (1.8 µM particle size). Injection volume was 2 µL. Initial mobile phase composition for both positive and negative mode acquisition consists of 10% HPLC grade methanol (0 - 0.5 min), increasing to 90% (0.5 – 3.0 min), with hold time to 8 minutes for positive mode and to 6 minutes for negative mode. Re-equilibration time was 5 minutes for positive mode and 3 minutes for negative mode. The TOF mass spectrometer was operated with a gas flow of 11 L/min, with gas temperature of 325°C and nebulizer pressure of 40 psi.

Morphine-d_6, methamphetamine-d_{14} and diazepam-d_5 were selected as internal standards for positive mode acquisition. The retention times for these deuterated analogs span the length of acquisition run time, with early, mid and late elution times, respectively. These internal standards are also representative of the three classes of drugs most-identified in case specimens (excluding cannabinoids) analyzed in the laboratory. Hexobarbital was selected as the internal standard for acquisition in negative mode, used in analysis of amobarbital/pentobarbital, valproic acid, salicylic acid, ibuprofen and topiramate.

An in-house database of more than 160 target compounds was created for use in qualitative analysis of LC-TOF-MS data. Injections of certified reference materials, or standard solutions prepared from certified reference materials, were analyzed to establish specific retention times for target compounds and internal standards. Target compound names, chemical formulas, molecular weights and retention times were recorded in the database. The in-house database was intended as the primary data analysis method for specimen testing. Where analysis against the in-house database yields negative results for a case specimen or case history indicates

a drug of interest not included in the in-house database, the data may be analyzed using the Forensic Toxicology Agilent library. This library was purchased from Agilent, the manufacturer of the LC-TOF-MS instrument, and includes more than 6,000 target compounds. Identification of target compounds from the LC-TOF-MS screening method was based on chromatography, retention time difference (from database), ppm difference (from exact mass), isotopic abundance and isotope spacing. The data analysis software uses an algorithm to calculate a final score from those factors above (except chromatography), with their respective weighted contribution to the overall score.

Validation of the test method was performed, including evaluation of limits of detection (LOD) for target compounds, precision (response) at LOD and 3x LOD, carryover, and xenobiotic specificity. Investigation of ion suppression/enhancement, alternative matrix evaluation (liver homogenate, serum, urine) were also performed. Case comparison testing was performed on over 400 forensic case specimens that were previously analyzed in 2017 using current immunoassay and basic drug GC-MS/NPD screening procedures, with concurrent comparison testing additionally performed on a selection of case specimens in 2018.

Limits of detection (LOD) were determined for target compounds, based on those factors listed above which contribute to the final score. Further evaluation was performed to confirm LOD performance. Within-batch (WBP, $n = 3$) and between-day (BDP, $n = 9$) precision was determined through analysis of replicates of whole blood pools prepared at LOD and 3x the LOD (expected positive control level) concentrations. Three replicates at each level were extracted and analyzed on three different days, and precision was evaluated for the response (peak area). Results of precision studies show coefficients of variation (CVs) of < 20% for target compounds, with the exception of lorazepam, methadone and diphenhydramine. Internal standard CVs were

< 20%. High CVs for lorazepam, methadone and diphenhydramine did not affect overall recovery or ability to identify these compounds.

Carryover was investigated at concentrations up to 5 mg/L for amines, opiates, benzodiazepines, methadone, zolpidem, cocaine and other basic compounds and at 100 mg/L for gabapentin, barbiturates, acidic and neutral compounds. Results showed carryover was possible at > 1 mg/L for oxycodone and sertraline, and > 2 mg/L for quetiapine, trazodone and tricyclic antidepressants. Ion suppression/enhancement studies showed internal standard suppression/enhancement was < 25% and CV < 15%. Target compound ion suppression/enhancement for target compounds was < 25% and CV < 15%, with the exception of bupropion and morphine/hydromorphone. Examination of LOD and 3x LOD results for these compounds showed no negative effects from suppression > 25% and/or CV > 15%.

Matrix evaluation results showed serum/plasma and urine specimens yield recoveries similar to that of blood. Liver homogenate results indicate low recoveries, compared to blood, for homogenate prepared to a 1:5 dilution factor. Performance of homogenate prepared to a 1:10 dilution factor yielded recoveries similar to blood. Spleen squeeze specimens must be analyzed with caution, with attention paid to both specimen quality and consistency of extracts prior to analysis on the instrument.

Overall, case comparison studies demonstrated that LC-TOF-MS analysis confirms qualitative results from initial testing of case specimens. Screening with LC-TOF-MS was able to detect compounds not detected in the laboratory's current initial screening protocols of immunoassay and basic drug screening by GC-MS/NPD, the most notable compounds being buprenorphine, gabapentin and 6-AM. Studies also demonstrated identification of common target compounds at lower concentrations than with current screening protocols. For example,

with an immunoassay cutoff concentration of 100 ng/mL for benzodiazepines, 50 ng/mL for opiates and 200 ng/mL for amphetamines, specimens containing these compounds do not routinely flag positive for lorazepam, alprazolam, clonazepam, barbiturates, cocaine metabolite, 6-AM and amphetamines. These compounds are not detected, or detected only at high concentrations, using the basic drug screen by GC-MS/NPD. Target compounds including zolpidem, methadone, tricyclic antidepressants, selective serotonin reuptake inhibitors, diphenhydramine, dextromethorphan and tramadol are detected at significantly lower concentrations using LC-TOF-MS than with the basic drug screen by GC-MS/NPD.

Case comparison analyses included positive controls at 3x LOD concentrations and a semi-quant control, consisting of compounds likely to require quantitative analysis at a dilution (e.g., morphine, oxycodone, methamphetamine, methadone) and compounds for which an estimated concentration aids in determining whether the level is > LOQ of the confirmation method (e.g., citalopram, fluoxetine, sertraline), was also evaluated. Results show that semi-quant analysis provides accurate estimated concentrations for most compounds, which will be useful in directing confirmation testing of casework specimens.

Comparison of required sample volume and preparation/analysis time between current specimen protocols for screening and the new LC-TOF-MS method demonstrate a marked improvement in testing efficiency. Analysis by immunoassay and basic drug GC-MS/NPD requires a combined sample volume of 2 mL, with a combined sample preparation time of 5 hours, instrument acquisition time of up to 20 hours and data analysis/evaluation time of up to 4 hours. The LC-TOF-MS method requires a sample volume of 0.2 mL, with sample preparation time of 2.5 hours and data analysis time of 3-4 hours.

Validation of the test method and use of the test method in case comparison studies have demonstrated this new LC-TOF-MS test method will enable the laboratory to perform comprehensive initial screening of case specimens, using minimal sample volume. Use of the method will also increase efficiency in use of time and materials. Training of forensic scientists on use of the new method, including software and data analysis, has been a time-intensive endeavor and is continuing. Training of supervising scientists and management on the review of data and reporting is also continuing.

IMPLICATIONS

The goal of this project was to produce a broad LC-TOF-MS screening method to replace less efficient and more costly screening approaches that we and many other forensic toxicology laboratories utilize. The validated method definitely has the potential for reducing costs in the long-term and reducing case backlogs and/or case turnaround. Based on the results of this project, other government toxicology laboratories can take advantage of the in-depth method development, optimization and validation work performed and can use our results to guide them in their testing and policy decisions. Indeed, when presenting the results of our project at an international/national forensic toxicology conference (SOFT/TIAFT, January 2018), the presenter was approached by nearly two dozen individuals/laboratories asking questions on how the method worked and how they should get started using this technology, or if they had recently purchased this technology, how best to move forward with method development and optimization. Since returning from the conference, our laboratory has been in contact with approximately 15 individuals/laboratories asking for further results, guidance and information.