Document Title:	**Forensic Toxicological Screening/Confirmation of 500+ Designer Drugs by LC-QTOF-MS and LC-QqQ-MS Analysis**
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Document Number:	**253327**
Date Received:	**August 2019**
Award Number:	**2014-R2-CX-K006**

Final Summary Overview

Agency: National Institute of Justice, Office of Justice Programs

Award Number: 2014-R2-CX-K006

Project Title: Forensic Toxicological Screening/Confirmation of 500+ Designer Drugs by LC-QTOF-MS and LC-QqQ-MS Analysis

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Submission Date: January 14, 2019

Recipient Organization:

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Award Period: 1/1/2015 - 12/31/2018

Signature of Submitting Official:

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Purpose

Novel psychoactive substances (NPS), also known as “designer drugs”, are derivatives of controlled substances that are sold on the street in an attempt to circumvent the legal restrictions placed on scheduled drugs. Chemical modifications in these drugs can be very subtle, leading to virtually unlimited structural variation. Consequently, there are many hundreds of such entities that have been identified to date. Traditional drug screening methods such as immunoassay are unable to detect most designer drugs, a situation exacerbated by rapid drug introduction and changing street profile. With the ongoing rise in novel drug popularity and increasing reports of adverse health effects of designer drugs, there is a clear need within the forensic toxicology community for robust, rapid, and comprehensive screening and confirmatory analytical methods for such compounds.

Liquid chromatography (LC) coupled to high-resolution, high-accuracy mass spectrometry (HRMS) is increasingly being employed for drug screening and for detection/identification of unknowns. In addition, because of its high sensitivity and applicability to targeted analysis, LC coupled to triple quadrupole (QqQ) MS has become an established methodology in many forensic laboratories, primarily for confirmatory analysis. Despite this progress, there are currently no validated HRMS or QqQ-MS based analytical methods capable of comprehensively screening large numbers of NPS in forensic specimens. Due to this lack of analytical capability, there is little objective information available on the number and identity of individual designer drugs actually being abused by various user subpopulations. Development of such methodologies would represent a significant contribution to advancing the science and practice of forensic toxicology for this important class of drugs.

Project Design and Methods

Task 1 involved the construction of a designer drug master database, a high-resolution, high mass accuracy QTOF-MS/MS mass spectral library, and a QqQ triggered MRM database including up to 10 transitions per compound for a minimum of 500 individual NPS entities and metabolites. Task 2 involved comparison of dilute-and-shoot-, crash-and-shoot-, on-line and classical SPE-, and "QuEChERS" (Quick, Easy, Cheap, Effective, Rugged, and Safe)-based extraction methods for reliable recovery of NPS from urine and whole blood. Task 3 of this project involved formal validation of LC-QTOF-MS and LC-QqQ-MS approaches for comprehensive screening/confirmation of 500+ NPS, including analysis of blind spiked and authentic urine and whole blood specimens. An additional goal was to explore 2D-LC coupled to QTOF-MS for the separation and analysis of isomeric and structurally related NPS.

Illustrative data generated in the project are included in the Appendix to this report.

Data Analysis and Results

***Standards*:** A total of 826 individual NPS standards, primarily from the synthetic cannabinoid and stimulant/hallucinogen classes, in addition to a number of designer opioids and other compounds, were acquired for the project. Numerous metabolites of parent drugs were included in this total, in addition to ~100 deuterated compounds for use as internal standards. All standards were prepared as 10 ppm stock solutions, which were further diluted to prepare 1 ppm working solutions.

***LC-QqQ-MS Data Collection and Method Validation*:** All standards were analyzed by flow injection analysis (FIA) by QqQ-MS using the 1 ppm working dilution. Diluted standards were individually injected directly into an Agilent 6460 series triple quadrupole

(QqQ) mass spectrometer with Jet Stream ESI ion source using an Agilent 1290 Infinity Series Binary Pump system. Data were collected in positive ion mode using an isocratic mobile phase. If FIA was successful, the standards were then analyzed using Optimizer software, which searched for 4 to 10 product ion transitions that were analyzed via an Optimizer Report. The report included precursor ion, fragmentor voltage, product ions identified, collision energies, and abundances.

All compounds that had four or more transitions with ion abundances above 1000 counts were then separated by gradient elution LC with a 16-min run time to obtain standardized retention times. A Zorbax Eclipse Plus RRHD C18 column (3.0 x 100 mm, 1.8 µm particle size) was used with temperature held at 40°C. All compounds were analyzed using a dynamic multiple reaction monitoring (dMRM) method that specifically targeted two transitions for each compound to trigger collection of additional MRM transitions, up to 10 total if higher specificity is needed. All transitions used were chosen from Optimizer results and were cross-checked for integrity against QTOF-MS data for the same compounds.

Determination of standardized retention times for all 826 NPS standards was completed. To fully validate the method, calibration curves were created for each drug standard. Since completing individual calibration curves for each of the 826 compounds would be extremely time consuming, an approach using standard calibration mixes was adopted. Criteria for individual mixes included the presence of unique transitions for each component, no co-eluting compounds, and a minimum of 0.2 min between compound peaks in the same mixture. Validation mixes were composed of 20 to 65 individual drug compounds each, with 15 mixes needed for validation of all analytes.

Matrix calibrators were created using commercially available certified blank urine. Seven different calibration levels were chosen for method validation; 1, 2, 5, 10, 20, 50,

and 100 ppb. All calibrators also contained an internal standard “supermix” made up of 22 deuterated standards representing multiple drug classes. Full validation included determination of LOD, LOQ, bias, precision, linearity, selectivity, freeze-thaw stability, and matrix effects. Validation, including determination of LOD, LOQ, bias, precision, linearity, selectivity, freeze-thaw stability, and matrix effects, was completed according to SWGTOX and OSAC guidelines for eight mixes; the remaining mixes will be validated under other funding sources. Data for the majority of compounds in each mix fit well within acceptable ranges in SWGTOX/OSAC guidelines.

Screening of blind-spoked and authentic specimens using the validated LC-QqQ-MS method, one of the components of Task 3, was not completed, but will be undertaken with other funding sources.

***Comparison and Optimization of Extraction Methods*:** Extraction of NPS from blood or urine by the five selected approaches was compared using two of the NPS mixes described above. Dilute-and-shoot was accomplished by diluting urine samples with water (1:5 dilution) followed by LC-QqQ-MS analysis. For crash-and-shoot, 600 µL of cold acetonitrile (-20˚C) was added to 200 µL of whole blood, vortexed, and centrifuged for 5 min at 7000 rpm. Supernatant was removed and added to an LC vial, evaporated to dryness under N_2, and reconstituted in 200 µL of methanol. Classical SPE was performed with a method previously developed in this laboratory using a positive pressure SPE apparatus and Agilent Bond Elute Plexa PCX cartridges. Online SPE utilized an Agilent 1290 Flex Cube LC unit with a Bond Elute (BE) online polymeric sorbent material (PLRP-S) cartridge. QuEChERS utilized an in-house developed mini one-pot kit. Samples were added to pre-weighed components (acetonitrile, $MgSO_4$, NaCl, PSA, C18), shaken by hand, vortexed, and centrifuged at 7000 rpm for 5 min,

followed by recovery of the supernatant for N_2 dry down and analysis.

The five different extraction procedures were evaluated and compared using two-way ANOVA to assess significant differences. If the ANOVA showed a significant difference, a Tukey HSD test was used to determine which specific methods were significantly different. Findings demonstrated that NPS recoveries from urine with QuEChERS and dilute-and-shoot were not statistically different, while recoveries from blood were significantly higher with QuEChERS than with crash-and-shoot. QuEChERS, when used for both blood and urine, showed a decrease in matrix effects for all classes of NPS when compared to crash/dilute and shoot. Online SPE provided low recoveries for many classes of NPS, especially synthetic cannabinoids, and was not evaluated further. Total time required from extraction start to analysis varied from 5 min (dilute-and-shoot) to 3 h (classical SPE), while costs varied from relatively inexpensive (dilute-and-shoot and QuEChERS) to expensive (online SPE and classical SPE). Although dilute-and-shoot was quickest and most cost effective, it is a crude method that can leave matrix components which can damage instrumentation and lead to unwanted ion suppression and enhancement. In conclusion, results showed that QuEChERS provided the best combination of extraction capability, elimination of matrix effects, time, and cost for application to NPS analysis.

***LC-QTOF-HRMS Spectral Data Collection and Method Validation*:** As with QqQ-MS/MS, collection of spectral data for the QTOF-MS/MS spectral library was done via FIA using an Agilent 6530 series QTOF MS with Jet Stream ESI ion source coupled to an Agilent 1290 Infinity Series Binary Pump system. A 50:50 aqueous/organic isocratic mobile phase system was used to infuse the analytes. Compounds were fragmented at three standard collision energies (10, 20, and 40 eV) to produce characteristic CID

spectra.

Mass spectra were reviewed using MassHunter Qualitative Analysis software to ensure that base peak counts for each spectrum were above a 1000 count threshold. Mass accuracy was assessed for each compound using the “Find by Formula” (FBF) algorithm, with mass accuracy within 5 ppm or less considered acceptable. The compound database and spectral library were curated using MassHunter Personal Computer Database Library (PCDL) software (Agilent Technologies). Compound information included common name, IUPAC name, 2D structure, accurate mass, molecular formula, and CAS/ChemSpider numbers when available. The spectral data collected during FIA was input into the PCDL for each compound, providing each with three characteristic fragmentation patterns.

Collection of retention times on the LC-QTOF-MS utilized an Agilent Zorbax Eclipse Plus C_{18} RRHD column with gradient elution, with a run time of 20 min. Spectral data and retention time information were imported into the in-house PCDL for all 826 compounds. In order to reduce time and required resources, QTOF method validation utilized the same mixes that were employed in the LC-QqQ-MS validation procedure. In order to reduce time and required resources, QTOF method validation utilized the same mixes that were employed in the LC-QqQ-MS validation procedure. Although some retention times differed slightly from those on the LC-QTOF-MS instrument, the high resolution, high mass accuracy capabilities of the LC-QTOF enabled the differentiation of any potential co-eluting compounds without the need to reformulate mixes.

Calibration curves were created using seven different calibration levels: 2, 5, 10, 20, 50, 100, and 120 ppb. Each calibrator uses an internal standard “supermix” made up of 22 deuterated standards representing multiple drug classes, each at a concentration of 40 ppb. Calibration curves were completed with mixtures in methanol,

followed by tests with the mixtures in blank urine matrix, using a “dilute-and-shoot” approach and a 1:5 dilution with aqueous mobile phase injected directly in the instrument. Calibrations were linear within the selected range for all components of all three mixtures.

As a proof-of-concept, full validation according to SWGTOX/OSAC guidelines was performed for three of the NPS mixes. Parameters addressed in the method validation included accuracy (bias) and precision, limit of detection and quantitation (LOD and LOQ), recovery, reproducibility, carryover, and stability, including a freeze/thaw study. All bias values were within ±10%. Overall precision values were all within ±10% CV. The within-run and between-run precision values were all within ±20%, with the majority within ±10%. Calculated LODs for components of the three mixes ranged from 0.1 to 2.9 ng/mL, with most values ≤1 ng/mL. Calculated LOQs ranged from 0.3 to 8.8 ng/mL, with most values ≤5 ng/mL. In the freeze-thaw experiment, most analytes fell within ±20% bias with only a few compounds at the low concentration having a bias value greater than 20%. Using both qualitative and quantitative analysis software, it was determined that there was no significant carryover of any compounds in any of the mixes. Finally, significant matrix effect (ion suppression) was noted for some analytes in urine matrix. The matrix effects exhibited were noted but did not impact validation of the method for these compounds, as the critical validation parameters of the compounds prepared in urine were still well within acceptable ranges. However, the results of the matrix effect studies do indicate that more thorough sample preparation for urine specimens containing NPS could ultimately improve analytical performance.

***Analysis of spiked and authentic specimens*:** For further assessment of the validated LC-QTOF-MS screening approach, two sets of 20 blind-spiked (with a single

NPS) or blank specimens were prepared in diluted urine. NPS levels in Set 1 samples were either 2 or 20 ng/mL; specimens in Set 2 were at 200 ng/mL. Two data acquisition methods were tested; Full Scan MS and Auto MS/MS. The same LC, ESI source, and scan parameters were used for each acquisition method. Full Scan MS collected all ion data and did not subject the ions to any collision energy. For Auto MS/MS, fragmentation was performed at three collision energies of 10, 20, and 40 eV. Agilent MassHunter Qualitative Analysis software was used to perform library and database searches for data collected using both Full Scan MS and Auto MS/MS modes, using the compound database and libraries previously created.

Screening of Set 1 resulted in positive identification of the NPS or blank specimen for 13/20 specimens, while screening of the Set 2 samples resulted in 18/20 correctly identified. Three of the specimens in Set 2 that were not initially identified were included on a “hit list” of possible identifications. Closer analysis of the ion fragmentation patterns for these specimens, as well as consideration of the retention times included as part of the in-house database, ultimately resulted in a correct identification. Although not all of the compounds in the blind-spiked specimens were correctly identified, the qualitative screening results do confirm the utility of the compound database and HRMS spectral library for rapid identification of NPS in human urine specimens. It is important to note that the HRMS library used in the above study was created in-house and has not been curated to remove background ions, as curation of the library was not included in the scope of the present research.

Screening of authentic specimens using the validated LC-QTOF-MS method, one of the components of Task 3, was not completed, but will be undertaken with other funding sources.

***Studies of 2D-LC Separation of Co-Eluting NPS*:** Investigation into the use of two-dimensional liquid chromatography (2D-LC) in order to better separate isobaric and co-eluting, chemically similar synthetic cannabinoids (SC) was completed. For this study, three different mixes of co-eluting NPS were created: one mixture of isobaric (*i.e.,* same exact mass) compounds and two mixes of non-isobaric SC with similar retention times.

Considerable method development work resulted in the selection of a combination of two reversed-phase columns to provide the two separation dimensions; an Agilent Poroshell 120 Bonus-RP column in the first dimension (^{1}D) and a Supelco Ascentis Express Biphenyl column in the second dimension (^{2}D). The (A) mobile phase used in both dimensions was HPLC water with 0.1% trifluoroacetic acid (TFA). The (B) mobile phase used in ^{1}D was acetonitrile:water (95:5 *v/v*) and in ^{2}D was methanol:water (95:5 *v/v*). A multistage gradient for ^{1}D was utilized with a run time of 45 min. The flow rate was 0.1 mL/min. The gradient used in ^{2}D was a shifted gradient with a flow rate of 0.55 mL/min. The gradient stop time was 0.95 min and the modulation time was 1.15 min.

2D-LC of CE Mix 1 resulted in excellent separation of the JWH-019 2-, 5-, and 6-fluorohexyl isomers with slight co-elution of the 3- and 4-fluorohexyl isomers. 2D-LC of CE Mix 2 resulted in complete separation of PB-22, JWH 203, and XLR-12, with slight co-elution of MAM-2201 N-(2-fluoropentyl) isomer and JWH 080. Finally, 2D-LC separation of CE Mix 3 yielded complete resolution of all five cannabinoid components.

Major Findings

LC retention times and optimized MS/MS transition determinations were determined for all 826 NPS standards on the LC-QqQ-MS instrument. A comprehensive NPS compound database was produced and LC retention times and HRMS spectral libraries at three different collision energies were determined for all 826 NPS

standards on the LC-QqQ-MS instrument.

A calibration approach using mixes of non-coeluting NPS substantially reduced the time and effort required to validate the LC-QqQ-MS and LC-QTOF-MS screening/confirmatory methods for a large number of NPS. Full validation of the LC-QqQ-MS and LC-QTOF-MS screening methods was completed for eight and three of 15 NPS mixes, respectively, as a proof-of-concept.

Analysis of blind-spiked urine samples using the validated LC-QTOF-MS method demonstrated its utility for NPS screening purposes.

Comparison of five different sample processing/extraction methods for LC-QqQ-MS screening/confirmation of NPS has proved important for further optimizing forensic toxicological analysis of these important compounds.

Comprehensive, on-line 2D-LC was shown to be a potentially valuable technique for the separation of isomeric and structurally related NPS.

Implications for Criminal Justice Policy and Practice in the United States

NPS ("designer drugs") are important abused substances that represent a challenge to forensic toxicology and the criminal justice system with regard to detection and confirmation. There is a critical need in forensic toxicology for reliable and comprehensive screening assays for multiple NPS in a variety of human specimens for both ante- and post-mortem investigation. This project provides important data that will lead to an improved ability to identify multiple NPS entities in human forensic specimens. Data and technologies developed from this project will allow crime laboratories to rapidly screen for and confirm the presence of hundreds of such drug entities in forensic toxicological specimens.