The author(s) shown below used Federal funds provided by the U.S. Department of Justice and prepared the following final report:

Document Title: Enhanced Forensic Mass Spectrometry Methods

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Document No.: 225532

Date Received: January 2009

Award Number: 2005-IJ-CX-K014

Final Technical Report

Enhanced Forensic Mass Spectrometry Methods

Grant number 2005-IJ-CX-K014

Submitted December 23, 2008 by Patrick S. Callery

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Abstract

As stated in the NIJ sponsored report entitled, *Forensic Sciences: Review of Status and Needs*, "Methods research, development, testing, and evaluation (RDT&E) activities are vital to the provision of effective forensic science laboratory services." The main goal of this proposal is to develop a better, faster and potentially less expensive method for confirming the identity of chemicals of forensic interest, and to facilitate the broad dissemination of the method for use by forensic chemists and toxicologists. While mass spectrometric methods are used for the identification of chemicals, especially of controlled substances, most require chromatographic sample purification steps. Our objective is to develop simple, robust, rapid, sensitive, specific and cost effective direct mass spectrometric methods for the identification of controlled and toxic substances that minimize sample clean-up procedures. Enhanced MS methods relevant to the practice of forensic chemistry and toxicology are anticipated from this proposal. Single stage mass spectra continue to be the cornerstone of chemical identification. Methods that apply multistage mass spectra, such as MS/MS, are well established. MS/MS/MS (MS^3) is available on competitively priced ion trap mass spectrometers. The application of further stages of fragmentation provide increased confidence in structure assignments of chemicals plus the potential for decreased need for extensive sample preparation. Multistage MS provides identification methods that take less time to perform, decrease hazardous solvent use, obviate derivatization steps, and provide chemical confirmation of substance identity. We developed methods that utilize MS^3 techniques and published the results. Ion suppression concerns were addressed through the use of stable isotope labeled internal standards. Methods were developed jointly at West Virginia University and the Office of the Chief Medical Examiner of West Virginia. The methods developed are publicly available through publication and presentations at regional and national meetings of forensic scientists.

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Executive Summary:

The main goal of this proposal is to develop a better, faster and potentially less expensive method for confirming the identity of chemicals of forensic interest, and to facilitate the broad dissemination of the methods for use by forensic chemists and toxicologists. Mass spectrometric methods are recognized as the best methods for the identification of chemicals, especially of controlled substances. Most MS methods require chromatographic sample purification steps. We propose to develop simple, robust, rapid, sensitive, specific and cost effective direct mass spectrometric methods for the identification of controlled and toxic substances that minimize sample clean-up procedures. Forensic chemists and toxicologists can be more effective in their work if the analytical methods employed can be completed accurately and precisely in less time than current methods. Enhanced MS methods relevant to the practice of forensic chemistry and toxicology are anticipated from this proposal. Single stage mass spectra continue to be the cornerstone of chemical identification. In recent years, methods that apply multistage mass spectra, such as MS/MS, have become well established. Applications of further stages of fragmentation provide increased confidence in structure assignments of chemicals and also provide the potential for decreased need for extensive sample preparation. Multistage MS provides the opportunity to develop identification methods that take less time to perform, decrease hazardous solvent use, obviate derivatization steps, and provide chemical identification. Ion suppression issues are diminished through the use of stable isotope labeled internal standards. Since further consecutive stages of mass spectrometric fragmentation, including MS/MS/MS (MS^3), are available on competitively priced ion trap mass spectrometers, we propose to develop methods that utilize MS^3 techniques and to disseminate the results through publications and presentations. Completed work focuses on detection of fentanyl in urine. Current work extends the methodology to the confirmation of identification of multiple drugs in blood. Methods have been developed and continue to be developed jointly at West Virginia University and the Office of the Chief Medical Examiner of West Virginia. The methods development work has led to two publications,

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one case note, and four abstracts of presentations at scientific meetings.

Main Body of the Final Technical Report

I. Introduction:

1. **Statement of the problem**:

Confirmation of the identity of substances of forensic interest can be an expensive, time-consuming process. New structure confirmation methods that are more efficient and effective than current methods are needed.

2. **Literature Citations and Review**

Background:

Forensic mass spectrometry provides a basis for high quality analyses of evidence. In many cases, mass spectrometry provides the gold standard for chemical identification. Identification of controlled substances prior to, or after ingestion, almost always requires mass spectrometric methods. The MS instrument of choice has been a quadrupole mass spectrometer (MS) interfaced with a gas chromatograph (GC). Millions of forensic cases involving controlled substances have been identified and quantified by GC-MS. GC-MS is reliable, highly sensitive, and well-established in the literature. Good libraries of standards are available, lower cost, ease of operation, and acceptability in the courtroom are additional advantages. However, there are significant limitations to GC-MS. Not all substances of interest have sufficient volatility to be separated and detected by GC-MS. For some less-volatile analytes, reagents are available that can convert the substance into a suitably volatile derivative. Chemicals having low volatility or poor chromatography properties are converted to higher molecular weight derivatives exhibiting better chromatographic characteristics. GC-MS assays are often cited as the gold standard, but can be time consuming when compared to more direct methods that do not require sample purification or derivatization reactions.

Liquid chromatography/mass spectrometry (LC-MS) has a role of increasing importance in forensic analysis. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) of analytes in single stage mass spectrometers provides useful molecular weight information, although

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further fragmentation is generally not produced. Fragmentation obtained from electron ionization GC-MS serves as the basis of library mass spectra comparisons for chemical identification. Sequential, tandem multistage mass spectral analyses known as MS/MS also provides fragmentation information. MS/MS is an established mass spectrometry technique that has potential for new applications to forensic analyses in the day-to-day operation of crime labs (1-3). Fragmentation of product ions formed by MS/MS leads to an additional stage of fragmentation called MS/MS/MS (MS^3). MS^3 spectra provide a means to confirm the identity of chemicals because the probability that different chemicals of the same molecular weight will have the identical MS^3 spectrum is extremely low except for certain known stereoisomers or regioisomers. A systematic approach to the determination of the usefulness of MS^3 spectra for forensic analysis is needed, and is the subject of this proposal.

Mass spectrometry is a category A analytical technique (4) which can be applied to virtually all drugs. Mass spectra can be interpreted for structure identification of unknown drugs. Further fragmentation of mass spectral product ions provides additional structure information characteristic of the analyte. There are several limitations to mass spectrometry. These include the inability to discriminate between enantiomers, most diastereomers, and salt forms of drugs. Another disadvantage of mass spectrometry is that some fragmentation patterns of drugs of similar structure may be identical. Draft guidelines for mass spectrometry-based qualitative analytical methods proposed by the SWGDRUG in January 2005 (4) outline the advantages and disadvantages of mass spectrometry in drug analysis, discuss the sample preparation and instrument parameters, and the performance characteristics including selectivity, matrix effects, recovery, accuracy, range (limit of detection, limit of quantification), robustness and ruggedness, as well as quality control.

A disadvantage of LC-MS is the potential for signal suppression by co-eluting ions from contaminating substances (5,6). Ion suppression is a problem because of the potential of causing false negative results. The best method for decreasing ion suppression is to remove the source of the ions causing the suppression by isolating the unknown from matrix components. The approaches to decreasing ion suppression usually involve extensive sample preparation steps. In our preliminary

studies, we have found that ion suppression occurs in urine samples thereby affecting the limit of detection of the drugs under study to about 50 ng. To address this problem, we propose to systematically evaluate the impact of ion suppression on the identification of drugs in urine and to develop approaches that use stable isotope internal standards to control ion suppression.

The SWGDRUG report on Methods of Analysis/Drug Identification, Part III B recommends as a minimum standard that when a validated Category A technique is used at least one other technique (from either Category A, B, or C) must be used (Section 3.1). According to Section 3.1.1, this combination must identify the specific drug present and must preclude a false positive identification. Section 3.1.3 requires that Category A techniques have data that are reviewable, for example, printed spectra. SWGDRUG July 2004 Part IV B Quality Assurance/Validation of Analytical Methods covers the definition and purpose of validation, analytical scheme, individual laboratory responsibility, operational environment, documentation, and recommendation. The general validation plan covers the purpose/scope, performance specification, process review, analytical method, reference materials, performance characteristics, selectivity, matrix effects, recovery, accuracy, precision (repeatability/reproducibility), trueness, range, limit of detection, limit of quantification, linearity (for quantitative methods), robustness, ruggedness, uncertainty (for quantitative methods), and quality control.

The identification of substances is a key component of forensic analytical toxicology (7). Multistage mass spectrometry is a technique that can serve as a basis for qualitative and quantitative measurement of substances (1-4,8,9). Sample introduction for mass spectrometry is usually by direct injection or through interfacing with a chromatographic inlet such as gas chromatography, liquid chromatography or capillary electrophoresis. Comparing costs with chromatography-based systems, such as GC-MS or LC-MS, the cost per analysis is lower for the direct injection method described in this report. Lower costs result from avoiding costs for chromatography supplies, instrumentation, and maintenance costs. On the other hand, the direct method has higher costs associated with purchase of stable isotope labeled internal standards. The robustness and reproducibility of MS/MS data has been improved to the point where library searches of product ion spectra may be available in the future (3).

Until such libraries can be validated and made available, multistage mass spectral analyses must rely on careful comparison to standards using standardized instrument conditions.

3. **Rationale for the Research:**

The aim of this research is to develop simple, rapid, and validated mass spectrometric assay methods for the forensic confirmation of the identification of drugs and to disseminate validated methods through publications and presentations to the forensic science community.

II. Methods

Methods, materials, and procedures were adapted from Peer, C.J., Shakleya, D.M., Younis, I.R., Kraner, J.C. and Callery, P.S. (2007) "Direct-injection mass spectrometric method for the rapid identification of fentanyl and norfentanyl in postmortem urine of six drug-overdose cases." *J Anal Tox.* **31**, 515-522 (reference 12), and from Peer, C.J., Clay, D.J., Glover, H.L., Renninger, K.L., Kraner, J.C., and Callery, P.S. (2008) "Direct injection mass spectrometric confirmation of multiple drugs in overdose cases from postmortem blood using ESI-MS-MS and MS[3]." *J Anal Tox.* **32**, 705-708 (2008). Some of the methods details in reference 13 are provided below.

Methanol, acetonitrile, ammonium hydroxide, and formic acid were purchased from Fisher Chemical (Pittsburgh, PA) and were of HPLC or MS grade. A Finnigan LCQ DECA ESI ion trap mass spectrometer using Xcalibur software (Thermo Fisher Scientific, Waltham, MA) was used to produce all MS[3] spectra. N_2 from a nitrogen gas generator (Parker-Balston, Haverhill, MA) at 45 psi was used as the sheath gas. Other mass spectrometers used included a Waters-Micromass ZMD ESI single quadrupole mass spectrometer and a Waters-Micromass Quattro ESI triple quadrupole mass spectrometer. Deuterium-labeled drugs were purchased from Cerilliant (Round Rock, TX) as 100 µg/mL free base equivalents in methanol: acetaminophen-d_4, alprazolam-d_5, benzoylecgonine-d_3, cocaine-d_3, diazepam-d_5, diphenhydramine-d_3, ecgonine methyl ester-d_3, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine-d_3 (EDDP-d_3) perchlorate, fentanyl-d_5, fluoxetine-d_6 oxalate, hydrocodone-d_3, methadone-d_3, norfentanyl-d_5

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oxalate, nortriptyline-d$_3$ HCl, promethazine-d$_3$ HCl, and zolpidem-d$_6$. Drug standards were purchased

from Cerilliant as 1 mg/mL free base equivalents in methanol: acetaminophen, alprazolam,

benzoylecgonine, carisoprodol, citalopram HBr (100 µg/mL), cocaine HCl, cyclobenzaprine HCl,

dextromethorphan, diazepam, ecgonine methyl ester, EDDP perchlorate, fentanyl HCl, fluoxetine HCl,

hydrocodone, ibuprofen, meprobamate, methadone HCl, promethazine, quetiapine hemifumarate,

norfentanyl oxalate, nortriptyline, venlafaxine HCl, and zolpidem hemitartrate. Postmortem whole blood

from cases of confirmed multi-drug overdose deaths was obtained at autopsy by the WV OCME and

aliquots were provided for study.

Sample Preparation, Urine Samples (12): Aliquots of 500 µL of urine were spiked with

deuterium labeled internal standard (10 ng/mL final concentration) and vortexed for 1 minute. The 10

ng/mL concentration is the recommended cut-off detection limit for fentanyl and other narcotics set by

the SOFT Drug-Facilitated Sexual Assault Committee (10). Samples were centrifuged for 5 minutes at

13,000 rpm on a desktop centrifuge (Eppendorf, Westbury, NY). The supernatant was applied to a Sep-

Pak® Plus C18 cartridge (Waters) preconditioned with 2 mL of methanol followed by 2 mL of water. The

cartridge was washed with 300 µL of water followed by 300 µL of a (95:5) water:methanol solution.

Analyte and internal standard were eluted with 1 mL of 0.1% formic acid in methanol. The 1mL eluent

was vortexed for 1 minute and a 100 µL aliquot was diluted to 500 µL with 0.1% formic acid in

methanol. Standard curves were generated consisting of 0, 10, 25, 50, 100, 200, and 300 ng/mL fentanyl

(each with 10 ng/mL d$_5$-fentanyl) by addition of 10 µL of the appropriate concentration of fentanyl

working solution to 1 mL of control urine and prepared as mentioned above. For fentanyl, the standard

curve was run on the triple quadrupole to confirm quantitative measurements from the ratio of *m/z*

337→188:*m/z* 342→188.

Sample Preparation, Blood Samples (13): A 200 µL aliquot of whole blood from each case

sample was spiked with deuterated internal standards of analytes confirmed by the WV OCME. The

blood concentration of all internal standards was 10 ng/mL. The blood concentration of all internal

standards was 10 ng/mL by the addition of 10 µL of each 1 µg/mL deuterated internal standard working solution prepared in methanol. This is consistent with the recommended minimal detectable concentrations proposed by the SOFT Drug-Facilitated Sexual Assault Committee (10), by the addition of 10 µL of each 1 µg/mL deuterated internal standard working solution prepared in methanol. Samples were vortexed for 1 minute prior to addition of 1 mL of acetonitrile to precipitate proteins and extract drugs, metabolites, and deuterium-labeled internal standards. Samples were again vortexed for 1 minute and then immediately centrifuged on a desktop centrifuge (Eppendorf, Westbury, NY) for 5 minutes at 13,000 rpm. A 50 µL aliquot of the supernatent from each case was added to 200 µL of 0.1 % formic acid in methanol, then directly injected into the ESI mass spectrometer operated in the positive ion mode using a syringe with a flow rate of 7 µL/min. A separate 50 µL aliquot for case 5 was added to 200 µL of 0.1 % NH_4OH in methanol to deprotonate ibuprofen and then injected into the mass spectrometer for analysis in the negative ion mode.

Working solutions of each drug, metabolite and internal standard from Cerilliant were prepared in methanol. Case-specific standard samples prepared in water were created with analyte and internal standard concentrations of 100 ng/mL and 10 ng/mL, respectively, to optimize conditions for each MS/MS and MS^3 transition. Seven case-specific MS/MS methods using 61 selected reaction monitoring (SRM) transitions for 65 total drugs, metabolites, and internal standards were developed to confirm the identity of each drug previously quantified by the WV OCME as ≥ 10 ng/mL in blood. There were four SRM transitions that fragmented two different parent drugs: The SRM of *m/z* 285 in Case 7 monitored both promethazine and diazepam; Case 8 contained one SRM of *m/z* 278 that fragmented both amitriptyline and venlafaxine and one SRM of *m/z* 264 that fragmented both nortriptyline and norvenlafaxine; Case 10 contained one SRM of *m/z* 285 that fragmented both promethazine and diazepam. Cases 10 and 11 were split into two methods to allow for optimal SRM conditions due to the larger number of transitions monitored. Run times were 5 minutes with each SRM transition consisting of 3 microscans over a 200 ms scan time.

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Five case-specific MS^3 methods were developed for cases 7-11 using 29 consecutive reaction monitoring (CRM) transitions on 29 parent drugs and metabolites in the five cases. CRM transitions were optimized to obtain the maximum signal for the MS^3 product ion resulting from fragmentation of the precursor ion and the major MS/MS product ion. Parent drugs not included in MS^3 analyses were ibuprofen, which did not have a reliable MS^3 product ion as determined by standards, and acetaminophen in case 8, whose MS/MS product ion was poorly detected. The MS^3 method run times were 2 minutes, with each CRM consisting of 3 microscans over 200 ms scan times. Table 4 contains the *m/z* values for precursor ions and MS/MS product ions of all 61 SRM transitions (representing 65 ions) and also the MS^3 *m/z* values for those 29 analytes. Mass spectrometer instrument conditions were consistent for each case method as follows: heated capillary temperature was set at 220 ˚C, capillary voltage at 20 V, spray voltage at 5.2 kV, sheath gas flow rate at 40 arbitrary units, SRM collision energies ranged from 25-40% for all MS/MS transitions, and CRM collision energies ranged from 25-35% for all MS^3 transitions.

Six case histories (cases 1-6) were reported in Peer, C.J., Shakleya, D.M., Younis, I.R., Kraner, J.C. and Callery, P.S. (2007) Direct-injection mass spectrometric method for the rapid identification of fentanyl and norfentanyl in postmortem urine of six drug-overdose cases. *J Anal Tox.* **31**, 515-522.

III. Results

1. **Statement of Results**

Confirmation of fentanyl and norfentanyl in urine of overdose cases by MS/MS. Urine and blood urine samples from six overdose cases involving fentanyl were obtained at autopsy by the Office of the Chief Medical Examiner of West Virginia. Blood and urine samples were screened for drugs and alcohol by the WV OCME or by NMS Labs. LC-MS was used by the WV OCME to identify and quantify drugs of abuse present in the blood in cases 1, 4, and 6 (Table 1). NMS Labs (Willow Grove, PA) identified and quantified any drugs present in cases 2, 3, and 5 using LC-MS/MS (Table 1). The analyses performed by the WV OCME and NMS Labs were conducted prior to this study and their work was included for comparative purposes. Therefore, their materials and methods were not included in this study.

Case Samples (cases 1-6): Fentanyl and d_5-fentanyl (*m/z* 337 and 342, respectively) were identified in urine for each of the cases using an ion trap mass spectrometer. Ratios were calculated based on the total ion chromatogram peak area of the MS^2 product ion from fentanyl:d_5-fentanyl (*m/z* 337→188:*m/z* 342→188) in order to provide an estimate of fentanyl urine concentrations. If the ratios of the case samples were above 1.0, the amount of fentanyl present in the postmortem urine was greater than 10 ng/mL because the internal standard concentration in each sample was 10 ng/mL. To test this, a negative control was run for case samples that were not spiked with internal standard. The *m/z* 342→188 transition was not detectable in urine samples that were not spiked with d_5-fentanyl. It was determined that this method can selectively confirm the identity of individual compounds based on monitoring product ions. In a second run, the MS^2 quantifier ion was fragmented using the same collision parameters to obtain the transition of *m/z* 188→105, where *m/z* 105 was identified as a qualifier for further confirmation of structure.

Urine samples run on the ion trap instrument were also analyzed on a triple quadrupole mass spectrometer for the identification and quantification of fentanyl. There were no apparent difficulties in transferring the direct injection method to a second type of MS/MS instrument. A seven-point calibration curve ranging from 0-300 ng/mL fentanyl ($r^2 > 0.99$) was used for quantitation. Fentanyl was structurally identified in all six cases above the LOD of 1 ng/mL, however only five of the six urine samples contained greater than the LOQ of 10 ng/mL (Table 2). Quantification of fentanyl by monitoring the MH^+ in the MS stage was unreliable due to substantial background. Less signal contamination was observed in MS/MS analyses, and methodology was developed to monitor the *m/z* 337→188 and *m/z* 342→188 transitions for the detection of fentanyl. Norfentanyl was confirmed in five of six cases using the ion trap mass spectrometer to detect the most abundant MS/MS product ion (*m/z* 84) as a qualifier ion.

Relative Ion Suppression: To assess ion suppression in each of the case samples, the mean peak area from the D_5-fentanyl MS^2 product ion (*m/z* 342→188) in each case was compared to the mean peak area of the D_5-fentanyl MS^2 product ion (*m/z* 342→188) in standard curve samples from water. The peak area values are the mean of four intra-day runs on the triple quadrupole mass spectrometer. Relative ion

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suppression was calculated as a percent difference between mean peak areas using Equation 1:

$$\frac{A - B}{A} * 100$$ (Eq. 1), where A = mean peak area at 10 ng/mL in water, and B = mean peak area at 10

ng/mL for each individual case. Ion suppression was present in all but one case sample (Table 3). Good

linearity of the standard curve indicated that the ratios were not perturbed by analyte concentration within

the range of 10-300 ng/mL suggesting that ion suppression did not alter fentanyl/D_5-fentanyl ratios (11).

Confirmation of multiple drugs in overdose cases from postmortem blood using ESI-MS-MS and MS^3 (13). Whole blood from five cases of confirmed multi-drug overdose deaths was obtained at autopsy

by the WV OCME. Each case was screened for drugs of abuse by enzyme multiplied immunoassay

technique (EMIT) and then analyzed by GC-MS or LC-MS to confirm the identity and quantify drugs and

metabolites present. There were 40 total drugs and metabolites identified in the five cases. Drugs that

were present in more than one case were methadone, diazepam, promethazine, acetaminophen,

alprazolam, dextromethorphan, and the cocaine metabolites benzoylecgonine and EME.   The

concentration of each analyte that was quantified by the WV OCME as $\geq$ 10 ng/mL in blood is provided

in Table 4. Direct injection MS/MS was performed on the five cases to confirm the identity of each

analyte based on the presence of its major MS/MS product ion relative to that of its corresponding

internal standard, which is thought to compensate for extraction efficiency and ion suppression.

The major MS/MS product ions were detected in 39 of the 40 analytes (parent drugs and

metabolites) and 24 of the 25 deuterated internal standards were detected (Table 4). Acetaminophen in

case 2 was not quantified because acetaminophen-d_4 was not detected. Total analysis time from thawed

samples to end of the seven case-specific MS/MS methods at 5 minutes each was less than one hour.

Analyses were performed in triplicate to verify results. Table 4 displays the mass to charge ratio (*m/z*) of

the precursor ion (MH$^+$) and the major MS/MS product ion as well as the proposed neutral loss of the

molecule resulting from the SRM fragmentation.

To ensure the selectivity of this method, for confirming the identity of each analyte and internal standard, a standard solution containing the deuterated internal standards was analyzed by the seven case-specific MS/MS methods. The internal standard signals were identified by the appearance of their major MS/MS product ions for all 25 internal standard SRM transitions. Conversely, a case sample of blood was prepared without spiking the corresponding deuterated internal standards to check for cross-talk or contaminating substances. The results of this experiment identified the major MS/MS product ions from the SRM transitions of the analytes, but not those of the internal standards. These spiking experiments suggest that MS/MS analyses provide selectivity and specificity that is sufficient to confirm the identity of the drugs and metabolites based on their unique MS/MS fragmentation patterns.

A second confirmation step was performed on 29 analytes based on their MS^3 fragmentation patterns optimized from standard samples. The 29 analytes excluded deuterated internal standards, metabolites (except cocaine metabolites in case 9 where no parent cocaine was detected by the WV OCME), ibuprofen in case 11, which did not provide a reliable MS^3 product ion as determined from standards, and acetaminophen in case 8, which was not determined due to poor detection of the drug and acetaminophen-d_4 using MS/MS. Table 4 displays the *m/z* ratios of the major product ion resulting from MS^3. All but 3 analytes, namely alprazolam in case 9, carisoprodol in case 10, and fluoxetine in case 11, were identified by their MS^3 product ions.

2. Tables and Figures

Table 1. Blood Concentrations of Fentanyl and Norfentanyl in Overdose Cases as Determined by LC-MS and LC-MS/MS (from reference 12)

Case	Fentanyl conc. (ng/mL)	Norfentanyl conc. (ng/mL)
1	14	Not Detected
2	6.8	1.7
3	39	Not Detected
4	5.2	3.0
5	10	28
6	15	Not Detected

Blood concentrations of fentanyl and norfentanyl in cases 1, 4, and 6 were measured by the Office of the Chief Medical Examiner of West Virginia using LC-MS and for cases 2, 3, and 5 by NMS Labs using LC-MS/MS.

Table 2. Urine Concentrations of Fentanyl and Norfentanyl in Overdose Cases as Determined by Direct-Injection Mass Spectrometry (from reference 12).

Case	Fentanyl conc. (ng/mL) $\pm$ RSD	Norfentanyl conc. (ng/mL)
1	185 ± 7.00	12.3
2	30.7 ± 5.80	10.3
3	93.7 ± 6.69	19.2
4	141 ± 6.78	21.4
5	< 10	18.7
6	45.7 ± 5.46	< 10

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Fentanyl was identified using both triple quadrupole and ion trap mass spectrometers using ESI in the

positive ion mode. Values are the mean peak areas of *m/z* 188 over four intra-day runs. Norfentanyl was

identified by selected ion recording of MH$^+$ ions on a single quadrupole and ion trap mass spectrometers.

Deuterium-labeled internal standards of fentanyl and norfentanyl were used at the recommended detection

limit of 10 ng/mL (10), which was also the limit of quantification.

Table 3. Ion Suppression in Urine Samples from Six Cases (from reference 12).

Case #	Relative Ion Suppression
1	76.3 %
2	34.1 %
3	57.4 %
4	-26.4 %
5	87.9 %
6	49.8 %

Ion suppression was calculated as the percent difference between the mean D$_5$-fentanyl MS^2 product ion

signals from each case compared to that of the 10 ng/mL standard curve samples in water. Ion

suppression was calculated as a percent difference between the two values using $\frac{A-B}{A}*100$, where A

is the mean peak area of the 10 ng/mL internal standard in the water and B is the mean peak area of the 10

ng/mL internal standard in the case samples. Negative percentages are the result of signal enhancement,

which implies that control urine contained more contaminants than the case sample urine.

Table 4. Drugs detected in blood from five cases (from reference 13).

Drug	Conc. (µg/mL)	MH⁺ m/z	MS/MS m/z	Proposed MS/MS Neutral Loss	MS³ m/z
Case 7					
Methadone	0.1	310	265	Dimethylamine	247
Methadone-d$_3$	-	313	268	Dimethylamine	-
Dextromethorphan	0.61	272	215	N-methylaziridine	147
Quetiapine	10.3	384	253	C$_6$H$_{13}$O$_2$N [c]	222
Diazepam	0.04	285	257	CO	228
Diazepam-d$_5$	-	290	262	CO	-
Nordiazepam	0.14	271	243	CO	-
Promethazine	0.31	285	240	Dimethylamine	199
Promethazine-d$_3$	-	288	240	Dimethylamine-d$_3$	-
Case 8					
Fentanyl	0.019	337	188	Propionylanilide	105
Fentanyl-d$_5$	-	342	188	Propionylanilide-d$_5$	-
Zolpidem	0.1	308	264	Dimethylamine	249
Zolpidem-d$_6$	-	314	264	Dimethylamine-d$_6$	-
Acetaminophen	20.1	152	110	Ketene	-
Acetaminophen-d$_4$	-	156	114	Ketene	-
Amitriptyline	0.36	278	233	Dimethylamine	218
Nortriptyline	0.1	264	233	Methylamine	-
Nortriptyline-d$_3$	-	267	233	Methylamine-d$_3$	-
Venlafaxine	1.21	278	260	H$_2$O	215
Norvenlafaxine	3.87	264	246	H$_2$O	-
Cyclobenzaprine	0.12	276	231	Dimethylamine	216
Case 9					
Hydrocodone	0.06	300	199	n.d.	172
Hydrocodone-d$_3$	-	303	199	n.d.	-
Alprazolam	0.03	309	281	N$_2$	205
Alprazolam-d$_5$	-	314	286	N$_2$	-
Benzoylecgonine	0.56	290	168	Benzoic acid	150
Benzoylecgonine-d$_3$	-	293	171	Benzoic acid	-
EME [d]	0.04	200	182	H$_2$O	150
EME-d$_3$	-	203	185	H$_2$O	-
Citalopram	0.15	325	262	n.d.	109
Acetaminophen	5.36	152	110	Ketene	92
Acetaminophen-d$_4$	-	156	114	Ketene	-
Case 10					
Alprazolam	0.04	309	281	N$_2$	205
Alprazolam-d$_5$	-	314	286	N$_2$	-
Diphenhydramine	0.39	256	167	Dimethylaminoethanol	152
Diphenhydramine-d$_3$	-	259	167	Dimethylaminoethanol-d$_3$	-
Diazepam	0.09	285	257	CO	228

Diazepam-d$_5$	-	290	262	CO	-
Nordiazepam	0.02	271	243	CO	-
Promethazine	0.02	285	240	Dimethylamine	199
Promethazine-d$_3$	-	288	240	Dimethylamine-d$_3$	-
Carisoprodol	0.06	261	200	Carbamic acid	115
Meprobamate	2.46	219	158	Carbamic acid	97
Acetaminophen	34.5	152	110	Ketene	92
Acetaminophen-d$_4$	-	156	114	Ketene	-
Case 11					
Cocaine	0.1	304	182	Benzoic acid	150
Cocaine-d$_3$	-	307	185	Benzoic acid	-
Benzoylecgonine	0.71	290	168	Benzoic acid	-
Benzoylecgonine-d$_3$	-	293	171	Benzoic acid	-
EME [d]	0.08	200	182	H$_2$O	-
EME-d$_3$	-	203	185	H$_2$O	-
Methadone	0.49	310	265	Dimethylamine	247
Methadone-d$_3$	-	313	268	Dimethylamine	-
EDDP [d]	0.1	278	249	n.d.	-
EDDP-d$_3$	-	281	249	n.d.	-
Dextromethorphan	0.23	272	215	N-methylaziridine	147
Alprazolam	0.08	309	281	N$_2$	205
Alprazolam-d$_5$	-	314	286	N$_2$	-
Diazepam	0.18	285	257	CO	228
Diazepam-d$_5$	-	290	262	CO	-
Nordiazepam	0.21	271	243	CO	-
Fluoxetine	0.97	310	148	4-Trifluoromethylphenol	119
Fluoxetine-d$_6$	-	316	154	4-Trifluoromethylphenol	-
Norfluoxetine	0.83	296	253	n.d.	-
Ibuprofen [e]	3.91	205	161	CO$_2$	-

[a] Selected Reaction Monitoring (SRM) conditions for all MS/MS transitions ranged between 25-40% collision energies.

[b] Consecutive Reaction Monitoring (CRM) conditions for all MS^3 transitions ranged between 25-35% collision energies.
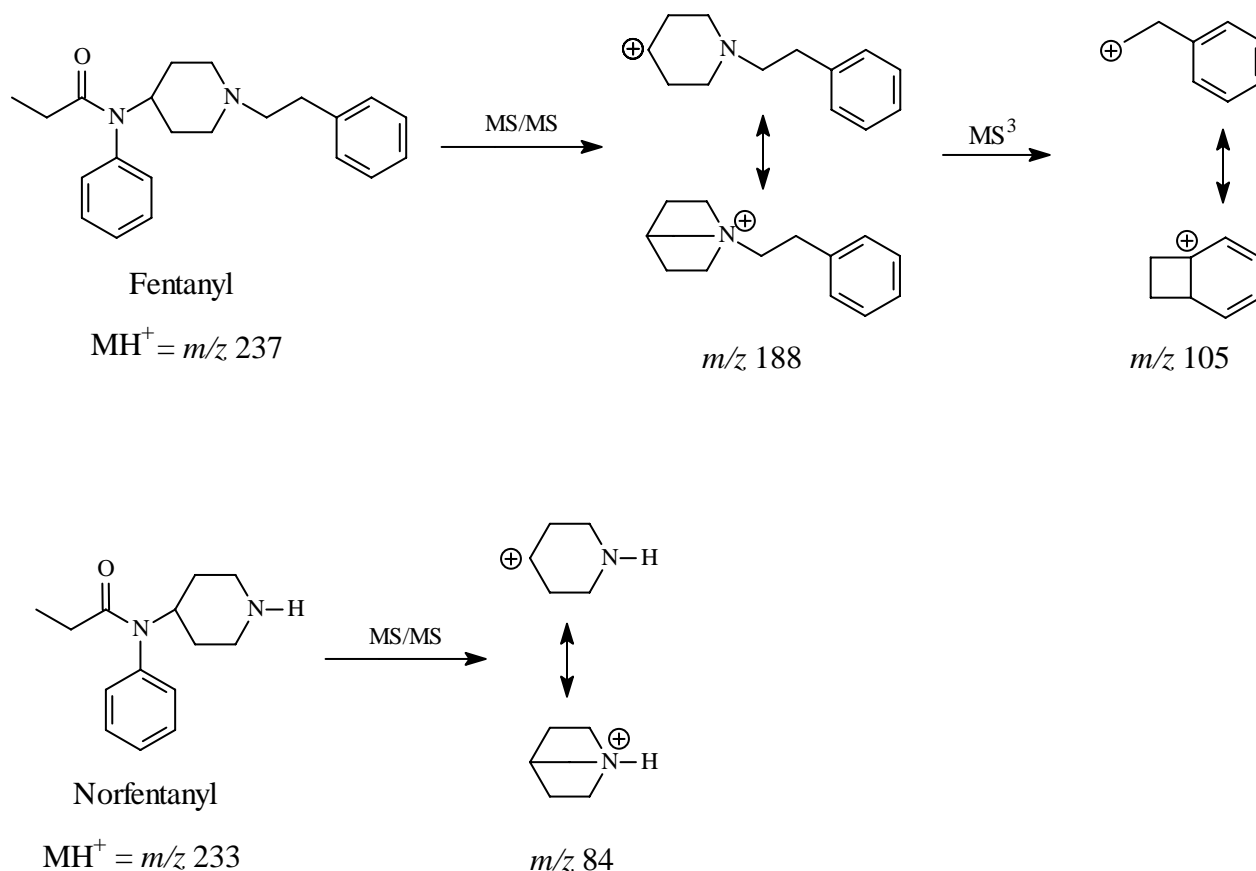
[c] *N*-[2-(3-hydroxypropyloxy)ethyl]aziridine

[d] Abbreviations: EME: ecgonine methyl ester; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

[e] Ibuprofen was analyzed in negative ion mode

n.d.: MS/MS neutral loss structure not proposed

Figure 1. Proposed Fragmentation Pattern of Fentanyl and Norfentanyl (from reference 12).

The fentanyl MS/MS transition m/z 337→188 is consistent with a neutral loss of N-phenylpropanamide.

The MS^3 transition m/z 188→105 forms the phenylethyl cation. MS/MS fragmentation of norfentanyl

yields a neutral loss of N-phenylpropanamide. The fragmentation pattern of the deuterium-labeled

compounds added support for the proposed products.

IV. Conclusions

1. **Discussion of findings (adapted from references 12 and 13):**

   Method for confirming the identity of fentanyl and norfentanyl in urine.

   The purpose of this study was to develop a simple and rapid mass spectrometric method for

confirming the identity of fentanyl and norfentanyl in forensic urine samples. Although not as sensitive

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for quantitative purposes as GC-MS and LC-MS, chromatography-free mass spectrometry quickly and accurately identified and quantified fentanyl and norfentanyl in urine. Total analysis time for the six case samples was less than one hour.

Electrospray ionization and related ionization methods are soft ionization techniques that produce intense protonated molecular ion species (MH^+) and little fragmentation. For pure samples, MH^+ ions provide useful molecular weight information. The intensity of the signal is proportional to the amount of compound present and through the use of appropriate internal standards, quantitative analytical methods can be developed based on monitoring the intensity of MH^+ ions. The analysis of low dose drugs, such as fentanyl in urine, simply monitoring MH^+ ion intensity may not be adequate for analyte identification. To reach acceptable selectivity, either extensive sample preparation is necessary, or further fragmentation of MH^+ ions (MS^n) is required to reliably confirm the identity of fentanyl. To address this selectivity issue, an identification method for fentanyl was developed based on detection of product ions formed from fragmentation of the fentanyl MH^+ ion. Fentanyl is rapidly metabolized and the major metabolite, norfentanyl, is also a urinary marker for fentanyl.

In this study, fentanyl concentrations in standards and case samples were measured based on the MS/MS product ions rather than the precursor ions for several reasons. First, in the MS stage, there are many co-eluting compounds in urine that are in the mass range of m/z 300-400 that can decrease the analyte and internal standard signal intensities (m/z 337 and 342, respectively). Ion suppression is more of a problem for the direct injection method than for traditional LC/MS methods. Better signal to noise ratios are obtained from MS/MS ions because product ions are more clearly visible and readily quantified in comparison to quantification using the measurement of MH^+ ions. This provides selective structural identification because the probability of co-eluting contaminants that weigh m/z 337 or 342 and also fragment to m/z 188 is extremely small. When m/z 188 appears in the spectrum window, it is confidently assessed to be the result of fragmentation of fentanyl or D_5-fentanyl, depending on which precursor ion is being fragmented, because the MS^2 isolation window is focused only on a single precursor ion with a width of 1.5 mass units.

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Three different mass spectrometers representing two manufacturers were used in this study to increase the general applicability of the method. Triple quadrupole mass spectrometers are capable of performing MS/MS, while ion traps are capable of performing multistage MS (MS^n). The ion trap MS was used to confirm the identity of fentanyl, norfentanyl, and their deuterated standards by the appearance of their MS/MS product ions. In the case of fentanyl, the MS^2 product ion is capable of being fragmented further, thus the MS^3 product (*m/z* 188→105) served as a qualifier to provide further structural confirmation. The triple quadrupole MS was used to confirm the identity and to quantify fentanyl and D_5-fentanyl based on a ratio of the intensities of product ions and deuterated isotopomer ions. A calibration curve was created over the range of 0-300 ng/mL fentanyl ($r^2>0.99$) based on the ratios of *m/z* 337→188:*m/z* 342→188 through standard samples prepared in control urine. The purpose of this curve was to verify the reliability of the ratios for providing a quantitative assessment of fentanyl concentration. Thus, for laboratories running many samples daily, a single calibration curve would be sufficient to ensure the ability to estimate fentanyl concentrations and the remaining samples for that day can be quantified based on the ratios to internal standards. The ability to apply the same method to two different mass spectrometers increased the applicability of this technique. The single quadrupole MS is capable of analyses of ions in only the MS stage, so to increase the applicability of the method to a variety of instruments, a single quadrupole was used to demonstrate the ability to detect norfentanyl, whose MH^+ ion is a better quantifier than its MS/MS ion.

In analyses of urine samples by ESI mass spectrometry, signal sensitivity was compromised by ion suppression, which is common in techniques that use ESI to transfer charged ions in solution to the gas phase for detection (14). High analyte concentration can also suppress the signal of the co-eluting internal standard in ESI-MS, however such signal suppression was shown to have no effect on the slopes of calibration curves and thus did not affect quantification (10). To estimate the extent of ion suppression occurring in this study, a percent suppression of each case sample internal standard signal was compared to that of the standard curve points from water. Although signals were relatively suppressed in all but one case sample, the ratios of fentanyl:internal standard did not change and thus ion suppression did not

hinder identification or quantification of analyte. This demonstrated that extensive sample preparation is not necessary for rapid identification of drugs of abuse in urine.

Conclusions. Chromatography-free mass spectrometry provides structural identification of fentanyl and norfentanyl in urine with an estimate of concentration by a method that is less time consuming than LC-MS/MS methodologies. The specificity of MS/MS transitions suggests that this method can be expanded to the analysis of a broad range of drugs of abuse in urine that show suitable mass spectra fragmentation patterns. This method is useful in detecting very small amounts of fentanyl in urine, arising from either therapeutic or illicit use.

The method for fentanyl was modified and extended to the confirmation of multiple drugs in whole blood. By using a direct injection multi-stage (MS/MS and MS^3) mass spectrometric method, the presence of all but one of the 40 drugs and metabolites that were previously identified and quantified in blood at $\geq$ 10 ng/mL by GC-MS or LC-MS analytical methods by the WV OCME was confirmed. Acetaminophen in case 8 was the only analyte not determined because the acetaminophen-d_4 MS/MS product ion was not detected. The MS^3 confirmation step was performed on 29 analytes, where 26 analytes were confirmed based on the presence of their major MS^3 product ions. This simple and rapid mass spectrometric method provided sufficient selectivity and sensitivity to confirm drugs and metabolites present in postmortem whole blood based on the presence of their MS/MS and/or MS^3 product ions.

There are disadvantages to simplifying the method by excluding separation steps. In the absence of the selectivity provided by chromatographic separations, the direct injection method has diminished capability for both unequivocal identification and highly accurate quantification. More accurate and precise measurements and more confident structure identification would require high resolution mass spectrometers that can provide exact mass information. Unit mass resolution mass spectrometers of the types usually found in forensic laboratories are not as selective as higher resolution mass spectrometers.

Application of the direct injection method is a confirmation level that requires prior knowledge of suspected drugs and the availability of a stable isotopically labeled standard. There is the potential that many drugs would be missed using this technique.

2. **Implications for policy and practice.**

There are implications of these results in terms of the practice of crime labs and medical examiners offices in the identification of substances of forensic interest in urine and blood. The method saves time and money when compared to current LC/MS methods for the same substances. The method involves the direct injection of samples into the mass spectrometer which takes a shorter time to complete than existing LC/MS methods and is simpler to accomplish because a liquid chromatography step is not a requirement. A weakness of the method is that it is less sensitive than existing methods because ion suppression diminishes analyte signal. Ion suppression also reduces the quantification efficiency of measurement of substances. Another weakness is the potential presence of contaminating substances that coincidentally have the same nominal mass and product ion as observed for the analyte. Chromatographic separation of contaminants reduces the risk of false positives, which gives LC-MS/MS methods an advantage over direct injection mass spectrometry.

3. **Implications for further research**.

Recommendations for further research include a more complete validation of the method including interlaboratory evaluation, determination of robustness of the method, and evaluation of potential changes in instrument maintenance schedules resulting from contamination of the mass spectrometer inlet from the direction injection technique. Another significant area of further research is to evaluate the application of higher resolution mass spectrometers in forensic science. High resolving power mass spectrometers are capable of reducing the influence of contaminating substances on analytical methods by focusing only on the exact mass of the analyte. Ideally, all forensic mass spectrometry should be done with exact mass determinations. To date, high resolution mass spectrometers are only available to a few forensic toxicologists.

V. References

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VI. Dissemination of Research Findings

Publications:

Peer, C.J., Callery, P.S., Kraner, J.C. and Shakleya, D.M. Identification of 8 Drugs in 15 Minutes from Postmortem Urine by Direct Infusion MS/MS. Case Note Published in May 2007 Issue of *ToxTalk,* a newsletter published by the Society of Forensic Toxicologists.

Peer, C.J., Shakleya, D.M., Younis, I.R., Kraner, J.C. and Callery, P.S. (2007) Direct-injection mass spectrometric method for the rapid identification of fentanyl and norfentanyl in postmortem urine of six drug-overdose cases. *J Anal Tox.* **31**, 515-522.

Peer, C.J., Clay, D.J., Glover, H.L., Renninger, K.L., Kraner, J.C., and Callery, P.S. (2008) Direct injection mass spectrometric confirmation of multiple drugs in overdose cases from postmortem blood using ESI-MS-MS and MS^3. *J Anal Tox*. **32**, 705-708.

Abstracts of presentations:

Cody J. Peer, Diaa M. Shakleya, Islam R. Younis, James C. Kraner, and Patrick S. Callery. Direct-Injection Mass Spectrometric Method for the Rapid Identification of Fentanyl and Norfentanyl in Postmortem Urine of Six Drug-Overdose Cases. Society of Forensic Toxicologists (SOFT) Annual Meeting, October 14-19, 2007, Chapel Hill, NC.

Cody J. Peer, Diaa M. Shakleya, Islam R. Younis, James C. Kraner, and Patrick S. Callery. Direct-Injection Mass Spectrometric Method for the Rapid Identification of Fentanyl and Norfentanyl in Postmortem Urine of Six Drug-Overdose Cases. 55th Annual Meeting of the American Society for Mass Spectrometry (ASMS), June 3-7, 2007, Indianapolis, IN.

Diaa M. Shakleya, James C. Kraner, Cody J. Peer, and Patrick S. Callery. Identification of Fentanyl in Urine From Drug Abuse Cases Using a Direct Multistage Mass Spectrometry Method. 39th Annual Mid-Atlantic Graduate Student Symposium in Medicinal Chemistry (MAGSS), Columbus, OH, June 2006.

Diaa M. Shakleya, James C. Kraner, and Patrick S. Callery, Identification of Fentanyl in Urine from Drug Abuse Cases Using a Direct Multistage Mass Spectrometry Method. American Academy of Forensic Sciences, 2006.

Acknowledgments