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Reproducibility assessment for a broad spectrum drug screening method from urine using liquid chromatography time-of-flight mass spectrometry

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ABSTRACT

During the reproducibility validation for a time-of-flight (TOF) high-resolution mass spectrometry (HRMS) method set up to detect 61 drugs of abuse commonly encountered in the toxicology laboratory, it was noticed that, a number of compounds were not identified correctly during the between run analysis; the most difficult compounds to identify were norpropoxyphene, morphine, norbuprenorphine, nortriptyline, EDDP and tramadol. In subsequent patient comparison studies, screening a panel of 338 analytes, the TOF-HRMS method correctly identified 211 analytes over two runs, but did not identify 127. A total of 11 false positive results were identified by manual review of the data to be the result of confirmation ion signal-to-noise ratio(s) < 3, although one false positive that was difficult to resolve (i.e., identification of maprotiline as amitriptyline) was due to similar fragment ions and retention times. The TOF-HRMS method showed reasonable agreement with LC–MS/MS results, but there were a number of discrepant results. Additionally, the TOF-HRMS did detect five compounds missed by the LC–MS/MS methods. This extensive validation effort highlights the difficulty of analysis for certain compounds that are likely to require additional follow up prior to reporting a positive result, especially at low and high concentrations, regardless of the type of instrumentation involved.

1. Introduction

Urine drug screening is among one of the most widely practiced procedures in the clinical toxicology laboratory. While immunoassays are typically used as an initial screen, confirmation is generally required to be made with liquid chromatography coupled to at least a unit resolution (i.e., low resolution) tandem mass spectrometer (LC–MS/MS) [1]. LC–MS/MS confirmation is made by comparing retention time (or relative retention time) and ion ratios between one or more pairs of precursor and product ion(s) detected in multiple reaction monitoring (MRM) mode [2]. A primary limitation of MRM-based analytical platforms is that they are restricted to a fixed panel of targeted analytes and are unable to perform non-targeted screening. Recently, high-resolution mass spectrometry (HRMS), including techniques such as time-of-flight mass spectrometry (TOF-MS), has been proposed as an alternative that would allow non-targeted drug screening [3,4]. Besides having the ability to detect a wide range of compounds, HRMS has a much greater specificity than immunoassays, and, therefore, does not require secondary confirmation.

In previous studies, we used a HRMS instrument to identify several novel psychoactive substances encountered in our emergency department [5,6], however, rigorous method validation is required to avoid false results [7,8]. To enable non-targeted screening our laboratory and others have utilized an “all-ions” approach for compound identification with fragment ions created in the collision cell [7,9–11]. From previous study we have determined that retention time, a precursor ion, and at least one fragment ion are necessary for positive analyte identification [7]. However, our initial study was limited in scope having only evaluated single spiked concentrations and patient comparisons; a more comprehensive analysis would have included run-to-run variability, which is an important consideration when evaluating acceptability criteria.

The resolving power of HRMS has made it possible to measure *m/z* to four decimal places, providing information that can aid in calculation of elemental composition of unknowns. However, the variability in exact mass measurements, especially when analyzing samples with a complex biological matrix, has not been widely reported. Understanding the variation in exact mass measurements that would be expected for an assay routinely used in a clinical setting is important since it is a critical parameter used for compound identification. Here,

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we rigorously test the reproducibility and reliability of a broad spectrum TOF-HRMS method by performing within run and between run precision studies at multiple drug concentrations for 61 different drugs, and demonstrate, by varying the exact mass tolerance of our HRMS, how the variability in exact mass measurements caused by the analysis matrix can affect analyte identification. Carryover and patient comparison studies were also conducted in order to characterize the false positive and false negative rates using specimens submitted from various sources.

2. Materials and methods

2.1. LC conditions

LC conditions were as previously described [7]. Briefly, ultra-performance liquid chromatography (UPLC; Waters) with a BEH C_{18} guard column (1.7 μm, 5 mm) and a BEH C_{18} analytical column (1.7 μm, 2.1 × 150 mm) was used for separation. Flow rate was 0.4 mL/min at a column temperature of 50 °C. Two mobile phases were used: mobile phase A (5 mmol/L ammonium formate, pH 3) and B (0.1% formic acid in acetonitrile). A gradient was used for elution starting with 5% B for 0.5 min, increased linearly up to 50% B at 6 min, and then to 95% B at 7 min where it was held for 2 min, followed by re-conditioning of the column at 5% B at 9.05 min and held for 2 min. Total injection-to-injection time for one run was 14 min.

2.2. TOF MS conditions

TOF MS conditions were as previously described [7]. Briefly, we used the Xevo G2 TOF from Waters with a resolution of 20,000 (full width at half maximum at *m/z* 400); capillary voltage, 0.8 kV; cone voltage, 20 V; extraction cone, 4 V; source block temperature, 130 °C; desolvation temperature, 550 °C; gas flow, 25 L/h; desolvation gas flow 850 L/h. Instrument calibration was performed using 5 mmol/L sodium formate in 90:10 2-propanol:water at weekly intervals (or more frequently if needed). Data was acquired in profile mode through MassLynx software v4.1, SCN 869 (Waters) without real-time mass correction. The MS method consisted of 3 functions: 1) acquisition of data over the 50–650 *m/z* range with 6 eV collision energy (low energy); 2) acquisition of data over a 50–650 *m/z* range with a collision energy ramp of 10–50 eV (high energy); 3) acquisition of lockmass data over 50–650 *m/z* range. To process data we used UNIFI v1.7.1 (Waters), which involved mass correction by using leucine-enkephalin with each sample. The UNIFI database for the 61 analytes was built from the Waters Toxicology Library and included information about molecular formula, fragment ions and retention time for each analyte.

2.3. Method comparison studies

We used two different sample sources during validation: 1) compounds were divided into six groups as previously described (10 drugs/group with the last group having 11 drugs) [7] and spiked into drug free urine (UTAK laboratories, CA) at three different concentrations of 100 ng/mL, 1000 ng/mL and 5000 ng/mL; 2) patient samples that were confirmed positive using our in-house LC-MS/MS method and additional patient samples that were sent to us from the University of California, San Francisco (UCSF).

Our in-house confirmation included MRM-based LC–MS/MS (Waters UPLC-Xevo TQ-S) methods that are routinely used to confirm immunoassay urine drug screens. Confirmation methods from UCSF included 5600 ABSciex QTOF, 3200 LC–MS/MS, Thermo Exactive Orbitrap and patient prescription records [12]. Lastly, a few samples that we were not able to confirm in-house or through UCSF were sent out to NMS (Willow Grove, PA) or ARUP labs (Salt Lake City, Utah) for targeted LC-MS/MS analysis.

2.4. Sample preparation

Sample preparation was as previously described [7]. Briefly, 200 μL of urine were used for each sample. To this was added 400 μL of deionized water, 100 μL of internal standard solution (1000 ng/mL mix of amphetamine-D5, codeine-D3, diazepam-D5, oxazepam-D5 and venlafaxine-D6 in methanol), and 300 μL of β-glucuronidase solution (5000 U/mL from Helix pomatia; Sigma-Aldrich, CA) prepared in 1.0 mol/L sodium acetate buffer (pH 5). The mixture was incubated at 50 °C for 90 min and centrifuged at 2010*g* for 10 min. 20 μL of the supernatant were injected for analysis.

For between run studies, samples were prepared fresh each day from a stock solution that was stored at −20 °C for up to two months. For patient comparison studies, in-house samples were run within two months of storage.

2.5. Validation protocol

2.5.1. Spiking studies

Within and between run validation studies consisted of five injections of each sample within the same day and 20 injections of each sample over 20 days, respectively. Three different concentrations of drugs were spiked into drug free urine: 100 ng/mL, 1000 ng/mL and 5000 ng/mL. The 61 compounds were divided into six groups with the first five groups containing 10 drugs per group and the last group containing 11 drugs.

2.5.2. Carry-over studies

Carry-over studies were performed by spiking the 61 compounds (divided into six groups, as described above) at 30,000 ng/mL and analyzing them in the following order: Blank2 → Blank1 → High1 → High2 → Blank1 → Blank 2 → Blank3 → Blank 1. Where "Blank" refers to drug free urine with no drugs spiked, and "High" refers to drug free urine spiked with 30,000 ng/mL of drug. Each "Blank" refers to a different preparation of drug free urine.

2.5.3. Patient comparison studies and proficiency testing samples

Using an IRB approved protocol (UCSD HRPP protocol number 90188), a total of 112 patient samples were collected from existing clinical specimens. Patient samples were run twice. Run number one analyzed specimens in the forward direction (e.g., sample #1 → 20) while run number two (same sample preparation procedure) analyzed specimens in the reverse direction (e.g., sample #20 → 1). Both runs (i.e., run #1 and run #2) were performed on the same day for the selected batch of samples; with run #2 immediately following run #1. Proficiency testing samples were from previous challenges (i.e., year 2013–2015).

2.5.4. Sample set-up & identification criteria

For each sample batch, the following set-up was used: (1) Wash, (2) System Suitability Test (SST), (3) Negative QC, (4) Positive QC, (5) Samples, (6) Negative QC.

Wash: 10% methanol in LC–MS grade water. SST: all five internal standards described above were spiked at 1000 ng/mL in 10% methanol in LC–MS grade water. Negative QC: drug free urine (UTAK). Positive QC: UTAK custom made in drug free urine (codeine 300 ng/mL, doxepin 300 ng/mL, norhydrocodone 300 ng/mL, ketamine 300 ng/mL, meprobamate 300 ng/mL, methylphenidate 300 ng/mL, morphine-3-β-D-glucuronide 486 ng/mL, oxazepam glucuronide 486 ng/mL, phencyclidine 300 ng/mL, norpropoxyphene 300 ng/mL).

The criteria for a positive identification were as follows: retention time match within 0.2 min, accurate mass of precursor ion within 5 ppm, at least one fragment with 10 ppm and detector counts ≥200. Any compounds identified as false positive also met all of the above criteria. UNIFI v1.7.1 was used for data processing. UNIFI involved mass correction by use of leucine encephalin and used the "all in the RT

Table 1
Within run validation.

Group 1	Amphetamine, d5	Codeine	Codeine, d3	Diazepam, d5	Doxepine	Ketamine	Meprobamate	Methylphenidate	Morphine	Norhydrocodone	Norpropoxyphene	Oxazepam	Oxazepam, d5	Phencyclidine (PCP)	Venlafaxine, d6
100 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	4	5	0	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**4**	**5**	**0**	**5**	**5**	**5**	**5**
3 ppm	4	5	5	5	5	5	5	5	4	4	0	5	5	5	5
1 ppm	0	2	3	5	4	1	3	4	2	0	0	5	3	3	5
1000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**4**	**5**	**5**	**5**	**5**
3 ppm	5	5	5	5	5	5	5	5	5	5	2	5	5	5	5
1 ppm	0	5	4	3	4	4	1	5	4	5	2	3	3	2	3
5000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	4	5	5	5	5	5	5	4	5	5	5	5	5	5	5
1 ppm	0	4	3	3	1	3	3	3	3	4	4	4	3	4	4

Group 2	Amphetamine, d5	Codeine, d3	Cyclobenzaprine	Diazepam, d5	Diltiazem	Fentanyl	Fentanyl, Nor	Hydromorphone	Methamphetamine	Methylone	Norbuprenorphine	Norpropoxyphene	Oxazepam, d5	Propoxyphene	Venlafaxine, d6
100 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	4	5	5	5	5	5	5	5	4	5	5	5	5	4	5
1 ppm	1	4	3	5	3	3	4	3	2	4	3	2	3	4	5
1000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	0	2	4	2	4	5	5	4	1	2	5	5	4	5	4
5000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	4	5	5	5	4	5	5	5	5	5	5	5	5	5	5
1 ppm	1	2	1	3	2	3	3	4	2	5	4	5	4	4	4

Group 3	Amphetamine, d5	Codeine, d3	Dextromethorphan	Diazepam, d5	MDMA	Methadone	Oxazepam, d5	Oxycodone	Oxycodone, Nor	Promethazine	Tapentadol	Venlafaxine, d6	Zaleplon	Zopiclone
100 ng/mL														
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	4	5	5	5	5	5	5	5	4	5	5	5	5	5
1 ppm	1	3	3	3	2	2	2	4	2	2	5	3	4	2
1000 ng/mL														
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	4	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	0	3	3	4	1	2	4	3	3	4	3	4	5	1
5000 ng/mL														
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	1	3	2	2	5	4	3	3	2	5	4	1	4	5

Group 4	Amitriptyline	Amphetamine, d5	Benzoylecgonine	Clonazepam, 7-amino	Codeine, d3	Diazepam, d5	Flunitrazepam	Lorazepam	MDPV	Morphine, 6 Monoacetyl	Nortriptyline	Oxazepam, d5	Oxymorphone	Quetiapine	Venlafaxine, d6
100 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	1	5	5	5	5	5	5	5	5	5	5	4	5	5
1 ppm	3	0	2	2	2	3	3	4	2	1	3	2	3	4	3
1000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	2	5	5	5	5	5	5	5	5	5	5	4	5	5
1 ppm	5	0	2	4	2	3	4	4	5	4	5	5	3	3	3
5000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	0	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	3	0	4	5	4	4	5	1	3	3	3	2	3	3	2

Group 5	Amphetamine	Amphetamine, d5	Atenolol	Citalopram/ Escitalopram	Codeine, d3	Diazepam, d5	EDDP	Meperidine	Mephedrone	Metoprolol	Nordiazepam	Oxazepam, d5	Trazodone	Venlafaxine, d6	Verapamil
100 ng/mL															
10 ppm	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**4**	**5**	**5**	**5**	**5**	**5**	**1**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	3	5	5	5	5	5	0	5	5	5	5	5	5	5	5
1 ppm	1	1	3	5	3	4	0	4	3	3	4	4	4	3	4
1000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	4	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	1	1	5	4	3	4	5	2	5	3	5	5	4	4	3
5000 ng/mL															
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	2	0	4	4	3	4	4	4	3	5	2	3	4	4	3

Group 6	Alprazolam, hydroxy	Amphetamine, d5	Buprenorphine	Carisoprodol	Codeine, d3	Diazepam	Diazepam, d5	Hydrocodone	MDA	MDEA	Oxazepam, d5	Propranolol	Temazepam	Venlafaxine, d6	Zolpidem	Tramadol
100 ng/mL																
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	0
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**0**
3 ppm	5	4	5	5	5	5	5	4	3	5	5	5	5	5	5	0
1 ppm	4	2	3	4	3	4	4	3	0	4	4	4	2	4	4	0
1000 ng/mL																
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	4	3	3	5	2	4	4	5	1	3	4	3	4	0	4	5
5000 ng/mL																
10 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5 ppm	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
3 ppm	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1 ppm	4	4	4	5	4	4	4	3	5	0	4	1	4	2	1	5

Samples were injected for a total of 5 times at three concentrations (100 ng/mL, 1000 ng/mL and 5000 ng/mL). A result of 5 indicates that the drug was identified 100% of the times (5/5). Bold results indicate the identification criteria that were used when analyzing patient samples (tr = ± 0.2 min, 5 ppm error for precursor and at least one fragment).

window" setting to target peaks by retention time.

Criteria for batch validation were as follows: SST – 5/5 (100%) compounds identified; Negative QC – no compounds identified; Positive QC – 7 or more compounds identified.

3. Results

3.1. Spiking studies

3.1.1. Within run

To assess method reproducibility we performed five injections for each of the samples from the six groups (i.e., 61 total compounds) on the same day and at three different concentrations (i.e., 100 ng/mL, 1000 ng/mL and 5000 ng/mL). When using a 5 ppm mass error

Table 2
Between run validation.

Group 1	Amphetamine, d5	Codeine	Codeine, d3	Diazepam, d5	Doxepine	Ketamine	Meprobamate	Methylphenidate	Morphine	Norhydrocodone	Norpropoxyphene	Oxazepam	Oxazepam, d5	Phencyclidine (PCP)	Venlafaxine, d6
100 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	17	20	0	20	20	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**17**	**20**	**0**	**20**	**20**	**20**	**20**
3 ppm	19	20	20	20	20	20	12	19	16	20	0	19	20	20	20
1 ppm	8	12	12	15	13	9	2	11	11	11	0	14	10	13	12
1000 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	19	20	17	20	20	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**14**	**20**	**20**	**20**	**20**
3 ppm	19	20	20	19	19	19	20	19	19	20	12	20	20	19	18
1 ppm	8	12	14	9	8	13	12	9	15	12	7	12	11	8	10
5000 ng/mL															
10 ppm	20	19	19	20	20	20	20	20	19	20	18	20	20	20	20
5 ppm	**20**	**19**	**19**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**17**	**20**	**20**	**20**	**20**
3 ppm	18	19	19	20	17	17	20	17	19	19	17	19	20	18	20
1 ppm	8	6	12	12	6	8	8	9	16	9	7	12	13	9	14

Group 2	Amphetamine, d5	Codeine, d3	Cyclobenzaprine	Diazepam, d5	Diltiazem	Fentanyl	Fentanyl, Nor	Hydromorphone	Methamphetamine	Methylone	Norbuprenorphine	Normeperidine	Oxazepam, d5	Propoxyphene	Venlafaxine, d6
100 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	20	15	20	20	18	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**15**	**20**	**20**	**18**	**20**
3 ppm	20	20	20	20	19	20	19	20	20	20	15	20	20	18	19
1 ppm	10	9	13	9	12	10	11	13	13	11	10	10	14	12	11
1000 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	20	19	20	20	20	20
5 ppm	**19**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**20**	**20**	**20**
3 ppm	18	20	20	20	20	20	20	20	18	20	19	20	20	20	18
1 ppm	11	14	11	14	11	12	9	8	11	11	14	12	14	17	11
5000 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**18**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	19	20	17	20	16	16	18	20	19	20	20	18	20	20	17
1 ppm	11	13	7	11	6	6	7	9	12	7	13	6	13	15	8

Group 3	Amphetamine, d5	Codeine, d3	Dextromethorphan	Diazepam, d5	MDMA	Methadone	Oxazepam, d5	Oxycodone	Oxycodone, Nor	Promethazine	Tapentadol	Venlafaxine	Venlafaxine, d6	Zaleplon	Zopiclone
100 ng/mL															
10 ppm	20	20	20	20	20	19	20	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	20	20	20	20	20	19	20	20	20	20	20	20	20	20	20
1 ppm	15	11	10	13	12	13	10	14	15	7	17	15	12	10	13
1000 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	19	20	17	19	19	17	20	19	20	19	18	20	20	18	20
1 ppm	8	6	6	11	12	7	11	12	9	13	10	10	12	13	11
5000 ng/mL															
10 ppm	20	20	20	20	20	18	20	19	19	20	20	20	20	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**18**	**20**	**19**	**19**	**20**	**20**	**20**	**19**	**20**	**20**
3 ppm	20	20	19	19	18	17	20	19	19	19	17	13	19	20	20
1 ppm	8	11	4	12	10	7	15	11	9	11	4	2	11	12	13

Group 4	Amitriptyline	Amphetamine, d5	Benzoylecgonine	Clonazepam, 7-amino	Codeine, d3	Diazepam, d5	Flunitrazepam	Lorazepam	MDPV	Monoacetyl Morphine, 6	Nortriptyline	Oxazepam, d5	Oxymorphone	Quetiapine	Venlafaxine, d6
100 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	19	20	20	19	19	18
5 ppm	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**20**	**19**	**19**	**18**
3 ppm	20	19	20	20	20	20	19	19	20	18	20	20	18	19	18
1 ppm	12	11	12	15	9	11	15	12	13	3	11	11	12	12	12
1000 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	19	20	19	19	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**19**	**19**	**20**	**20**
3 ppm	19	20	20	20	20	19	20	20	19	19	20	19	19	20	20
1 ppm	12	8	11	17	11	13	11	15	9	11	10	16	14	16	10
5000 ng/mL															
10 ppm	20	20	20	20	20	20	20	20	20	19	16	20	19	20	20
5 ppm	**18**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**19**	**16**	**20**	**19**	**20**	**20**
3 ppm	17	19	19	20	20	20	20	20	18	19	13	20	18	18	20
1 ppm	7	11	6	14	11	12	9	15	8	11	6	11	12	8	11

Group 5	Amphetamine	Amphetamine, d5	Atenolol	Citalopram/ Escitalopram	Codeine, d3	Diazepam, d5	EDDP	Meperidine	Mephedrone	Metoprolol	Nordiazepam	Oxazepam, d5	Trazodone	Venlafaxine, d6	Verapamil
100 ng/mL															
10 ppm	20	20	19	19	20	20	11	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**19**	**19**	**20**	**20**	**6**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	20	20	19	18	20	20	5	20	20	20	20	20	20	20	20
1 ppm	12	15	11	7	12	12	2	10	12	9	8	11	13	10	8
1000 ng/mL															
10 ppm	20	20	19	19	20	20	20	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**19**	**19**	**20**	**20**	**18**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	20	20	19	18	20	19	13	18	18	19	20	20	20	18	20
1 ppm	10	10	10	6	14	14	6	11	14	13	12	11	11	11	8
5000 ng/mL															
10 ppm	20	20	19	20	20	20	20	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**19**	**20**	**20**	**20**	**19**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	20	20	17	19	20	20	18	17	16	13	20	20	20	20	19
1 ppm	9	11	11	3	12	11	11	4	6	5	9	16	8	6	7

Group 6	Alprazolam, hydroxy	Amphetamine, d5	Buprenorphine	Carisoprodol	Codeine, d3	Diazepam	Diazepam, d5	Hydrocodone	MDA	MDEA	Oxazepam, d5	Propranolol	Temazepam	Tramadol	Venlafaxine, d6	Zolpidem
100 ng/mL																
10 ppm	20	20	20	20	20	19	20	20	18	20	20	20	20	1	20	20
5 ppm	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**20**	**18**	**20**	**20**	**20**	**20**	**1**	**20**	**20**
3 ppm	19	20	20	19	20	18	20	20	18	20	20	20	20	1	20	17
1 ppm	12	9	11	10	15	9	10	13	7	12	12	13	12	1	8	8
1000 ng/mL																
10 ppm	20	20	20	19	20	20	20	20	20	20	20	20	20	20	20	20
5 ppm	**20**	**20**	**20**	**19**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
3 ppm	20	19	20	19	20	20	20	20	19	20	19	20	19	20	19	19
1 ppm	9	11	10	9	16	9	13	15	15	13	12	8	11	10	14	10
5000 ng/mL																
10 ppm	20	20	20	18	20	20	20	20	20	19	20	20	20	20	20	20
5 ppm	**20**	**20**	**20**	**18**	**20**	**20**	**20**	**20**	**20**	**19**	**20**	**20**	**20**	**18**	**20**	**19**
3 ppm	20	18	20	18	20	18	19	19	20	14	20	16	20	13	20	16
1 ppm	15	8	12	12	12	9	12	14	12	3	18	6	12	3	10	6

Samples were injected for a total of 20 times at three concentrations over twenty days. A result of 20 indicates that the drug was identified 100% of the times (20/20). Bold results indicate the identification criteria that were used when analyzing patient samples (tr = ± 0.2 min, 5 ppm error for precursor and at least one fragment).

tolerance, the only compounds that did not achieve a 100% identification rate were: norpropoxyphene (missed 5/5 times at 100 ng/mL and missed 1/5 times at 1000 ng/mL), morphine (missed 1/5 at 100 ng/mL), amphetamine-D5 (missed 1/5 at 1000 ng/mL), amphetamine (missed 1/5 times at 100 ng/mL), EDDP (missed 4/5 times at 100 ng/mL) and tramadol (missed 5/5 times at 100 ng/mL), Table 1. To understand the impact of precursor mass error tolerance on these results we tightened the mass error window to 1 and 3 ppm, or widened it to 10 ppm. Widening the mass error window to 10 ppm increased the number of positive identifications (Table 1). Alternately, reducing the mass tolerance error to 1 and 3 ppm increased the false negative rate (Table 1).

3.1.2. Between run

As an additional measure of variability and reliability, the samples analyzed above, encompassing 61 compounds at three concentration levels, were analyzed once per day for twenty days to evaluate the between run variability with TOF-HRMS. Using a 5 ppm mass error tolerance, as for the within run study, norpropoxyphene, EDDP and tramadol were frequently not detected, especially at the lowest concentration of 100 ng/mL (Table 2). When compared to the within run study results, there were a larger number of analytes that were not identified at 100% for the between run study (Table 2). We focused on analytes that were missed more than 2/20 times in any of the three spiked concentrations; the following compounds stood out:

norpropoxyphene, morphine, norbuprenorphine, nortriptyline, EDDP, and tramadol. The identification rate greatly improved when the concentration of these analytes was increased from 100 ng/mL to 5000 ng/mL (Table 2), as seen for norpropoxyphene, EDDP and tramadol. However, in the case of nortriptyline, the identification rate was reduced at the higher concentration (from 20/20 at the concentration of 100 ng/mL and 1000 ng/mL to 16/20 at 5000 ng/mL). As for the within run validation, we also reduced the mass error tolerance below 5 ppm here and noted a significant increase in the number of false negative results, while increasing the mass error tolerance increased the number of positive identifications.

3.1.3. False positive results during within and between run validation

We did not observe any false positives during the within run validation. However, the following false positives were observed during the between run validation studies: MDA (n = 39), Norpropoxyphene (n = 3), Hydromorphone (n = 3), EDDP (n = 2), Oxycodone (n = 2) and MDMA (n = 1) (data not shown).

3.1.4. Carry-over studies

Samples were spiked at 30,000 ng/mL and run as described in the Methods section. We did not observe any carry-over to the blank sample, which was run immediately following the two samples containing the high concentration of analytes. However, analytes such as: EDDP, MDA, Morphine, MDMA and Meprobamate were observed as false positives during the second injection of the sample at high concentration (i.e., 30,000 ng/mL). None of these compounds were spiked into the high concentration samples.

3.2. Patient comparison studies

We analyzed 112 patient specimens for the patient comparison study. All samples were analyzed by a confirmatory method as described in the Methods section, and in addition they were run by the TOF-HRMS broad spectrum screen method twice. A total of 338 analytes were detected between the TOF-HRMS and the confirmatory methods. Of these, 211 analytes were detected by both the confirmatory methods and the TOF-HRMS method during both runs (Supplemental Table 1), leaving 127 analytes that were discrepant between the confirmation method and either TOF-HRMS run #1 or run #2 (Supplemental Table 2). Fig. 1 shows the breakdown of the discrepant findings. Of the 127 discrepant results, a total of 81 findings were missed by the TOF run #1 and a total of 85 findings were missed by the TOF run #2 with a total of 55 findings missed by both runs. A total of 56 results were identified between both runs of TOF-HRMS with 29 findings in run #1 and 27 findings in run #2. Ten results were false positive by TOF run #1 versus two results for the TOF-HRMS run #2 with a grand total of 11 false positives between the two runs due to one drug (methamphetamine) being detected in run #2 (Fig. 1), but not in run #1 (Supplemental Table 2). Five results were identified to be true positives by TOF-HRMS run #1 versus two for TOF run #2. An identification was determined to be “true positive” by TOF-HRMS if it met the following conditions: the concentration of the drug was just below the threshold of quantification by LC–MS/MS, the drug in question had the correct metabolite pattern, the drug was consistent with the patient's prescription, the drug had the correct ion ratios, retention time and good signal-to-noise (S/N ≥ 10). For a list of specific findings refer to Supplemental Table 2.

3.3. Proficiency testing results

A total of 89 CAP proficiency testing samples were run. UC San Diego-TOF method missed only one compound (i.e., Tramdol) and identified two false positives (i.e., amitriptyline and EDDP). A list of the specific compounds can be found on Supplemental Table 3.

4. Discussion

Several reports have illustrated the usefulness of HRMS in the investigation of clinical and forensic toxicology cases [13–15]. Studies have found good agreement between HRMS instruments and routine LC–MS/MS, however, a limitation of these studies has been that samples were only analyzed once [7,12]. In HRMS, mass accuracy is the main criterion used to identify analytes, because of this we evaluated the mass error tolerance. In addition, we assessed the reliability and the reproducibility of our TOF-HRMS method by performing multiple injections for a within and between run validation study, as well as patient comparison study.

Within run analysis, where most compounds were identified at a rate of 100% at three different concentrations (Table 1), provided a better rate of identification for our analyte panel than between run, where several compounds were not identified (Table 2). Two examples where detection was made within run, but missed between run were norbuprenorphine and MDA. Within run, at a concentration of 100 ng/mL, norbuprenorphine and MDA were both identified 100% of the time (Table 1), but between run they were identified 75% and 90% of the time, respectively (Table 2). It was also observed, that the rate of compound identification increased with increasing analyte concentration (Tables 1 and 2) for both within and between run analyses. This was illustrated dramatically for norpropoxyphene and tramadol; norpropoxyphene was identified 0/20 times at a concentration of 100 ng/mL, and 17/20 times at 5000 ng/mL (Table 2); tramadol was identified 1/20 times at 100 ng/mL and 20/20 at 1000 ng/mL and 5000 ng/mL (Table 2).

The primary reason for failed identification was insufficient fragments; retention time shifts were not found to be a factor. Occasionally, a mass error above 5 ppm would cause a compound to go undetected. For example, norpropoxyphene, in Group 1 of Table 2, at 1000 ng/mL it was identified 14/20 times using the 5 ppm mass error cut-off, however, increasing the mass error to 10 ppm increased the identification to 17/20 times. As expected, reducing the mass error tolerance to 3 or 1 ppm reduced the identification frequency (Tables 1 and 2). For most compounds, the standard deviation of the mass error varied between 1 and 3 ppm (data not shown), explaining the lower rate of identification with a mass error tolerance of 3 or 1 ppm and the higher rate of identification with a mass error tolerance of 10 ppm.

False positive results were observed during the between run validation study, the most prominent culprit being MDA. In general, false positives had the following criteria in common: low peak intensity (i.e., usually less than 500 detector counts instead of tens of thousands), and reduced fragment presence in combination with signal-to-noise ratios < 3. However, the occurrence of false positives could be eliminated with analysis software that allowed specification for a minimum signal-to-noise of the fragment ion(s), or by manual review of the data.

An unusual finding was that nortriptyline was only identified 16/20 times at a concentration of 5000 ng/mL. At this concentration a peak was clearly present with a signal-to-noise greater than 100:1, but the software integrated the incorrect peak. We suspect that this occurred because the high concentration distorted the mass accuracy.

We did not observe any carry-over effects when spiking compounds up to 30,000 ng/mL. However, on the second injection of the high concentration of the sample, we did see some false positive results for analytes that were not part of the target group. These false positives had the same characteristics as discussed above (i.e., low intensity, with fragment ion S/N < 3) and could be confirmed as false positives on manual evaluation, except in the case of morphine. The suspected “false positive” morphine had good signal-to-noise (i.e., > 20) along with characteristic fragments. It was subsequently shown that morphine was a degradation product of 6-monoacetylmorphine, a compound present in our test specimens, so, finally, morphine was not classified as a false positive since it was, in fact, present in the sample. The false positive amitriptyline in the CAP sample was due to the presence of maprotiline,

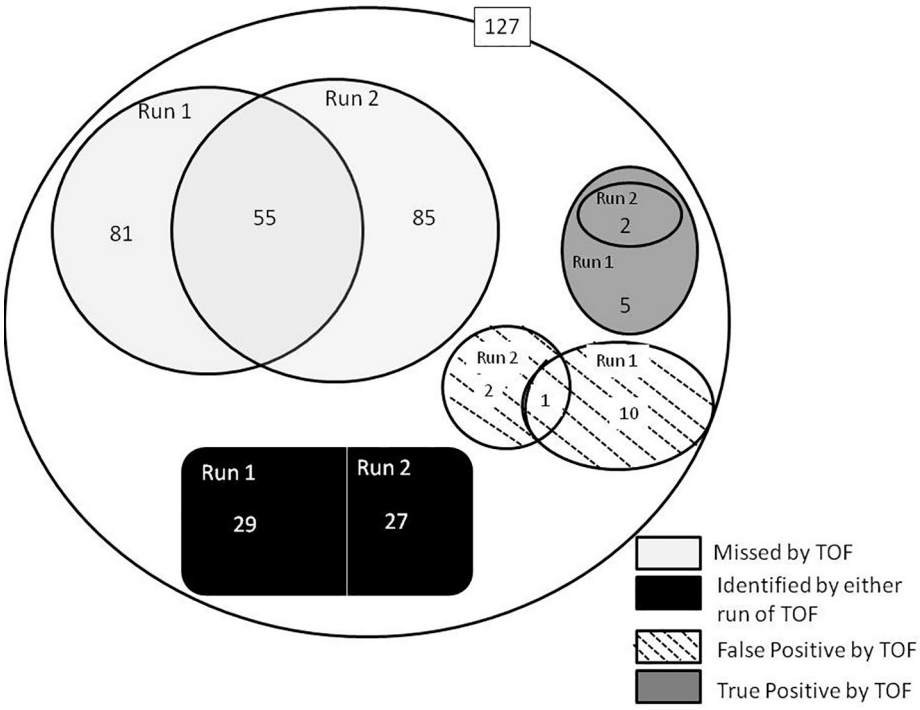

Fig. 1. Schematic representation of the discrepant findings between the TOF-HRMS and the confirmatory LC–MS/MS method (including samples from UCSD and UCSF). A total of 127 discrepant findings were identified. Of these 81 were false negative by TOF run #1 and 85 were false negative by TOF run #2. Ten findings were false positive by TOF run #1 and 2 findings were false positive by TOF run #2. Of the 127 total findings, 29 were identified only by TOF-HRMS run #1 (also identified by confirmatory method) and 27 were identified only by TOF-HRMS run #2 (also identified by confirmatory method). Five findings were identified as true positive by TOF-HRMS run #1 (missed by confirmatory method) and two findings were identified as true positive by TOF-HRMS run #2 (missed by confirmatory method).

which had the same retention time and same exact mass for the precursor ion. The false positive EDDP in the CAP samples had a fragment that was also identified by the LC-MS/MS, but confirmation failed due to incorrect ion ratios.

For our patient comparison studies TOF-HRMS correctly identified 211 analytes (over both runs) against LC–MS/MS. A total of 127 discrepant results were observed between the TOF-HRMS and the LC–MS/MS (Fig. 1 & Supplemental Table 2). A total of 81 false negative results were observed by TOF-HRMS during run #1 and a total of 85 false negative results were observed during run #2. The main reasons that a compound was missed during the patient comparison studies were as follows (listed by frequency of occurrence): 45%, missing fragment ion; 19%, the mass accuracy was above 5 ppm; 12%, there was a missing fragment and in addition the detector counts were below 200; 9%, the compound was not detected by UNIFI even after removing all filters; 8%, the UNIFI software chose the wrong peak to integrate for analysis and the remaining 7%, there was a missing fragment and a mass error above 5 ppm. It is difficult to determine exactly why there were so many false negative results, but it is likely due to the fact that the LC–MS/MS methods (limit of quantification 5–100 ng/mL) were more sensitive that the TOF-HRMS assay (limit of detection of 100–500 ng/mL) we were evaluating. With a more sensitive TOF-HRMS instrument, and cleaner sample preparation, it is likely that there would be fewer false negative results.

In addition, TOF-HRMS had 10 false positive results in run #1 and two false positive results in run #2. Overall, 29 analytes were identified by TOF-HRMS in run #1 and another 27 different analytes were identified in run #2. However, there were a number of true positive results identified by the TOF-HRMS that were not reported as positive by the LC-MS/MS confirmatory method (i.e., five results in run #1 and two results in run #2). Although LC-MS/MS identified the drug peak, the concentrations were below the cut-off for reporting a positive.

An additional advantage of the TOF-HRMS, when used for drug screening, is the ease by which new drugs can be added and validated, as compared to immunoassay analyzers where the lab is required to rely on FDA clearance for each kitted analyte or group of analytes. Furthermore, TOF-HRMS offers improved sensitivity and selectivity compared to traditional immunoassays. However, TOF-HRMS does have limitations that were highlighted during this validation – specifically there were some key analytes that were difficult to identify. As shown previously [7], and in this investigation, in order to reduce false negative results mass error tolerance must be increased to greater than 5 ppm. This leads to implementation of additional measures needed to reduce false positives.

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Conflict of Interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.clinms.2018.02.002.

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