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Liquid Chromatography High-Resolution TOF Analysis: Investigation of MS^E for Broad-Spectrum Drug Screening

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BACKGROUND: High-resolution mass spectrometry (HRMS) has the potential to supplement other drug screening platforms used in toxicology laboratories. HRMS offers high analytical specificity, which can be further enhanced by incorporating a fragment ion for each analyte. The ability to obtain precursor ions and fragment ions using elevated collision energies (MS^E) can help improve the specificity of HRMS methods.

METHODS: We developed a broad-spectrum screening method on an ultraperformance liquid chromatography TOF mass spectrometer (UPLC-TOF-MS) using the MS^E mode. A diverse set of patient samples were subjected to a simple dilute, hydrolyze, and shoot protocol and analyzed in a blind manner. Data were processed with 3 sets of criteria with increasing stringency, and the results were compared with the reference laboratory results.

RESULTS: A combination of retention time match ($\pm$0.2 min), a protonated analyte, and fragment ion mass accuracy of $\pm$5 ppm produced zero false-positive results. Using these criteria, we confirmed 92% (253/275) of true positives. The positive confirmation rate increased to 98% (270/275) when the requirement for a fragment ion was dropped, but also produced 53 false positives. A total of 136 additional positive drug findings not identified by the reference methods were identified with the UPLC-TOF-MS.

CONCLUSIONS: MS^E provides a unique way to incorporate fragment ion information without the need of precursor ion selection. A primary limitation of requiring a fragment ion for positive identification was that certain drug classes required high-energy collisions, which formed many fragment ions of low abundance that were not readily detected.

In the early part of the last decade, the toxicology community witnessed the arrival of high-resolution mass spectrometry (HRMS)[3] *(1–3)*. The number of reports on HRMS in toxicology laboratories is steadily increasing *(4)*. HRMS offers some unique advantages over low-resolution MS and/or tandem MS (MS/MS). HRMS can provide greater specificity due to its increased resolving power. An HRMS instrument also provides the opportunity for retrospective analysis, since data are typically acquired over a wide mass range in a full scan mode. Unlike targeted acquisitions such as multiple reaction monitoring (MRM), one can obtain and analyze HRMS data to detect "unknown unknowns" present in a sample without having reference standards.

During the introductory period of HRMS in toxicology laboratories, use of accurate mass with a relatively wide ($\pm$20 ppm) mass error window was reported for identifying unknowns *(2)*. Under such conditions, false-positive results could occur because endogenous analytes and environmental contaminants in a complex biological matrix might fall within the mass error window used for analysis. Other reports demonstrated that the number of false positives could be reduced by narrowing the mass error window *(5)*, incorporating the metabolic pattern *(6)*, and using isotope patterns *(7)*. Comparison of ultra-HRMS (resolution 100 000 at m/z 200) with HRMS (resolution 10 000 at m/z 200) has also been reported for toxicological screening purposes *(8)*. That report stated that under the given conditions, ultra-HRMS did not lower the number of false-positive results compared with HRMS. The number of false-positive results also depends on how the collected data are analyzed. In general, numbers of false-positive results go up in proportion to the number of entries in a database being searched *(9, 10)*.

Fragment ion information can be used to increase the analytical specificity and hence lower the false-

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[3] Nonstandard abbreviations: HRMS, high-resolution mass spectrometry; MRM, multiple reaction monitoring; CID, collision-induced dissociation; MS^E, mass spectrometry using elevated collision energies; DHS, dilute, hydrolyze, and shoot; UPLC, ultraperformance liquid chromatography; DI, deionized; MDA, 3,4-methylenedioxyamphetamine.

positive rate. Collision-induced dissociation (CID) is commonly used to obtain fragment ion information. CID spectra can be obtained by performing dissociation in the ion source (source-CID) or in a collision cell. Use of source-CID has been reported in confirming the positive findings in hair sample analysis *(11)*. The CID spectra obtained by this approach are less reproducible because (*a*) it does not involve precursor ion selection and (*b*) there are many factors that can influence fragmentation of ions in the source such as source gas pressure, source voltages, coeluting compounds, neutral particles, temperature, and solvents.

There are 2 ways to carry out CID in a collision cell. In the first approach, before colliding ions in a collision cell, precursors of interest can be selected in a quadrupole that provides information on ion lineage (e.g., a certain fragment ion originated from a selected precursor ion). Pavlic et al. used a hybrid tandem quadrupole TOF MS analyzer to develop a library of product ion spectra at various collision energies in the collision cell *(12)*. Information-dependent fragmentation of qualifying analytes has also been used *(1, 13)*. It involves a preselection criterion for identifying ions for fragmentation. During data acquisition, any ion that meets or exceeds the set intensity threshold is selected for fragmentation, producing specific diagnostic ions for identification purposes.

The second approach to performing CID in a collision cell involves sending all ions generated in the ion source into the collision cell. These ions can be subjected to low-energy collisions to obtain protonated analytes or elevated energy collisions to form fragment ions (MS^E). A primary advantage of CID in a collision cell compared with source CID is that most of the neutral molecules do not reach the collision cell. This approach provides a true broad-spectrum screen, since no ions are deliberately excluded. MS^E combines fragment ion information into algorithms for identifying unknowns *(14)*. MS^E uses a collision cell before *m*/*z* separation and acquires data in a dual-scanning mode. The first scan applies a fixed low voltage to the collision cell. Because ions do not undergo significant fragmentation at this low collision energy, this scan generally provides intact protonated ion information for all analytes. A second high-energy scan alternates with the low-energy scan to acquire data with a collision energy ramp. All ions entering the collision cell experience the collision energy ramp and undergo fragmentation to provide fragment ion information.

In this investigation, we set out to answer the following practical questions before implementing this methodology in a toxicology lab: (*a*) What are the best set of data processing criteria for producing results comparable to reference methods? (*b*) Can MS^E match the performance of traditional MRM-based LC-MS/MS methods with a variety of matrices? (*c*) Can MS^E function with a dilute, hydrolyze, and shoot (DHS) method? and (*d*) Does fragment ion information improve the analytical specificity of this methodology?

Materials and Methods

Details about materials, TOF-MS lock spray settings, unit resolution LC-MS/MS conditions, and analyte selection are available in the Supplemental Data, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue8.

LC conditions

Chromatographic separation was achieved by ultraperformance liquid chromatography (UPLC) (Waters Corp.). We used a UPLC BEH C_{18} guard column (1.7 μm, 5 mm) and an analytical column (1.7 μm, 2.1 $\times$ 150 mm) with flow rate of 0.4 mL/min at 50 °C. Mobile phase A and B were 5 mmol/L ammonium formate (pH 3) and 0.1% formic acid in acetonitrile, respectively. Five percent B was maintained for initial 0.5 min, increased in a linear fashion to 50% B at 6 min, and then to 95% B at 7 min, where it was held for 2 min. Reconditioning of the columns was achieved by dropping the gradient to 5% B at 9.05 min and maintaining it for 2.0 min.

TOF MS conditions

We used a TOF-MS (Xevo G2 TOF; Waters Corp.) with a resolution of approximately 20 000 (full width at half maximum at *m*/*z* 400); cone voltage, 20 V; capillary voltage, 0.8 kV; source block temperature, 130 °C; and desolvation gas flow 1000 L/h at 450 °C. Data were acquired in profile mode with MassLynx software, version 4.1, SCN 862 (Waters Corp.) without real-time mass correction. The MS method consisted of 3 functions. Function 1 acquired data over the 50- to 650-*m*/*z* range with a 6-eV collision energy (low energy). Function 2 acquired sample data over a 50- to 650-*m*/*z* range with a collision energy ramp of 10–50 eV (high energy). Function 3 acquired lock mass data over a 50- to 650-*m*/*z* range. UNIFI, version 1.6.1 (Waters Corp.), was used for data processing, which involved mass correction by use of leucine enkephalin data acquired simultaneously with each sample. The database was built for 61 analytes and included the retention time, molecular formula, and fragment ion information for each analyte.

A separate MRM-based LC-MS/MS (UPLC-Xevo TQ-S) was developed specifically for confirming positives tentatively identified by the UPLC-TOF-MS method that were not identified by the reference laboratories. The tandem instrument used was more analytically sensitive than the UPLC-TOF-MS under in-

vestigation. We used 2 MRM transitions for each analyte, and the sample preparation and LC conditions used for these analyses were exactly the same as those used in UPLC-TOF-MS analyses. Other details of the MRM-based LC-MS/MS method are presented in the online Supplemental Data.

SAMPLE SOURCES

Our goal was to obtain samples from different sources that would represent the variety of matrices commonly encountered by toxicology laboratories. We obtained 4 sets of samples from 3 different sources. These 4 sets were clinical toxicology samples that screened positive for opiates (set 1) and benzodiazepines (set 2), postmortem samples (set 3), and samples from pain management patients (set 4). These samples had previously been analyzed by LC-MS/MS or GC-MS using MRM or selected ion monitoring methods, respectively. The results of the initial analyses were kept blind until completion of UPLC-TOF-MS analysis.

SAMPLE PREPARATION

We added 400 μL deionized (DI) water to a vial followed by 100 μL internal standard solution (1 ng/μL mix of amphetamine D_5, codeine-D_3, diazepam-D_5, oxazepam-D_5, and venlafaxin-D_6) in methanol and 200 μL urine sample. We added 300 μL β-glucuronidase solution (5000 U/mL) prepared in a 1.0-mol/L sodium acetate buffer (pH 5) and mixed the solution by inversion. The mixture was incubated at 50 °C for 90 min followed by cooling and centrifugation at 2010*g* for 10 min. Supernatant (20 μL) was injected for analysis.

MATRIX EFFECT

We selected samples from 13 different patients because they had abnormal color (red, brown, black, etc.) and/or because of turbidity (clear, slightly turbid, extremely turbid). These samples were presumed to be indicative of a greater matrix complexity than would commonly be encountered. We also obtained 5 samples from healthy volunteers to represent samples that are more typically encountered in clinical practice. To study matrix effects specifically, these 18 samples were analyzed by DHS for the presence of all 61 analytes to evaluate if they contained any of the compounds of interest. These samples (and DI water) were then spiked with a mixture of all 61 analytes at 100- and 1000-ng/mL concentrations. In addition to these 18 samples, we analyzed 61 samples during the correlation study, which also represented a variety of different urine matrices. Matrix effect was calculated by the following formula; 100 − [(response in samples/response from spiked DI water) × 100]. Hence, a higher value indicated greater ion suppression.

SPECIFICITY

Six groups of compounds were spiked at 500 ng/mL in the starting gradient matrix (95:5 mobile phase A:B) and analyzed on UPLC-TOF-MS. This experiment was performed to establish target retention times, identify potential fragment ions, and determine the specificity for each analyte.

Results and Discussion

DHS

Compared to other techniques, the DHS methodology is fast, allows for a 1-vial procedure, and has low cost of consumables per sample *(15)*. Broad-spectrum screening methods require that none of the analytes are lost during the sample preparation. With techniques such as liquid-liquid extraction and solid-phase extraction, there is a risk of losing an analyte during the isolation steps. Because DHS has no purification steps, it avoids potential loss of analytes during sample preparation. However, there are several drawbacks associated with the DHS methodology used in this investigation. In our method, addition of enzyme for hydrolysis increases the amount of matrix in the sample. Because we did not separate matrix from the analytes, unwanted matrix could coelute with analytes of interest, causing ion suppression. Matrix in the sample can also coat stationary-phase particles in the analytical column, leading to decreased interaction between the analyte molecules and column particles. As the number of injections on a column increases, more matrix is deposited, causing partial column blockage that results in increased solvent back-pressure and decreased column life. In our experience, columns last for 300–400 injections.

MATRIX EFFECT

Previous publications that used HRMS and libraries with hundreds to thousands of compounds did not perform matrix-effect studies *(2, 5–7, 16)* on each analyte in the library. In our experience, having a larger database with no matrix effects studies severely limits the utility of these databases. We were concerned about matrix effects using DHS and thus selected samples from 13 different patients representing worst-case scenarios and 5 healthy volunteer samples for spiking experiments. All 5 samples from healthy volunteers were found to be negative for the 61 analytes. The 13 patient samples showed a total of 13 positive results for 10 different analytes. These 13 positives were excluded from the matrix effect study, e.g., if a sample was positive for morphine, then after spiking 61 analytes in that sample, the responses of

Table 1. List of analytes studied and matrix effects in specimens obtained from healthy individuals (effect 1) and patient's specimens selected because of their abnormal color and turbidity (effect 2).[a]

	Matrix effect 1, %			Matrix effect 2, %		
Analyte	Min	Max	Median	Min	Max	Median
6-Acetylmorphine	27	64	53	45	88	75
7-Aminoclonazepam	8	52	44	36	85	66
Amitriptyline	−11	14	5	18	51	40
Amphetamine	11	54	42	46	95	74
Atenolol	20	47	38	33	91	67
Benzoylegonine	14	53	46	33	81	67
Buprenorphine	−33	12	−12	−3	44	29
Carisoprodol	−4	26	15	24	59	46
Citalopram	22	39	34	41	73	57
Codeine	4	53	33	5	100	65
Cyclobenzaprine	−17	21	10	17	63	39
Dextromethorphan	18	38	32	24	73	54
Diazepam	−20	1	−7	7	44	25
Diltiazem	−9	14	2	12	51	36
Doxepine	8	27	18	21	65	46
EDDP[b]	−15	16	7	21	51	41
Fentanyl	10	26	17	11	70	48
Flunitrazepam	12	32	29	28	95	57
Hydrocodone	33	80	69	68	94	84
Hydromorphone	32	59	57	38	85	75
Ketamine	34	66	48	32	79	71
Lorazepam	−3	34	20	20	60	47
MDA	75	87	79	−79	94	72
MDEA	10	39	35	24	81	57
MDMA	14	56	49	57	99	79
MDPV	−10	22	17	21	78	49
Meperidine	6	30	23	31	76	55
Mephedrone	17	60	40	27	77	59
Meprobamate	−8	31	27	37	80	55
Methadone	−14	17	14	19	50	41
Methamphetamine	16	50	45	57	99	77
Methylone	3	41	31	32	80	61
Methylphenidate	4	28	23	37	79	50
Metoprolol	4	29	27	22	67	51
Morphine	16	61	50	36	97	76
Norbuprenorphine	16	53	49	26	77	67
Nordiazepam	12	29	25	19	53	42
Norfentanyl	43	69	55	27	84	70
Norhydrocodone	81	92	88	75	100	95
Normeperidine	11	28	23	30	76	57
Noroxycodone	49	72	66	54	93	83

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Table 1. List of analytes studied and matrix effects in specimens obtained from healthy individuals (effect 1) and patient's specimens selected because of their abnormal color and turbidity (effect 2).[a] *(Continued from page 1118)*

	Matrix effect 1, %			Matrix effect 2, %		
Analyte	Min	Max	Median	Min	Max	Median
Norpropoxyphene	−2	100	66	50	100	100
Nortriptyline	−13	16	13	15	58	43
Oxazepam	3	34	29	25	67	53
Oxycodone	29	68	59	66	98	84
Oxymorphone	40	72	62	59	94	87
Phencyclidine	4	27	23	28	78	52
Promethazine	−1	29	22	22	61	48
Propoxyphene	−16	100	9	18	100	43
Propranolol	10	35	27	33	80	55
Quetiapine	2	36	8	3	52	28
Tapentadol	7	34	33	38	89	56
Temazepam	−17	25	16	13	50	38
Tramadol	−1	25	17	24	63	50
Trazadone	−5	66	58	22	82	64
Venlafaxine	0	46	37	26	80	62
Verapamil	−20	13	4	5	42	27
Zaleplon	4	42	35	28	78	65
Zolpidem	−19	11	7	10	57	37
Zopiclone	5	56	42	25	86	70
α-Hydroxyalprazolam	−4	42	30	27	69	54

[a] Matrix effect formula: 100 − [(response in specimen/response from spiked DI water) × 100]. Negative matrix effect indicates an increase in the response.
[b] EDDP, 2-ethylidine-1,5dimethyl-3,3-diphenylpyrrolidine; MDEA, 3,4-methylenedioxy-*N*-ethylamphetamine; MDMA, 3,4-methylenedioxy-*N*-methylamphetamine; MDPV, methylenedioxypyrovalerone.

all analytes, except morphine, were investigated for matrix effect.

Spiking experiment. Typical urine drug concentrations observed in intoxication cases in the emergency department are often in the microgram-per-milliliter or higher range. Analytes can be detected with relative ease, even in the presence of ion suppression, if present at a sufficiently high concentration. However, matrix effects can be profound when the analyte concentration is relatively low; consequently, we decided to spike matrices at 100 and 1000 ng/mL. Because 100 ng/mL was the lowest concentration tested in human urine samples that gave positive results meeting our acceptance criteria, we consider this concentration to be our limit of detection for most drugs, with the exception of a few compounds where the limit of detection was between 100 and 1000 ng/mL.

DI water. To obtain signal intensity data without a matrix, DI water was spiked with all 61 analytes at 100 and 1000 ng/mL and processed with the DHS method. At 100 ng/mL, all analytes were identified with their respective fragment ions except morphine, norpropoxyphene, and tramadol. These 3 analytes did not show fragment ions in the absence of sample matrix. For the spiking experiment of 61 analytes at 1000 ng/mL, all analytes were detected with their respective fragment ions.

Healthy volunteer–spiked samples. All 5 samples were spiked at 100-ng/mL concentration with the 61 analytes and processed with the DHS method. The responses of each analyte were compared with the response from spiked DI water. Table 1 shows that there was a wide range of matrix effects in samples from healthy donors. We observed ion suppression that ranged from zero to 100%. In addition, we observed ion enhancement of up to 33%. Of the 61 analytes spiked into these 5 samples, buprenorphine (twice), norbuprenorphine (3 times), and norhydrocodone (4 times) failed to show a fragment ion, whereas meprobamate and propoxyphene failed once each to show

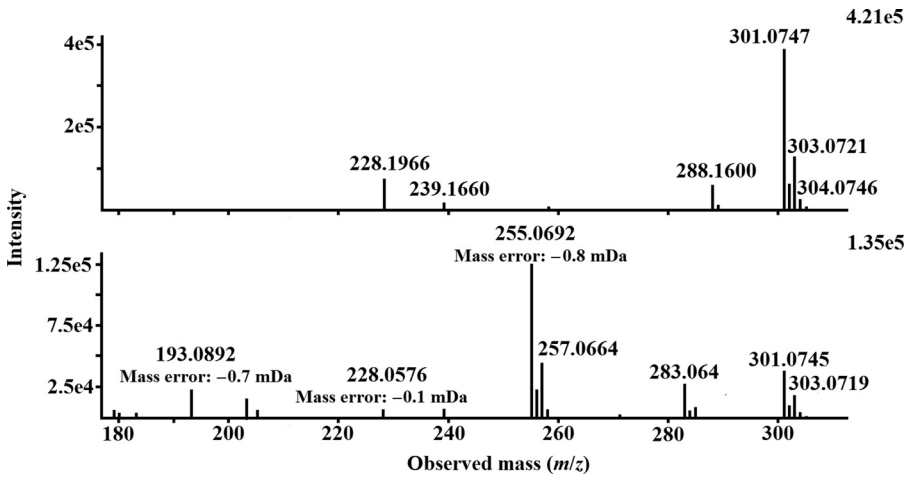

Fig. 1. Sample data processed by UNIFI show mass spectra of positively identified analyte (temazepam).

Top spectrum, low-energy scan mass spectrum showing an identified protonated analyte (*m/z* 301.0747) for temazepam; bottom spectrum, high-energy scan mass spectrum showing 3 identified fragment ions of temazepam at *m/z* 193.0892, 228.0576, and 255.0692.

related protonated analyte. As seen with the spiked DI water samples, tramadol and morphine failed to show fragment ions in all 5 samples. For spiking at 1000 ng/mL, all analytes (protonated analyte as well as at least 1 fragment) were detected in all 5 samples.

Spiked patient samples. Thirteen patient samples, selected because they were abnormally colored or had precipitates, were spiked at 100-ng/mL concentration and processed with the DHS method. Similar to samples from healthy donors, ion suppression varied widely in these samples. Thirty-seven of 61 analytes consistently showed fragments ions in all samples. Twenty-four of 61 analytes failed to show fragments in 1 or more samples. As in the DI water samples, morphine, tramadol, and norpropoxyphene failed to show fragments in all 13 samples. Fragmentation of tramadol led to only 1 fragment at *m/z* 58, which failed to be detected on a consistent basis. Because of its small *m/z*, it was difficult to distinguish from the background noise.

After spiking at 1000 ng/mL, all analytes showed their protonated adduct as well as fragment ions, except codeine (once), 3,4-methylenedioxyamphetamine (MDA) (4 times), methylphenidate (once), morphine (4 times), norhydrocodone (once), and tramadol (3 times) failed to show fragment ion.

ANALYTICAL SPECIFICITY

The 61 analytes were divided into 6 groups (5 × 10 and 1 × 11). Each group contained chromatographically resolved analytes. Data from each group were screened for 61 analytes. All spiked analytes showed positive results. We did not observe any (false) positives for analytes that were not spiked in respective groups.

PATIENT SAMPLE DATA ANALYSIS

ULPC-TOF (MS^E) data were analyzed with UNIFI software. The data were processed with a retention time window of ±0.2 min and ±20 ppm window and fragment ion with ±5 ppm mass accuracy. To ensure that matrix effects were not obscuring peaks, we verified that the protonated molecular ion of the 5 internal standards, which spanned the retention time of interest, were present at the expected retention time. The processed data were then sorted with various filters. We evaluated the following: (*a*) mass accuracy of ±20 ppm for protonated analyte combined with a retention time match of ±0.2 min; (*b*) mass accuracy of ±5 ppm for protonated analyte combined with a retention time match of ±0.2 min; (*c*) mass accuracy of ±5 ppm for protonated analyte combined with a retention time match of ±0.2 min and ≥1 fragment ion (±5 ppm); and (*d*) isotope cluster intensity and ppm match. Typical processed MS^E spectra are shown in Fig. 1.

Eleven samples (sample set 1) that were positive for various opiates by LC-MS/MS were analyzed by UPLC-TOF-MS DHS. The reference method identified 55 positives (Table 2). Table 2 also shows that, as the requirements for identification became more stringent, the number of false-positive UPLC-TOF-MS results decreased. For example, in the case of hydrocodone, there were 3 false-positive UPLC-TOF-MS

Table 2. Comparison of number of positive results for opiate specimens (sample set 1) obtained using different identification criteria.[a]

Opiate	Retention time			Reference positives	Comments
	20 ppm	5 ppm	5 ppm, fragment		
6-Acetylmorphine	3	3	3	3	
Codeine	4	4	4	4	
EDDP[b]	4	4	4	4	
Fentanyl	5	5	5	5	
Hydrocodone	5	4	2	2	
Hydromorphone	8	7	6	8	NF 106 ng/mL, NI 263 ng/mL
Methadone	4	4	4	4	
Morphine	7	7	7	7	
Norfentanyl	5	5	3	5	NF 79 and 95 ng/mL
Oxycodone	6	6	6	6	
Oxymorphone	9	9	6	7	NF 234 ng/mL
Total	60	58	50	55	

[a] Reference results obtained using MRM LC-MS/MS. Reference positives are based on the cutoff concentrations listed in online Supplemental Table 2.
[b] EEDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; NF, no fragment (for identified protonated analyte); NI, not identified (protonated analyte).

results when the only criteria used for identification were a retention time ±0.2 min and ±20 ppm protonated analyte match (5 matches vs 2 matches with the reference method). With requirements of a retention time ±0.2 min and ±5 ppm protonated analyte match, there were only 2 false-positive results, and when a fragment ion match (±5 ppm) was included, there were no false-positive results. This finding was consistent with all of the sample sets, and because these criteria provided the highest number of true positives with zero false positives, they became the identification criteria we used for the rest of the study. UPLC-TOF-MS was able to identify 91% (50/55) of the true positives in the opiate data set by use of the requirements of a ±5 ppm protonated analyte match, with a ±0.2 min retention time and a ±5 ppm fragment ion match (Table 2). By these criteria, a total of 5 false negatives were also encountered. In 4 cases, false negatives were due to absence of a fragment ion, whereas in 1 case, the analyte ion failed to be detected. There were 45 additional positives in these 11 samples (see online Supplemental Table 1). We confirmed these positives by an MRM LC-MS/MS (UPLC-Xevo TQ-S) assay developed specifically for this purpose.

Table 3. Comparison of number of positive results for benzodiazepine specimens (sample set 2) obtained using different identification criteria.[a]

Benzodiazepine	Retention time			Reference positives	Comments
	20 ppm	5 ppm	5 ppm, fragment		
7-Aminoclonazepam	3	2	2	3	NI 5.02 ppm error[b]
Lorazepam	3	3	3	3	
Nordiazepam	9	9	8	9	NF 24 ng/mL
Oxazepam	10	10	10	10	
Temazepam	9	9	9	9	
α-Hydroxyalprazolam	2	2	2	2	
Total	36	35	34	36	

[a] Reference results obtained using MRM LC-MS/MS. Reference positives are based on the cutoff concentration listed in online Supplemental Table 2.
[b] NI, not identified (protonated analyte); NF, no fragment (for identified protonated analyte).

Table 4. Comparison of number of positive results for postmortem specimens (sample set 3) obtained using different identification criteria.[a]

Analyte	Retention time			Reference positive	Comments
	20 ppm	5 ppm	5 ppm, fragment		
6-Acetylmorphine	11	9	6	8	2 NF[b]
Amphetamine	2	2	2	2	
Atenolol	1	1	1	1	
Benzoylecgonine	1	1	1	1	
Citalopram	1	1	1	1	
Codeine	9	8	8	8	
EDDP	1	1	1	1	
Fentanyl	2	2	1	1	
Hydrocodone	3	3	0	1	NF
Ketamine	1	1	1	1	
Meprobamate	1	1	1	1	
Methadone	1	1	1	1	
Methamphetamine	4	4	2	2	
Morphine	11	11	11	12	NF
Norfentanyl	3	3	1	1	
Norhydrocodone	1	1	1	1	
Oxycodone	4	4	3	3	
Oxymorphone	5	5	2	2	
Promethazine	1	1	1	1	
Propranolol	2	2	1	1	
Quetiapine	1	1	1	1	
Tramadol	4	4	2	2	
Total	70	67	49	53	

[a] Reference results obtained using GC/MS. Reference positives are based on the cutoff concentration listed in online Supplemental Table 2.
[b] NF, no fragment (for identified protonated analyte); EEDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

Ten patients' samples (sample set 2) positive for benzodiazepines by LC-MS/MS were analyzed by the UPLC-TOF-MS method. Ninety-four percent (34/36) of the true positives were confirmed by UPLC-TOF-MS (Table 3). One of the false negatives was due to mass error of 5.02 ppm, which was just above the set criteria of ±5 ppm. The other false negative for nordiazepam was due to its low concentration (24 ng/mL). Twenty-one additional drugs identified by UPLC-TOF-MS were subsequently confirmed by the LC-MS/MS method (see online Supplemental Table 1).

We also analyzed 15 postmortem urine samples (sample set 3). Ninety-two percent (49/53) of the true-positive results were confirmed by our analysis (Table 4). In 4 incidences, the analysis failed to produce fragment ion information. Those false negatives could be due to low concentration of analytes, ion suppression, or both. We could not confirm/hypothesize reasons for the lack of the fragment ions because only qualitative results were available from the reference laboratory for this data set. The UPLC-TOF-MS method identified 38 additional drugs that were not identified by the reference method (see online Supplemental Table 1). All of these additional positives were confirmed by LC-MS/MS.

Twenty-five pain management samples (sample set 4) had a total of 131 true positives (Table 5) for 38 different analytes. The UPLC-TOF-MS analysis confirmed 92% (120/131) of the true positives reported. There were 38 additional positives tentatively identified with the UPLC-TOF-MS method that were not initially detected with the reference method (see online Supplemental Table 1). These additional positives were confirmed by a targeted LC-MS/MS.

As expected, the number of false positives decreased as we used more stringent identification criteria. Use of a wider mass accuracy window (±20 ppm)

Table 5. Comparison of number of positive results for pain management specimens (sample set 4) obtained using different identification criteria.[a]

Analyte	Retention time			Reference positives	Comments
	20 ppm	5 ppm	5 ppm, fragment		
6-Acetylmorphine	1	1	1	2	NI 30 ng/mL[b]
7-Aminoclonazepam	4	4	3	4	NF 234 ng/mL
Amitriptyline	2	2	2	2	
Amphetamine	4	4	3	3	
Benzoylegonine	9	7	3	3	
Buprenorphine	4	4	3	3	
Carisoprodol	5	4	2	2	
Codeine	3	2	2	2	
Cyclobenzaprine	3	3	3	3	
EDDP	5	4	3	3	
Fentanyl	3	3	2	3	NF 3 ng/mL
Hydrocodone	5	5	5	5	
Hydromorphone	6	6	6	6	
Ketamine	1	1	1	1	
Lorazepam	2	2	2	2	
Meperidine	2	2	1	1	
Meprobamate	4	3	3	3	
Methadone	4	4	4	4	
Methamphetamine	2	1	1	1	
Methylone	5	5	2	2	
Methylphenidate	2	2	2	2	
Morphine	7	5	5	6	NF 128 ng/mL
Norbuprenorphine	8	4	2	4	NF 56 ng/mL; NI 76 ng/mL
Nordiazepam	4	4	3	3	
Norfentanyl	3	2	2	2	
Norhydrocodone	5	5	3	4	1 NF 635 ng/mL
Noroxycodone	14	11	10	10	
Nortriptyline	2	2	2	2	
Oxazepam	4	4	4	4	
Oxycodone	11	10	10	10	
Oxymorphone	10	10	9	10	NF 349 ng/mL
Phencyclidine	1	1	1	1	
Tapentadol	4	4	2	2	
Temazepam	3	3	3	3	
Tramadol	4	2	2	2	
Venlafaxine	1	1	1	1	
Zolpidem	2	2	2	2	
α-Hydroxyalprazolam	7	7	5	8	NF 34 and 39 ng/mL; NI 31 ng/mL
Total	166	146	120	131	

[a] Reference results obtained using MRM LC-MS/MS. Reference positives are based on the cutoff concentration listed in online Supplemental Table 2.
[b] NI, not identified (protonated analyte); NF, no fragment (for identified protonated analyte); EEDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

with retention time gave the most false positives (57), whereas a ±5 ppm with retention time match had 31 false positives, and the addition of a fragment ion (±5 ppm) reduced the false positives to zero. By the most stringent criteria for identification, in a total of 18 incidences the UPLC-TOF-MS analysis failed to obtain fragment ion information for 9 different analytes (6-acetylmorphine, hydrocodone, hydromorphone, morphine, norbuprenorphine, norfentanyl, norhydrocodone, oxymorphone, tramadol) even at relatively higher concentrations. We believe that matrix effect is the likely reason that these true positives failed to show fragment ions with our analysis. These analytes had relatively high (>60%) ion suppression in our matrix effect studies, except tramadol. Tramadol shows only 1 fragment in general at *m/z* 58.0652 and is usually found in the background noise owing to its smaller molecular weight. It is likely that the accurate mass of this ion is heavily influenced by the neighboring noise, knocking it out of the mass accuracy window. Also, all analytes that the UPLC-TOF-MS method had difficulty identifying, except tramadol and norfentanyl, have a morphine-like core structure and require high energy for fragmentation that generally results in many fragments with low signal-to-noise ratios. For most of these analytes, there are immunoassays readily available. In routine practice, most urine samples are screened by immunoassay panels before MS analysis. Consistent with the Society of Forensic Toxicologists guidelines for identification of drugs in biological samples, we consider a result reportable if we obtain a positive finding on immunoassay followed by retention time (±0.2 min) and a precursor ion (±5 ppm) match on UPLC-TOF-MS analysis *(17)*. We did not identify any additional positives related to analytes that should have been detected by the methods of the reference laboratories.

Addition of the isotope pattern can be useful in HRMS analyses *(7)*. We also investigated the usefulness of isotope pattern (intensity and mass accuracy of isotope cluster). For the method we developed, the results for isotope pattern did not add value, primarily because of the low signal-to-noise ratio for isotope clusters at lower limits of detection. For example, UPLC-TOF-MS analysis would confirm the presence of morphine by mass accuracy of ±5 ppm for protonated analyte with a retention time match of ±0.2 min and ≥1 fragment ion (±5 ppm) but would fail the isotope cluster intensity and mass accuracy criteria. Adding isotope cluster criteria did not improve the analysis compared with the criteria that included a retention time match (±0.2 min) combined with a parent ion (±5 ppm match) and at least 1 fragment ion (±5 ppm match).

Conclusions

In our experiments, fragment ion information was critical for eliminating false-positive results. MS^E incorporates fragment ion information in HRMS-based drug screening with an untargeted approach. By use of a collision energy ramp, MS^E eliminates the need to optimize collision energies for individual analytes. The combination of a low- and high-energy scan helps to unambiguously identify analytes. A limitation of MS^E is that some analytes, such as morphine, do not yield fragment ions of high abundance. DHS sample preparation worked well with our UPLC-TOF-MS method, showing 92% confirmation of true positives, with the ability to identify other drugs missed by the reference methods. The method we developed is fast, simple, and cheap, but is subject to ion suppression, especially when challenged with abnormal urine samples. Preliminary solid-phase extraction experiments demonstrated reduced recovery of certain drugs, with minimal improvement in matrix effects. Future studies should examine alternative sample preparation schemes to minimize matrix effects while retaining the ability to detect a wide variety of potential intoxicants.

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