Postmortem Drug Screening by Non-Targeted and Targeted Ultra-Performance Liquid Chromatography–Mass Spectrometry Technology

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Abstract

In the medical examiner setting, comprehensive drug screening is an essential analytical tool in the investigation of cause and manner of death. We have validated non-targeted and targeted screening assays for drugs and metabolites using ultra-performance liquid chromatography (UPLC) interfaced with mass spectrometry (MS) in single and tandem stages. For non-targeted screening by UPLC–MS electrospray interface, in-source fragmentation was used along with MS scanning (*m/z* 80–650) and library search for over 700 drug and metabolite analytes. Targeted detection of over 200 analytes by UPLC–MS–MS was performed with dual transition ion monitoring. Validation studies confirmed reproducibility of both mass spectra produced by in-source fragmentation and transition ion ratios by collision-cell dissociation. Lower limit of detection by UPLC–MS (10–150 ng/mL) and UPLC–MS–MS (1–50 ng/mL) was determined for a subset of drugs and correlated with extraction recovery and matrix effect. Drug findings by UPLC–MS and UPLC–MS–MS were compared with gas chromatography–mass spectrometry (GC–MS) screening in postmortem blood from 410 medical examiner cases with 1121 positive drug findings by all methods. Accuracy, based on results of confirmation testing, was high (98–99%) across all screening assays and detection sensitivity by GC–MS (71%), UPLC–MS (73%), and UPLC–MS–MS (76%) was determined. UPLC–MS plus UPLC–MS–MS screening resulted in the highest drug detection rate (95%) and provided optimal dual-screening for the postmortem casework.

Introduction

The goal of drug screening in postmortem toxicology is to detect and identify any suspected or unsuspected drug or metabolite in postmortem or antemortem specimens, and to direct drug confirmation and quantification analysis as needed in determining a potential drug-related or -caused fatality. Assay techniques that approach this goal are referred to as general unknown or systematic toxicological drug screens and optimally require capacity for non-targeted detection of drugs and metabolites over a wide range of molecular weight, polarity, lipid solubility, and thermal stability. Gas chromatography–mass spectrometry (GC–MS) is the most commonly used general unknown approach in postmortem toxicology, due largely to availability of well-developed mass spectral libraries. GC–MS screening, however, is technically limited in detection of non-volatile, polar, and thermally unstable compounds and may require specific chemical derivatization to overcome some of these analytical limitations. Immunoassay screening for drug classes is frequently used along with GC–MS screening, but positive immunoassay findings require further analysis for the identity of specific drugs or metabolites.

In both forensic and clinical toxicology, drug screening by liquid chromatography (LC)–MS technology continues to develop as a result of advancing instrumentation and mass spectral libraries. For single-stage MS analysis, the observation of reproducibility of in-source fragmentation (1–5), using electrospray ionization (ESI) on the same instrument or on different instruments with similar source design, lead to the development by several toxicology centers of ESI-MS libraries (2,6,7). Weinmann et al. (6) reported a library for single-stage MS detection of 400 drugs over an analytical acquisition time of 32 min, using SCIEX-API instrumentation and individual spectra attained with three positive ESI voltage energies. Using a longer chromatographic run time of 50 min, Marquet and coworkers (2) developed screening analysis for approximately 1300 drugs using a spectral library of dual composite-spectra from two positive and two negative ESI conditions. More recently, an ultra-performance liquid chromatography (UPLC)–MS screen method (7) was reported for identification of 500 analytes using a reduced chromatographic run time of 15 min with a library search of individual mass spectra from up to six positive and six negative in-source voltage energies. Tandem MS databases and spectral libraries have also been developed for use in clinical and forensic drug screening. Multiple reaction-monitoring (MRM) of targeted analytes have been re-

ported (8,9) and full mass spectra based on collision cell fragmentation have been applied in a number of information dependent acquisition algorithms using tandem MS with a quadrupole-linear ion-trap (10–15). The linear trap applications that employ an MRM survey scan followed by product-ion dependent scanning remain targeted in their approach (11,12,14), whereas applications using an enhanced mass scan mode in the survey scan qualify as non-targeted screening methods (10,13).

Along with the development of mass spectral libraries for drug screening by LC–MS technology, a few toxicology centers have compared these newer drug screening methods with the established GC–MS screening applications. In a study of 51 clinical sera with a total of 84 drug findings, a drug detection rate of 66% by GC–MS was compared with a 75% detection rate by liquid chromatography interfaced single stage MS screening (16). In 95 postmortem cases, Herrin et al. (17) found comparable detection rates between GC–MS and a targeted 114-drug screen method using liquid chromatography interfaced with quadrupole-linear ion-trap MS. In another small study of 36 clinical samples with a total of 130 drug findings, a detection rate of 93.8% was found by Sauvage and co-workers, using a linear ion trap approach with a non-targeted survey scan. The comparable detection rate by GC–MS in this study was only 64% (13).

We report the validation of UPLC–MS and UPLC–MS–MS screening assays in postmortem blood, along with a comparison to GC–MS screening in a relatively large cohort of medical examiner cases.

Materials and Methods

Chemicals and reagents

For preparation of quality control material and recovery studies, samples of 6-acetylmorphine, alprazolam, amphetamine, benzoylecgonine, 3,4-methylenedioxyphenyl-2-butanamine (BDB), citalopram, cocaethylene, cocaine, codeine, diazepam, dihydrocodeine, diltiazem, hydromorphone, hydrocodone, imipramine, methamphetamine, methadone, N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), morphine, nordiazepam, oxazepam, oxycodone, oxymorphone, phencyclidine, propoxyphene, pseudoephedrine, quetiapine, tramadol, and venlafaxine reference materials were obtained from Cerilliant (Round Rock, TX). Additional drug and metabolite reference material for mass spectral library and transition-ion ratio database development were obtained from Cerilliant and Alltech Applied Science (State College PA). Methapyrilene and hexobarbital were obtained from Alltech Applied Science. Ammonium acetate, polyethylene glycol (PEG 400), ACS-grade methanol, dibasic sodium phosphate, monobasic sodium phosphate, and HPLC-grade acetonitrile and methylene chloride were purchased from Fisher Scientific (Fairlawn, NJ). AR grade hydrochloric acid, ammonium hydroxide, acetic acid, and spectral-grade hexane were from Mallinckrodt (Paris, KY); ethyl acetate was from Anachemia (Champlain, NY). Mass calibration for UPLC–MS and UPLC–MS–MS analysis was performed with 2 mM ammonium acetate and 50 µg/mL PEG 400 in acetonitrile/water (50:50). Perfluorotributylamine (Varian, Walnut Creek, CA) was used for GC–MS mass calibration.

Solid-phase extraction of postmortem blood for all assays was performed with Clean Screen extraction columns (ZSDAU020) from United Column Technology (Bristol, PA). A mixed solvent with ammonium hydroxide/isopropanol/methylene chloride (2:20:80) was prepared for later elution of basic compounds from the extraction columns. For gradient UPLC analysis, mobile phase A (5 mM ammonium formate, pH 3.0) and mobile phase B (0.1% v/v formic acid in acetonitrile) were prepared along with a mobile phase mixture solution containing 87% mobile phase A and 13% mobile phase B that was used for sample reconstitution. Deionized water was produced by an ultrafiltration (AquaPure, Schenectady, NY) and reverse osmosis (Alpha Water Systems, Montague, NJ) treatment system.

Postmortem blood samples

Postmortem blood used in the comparison of UPLC–MS, UPLC–MS–MS, and GC–MS screening assays was obtained from 410 consecutive postmortem toxicology cases submitted to the Forensic Toxicology Laboratory at the Albany Medical Center. The decedent cohort included 299 males with an average age of 49 years (range < 1–94) and 111 females with an average age of 39 years (range < 1–89). Confirmation testing for all forensically significant drug findings was performed by case sample re-extraction and analysis by another qualitative or quantitative method. Low level caffeine, theophylline, cotinine, acetaminophen, diphenhydramine, chlorpheniramine, and dextromethorphan are not routinely confirmed, as well as atropine in resuscitation-attempt cases. Validated methods for confirmation of screen positive findings included targeted GC–MS analysis following derivatization or LC–MS analysis by single or tandem stage.

De-identified postmortem blood from a limited number of decomposition and non-decomposition cases were also used to assess extraction recovery and matrix effect in comparison to blood bank blood.

Extraction procedure for screening assays

Methapyrilene was added to 2 mL of postmortem and control blood as internal standard at a final concentration of 500 ng/mL. Hexobarbital was also added (500 ng/mL final concentration) for GC–MS analysis of the acid/neutral extracts. Following a 15-min sonication and addition of 4 mL of 0.1 M phosphate buffer (pH 6.0), samples were loaded onto extraction columns at an aspiration rate of 1 mL/min. Columns were washed sequentially with deionized water (2 mL), 0.1 M acetic acid (1 mL) and hexane (3 mL). Acid/neutral drugs were then eluted with ethyl acetate (3 mL). Columns were futher washed with methanol (2 mL) and basic drugs were eluted with mixed solvent (3 mL) into tubes containing 50 µL of 0.1 N HCl. For initial GC–MS analysis, the acid/neutral and basic eluates were separately dried under nitrogen and reconstituted with 60 µL of ethyl acetate. After injection of 1–2 µL for GC–MS analysis,

reconstituted acid/neutral and basic drug extracts were combined, dried down under nitrogen, and reconstituted with 100 µL of mobile phase mixture solution. A 10-µL injection volume of reconstituted extract was used for each UPLC–MS and UPLC–MS–MS analysis.

Quality control, limit of detection (LOD), and recovery studies

Quality control material extracted and analyzed along with case specimens was prepared in analyte-free whole blood obtained from the Albany Medical Center's Blood Bank. Positive control contained 250 ng/mL for amphetamine; 150 ng/mL for benzoylecgonine, methadone, morphine, nordiazepam, and propoxyphene; 100 ng/mL for imipramine; and 50 ng/mL for phencyclidine. Positive and negative controls were co-extracted with each batch of case specimens. Additionally, neat reference material was prepared for analysis by GC–MS to verify retention time and CI spectra for positive drug findings, and by LC–MS–MS to verify retention time and transition ion ratios. Analysis of these neat reference materials also allowed for run-to-run assessment of transition ion ratio stability in the LC–MS–MS analysis.

Experimental LOD studies for UPLC–MS and UPLC–MS–MS assays were performed in singlet by extraction and analysis of analyte-free blood bank blood supplemented with 1, 10, 50, 100, and 150 ng/mL each of 6-acetylmorphine, alprazolam, amphetamine, benzoylecgonine, BDB, citalopram, cocaethylene, cocaine, codeine, diazepam, dihydrocodeine, diltiazem, hydromorphone, hydrocodone, methamphetamine, MBDB, MDA, MDMA, morphine, nordiazepam, oxazepam, oxycodone, oxymorphone, pseudoephedrine, quetiapine, tramadol, and venlafaxine. Agents included in this study represent both low- and high-volume drugs found in our postmortem casework. The LOD was defined as the lowest concentration resulting in a positive drug finding based on assay criteria. Assay sensitivity for an expanded number of drugs was also assessed by analyzing the quantitative confirmation data from casework-positive screening results. An experiential minimum-detected concentration by UPLC–MS and UPLC–MS–MS screening was based on the lowest drug or metabolite concentration determined by confirmation testing. Although this limit is not experimentally determined and does not fully define the LOD it does provide additional sensitivity data based on actual casework.

Extraction recovery and matrix effect was evaluated using multiple sources of blood bank blood. Extraction and analysis of multiple blood samples by UPLC–MS–MS was performed at a concentrations of 100 ng/mL for combinations of the following drugs: alprazolam, amphetamine, benzoylecgonine, BDB, citalopram, cocaethylene, cocaine, codeine, diazepam, dihydrocodeine, diltiazem, hydromorphone, hydrocodone, methamphetamine, MBDB, MDA, MDMA, morphine, nordiazepam, oxazepam, oxycodone, oxymorphone, pseudoephedrine, quetiapine, tramadol, and venlafaxine. 6-Acetylmorphine was tested for recovery and matrix effect at a concentration of 10 ng/mL. For comparison with these extracted samples, additional samples with matching drug concentrations were prepared in mobile phase (non-extracted neat control) and in post-extracted sample matrices (post-extracted spiked sample) for direct analysis by LC–MS–MS. Percent extraction recovery was calculated as the ratio of response of matching sets of extracted samples and post-extracted spiked samples multiplied by 100. The percent change in transition ion abundance due to matrix effect was calculated from the ion count ratio of matching sets of post-extracted spiked samples and non-extracted neat controls multiplied by 100. Recovery and matrix effect were evaluated for each transition ion response.

Extraction recovery and matrix effect in blood bank blood was compared with postmortem blood from five decomposed and five non-decomposed cases. Samples were spiked with 100 ng/mL of citalopram, diltiazem, quetiapine, tramadol, and venlafaxine, followed by extraction and analysis. Matching non-extracted neat controls and post-extracted fortified samples were also prepared and analyzed as described.

UPLC–MS and UPLC–MS–MS analysis

Instrumentation used for UPLC–MS and UPLC–MS–MS analysis was an Acquity UPLC System with a TQ Detector. System software included MassLynx™ (4.1 SCN 714), ChromaLynx™, and TargetLynx™ (Waters). Chromatographic separation was performed with an HSS C_{18} 1.8 µm (2.1 × 150 mm) column (Waters) maintained at 50°C. A mobile phase gradient from 87%A and 13%B to 5%A and 95%B was used with a flow rate of 400 µL/min and a total analytical run time of 15 min.

MS conditions for UPLC–MS analysis included within-run ESI positive and negative modes (20 ms frequency), capillary voltage 3.5 kV, cone voltage 20 V to 95 V in 15 V increments (in-source CID), desolvation temperature 400°C, desolvation gas flow of 800 L/h, source temperature 150°C, acquisition range *m/z* 80–650 and scan speed > 7000 atomic mass units per second. An in-source CID mass spectral library of 710 (library size at time of study) was obtained from Waters with additional mass spectra library additions in-house. The in-house produced library included a number of drugs and metabolites not available in the commercial library. In addition, the in-house library included a number of drugs present in the commercial library, allowing for a limited comparison of spectral match using in-house versus externally produced libraries. Criteria for a positive drug finding include retention time within 0.3 minutes and an average library forward fit ≥ 650. Alternate criteria included a tentative library fit (450–649) plus identification of the molecular species and two major fragment ions.

MS–MS conditions for UPLC–MS–MS analysis include ESI positive ionization mode, capillary voltage (3.0 kV), desolvation temperature (400°C), desolvation gas flow (800 L/h), source temperature (150°C), and collision gas (argon) pressure closely maintained at 0.45 Pa. MRM was performed for 202 targeted drugs and metabolites using dual transition ions for all analytes except tramadol where only a single detectable transition was produced in the collision cell. Cone voltage and collision energy was optimized for each analyte transition, with a target transition-ion ratio established for use in criteria-based identification of all analytes except tramadol. European Union cri-

teria (18,19) were used for transition ion ratio monitoring, including a transition ion ratio within 20% of target ratio for transition ratios ≥ 0.50, within 25% for transition ratios between 0.20 and < 0.50, within 30% for transition ratios between 0.10 and < 0.20, and 50% for transition ratios < 0.10. Mass calibration was performed prior to each analytical run for both UPLC screening assays.

GC–MS analysis

Chromatographic separation was performed with Varian 3900 GC (Varian) instrumentation using a 5% diphenyl/95% dimethyl polysiloxane capillary column (Rxi-5ms, 15 m × 0.25-mm i.d., 0.25-µm film thickness, Restek, Bellfonte, PA) with a helium gas flow rate of 1 mL/min. A linear column temperature ramp over 19 min (75–300ºC) was employed along with an injection temperature of 250ºC. The GC was interfaced to a Saturn 2100T ion trap MS (Varian) operated in both electron impact (EI) and acetonitrile-chemical ionization (CI) modes. EI screening was performed with automatic gain control, a mass spectral scan range of *m/z* 40–650 and search with NIST/EPA/NIH 2008, Wiley Registry of Mass Spectral Data, Mass Spectral Library of Drugs, Poisons, Pesticides, Pollutants and Their Metabolites 2007 and in-house mass spectral libraries. Criteria for presumptive positive drug or metabolites finding included chromatographic retention time within 2% of co-analyzed reference analyte, forward fit ≥ 800 and both purity and reverse fit ≥ 500. All EI positive extracts were re-analyzed in CI mode. For CI analysis, automatic reaction control was used with mass spectral scan range of *m/z* 67–650 and positive CI criteria include identitification of protonated molecular ion and fragment ions consistent with a co-analyzed neat reference analyte. Switch to CI mode is accomplished with a single software commands and requires no hardware modification. Workstation software version 5.52 (Varian) was used for instrument data management.

Results and Discussion

Assay validation studies

A total ion chromatogram (TIC) for UPLC–MS analyzed positive control blood is shown in Figure 1. Overlapping TICs are displayed for each in-source CID collision energy and the candidate list shows match criteria (≥ 650) and retention time for drugs identified in the positive control pool. Significant mass spectral detail underlie the RIC as demonstrated in Figure 2 where the upper panel show the RIC for each cone voltage and representative mass spectra for ecgonine methylester at +20, +50, and +95 V are shown. The average library fit of 792

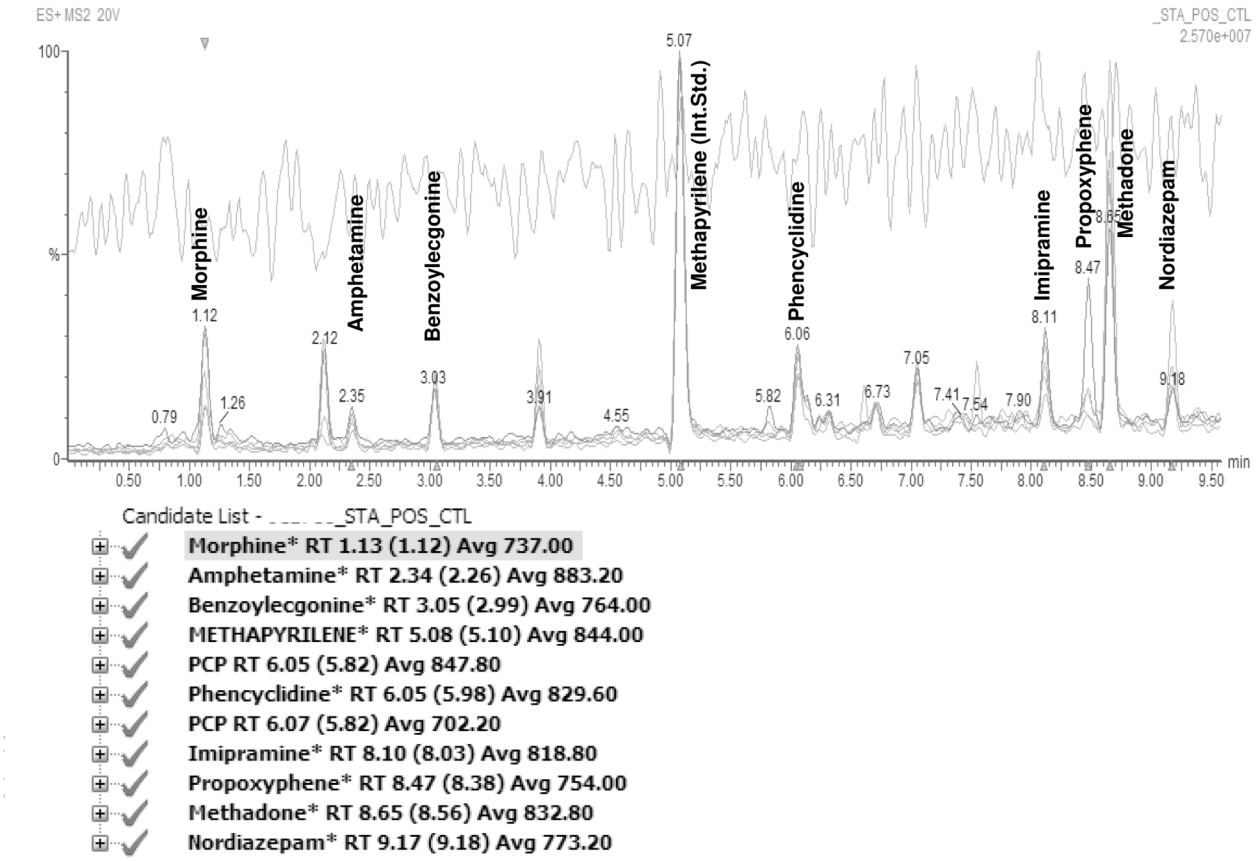

Figure 1. Total ion chromatograms (TICs) for analysis of positive blood control.

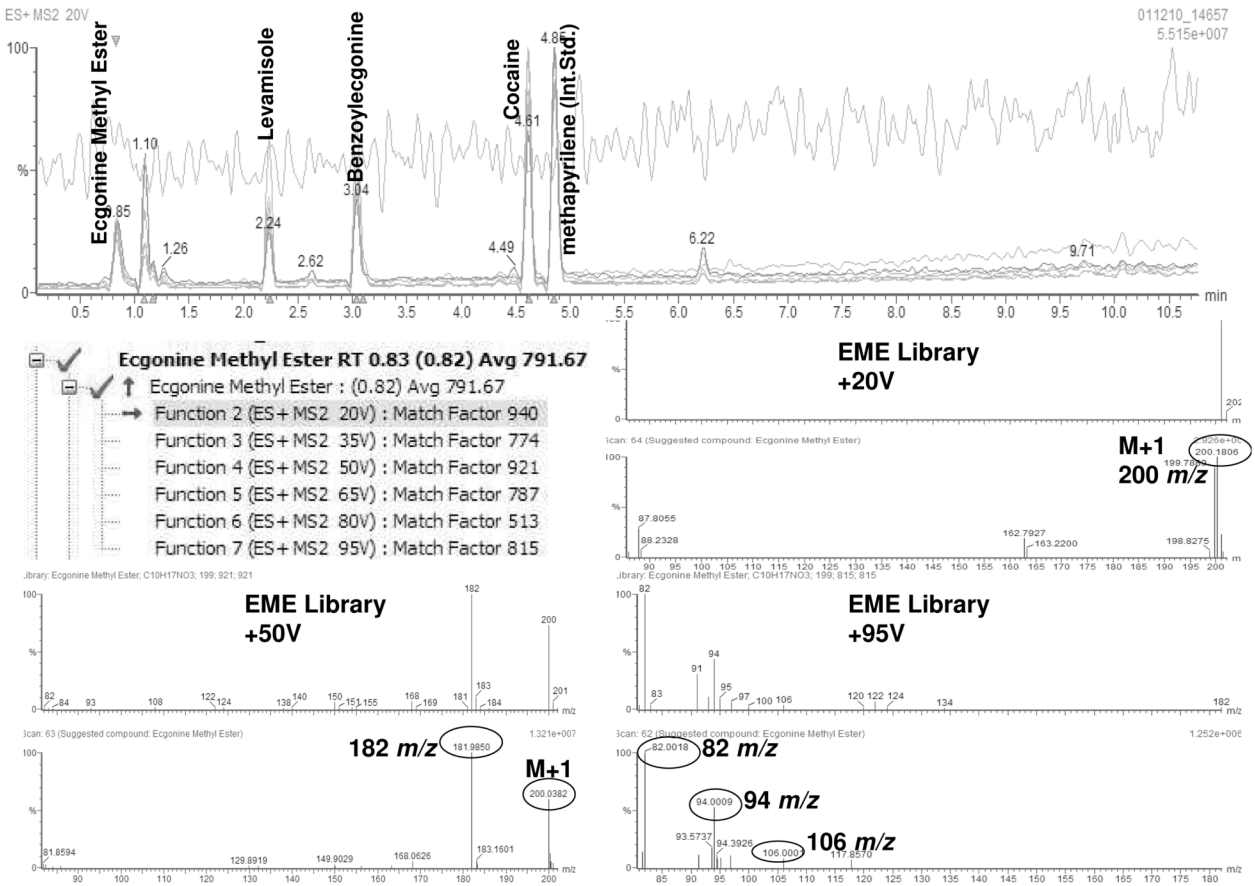

Figure 2. TICs and +20, +50, and +95 V ESI CID mass spectra for ecgonine methyl ester from analysis of postmortem blood screened positive for cocaine, cocaine metabolites, and levamisole.

represents a composite of fit criteria from each of the CID spectra. Each tentative (average fit 450–649) or positive (average fit ≥ 650) drug displayed in the TIC includes this level of mass spectral detail. In contrast with the single mass spectra generated from a 70 eV electron impact GC–MS analysis, the UPLC–MS screening assay is based upon a multi-spectra fit resulting from progressive fragmentation over incremental CID voltages. Mass spectra sets starting with a prominence of protonated molecular species at the lower cone voltages followed by increasing fragment-ions abundance at higher cone voltages is the general pattern offering specificity information for each analyte. In-source CID currently provides identity for 710 drugs and metabolites via this fragmentation pathway screening technique. Noteworthy in Figure 2 are additional unidentified TIC peaks that are detected by this non-targeted screening technology and allow for retrospective identification of unknown peaks through further development of in-house or external, adopted mass spectral libraries.

Transferability of mass spectral library data between the same instrument models was assessed by dual search of study extracts with both external and in-house generated libraries for seven analytes. Table I shows average mass spectral fit obtained by external and in-house library match and no statistically significant difference was found between library fit. The findings are consistent with reported stability of ESI CID for MS instruments with similar source design, and the results further support the multicenter use of large library repositories. Close control of mass calibration is, however, essential for reliable use of UPLC–MS spectral libraries and a mass calibration was routinely performed prior to each batch run.

Table I. Comparison of External and In-House Library Fit for UPLC–MS Analysis of Case Samples

Analyte (# of cases)	External Library Average Fit (± SD)	In-House Library Average Fit (± SD)
Amitriptyline (3)	738 (+45)	773 (+30)
Amphetamine (8)	711 (+62)	771 (+69)
Cocaethylene (3)	758 (+68)	773 (+12)
Cocaine (10)	795 (+56)	818 (+36)
Benzoylecgonine (16)	745 (+76)	753 (+70)
Fentanyl (6)	672 (+106)	725 (+126)
Quetiapine (6)	820 (+133)	816 (+42)

An MRM chromatogram for UPLC–MS–MS analysis of a positive control blood is displayed in Figure 3. The molecular transitions and abundance for each of the supplemented analytes are show in the upper table and MRM chromatograms for

#	Name	RT	1st MRM	1st Area	2nd MRM	2nd Area	Primary Flags	Actual Ratio	Expected Ratio	Failed Ratio?	S/N
1	112 Methapyrilene	5.04	262.2 > 217.2	444738	262.2 > 97	1173390	bb	0.379	0.360	NO	2902.184
2	110 Methadone	8.61	310.3>105.1	253352	310.3>265.2	337813	bb	0.750	0.830	NO	3943.783
3	87 Imipramine	8.08	281.2>57.8	139635	281.2>86.1	426119	bb	0.328	0.350	NO	107279.0...
4	14 Amphetamine	2.34	136.1 >119.1	104518	136.1 > 91.1	126305	bb	0.827	0.710	NO	1286127....
5	144 PCP	6.02	244.1> 85.7	71305	244.1>159.1	65474	bb	1.089	1.000	NO	6020.693
6	163 Propoxyphene	8.44	340.1>266.2	68386	340.1>58.2	114933	bb	0.595	0.492	NO	12693.604
7	20 Benzoylecgonine	3.03	290.1>105.1	65020	290.1>168.1	165329	bb	0.393	0.370	NO	3823.632
8	133 Nordiazepam	9.15	271.1>165.1	14295	271.1>140	19498	bb	0.733	0.700	NO	1393.665
9	123 Morphine	1.13	286.2>165.1	2733	286.2>153.1	2791	bb	0.979	0.877	NO	4573.224

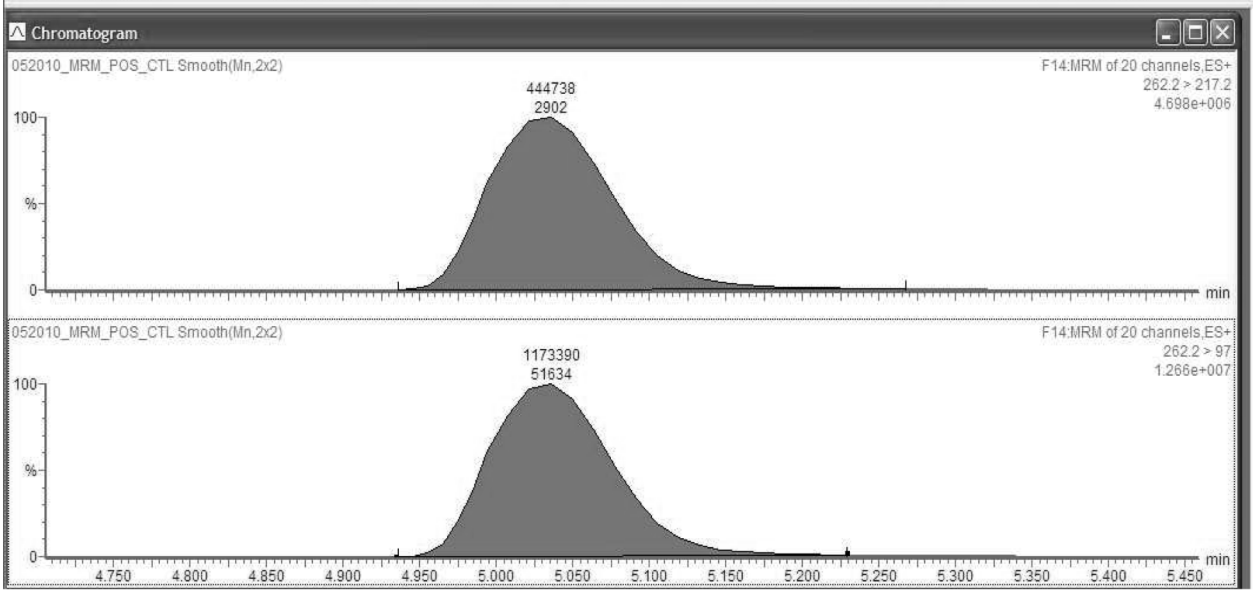

Figure 3. MRM results for positive control analysis and representative dual transition ion chromatograms for methapyrilene.

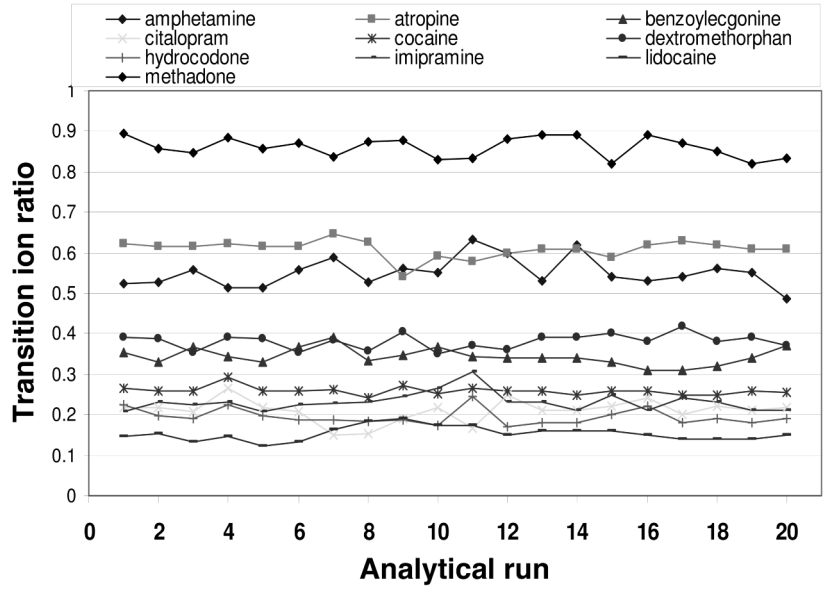

Figure 4. MRM transition ion ratio stability study over 20 runs.

the methapyrilene's dual transitions is shown in the bottom panel. The relative transition ion abundance (ion ratio) for the dual transition analysis of each analyte is shown in the upper table along with both the in-house established ion ratio and any failure to meet the European Union criteria for ion ration. In Figure 4, the reproducibility of in-house ion ratios is shown for 10 representative analytes across 20 UPLC–MS–MS analytical batch runs performed during an approximate six month period of routine operation. A major variable effecting ion ratio is the collision gas pressure in the CID quadrapole. The between run ratios shown in Figure 4 are maintained within the European Union criteria, documenting the stability of ion ratios achievable when collision gas pressure is close to the target of 4.5 mbar, as maintained during this study.

Matrix effect (i.e., ion suppression or enhancement) and extraction efficiency can influence the sensitivity of the

screening assays, and a study of these effects was performed with a subset of screened drugs as described in the materials and methods section. The upper panel in Figure 5 shows the extraction recovery of the transition ions for each analyte tested. Average transition ion recovery ranged higher than 80% for all drugs, except pseudoephedrine, morphine, and 6-acetylmorphine with extraction recovered in the range of 60–80%. As shown in the lower panel, matrix effect response was within 20% of the non-extracted neat control for all analytes except morphine and oxymorphone. Ion suppression or enhancement at the liquid chromatography ESI interface has been previously reported and has been attributed to interference from unretained polar compounds from the extraction matrix (20–22). The column-front elution of morphine and oxymorphone with respective retention times of 1.05 and 1.15 min may explain the ion suppression of these opiate.

In a study of antipsychotic drugs by LC–MS–MS, Saar and co-workers (23) reported considerable difference in extraction efficiency and in matrix effect between clinical and postmortem blood, especially in cases of postmortem decomposition. Additional extraction recovery and matrix effect studies were therefore performed comparing blood bank blood to postmortem blood from non-decomposed and decomposed cases (Tables II and III). As shown in Table II, recoveries of all tested-drugs in the five non-decomposed cases were within ±20% percent of the mean value for these cases, and recovery across all tested-drugs averaged 2–15% lower than the mean recovery in blood bank blood. The relative standard deviation indicated greater variability in extraction recovery for both decomposed and non-decomposed blood compared to blood bank blood, consistent with the greater variability of matrix in postmortem blood. In decomposed blood one of the cases (case 8) was a 20% outlier that showed lower recovery of diltiazem, quetiapine, and citalopram, and the average drug recovery in blood from decomposed cases ranged from 5 to 25% lower than recovery for blood bank blood. In this study

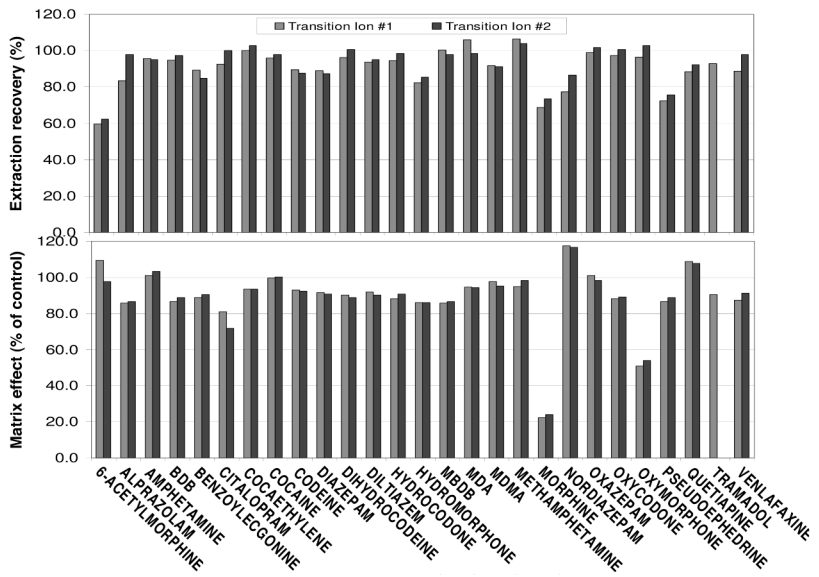

Figure 5. Extraction recovery and matrix effect for selected analytes determined by singlet UPLC–MS–MS analysis.

Table II. Percent Extraction Recovery of Drugs in Blood Bank Blood Compared With Postmortem Blood from Decomposed and Nondecomposed Cases

Sample Type		Diltiazem		Quetiapine		Citalopram		Venlafaxine		
		Ion 1	Ion 2	Ion 1	Ion 2	Ion 1	Ion 2	Ion 1	Ion 2	Tramadol Ion 1
Blood bank blood	pool mean ± SD	93.6 ± 3.1	95.1 ± 6.5	88.3 ± 1.0	92.2 ± 0.9	92.5 ± 0.2	100.1 ± 2.3	88.7 ± 0.2	97.8 ± 3.1	92.9 ± 1.9
Postmortem blood Nondecomposed	Case 1	98.1	94.7	82.1	89.4	90	87.2	85.2	96.8	88.6
	Case 2	88.6	87	80.7	77.3	97.3	92.7	88.7	91.8	97.1
	Case 3	87.2	87.4	77	80.7	92.5	91.1	84.9	88.7	88.9
	Case 4	67.6	69	54.6	56.3	63.4	63.8	75.6	71.8	63.9
	Case 5	99.7	104.6	88.8	88.4	94.8	93.3	98.7	96.5	95
Mean ± SD	Cases 1–5	88.2 ± 12.8	88.5 ± 13.1	76.6 ± 13.0	78.4 ± 13.4	87.6 ± 13.8	85.6 ± 12.4	86.6 ± 8.3	89.1 ± 10.3	86.7 ± 13.3
Postmortem blood Decomposed	Case 6	87.9	88.7	85.5	89.5	78.9	79.2	78.9	85.6	90.2
	Case 7	99.5	99.9	92.6	95.1	88	93.3	95.3	98.1	83.9
	Case 8	50.4	49.4	48.2	47.9	41.2	42.0	75.2	78.2	78.8
	Case 9	79.3	75.1	75.5	70.7	73.1	78.2	77.2	83.4	84.6
	Case 10	89.7	85.2	81.2	82.4	83.1	82.7	86.9	94.6	92.9
Mean ± SD	Cases 6–10	81.4 ± 18.7	79.7 ± 19.1	76.6 ± 17.1	77.1 ± 18.7	72.9 ± 18.5	75.1 ± 19.4	82.7 ± 8.3	88 ± 8.2	86.1 ± 5.6

Table III. Percent Change in Transition Ion Count Due to Matrix Effect in Blood Bank Blood Compared With Postmortem Blood from Decomposed and Nondecomposed Cases

Sample Type		Diltiazem		Quetiapine		Citalopram		Venlafaxine		
		Ion 1	Ion 2	Ion 1	Ion 2	Ion 1	Ion 2	Ion 1	Ion 2	Tramadol Ion 1
Blood bank blood	pool mean ± SD	91.9 ± 2.2	90.1 ± 4.2	108.8 ± 3.6	107.7 ± 2.1	81.0 ± 0.2	71.7 ± 2.9	87.4 ± 0.2	91.4 ± 2.9	90.6 ± 3.3
Postmortem blood Nondecomposed	Case 1	89.9	93.2	113.3	111.9	84.8	82.3	92.1	90.1	89.9
	Case 2	97.3	99.2	115.8	118.0	79.1	76.7	86.4	92.8	85.2
	Case 3	95.0	97.3	124.2	119.2	80.8	78.9	90.5	91.0	91.0
	Case 4	90.5	90.4	104.1	102.4	82.1	75.6	82.2	90.6	91.9
	Case 5	85.6	83.7	111.6	109.8	81.2	76.9	82.9	87.4	87.2
Mean ± SD	Cases 1–5	91.7 ± 4.6	92.7 ± 6.1	113.8 ± 7.3	112.3 ± 6.8	81.6 ± 2.1	78.1 ± 2.6	86.8 ± 4.4	90.4 ± 2.0	89.0 ± 2.8
Postmortem blood Decomposed	Case 6	85.2	84.9	105.3	101.3	73.0	71.8	80.2	84.2	80.5
	Case 7	89.1	91.1	113.2	113.4	83.1	78.9	79.1	84.6	83.7
	Case 8	75.8	76.8	97.1	98.9	78.8	76.5	69.5	73.3	72.9
	Case 9	86.1	92.1	112.5	115.3	76.6	73.1	72.7	79.9	73.0
	Case 10	91.2	93.0	125.8	122.4	86.0	83.3	83.9	86.6	85.1
Mean ± SD	Cases 6–10	85.5 ± 5.9	87.6 ± 6.8	110.8 ± 10.6	110.3 ± 9.9	79.5 ± 5.2	76.7 ± 4.6	77.1 ± 5.9	81.7 ± 5.3	79.0 ± 5.8

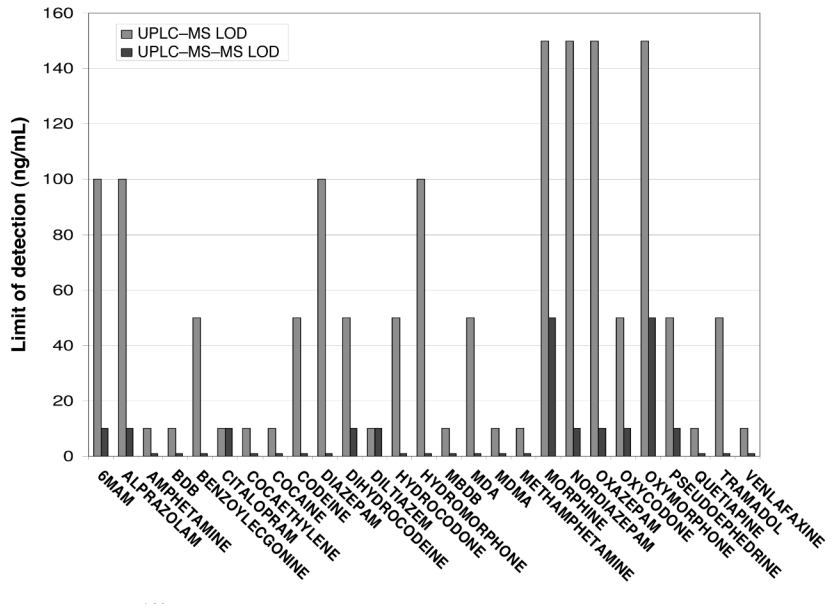

Figure 6. Limit of detection determined by experimental method for UPLC–MS and UPLC–MS–MS assays.

we find that recovery in both blood bank and postmortem blood is significantly higher than reported by others (23) but we agree that interpretive caution should be taken in the screening of blood from decomposed cases where a loss in extraction recovery may occur. The study of matrix effect as reported in Table III did not show any significant difference in the analysis of postmortem blood from either non-decomposed or decomposed cases in comparison to blood bank blood, and thus decomposition is not a major contributor to ion suppression or enhancement for the drugs tested.

LOD studies shown in Figure 6 were performed to determine the effect of factors, including matrix and extraction efficiency, on assay sensitivity. LOD for UPLC–MS–MS analysis ranged from 1 to 50 ng/mL with the higher detection limits correlating with ion suppression effects. UPLC–MS analysis showed higher LODs ranging from 10 to 150 ng/mL. Experiential data (not shown) from case studies, where quantitative confirmation testing has been performed, showed a similar range of sensitivity for additional drugs and metabolites included in the screening assays.

Postmortem case studies

The performance of GC–MS, UPLC–MS, and UPLC–MS–MS screening for specific drugs and metabolites was compared for postmortem blood obtained from 410 consecutive medical examiner toxicology cases submitted to the Forensic Toxicology Laboratory at the Albany Medical Center. A total of 140 specific drug and metabolite compounds were detected with varying frequency, giving a total of 1121 positive drug and metabolite findings with combined screening by the three methods. Table IV shows the drugs and metabolites detected for individual and combined screening approaches. UPLC–MS–MS screening showed significantly higher detection rates for drugs including acetaminophen, atropine, benzoylecgonine, fentanyl, haloperidol, metoprolol, morphine, quetiapine, and trazodone. There were a few cases of UPLC–MS–MS screening where UPLC–MS

Table IV. Specific Drug and Metabolite Positive Findings for All, Individual, and Combined Screening Methods

Drug or Metabolite	All	GC–MS	UPLC–MS	UPLC–MS–MS	GC–MS + UPLC–MS	GC–MS + UPLC–MS–MS	UPLC–MS UPLC–MS–MS
Acetylmorphine	3	2	2	1	2	3	3
7-Aminoclonazapam	5	0	4	4	4	4	5
Acetaminophen	16	2	0	16	3	16	16
Adrafinil	4	1	4	NI*	4	1	4
Ajmaline	1	0	1	NI	1	0	1
Allopurinol	1	0	1	NI	1	0	1
Alprazolam	15	12	6	10	14	15	12
Amidopyrine	1	1	1	NI	1	1	1
Amiodarone	15	0	15	10	15	11	15
Amiodarone, desethyl	3	0	3	NI	3	0	3
Amitriptyline	10	10	9	8	10	10	10
Amphetamine	2	0	2	1	2	1	2
Aripiprazole	7	0	3	7	4	7	7
Atenolol	8	0	1	7	4	7	8
Atropine	123	81	106	115	111	121	123
Benzoylecgonine	36	15	31	35	31	35	36
Benztropine	7	7	5	7	7	7	7
Buprivicane	2	2	1	2	2	2	2
Bupropion	6	6	4	6	6	6	6
Carbamazepine	2	2	2	2	2	2	2
Cetirizine	3	3	3	1	3	3	3
Chlordiazepoxide	2	2	2	1	2	2	2
Chlorpheniramine	7	6	6	4	7	6	7
Chlorpromazine	3	3	2	3	3	3	3
Chlorthalidone	1	0	1	NI	1	0	1
Citalopram	26	26	25	22	26	26	25
Citalopram, methyl	16	10	16	0	16	10	16
Clozapine	2	2	2	NI	2	2	2
Clozapine, desethyl	1	1	1	NI	1	0	1
Clozapine, desmethyl	1	0	1	NI	1	0	1
Cocaethylene	13	13	6	7	13	13	11
Cocaine	28	27	18	28	27	28	28
Codeine	3	3	2	3	3	3	3
Cyclobenzaprine	15	13	8	12	13	15	15
Cyproheptadine	1	1	0	NI	1	1	0
Dehydronifedipine	1	0	1	NI	1	0	1
Dextromethorphan	22	20	16	21	20	22	22
Diazepam	26	26	15	24	26	26	24
Dicyclomine	1	1	0	NI	1	1	0
Dihydocodeine	13	11	10	11	13	13	12
Diltiazem	9	5	7	9	8	9	9
Diphenhydramine	42	35	37	42	38	42	42
Dipyridamol	1	0	1	NI	1	0	1
Donepezil	1	1	1	NI	1	1	1
Doxepin	2	1	2	2	2	2	2
Doxylamine	11	8	7	7	9	10	10
Droperidol	1	0	0	1	0	1	1
Duloxetine	2	1	1	1	2	2	1
EDDP	14	14	10	13	14	14	14
Ecgonine methylester	2	0	2	NI	2	0	2
Etomidate	1	0	1	NI*	1	0	1

* NI = not included in the MRM.

Table IV (continued). Specific Drug and Metabolite Positive Findings for All, Individual, and Combined Screening Methods

Drug or Metabolite	All	GC–MS	UPLC–MS	UPLC–MS–MS	GC–MS + UPLC–MS	GC–MS + UPLC–MS–MS	UPLC–MS UPLC–MS–MS
Fentanyl	24	19	14	23	19	24	23
Fluconazole	4	3	4	NI	4	3	4
Flumazenil	1	0	1	NI	1	0	1
Fluoxetine	14	11	11	11	13	13	14
Fluoxetine, desmethyl	5	4	5	NI	5	4	5
Gabapentin	7	4	1	4	4	7	5
Guaiphenesin	2	1	2	NI	2	1	2
Haloperidol	5	1	3	5	5	5	5
Haloperidol, dihydro	1	0	1	NI	-1	0	1
Hydrocodone	24	23	14	18	23	24	20
Hydromorphone	2	0	2	0	2	0	2
Hydroxyzine	16	3	15	15	15	15	16
Ibuprofen	3	3	0	NI	3	3	0
Irbesartan	3	3	3	NI	3	3	3
Ketamine	1	1	1	1	1	1	1
Lamotragine	8	8	8	6	8	8	8
Levamisole	6	5	6	NI	6	5	6
Levetiracetam	10	9	7	10	9	10	10
Lidocaine	32	32	28	31	32	32	31
Lorazepam	2	1	0	1	1	2	1
Loxapine	1	1	0	1	1	1	1
MDA	2	0	1	2	1	2	2
MDMA	7	5	6	6	6	7	7
Meclizine	6	6	4	0	6	6	5
Medofinil	1	0	1	NI	1	0	1
Meloxicam	3	0	3	2	3	2	3
Meprobamate	2	2	0	2	2	2	2
Metalaxone	2	2	0	NI	2	2	0
Methadone	17	16	16	16	16	17	17
Methamphetamine	2	0	1	1	1	1	2
Methylphenidate	2	2	2	2	2	2	2
Metoclopramide	6	5	6	6	6	6	6
Metoprolol	32	12	21	29	23	30	32
Midazolam	11	11	6	11	11	11	11
Milnacipran	2	0	2	NI	2	0	2
Mirtazapine	6	6	4	3	6	6	5
Modafinil	3	3	3	NI	3	3	3
Modafinil acid	3	1	3	NI	3	1	3
Modafinil Sulfone	1	0	1	NI	1	0	1
Morphine	22	15	11	19	20	21	20
Nadolol	1	0	0	NI	0	0	1
Naloxone	8	2	8	5	8	6	8
Naphazoline	1	0	1	NI	1	0	1
Nifedipine	1	1	0	NI	1	1	0
Norbuprenorphine	1	0	1	0	1	0	1
Nordiazepam	25	25	11	22	25	25	22
Norpropoxyphene	6	6	3	5	6	6	6
Nortriptyline	12	9	10	6	12	9	12
Olanzapine	7	6	4	3	7	7	4
Orphenadrine	2	2	2	NI*	2	2	2
Oxcarbazepine	3	3	3	3	3	3	3

* NI = not included in the MRM.

showed higher rates of detection, such as 6-acetylmorphine screening where two cases were missed by UPLC–MS–MS. In these missed cases, the UPLC–MS–MS transition ions were present and abundant, but the ion-ratio criteria were not met. GC–MS did demonstrated higher sensitive for the one pentobarbital and three phenobarbital positive cases. The transition ions for barbiturates were not monitored by the UPLC–MS–MS screening, but the barbiturates were also not detected by the non-targeted UPLC–MS method due possibly to inadequate electrospray ionization at the low pH conditions of the mobile phase.

Overall screening sensitivity and specificity in the postmortem cases was compared as shown in Table V. Drug detection rate for individual methods ranged from 71 to 76%, with highest detection by UPLC–MS–MS analysis. The non-targeted GC–MS and UPLC–MS methods identified a more comprehensive range of drugs and metabolites than the UPLC–MS–MS method where 46 of the identified drug and metabolite agents

Table IV (continued). Specific Drug and Metabolite Positive Findings for All, Individual, and Combined Screening Methods

Drug or Metabolite	All	GC–MS	UPLC–MS	UPLC–MS–MS	GC–MS + UPLC–MS	GC–MS + UPLC–MS–MS	UPLC–MS UPLC–MS–MS
Oxycodone	26	22	20	20	25	26	23
Oxymorphone	2	0	1	2	1	2	2
Papaverine	4	1	2	4	2	4	4
Paroxetine	6	5	2	4	5	6	5
Pentobarbital	1	1	0	NI	1	1	0
Phenobarbital	3	3	0	NI	3	3	0
Phenothiazine	1	1	0	NI	1	1	0
Phenylpropanolamine	2	0	1	2	1	2	2
Phenytoin	3	3	0	2	3	3	2
Piroxicam	1	1	1	NI	1	1	1
Prazosine	1	0	1	NI	1	0	1
Primadone	1	1	1	1	1	1	1
Promethazine	3	3	1	1	3	3	2
Propafenone	2	0	1	1	1	1	2
Propoxyphene	8	8	4	7	8	8	7
Propranolol	1	0	1	1	1	0	1
Pseudoephedrine	8	2	7	7	7	7	8
Quetiapine	15	8	11	14	11	15	15
Quinidine	6	2	2	6	2	6	6
Resperidone	5	0	3	4	3	4	5
Resperidone, hydroxy	3	0	3	NI	3	0	3
Salbutamol	2	0	1	2	0	2	2
Sertraline	9	9	6	8	9	9	8
Sertraline, desmethyl	4	4	3	NI	4	4	0
Temazepam	3	2	2	3	3	3	3
Theophylline	16	6	15	3	15	10	15
Ticlopidine	2	2	0	NI	2	2	0
Topiramate	4	4	4	NI	4	4	4
Tramadol	7	7	6	6	7	7	6
Trazodone	17	10	13	17	14	17	17
Trihexphenidyl	2	2	0	1	2	2	1
Trimethoprim	3	3	2	3	3	3	3
Urapidol	1	0	1	NI	1	0	1
Venlafaxine	7	6	6	7	6	7	7
Venlafaxine, desmethyl	5	4	5	NI	5	4	5
Verapamil	2	2	2	2	2	2	2
Verapamil, desmethyl	3	2	3	NI	3	2	3
Warfarin	5	5	5	NI	5	0	5
Zolpidem	21	21	18	19	20	21	21

* NI = not included in the MRM.

Table V. Drug and Metabolite Detection Rate and Positive Predictive Value for Individual and Combined Screening Methods

	GC–MS	UPLC–MS	UPLC–MS–MS	GC–MS + UPLC–MS	GC–MS + UPLC–MS–MS	UPLC–MS UPLC–MS–MS
Drugs detected	796	819	856	1011	1033	1060
Detection sensitivity	71.0%	73.1%	76.4%	90.20%	92.20%	94.60%
No. of screen-positive confirmation tests performed	785	649	674	816	816	805
No. of confirmed screen-positive tests	774	646	665	804	799	794
Positive predictive value	98.60%	99.50%	98.7%	98.50%	97.90%	98.60%

were not included in the multiple reaction monitoring by UPLC–MS–MS. In the subset of monitored analytes included in the targeted UPLC–MS–MS method, a total of 1022 drugs were detected across the 3 screening methods with UPLC–MS–MS providing the highest detection rate of 84% compared to a detection rates of 72% for either GC–MS or UPLC–MS. Although a greater analytical sensitivity was observed for targeted LC–MS–MS, it should be noted that a derivatization method for GC–MS analysis was not used in this study but may have improved GC–MS assay sensitivity, especially for polarity and thermal instability agents.

The accuracy of screening by each method was assessed by concordance with confirmation testing results. Positive predictive value was evaluated for all screen-positive findings where confirmation testing was performed and was defined as the percentage of screen-positive agents that were also confirmed as positive. Table V shows that the predictive value of a positive screening result was high (98.6–99.5%) across the three screening methods. The value of combined screening was also evident from our findings as summarized in Table V, with dual combinations of methods increasing drug and metabolite detection to a range of 90–95%. Combination of non-targeted GC–MS or LC–MS analysis with targeted screening by LC–MS–MS resulted in best overall detection rates with the highest detection rate of over 95% attained with combined UPLC–MS and UPLC–MS–MS screening.

Conclusions

UPLC–MS and UPLC–MS–MS methods have been validated and are now in routine use in the postmortem drug screening program at the Albany Medical Center. The incorporation of combined UPLC–MS and UPLC–MS–MS screening into routine practice has added 30 min of analytical run time per case but has also resulted in a significant improvement in postmortem drug detection. Non-targeted UPLC–MS analysis also allows for retrospective identification of drugs and metabolites in concert with the continuing development of the mass spectral library. In addition, customization of analytes monitored by UPLC–MS–MS analysis based upon casework findings may further improve the contribution of postmortem drug testing to cause and manner of death determination.

References

1. L.J. Josephs. Characterization of over-the-counter cough/cold medications by liquid chromatography/electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **9:** 1270–1274 (1995).
2. P. Marquet, N. Venisse, É. Lacassie, and G. Lachâtre. In-source CID mass spectral libraries for the 'general unknown screening of drugs and toxicants. *Analusis* **28:** 925–934 (2000).
3. J.M. Hough, C.A Haney, R.D. Voyksner, and R.D. Bereman. Evaluation of electrospray transport CID for the generation of searchable libraries. *Anal. Chem.* **72:** 2265–2270 (2000).
4. A. Schreiber, J. Efer, and W. Englewald. Application of spectral libraries for high-performance liquid chromatography–atmospheric pressure ionisation mass spectrometry to the analysis of pesticide and explosive residues in the environment. *J. Chromatogr. A* **869:** 411–425 (2000).
5. W. Weinmann, M. Stoertzel, S. Vogt, and J. Wendt. Tune compounds for electrospray ionization/in-source collision-induced dissociation with mass spectral library searching. *J. Chromatogr. A* **926:** 199–209 (2001).
6. W. Weinmann, A. Weidemann, B. Eppinger, and M. Renz. Screening for drugs in serum by electrospray ionization/collision-induced dissociation and library searching. *Am. Soc. Mass Spectrom.* **10:** 1028–1037 (1999).
7. L. Humbert, F. Grisel, C. Richeval, and M. Lhermitte. Screening of xenobiotics by ultra-performance liquid chromatography–mass spectrometry using in-source fragmentation at increasing cone voltages: library constitution and an evaluation of spectral stability. *J. Anal. Toxicol.* **34:** 1–10 (2010).
8. W. Weinmann, N. Lehmann, M. Renz, A. Wiedemann, and M. Svoboda. Screening for drugs in serum and urine by LC/ESI/CID-MS and MS/MS with library searching. *J. Forensic Sci.* **42:** 202–208 (2000).
9. M. Gergov, I. Ojanperä, and E. Vuori. Simultaneous screening for 238 drugs in blood by liquid chromatography–ionspray tandem mass spectrometry with multiple-reaction monitoring. *J. Chromatogr. B* **795:** 41–53 (2003).
10. P. Marquet, F. Saint-Marcoux, T.N. Gamble, and J.C.Y. Leblanc. Comparison of a preliminary procedure for the general unknown screening of drugs and toxic compounds using a quadrupole-linear ion-trap mass spectrometer with a liquid chromatography–mass spectrometry reference technique. *J. Chromatogr. B* **789:** 9–18 (2003).
11. C.A. Mueller, W. Weinmann, S. Dresen, A. Schreiber, and M. Gergov. Development of a multi-target screening analysis for 301 drugs using a QTrap liquid chromatography/tandem mass spectrometry system and automated library search. *Rapid Commun. Mass Spectrom.* **19:** 1332–1338 (2005).
12. S. Dresen, J. Kempf, and W. Weinmann. Electrospray-ionization MS/MS library of drugs as database for method development and drug identification. *Forensic Sci. Int.* **166:** 86–91 (2006).

13. F.-L. Sauvage, F Saint-Marcoux, B. Duretz, D. Deporte, G. Lachatre, and P. Marquet. Screening of drugs and toxic compounds with liquid chromatography–linear ion trap tandem mass spectrometry. *Clin. Chem.* **52:** 1735–1742 (2006).
14. S. Dresen, N. Ferreirós, H. Gnann, and R. Zimmermann. Detection and identification of 700 drugs by multi-target screening with a 3200 Q TRAP LC–MS/MS system and library searching. *Anal. Bioanal. Chem.* **396:** 2425–2434 (2010).
15. S. Dresen, M. Gergov, L. Politi, C. Halter, and W. Weinmann. ESI-MS/MS library of 1,253 compounds for application in forensic and clinical toxicology. *Anal. Bioanal. Chem.* **395:** 2521–2526 (2009).
16. F. Saint-Marcoux, G. Lachâtre, and P. Marquet. Evaluation of an improved general unknown screening procedure using liquid-chromatography–electrospray-mass spectrometry by comparison with gas chromatography and high-performance liquid-chromatography–diode array detection. *J. Am. Soc. Mass Spectrom.* **14:** 14–22 (2003).
17. G.L. Herrin, H.H. McCurdy, and W.H. Wall. Investigation of an LC–MS–MS (QTrap) method for the rapid screening and identification of drugs in postmortem toxicology whole blood samples. *J. Anal. Toxicol.* **29:** 599–606 (2005).
18. European Union Decision2002/657/EC 17.8.2002. *Off. J. Eur. Commun.* **221:** 8–36 (2002).
19. L. Rivier. Criteria for the identification of compounds by liquid chromatography–mass spectrometry and liquid chromatography–multiple mass spectrometry in forensic toxicology and doping analysis. *Anal. Chim. Acta* **492:** 69–82 (2003).
20. E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, and J.R. Mazzeo. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J. Chromatogr. B* **852:** 22–34 (2007).
21. C. Müller, P. Schäfer, M. Störtzel, S. Vogt, and W. Weinmann. Ion suppression effects in liquid-electrospray-ionization transport region collision induced dissociation mass spectrometry with different serum extraction methods for systematic toxicological analysis with mass spectra libraries. *J. Chromatogr. B* **733:** 47–52 (2002).
22. R. Diems, M.A. Huestis, W.E. Lambert, and C.M. Murphy. Matrix effect in bio-analysis of illicit drugs with LC–MS/MS: influence of ionization type, sample preparation, and biofluid. *J. Am. Soc. Mass Spectrom.* **14:** 1290–1294 (2003).
23. E. Saar, D. Gerostamoulos, and O.H. Drummer. Comparison of extraction efficiencies and LC–MS–MS matrix effects using LLE and SPE methods for 19 antipsychotics in human blood. *Anal. Bioanal. Chem.* **393:** 727–734 (2009).

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