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Special Issue

Multi-drug and Metabolite Quantification in Postmortem Blood by Liquid Chromatography–High-Resolution Mass Spectrometry: Comparison with Nominal Mass Technology

Thomas G. Rosano[1]*, Seo Na[1], Kenneth Ihenetu[2], Thomas A. Swift[1] and Michelle Wood[3]

[1]Forensic Toxicology Laboratory, Department of Pathology and Laboratory Medicine, Albany Medical Center Hospital and College, 43 New Scotland Avenue, Albany, NY 12208, USA, [2]Department of Health Sciences, Albany College of Pharmacy and Health Sciences, Albany, NY, USA, and [3]Waters Corporation, MS Technologies Centre, Atlas Park, Wythenshawe, Manchester M22 5PP, UK

*Author to whom correspondence should be addressed. Email: rosanot@mail.amc.edu

High-resolution mass spectrometry (HRMS) is being applied in postmortem drug screening as an alternative to nominal mass spectrometry, and additional evaluation in quantitative casework is needed. We report quantitative analysis of benzoylecgonine, citalopram, cocaethylene, cocaine, codeine, dextromethorphan, dihydrocodeine, diphenhydramine, 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine, hydrocodone, hydromorphone, meperidine, methadone, morphine, oxycodone and oxymorphone in postmortem blood by ultra-performance liquid chromatography (UPLC)–MS^E/time-of-flight (TOF). The method employs analyte-matched deuterated internal standardization and MS^E acquisition of precursor and product ions at low (6 eV) and ramped (10–40 eV) collision energies, respectively. Quantification was performed using precursor ion data obtained with a mass extraction window of ±5 ppm. Fragment and residual precursor ion acquisitions at ramped collision energies were evaluated as additional analyte identifiers. Extraction recovery of >60% and matrix effect of <20% were determined for all analytes and internal standards. Defined limits of detection (10 ng/mL) and quantification (25 ng/mL) were validated along with a linearity analytical range of 25–3,000 ng/mL ($R^2 > 0.99$) for all analytes. Parallel UPLC–MS^E/TOF and UPLC–MS/MS analysis showed comparable precision and bias along with concordance of 253 positive ($y = 1.002x + 1.523$; $R^2 = 0.993$) and 2,269 negative analyte findings in 159 postmortem cases. Analytical performance and correlation studies demonstrate accurate quantification by UPLC–MS^E/TOF and extended application of HRMS in postmortem casework.

Introduction

Drug and metabolite (analytes) findings in medical examiner cases often require quantitative analysis for interpretive assistance in determination of cause and manner of death. The proliferation of pharmaceutical and designer agents identified in drug-related deaths, as well as the increase in analyte detection by newer screening methods (1, 2), has added to the quantitative work in postmortem toxicology laboratories. In an effort to improve workflow and reduce the number of analytical methods, multi-analyte testing has been used in forensic laboratories for simultaneous quantification of frequently detected analytes as well as for expanded drug classes.

Liquid chromatographic separation by either high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) technology coupled with MS/MS detection in selective reaction monitoring (SRM) mode is a widely used analytical technique in multi-analyte quantification. SRM employs selective monitoring of precursor–product ion transitions, and multiple transitions are now used routinely in practice to improve specificity of analysis. Dedicated scanning time is needed for each transition ion during the MS/MS duty cycle in order to achieve adequate chromatographic peak resolution and a sufficient number of data points to achieve reproducible quantitative analysis. Multi-analyte analysis by SRM, therefore, requires careful selection of precursor–product transitions, optimization of respective collision energies and use of retention time-based SRM windows for multi-analyte subsets. Investigation of high-resolution mass spectrometry (HRMS) as an alternative technology for multi-analyte quantification is warranted in light of enhanced selectivity of accurate mass analysis, availability of full-spectrum data without duty cycle limitation and growing access to the technology.

Application of HRMS in forensic toxicology is currently focused on drug screening where instrumental gains in ion resolution and mass accuracy allow sensitive and comprehensive analyte detection (1, 3–16), along with the potential for elemental and structural identification of agents (17–19). In contrast to MS/MS detection in SRM mode, HRMS analysis is performed primarily in a non-targeted single-stage mode using post-acquisition extraction of analyte and internal standard ions. The gain in mass resolution allows post-acquisition selection of quantifier and qualifier ions through a narrow mass extraction window (MEW) that cannot be achieved by nominal mass detection. The multiplex acquisition of a full mass spectrum in single-stage mode also eliminates the need for analyte-specific MS/MS method development and duty cycle limitations.

Quantitative application of HRMS coupled with either HPLC or UPLC has already been demonstrated in other areas of drug testing using the non-targeted acquisition and ion extraction approach. Zhang *et al.* (20) evaluated quantitative HPLC–HRMS analysis of five drugs in plasma, using an early-generation HRMS technology and a relatively large MEW of ±100 millimass units (mmu). With only a 5-fold narrowing of the ±500 mmu MEW achievable by nominal mass analysis, the HRMS method showed a limited dynamic range due to detector saturation. With advances in HRMS instrumentation, O'Connor *et al.* (21) were able to narrow the MEW to ±25 mmu in their UPLC–HRMS analysis of 14 test compounds in a drug turnover study that showed quantitative data equivalent to HPLC–MS/MS analysis.

A reduction in endogenous ion interference with progressive narrowing of the MEW has been demonstrated in a number of studies (21–23). Criteria for optimizing a ppm-normalized MEW in order to achieve efficient mass resolution from endogenous interferences, along with maximizing acquisition of analyte ions, have been reported (24). Using the range of HRMS

resolution achieved with Orbitrap and time-of-flight (TOF) technology, Kaufmann *et al.* (25) found equal or slightly better quantitative performance with UPLC–HRMS analysis of drugs in tissue when compared with UPLC–MS/MS. Additional studies by this group showed that analytes with poor fragmentation efficiency that limited the abundance of transition product ions measured by nominal mass SRM analysis were more reproducibly quantified with HRMS, where quantitation was selectively performed with the molecular species (26). Accurate quantification of veterinarian drugs by UPLC–HRMS has also been reported (27), and additional comparative studies of multi-drug quantification in plasma by HPLC–HRMS versus HPLC–MS/MS analysis have further demonstrated equivalency in analytical performance, including a comparable linear dynamic range (28–30). As a result of these studies and the increasing availability of HRMS detection in the drug discovery industry, a paradigm shift in mass detection from nominal to high resolution has been reported in a number of bio-analytical reviews (31, 32).

A novel HRMS ion acquisition technique initially reported by Plumb *et al.* (33) called MS^E allows dual ion acquisition with low and high collision-induced dissociation energies for optimum detection of precursor and product ions in single-stage mode. MS^E acquisition of a molecular quantification ion and its isotopes occurs at low collision energy, along with co-acquisition of residual molecular ion and fragment ions at increasing collision energies. To date, there are few reports of quantitative application of HRMS with MS^E acquisition forensic casework (34), and additional analytical performance studies along with comparative evaluation in postmortem matrices are needed. We report the development of a UPLC–MS^E/TOF method for simultaneous quantification of 16 analytes in postmortem blood. Analytes include a panel of opiate/opioid agents along with other drugs frequently confirmed and quantified in the Forensic Toxicology Laboratory at the Albany Medical Center. The validation studies include comparison of method precision, accuracy and correlation with UPLC–MS/MS analysis.

Materials and methods

Chemicals, reagents and quality control material

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, USA). AR grade hydrochloric acid, ammonium hydroxide and acetic acid were from Mallinckrodt, Inc. (Paris, NY, USA). Formic acid and leucine enkephalin were obtained from Sigma Aldrich (St Louis, MO, USA). Deionized water was produced by combined ultra-filtration (AquaPure, Schenectady, NY, USA) and reverse osmosis (Alpha Water Systems, Montague, NJ, USA) treatment.

For preparation of blood-based calibrator and control material, separate sources of reference material for benzoylecgonine, citalopram, cocaethylene, cocaine, codeine, dextromethorphan, dihydrocodeine, diphenhydramine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), hydrocodone, hydromorphone, meperidine, methadone, morphine, oxycodone and oxymorphone reference material were obtained from Cerilliant (Round Rock, TX, USA) and Alltech Associates, Inc. (Deerfield, IL, USA) and were stored at −10°C. Multi-analyte reference stock solutions were prepared in methanol at analyte concentrations of 10,000 ng/mL and stored at −10°C. Working calibrators were prepared by supplementing negative blood bank blood at analyte concentrations of 25, 50, 150, 500, 1,000, 2,000 and 3,000 ng/mL, and additional negative blood bank blood was supplemented at concentrations of 10 and 5 ng/mL for assessment of lower limit of detection. For assessment of carryover and dilution-integrity negative blood bank blood was spiked at a concentration of 5,000 ng/mL. Quality control material was prepared by supplementing negative blood bank blood with an alternate stock solution of multi-analytes at concentrations of 40, 125, 1,600 and 2,500 ng/mL. Deuterated internal standards obtained from Cerilliant and used in the analysis included benzoylecgonine D^3, citalopram D^6, cocaethylene D^3, cocaine D^3, codeine D^3, dextromethorphan D^3, dihydrocodeine D^6, diphenhydramine D^3, EDDP D^3, hydrocodone D^3, hydromorphone D^3, meperidine D^4, methadone D^3, morphine D^3, oxycodone D^3 and oxymorphone D^3. A combined stock of deuterated internal standards was prepared in methanol at a concentration of 5,000 ng/mL and stored at −10°C. Additional deuterated internal standards, codeine D^6 and hydrocodone D^6, were obtained from Cerriliant and used in the evaluation of available diagnostic fragment ions during single-stage UPLC–MS^E/TOF analysis.

Mass calibration for UPLC–MS/MS analysis was performed with 2 mM ammonium acetate and 50 mg/L PEG 400 in

Table I
Summary of molecular elemental composition and exact mass of molecular, isotope and fragmentions

Analytes	Elemental composition	Molecular ion (M)	M + 1 isotope	M + 2 isotope	Fragment ion 1	Fragment ion 2	Fragment ion 3
Benzoylecgonine	$C_{16}H_{19}NO_4$	290.1392	291.1425	292.145	168.1025	82.0657	150.0919
Citalopram	$C_{20}H_{21}FN_2O$	325.1716	326.1748	327.1778	109.0454	262.1032	234.0719
Cocaethylene	$C_{18}H_{23}NO_4$	318.1705	319.1738	320.1764	196.1338	82.0657	150.0919
Cocaine	$C_{17}H_{21}NO_4$	304.1549	305.1582	306.1607	182.1181	82.0657	150.0919
Codeine	$C_{18}H_{21}NO_3$	300.16	301.1632	302.166	199.0759	243.1021	282.1494
Dextromethorphan	$C_{18}H_{25}NO$	272.2015	273.2046	274.2077	215.1436	171.0804	213.1279
Dihydrocodeine	$C_{18}H_{23}NO_3$	302.1756	303.1789	304.1816	199.0759	227.1072	245.1178
Diphenhydramine	$C_{17}H_{21}NO$	256.1701	257.1737	258.1763	167.0861	152.0626	165.0704
EDDP	$C_{20}H_{23}N$	278.1909	279.1942	280.1974	186.1283	234.1283	249.1517
Hydrocodone	$C_{18}H_{21}NO_3$	300.16	301.1632	302.166	199.0759	171.0809	241.0862
Hydromorphone	$C_{17}H_{19}NO_3$	286.1443	287.1476	288.1503	185.0603	227.0706	199.0759
Meperidine	$C_{15}H_{21}NO_2$	248.1651	249.1683	250.1711	220.1338	174.1283	131.0861
Methadone	$C_{21}H_{27}NO$	310.2171	311.2204	312.2235	265.1592	223.1123	195.1174
Morphine	$C_{17}H_{19}NO_3$	286.1443	287.1476	288.1503	201.0916	165.0704	229.0865
Oxycodone	$C_{18}H_{21}NO_4$	316.1549	317.1582	318.1608	298.1443	256.1338	241.1103
Oxymorphone	$C_{17}H_{19}NO_4$	302.1392	303.1425	304.1451	284.1287	227.0946	242.1181

acetonitrile–water (50 : 50 v/v), and for UPLC–MS^E/TOF, it was performed with 5 mM sodium formate in 2-propanol–water (90 : 10 v/v). Lock mass calibrant for UPLC–MS^E/TOF analysis was leucine enkephalin at a stock concentration of 400 mg/L in water with storage of aliquots at −20°C for up to 6 months. Working leucine enkephalin (2 mg/L) was prepared by further dilution of the stock solution with acetonitrile–water (50 : 50 v/v) containing 0.1% formic acid. A mixed solvent with ammonium hydroxide–isopropanol–methylene chloride (2 : 20 : 80 v/v/v) was prepared for extraction-column elution of analytes. For all 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)

Table II

Summary of UPLC–MS/MS transition ions, cone voltages (V) and collision energies (eV) for analytes and their deuterated internal standards

Analytes	Analyte transition 1	Cone (V)	CE (eV)	Analyte transition 2	Cone (V)	CE (eV)	Int. std transition	Cone (V)	CE (eV)
Benzoylecgonine	290.1 > 168.1	30	20	290.1 > 105.1	30	33	293.1 > 171.0	30	20
Citalopram	325.2 > 109.1	40	25	325.2 > 262.1	40	25	331.2 > 109.1	40	25
Cocaethylene	318.2 > 196.2	35	25	318.2 > 82.1	35	35	321.2 > 199.2	35	25
Cocaine	304.2 > 182.2	45	18	304.2 > 105.1	45	30	307.2 > 185.1	40	20
Codeine	300.1 > 215.1	50	25	300.1 > 199.2	50	27	303.1 > 215.1	50	25
Dextromethorphan	272.2 > 171.1	40	30	272.2 > 213.2	40	30	275.2 > 171.1	40	30
Dihydrocodeine	302.3 > 199.1	55	34	302.3 > 201.2	55	30	308.2 > 202.0	50	35
Diphenhydramine	256.2 > 167.1	20	20	256.2 > 152.1	20	35	259.2 > 167.0	20	15
EDDP	278.2 > 234.2	40	20	278.2 > 186.2	40	25	281.1 > 234.2	40	20
Hydrocodone	300.1 > 199.1	50	40	300.1 > 171.1	50	55	303.1 > 199.1	50	30
Hydromorphone	286.1 > 185.1	55	30	286.1 > 157.1	55	38	289.1 > 185.1	55	30
Meperidine	248.2 > 174.1	35	19	248.2 > 220.1	35	20	252.1 > 178.1	40	20
Methadone	310.3 > 265.2	25	14	310.3 > 105.1	25	32	313.3 > 268.2	22	22
Morphine	286.1 > 165.1	55	40	286.1 > 153.1	55	40	289.2 > 165.0	55	35
Oxycodone	316.1 > 241.2	35	30	316.1 > 256.2	35	30	319.2 > 301.1	35	25
Oxymorphone	302.2 > 227.2	35	30	302.2 > 284.2	35	30	305.2 > 287.2	35	30

Dwell time of 0.010 s was used for all transitions.

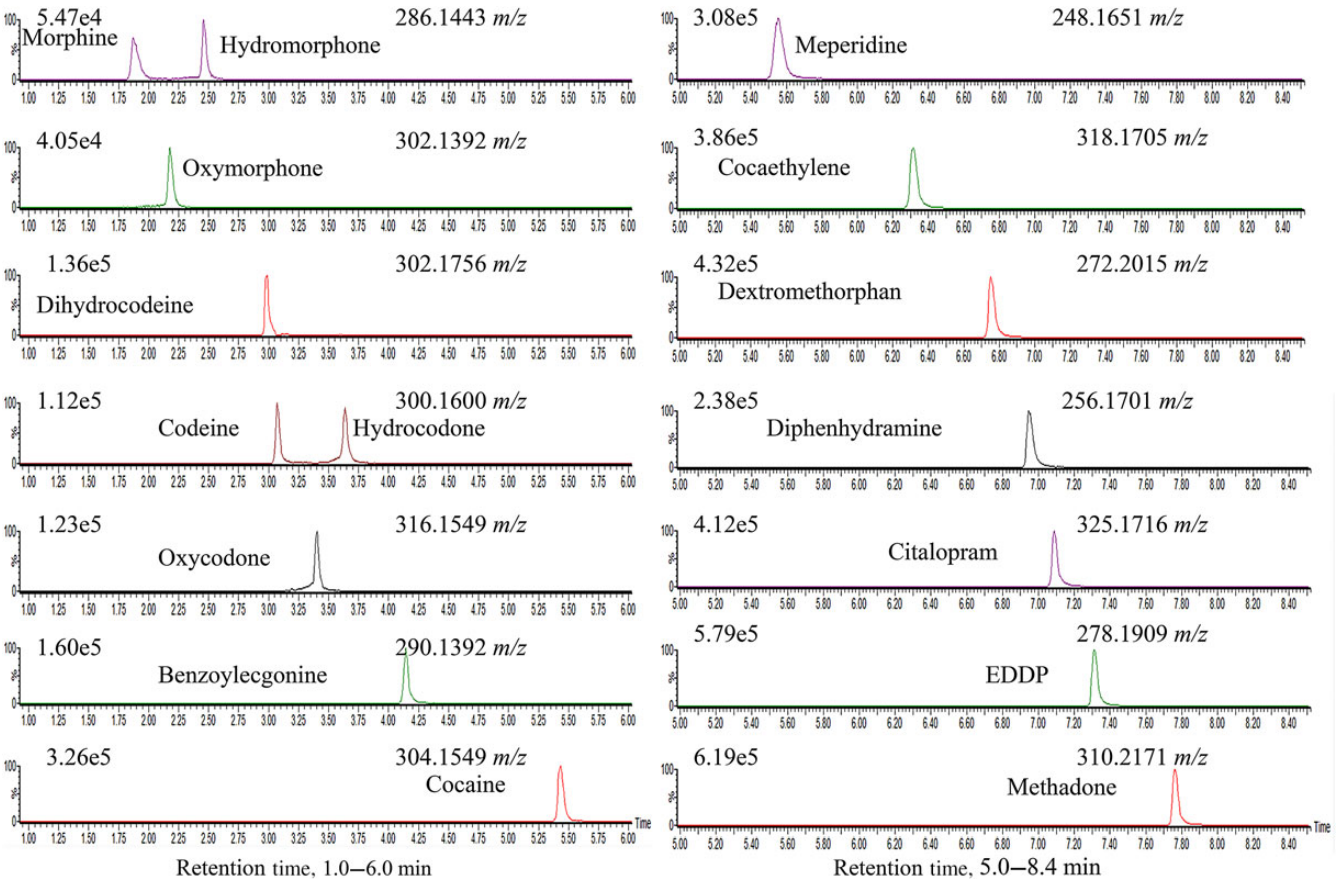


Figure 1. Extracted ion chromatograms (±5 ppm) of molecular precursor ions acquired in function 1 by UPLC–MS^E/TOF analysis of analyte-negative blood supplemented with 16 analytes at a concentration of 500 ng/mL. The exact mass and ion counts are also shown.

were prepared along with a starting mobile phase solution containing 98% mobile phase A and 2% mobile phase B.

Medical examiner case samples

Postmortem cases used in the comparison quantification by UPLC–MS^E/TOF and UPLC–MS/MS analysis had initially screened positive for analytes based on qualitative UPLC–MS and UPLC–MS/MS methods previously described (1, 2) and had been subsequently confirmed by the validated, quantitative UPLC–MS/MS method that is used routinely in the Forensic Toxicology Laboratory at the Albany Medical Center. The discarded extracts from UPLC–MS/MS quantification were de-identified and reanalyzed by UPLC–MS^E/TOF in accordance with an Albany Medical College IRB reviewed protocol.

Analytical methods

Extraction procedure

Blood samples were extracted using Clean Screen extraction columns (ZSDAU020) obtained from United Column Technology (Bristol, PA) and preconditioned sequentially with 3 mL methanol, 3 mL water and 1 mL 0.1 M phosphate buffer (pH 6.0). Stock solution of deuterated internal standard (30 μL) was added to 1 mL of blood calibrators, controls and case samples followed by sonication for 15 min. Following addition of 2 mL of 0.1 M phosphate buffer with any needed adjustment to pH 6.0 ± 0.5, the samples were loaded onto extraction columns at an aspiration rate of 1 mL/min, followed by sequential washing with 2 mL deionized water, 2 mL 0.1 N HCl and 2 mL methanol, allowing complete aspiration of each washing with final full vacuum drying for 15 min. Analytes were eluted from the columns with 3 mL of eluting solvent. Dried extracts were reconstituted with 150 μL of starting mobile phase mixture and analyzed by the standard UPLC–MS/MS method followed by UPLC–MS^E/TOF.

UPLC–MS^E/TOF method

UPLC–MS^E/TOF analysis was performed using a Waters Acquity UPLC interfaced with a hybrid quadrupole TOF mass spectrometry (Waters Xevo™ G2 QTOF) using electrospray ionization (ESI) in positive mode. MassLynx™ (4.1 SCN 802) was used for control of systems operation. UPLC separation was performed on a trifunctional C18 alkyl-bonded stationary phase column (HSS T3, 1.8 μm, 2.1 × 50 mm Waters) maintained at 30°C. After 2.5 min of isocratic mobile phase conditions (2% B), linear gradients for 2.5 min (10% B), 5.5 min (22% B), 7.5 min (42% B), 8 min (95% B) and 10 min (98% B) were used with a return to isocratic 2% B conditions at 12 min. A 500-μL flow rate per

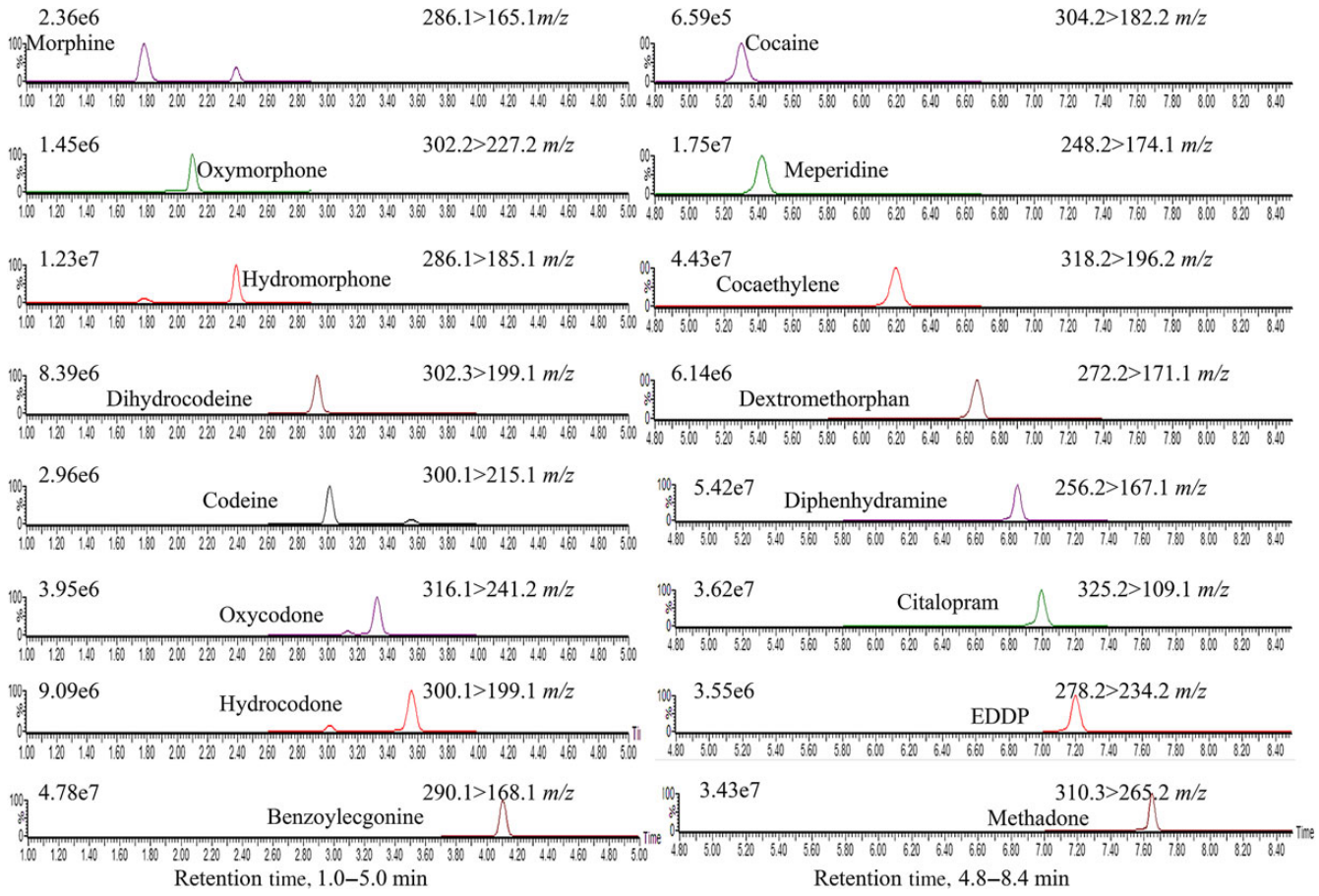


Figure 2. SRM ion chromatogram of quantifier transition ion used for quantitative analysis of 16 analytes by UPLC–MS/MS analysis.

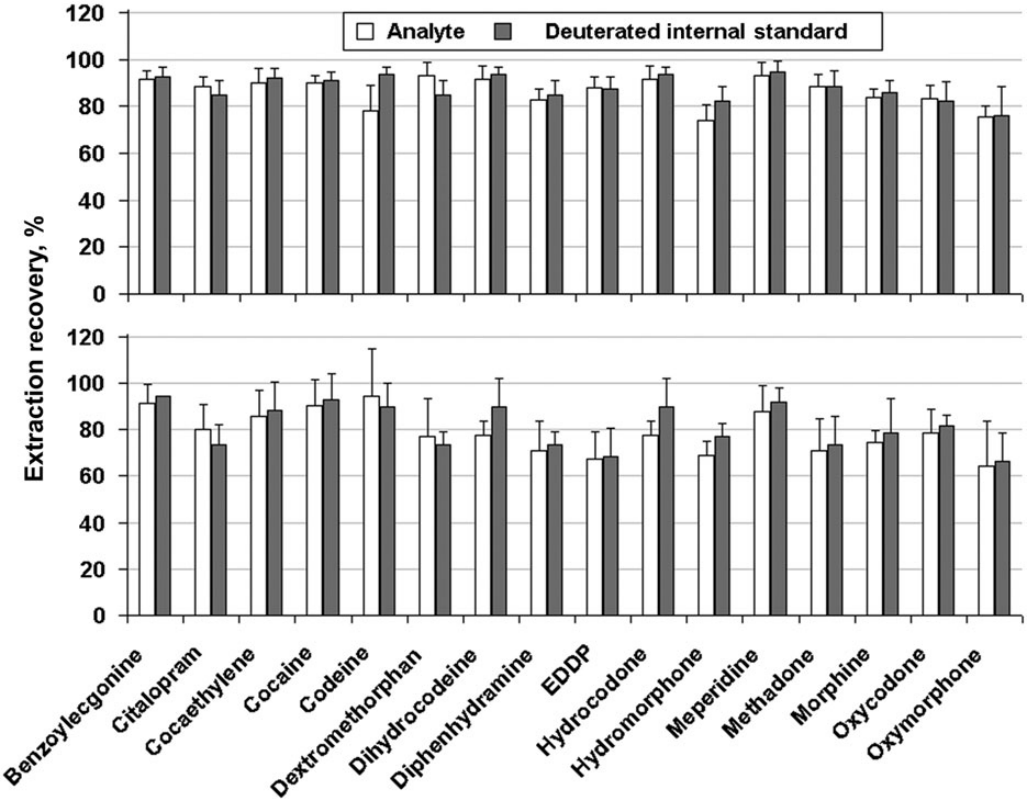


Figure 3. Solid phase extraction efficiency determined by UPLC–MS^E/TOF analysis of 16 analytes and respective deuterated internal standards at analyte concentrations of 100 ng/mL (lower panel) and 1,000 ng/mL (upper panel) with SD error bars.

minute and total analytical run time of 15 min were used. Xevo™ G2 QTOF with a working mass resolution of ~20,000 full-width half-maximum was operated with a capillary voltage (1.0 kV), sample cone voltage (20 V), desolvation temperature (500°C), desolvation gas flow (600 L/h), source temperature (120°C) and collision gas (argon) pressure closely maintained at about 0.4 Pa. Detector check, lock mass check and mass calibration were performed prior to each analysis batch.

Ion acquisition was performed at a rate of 10 spectra per second in centroid mode from m/z 50–1,000 using MS^E functions. MS^E function 1 was acquired with collision energy of 6 eV and MS^E function 2 ramped from 10 to 40 eV. A second ion source was used for baffled entry of the reference solution (leucine enkephalin) at a flow rate of 5 µL/min and a 25-s interval acquisition (function 3). Data analyzed by TargetLynx™ software (Waters) used molecular ion and deuterated molecular ion data acquired in function 1 for quantification. Weighted (1/X) least squares regression analysis was employed as the calibration model and acceptance criteria included a coefficient of determination (R^2) of ≥0.99, each calibrator within 20% of regression line and quality control results within 20% of target. Identification criteria included analyte retention time within 0.3 min of a reference analyte analysis, detection of molecular ion and molecular ion isotopes M + 1 and M + 2 along with available diagnostic fragment ions, all within ±5 ppm of exact mass. Table I lists the exact mass of molecular, isotope and major fragment ions. Exact mass of molecular and isotope ions was determined from elemental composition and atomic frequency of isotopes in nature. Determination of exact mass for the fragment ions required the analysis of experimental mass spectra using elemental composition and MassFragment™ software (Waters).

UPLC–MS/MS method

UPLC–MS/MS analysis was performed with a Waters (Milford, MA) Acquity UPLC system interfaced with a nominal mass TQ Detector, using ESI in positive ion mode. MassLynx™ (4.1 SCN 714) was used for systems operations. UPLC column, temperature, mobile phase gradient and flow rate conditions were identical to the UPLC–MS^E/TOF method described above. MS/MS instrumentation conditions include capillary voltage (3.0 kV), desolvation temperature (500°C), desolvation gas flow (1,200 L/h), source temperature (150°C) and collision gas (argon) pressure closely maintained at about 0.4 Pa. SRM with two transitions ions per analyte and one transition per internal standard was used with a dwell time of 0.01 s per transition and six retention-based scanning windows. Cone voltage and collision energy conditions for analyte and internal standard transition ions are shown in Table II. Data acceptance criteria included calibration curve $R^2 \geq 0.99$, individual calibrators within 20% of regression line, quality control results within 20% of target and an acceptable ratio of transition ions based upon European Union criteria (35, 36). Mass calibration was performed prior to each analytical run. The TargetLynx™ calibration model and criteria were the same as described for the UPLC–MS^E/TOF method.

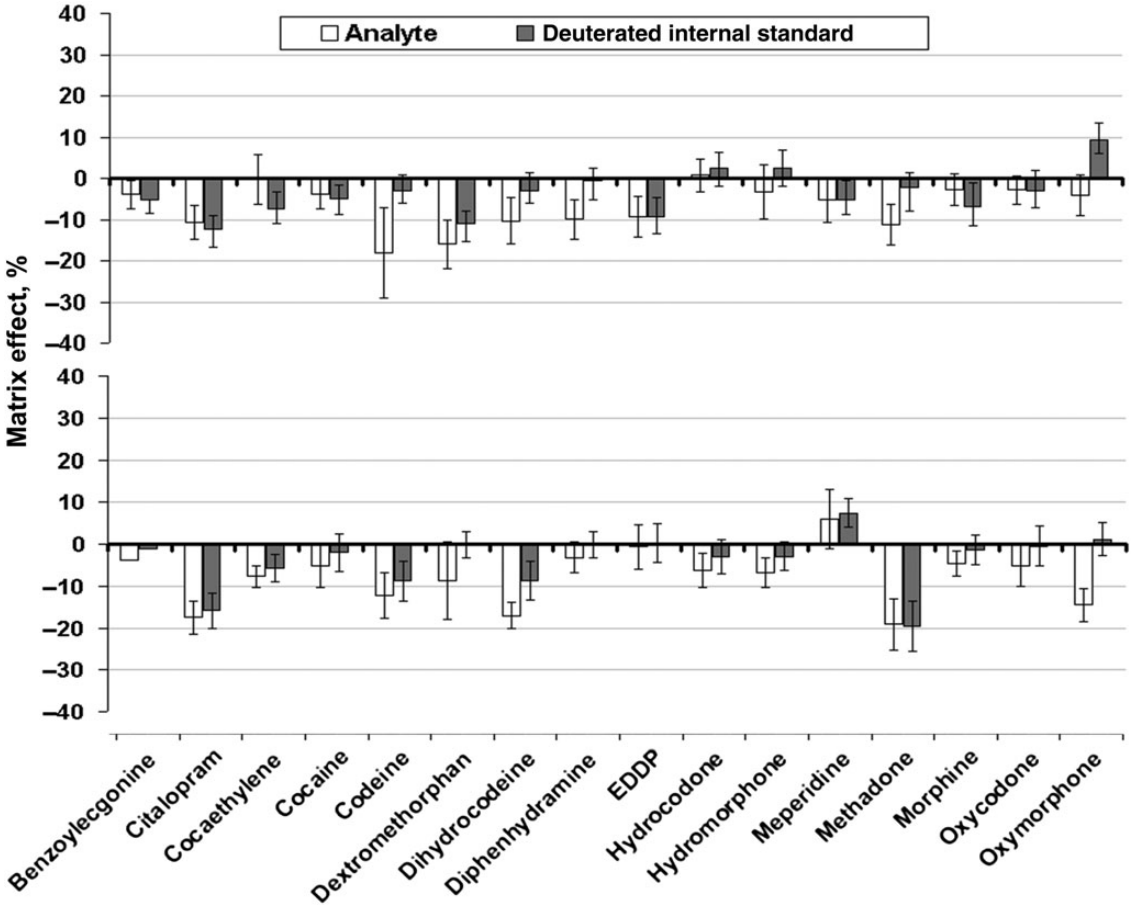


Figure 4. Blood matrix effect by UPLC–MS^E/TOF analysis of 16 analytes and respective deuterated internal standards at analyte concentrations of 100 ng/mL (lower panel) and 1,000 ng/mL (upper panel) with SD error bars.

UPLC–MS^E/TOF method validation plan

The validation plan was based primarily upon the SWGTOX guidelines that have been recently published (37). Column recovery and matrix effects for UPLC–MS^E/TOF analysis were assessed at concentrations of 100 and 1,000 ng/mL for both analytes and deuterated internal standards. Blood from six donors without a history of using the drugs tested for in the assay were obtained and verified as negative by analysis. A portion of each blood pool was supplemented before extraction with reference analytes and deuterated internal standards at concentrations of 100 and 1,000 ng/mL prior to analysis as pre-spiked blood. Neat samples were prepared in starting mobile phase with reference analytes and deuterated internal standards also at concentrations of 100 and 1,000 ng/mL. The pre-spiked blood samples were extracted along with negative blood samples that were supplemented after the extraction with equivalent 100 and 1,000 ng/mL concentrations of analytes and internal standards (post-spiked samples). All pre-spiked, neat and post-spiked samples were co-analyzed by UPLC–MS^E/TOF with analysis of molecular ion area data (acquisition 1) for each analyte and internal standard. Percent column recovery of analytes and internal standards for each blood matrix was calculated by multiplying the corresponding ratio of pre-spiked to post-spiked ion area by 100. Percent matrix effect for each analyte and internal standard was determined by the following ion area calculation: (post-spiked area/neat ion areas − 1) × 100. The mean and standard deviation for column recovery and ionization matrix effect was calculated for the six matrices. Extraction recovery >50% along with ion suppression or enhancement of <25% was considered acceptable.

The limit of quantitation (LOQ) range of the method (25–3,000 ng/mL) was defined by a seven-point calibration model and was evaluated in multiple batch analyses using criteria for least squares regression ($R^2 > 0.99$), control sample analysis (≤20% of target concentration) and individual calibrator deviation from regression line (≤20%). An limit of detection (LOD) of 10 ng/mL for all analytes was administratively defined as 40% of the lowest calibrator concentration. The administrative LOD was evaluated in replicate by analysis, on each batch run ($N = 12$), of analyte-negative blood bank blood supplemented with at the LOD concentration for all analytes. Acceptable detection of analytes at the LOD threshold was based on the analyte detection criteria described in the 'Materials and methods' section.

Endogenous interferences were evaluated in blood from six volunteers who were not currently using the drugs tested for in the method. Analysis of volunteer blood with and without internal standard addition was performed to evaluate for both analyte and internal standard interference. In addition, blood

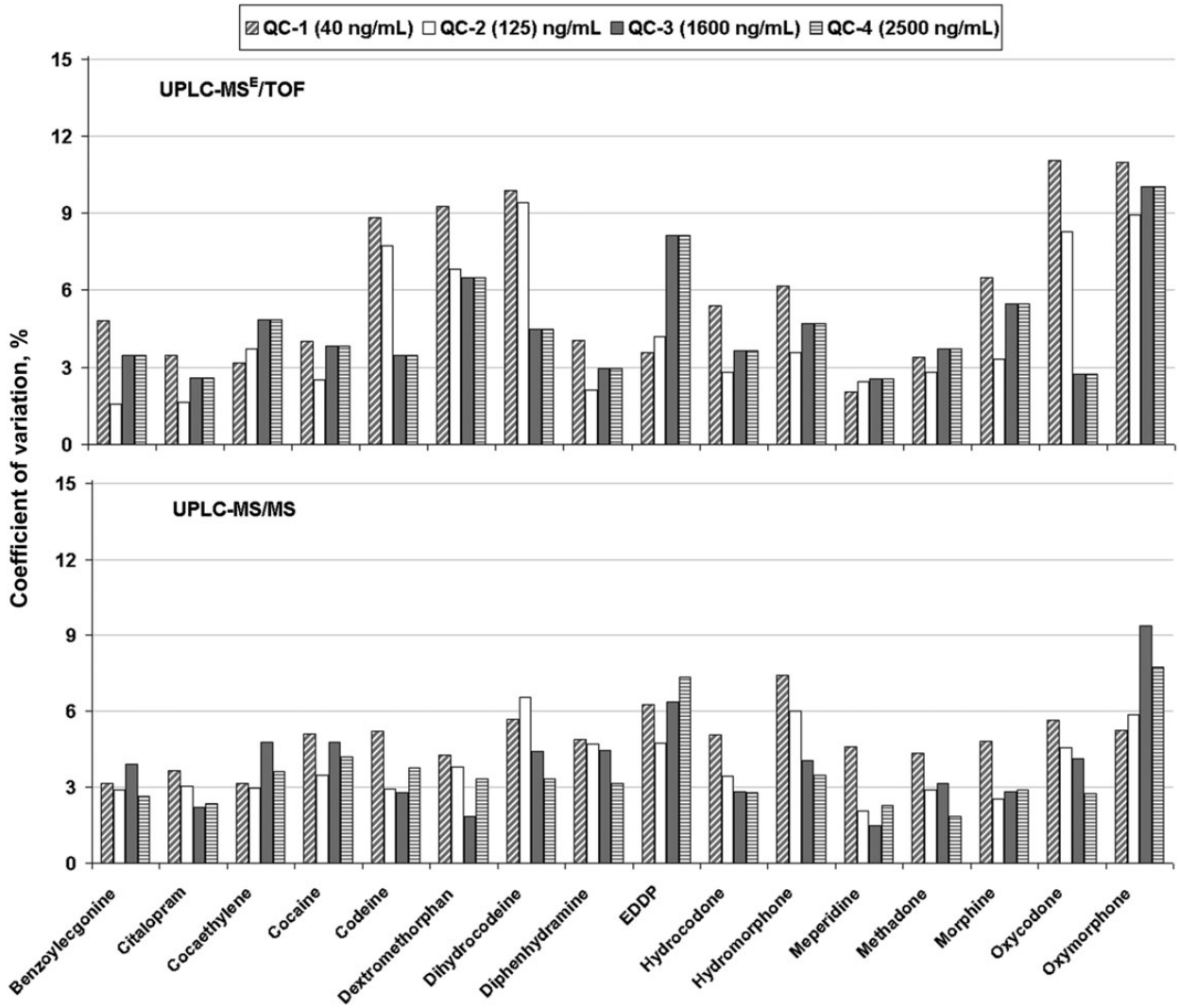


Figure 5. Comparative UPLC–MS^E/TOF and UPLC–MS/MS precision with analysis of multi-concentration blood controls prepared from weighed-in reference material.

from 159 postmortem cases with 2,269 analyte-negative findings by the routine UPLC–MS and UPLC–MS/MS screening methods were analyzed by UPLC–MS^E/TOF for assessment of interference. Carryover was assessed by analysis of an analyte-negative sample immediately following the analysis of the blood bank blood spiked at a concentration of 5,000 ng/mL. Presence of interference and carryover was based upon the analyte detection criteria described in the 'Materials and methods' section.

Assay precision and bias were determined by replicate analysis ($N = 16$) of multi-analyte quality control material prepared from reference material as described in the 'Chemical, reagents and quality control material' section. Acceptance criteria for precision (<15% CV) and bias (<20%) were used. Dilution integrity was assessed by analysis of blood bank blood spiked with target concentrations of 3,000 ng/mL with 2-fold dilution and 5,000 ng/mL with 5-fold dilution. Acceptable analyte recovery was ± 20% of target concentration following a dilution factor correction. Calibration model and criteria are described in the 'UPLC–MS^E/TOF method' section.

Results and discussion

Chromatographic conditions were optimized for the separation of morphine from hydromorphone and codeine from hydrocodone, because these analyte pairs share molecular and fragment ion masses. Figure 1 shows representative extracted ion chromatograms for each analyte with the achieved separations of morphine, hydromorphone, codeine and hydrocodone. Ion extraction chromatograms were generated with a MEW of ±5 ppm that was optimized during method development by parallel analysis of analytical batch run data using progressive reduction in MEW of ±25, ±10, ±5, ±4 to ±2.5 ppm. Ion extraction with MEW of ±5 ppm resulted in optimum selectivity for post-acquisition analysis of data based on ion area, signal-to-noise ratio, peak shape and residual plots for low-concentration calibrators. The optimized MEW of ±5 ppm is consistent with both mass error studies previously reported by several authors (1) and a study by Xia *et al.* (24), which determined that selectivity for HRMS with 20,000 resolution power is highest when a ±5 ppm MEW is used. The ion extraction chromatograms do not show significant endogenous peaks and compare well with the

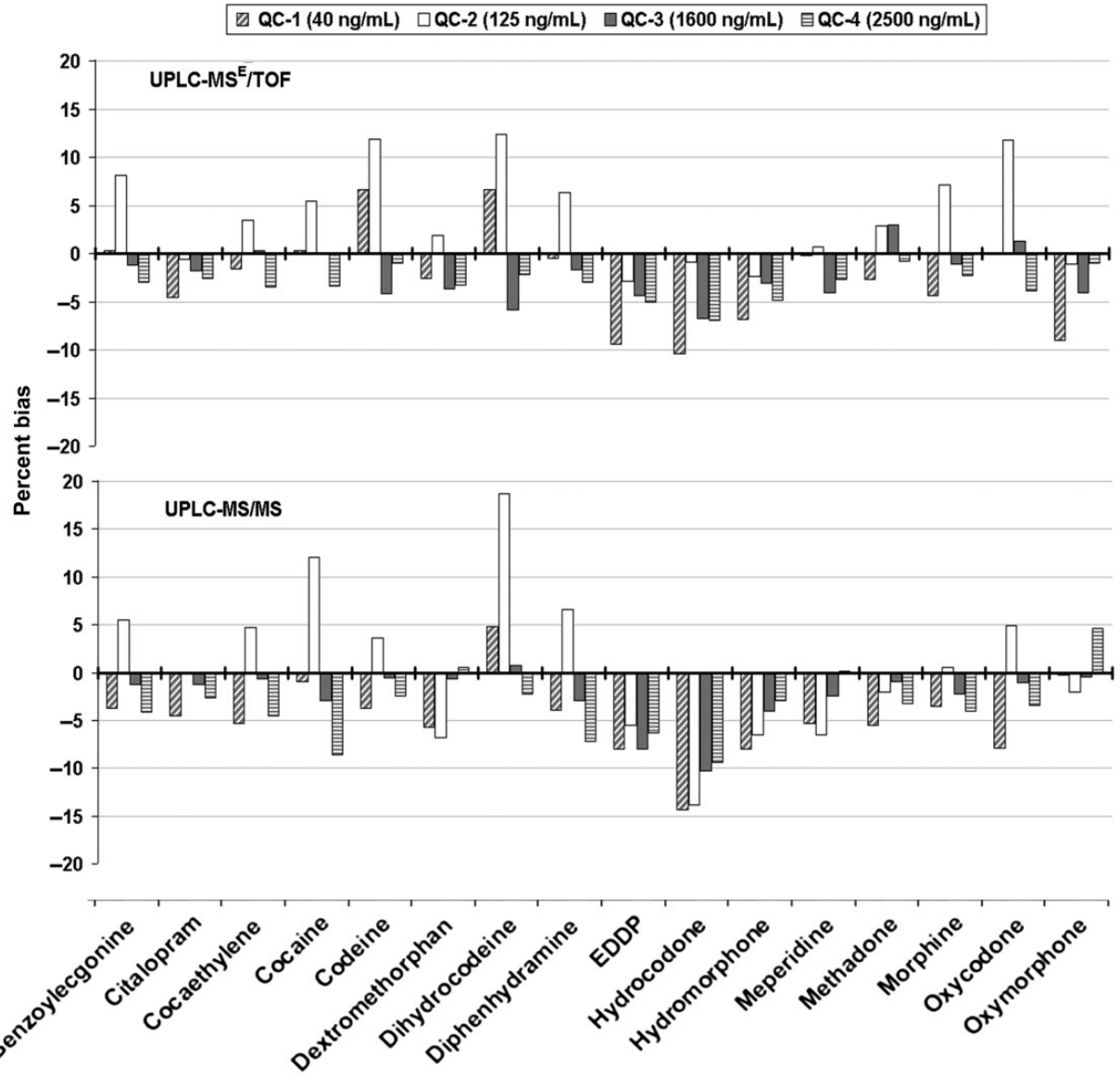


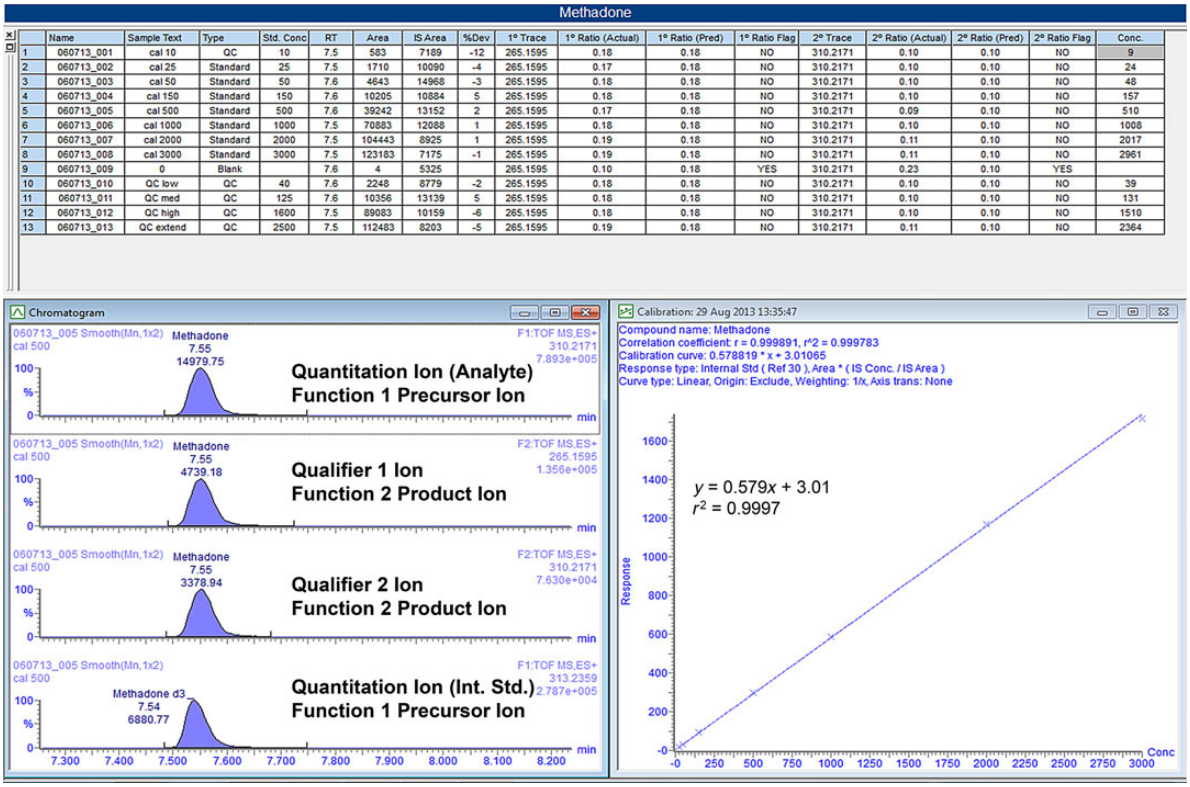
Figure 6. Assay bias plots based on recovery by UPLC–MS^E/TOF and UPLC–MS/MS analysis of blood controls prepared from weighed-in reference material.

selectivity of transition ion chromatograms for analytes by UPLC–MS/MS, as shown in Figure 2.

Solid phase extraction of analytes with a mixed mode column bed was also optimized during method development. The acidity of the column-wash prior to analyte elution significantly influences the recovery of benzoylecgonine, which is a zwitterion with positive (pK_a 9.14) and negative (pK_a 3.15) ionizable groups and an isoelectric point of 6.49. Under weak acid wash (pH 4.5) conditions, over 90% of benzoylecgonine and its deuterated internal standard are lost in the methanol wash prior to the analyte elution step. Reducing the pH of the wash to pH 1.0 in the final method conditions increased extraction recovery for benzoylecgonine to greater than 90%, with greater than 70% recovery of all analytes and internal standard as shown in Figure 3.

Matrix effects were minimized by adjustment of mobile phase conditions. Prior experience with a general unknown screening method revealed enhanced ion suppression with early and late eluting drugs and metabolites (1). Modification of mobile phase conditions with increasing retention of the early eluters, morphine and hydromorphone, showed a decrease in ion suppression for these analytes (data not shown). With the use of a gradient containing a low organic content early in the program and a slowly progressive increase followed by a final rapid increase, analyte retention early or late in the gradient was avoided. Figure 4 shows an acceptable ion suppression or enhancement effect for both analytes and internal standards within 20% of neat response with use of the finalized mobile phase conditions.

Precision, accuracy and calibration performance of UPLC–MS^E/TOF and UPLC–MS/MS analysis were compared. For precision and accuracy, replicate analysis ($N = 16$) of blood control pools with targeted concentrations spanning the dynamic range of the methods was performed. Figure 5 shows the comparative coefficients of variation at target concentrations of 40, 125, 1,600 and 2,500 ng/mL. Both methods met the precision



	Name	Sample Text	Type	Std. Conc	RT	Area	IS Area	%Dev	1º Trace	1º Ratio (Actual)	1º Ratio (Pred)	1º Ratio Flag	2º Trace	2º Ratio (Actual)	2º Ratio (Pred)	2º Ratio Flag	Conc.
1	060713_001	cal 10	QC	10	7.5	583	7189	-12	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	9
2	060713_002	cal 25	Standard	25	7.5	1710	10090	-4	265.1595	0.17	0.18	NO	310.2171	0.10	0.10	NO	24
3	060713_003	cal 50	Standard	50	7.6	4643	14968	-3	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	48
4	060713_004	cal 150	Standard	150	7.6	10205	10884	5	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	157
5	060713_005	cal 500	Standard	500	7.6	39242	13152	2	265.1595	0.17	0.18	NO	310.2171	0.09	0.10	NO	510
6	060713_006	cal 1000	Standard	1000	7.5	70883	12088	1	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	1008
7	060713_007	cal 2000	Standard	2000	7.5	104443	8925	1	265.1595	0.19	0.18	NO	310.2171	0.11	0.10	NO	2017
8	060713_008	cal 3000	Standard	3000	7.5	123183	7175	-1	265.1595	0.19	0.18	NO	310.2171	0.11	0.10	NO	2961
9	060713_009	0	Blank		7.6	4	5325		265.1595	0.10	0.18	YES	310.2171	0.23	0.10	YES	
10	060713_010	QC low	QC	40	7.6	2248	8779	-2	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	39
11	060713_011	QC med	QC	125	7.6	10356	13139	5	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	131
12	060713_012	QC high	QC	1600	7.5	89083	10159	-6	265.1595	0.18	0.18	NO	310.2171	0.10	0.10	NO	1510
13	060713_013	QC extend	QC	2500	7.5	112483	8203	-5	265.1595	0.19	0.18	NO	310.2171	0.11	0.10	NO	2364

Figure 7. Representative calibration and control data for methadone analysis.

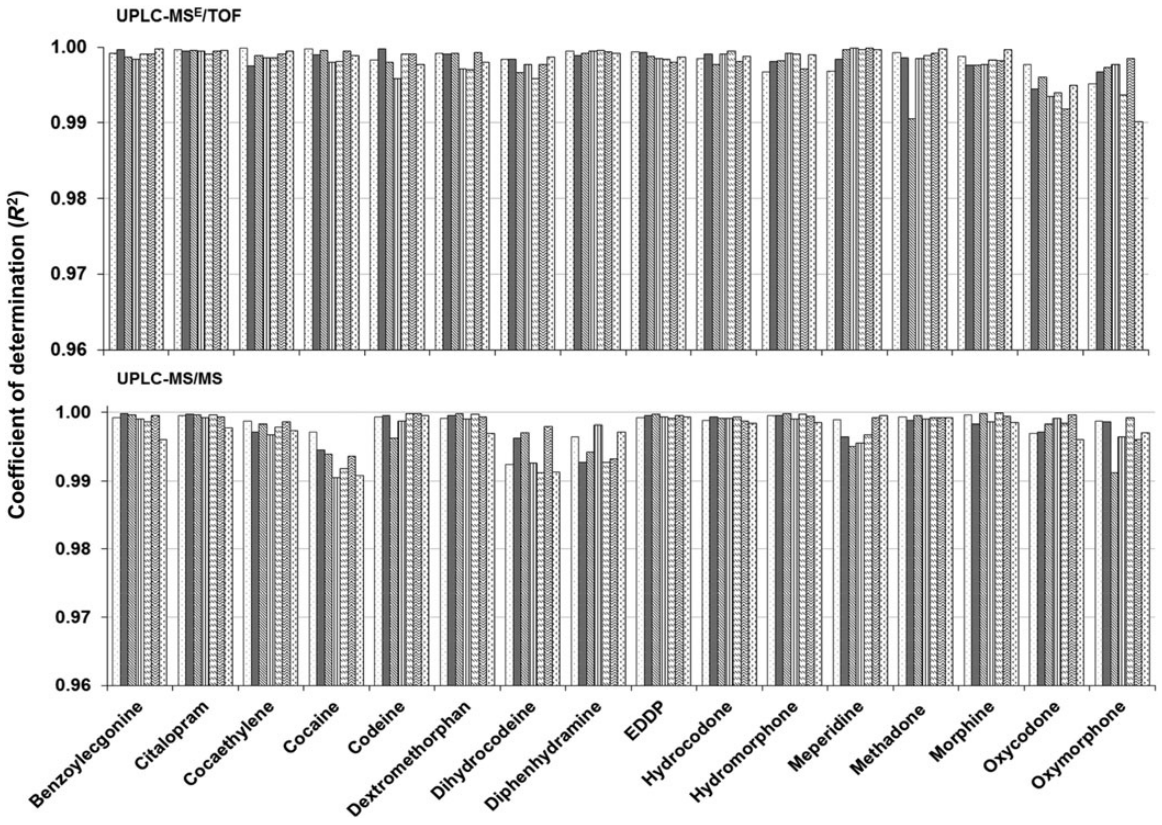


Figure 8. Coefficient of determination (R^2) for multiple analytical calibration curves for each analyte.

criteria in the validation plan with higher variation across low and high concentration for several analytes by UPLC–MS^E/TOF analysis. Bias plot data for both methods were comparable and within validation criteria across all analyte concentration as shown in Figure 6. Representative calibration data and plot are shown as part of a methadone analysis report in Figure 7. Percent deviation of residuals for each calibrator is within validation criteria along with a coefficient of determination (R^2) greater than 0.99. Figure 8 shows calibration performance (R^2) for all analytes across seven consecutive analytical runs with parallel analysis by UPLC–MS^E/TOF and UPLC–MS/MS. The findings confirm linearity of UPLC–MS^E/TOF and UPLC–MS/MS over the calibration range selected for the analysis. Dilution linearity, carryover and administrative LOD assessments also met the validation plan criteria for all analytes.

Analyte concentrations determined in postmortem blood are tabulated in Table III showing comparable UPLC–MS^E/TOF and UPLC–MS/MS results. For analytes above the lower limit of quantitation by both methods, regression analysis was performed and shown in Figure 9. Between-method quantification showed no significant difference on either a statistical or forensically interpretive basis. In 22 of the comparative quantifications, as shown Table III, the analyte concentration was between the LOD and LOQ and reported as <25 ng/mL for at least one of the methods. These low concentration detections also showed close comparison between methods, further indicating the validity of analysis

Table III
Analyte concentration (ng/mL) in postmortem blood by comparative UPLC–MS^E/TOF and UPLC–MS/MS analysis

TOF	MS/MS	TOF	MS/MS	TOF	MS/MS	TOF	MS/MS	TOF	MS/MS	TOF	MS/MS
Benzoylecgonine		Citalopram		Dextromethorphan		EDDP		Morphine		Oxycodone	
3,782	3,959	105	101	27	36	41	40	36	34	1,379	1,055
1,537	1,601	872	848	79	79	256	263	134	137	791	763
137	137	157	150	88	88	46	47	254	260	131	142
28	34	172	168	76	72	51	49	325	366	107	114
1,401	1,501	978	932	193	256	28	<25	787	805	732	664
87	82	278	267	77	116	92	99	398	411	627	563
2,330	2,389	213	203	145	140	39	40	316	305	474	533
99	105	1,603	1,634	39	41.8	<25	<25	512	537	138	162
1,811	1,756	286	282	79	75			119	124	983	1,092
155	108	288	286	94	92	Hydrocodone		494	496	176	195
25	28	103	104	751	691			153	156	30	28
78	88	328	280	<25	<25	179	184	24	23	42	41
1,409	1,507	711	684	34	26	92	100	183	184	634	662
<25	<25	637	645	36	29	84	93	674	734	38	29
<25	<25	269	259			45	49	30	25	34	28
830	800	148	152	Diphenhydramine		383	418	233	263	112	120
406	403	393	404			<25	<25	379	435	1,958	1,893
421	417	1,162	1,151	68	59	488	443	309	358	1,365	1,169
37	37	568	568	760	776	351	334	92	95	216	233
1,499	1,527	252	265	1,515	1,543	102	115	208	228	86	101
83	90	39	41	441	392	392	325	230	257	404	399
36	36	599	601	976	1,083	175	178	1,410	1,609	827	752
2,349	2,267	509	520	103	86	82	85	46	46	1,109	1,026
44	46	163	156	378	339	61	71	154	177	591	561
1,522	1,585	129	129	236	197	46	48	150	169	31	38
1,161	1,154	32	33	1,280	1,323	559	485	92	106	61	71
247	256	766	748	185	163	31	31	406	451	40	41
1,265	1,249			42	45	39	44	92	106	63	89
1,325	1,407	Cocaethylene		26	26	36	31	27	26	<25	<25
1,610	1,687			1,195	1,162	35	42	145	161	1,358	1,363
115	115	36.0	40	26	25			115	127	71	108
43	41	146	143	503	516	Hydromorphone		575	602		
1,121	1,203	104	109	618	633			428	451	Oxymorphone	
		62	60	60	59	2,395	2,305	505	547		
Cocaine		34	34	219	227	297	273	366	383	98.9	95
		176	167	39	37	148	148	145	160	<25	<25
622	556	<25	26	2,437	2,559	30	<25	379	408	<25	<25
237	212			139	128	133	89	626	688	<25	<25
148	136	Codeine						399	414	31.0	28
236	213			Dihydrocodeine		Methadone		1,507	1,548	57.5	59
174	158	<25	<25					72	82	65	57
15	20	29	<25	50	47	60	59	174	213	<25	<25
30	32	94	123	<25	<25	224	215	52	53	<25	<25
87	83	71	67	25	<25	63	64	603	622	<25	<25
252	236	44	66	233	306	226	220	134	152	<25	<25
416	408			43	75	145	141	48	48		
76	75			25	<25	295	307	120	140		
171	158			237	222	315	328	63	89		
396	367			25	36	603	601	36	30		
121	119			129	122	150	155	58	45		
49	46			222	188	1,283	1,301	55	51		
<25	<25					268	226	<25	<25		
217	208							526	487		
139	139					Meperidine		53	47		
								136	153		
						64	69				

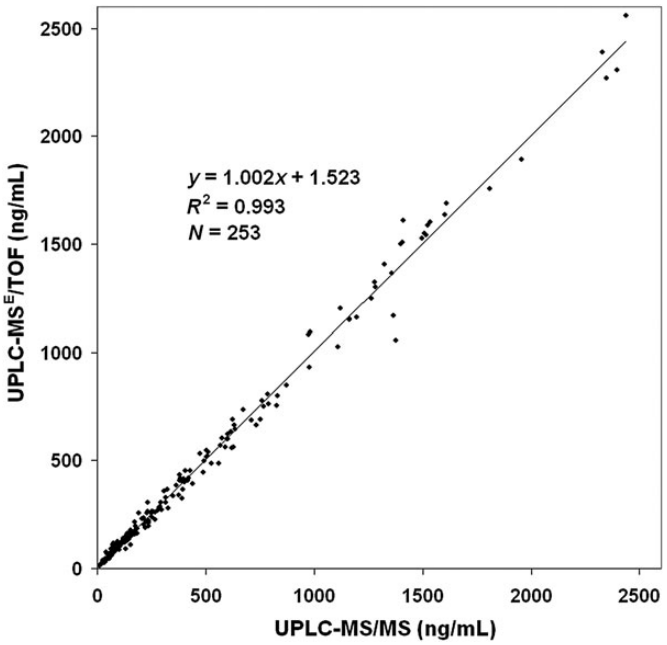


Figure 9. Comparison of all analyte quantifications by UPLC–MS^E/TOF and UPLC–MS/MS analysis of medical examiner case bloods showing linear regression analysis results.

by UPLC–MS^E/TOF in postmortem blood over the dynamic range selected for the analysis. Interference studies in multiple volunteer blood specimens, as well as in 159 postmortem blood specimens that were analyte-negative by initial qualitative UPLC–MS and UPLC–MS/MS testing, showed no detectable analyte by either quantitative UPLC–MS/MS or UPLC–MS^E/TOF analysis.

Fragment ion information available in UPLC–MS^E/TOF analysis was also evaluated. Single-stage MS^E/TOF analysis may result in shared fragment ions by analytes and their deuterated internal standard once the deuterium labeling is lost in the collision-induced dissociation pathway. Comparative mass spectral analysis of analyte and their deuterated internal standard was performed to identify deuterium-deficient fragments. Deuterium was lost early in the fragmentation pathway for citalopram D^6, codeine D^3, dextromethorphan D^3, diphenhydramine D^3, hydrocodone D^3, hydromorphone D^3 and morphine D^3, resulting in common fragment masses for analyte and internal standard pairs. Therefore, use of fragment ions as additional identifier ions for these analytes may require analysis without internal standard or use of alternate internal standards with additional side chain deuteration or C^{13} labeling. Evaluation of codeine D^6 and hydrocodone D^6 showed that all fragment ions listed for codeine and hydrocodone in Table I are unique and available as identifier ions when the D^6-labeled internal standards are employed. For analytes with unique fragment ions, the ratio of fragment ion area in acquisition 2 to the quantifier (molecular) ion area in acquisition 1 was reproducible. Figure 7 shows representative ion ratio data for one of the major fragment ions of methadone with good reproducibility across all calibrators and control material analyses. MS^E/TOF detection also provided additional molecular identity data not available with nominal mass. Dual acquisition of molecular ion with ramped-high and low collision energy shows a reproducible and analyte-characteristic ratio. Figure 7 shows a representative molecular ion ratio of acquisition 2 to acquisition 1 for methadone. Dual acquisition of molecular ion may serve as new and additional qualifiers ions in HRMS quantitative analysis, but further investigation and standardization are needed prior to application in forensic toxicology practice. Molecular ion isotope masses such as M + 1 and M + 2 are also available with HRMS analysis and can be evaluated in relationship to the known ratio of molecular isotopes in nature. Isotope data may also be useful in analyte identification criteria.

Conclusions

A UPLC–MS^E/TOF method has been developed and validated for multi-analyte quantification in postmortem blood. The study shows accurate quantification by UPLC–MS^E/TOF analysis, comparable to SRM analysis. HRMS detection has the selectivity of high mass resolution and the availability of full-spectrum data acquisition with limited need for optimization of ion acquisition as compared with MS/MS analysis. Our experience shows that multi-analyte quantification improves work flow in the forensic toxicology laboratory and that HRMS may provide not only a sensitive and specific general unknown screening method, but also an alternative to the nominal mass detection for quantitative forensic casework.

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