Published in final edited form as:

Anal Bioanal Chem. 2009 April ; 393(8): 1957–1965. doi:10.1007/s00216-009-2661-8.

Simultaneous quantification of nicotine, opioids, cocaine, and metabolites in human fetal postmortem brain by liquid chromatography tandem mass spectrometry

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Abstract

A validated method for simultaneous LCMSMS quantification of nicotine, cocaine, 6-acetylmorphine (6AM), codeine, and metabolites in 100 mg fetal human brain was developed and validated. After homogenization and solid-phase extraction, analytes were resolved on a Hydro-RP analytical column with gradient elution. Empirically determined linearity was from 5–5,000 pg/mg for cocaine and benzoylecgonine (BE), 25–5,000 pg/mg for cotinine, ecgonine methyl ester (EME) and 6AM, 50–5000 pg/mg for *trans*-3-hydroxycotinine (OH-cotinine) and codeine, and 250–5,000 pg/mg for nicotine. Potential endogenous and exogenous interferences were resolved. Intra- and inter-assay analytical recoveries were $\geq 92\%$, intra- and inter-day and total assay imprecision were $\leq 14\%$ RSD and extraction efficiencies were $\geq 67.2\%$ with $\leq 83\%$ matrix effect. Method applicability was demonstrated with a postmortem fetal brain containing 40 pg/mg cotinine, 65 pg/mg OH-cotinine, 13 pg/mg cocaine, 34 pg/mg EME, and 525 pg/mg BE. This validated method is useful for determination of nicotine, opioid, and cocaine biomarkers in brain.

Keywords

LCMSMS; Brain; Fetal; Cocaine; Opiate; Nicotine

Introduction

In utero exposure to tobacco and drugs has adverse effects on fetal development. Maternal tobacco smoking and drug use can lead to deficits early in postnatal life [1, 2], including low birth weight [3], sudden infant death syndrome (SIDS) [4, 5], poor attention [6], and neonatal abstinence syndrome [6]. According to data collected in the National Survey on Drug Use and Health (2003), 9.8, 18, and 4.3% of pregnant women admitted drinking alcohol, using tobacco and/or illicit drugs within the last month, respectively [7]. Another recent study showed that 17% of women denying cocaine use had positive maternal or newborn biological specimens [8]. Consequently, sensitive and specific bioanalytical methods are necessary to quantify biomarkers of in utero drug exposure.

Several common neonatal matrices available for monitoring prenatal drug exposure include meconium [9–12], hair [13], urine [14], plasma [15], breast milk [16], placenta [17, 18], and

umbilical cord [19, 20]. However, when postmortem investigations are needed to determine potential cause and manner of death, and for research-linking changes in gene expression with drug exposure, analysis of tissue, specifically brain, may provide valuable information [21–24]. With microarray analysis, Lehrmann et al. [24] found that humans who used cocaine, cannabis, and/or phencyclidine had decreased transcription of calmodulin-related genes and increased transcription of genes related to lipid/cholesterol and Golgi/endoplasmic reticulum function. When analysis of postmortem fetal brain is required, specimen size is greatly limited.

Methods for isolating drugs in brain have employed liquid/ liquid [25–27] or solid-phase extraction (SPE) [28–36]. SPE offers advantages over liquid/liquid including ease of use, greater selectivity, high recovery, and decreased solvent volumes. Liquid chromatography mass spectrometry (LCMS) [37, 38] and gas chromatography mass spectrometry (GCMS) [25, 28, 29, 31, 33, 34, 39] are the most common analytical methods for detection and quantification of drugs in tissues, including brain. Hernandez et al. [40] utilized 1 g of brain and focused primarily on cocaine, benzoylecgonine and cocaethylene. Moore et al. [35] developed an LCMS method for quantification of cocaine and metabolites in 1 g of brain tissue following a lipase digestion procedure prior to SPE. The current method improves on previously reported techniques by reducing the amount of brain tissue to 100 mg, simultaneously quantifies three important drug classes and increases sensitivity. Selectivity, sensitivity, limits of detection (LOD), and quantification (LOQ), linearity, imprecision, analytical recovery, extraction efficiency, carryover effect, matrix effect, matrix interference and stability studies were determined during method validation. This assay will be employed in studies of DNA expression in postmortem fetal brains following prenatal drug exposure.

Experimental

Reagents

(3'R, 5'S)-OH-cotinine (10 mg powder) and OH-cotinine-*d*₃ (1 mg powder) were purchased from Toronto Research Chemicals (North York, Ontario, Canada), and (–)-nicotine (1 mg powder) and formic acid were obtained from Sigma (St. Louis, MO, USA). (–)-Cotinine (1 mg/mL in methanol), (±)-cotinine-*d*₃, (100 µg/mL in methanol) (±)-nicotine-*d*₄ (100 µg/mL in methanol), ecgonine methyl ester (EME) (1 mg/mL in methanol), ecgonine methyl ester-*d*₃ (EME-*d*₃) (100 µg/mL in methanol), cocaine (1 mg/mL in methanol), cocaine-*d*₃ (100 µg/mL in methanol), codeine (1 mg/mL in methanol), codeine-*d*₆ (100 µg/mL in methanol), benzoylecgonine (BE) (1 mg/mL in methanol), benzoylecgonine-*d*₈ (BE-*d*₈) (100 µg/mL in methanol), morphine (1 mg/mL in methanol), morphine-*d*₃ (100 µg/mL in methanol), 6-acetylmorphine (6AM) (1 mg/mL in methanol), and 6-acetylmorphine-*d*₆ (6AM-*d*₆) (100 µg/mL in methanol) were acquired from Cerilliant (Austin, TX, USA). Water, acetonitrile, sodium phosphate dibasic, sodium phosphate monobasic, sodium acetate, hydrochloric acid, dichloromethane, 2-propanol, and ammonium hydroxide were from J.T. Baker (Philipsburg, NJ, USA) and methanol from Fisher Chemical (Pittsburgh, PA, USA). All solvents and reagents were HPLC or ACS grade. CleanScreen solid-phase extraction columns, part ZSDAU020, were purchased from United Chemical Technologies (Bristol, PA, USA).

Sodium phosphate buffer (pH 6.0±0.05) was prepared with 438.5 mL 0.1 M sodium monophosphate and 61.5 mL 0.1 M sodium dibasic phosphate, with a final volume of 1,000 mL of water for a 0.1 M solution. Elution solvent (methylene chloride:isopropanol:concentrated ammonium hydroxide, 78:20:2 v/v/v) was prepared fresh daily.

Postmortem brain for calibrators and quality control (QC) samples—

Postmortem brain tissue was procured from the brain repository of the Clinical Brain

Disorders Branch (National Institute on Mental Health, National Institutes of Health). Human brain specimens were obtained during routine autopsy through the Offices of the Chief Medical Examiner of the District of Columbia and Northern Virginia with the informed consent of next of kin. Brain was stored at -80°C prior to analysis.

Drug-free brain matrix was prepared in a 15 mL polypropylene centrifuge tube by homogenization of 100 mg of brain tissue in 5 mL of pH 6, 0.1 M sodium phosphate buffer with an Ultrasonic Processor (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) at 60 A, 60% duty cycle for 2 to 3 min. Tubes were cooled in an ice bath during homogenization. Homogenates were centrifuged at $1,850\times g$ for 10 min.

Calibrator and QC sample preparation—For calibrators, a stock solution of $100\mu\text{g/mL}$ of each analyte was prepared in methanol and stored at -20°C until use. Working solutions, 0.05, 0.5, and $5\mu\text{g/mL}$, were prepared by independent dilution with methanol. Blank postmortem human brain samples (100 mg) were fortified with aliquots of working solutions to yield a nine-point calibration curve (5, 25, 50, 125, 250, 500, 1,250, 2,500, and 5,000 pg/mg) for BE and cocaine, eight points (25, 50, 125, 250, 500, 1,250, 2,500, and 5,000 pg/mg) for EME, 6AM, and cotinine, seven points (50, 125, 250, 500, 1,250, 2,500, and 5,000 pg/mg) for morphine, codeine, and OH-cotinine, and five points (250, 500, 1,250, 2,500, and 5,000 pg/mg) for nicotine.

QC samples were prepared in a similar way from a different lot than utilized for calibrators. Blank postmortem human brain samples were fortified with aliquots of QC working solutions to yield 15, 350, and 3,500 pg/mg for BE and cocaine, 35, 350, and 3,500 pg/mg for EME, 6AM, and cotinine, 75, 350, and 3,500 pg/mg for morphine, codeine, and OH-cotinine, and 350, 1,500, and 3,500 pg/mg for nicotine (low, medium, and high, respectively) QC samples.

Individual deuterated internal standard stock $100\mu\text{g/mL}$ solutions of BE- d_8 , cocaine- d_3 , EME- d_3 , 6AM- d_6 , cotinine- d_3 , morphine- d_3 , codeine- d_6 , OH-cotinine- d_3 , and nicotine- d_4 were prepared in methanol and stored at -20°C until use. Three mixtures of internal standard working solutions were prepared, A containing $5\mu\text{g/mL}$ nicotine- d_4 , B $5\mu\text{g/mL}$ morphine- d_3 , codeine- d_6 , and OH-cotinine- d_3 , and C $1\mu\text{g/mL}$ BE- d_8 , cocaine- d_3 , EME- d_3 , 6AM- d_6 , and cotinine- d_3 . Internal standard working solution was added to 100 mg human brain, yielding a final brain homogenate concentration of 250 pg/mg for BE, cocaine, EME, 6AM, and cotinine, 500 pg/mg for morphine, codeine, and OH-cotinine, and 1,250 pg/mg for nicotine. Quantification was accomplished by comparing peak area ratios of target analytes to internal standards. Data were fit to a linear least-squares regression curve with a weighting factor of $1/x$. Concentrations of each calibrator were calculated against the calibration curve and were required to quantify within $\pm 20\%$ of target.

Postmortem fetal brain—Postmortem fetal brain was obtained from the National Institute of Child Health and Human Development at the University of Maryland at Baltimore Brain and Tissue Bank for Development Disorders. Brain tissue was procured under a protocol approved by the University of Maryland at Baltimore and the Maryland Department of Hygiene and Mental Health Institutional Review Boards, and donated under the terms of a Material Transfer Agreement.

Specimen preparation—Fetal brain (100 mg) was homogenized in 5 mL of pH 6 sodium phosphate for 2 min. $25\mu\text{L}$ from internal standard mixtures A and C and $10\mu\text{L}$ mixture B were added prior to homogenization. Homogenates were centrifuged and supernatants subjected to SPE.

CleanScreen DAU solid-phase extraction columns were conditioned with 3 mL methanol, 3 mL water, and 1 mL 100 mM sodium phosphate buffer, pH 6. Specimens were poured onto columns and allowed to flow by gravity alone. Columns were washed with 3 mL water, dried under vacuum for 1 min, followed by 1.5 mL 100 mM hydrochloric acid, dried for 5 min, and washed with 3 mL methanol followed by 5 min drying. Analytes were eluted with freshly prepared 5 mL dichloromethane:isopropanol:ammonium hydroxide (78:20:2, v/v/v). Eluates were dried under nitrogen at 40°C after addition of 100 µL 1% hydrochloric acid in methanol (v/v) to prevent evaporation of nicotine and other analytes. Samples were reconstituted in 1 mL water with 0.1% formic acid (v/v) and transferred to glass autosampler vials.

LCMSMS—Liquid chromatography–mass spectrometry/mass spectrometry (LCMSMS) experiments were performed with the MDS Sciex API 3200 QTrap® triple quadrupole/linear ion trap mass spectrometer with a TurboIonSpray source (Applied Biosystems, Foster City, CA, USA). The HPLC system consisted of Shimadzu LC-20AD pumps and SIL-20AC autosampler (Columbia, MD, USA). Analyst software version 1.4.1 was utilized for acquisition and data analyses.

Chromatographic separation was performed on a Synergi Hydro-RP column (75×2.0 mm, 4 µm), protected by a guard column with identical packing material (4×2.0 mm; Phenomenex, Torrance, CA, USA). Gradient elution occurred with (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 200 µL/min. The gradient program included 5% B to 50% B over 3 min, 3–7 min hold at 50% B, and 50% B to 5% B over 3 min. The column oven and auto-injector sample tray were maintained at 25 and 4°C, respectively.

MS data were collected in positive ion mode, with optimized Turbo IonSpray-MS parameter settings shown in Table 1. The following transitions were monitored: m/z 163 to 132 and 84 for nicotine, m/z 177 to 80 and 98 for cotinine, m/z 193 to 80 and 134 for OH-cotinine, m/z 304 to 182 and 82 for cocaine, m/z 290 to 168 and 105 for BE, m/z 200 to 182 and 82 for EME, m/z 286 to 152 and 165 for morphine, m/z 300 to 152 and 165 for codeine and m/z 328.3 to 165 and 152 for 6AM. The underlined transitions were used as quantifier ions.

Data analysis—Peak area ratios of target analytes and respective internal standards were calculated at each concentration. The most abundant transition for each analyte was used for quantification; the second transition served as qualifier (Table 1).

Method validation—Selectivity, sensitivity, LOD and LOQ, linearity, imprecision, analytical recovery, extraction efficiency, carryover effect, matrix effect, matrix interference and stability were evaluated. Method validation was accomplished in 4 days with four unique assays.

Selectivity of the method was assessed by analysis of ten unique blank brain specimens. 100 mg of each blank brain specimen was homogenized in 5 mL of pH 6 sodium phosphate and subjected to solid-phase extraction. The final reconstituted specimens were analyzed for potential interferences from endogenous substances. In addition, potential interferences from commonly used drugs and minor tobacco alkaloids were evaluated by fortifying drugs into low-concentration QC samples. Final interferent concentrations were 1 µg/mL of Δ^9 -tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, hydrocodone, hydromorphone, oxycodone, noroxycodone, oxymorphone, noroxymorphone, diazepam, lorazepam, oxazepam, alprazolam, imipramine, clomipramine, fluoxetine, norfluoxetine oxalate, paroxetine maleate, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine,

chlorpheniramine, brompheniramine, aspirin, acetaminophen, phencyclidine, amphetamine, methamphetamine, anabasine and anatabine. No interference was noted if all analytes quantified within $\pm 20\%$ of target concentrations. Specificity also was assessed by relative retention time and quantifier and qualifier transition peak area ratios. Transition peak area ratios for quality control and authentic specimens were required to be within $\pm 20\%$ of the average calibrator transition peak area ratio.

Sensitivity of the method was determined with decreasing analyte concentrations in drug-fortified blank brain. LOD was the lowest concentration with signal to noise ratios of at least 3:1 for both transition ions, good chromatography and appropriate retention time, and LOQ incorporated these features plus signal to noise ratios of 10:1 for quantifier and qualifier transitions and quantification within $\pm 20\%$ of target. Linearity was determined by calculating the regression line by the method of least-squares and expressed by the correlation coefficient (r^2). The r^2 value for the full curve was required to be greater than 0.99 for each analyte on every analysis. A $1/x$ weighting was applied, and linearity was determined with at least five calibrators, not including the blank matrix. Concentrations of each calibrator were required to be within $\pm 20\%$ of target when calculated against the full curve.

Intra-day imprecision and analytical recovery were determined with five replicates at three concentrations across the linear dynamic range. Inter-day imprecision and analytical recovery were evaluated for five replicates analyzed on four separate days ($n=20$). The guidelines given by Krouwer and Rabinowitz [41] were followed for calculation of pooled intra-day, inter-day and total imprecision. Imprecision was expressed as % relative standard deviation (%RSD) of the calculated concentrations. Analytical recovery was determined by comparing the mean result for analyses to the nominal concentration. Dilution integrity or accuracy of quantification of a diluted sample, was evaluated by performing a 1:2 dilution of two times the highest point of the calibration curve with blank matrix and comparing the quantitative result with nominal concentration ($n=3$).

Extraction efficiency, matrix effect, and process efficiency were evaluated with the three-set system described by Matuszewski et al. [42]. In the first set, brain samples were fortified with analytes and internal standards prior to solid-phase extraction. In set 2, brain samples were fortified with analytes and internal standards after solid-phase extraction, and the third set contained "neat" analytes and internal standards in mobile phase. There were five replicates in each set. Extraction efficiency, expressed as a percentage, was calculated by dividing average peak areas of set 1 by set 2. Matrix effect was calculated by dividing the average peak area of set 2 by set 3. Process efficiency was calculated by dividing the average peak area of set 1 by set 3. The value was converted to a percentage and subtracted from 100 to represent the amount of signal suppressed/enhanced by the presence of matrix. To evaluate whether cocaine and 6AM were stable during homogenization and extraction, blank brain homogenates were individually fortified with cocaine or 6AM at the three QC concentrations in triplicate and compared to neat solutions prepared in a mobile phase. Acceptable carryover was defined as no quantifiable transition peaks in a brain sample injection immediately following a sample containing two times the upper LOQ.

Stability was evaluated with human brain fortified with analytes of interest at the three QC concentrations ($n=5$). Short-term temperature stability was evaluated for human brain stored for 24 h at room temperature, 72 h at 4°C , 72 h on the autosampler (15°C), and after three freeze–thaw cycles at -20°C . On the day of the analysis, internal standard was added to each sample and specimens analyzed as described. Calculated concentrations of stability specimens were compared to QC samples prepared on the day of the analysis. QC samples

stored on the autosampler for 72 h were reassessed and compared to original values, both sets calculated against the original calibration curve.

Results and discussion

This validated method is the first LCMSMS analytical procedure to simultaneously quantify three important drug classes, nicotine, opiates, cocaine and metabolites in human brain. Due to serious health consequences from environmental tobacco smoke along with drug misuse, methods for determination of nicotine and other drugs in biological specimens are needed. The analytical challenge of developing a validated method for simultaneous quantification of multiple drug analytes in human brain required optimization of important factors which included, a technique for successful tissue disruption coupled with an efficient extraction methodology. This was addressed by brief ultrasonic homogenization of 100 mg of tissue in pH 6 sodium phosphate buffer followed by high centrifugation at 4°C. Homogenization was rapid and permitted efficient cleaning and changeover between specimens. Effective tissue homogenization and production of a compact pellet for easy decanting allowed direct application of the supernatant to the SPE column without clogging.

Analytes eluted within 6 min (Fig. 1), with a total chromatographic run time of 10 min. Representative chromatograms of extracted blank brain, low QC, and a positive postmortem brain specimen are shown in Fig. 1. Stability of the chromatographic method was evaluated by calculating retention time variabilities. Percent relative variation for retention times was $\leq 0.1\%$ for all analytes over 30 consecutive injections (Table 1). Blank samples injected after the highest calibrator did not show any carryover.

Mass spectrometric (MS) optimization was performed by direct infusion of single analytes of interest. For maximum sensitivity of MS analysis, fragmentor voltages were chosen for each ion product (Table 1). Table 1 also includes quantitative and qualifying ions, and retention times for all analytes.

Deuterated internal standards (($\pm$)-nicotine- d_4 , cotinine- d_3 , OH-cotinine- d_3 , cocaine- d_3 , EME- d_3 , BE- d_3 , 6AM- d_3 , morphine- d_3 , and codeine- d_3) were employed to minimize loss during specimen preparation and to compensate for matrix ion suppression or enhancement. LOD and LOQ are found in Table 3. Linear calibration curves were obtained with an average correlation coefficient (r^2 , weighting factor $1/x$, $n=5$) of >0.99 (Table 2) for all analytes in human brain.

No endogenous signal was observed in ten blank human brain specimens, demonstrating selectivity of the method. No interferences were noted for 35 common over-the-counter and abused drugs added to low QC samples; all samples quantified within $\pm 20\%$ of target with acceptable transition ratios. We also evaluated anabasine and anatabine, minor alkaloids in tobacco, at a concentration of $1\mu\text{g/mL}$. Neither drug interfered with quantification of any analyte of interest.

Table 3 details analytical recovery and imprecision data. Analytical recovery of each analyte at low, medium and high QC concentrations ranged from 94 to 114% of target ($n=20$) for inter-assay and 92 to 114.4% for intra-assay ($n=5$). Inter-, intra-day, and total assay imprecision at three QC concentrations were $\leq 14\%$ RSD. Dilution integrity proved to be $>90\%$ for all analytes. Mean extraction efficiency for the low, medium and high QC samples ($n=5$) ranged from 56.8 to 105.7%, 58.5 to 108.6%, and 65 to 108%, respectively (Table 4). Matrix effect was investigated by comparing analyte areas in brain samples fortified after SPE to neat analyte areas in mobile phase A ($n=5$). Mean matrix effect in five different brain pools ranged from 9.2 to 84.2%, 21.7 to 84%, and 3 to 77.6%, for low, medium and high QC, respectively. Mean process efficiency for all analytes ranged from 56.8 to 108.6%, at

three QC concentrations. Brain tissue with high lipid content is a highly complex matrix and, although specimen preparation was more extensive and solid-phase extraction was performed, matrix effect was not eliminated. The presence of deuterated internal standards for each analyte minimized the effect. Sensitivity was good with LOQs from 5 to 250 pg/mg, lower than those previously reported [35, 39]. In addition, recovery and precision were not negatively affected by matrix effect.

Quantification of BE in cocaine and morphine in 6AM-fortified samples evaluated the stability of these analytes during the experimental process; there was less than 0.1% hydrolysis of cocaine and 6AM. No analyte peaks were detected in the sample injected after a specimen containing two times the upper LOQ, demonstrating a lack of carryover under these conditions.

Analyte stability is found in Table 5. Percent loss of analytes at three QC concentrations was less than 17.7% at four storage conditions, indicating stability at room temperature for 24 h, 72 h at 4°C, 72 h on the autosampler (15 °C), and after three freeze–thaw cycles at –20 °C.

Clinical application

Nicotine, opiates, cocaine and metabolite extracted ion chromatograms in a postmortem fetal brain specimen are illustrated in Fig. 1. This fetal brain specimen included 40 pg/mg cotinine, 65 pg/mg OH-cotinine, 13 pg/mg cocaine, 34 pg/mg EME, and 525 pg/mg BE. From these data, it is apparent that the mother smoked tobacco or was exposed to tobacco in her environment, although parent nicotine was not present above 250 pg/mg. It appears that cotinine and OH-cotinine are good biomarkers in brain for in utero nicotine exposure. Three biomarkers of maternal cocaine exposure also were evident. In this case, the BE concentration was more than 15 times as great as either the cocaine and EME concentrations, suggesting that BE is the best biomarker of prenatal cocaine exposure in brain. We demonstrated that BE production from cocaine was less than 0.1% in the analytical procedure.

Conclusion

A sensitive and specific LCMSMS procedure for the simultaneous detection and quantification of nicotine, opiates, cocaine and metabolites in human postmortem fetal brain is presented. The method provides sufficient analytical sensitivity to allow quantification of drugs in small amounts of tissue. The assay employs a rapid and efficient tissue homogenization with an ultrasonic probe followed by SPE. The method can identify in utero drug exposure, help in understanding changes in gene expression with drug exposure, forensic toxicology investigations, and pharmacokinetic studies.

Acknowledgments

The authors are indebted to Dr. Ronald Zielke, Mr. Robert Vigoritto, Mr. Robert Johnson of the NICHD Brain and Tissue Bank for Development Disorders. Also, the authors are thankful to Thomas Hyde, MD, PhD, Dr. Joel Kleinman, PhD, and Elin Lehrmann, PhD. Funding was from the National Institutes of Health, Intramural Research Programs, National Institute on Drug Abuse and National Institute of Mental Health.

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Fig. 1.

Extracted ion chromatograms for nicotine (m/z 132.2), cotinine (m/z 80.1), *trans*-3'-hydroxycotinine (m/z 80.2), cocaine (m/z 182.3), ecgonine methyl ester (m/z 182.2), benzoylecgonine (m/z 168.3), morphine (m/z 152), 6-acetylmorphine (m/z 165.2) and codeine (m/z 151.9). **a** Blank postmortem human brain, **b** Blank postmortem human brain fortified with low quality control of each analyte, and **c** Postmortem human fetal brain containing 40 pg/mg for cotinine, 65 pg/mg for OH-cotinine, 13 pg/mg for cocaine, 34 pg/mg for EME, and 525 pg/mg for BE

Liquid chromatography–tandem mass spectrometry parameters for nicotine, opiates, cocaine and metabolites in postmortem human brain

Table 1

Compounds	Q1 mass (amu)	Q3 mass (amu)	Dwell time (msec)	Declustering potential (volts)	Entrance potential (volts)	Collision entrance potential (volts)	Collision energy (volts)	Cell exit potential (volts)	Retention time ($\pm$ SD) N=30
Nicotine	163.3	132.2	150	35	10	12	21	4	1.875 ($\pm$ 0.034)
Nicotine- <i>d</i> ₄	167.3	84.2	150	35	12	12	29	4	1.864 ($\pm$ 0.019)
		136.2	150	36	4	12	21	4	
Cotinine	177.2	121.2	150	36	4	12	29	4	2.147 ($\pm$ 0.025)
		98.2	150	41	10	12	29	4	
Cotinine- <i>d</i> ₃	180.2	80.1	150	41	10	12	33	4	2.235 ($\pm$ 0.029)
		101.2	150	21	4	12	31	4	
OH-cotinine ^a	193.2	80.1	150	21	4	12	33	4	1.673 ($\pm$ 0.045)
		134.1	150	46	4	12	27	4	
OH-cotinine- <i>d</i> ₃ ^b	196.2	80.2	150	46	4	12	37	4	3.224 ($\pm$ 0.076)
		134.1	150	46	11	14	27	4	
Morphine	286.3	80.2	150	46	11	14	43	4	3.181 ($\pm$ 0.057)
		152.0	150	66	3	16	71	4	
Morphine- <i>d</i> ₃	289.3	165.0	150	66	3	16	51	4	4.293 ($\pm$ 0.031)
		152.0	150	66	4	20	69	4	
6AM ^c	328.2	165.0	150	66	4	20	53	4	4.284 ($\pm$ 0.016)
		152.2	150	66	8	14	79	4	
6AM- <i>d</i> ₆ ^d	334.2	165.2	150	66	8	14	53	4	4.103 ($\pm$ 0.034)
		152.2	150	66	10	16	80	4	
Codeine	300.3	165.2	150	66	10	16	51	4	4.090 ($\pm$ 0.003)
		151.9	150	50	7	14	91	4	
Codeine- <i>d</i> ₆	306.3	165.2	150	50	7	14	53	8	4.922 ($\pm$ 0.023)
		151.9	150	52	4	20	81	4	
Cocaine	304.3	165.2	150	52	4	20	53	4	
		182.3	150	51	3	16	25	4	
		82.3	150	51	3	16	37	4	

Compounds	Q1 mass (amu)	Q3 mass (amu)	Dwell time (msec)	Declustering potential (volts)	Entrance potential (volts)	Collision entrance potential (volts)	Collision energy (volts)	Cell exit potential (volts)	Retention time (±SD) N=30
Cocaine- <i>d</i> ₃	307.3	185.3	150	41	5	18	27	4	4.913 (±0.037)
EME ^e	200.2	85.3	150	41	5	18	43	4	1.583 (±0.018)
		182.2	150	36	9	12	25	4	
EME- <i>d</i> ₃ ^f	203.2	82.2	150	36	9	12	35	4	1.585 (±0.022)
		185.2	150	41	11	12	25	4	
BE ^g	290.2	85.2	150	41	11	12	33	4	4.647 (±0.009)
		168.3	150	41	10	20	25	4	
BE- <i>d</i> ₈ ^h	298.2	105.2	150	41	10	20	43	4	4.616 (±0.044)
		171.3	150	46	3	20	27	4	
		110.2	150	46	3	20	41	4	

- ^a *trans*-3'-hydroxycotinine
^b *trans*-3'-hydroxycotinine-*d*₃
^c 6-Acetyl morphine
^d 6-Acetyl morphine-*d*₆
^e Ecgonine methyl ester
^f Ecgonine methyl ester-*d*₃
^g Benzoyl ecgonine
^h Benzoyl ecgonine-*d*₈

Table 2

Limits of detection (LOD), limits of quantification (LOQ), and calibration curve results ($N=5$) for nicotine, opiates, cocaine and metabolites in postmortem human brain by liquid chromatography-tandem mass spectrometry

Analyte	LOD (pg/mg)	LOQ (pg/mg)	ULOQ ^a (pg/mg)	Y-intercept mean±SD	Slope mean±SD	R ²
Nicotine	125	250	5,000	-0.01362±0.00950	0.6726±0.06673	0.9986
Cotinine	12.5	25	5,000	-0.41951±0.44144	14.124±16.607	0.9982
OH-Cotinine	25	50	5,000	-0.00942±0.02268	1.276±0.1950	0.9988
Cocaine	2.5	5	5,000	0.02786±0.01693	0.7872±0.0813	0.9994
EME	12.5	25	5,000	0.01569±0.01862	1.07±0.0533	0.9994
BE	2.5	5	5,000	0.0106±0.01041	0.8678±0.0278	0.9996
Morphine	25	50	5,000	0.04164±0.05081	0.9476±0.1765	0.9979
6AM	12.5	25	5,000	0.01952±0.02895	0.5752±0.3090	0.9986
Codeine	25	50	5,000	-0.00472±0.01540	1.09±0.0570	0.9983

^aUpper limit of quantification

Table 3

Intra- and inter-assay recovery and imprecision for nicotine, opiates, cocaine, and metabolites in postmortem human brain

Analyte	Expected conc. (pg/mg)	Analytical recovery (% of target)			Imprecision (N=20, %RSD)		
		Intra-assay N=5		Inter-assay N=20	Pooled Intra-day	Inter-day	Total
		Mean	Range				
Nicotine	350	109.7	(99.3–113)	104.9	(83.5–120)	5.9	6.9
	1,500	108.8	(101–115)	109.3	(92.8–116)	6.1	0.0
	3,500	114.4	(109–120)	109.1	(94.1–120)	5.7	0.0
Cotinine	35	111.4	(104–118)	113.9	(104–119)	5.6	0.0
	350	95.5	(90.6–105)	95.9	(81.3–116)	7.7	4.5
	3,500	106.5	(99.7–116)	102.8	(93.6–116)	5.5	2.1
OH-cotinine	75	101.8	(81.2–110)	103.9	(81.2–119)	11.9	0.0
	1,500	95.6	(84.9–103)	94.1	(8.5–105)	7.7	1.8
	3,500	97.0	(85.6–103)	98.2	(85.6–110)	5.4	0.0
Cocaine	15	110.8	(107–117)	111.8	(93.5–120)	10.5	0.0
	35	103.0	(101–105)	104.8	(97.3–116)	3.9	3.6
	3,500	101.0	(95.8–106)	101.9	(91.5–118)	5.9	4.4
EME	35	109.1	(95.3–117)	103.1	(82.7–117)	11.1	0.0
	350	106.4	(100–113)	102.2	(91.5–113)	2.6	3.0
	3,500	107.8	(101–113)	103.7	(90.3–113)	5.4	3.7
BE	15	107.5	(91.5–116)	109.5	(91.5–120)	10.5	0.0
	350	105.0	(102–115)	103.4	(98.3–111)	5.2	2.0
	3,500	102.2	(93.9–106)	105.9	(93.9–114)	4.7	0.0
Morphine	75	96.7	(82.8–116)	100.7	(82.8–116)	11.6	8.2
	1,500	101.8	(83.8–110)	97.8	(80.2–120)	12.4	3.6
	3,500	99.0	(93.9–105)	99.8	(82.1–114)	10.5	3.1
6AM	35	113.0	(106–120)	105.6	(80.9–120)	10.1	8.3
	350	100.0	(87.3–105)	100.9	(84.7–120)	6.7	6.8
	3,500	96.4	(93–104)	103.1	(86.7–118)	7.7	5.8
Codeine	75	104.1	(84.6–119)	102.3	(80.5–120)	11.4	2.8
	1,500	92.0	(81.5–104)	99.7	(81.5–113)	7.4	3.7
							8.3

Analyte	Expected conc. (pg/mg)	Analytical recovery (% of target)				Imprecision (N=20, %RSD)		
		Intra-assay N=5		Inter-assay N=20		Pooled Intra-day	Inter-day	Total
		Mean	Range	Mean	Range			
	3,500	93.7	(91–98.6)	96.5	(85–113)	5.0	5.3	7.3

Table 4

Extraction efficiency and matrix effect for nicotine, opiates, cocaine, and metabolites extracted from postmortem human brain at three quality control concentrations across the linear dynamic range of the assay

Analyte	Extraction efficiency (%; N=5)			Matrix effect (% of signal suppressed; N=5)		
	Low	Medium	High	Low	Medium	High
Nicotine	93.8	96.6	71.1	59.1	38.3	12.9
Nicotine- <i>d</i> ₄	82.4	91.3	95.6	19.6	48.7	8.0
Cotinine	56.8	58.5	64.9	47.5	74.6	17.8
Cotinine- <i>d</i> ₃	95.3	97.5	83.6	22.9	21.7	3.0
<i>trans</i> -3'-Hydroxycotinine	82.3	98.8	96.1	22.3	34.6	21.0
<i>trans</i> -3'-Hydroxycotinine- <i>d</i> ₃	67.2	99.9	81.2	12.1	39.3	19.5
Cocaine	78.2	85.0	90.3	9.2	28.7	10.2
Cocaine- <i>d</i> ₃	102.7	103.4	101.5	17.6	27.7	24.2
Egonine methyl ester	96.2	97.1	93.7	63.7	70.6	49.8
Egonine methyl ester- <i>d</i> ₃	94.3	88.0	80.9	70.5	65.7	43.8
Benzoylcegonine	91.9	94.3	95.6	35.6	31.4	28.5
Benzoylcegonine- <i>d</i> ₉	90.7	93.9	95.9	18.2	28.4	27.2
Morphine	96.8	91.1	97.6	78.5	84.0	76.4
Morphine- <i>d</i> ₃	79.6	108.6	97.4	83.1	84.0	72.0
6-Acetylmorphine	90.1	91.2	97.8	84.2	37.6	77.6
6-Acetylmorphine- <i>d</i> ₆	86.4	77.5	108.1	75.9	72.8	67.9
Codeine	95.0	98.8	95.3	72.4	60.6	49.0
Codeine- <i>d</i> ₆	105.7	95.1	97.0	52.7	35.9	28.5

Table 5

Stability of nicotine, opiates, cocaine, and metabolites in postmortem human brain at different specimen preparation conditions

Stability condition	% Analyte decrease									
	N=5	Nicotine	Cotinine	OH-cotinine	Cocaine	EME	BE	Morphine	6AM	Codeine
24 h RT ^a										
Low		2.7	2.0	0.4	15.5	5.2	12.8	13.3	9.7	2.8
Medium		1.9	17.7	2.3	0.7	4.9	2.4	3.0	0.7	8.3
High		8.1	12.2	1.1	1.9	5.3	0.8	5.2	3.5	0.6
72 h 4°C										
Low		13.1	5.1	11.6	6.7	17.4	8.6	7.9	9.3	4.9
Medium		4.9	6.1	7.6	6.3	6.8	9.6	11.4	6.4	7.0
High		6.7	9.0	7.1	1.7	3.6	2.5	8.3	0.9	0.5
72 h 15°C Autosampler										
Low		6.6	12.7	2.0	10.3	3.4	3.5	5.4	10.2	11.3
Medium		2.9	15.0	0.6	2.6	2.4	0.7	2.0	1.7	1.8
High		8.0	15.7	4.2	2.2	5.6	1.1	5.9	2.5	1.2
3 freeze-thaw cycles										
Low		2.9	2.4	4.7	2.1	6.2	1.8	3.9	12.8	3.7
Medium		1.5	9.4	1.5	10.0	6.4	1.2	2.6	15.5	7.9
High		6.9	5.3	3.3	12.1	3.9	7.1	7.3	17.3	2.4

^aRoom temperature