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High-throughput simultaneous analysis of buprenorphine, methadone, cocaine, opiates, nicotine, and metabolites in oral fluid by liquid chromatography tandem mass spectrometry

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Abstract

A method for simultaneous determination of buprenorphine (BUP), norbuprenorphine (NBUP), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), anhydroecgonine methyl ester (AEME), morphine, codeine, 6-acetylmorphine (6AM), heroin, 6-acetylcodeine (6AC), nicotine, cotinine, and *trans*-3'-hydroxycotinine (OH-cotinine) by liquid chromatography tandem mass spectrometry in oral fluid (OF) was developed and extensively validated. Acetonitrile (800 μ L) and OF (250 μ L) were added to a 96-well Isolute-PPT+protein precipitation plate. Reverse-phase separation was achieved in 16 min and quantification was performed by multiple reaction monitoring. The assay was linear from 0.5 or 1 to 500 μ g/L. Intraday, interday, and total imprecision were less than 13% ($n=20$), analytical recovery was 92–114% ($n=20$), extraction efficiencies were more than 77% ($n=5$), and process efficiencies were more than 45% ($n=5$). Although ion suppression was detected for EME, cocaine, morphine, 6AC, and heroin (less than 56%) and enhancement was detected for BE and nicotine (less than 316%), deuterated internal standards compensated for these effects. The method was sensitive (limit of detection 0.2–0.8 μ g/L) and specific (no interferences) except that 3-hydroxy-4-methoxyamphetamine interfered with AEME. No carryover was detected, and all analytes were stable for 24 h at 22 °C, for 72 h at 4 °C, and after three freeze–thaw cycles, except

cocaine, 6AC, and heroin (22–97% loss). The method was applied to 41 OF specimens collected throughout pregnancy with a Salivette® OF collection device from an opioid-dependent BUP-maintained pregnant woman. BUP ranged from 0 to 7,400 µg/L, NBUP from 0 to 71 µg/L, methadone from 0 to 3 µg/L, nicotine from 32 to 5,020 µg/L, cotinine from 125 to 508 µg/L, OH-cotinine from 11 to 51 µg/L, cocaine from 0 to 419 µg/L, BE from 0 to 351 µg/L, EME from 0 to 286 µg/L, AEME from 0 to 7 µg/L, morphine from 0 to 22 µg/L, codeine from 0 to 1 µg/L, 6AM from 0 to 4 µg/L, and heroin from 0 to 2 µg/L. All specimens tested negative for EDDP and 6AC. This method permits a fast and simultaneous quantification of 16 drugs and metabolites in OF, with good selectivity and sensitivity.

Keywords

Oral fluid; Liquid chromatography tandem mass spectrometry; Buprenorphine; Bioanalytical methods; Forensics/toxicology

Introduction

Oral fluid (OF) is an important alternative matrix to urine or blood for detecting recent drug consumption. The main advantages of monitoring drug intake with OF testing are an easy, noninvasive collection under direct observation, reduced adulteration opportunity, and a shorter drug detection window reflecting recent consumption. Disadvantages include the small amount of sample available, especially after stimulant use, low concentrations requiring high sensitivity, and difficulty in result interpretation. The OF-to-blood drug concentration ratio depends upon the pH of blood and OF, the degree of protein binding, the OF excretion rate, and the drug's lipid solubility, pK_a , and molecular weight [1, 2].

Owing to the small amount of OF available (less than 1 mL), multianalyte methods are recommended. Liquid chromatography tandem mass spectrometry (LCMSMS) allows simultaneous analysis of analytes of different polarity, without derivatization, and with good sensitivity. Procedures exist for simultaneous analysis of amphetamines, opiates and cocaine [3, 4], and other drugs, such as phencyclidine [5], cannabinoids, ketamine and phencyclidine [6], and benzodiazepines [7]. Several methods [8–11] included compounds required for the DRUID (driving under the influence of drugs, alcohol, and medicines) Project [12], and Dams et al. [13] included numerous cocaine and opioid metabolites other than methadone. These methods were developed in neat OF [3, 6, 9, 13], OF and Omni-Sal® buffer [7], Intercept® buffer [4, 8], Quantisal® buffer [5], and Statsure® buffer [10, 11]. The elution buffers improve drug recovery from the device and the stability of the analytes; however, the buffers' components may contribute to matrix effects (ion suppression or enhancement) and the sensitivity must be high owing to dilution of drugs in OF by the buffer. Nicotine and metabolites were analyzed previously by LCMSMS [14–16], but to date have not been included in a multianalyte assay.

This LCMSMS method was developed to monitor buprenorphine (BUP) compliance, determine tobacco-smoking status, identify relapse to opiates and cocaine, and methadone diversion in opioid-dependent pregnant women receiving BUP pharmacotherapy. This extensively validated method permits a fast and simultaneous quantification of 16 drugs and metabolites in just 250 µL OF, an alternative matrix with increasing utility, and high selectivity and sensitivity for identifying drug exposure.

Materials and methods

Chemicals and materials

Morphine, codeine, 6-acetylmorphine (6AM), heroin, 6-acetylcodeine (6AC), cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), anhydroecgonine methyl ester (AEME), nicotine, cotinine, BUP, norbuprenorphine (NBUP), methadone, and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) standards and morphine-*d*₆, 6AM-*d*₆, heroin-*d*₉, cocaine-*d*₃, BE-*d*₈, EME-*d*₃, nicotined₄, cotinine-*d*₃, BUP-*d*₄, NBUP-*d*₃, methadone-*d*₃, and EDDP-*d*₃ internal standards (IStd) (1 mg/mL or 100 µg/mL ampoules) were obtained from Cerilliant (Round Rock, TX, USA). Morphine, codeine, 6AM, 6AC, cocaine, BE, EME, BUP, and methadone quality controls (QC) and 6AC-*d*₃ and AEME-*d*₃ IStd (1 mg/mL ampoules) were obtained from Lipomed (Cambridge, MA, USA). *trans*-3'-Hydroxycotinine (OH-cotinine) and OH-cotinine-*d*₃ were purchased from Toronto Research Chemicals (North York, ON, Canada). Heroin, AEME, nicotine, cotinine, NBUP, and EDDP QC samples were prepared from a different lot from Cerilliant, when possible, or from a different vial, with preparation on different days than for the calibrators. OH-cotinine QC samples were prepared on different days than for the calibrators. Reagent grade formic acid was from Sigma Chemicals (St. Louis, MO, USA). All solvents were high performance liquid chromatography grade. Protein precipitation was performed in a 96-well Isolute PPT +protein precipitation plate (Biotage, Charlottesville, VA, USA). Different pools of drug-free human OF were obtained from volunteers expectorating into polypropylene tubes.

Apparatus

LCMSMS analysis was performed with a Shimadzu liquid chromatography system (Kyoto, Japan) interfaced to a 3200 QTrap (Applied Biosystems/MDS Sciex, Foster City, CA, USA) with an electrospray ion source. The Shimadzu system consisted of an LC-20AD binary pump, a DGU-20A3 degasser, a SIL-20AD autosampler, and a CTO-20A column oven. Data acquisition, peak integration, and calculation were performed by a computer workstation running Analyst™ 1.4.1. Sample centrifugation was performed with an Eppendorf 5804R centrifuge (Hamburg, Germany). Solvent evaporation was carried out with a MiniVap from Porvair Sciences (Leatherhead, UK).

Calibrator, quality control, and specimen preparation

OF samples from expectoration were centrifuged for 10 min at 7,232 *g*. A nine- or ten-point calibration curve (0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 500 µg/L) was prepared by adding 25 µL working calibrator and 30 µL IStd solution (0.1 mg/L of 16 deuterated analogs) to 250 µL blank OF. Low-, medium-, and high-concentration QC samples were prepared at 1.5, 30, and 300 µg/L for codeine, 6AM, 6AC, heroin, BE, cocaine, cotinine, OH-cotinine and BUP and at 3, 30, and 300 µg/L for morphine, EME, AEME, nicotine, NBUP, methadone, and EDDP. QC samples were prepared by adding 25 µL working QC solution and 30 µL IStd solution to 250 µL blank OF. For authentic specimens, 30 µL IStd solution was added to 250 µL OF.

Specimen cleanup

OF (250 µL) was fortified with IStd solution (30 µL). In a 96-well Isolute PPT+protein precipitation plate, 800 µL cold acetonitrile and the sample (250 µL OF fortified with IStd) were added to each well and equilibrated for 10 min. No vortexing was required. The purified filtrate was collected by applying a vacuum for 10 min. The filtrate was dried under nitrogen at 45 °C, reconstituted in 100 µL 0.1% formic acid in water, and 20 µL was injected into the LCMSMS system.

Liquid chromatography

Chromatographic separation was achieved with a Synergi Polar-RP 80A (75 mm×2 mm, 4 µm) column with a 4 mm×2 mm identically packed guard column (Phenomenex, Torrance, CA) and gradient elution with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 200 µL/min. The initial mixture (95% mobile phase A, 5% mobile phase B) was maintained for 1 min, mobile phase B was increased from 5% to 90% over 8 min, 90% mobile phase B was held for 3 min, and then the mixture was returned to the initial conditions over 0.5 min, followed by a 3.5 min equilibration. The total run time was 16 min.

Mass spectrometry

Mass-spectrometric data were acquired in positive electro-spray ionization mode with the following parameters: IonSpray voltage 5,500 V; temperature 550 °C; curtain gas 10; ion source gas 1:50; and ion source gas 2:55. The curtain and ion source gases were nitrogen. Data were recorded in multiple reaction monitoring mode. Tandem mass spectrometry (MSMS) optimization was established by directly infusing 100 µg/L of each analyte in methanol directly. The precursor ions, product ions, and LCMSMS parameters are displayed in Table 1. The MSMS method was divided into three acquisition windows to increase the dwell time, improve the sensitivity, and achieve a minimum of 12 points per peak.

Validation

The validation parameters included linearity, the limit of detection (LOD), the limit of quantification (LOQ), imprecision, analytical recovery, extraction efficiency, process efficiency, matrix effect, selectivity, carryover, dilution integrity, and stability studies. The linearity was determined by least-squares regression with 1/*x* weighting. Acceptable linearity was achieved when the coefficient of determination was at least 0.99 and the calibrators were quantified within ±20% at the LOQ and ±15% at other concentrations. The LOD and LOQ were evaluated with decreasing analyte concentrations in drug-fortified OF. The LOD was defined as the lowest concentration with acceptable chromatography, the presence of all transitions with signal-to-noise ratios of at least 3, and a retention time within ±0.2 min of the average retention time of the calibrator. The LOQ was the lowest concentration that met the LOD criteria and a signal-to-noise ratio of at least 10, imprecision lower than 20%, and analytical recovery between 80 and 120%.

The imprecision and analytical recovery were determined at three concentrations by analyzing five replicates on four different days (*n*=20). Imprecision, expressed as the coefficient of variation of the measured values, was expected to be less than 15%. The guidelines given by Krouwer and Rabinowitz [17] were followed for the calculation of pooled intraday, interday, and total imprecision. The analytical recovery was evaluated as the percentage of the target concentration (*n*=20) with a required criterion of 85–115%.

The extraction efficiency for each analyte was measured at each QC concentration. Blank OF was fortified with QC and IStd solutions before and after solid-phase extraction. The percentage extraction efficiency from OF was expressed as the mean analyte area of samples (*n*=5) fortified with control solution before extraction divided by the mean area of samples (*n*=5) with the control solution added after extraction. The matrix effect was assessed by comparing the analyte peak areas in ten different blank extracted OF specimens fortified with QC and IStd solutions after extraction with the peak areas of samples at the same nominal concentrations prepared in mobile phase A (neat). The process efficiency examined the overall effect of the extraction efficiency and the matrix effect on the quantification of the analytes of interest. It was determined by comparing the mean analyte peak areas of five

samples fortified before extraction with the mean peak areas of five neat samples prepared in the mobile phase at the same concentration.

Interferences from endogenous matrix components were evaluated by analyzing OF samples from ten healthy non-drug-consuming volunteers fortified only with IStd solution. Endogenous interferences were considered insignificant if analytes were not detected in these ten OF samples. The method specificity was demonstrated by adding high concentrations (500 µg/L) of potentially interfering licit and illicit drugs to low-concentration QC samples. The following drugs and metabolites were examined: amphetamine, methamphetamine, *p*-hydroxyamphetamine, *p*-hydroxymethamphetamine, norephedrine, hydroxynorephedrine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine, 3-hydroxy-4-methoxyamphetamine (HMA), 3-hydroxy-4-methoxymethamphetamine, Δ^9 -tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, diazepam, lorazepam, oxazepam, alprazolam, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine, chlorpheniramine, brompheniramine, acetylsalicylic acid, acetaminophen, and phencyclidine. Sufficient specificity was achieved if the analytes were quantified within $\pm 15\%$ of the low QC concentrations.

Lack of carryover was demonstrated by injecting IStd-fortified blank OF immediately after a sample had been spiked with all analytes at 1,000 µg/L, 2 times the upper LOQ. Carryover was considered negligible if the measured concentration was below the LOQ. The dilution integrity was evaluated by diluting OF samples ($n=2$) containing 1,000 µg/L of each analyte with blank OF to achieve 1:10 and 1:100 dilutions. IStd was added to diluted samples that were extracted as described. Dilution integrity was achieved if samples were quantified within $\pm 15\%$ of 100 µg/L (dilution 1:10) or of 10 µg/L (dilution 1:100).

Analyte stability was evaluated for drug-fortified OF stored at room temperature (22 °C) for 24 h, in a refrigerator (4 °C) for 72 h, and after three freeze–thaw cycles (frozen for 24 h, thawed, and kept at room temperature for 3 h). OF samples were fortified with analyte mixture 1 (codeine, heroin, cocaine, nicotine, BUP, and methadone) and with analyte mixture 2 (morphine, 6AM, 6AC, BE, EME, AEME, cotinine, OH-cotinine, NBUP, and EDDP) at three concentrations (low, medium, and high QC concentrations) in triplicate. Stability was considered acceptable if QC samples were quantified within $\pm 15\%$ of the target.

Identification criteria

Identification criteria included a retention time within ± 0.2 min of the average calibrator retention time, the presence of two transitions, and relative ion intensities (percentage of the base peak) within $\pm 20\%$ if the relative ion intensity was more than 50%, $\pm 25\%$ if it was 20–50%, $\pm 30\%$ if it was 10–20%, and $\pm 50\%$ if it was 10% or less [18]. Relative ion intensities were calculated on the basis of ion ratios (quantification transition divided by qualifier transition). These values were compared with the mean relative ion intensity of all calibrators.

Method application

The method was applied to the analysis of OF specimens collected thrice weekly throughout pregnancy from an opioid-dependent pregnant woman receiving BUP treatment during participation in a Johns Hopkins Bayview Medical Center and National Institute on Drug Abuse Institutional Review Board approved research project. Subjects provided written informed consent to participate. Specimen collection was performed using Sarstedt Salivette® collection devices (Nümbrecht, Germany). For sample collection, the Salivette®

swab was placed into the mouth and chewed for approximately 45 s. The swab was placed into the insert in the collection tube, centrifuged, and the insert with the swab removed to recover clear OF [19]. OF specimens were collected and stored in a freezer at -20°C until analysis.

Salivette® recovery

Analyte recovery from the cotton swab of the Sarstedt Salivette® collection device was evaluated at three QC concentrations. In a borosilicate tube, 1 mL of blank OF was spiked with QC standards and vortexed. The cotton swab absorbed all of the OF, and was centrifuged for 10 min. OF (250 μL) was analyzed as described. These samples were compared with samples prepared in the same way but without the cotton swab step.

Results

The linearity of the analyte-to-IStd peak area ratio versus the theoretical concentration was verified in OF with $1/x$ -weighted linear regression. The curvature tested on a set of four calibration curves yielded determination coefficients (r^2) above 0.99, with residuals within $\pm 20\%$ at the LOQ and $\pm 15\%$ at all other calibrator concentrations. The LOD ranged between 0.2 and 0.8 $\mu\text{g/L}$, and the LOQ was 0.5 or 1 $\mu\text{g/L}$ depending on the analyte. These results are summarized in Table 2. Figure 1 shows chromatograms of the quantification transition at the low QC concentration for all analytes.

Imprecision and analytical recovery results were satisfactory for all concentrations tested (Table 3). Extraction efficiencies for all analytes ranged from 77.3 to 116.0%, and process efficiencies ranged from 45.4 to 358.3%. The matrix effect ranged from 0.1 to 315.5%, with variation between ten different OF of less than 29.4%. The data are shown in Table 4.

Under the conditions described, no interference with any extractable endogenous compound in OF was observed. The method selectivity was demonstrated by adding high concentrations (500 $\mu\text{g/L}$) of 28 potentially interfering licit and illicit drugs, and metabolites to low-concentration QC samples. All test samples were quantified within $\pm 15\%$ of the target, indicating no interference with the 16 analytes of interest, except HMA interfered with the correct identification of AEME; false-negative AEME test results could be a consequence. No analyte was detected in a blank sample injected immediately following analysis of a 1,000 $\mu\text{g/L}$ sample, indicating no carryover at this concentration.

The ability of the method to accurately quantify specimens containing high concentrations of analytes was evaluated by diluting 1:10 and 1:100 samples at 1,000 $\mu\text{g/L}$ ($n=2$) with blank OF; 225 μL blank OF was added to 25 μL fortified sample to achieve a 1:10 dilution, and analyzed; 990 μL blank OF was added to 10 μL fortified sample to achieve a 1:100 dilution, and 250 μL of this sample was analyzed. Samples were quantified within 15% of 100 or 10 $\mu\text{g/L}$, confirming dilution integrity.

The analytes were stable when stored at room temperature (22°C) for 24 h, refrigerated at 4°C for 72 h, and after three freeze–thaw cycles, with a loss of less than 19.2%, except for cocaine at room temperature, 6 AC for the low QC concentration at every storage condition, and all heroin concentrations at every storage condition. The results are shown in Table 5. The recovery of the analytes from the cotton swab of the Salivette® collection device was more than 73.3%, except for methadone (47.4–50.8%), EDDP (64.8–65.6%), BUP (20.2–21.7%), and NBUP (47.4–59.5%).

Forty-one OF specimens collected by the Salivette® device from a BUP-maintained opioid-dependent pregnant woman throughout pregnancy were analyzed. Of these, 85.4% were

positive for BUP, 61.0% for NBUP, 4.9% for methadone, 68.3% for cocaine, 97.6% for BE, 24.4% for EME, 29.3% for AEME, 29.3% for morphine, 4.9% for codeine, 19.3% for 6AM, and 7.3% for heroin. All were positive for nicotine and metabolites, and negative for EDDP and 6AC. The median and range of concentrations were 170 and 0–7,400 µg/L for BUP, 4.1 and 0–71.3 µg/L for NBUP, 0 and 0–3.1 µg/L for methadone, 187 and 31.6–5,020 µg/L for nicotine, 240 and 125–508 µg/L for cotinine, 33.8 and 10.7–51 µg/L for OH-cotinine, 2.5 and 0–419 µg/L for cocaine, 8.4 and 0–351 µg/L for BE, 0 and 0–286 µg/L for EME, 0 and 0–6.8 µg/L for AEME, 0 and 0–22.2 µg/L for morphine, 0 and 0–1.1 µg/L for codeine, 0 and 0–3.9 µg/L for 6AM, and 0 and 0–1.8 µg/L for heroin.

Discussion

A simple and fast method for the sensitive (0.5 or 1 µg/L LOQ) quantification of BUP, methadone, opiates, cocaine, nicotine, and their metabolites in only 250 µL OF was developed and fully validated. The assay fulfilled compound identification criteria suggested by the European Union (EU) [18] and the Food and Drug Administration (FDA) [20]. These guidelines [18, 20] require chromatographic separation, a minimum of two transitions per analyte if LCMSMS is employed or three characteristic ions if liquid chromatography mass spectrometry (LCMS) (single mass spectrometry) is employed, and acceptable relative ion intensities. In the literature, several examples of false-positive results have been reported with utilization of a single transition [21, 22], demonstrating the importance of following these confirmation guidelines.

Cocaine and BE were included in all previously published methods [3–11, 13], but EME appears in only two methods [7, 13] and AEME in one method [13]. The inclusion of AEME may be important to differentiate crack cocaine consumption from other cocaine consumption [23]. In addition, the LOQ for EME and AEME in the present method (1 µg/L) are lower than those reported in the previous assays, with LOQ of 10 µg/L [13] and 5 µg/L [7]. Furthermore, the EU [18] and FDA [20] confirmation criteria (two transitions, correct ion ratio) were not fulfilled in the previous methods because only one transition was monitored.

With regard to opiates, the analytes normally included are morphine, codeine, and 6AM [3–11]. In our study, we decided to include also heroin and 6AC as markers of illicit heroin use [24]. Dams et al. [13] analyzed numerous opiate metabolites, including heroin and 6AC, but with higher LOQ (1 µg/L) than the present method (0.5 µg/L).

BUP was included in three previous methods [4, 8, 11], with LOQ similar to [11] or higher than, 1.2 µg/L [8] and 5 µg/L [7], the LOQ of the present method (0.5 µg/L). Its metabolite, NBUP, was analyzed only by Wylie et al. [7], but at a higher LOQ (5 µg/L), and only one transition was monitored. Methadone was included in several methods [7–11, 13], but EDDP was included in only two methods [7, 13]. The EDDP LOQ achieved was similar to, 1 µg/L [13], or higher than, 5 µg/L [7], the LOQ of the present method, and only one transition was monitored.

LCMS and LCMSMS methods for the quantification of nicotine and metabolites in OF were published previously [14–16] with LOQ similar to [14, 16] or better than [14–16] that of the present method, depending on the compound. However, these methods employed a larger specimen volume, 0.5 mL [14] or 1 mL [15, 16], compared with 250 µL in the present method, and only one transition was monitored for OH-cotinine [15] or just one ion in selected ion monitoring mode for nicotine and cotinine [16].

Owing to the simple sample preparation (protein precipitation plate), the extraction efficiency was more than 77% for all compounds; however, a matrix effect, ion suppression

or enhancement, was detected for several analytes. Cocaine, morphine, 6AC, and heroin showed ion suppression up to 56.4%, and BE and nicotine showed ion enhancement up to 315.5%. Deuterated analogs of each compound were used as an IStd, to compensate for these effects. In the literature, four articles address measurement of drugs in neat OF [3, 6, 9, 13]. Dams et al. [13] and Sergi et al. [6] also employed a protein precipitation cleanup procedure, and no matrix effect was detected. Dams et al. [13] evaluated the matrix effect only for morphine, investigating just one OF source, with the postcolumn infusion method. Sergi et al. [6] compared the instrumental calibration curve ("calibration curve in solvent") with calibration curves obtained from blank extracts spiked with analytes ("calibration curve in matrix"). Unfortunately, the number of different sources investigated was not reported. Mortier et al. [3] and Concheiro et al. [9] employed solid-phase extraction as the cleanup procedure. Mortier et al. [3] did not observe any matrix effect with five different sources, and Concheiro et al. [9] observed ion suppression of up to 56% ($n=10$), despite performing solid-phase extraction.

No endogenous (ten different sources) or exogenous (28 licit and illicit drugs and metabolites) interferences were detected with this method, except that HMA interfered with AEME. OF spiked at 500 $\mu\text{g/L}$ HMA gave a signal equivalent to 10 $\mu\text{g/L}$ AEME. The slightly different retention times, and the absence of the qualification transition, avoided the misidentification of the peak, but a correct AEME quantification is not possible when high HMA concentrations are present. HMA is a minor MDMA metabolite, but it is not yet known what OF HMA concentrations may be present after MDA administration.

No significant losses were observed for all compounds in neat OF under different storage conditions (24 h at 22 °C, 72 h at 4 °C, three freeze–thaw cycles), except for cocaine at room temperature (33.1% loss), 6 AC for the low QC concentration at every storage condition (25.8% loss), and heroin at every storage condition (up to 96.9% at 22 °C). Good stability in neat OF was previously reported after three to five freeze–thaw cycles [6, 13], for 24 h at 22 °C [13], and for 1–2 months at –20 °C [6]. Dams et al. [13] did not observe any cocaine loss at room temperature, and heroin was stable for 24 h at room temperature and after three freeze–thaw cycles in neat OF; however, the stability was studied in only one fortified OF at one concentration. The pH of neat OF, which could affect analyte stability, was not measured in any study.

OF was collected with the Salivette® device in this study. The recovery of the analytes from the cotton swab of the Salivette® collection device was more than 73.3%, except for methadone, EDDP, BUP, and NBUP, with recoveries between 20.2 and 65.6%. In accordance with these results, Dams et al. [13] also reported good recoveries from the Salivette® device for opiates, cocaine, and metabolites. However, Langel et al. [19] reported low recoveries for cocaine (33.3%), morphine (35.2%), and codeine (39.0%). No data from the Salivette® device for methadone, EDDP, BUP, and NBUP recovery were published previously.

As a proof of method, 41 OF specimens collected throughout pregnancy from a BUP-maintained opioid-dependent pregnant woman were analyzed. As expected, BUP was detected in a high percentage of specimens (85.4%), achieving high concentrations (up to 7,400.0 $\mu\text{g/L}$) probably owing to oral contamination. NBUP was detected in 61.0% of specimens with concentrations up to 71.3 $\mu\text{g/L}$. Although the subject was to be dosed daily with 16 mg BUP, several OF specimens tested negative for BUP and NBUP, perhaps owing to different specimen collection times or missed doses. Relapse to opiates and cocaine was detected in 29.3 and 97.6% of specimens, respectively. Methadone diversion was detected in 4.9% of specimens, however the methadone concentrations were low (1.2–3.1 $\mu\text{g/L}$).

Nicotine and metabolites were detected in all specimens, indicating tobacco consumption throughout pregnancy.

Conclusion

A fast and simple method for the simultaneous analysis by LCMSMS of BUP, methadone, opiates, cocaine, nicotine, and their metabolites in only 250 μ L OF was developed. Protein precipitation was performed with a protein precipitation plate that allows the simultaneous analysis of 96 specimens. The method was fully validated, showing good sensitivity (LOQ 0.5–1 μ g/L) and specificity, and fulfilled FDA [20] and EU [18] confirmation criteria (two transitions, correct ion ratio). This method should be highly useful for OF quantification of illicit drugs, opioid-dependence pharmacotherapies, and tobacco markers in clinical and forensic toxicology laboratories.

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Fig. 1. Liquid chromatography tandem mass spectrometry chromatograms of an oral fluid sample spiked at the low quality control concentration, 1.5 µg/L for codeine, 6-acetylmorphine (6AM), 6-acetylcodeine (6AC), heroin, benzoylecgonine (BE), cocaine, cotinine, *trans*-3'-hydroxycotinine (OH-cotinine), and buprenorphine (BUP), and 3 µg/L for morphine, ecgonine methyl ester (EME), anhydroecgonine methyl ester (AEME), nicotine, norbuprenorphine (NBUP), methadone, and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Quantitation transitions of analytes monitored in the first (a), second (b), and third (c) acquisition windows are included

Table 1

Liquid chromatography tandem mass spectrometry parameters, retention times (*RT*), and internal standards (*IStd*)

Analyte	MRM transition ^a	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)	RT (min)	IStd
EME	<u>200.2→182.2</u>	36	9	12	25	4	2.2	EME- <i>d</i> ₃
	<u>200.2→82.2</u>	36	9	12	35	4		
EME- <i>d</i> ₃	<u>203.3→185.3</u>	41	11	12	25	4	2.2	
	<u>203.3→85.2</u>	41	11	12	35	4		
Nicotine	<u>163.2→132.2</u>	36	5.5	10	21	4	2.2	Nicotine- <i>d</i> ₄
	<u>163.2→80.1</u>	36	5.5	10	25	4		
Nicotine- <i>d</i> ₄	<u>167.2→136.1</u>	36	3.5	12	21	4	2.2	
	<u>167.2→84.0</u>	36	3.5	12	21	4		
OH-cotinine	<u>193.2→80.0</u>	46	3.5	12	37	4	2.2	OH-cotinine- <i>d</i> ₃
	<u>193.2→134.0</u>	46	3.5	12	27	4		
OH-cotinine- <i>d</i> ₃	<u>196.2→80.0</u>	46	10.5	14	43	4	2.2	
	<u>196.2→134.0</u>	46	10.5	14	27	4		
Cotinine	<u>177.2→80.1</u>	41	10	12	33	4	2.8	Cotinine- <i>d</i> ₃
	<u>177.2→98.1</u>	41	10	12	29	4		
Cotinine- <i>d</i> ₃	<u>180.2→80.2</u>	21	7	12	33	4	2.8	
	<u>180.2→101.2</u>	21	7	12	31	4		
AEME	<u>182.2→122.0</u>	41	7	14	27	4	3.8	AEME- <i>d</i> ₃
	<u>182.2→150.1</u>	41	7	14	23	4		
AEME- <i>d</i> ₃	<u>185.2→125.1</u>	31	7	12	29	4	3.8	
	<u>185.2→153.1</u>	31	7	12	27	4		
Morphine	<u>286.3→152.0</u>	66	3	16	71	4	4.7	Morphine- <i>d</i> ₆
	<u>286.3→165.2</u>	66	3	16	51	4		
Morphine- <i>d</i> ₆	<u>292.2→201.0</u>	61	3.5	22	25	4	4.7	
	<u>292.2→211.1</u>	61	3.5	22	43	4		
Codeine	<u>300.3→151.9</u>	50	6.5	14	91	4	5.8	Codeine- <i>d</i> ₆
	<u>300.3→165.1</u>	50	6.5	14	53	8		
Codeine- <i>d</i> ₆	<u>306.3→152.2</u>	52	3.5	22	81	4	5.8	

Analyte	MRM transition ^a	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)	RT (min)	ISId
6AM	306.3→165.2	52	3.5	22	53	4		
	328.2→165.2	66	7.5	14	53	4	6.1	6AM-d ₆
6AM-d ₆	328.2→152.2	66	7.5	14	79	4		
	334.3→165.2	66	10	16	51	4	6.1	
	334.3→152.0	66	10	16	80	4		
BE	290.2→168.3	41	9.5	22	25	4	6.4	BE-d ₈
	290.2→105.1	41	9.5	22	43	4		
BE-d ₈	298.3→171.3	46	3	20	27	4	6.4	
	298.3→110.0	46	3	20	41	4		
6AC	342.2→225.5	71	4.5	20	41	4	6.9	6AC-d ₃
	342.2→282.2	71	4.5	20	41	4		
6AC-d ₃	345.2→225.2	61	6	14	33	6	6.9	
	345.2→285.2	61	6	14	27	4		
Heroin	370.1→268.2	76	7	14	39	4	6.9	Heroin-d ₉
	370.1→328.1	76	7	14	35	4		
Heroin-d ₉	379.2→272.2	61	3.5	18	39	4	6.9	
	379.2→335.3	61	3.5	18	37	4		
NBUP	414.3→340.1	86	12	26	43	4	7.1	NBUP-d ₃
	414.3→326.0	86	12	26	40	5		
NBUP-d ₃	417.3→343.4	86	12	16	39	4	7.1	
	417.3→326.0	86	12	22	43	4		
Cocaine	304.3→182.3	51	3	16	25	4	7.2	Cocaine-d ₃
	304.3→82.1	51	3	16	37	4		
Cocaine-d ₃	307.3→185.3	41	5	18	27	4	7.2	
	307.3→85.2	41	5	18	43	4		
BUP	468.3→396.2	86	12	18	53	6	8.1	BUP-d ₄
	468.3→414.3	86	12	18	45	12		
BUP-d ₄	472.5→400.1	86	11	24	61	6	8.1	
	472.5→415.3	86	11	24	45	8		
EDDP	278.2→234.1	61	4	16	43	4	8.6	EDDP-d ₃

Analyte	MRM transition ^a	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)	RT (min)	IStd
EDDP- <i>d</i> ₃	278.2→249.2	61	4	16	33	4		
	<u>281.3→234.1</u>	61	9.5	24	31	4	8.6	
	281.3→249.2	61	9.5	24	31	4		
Methadone	<u>310.3→265.2</u>	31	4	14	19	4	8.7	Methadone- <i>d</i> ₃
	310.3→223.3	31	4	14	27	4		
Methadone- <i>d</i> ₃	<u>313.3→268.1</u>	31	4.5	16	19	4	8.7	
	313.3→226.2	31	4.5	16	27	4		

MRM multiple reaction monitoring, DP declustering potential, EP entrance potential, CEP collision cell entrance potential, CE collision energy, CXP collision cell exit potential, EME ecgonine methyl ester, OH-cocaine *trans*-3'-hydroxycocaine, AEME anhydroecgonine methyl ester, 6AM 6-acetylcodeine, BE benzoylcodeine, NBUP norbuprenorphine, BUP buprenorphine, EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

^aThe quantification transition is *underlined*.

Table 2Limits of detection (*LOD*), limits of quantification (*LOQ*), and linearity results

Analyte	LOD (µg/L)	LOQ (µg/L)	Linearity (µg/L)	Intercept ± SD (n=4)	Slope ± SD (n=4)	r ² ± SD (n=4)
EME	0.8	1	1–500	−0.0062±0.0074	0.0379±0.0040	0.9954±0.0021
AEME	0.8	1	1–500	0.0005±0.0028	0.0395±0.0047	0.9942±0.0023
BE	0.4	0.5	0.5–500	0.0032±0.0044	0.0328±0.0031	0.9965±0.0021
Cocaine	0.4	0.5	0.5–500	0.0107±0.0021	0.0447±0.0051	0.9967±0.0022
Morphine	0.5	1	1–500	0.0100±0.0437	0.1605±0.0085	0.9958±0.0015
Codeine	0.4	0.5	0.5–500	−0.0050±0.0020	0.0490±0.0025	0.9952±0.0036
6AM	0.4	0.5	0.5–500	0.0072±0.0056	0.0482±0.0032	0.9963±0.0021
6AC	0.4	0.5	0.5–500	0.0015±0.0026	0.0378±0.0081	0.9965±0.0017
Heroin	0.4	0.5	0.5–500	−0.0020±0.0060	0.0453±0.0082	0.9952±0.0026
Nicotine	0.5	1	1–500	0.0073±0.0032	0.0436±0.0043	0.9970±0.0015
Cotinine	0.4	0.5	0.5–500	0.0074±0.0027	0.0599±0.0043	0.9960±0.0031
OH-cotinine	0.4	0.5	0.5–500	0.0011±0.0021	0.0321±0.0014	0.9956±0.0027
Methadone	0.2	1	1–500	0.0297±0.0042	0.0336±0.0038	0.9968±0.0006
EDDP	0.2	1	1–500	0.0428±0.0082	0.0610±0.0057	0.9965±0.0016
BUP	0.4	0.5	0.5–500	0.0191±0.0294	0.0686±0.0046	0.9959±0.0032
NBUP	0.8	1	1–500	0.0178±0.0057	0.0336±0.0044	0.9959±0.0033

SD standard deviation

Table 3

Pooled intraday, interday, and total imprecision, and analytical recovery

Analyte	Pooled intraday imprecision (n=20; CV, %)			Interday imprecision (n=20; CV, %)			Total imprecision (n=20; CV, %)			Analytical recovery (n=20, % target)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
EME	10.1	7.5	4.5	0.0	4.1	9.2	10.1	8.5	10.2	104.1	103.4	101.7
AEME	7.1	6.2	5.9	0.0	9.4	9.2	7.1	11.3	10.9	105.7	102.1	99.6
BE	9.2	4.1	5.5	0.0	7.6	8.2	9.2	8.6	9.9	105.1	97.0	94.7
Cocaine	7.3	5.5	6.1	0.0	6.4	4.2	7.3	8.5	7.4	104.4	103.9	96.5
Morphine	7.8	6.9	6.6	5.0	0.0	6.4	9.3	6.9	9.2	105.4	104.5	99.3
Codeine	6.1	5.4	7.8	2.7	6.7	7.4	6.7	8.6	10.7	108.7	104.3	100.0
6AM	7.6	4.6	6.7	0.0	5.7	9.4	7.6	7.4	11.5	107.8	104.9	101.8
6AC	9.5	6.0	7.1	3.2	4.4	5.2	10.0	7.5	8.8	102.2	94.4	92.4
Heroin	6.4	4.4	6.0	0.0	5.7	7.3	6.4	7.2	9.5	109.0	107.3	104.8
Nicotine	9.7	5.6	7.4	2.1	7.8	8.9	9.9	9.6	11.6	108.0	104.4	98.9
Cotinine	8.8	5.0	7.4	2.3	7.3	8.4	9.1	8.8	11.2	106.4	103.8	99.6
OH-cotinine	6.3	6.1	6.9	11.6	6.2	8.3	13.2	8.7	9.3	105.4	99.8	98.7
Methadone	7.9	4.0	6.4	0.0	2.3	8.7	7.9	4.6	10.8	106.3	110.3	103.2
EDDP	5.6	3.7	5.4	7.3	0.0	3.1	9.2	3.7	6.2	108.4	114.3	107.3
BUP	8.1	5.1	5.8	6.9	9.8	6.8	10.7	11.1	8.9	104.1	100.1	98.7
NBUP	8.8	6.8	7.6	5.2	7.5	8.2	10.2	10.1	11.2	97.8	100.7	99.9

CV coefficient of variation

Table 4

Extraction efficiency, process efficiency, and matrix effect

Analyte	Extraction efficiency (%) (n=5)			Process efficiency (%) (n=5)			Matrix effect (CV, %) (n=10)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
EME	91.4	77.3	89.5	49.3	87.2	89.0	-46.0 (20.5)	6.5 (15.1)	-1.9 (12.6)
AEME	92.3	85.6	96.2	68.8	103.7	106.3	-26.5 (14.1)	15.7 (11.2)	-3.0 (17.8)
BE	90.6	93.9	110.2	307.6	309.8	210.4	238.9 (12.6)	225.7 (12.2)	82.6 (19.3)
Cocaine	104.0	100.6	110.1	45.4	54.2	66.9	-56.4 (10.1)	-46.8 (16.6)	-39.9 (13.2)
Morphine	90.4	91.0	105.9	67.3	54.7	70.2	-26.8 (15.9)	-40.2 (14.3)	-39.8 (17.0)
Codeine	87.6	90.6	99.6	73.0	83.7	91.8	-19.4 (9.4)	-12.2 (13.5)	-12.7 (14.2)
6AM	91.6	85.1	104.3	97.6	94.7	107.5	3.9 (11.4)	9.2 (12.9)	-2.5 (16.7)
6AC	102.5	104.1	116.0	52.9	63.9	62.9	-47.1 (12.4)	-39.8 (18.6)	-46.9 (18.7)
Heroin	94.4	94.3	109.2	54.4	59.8	55.8	-43.2 (16.9)	-36.2 (17.5)	-49.3 (17.3)
Nicotine	92.4	85.3	93.9	309.3	358.3	142.7	232.7 (29.4)	315.5 (20.0)	71.6 (21.5)
Cotinine	88.5	91.5	102.0	93.4	95.3	91.1	1.2 (16.1)	-0.5 (12.9)	-4.7 (12.1)
OH-cotinine	93.8	89.0	91.0	94.8	75.2	67.8	7.9 (18.9)	-14.9 (15.1)	-22.0 (18.2)
Methadone	90.9	78.8	97.9	104.7	111.6	116.6	17.3 (9.2)	41.4 (7.3)	20.1 (8.1)
EDDP	93.1	85.4	103.1	114.5	188.2	108.5	26.5 (9.3)	123 (8.4)	6 (6.9)
BUP	90.2	83.7	90.8	89.7	91.2	95.7	0.1 (9.7)	8.1 (10.1)	5.0 (12.5)
NBUP	93.0	88.8	111.9	74.6	81.9	110.4	-18.5 (15.3)	-9.5 (15.6)	-1.8 (17.8)

Table 5

The stability in neat oral fluid under different storage conditions: room temperature for 24 h; 4 °C for 72 h; three freeze-thaw cycles

Analyte	Room temperature 24h (n=3, % difference)			4 °C 72h (n=3, % difference)			3 freeze-thaw cycles (n=3, % difference)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
EME	-19.2	-4.3	-4.4	-13.5	4.2	-5.4	-14.0	17.4	-6.0
AEME	-12.8	13.6	8.5	-10.1	12.3	11.1	-13.5	15.7	9.5
BE	-8.5	6.4	11.0	-9.9	-2.2	-4.1	-11.6	-3.6	-5.2
Cocaine	-33.1	-24.6	-27.1	-22.2	-6.7	-11.3	-17.2	-1.4	-8.4
Morphine	-0.5	10.3	3.5	4.3	4.7	5.9	-4.3	10.4	8.7
Codeine	-6.3	16.1	-1.4	-1.8	12.1	-2.1	-2.7	11.3	11.0
6AM	-5.7	-8.8	-15.2	-3.6	-5.3	-7.7	-7.8	-7.7	-9.0
6AC	-23.6	-3.7	-10.7	-25.8	-3.4	-10.8	-23.0	-2.0	-8.0
Heroin	-76.3	-95.8	-96.9	-41.6	-79.9	-85.1	-12.3	-55.5	-55.7
Nicotine	-9.2	13.0	5.5	-7.7	8.4	6.1	3.0	8.3	4.6
Cotinine	-0.2	14.6	18.4	-5.4	13.6	18.7	2.9	15.7	13.7
OH-cotinine	6.7	19.1	19.1	6.0	9.6	15.0	2.9	11.5	14.9
Methadone	-14.3	4.4	5.2	-15.9	4.3	6.4	-17.2	0.5	6.7
EDDP	-1.4	2.6	7.0	-4.6	-0.8	8.3	-3.7	-2.1	3.9
BUP	-0.5	15.9	0.5	18.5	3.6	-6.5	20.1	-0.8	-3.7
NBUP	-13.8	11.3	-3.5	2.9	0.5	-8.3	-11.7	-3.7	-0.1

% difference percentage difference from fresh controls