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Optimization and validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of nicotine, cotinine, *trans*-3'-hydroxycotinine and norcotinine in human oral fluid

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Abstract

An analytical procedure was developed and validated for the simultaneous identification and quantification of nicotine, cotinine, *trans*-3'-hydroxycotinine, and norcotinine in 0.5 mL of human oral fluid collected with the Quantisal™ oral fluid collection device. Solid phase extraction and liquid chromatography-tandem mass spectrometry with multiple reaction monitoring were utilized. Endogenous and exogenous interferences were extensively evaluated. Limits of quantification were empirically identified by decreasing analyte concentrations. Linearity was from 1 to 2,000 ng/mL for nicotine and norcotinine, 0.5 to 2,000 ng/mL for *trans*-3'-hydroxycotinine, and 0.2 to 2,000 ng/mL for cotinine. Correlation coefficients for calibration curves were >0.99 and analytes quantified within ±13% of target at all calibrator concentrations. Suitable analytical recovery (>91%) was achieved with extraction efficiencies >56% and matrix effects <29%. This assay will be applied to the quantification of nicotine and metabolites in oral fluid in a clinical study determining the most appropriate nicotine biomarker concentrations differentiating active, passive, and environmental nicotine exposure.

Keywords

Nicotine; Cotinine; *trans*-3'-Hydroxycotinine; Norcotinine; Oral fluid; Tobacco biomarkers; LCMSMS; Saliva

Introduction

Oral fluid is an important alternative matrix to blood and urine for monitoring drug and tobacco exposure; collection is simple and noninvasive and can be performed by nonmedical personnel. The matrix is relatively clean and offers measurement of unbound, pharmacologically active drug concentrations. Drug detection windows in biological

specimens are determined by multiple factors including dose, route of administration, cutoff concentrations, specimen dilution, oral fluid flow, pH, and type of collection device. In addition, specimen collection can have a serious impact on analytical findings, and volumes of oral fluid tend to be low restricting the number and scope of analyses [1]. Oral fluid can be collected by expectoration or the passive drool technique, but generally oral fluid collection devices are utilized. The amount and variability of oral fluid collected varies widely across devices, as do the buffers employed to achieve recovery of drugs from device components [2, 3]. The buffers and surfactants in each collection device are proprietary and may interfere with drug measurement due to suppression or enhancement of analyte ionization. Thus, there is a need for sensitive and specific assays optimized for each type of oral fluid collection device.

Nicotine is a major component of tobacco and the addictive substance in cigarette smoke. Active and passive tobacco smoking are major causes of morbidity [4]. Nicotine is absorbed through the skin and mucosal lining of the mouth and nose and by inhalation through the lungs of active and passive smokers [5]. The drug is extensively metabolized with the rate and pattern of metabolism varying among individuals. Cotinine, a primary metabolite of nicotine, is formed by oxidation by hepatic cytochrome P450 CYP2A6, with further metabolism to *trans*-3'-hydroxycotinine (OH-cotinine) and other minor metabolites including norcotinine [5]. Nicotine in plasma has a short half-life ($t_{1/2}$) of 1–2 h [1] with longer half-lives for cotinine (18–20 h) and OH-cotinine (4–8 h) [5, 6]. Thus, the latter two analytes are superior biomarkers for identifying tobacco exposure.

Expired carbon monoxide (CO), a well-characterized biomarker of tobacco smoking, has a half-life of 2–8 h [7], indicating relatively recent exposure. CO measurements are noninvasive and inexpensive and can be performed rapidly, but CO's short half-life limits its usefulness for documenting tobacco abstinence.

Methods for nicotine and metabolite quantification in biological specimens include radioimmunoassay [8], enzyme-linked immunoassay [9], gas chromatography [10], gas chromatography mass spectrometry (GCMS) [11], high-performance liquid chromatography [11], and liquid chromatography-tandem mass spectrometry (LCMSMS) [12–16]. Generally, liquid–liquid extraction or solid phase extraction (SPE) is required for biological specimen extraction and concentration prior to chromatography. Cotinine, the analyte with the longest half-life, has been quantified in blood [17], saliva [18, 19], brain [20], semen [21], and urine [19]. Enzyme immunoassay estimated cotinine in urine, blood, and oral fluid of smokers and nonsmokers with a 15-ng/mL cutoff [22]. Kim et al. utilized SPE and GCMS for analysis of nicotine, cotinine, norcotinine, and OH-cotinine in human oral fluid with a limit of quantification (LOQ) of 5 ng/mL and linearity from 5 to 1,000 ng/mL [18].

Few LCMSMS methods are available for the analysis of nicotine and metabolites in oral fluid collected by expectoration or with sponge absorption methods and none for oral fluid diluted with buffer and surfactants [19, 23, 24]. Kataoka and colleagues describe a SPE quantitative LCMSMS method for nicotine, cotinine, norcotinine, anabasine, and anatabine in oral fluid with limits of quantification of 0.5 ng/mL and linearity between 0.5 and 20 ng/mL [19]. However, OH-cotinine and norcotinine were not included, and selected ion monitoring mode, a less selective procedure, was employed. Another LCMSMS method was reported by Bentley et al. for the assay of cotinine and OH-cotinine with LOQ of 0.05 and 0.1 ng/mL, respectively. Oral fluid was collected from smokers by expectoration into sterile Salivette tubes followed by SPE and LCMSMS.

We present for the first time an LCMSMS multiple reaction monitoring (MRM) validated analytical procedure for the simultaneous quantification of nicotine, cotinine, OH-cotinine,

and norcotinine in 0.5 mL human oral fluid collected with the Quantisal™ device. The Quantisal device collects 1.0±0.1 mL of oral fluid, has a volume indicator to indicate adequate collection, and buffers and surfactants to improve recovery of drugs from the device. This new LCMSMS method will support clinical studies comparing the efficacy of monitoring tobacco exposure by CO, oral fluid, and urine.

Experimental

Reagents

(*R,S*)-OH-Cotinine (10 mg powder), OH-cotinine-*d*₃ (1 mg powder), (*R,S*)-norcotinine (10 mg powder), and (*R,S*)-norcotinine-*d*₄ (5 mg powder) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). (*S*)-Cotinine (1 mg/mL in methanol), (*S*)-cotinine-*d*₃ (100 µg/mL in methanol), and (*S*)-nicotine-*d*₄ (100 µg/mL in methanol) were acquired from Cerilliant (Austin, TX, USA). (*S*)-Nicotine (1 mg powder) and formic acid were obtained from Sigma (St. Louis, MO, USA). Water, acetonitrile, sodium phosphate dibasic, sodium phosphate monobasic, 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 LC or ACS grade. CleanScreen SPE columns, part ZSDAU020, were purchased from United Chemical Technologies (Bristol, PA, USA). Blank human oral fluid pools were tested prior to use to ensure absence of analytes of interest or endogenous interferences. Quantisal™ collection devices and buffer were from Immunalysis Corporation (Pomona, CA, USA).

Sodium phosphate buffer (0.1 M, pH6.0±0.05) was prepared with 0.1 M sodium monophosphate and 0.1 M sodium dibasic phosphate. Elution solvent (methylene chloride/ isopropanol/concentrated ammonium hydroxide, 78:20:2 v/v/v) was prepared fresh daily.

Instrumentation

Tandem mass spectrometric analysis was performed on a MDS Sciex API 3200 QTrap® triple quadrupole/linear ion trap mass spectrometer with a TurboIonSpray source (Applied Biosystems, Foster City, CA, USA). The high-performance liquid chromatography system consisted of Shimadzu LC-20 AD pumps and SIL-20 AC autosampler (Columbia, MD, USA). Analyst software version 1.4.1 was employed for acquisition and data analysis. During method development, sonication was performed with a Branson 3510 Ultrasonicator (Danbury, CT, USA).

Calibrators and controls

For calibrators, a stock solution of 100 µg/mL of each analyte was prepared in methanol and stored at -20°C until use. Working solutions, ranging from 2 to 20,000 ng/mL, were prepared by dilution with methanol. Individual deuterated internal standard (ISTd) (nicotine-*d*₄, cotinine-*d*₃, OH-cotinine-*d*₃, and norcotinine-*d*₄) stock 10,000 ng/mL solutions were prepared in methanol and stored at -20°C until use. Blank human oral fluid samples (500 µL) in 2 mL 0.1M phosphate buffer and 1.5 mL Quantisal™ buffer were fortified with 50 µL of 500 ng/mL ISTd and 20 µL aliquots of working calibrator solutions. A ten-point calibration curve (0.2, 0.5, 1, 5, 10, 50, 100, 500, 1,000, and 2,000 ng/mL) for cotinine, nine-point (0.5, 1, 5, 10, 50, 100, 500, 1,000, and 2,000 ng/mL) for OH-cotinine, and eight-point (1, 5, 10, 50, 100, 500, 1,000, and 2,000 ng/mL) for nicotine and norcotinine were utilized. Quality control (QC) samples were prepared in a similar manner from different vials from the same manufacturer. Blank human oral fluid (500 µL) in 2 mL 0.1M phosphate buffer and 1.5 mL Quantisal™ buffer was fortified with 50 µL of 500 ng/mL ISTd and 20 µL aliquots of QC working solutions to yield 0.6, 60, and 600 ng/mL samples for cotinine, 1.5,

60, and 600 ng/mL for OH-cotinine, and 3, 60, and 600 ng/mL for nicotine and norcotinine (low, medium, and high QC, respectively).

Quantification was accomplished by comparing peak area ratios of target analytes to IStd. Data were fit to a linear least-squares regression curve with $1/x$ weighting.

Specimen preparation

Authentic oral fluid specimens collected with the Quantisal™ oral fluid collection device contained 1.0 ± 0.1 mL oral fluid and 3.0 mL Quantisal™ buffer, yielding a 1:4 dilution. After completion of oral fluid collection, the device was placed in the vial containing buffer and centrifuged to release oral fluid from the collection pad into the buffer. The oral fluid and buffer mixture was frozen at -20°C until analysis. Two milliliters (equivalent to 0.5 mL oral fluid) was vortexed with 50 μL IStd and 2 mL 0.1 M sodium phosphate buffer, pH6 in a 15 mL polypropylene tube prior to SPE.

Solid phase extraction

CleanScreen DAU SPE columns were conditioned with 3 mL methanol, 3 mL water, and 1 mL 0.1 M sodium phosphate buffer, pH6. Samples were loaded onto columns 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, drying for 5 min, and finally washing 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 reduce evaporative loss. Samples were reconstituted in 200 μL 0.1% formic acid (v/v) and transferred to glass autosampler vials. Twenty microliters was injected into the LCMSMS.

Liquid chromatography-tandem mass spectrometry

Chromatographic separation was performed on a Synergi Polar RP column (100 $\times$ 2.0 mm, 2.5 μm), protected by a guard column with identical packing material (4 $\times$ 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 250 $\mu\text{L}/\text{min}$. The gradient program progressed from 5% to 50% B over 3 min, 3–4 min hold at 50% B, and 50% B to 5% B over 1 min. The analytical column was re-equilibrated for an additional 2 min, yielding a total chromatographic run time of 7 min. Column oven and auto-injector sample tray were maintained at 25 and 415°C , respectively. Mass spectrometric data were collected in positive ion mode, with optimized TurboIonSpray-MS parameter settings shown in Table 1. Source parameters were 35 psi curtain gas, 40 psi auxiliary gas, 70 psi nebulizer gas, medium collision gas, 3.0 μA nebulizer current, and 450°C source temperature after flow injection analysis source optimization. Quadrupoles 1 and 3 were set to unit resolution. The following transitions were monitored: m/z 163.2 to 132.2 and 84.2 for nicotine, m/z 167.2 to 136.1 and 121.0 for nicotine- d_4 , m/z 177.2 to 80.1 and 98.1 for cotinine, m/z 180.2 to 101.2 and 80.2 for cotinine- d_3 , m/z 193.2 to 80.2 and 134.0 for OH-cotinine, m/z 196.2 to 134.1 and 79.9 for OH-cotinine- d_3 , m/z 163.2 to 80.2 and 118.2 for norcotinine, and m/z 167.2 to 139.2 and 84.2 for norcotinine- d_4 . The italicized transitions were the quantifier ions.

Data analysis

Analyte concentrations were determined by calculating the ratio of each analyte's peak area relative to its IStd peak area. Calibrator analysis in each batch employed linear regression analysis for all calibrators. Mean $\pm$ SD slope and y-intercept from four calibration curves are reported in Table 2. The most abundant transition for each analyte was employed for

quantification; the second transition served as a qualifier (Table 1). Transition peak area ratios were required to be within ± 20 of the mean calibrator transition peak area ratio.

Method validation

Selectivity, sensitivity, limits of detection (LOD) and LOQ, linearity, imprecision, analytical recovery, extraction efficiency, matrix effect, process efficiency, carryover effect, dilution integrity, and stability were evaluated. Method validation was accomplished in 4 days with four unique assays.

Selectivity of the method was assessed by analyses of ten oral fluid specimens from different individuals. Each blank sample was fortified with IStd, extracted, and 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 $\mu\text{g/mL}$ cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine, Δ^9 -tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, morphine, normorphine, morphine-3- β -D-glucuronide, morphine-6- β -D-glucuronide, codeine, norcodeine, 6-acetylmorphine, 6-acetylcodeine, hydrocodone, hydromorphone, oxycodone, noroxycodone, oxymorphone, noroxymorphone, diazepam, lorazepam, oxazepam, alprazolam, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine, chlorpheniramine, brompheniramine, aspirin, acetaminophen, phencyclidine, nitrazepam, flunitrazepam, temazepam, nordiazepam, amphetamine, methamphetamine, anabasine, and anatabine. No interference was noted if analytes in the low QC quantified within $\pm 15\%$ of target concentrations. Specificity also was assessed by relative retention time and qualifier/quantifier transition peak area ratios. Transition peak area ratios for QC and authentic specimens were required to be within $\pm 15\%$ of the mean calibrator transition peak area ratio.

Sensitivity of the method was evaluated by empirically determining the lowest concentration with a signal-to-noise ratio of at least 3:1 (LOD) and 10:1 (LOQ) for quantifier and qualifier transitions. Acceptable chromatography and retention time also were required. Linearity of the method was investigated 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. Method linearity was determined with at least eight calibrators with a weighting factor of $1/x$. Concentrations of each calibrator were required to be within $\pm 15\%$ of target for calibrators when calculated against the full curve, except for the LOQ, for which $\pm 20\%$ was acceptable.

Intra-day imprecision and analytical recovery were determined from five replicates at three different concentrations. Inter-day imprecision and analytical recovery were evaluated on four different runs with five replicates in each run, analyzed on four separate days ($n=20$). Imprecision was expressed as percent relative standard deviation of the calculated concentrations. The guidelines of Krouwer and Rabinowitz [25] were followed for the calculation of pooled intra-day, inter-day, and total imprecision. Analytical recovery was determined by comparing the mean result for all analyses to the nominal concentration value.

Extraction efficiency and matrix effect were evaluated on five different oral fluid lots with the three-set system described by Matuszewski et al. [26]. In the first set, oral fluid samples were fortified with analytes and IStd prior to SPE. In set 2, oral fluid samples were fortified with analytes and IStd after SPE, and the third set contained "neat" analytes and IStd in mobile phase. There were five replicates in each set. Extraction efficiency, expressed as a percentage, was calculated by dividing mean peak area of set 1 by set 2. Absolute matrix effect was calculated by dividing the mean peak area of the analyte in set 2 by the mean

analyte area in set 3, and process efficiency was determined by dividing mean peak area of set 1 by set 3. Results were converted to percentages and subtracted from 100. Acceptable carryover was defined as no quantifiable transition peaks in a blank oral fluid sample containing IStd immediately following a sample containing two times the upper LOQ. Dilution integrity was evaluated by diluting oral fluid samples ($n=3$) containing 4,000 ng/mL of each analyte with blank oral fluid to achieve a 1:4 dilution. IStd was added, and samples were extracted as described. Dilutional integrity was maintained if specimens quantified within $\pm 15\%$ of 1,000 ng/mL.

Stability was evaluated with human oral fluid fortified with analytes of interest at three QC concentrations ($n=5$). Short-term temperature stability was evaluated for human oral fluid 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 analysis, IStd was added to each specimen and analyzed as described. Calculated concentrations of stability specimens were compared to QC samples prepared on the day of analysis. Autosampler stability was assessed by re-injecting QC specimens after 72 h and comparing calculated concentrations to results obtained from the original calibration curve.

Clinical application

Oral fluid specimens were collected in a National Institute on Drug Abuse, Institutional Review Board-approved protocol, and participants provided written informed consent. Participants were regular tobacco smokers, and specimens were collected at various times after the last cigarette.

Results

Analytes were adequately resolved within 4 min (Fig. 1), with a total chromatographic run time of 7 min. Stability of the LC method was evaluated by calculating retention time variabilities. Standard deviations for retention times were ≤ 0.18 min for all compounds over 30 consecutive injections (Table 1). Mass spectrometric (MS) optimization was performed by direct infusion of analytes of interest. Optimized parameters and fragmentor voltages were chosen for each ion product to maximize sensitivity (Table 1). Quantification was based on the intensity of analyte product ions. Ions with the highest m/z values for each analyte and minimum background interferences were selected, providing better peak shapes for quantification.

Linearity was assessed over four different runs. As shown in Table 2, all mean concentrations were well within acceptable limits of $\pm 15\%$ ($\pm 20\%$ at LOQ) of target concentrations when calculated against the full calibration curve. Mean correlation coefficient (r^2 , weighting factor $1/x$, $n=4$) was >0.99 for all analytes in human oral fluid (Table 2). Empirical determination of LOQ with decreasing concentrations of analytes achieved LOQs of 1 ng/mL for nicotine and norcotinine, 0.2 ng/mL for cotinine, and 0.5 ng/mL for OH-cotinine. Total ion chromatograms for analytes showed minimum interference from the oral fluid matrix. In addition, high recovery was achieved contributing to the low LOQ attained. Deuterated IStd, nicotine- d_4 , cotinine- d_3 , OH-cotinine- d_3 , and norcotinine- d_4 were employed to compensate for loss during specimen preparation and for matrix ion suppression or enhancement.

Selectivity of the assay for endogenous interferences was evaluated with ten nonsmoker oral fluid samples; no response exceeded the LOD for any analyte of interest. For exogenous interferences, we evaluated 43 commonly ingested over-the-counter, abused drugs and anabasine and anatabine, minor tobacco alkaloids, fortified in the low QC sample at a concentration of 1 $\mu\text{g/mL}$. No drugs interfered with quantification of analytes of interest

within $\pm 15\%$ of target and with transition ratios within $\pm 20\%$ of mean calibrator transition ratios.

Intra-batch analytical recovery and imprecision were determined in four separate batches with fortified blank oral fluid samples prepared at low, medium, and high QC concentrations, as shown in Table 3. For the five replicates at each level, analytical recovery was within $\pm 15\%$ for each analyte in each batch. Imprecision, expressed as %CV, was less than 5.5% for all analytes in each batch. Inter-batch analytical recovery and imprecision were determined from four batches as included in Table 3. Inter-batch analytical recovery was within $\pm 15\%$ of target with total and inter-batch imprecision less than 13.5%.

Extraction efficiencies ($n=5$) were estimated by comparing LCMSMS peak areas of unextracted and extracted samples. Mean extraction efficiencies for nicotine, cotinine, OH-cotinine, and norcotinine were 56.8–101.2% at three QC concentrations (Table 4). Mean matrix effect in five different oral fluid lots ranged from 0.5 to 23.8, 2.8 to 17.8, and 2.2 to 28.6, for low, medium, and high QC with $<10\%$ CV, respectively. Blank oral fluid samples injected after the highest calibrator did not demonstrate carryover. The ability of the method to accurately quantify specimens containing high concentrations of analytes was evaluated by diluting 4,000 ng/mL samples ($n=3$) with blank oral fluid to achieve a 1:4 dilution. All samples quantified within 13% of target (1,000 ng/mL), confirming dilution integrity.

Short-term temperature stability at three QC concentrations in blank oral fluid were within $\pm 15\%$ of expected, documenting stability in oral fluid at room temperature for 24 h, 72 h on the autosampler, 72 h at 4°C, and after three freeze–thaw cycles (Table 5).

The method was applied to the measurement of nicotine, cotinine, OH-cotinine, and norcotinine in five oral fluid specimens collected from different individuals. One participant's oral fluid specimen is shown in Fig. 1c. Oral fluid concentrations of nicotine, cotinine, OH-cotinine, and norcotinine in five subjects ranged from 1.6 to 1,440, 3.6 to 320, 5.2 to 53.1, and none detected to 133 ng/mL, respectively.

Discussion

Modern analytical instrumentation has revolutionized the identification and quantification of drugs and metabolites in forensic toxicology. In particular, LCMSMS has improved quantitative bioanalysis since the 1990s due to its inherent specificity, sensitivity, and speed. In addition, derivatization is avoided, and the technique is applicable to quantification of a wide range of drugs and metabolites in complex biological matrices. In the specific case of this new oral fluid assay, a single extraction permitted simultaneous quantification of four nicotine biomarkers at low concentrations (0.2 to 1 ng/mL).

Several chromatographic columns and elution solvents were evaluated to obtain optimal analyte separation. A Synergi Hydro column of 150 mm length and 4 μ m pore size was utilized initially to develop the chromatographic system for nicotine and metabolites. Irregular peak shape was observed for OH-cotinine. Another type of column, a Synergi Polar 150 mm 4 μ m, did not provide good resolution and sensitivity. However, the Synergi Polar column with a 2.5 μ m pore size and gradient mobile phase achieved separation of the four compounds in 4 min and re-equilibration within 3 min. The 20- μ L injection volume increased sensitivity without affecting chromatographic peak shape and was within the acceptable range for the 2.5 μ m pore size column. Formic acid provided the best chromatographic resolution and MS signal-to-noise ratio compared to other organic acids, such as acetic acid or trifluoroacetic acid. Stability of the LC method was evaluated by retention time variability over 30 injections. Relative standard deviation was less than 0.18 min, indicating good chromatographic stability.

Mass spectrometric optimization was performed by direct infusion of 100 ng/mL of single analytes of interest. Maximum sensitivity was achieved by selecting the best fragmentor voltage for each product ion (Table 1). Deuterated analogs were available for all analytes to compensate for matrix suppression or enhancement. Two precursor/product ion transitions were monitored for each analyte to improve analyte identification. Selection of MRM transitions were based on the best signal-to-noise ratios at the lowest QC concentrations to improve sensitivity and selectivity.

Preliminary experiments included analysis of ten different authentic oral fluid specimens to evaluate potential matrix interference with each MRM transition. There were no interfering peaks in any of the ten oral fluid samples with the four nicotine biomarkers. As shown in Table 2, correlation coefficients were ≥ 0.99 for all the analytes with all concentrations within $\pm 15\%$ of target when calculated against the full calibration curve.

Nicotine and metabolites' product ions were present in high abundance with TurboIonSpray ionization under these LCMSMS conditions (Table 1). As shown in Table 2, empirically determined detection limits ranged from 0.1 to 0.7 ng/mL with signal-to-noise ratios of 3:1; LOQs were 0.2 to 1 ng/mL. Matrix effect for the four analytes of interest was less than 30% with a %CV less than 10%. Stability studies demonstrated that there were no significant decreases in analyte concentrations during storage under the specified temperature conditions (Table 5).

Kataoka et al. reported LOQs of 0.5 ng/mL for nicotine, cotinine, nornicotine, anabasine, and antabine in oral fluid collected in Salisoft tubes containing a polypropylene-polyethylene sponge without buffer dilution; however, validation data for matrix effect, carryover, dilution integrity, and stability were not included [19]. Byrd et al. reported higher LOQs for nicotine (2 ng/mL) and cotinine (20 ng/mL) from smokers' 100 μ L oral fluid collected directly by expectoration [24]. In this latter study, the method was validated only in rat serum, with partial validation in human oral fluid only for cotinine, as they indicated that cotinine was the only analyte of interest in oral fluid from smokers.

In the present method, oral fluid was collected with the Quantisal™ collection device containing a buffer with protein stabilizers and surfactants (content proprietary) that can interfere with ionization. The majority of oral fluid specimens collected in clinical investigations, drug treatment, insurance, and driving under the influence of drugs applications utilize oral fluid collection devices, which contain buffers to elute analytes from absorbent pads and plastic components and to stabilize analytes. We extensively validated our method for endogenous and exogenous interferences, analyte carryover, dilutional integrity, matrix effect, and short-term analyte stability in oral fluid, as well as sensitivity, imprecision, and linearity. Also, we utilized efficient SPE extraction methodology and deuterated ISTD to optimize low LOQs. To the best of our knowledge, this is the first LCMSMS validated method to simultaneously quantify nicotine, cotinine, OH-cotinine, and nornicotine from oral fluid collected with the Quantisal™ collection device. This new assay is a sensitive and selective tool that can be utilized for monitoring tobacco smoking and abstinence, quantifying nicotine and metabolites' oral fluid concentrations in clinical investigations of nicotine's cognitive, physiological, and subjective effects and brain activities, and as an objective measure of the efficacy of new nicotine dependency treatments.

Conclusion

Determination of nicotine and metabolite concentrations in biological fluids at low LOQs is of increasing interest in nicotine cessation and clinical investigations. Highly sensitive

assays are required to detect environmental tobacco exposure and to extend the window of drug detection in nicotine cessation programs. Full analytical method validation is required for reliable and accurate drug quantification. This LCMSMS procedure is simple and easy to perform and should be useful for routine monitoring of nicotine and metabolites in oral fluid.

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Fig 1.

Extracted ion chromatograms for nicotine (m/z 132.2, 84.2), cotinine (m/z 80.1, 98.1), *trans*-3'-hydroxycotinine (OH-cotinine) (m/z 80.2, 134), and norcotinine (m/z 80.2, 118.2). **a** Blank human oral fluid, **b** blank human oral fluid fortified with all analytes at the limit of quantification, **c** authentic oral fluid specimen

Table 1

Liquid chromatography-tandem mass spectrometry parameters for nicotine and metabolites in oral fluid

Analytes	Q1 mass (amu)	Q3 mass (amu)	Dwell time (ms)	Declustering potential (V)	Entrance potential (V)	Collision entrance potential (V)	Collision energy (V)	Cell exit potential (V)	Retention time (±SD) N=30
Nicotine	163.2	132.2	150	35	5.5	12	21	4	2.65 (±0.088)
Nicotine-d ₄	167.2	84.2	150	35	4.5	12	29	4	2.59 (±0.089)
		136.1	150	35	6	12	21	4	
Cotinine	177.2	121.0	150	35	6	12	29	4	3.11 (±0.186)
		98.1	150	41	3	12	29	4	
Cotinine-d ₃	180.2	80.1	150	41	3	12	33	4	3.15 (±0.084)
		101.2	150	36	3	12	31	4	
OH-Cotinine ^a	193.2	80.2	150	36	3	12	33	4	2.25 (±0.062)
		134.0	150	46	8	12	27	4	
OH-Cotinine-d ₃	196.2	80.2	150	46	8	12	35	4	2.24 (±0.059)
		134.1	150	46	10.5	14	27	4	
Norcotinine	163.2	79.9	150	46	10.5	14	38	4	2.38 (±0.066)
		118.2	150	46	10.5	12	29	4	
Norcotinine-d ₄	167.2	80.2	150	41	10	12	33	4	2.34 (±0.079)
		139.2	150	46	10.5	12	29	4	
^a <i>trans</i> -3'-Hydroxycotinine		84.2	150	46	10.5	12	33	4	

Table 2

Nicotine and metabolites in oral fluid by liquid chromatography-tandem mass spectrometry: limits of detection, limits of quantification, and calibration curve results ($N=4$)

Analyte	LOD (ng/mL)	LOQ (ng/mL)	ULOQ ^a (ng/mL)	Slope mean $\pm$ SD	y-Intercept mean $\pm$ SD	R ² mean $\pm$ SD
Nicotine	0.5	1	2,000	1.00 $\pm$ 0.0617	-0.00433 $\pm$ 0.1021	0.9997 $\pm$ 0.0003
Cotinine	0.1	0.2	2,000	0.895 $\pm$ 0.0538	0.09578 $\pm$ 0.0799	0.9996 $\pm$ 0.0003
OH-Cotinine ^b	0.3	0.5	2,000	0.97 $\pm$ 0.0396	0.00143 $\pm$ 0.0857	0.9998 $\pm$ 0.0001
Norcotinine	0.7	1	2,000	0.636 $\pm$ 0.0274	0.01724 $\pm$ 0.0723	0.9998 $\pm$ 0.0001

^a Upper limit of quantification

^b *trans*-3'-Hydroxycotinine

Table 3

Mean±SD intra- and inter-assay recovery and imprecision for nicotine and metabolites in oral fluid

Analyte	Expected concentration (ng/mL)	Analytical recovery (% of target)				Imprecision (N=20, %CV)		
		Intra-assay N=5		Inter-assay N=20		Pooled intra-day	Inter-day	Total
		Mean	Range	Mean	Range			
Nicotine	3	110.2±2.6	(80.7–116)	99.8±9.6	(80.7–119)	5.2	12.1	13.1
	60	119.4±3.5	(93.1–120)	106±8.8	(93.1–120)	2.9	0.0	7.6
	600	112.4±5.3	(105–117)	111.4±4.3	(108–118)	3.4	0.0	3.4
Cotinine	0.6	93.2±3.8	(88–103.3)	98.4±4.1	(88–103.3)	0.0	3.3	3.3
	60	91.6±3.6	(88–116)	97.2±7.9	(88–116)	3.8	7.5	8.5
	600	110.2±3.3	(103–113)	108.5±2.7	(103–113)	2.3	1.0	2.5
OH-Cotinine ^a	1.5	112.8±1.4	(92.7–115)	103.6±5.6	(92.7–115)	4.2	0.0	4.2
	60	115.8±2.2	(90.1–120)	101.2±9.2	(90.1–120)	2.4	9.9	10.2
	600	105.8±2.9	(99.5–110)	104.2±2.8	(99.5–109)	2.3	1.2	2.6
Norcotinine	3	109.1±2.1	(92.1–117)	102.7±6.7	(92.1–117)	4.3	6.3	7.6
	60	115.6±1.8	(85.7–119)	99.8±10.6	(85.7–117)	2.1	11.9	12.1
	600	109.0±2.4	(98.2–111)	105.4±4.3	(98.2–111)	1.8	0.0	1.8

^a *trans*-3'-Hydroxycotinine

Table 4

Mean±SD extraction efficiency and matrix effect for nicotine and metabolites in oral fluid

Analyte	Extraction efficiency (%; N=5)			Matrix effect (% of signal suppressed; N=5; %CV)		
	Low	Medium	High	Low	Medium	High
Nicotine	71.4±7.2	84.2±9.7	72.1±5.2	14.4 (7.3%)	11.6 (6.2%)	28.6 (5.5%)
Nicotine-d ₄	70.8±4.8	63.2±8.2	74.7±6.7	13.5 (9.4%)	14.9 (3.7%)	22.9 (5.2%)
Cotinine	76.9±3.7	91.0±5.6	93.3±11.2	23.8 (3.7%)	2.8 (7.2%)	8.1 (9.7%)
Cotinine-d ₃	67.2±4.3	81.9±9.3	95.5±9.4	19.6 (5.6%)	3.2 (8.4%)	4.8 (6.6%)
<i>trans</i> -3'-Hydroxycotinine	67.4±9.4	68.4±9.8	58.3±6.3	0.5 (9.3%)	6.0 (5.7%)	2.2 (5.4%)
<i>trans</i> -3'-Hydroxycotinine-d ₃	63.6±4.6	61.4±8.1	56.8±5.7	4.2 (8.3%)	6.7 (4.8%)	6.5 (6.2%)
Norcotinine	77.1±7.8	95.2±5.4	97.3±6.6	10.2 (9.6%)	17.8 (7.1%)	20.2 (6.8%)
Norcotinine-d ₄	90.4±11.2	101±7.8	101.2±4.3	8.6 (7.5%)	17.4 (8.3%)	15.0 (5.3%)

Table 5

Mean±SD stability of nicotine and metabolites in oral fluid under different specimen storage conditions

Storage condition (N=5)	% Analyte decrease			
	Nicotine	Cotinine	OH-Cotinine ^a	Norcotinine
24 h RT ^b				
Low	3.0±4.8	0.7±2.7	8.3±6.7	9.9±4.4
Medium	0.8±2.5	1.5±1.9	1.9±1.4	0.8±1.3
High	0.4±2.2	1.0±1.2	1.3±1.8	0.6±1.1
72 h 4°C				
Low	12.3±11.1	0.9±3.7	3.9±2.5	12.9±5.8
Medium	6.0±6.8	3.9±4.2	9.5±3.2	4.8±4.2
High	11.0±5.4	2.5±3.4	2.6±3.4	10.6±1.3
72 h 15°C on autosampler				
Low	7.5±9.5	5.8±5.2	4.3±7.9	9.0±5.5
Medium	2.3±3.3	1.3±11.7	5.6±5.1	4.3±4.2
High	0.7±4.9	1.2±3.8	9.5±3.5	1.1±4.8
3 Freeze–thaw cycles				
Low	2.4±4.2	3.8±8.2	1.6±7.7	1.7±6.1
Medium	5.4±7.4	1.6±2.4	2.3±3.4	2.0±2.2
High	2.3±6.3	1.5±5.7	2.5±1.3	0.9±1.9

^a *trans*-3'-Hydroxycotinine^b Room temperature