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Simultaneous determination of nicotine, cotinine, norcotinine, and *trans*-3′-hydroxycotinine in human oral fluid using solid phase extraction and gas chromatography–mass spectrometry

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Abstract

Nicotine is rapidly and extensively metabolized in humans. We present an analytical method to simultaneously quantify nicotine, cotinine, norcotinine, and *trans*-3′-hydroxycotinine in human oral fluid. Solid phase extraction (SPE) and GC/MS/EI with selected ion monitoring (SIM) were utilized. Linearity ranged from 5 to 1000 ng/mL of oral fluid; correlation coefficients for calibration curves were >0.99. Recoveries were 90–115% nicotine, 76–117% cotinine, 88–101% norcotinine, and 67–77% *trans*-3′-hydroxycotinine. Intra-assay precision and accuracy ranged from 1.6 to 5.7% and 1.6 to 17.8%, respectively. Inter-assay precision and accuracy ranged from 4.3 to 10.2% and 0 to 12.8%, respectively. Suitable precision and accuracy were achieved for the simultaneous determination of nicotine and three metabolites in the oral fluid of smokers. This assay is applicable to pharmacokinetic studies of nicotine, cotinine, and *trans*-3′-hydroxycotinine from tobacco smokers and can be utilized for routine monitoring of tobacco smoke exposure. 3-Hydroxycotinine requires additional investigation to determine its usefulness as a biomarker for tobacco smoke exposure.

Keywords: Nicotine; Cotinine; *trans*-3′-Hydroxycotinine; Norcotinine; Oral fluid

1. Introduction

Tobacco smoking was recognized as a major cause of mortality and morbidity when environmental tobacco smoke was found to be a human lung carcinogen by the U.S. Environmental Protection Agency in 1992. Tobacco smoke is a mixture of more than 4000 compounds, which is not easily characterized with respect to chemical composition, levels of exposure, and toxicity of constituents [1].

Nicotine, a major component in tobacco, also is a major addictive substance in cigarette smoke. It is absorbed through the skin and mucosal lining of the mouth and nose or by inhalation in the lungs by both active and passive smokers. Nicotine is extensively metabolized to a number of metabolites, but the rate or pattern of metabolism of nicotine varies among individuals. Cotinine is a primary metabolite of nicotine formed after C-oxidation by hepatic cytochrome P450 (CYP2A6) and is further metabolized by the same enzyme system to *trans*-3′-hydroxycotinine (3-hydroxycotinine) and to other minor metabolites including norcotinine [2,3]. Previous studies reported that nicotine has a relatively short half-life ($t_{1/2}$ = 1–2 h) [4]; however, since cotinine and 3-hydroxycotinine have longer half-lives (18–20 h and 4–8 h, respectively) than nicotine [4,5], these are considered appropriate biomarkers for evaluating environmental tobacco smoke exposure.

Due to serious health consequences from environmental tobacco smoke, methods for the determination of nicotine and its metabolites in biological samples are needed. Several methods were published including radioimmunoassay [6], enzyme-linked immunoassay [7], gas chromatog-

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raphy (GC) [8,9] or gas chromatography coupled to mass spectrometry (GC/MS) [10–15], high-performance liquid chromatography (HPLC) [16–18], or LC/MS/MS [19–21]. These techniques generally require liquid–liquid extraction (LLE) or solid phase extraction (SPE) for biological specimen clean-up and concentration prior to chromatography.

Because oral fluid collection is easy and non-invasive, oral fluid is useful for nicotine monitoring [12,13,20,22,23]. Toraño and van Kan [13] determined nicotine and cotinine in oral fluid by LLE followed by GC/MS with limits of quantification (LOQ) of 10 ng/mL and linearity between 10 and 3000 ng/mL for both analytes. Shin et al. [12] also used LLE and GC/MS for nicotine and cotinine in oral fluid and obtained an LOQ of 1 ng/mL and linearity in the range of 1–10 000 ng/mL. Bentley et al. [20] utilized an automated SPE and LC/MS/MS method for the assessment of low level environmental tobacco smoke exposure in oral fluid with LOQs of 0.05 and 0.10 ng/mL for cotinine and 3-hydroxycotinine, respectively, and a linear range of 0.020–10.0 ng/mL. In a recent review, Dhar [23] compared the ability of different biomarkers to determine smoking status, and the utility of different biological specimens and methods to document tobacco exposure. Oral fluid was determined to be the matrix of choice for the determination of nicotine and metabolites. GC/MS was preferred as the analytical method for monitoring smokers and LC/MS/MS for monitoring passive exposure in non-smokers.

In this study, we developed and validated a SPE procedure coupled to GC/MS for the simultaneous determination of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in human oral fluid for support of our clinical research studies. The method was applied to the determination of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in a series of oral fluid specimens from a pregnant woman smoker.

2. Experimental

2.1. Standards and reagents

Chemicals were obtained from the following sources: *S*(−)-nicotine, (−)-cotinine, and (±)-cotinine-d_3 (Cerilliant, Austin, TX); nicotine-d_3 salicylate salt (Sigma, St. Louis, MO); (*R*,*S*)-norcotinine, (3S, 5S)-3′-hydroxycotinine, and (±)-*trans*-3′-hydroxycotinine-d_3 (Toronto Research Chemicals, North York, Canada); *N*,*O*-*bis* (trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical, Rockford, IL). Solid phase extraction columns (Clean Screen® ZSDAU020, 200 mg–10 mL) were obtained from United Chemical Technologies (Bristol, PA). Methanol, methylene chloride, 2-propanol and acetonitrile were HPLC grade chemicals. All other chemicals were reagent grade.

2.2. Calibrators and controls

For the calibrator samples, three working solutions were prepared in methanol at the following concentrations: 0.1, 1, and 10 μg/mL for nicotine, cotinine, norcotinine, and 3-hydroxycotinine. Different methanolic solutions were prepared for quality control (QC) samples at the same concentrations as the standard working solutions. Calibrator and QC working solutions were made from different source lots. All working solutions were stored at −20 °C when not in use. Daily eight point calibration (5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL) samples for nicotine, cotinine, norcotinine, and 3-hydroxycotinine were prepared in blank oral fluid. Low, medium, and high QC samples also were prepared daily in blank oral fluid with concentrations of 16, 160, and 800 ng/mL (low, medium, and high, respectively) for all analytes.

The deuterated internal standard (nicotine-d_3, cotinine-d_3, and 3-hydroxycotine-d_3) working solution was 1 μg/mL in methanol and was stored at −20 °C when not in use.

2.3. Sample preparation

An aliquot (0.5 mL) of each oral fluid sample, QC sample, or calibration standard was mixed with 2 mL of 2 mol/L sodium acetate buffer (pH 5.5). Twenty-five microliters (corresponding to 50 ng/mL) of internal standard working solution was added to each sample prior to extraction. The SPE columns were preconditioned in the following order, 1 mL of methylene chloride:2-propanol:concentrated ammonium hydroxide (80:20:2, v/v/v), 3 mL of methanol, 3 mL of deionized water, and 2 mL of 2 mol/L sodium acetate buffer (pH 5.5). Each sample was loaded onto the SPE column and washed with 2 mL deionized water, 1.5 mL 0.2 mol/L hydrochloric acid and twice with 1 mL methanol. Analytes were eluted four times with 1 mL methylene chloride:2-propanol:concentrated ammonium hydroxide (80:20:2, v/v/v), eluates were combined, and 100 μL of 1% hydrochloric acid in methanol (v/v) was added prior to evaporation. Extracts were evaporated to dryness under a stream of nitrogen at 40 °C using a Zymark Turbovap® LV Evaporator. Extracted residues were reconstituted in 25 μL of acetonitrile and 25 μL of BSTFA (with 1% TMCS) and centrifuged at 2500 rpm for 10 min. The supernatant was transferred to autosampler vials and derivatized at 85 °C for 45 min. The derivatized extract (1 μL) was injected onto the GC/MS with electron impact (EI) selected ion monitoring (SIM) mode.

2.4. Gas chromatography–mass spectrometry

GC/MS analysis was performed using an HP6890 GC interfaced with HP5973 mass-selective detector, equipped with HP-5MS column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) with helium as the carrier gas at a flow rate of 1.0 mL/min. Samples were injected in the splitless mode

with the purge valve closed for 2 min. The oven temperature started at 70 °C for 1 min, followed by a temperature ramp of 30 °C/min to 190 °C, 5 °C/min to 230 °C, and 25 °C/min to 290 °C. The total separation time was 15.40 min. The temperature of the injection port was 250 °C and the GC interface was 290 °C. The ion source was kept at 230 °C and the quadrupole at 150 °C. SIM mode was used with a dwell time of 50 ms. The ions for each analyte were monitored in the following elution order (quantitative ions are indicated in parenthesis) for the derivatized analytes: nicotine-d_3, *m*/*z* (87), 165; nicotine, *m*/*z* (84), 162; cotinine-d_3, *m*/*z* (101), 179; cotinine, *m*/*z* (98), 176; norcotinine, *m*/*z* (234), 219; 3-hydroxycotinine-d_3, *m*/*z* (252), 147; and 3-hydroxycotinine, *m*/*z* (249), 144.

2.5. Data analysis

Calibration, using internal standardization, was done by linear regression analysis over a concentration range from 5 to 1000 ng/mL. Peak area ratios of target analytes and their respective internal standards were calculated for each concentration by MSD Chemstation software (v D.00.00). The data were fit to a linear least-squares regression curve with a weighting factor of 1/*x*.

2.6. Selectivity

Six blank oral fluid specimens from different nicotine abstinent individuals were extracted and analyzed for assessment of potential interferences from endogenous substances and from low concentrations of analytes that may be present due to passive smoke exposure. Three aliquots of each volunteer's oral fluid were prepared; nothing was added to the first aliquot, internal standard was added to the second aliquot, and internal standard and 5 ng/mL of each analyte were added to the third aliquot.

In addition, potential interferences from commonly used drugs were evaluated by adding compounds at concentrations of up to 10 000 ng/mL to low QC concentration samples (16 ng/mL). 100–10 000 ng/mL of methamphetamine, 2000 ng/mL of cannabidiol and cannabigerol, and 10 000 ng/mL of acetaminophen, amphetamine, caffeine, cocaine, codeine, dextromethorphan, diphenhydramine, ephedrine, ibuprofen, methadone, morphine, oxycodone, pseudoephedrine, and Δ^9-tetrahydrocannabinol were added individually to quality control samples containing 16 ng/mL nicotine, cotinine, norcotinine, and 3-hydroxycotinine.

2.7. Linearity and sensitivity

The 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). A 1/*x* weighting factor was applied, and linearity of each of the compounds was determined with at least eight concentration levels not including the blank matrix. The concentrations of each calibrator were within 20% of the target concentration when calculated against the full eight point calibration curve.

The sensitivity of the method was evaluated by determining the limits of detection (LOD) and LOQ. The LOD was defined as the lowest concentration at which the analyte ion signal-to-noise ratio (determined by peak height) was at least 3 and chromatography (peak shape and resolution), retention time (less than a 2% of the 50 ng/mL calibrator retention time), and ion ratio within ±20% of those of the 50 ng/mL calibrator were acceptable. The LOQ was defined as the lowest concentration that met all LOD criteria with a signal-to-noise ratio of at least 5 and acceptable precision and accuracy (relative standard deviation and percent difference, respectively, within ±20%). The peak heights of ions in the spiked 5 ng/mL calibrator were compared to the peak heights of ions in the adjacent baseline noise utilizing the Agilent MSD Chemstation software.

2.8. Precision and accuracy

Inter- and intra-assay precision and accuracy data for nicotine and metabolites were determined with the low, medium, and high QC samples. Intra-assay data were assessed by comparing data from within one run ($n = 10$). Inter-assay data were determined from a total of 34 samples of each control concentration over five separate runs (10 samples from the first run and 6 samples of each level in four additional runs). Precision was expressed as percent relative standard deviation (%R.S.D.), and accuracy was expressed as the percent difference from the expected value.

2.9. Recovery

The recovery for each analyte was determined at low, medium, and high concentrations ($n = 5$). One set of spiked oral fluid samples was extracted as described but the internal standard working solution was added just before evaporation. Samples were subsequently derivatized and analyzed. Another set of samples was prepared with neat analyte and internal standard working solutions that were subsequently evaporated, derivatized and analyzed. Recovery (%) was calculated by comparing the peak area ratios of analyte to internal standard for the extracted and unextracted samples.

2.10. Stability

Stability of spiked unextracted oral fluid samples was tested at low, medium, and high concentrations ($n = 3$) after three freeze-and-thaw cycles, at room temperature for 24 h, and at 4 °C for up to 72 h. Stability of analytes after derivatization also was examined. GC autosampler vials containing derivatized low, medium, and high quality control samples ($n = 3$) were stored at room temperature for up to 72 h following initial analysis. Concentrations of analytes in stored vials were compared to results obtained with freshly prepared quality control samples.

2.11. Clinical application

Serial clinical specimens ($n = 33$) were collected over 5 months from one opiate, cocaine and nicotine addicted pregnant woman enrolled in a methadone maintenance study. The protocol was approved by the Johns Hopkins Bayview Medical Center's and the National Institute on Drug Abuse's (NIDA) Institutional Review Boards. Informed consent was provided. Oral fluid was collected with the Salivette® cotton swab. The cotton swabs were placed between the cheek and gum or under the participant's tongue until saturated with oral fluid. The cotton swab was sometimes chewed to stimulate oral fluid production. After collection, the swab was centrifuged in conical tubes to release oral fluid from the cotton. Oral fluid was frozen at −20 °C until analysis.

3. Results and discussion

3.1. Method development

Nicotine, cotinine, and/or 3-hydroxycotinine have been utilized as biomarkers for recent exposure to environmental tobacco smoke in both active and passive smokers [24–30]. Since the half-life of nicotine ($t_{1/2} = 1$–2 h) is much shorter than those of its metabolites [4,5], analysis of metabolites provides advantages over monitoring the parent compound. This method used SPE for extraction of analytes from oral fluid in order to simplify analytical sample preparation and reduce time and solvent consumption in comparison to LLE. To prevent vaporization of nicotine during evaporation of the SPE extract, 100 μL of 1% hydrochloric acid in methanol (v/v) was added to the extracts in order to form the nicotine hydrochloride salt. Total GC/MS separation time was 15.4 min, with the retention order of nicotine, cotinine, norcotinine, and 3-hydroxycotinine.

3.2. Method validation

Blank oral fluid samples had peaks up to 50% of the area of the quantification ions for the 5 ng/mL calibrator (method LOQ) for nicotine, cotinine and norcotinine. However, ion ratios for these substances did not meet ion ratio criteria for establishing the identity of nicotine, cotinine and norcotinine. Despite this small contribution, samples spiked to contain 5 ng/mL of each of these analytes quantified within ±20% of target, substantiating our choice of 5 ng/mL as the method's LOQ.

The observed interference in blank oral fluid is most likely due to a combination of endogenous substances and nicotine and metabolites from passive smoke exposure. Low concentrations of nicotine, cotinine and 3-hydroxycotinine have been reported in biological specimens in non-smoking populations [19,20,27]. Etter et al. [27] found a median concentration for cotinine in oral fluid from non-smokers ($n = 97$) of 2.4 ng/mL by GC-nitrogen selective detector. Bentley et al. [20] compared oral fluid cotinine and 3-hydroxycotinine concentrations for participants with varying degrees of self-reported environmental tobacco smoke exposure by LC/MS/MS. Non-smoking participants with no recent exposure ($n = 18$) had 0.025–0.613 ng/mL cotinine and 0.050–0.225 ng/mL 3-hydroxycotinine concentrations in their oral fluid, while non-smoking participants with some exposure ($n = 6$) or living with a smoker ($n = 10$) had 0.2–1.3 ng/mL or 0.4–2.6 ng/mL cotinine and less than 0.7 ng/mL or 1.0 ng/mL 3-hydroxycotinine. It is difficult to obtain oral fluid that has no nicotine or metabolites due to the potential for passive smoke exposure and also, due to the presence of low concentrations of nicotine in food [31]. Extracted ion chromatograms obtained following the extraction of blank oral fluid (A), the same blank oral fluid sample spiked with analytes at the LOQ concentration (5 ng/mL) (B), and a participant's oral fluid specimen from the described clinical study (C) are shown in Fig. 1.

For exogenous interferences, we evaluated commonly used over-the-counter or abused drugs (2000 ng/mL of cannabidiol and cannabigerol; 10 000 ng/mL of acetaminophen, amphetamines, caffeine, cocaine, codeine, dextromethorphan, diphenhydramine, ephedrine, ibuprofen, methadone, morphine, oxycodone, pseudoephedrine, and Δ^9-tetrahydrocannabinol) by adding these drug concentrations to the low concentration QC samples. None of the above drugs interfered with quantification of the low QC samples (16 ng/mL). We also evaluated methamphetamine at concentrations ranging from 100 to 10 000 ng/mL. If the methamphetamine oral fluid concentration was greater than 200 ng/mL, nicotine ion ratios fell outside of established limits, producing a false negative nicotine result. However, cotinine, 3-hydroxycotinine and norcotinine were not affected. Thus, exposure to tobacco smoke would be evident, as long as other biomarkers were monitored.

Linearity was obtained with an average correlation coefficient (r^2; weighting factor, $1/x$, $n = 5$) of >0.99, over a dynamic range from 5 to 1000 ng/mL oral fluid for all analytes (Table 1). According to the criteria described in the Experimental Section, LOD and LOQ were established at 5 ng/mL (50 pg on column). At the LOQ, precision and accuracy for nicotine were 15.5% and 5.8%, for cotinine 11.0% and 2.5%, for norcotinine 11.0% and 4.5%, and for 3-hydroxycotinine 4.0% and 16.2%, respectively.

Tables 2 and 3 include precision and accuracy data for the method at three concentrations (low, medium, high) over the linear dynamic range. Intra-assay (within-run) precision and accuracy were determined by replicate analysis ($n = 10$) of QC samples. Intra-assay precision for all analytes proved to be less than 5.7% and accuracy <17.8% across the linear range of the assay. Inter-assay (between-run) precision and accuracy were assessed with 34 specimen samples at each QC concentration (33 for low QC due to an experimental error with one sample) on five separate runs and ranged from 4.3 to 10.2% and 0 to 12.8%, respectively, for all analytes at all three concentrations.

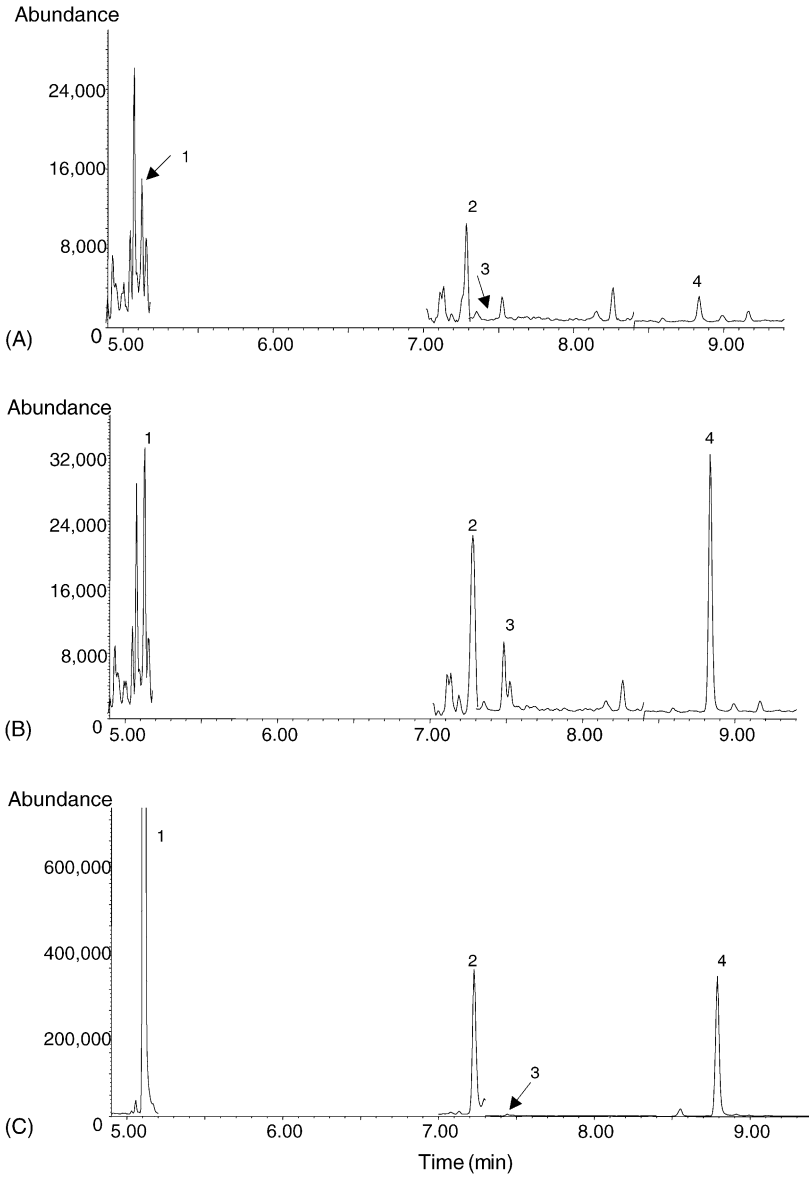

Fig. 1. Extracted ion chromatograms for nicotine (1, *m*/*z* 84), cotinine (2, *m*/*z* 98), norcotinine (3, *m*/*z* 234), and *trans*-3′-hydroxycotinine (4, *m*/*z* 249) in oral fluid. (A) Blank oral fluid, (B) blank oral fluid spiked with 5 ng/mL of each analyte, and (C) pregnant tobacco smoker's oral fluid containing 1236 ng/mL nicotine, 236 ng/mL cotinine, 6 ng/mL norcotinine, and 158 ng/mL *trans*-3′-hydroxycotinine.

Table 1
Limits of detection and quantification and calibration curves[a] for nicotine, cotinine, norcotinine, and 3-hydroxycotinine in oral fluid

Analyte	Internal standard	LOD[b] and LOQ[c] (ng/mL)	Equation[d]	r^2
Nicotine	Nicotine-*d3*	5	$y = 0.078(0.006)x + 0.096(0.122)$	0.999
Cotinine	Cotinine-*d3*	5	$y = 0.039(0.003)x + 0.049(0.092)$	0.999
Norcotinine	Cotinine-*d3*	5	$y = 0.025(0.002)x - 0.013(0.021)$	0.999
3-Hydroxycotinine	3-Hydroxycotinine-*d3*	5	$y = 0.039(0.002)x + 0.034(0.007)$	0.999

[a] $n = 5$.
[b] Limit of detection.
[c] Limit of quantification.
[d] Data are mean (S.D.).

Table 2
Intra-assay precision and accuracy for the determination of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in oral fluid ($n = 10$)

Analyte	Expected concentration (ng/mL)	Observed concentration (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
Nicotine	16	17.8 ± 0.5	2.9	11.0
	160	140.2 ± 5.7	4.1	12.4
	800	657.7 ± 17.0	2.6	17.8
Cotinine	16	16.4 ± 0.9	5.7	2.3
	160	157.4 ± 7.0	4.4	1.6
	800	751.6 ± 16.8	2.2	6.1
Norcotinine	16	17.8 ± 0.9	5.0	11.4
	160	179.7 ± 4.8	2.7	12.3
	800	890.1 ± 31.0	3.5	11.3
3-Hydroxycotinine	16	18.1 ± 1.0	5.7	13.0
	160	184.5 ± 6.2	3.4	15.3
	800	838.8 ± 13.6	1.6	4.8

Table 3
Inter-assay precision and accuracy for the determination of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in oral fluid from five separate runs

Analyte	n	Expected concentration (ng/mL)	Observed concentration (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
Nicotine	33	16	18.0 ± 1.0	5.6	12.8
	34	160	151.2 ± 12.5	8.3	5.5
	34	800	732.4 ± 74.9	10.2	8.4
Cotinine	33	16	16.0 ± 1.4	8.7	0.0
	34	160	155.8 ± 6.7	4.3	2.6
	34	800	782.7 ± 53.5	6.8	2.2
Norcotinine	33	16	18.0 ± 1.0	5.6	12.4
	34	160	174.6 ± 13.2	7.6	9.1
	34	800	877.1 ± 64.2	7.3	9.6
3-Hydroxycotinine	33	16	17.6 ± 1.4	7.9	10.0
	34	160	179.9 ± 9.4	5.3	12.4
	34	800	839.1 ± 57.8	6.9	4.9

Recoveries for all analytes ($n = 5$) were estimated by comparing GC/MS peak area ratios of unextracted and extracted samples (Table 4). Mean recoveries for nicotine, cotinine, norcotinine, and 3-hydroxycotinine ranged from 90.8 to 115.3%, 76.7 to 117.8%, 88.5 to 101.8%, and 67.0 to 77.2%, respectively, for the three QC concentrations of 16, 160, and 800 ng/mL. We achieved adequate but higher recoveries for the 16 ng/mL low quality control samples for nicotine and cotinine. Contributing factors could include low concentrations of these analytes from passive smoke exposure and/or interferences from endogenous substances below the LOQ concentration. As described earlier, low concentrations of

Table 4
Recoveries of nicotine, cotinine, norcotinine, and 3-hydroxycotinine from oral fluid ($n = 5$)

Analyte	Expected concentration (ng/mL)	Mean peak ratio of extracted samples	Mean peak ratio of non-extracted samples	Recovery (%)
Nicotine	16	0.95	0.82	115.3
	160	6.06	6.47	93.6
	800	28.72	31.64	90.8
Cotinine	16	0.42	0.36	117.8
	160	2.88	3.24	89.0
	800	12.78	16.67	76.7
Norcotinine	16	0.24	0.23	101.1
	160	2.35	2.31	101.8
	800	10.90	12.32	88.5
3-Hydroxycotinine	16	0.28	0.36	77.2
	160	2.41	3.39	71.1
	800	11.04	16.48	67.0

Table 5
Stability of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in oral fluid ($n = 3$) and stability of derivatized analytes at room temperature ($n = 3$)

Analyte	Expected concentration (ng/mL)	Freeze–thaw × 3 (%)	72 h at 4 °C (%)	24 h at RT (%)	Derivatized analytes for 24 h (%)
Nicotine	16	104.9	108.4	107.0	105.1
	160	101.0	103.2	91.9	101.5
	800	82.5	90.1	95.4	100.6
Cotinine	16	93.6	84.1	99.9	86.5
	160	96.4	102.9	95.5	97.8
	800	93.8	96.1	90.8	100.6
Norcotinine	16	98.6	100.1	109.6	99.1
	160	98.8	105.7	100.5	98.7
	800	96.9	96.2	91.1	101.8
3-Hydroxycotinine	16	95.6	94.6	100.8	98.3
	160	97.8	101.5	98.0	99.3
	800	93.5	96.9	96.8	99.5

nicotine and metabolites in oral fluid and other biological fluids from non-smokers are frequently found [19,20,27].

Analyte concentrations were stable in oral fluid after three freeze–thaw cycles, at 4 °C for 72 h, and at room temperature for 24 h (Table 5). Stability of derivatized analytes in capped GC autosampler vials at room temperature was assessed after 24, 48, and 72 h. The derivatized samples were stable (no significant differences by ANOVA) for up to 48 h, except the low QC concentration of cotinine, which was stable for 24 h (Table 5).

3.3. Application

The method was used to measure concentrations of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in 33 oral fluid samples collected over 5 months from one opiate, cocaine, and nicotine addicted pregnant woman enrolled in a methadone maintenance treatment study. One of the participant's oral fluid specimens from the described clinical study (C) is shown in Fig. 1. Oral fluid concentrations of nicotine, cotinine, 3-hydroxycotinine ranged from 75.6 to 3549.7, 63.1 to 279.9, and 28.6 to 203.6 ng/mL, respectively. Norcotinine was detected in six of 33 oral fluid samples with concentrations less than 10 ng/mL.

This new analytical method for the simultaneous measurement of nicotine, cotinine, norcotinine and 3-hydroxycotinine in oral fluid will be used to monitor tobacco smoking in pregnant women and will help determine the usefulness of 3-hydroxycotinine as a biomarker of tobacco exposure.

4. Conclusions

Our report describes a sensitive and specific GC/MS procedure for the simultaneous quantification of nicotine, cotinine, norcotinine, and 3-hydroxycotinine in oral fluid. SPE was paired with GC/MS/EI selected ion monitoring mode to achieve an accurate and precise quantification of nicotine and metabolites in this alternative matrix. This method should be useful for routine monitoring of nicotine exposure and for pharmacokinetic analyses. 3-Hydroxycotinine requires additional investigation to determine its usefulness as a biomarker for tobacco smoke exposure.

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