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Determination of the nicotine metabolites cotinine and *trans*-3′-hydroxycotinine in biologic fluids of smokers and non-smokers using liquid chromatography–tandem mass spectrometry: Biomarkers for tobacco smoke exposure and for phenotyping cytochrome P450 2A6 activity

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ABSTRACT

The nicotine metabolite cotinine is widely used to assess the extent of tobacco use in smokers, and secondhand smoke exposure in non-smokers. The ratio of another nicotine metabolite, *trans*-3′-hydroxycotinine, to cotinine in biofluids is highly correlated with the rate of nicotine metabolism, which is catalyzed mainly by cytochrome P450 2A6 (CYP2A6). Consequently, this nicotine metabolite ratio is being used to phenotype individuals for CYP2A6 activity and to individualize pharmacotherapies for tobacco addiction. In this paper we describe a highly sensitive liquid chromatography–tandem mass spectrometry method for determination of the nicotine metabolites cotinine and *trans*-3′-hydroxycotinine in human plasma, urine, and saliva. Lower limits of quantitation range from 0.02 to 0.1 ng/mL. The extraction procedure is straightforward and suitable for large-scale studies. The method has been applied to several thousand biofluid samples for pharmacogenetic studies and for studies of exposure to low levels of secondhand smoke. Concentrations of both metabolites in urine of non-smokers with different levels of secondhand smoke exposure are presented.

1. Introduction

Worldwide, tobacco-related diseases cause about 5 million premature deaths per year [1]. Most of these deaths occur in smokers, but smokeless tobacco use [2] and exposure to secondhand smoke in non-smokers also poses a significant health risk [3,4]. Most smokers in the United States say they want to quit, but the majority of them are unable to do so, in large part because of nicotine addiction [5].

Determining exposure to nicotine is of interest to researchers studying the effects of tobacco use on health, to clinicians who need an objective outcome measure for tobacco dependence treatment programs, to scientists studying exposure to secondhand smoke and its effects, and for numerous other areas of inquiry into the pharmacology and toxicology of nicotine and tobacco. A widely used approach for measuring exposure is determination of tobacco-derived biomarkers in biologic fluids [6–8]. In this regard, the nicotine metabolite cotinine is the most widely used, and has excellent specificity for both active use of tobacco and for secondhand smoke exposure [6,9,10], except in individuals using nicotine-containing medications [7]. Cotinine concentrations have been determined in a variety of biological matrices, including plasma, serum, urine, saliva, hair, and nails [6,9,11–15]. Saliva concentrations are highly correlated with plasma concentrations [16,17], and since obtaining saliva does not require venipuncture, saliva is the preferred biofluid for many studies. Urine concentrations are generally much higher than those in plasma or saliva [18], and for this reason urine analyses can provide greater sensitivity for assessing low level exposure.

trans-3′-Hydroxycotinine (3HC) is, in most individuals, the major metabolite of cotinine [19,20]. Its concentrations in urine generally exceed cotinine concentrations by 3- to 4-fold [19,20], but in plasma or saliva, cotinine concentrations are generally higher than those of 3HC [21,22]. Consequently, determination of 3HC, as well as cotinine, might provide a more sensitive measure of exposure, especially when urine is used. The conversion of cotinine to 3HC, as well as the conversion of nicotine to cotinine in humans is largely mediated by the liver enzyme cytochrome P450 2A6

Abbreviations: SHS, secondhand smoke; 3HC, *trans*-3′-hydroxycotinine; COPD, chronic obstructive pulmonary disease.

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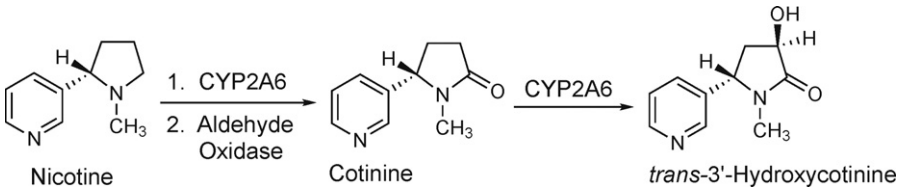

Fig. 1. Metabolism of nicotine to cotinine and *trans*-3′-hydroxycotinine.

(CYP2A6) (Fig. 1) [23,24]. Recently, we reported a high correlation between the ratio of 3HC to cotinine concentration in plasma and nicotine oral clearance. This ratio provides a convenient measure to phenotype individuals for CYP2A6 activity [25,26]. This method is being used for large-scale pharmacogenetic studies.

Numerous methods for determination of cotinine in biologic fluids have been reported, including gas chromatography (GC) [11,27], high performance liquid chromatography (HPLC) [28], gas chromatography–mass spectrometry (GC–MS) [11,29,30], liquid chromatography–mass spectrometry (LC–MS) [22,31–35], and immunoassay procedures [36–38]. Chromatographic and chromatographic–mass spectrometric methods have been used for determination of 3HC as well [11,39], but, to our knowledge, immunoassay methods have not. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) with triple-stage quadrupole instruments is widely used for low-level quantitation of basic drugs, their metabolites, and various endogenous substances in biologic fluids [40,41]. During the past few years, LC–MS/MS methods for determination of sub-nanogram per milliliter concentrations of nicotine and its metabolites have been reported [22,31–33,42].

As part of our studies of the pharmacology and toxicology of nicotine and tobacco, we required methods for determination of both cotinine and 3HC in various biological matrices. For studies of low-level secondhand smoke (SHS) exposure, methods with very high sensitivity were required, in order to achieve limits of quantitation of 0.1 ng/mL or lower. In addition, the methods had to be practical for analysis of large numbers of samples. This paper describes LC–MS/MS methods for simultaneous determination of low concentrations of cotinine and 3HC in human biofluids. Advantages of the methods include: (1) straight-forward extraction procedures that are convenient for large batches of samples; (2) excellent precision, accuracy, and sensitivity with lower limits of quantitation (LLOQ) ranging from 0.02 to 0.1 ng/mL for 1 mL volume samples; and (3) the methods have been applied to and validated for plasma, urine and saliva samples, the major biofluids that are used for tobacco smoke exposure assessment.

2. Materials and methods

2.1. Biofluid samples

Plasma, saliva, and urine samples were collected and analyzed for studies that have been reported elsewhere [43–45]. All studies received approval of the appropriate institutional review boards. Typically, 7 mL of blood is collected in vacutainer tubes containing 100 USP units of lithium heparin, then centrifuged to obtain plasma. Plasma is transferred to polypropylene cryogenic vials. Saliva is collected into 20 mL polypropylene vials. Prior to collection, subjects are asked to wash their mouths with water. If necessary to collect sufficient volume, subjects may chew on a piece of paraffin. However, it should be noted that stimulation of saliva flow may affect cotinine concentrations [46]. Urine is acidified to a pH of 2–3 with solid sodium bisulfate. Biofluid samples are stored at −20 °C until analysis.

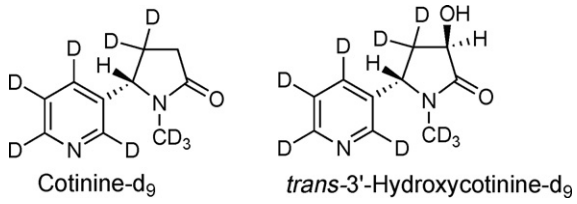

Fig. 2. Chemical structures of internal standards.

2.2. Reagents and standards

Cotinine perchlorate and *trans*-3′-hydroxycotinine perchlorate were synthesized as previously described [47,48]. The internal standards, cotinine-d_9 and *trans*-3′-hydroxycotinine-d_9 (Fig. 2) were synthesized by modification of published procedures [47–49]. The HPLC mobile phase was prepared from HPLC grade water and HPLC grade methanol from Burdick–Jackson. These were buffered using formic acid (ACS reagent grade) and ammonium formate (Certified) from Fisher Chemical Company (Pittsburgh, PA). Optima grade methylene chloride used for extractions, ACS reagent grade 60–62% perchloric acid, ACS reagent grade hydrochloric acid, and CP tripotassium phosphate monohydrate (Acros) were from Fisher.

2.3. Instrumentation

LC–MS/MS analyses were carried out with a Thermo Surveyor or Agilent 1200 HPLC interfaced to a Thermo-Finnigan TSQ Quantum Ultra triple-stage quadrupole mass spectrometer for analyses requiring maximum sensitivity, or using a Hewlett–Packard 1090 HPLC interfaced with a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer with an API2 ion source. Solvent evaporation was carried out using a Savant Automatic Environmental SpeedVac Model AES 2000.

2.4. Working standards and controls

Standards were prepared from the perchlorate salts of cotinine and 3HC in 0.01 M HCl/HPLC grade water, and concentrations were corrected to those of the free bases. Dilutions were made in 0.01 M HCl to prepare working standards ranging from 0.01 to 20 ng/mL for studies of SHS exposure, from 1 to 500 ng/mL analyses of smokers' plasma or saliva, and from 10 to 10,000 ng/mL for analyses of smokers' urine. Aqueous working standards were used because nicotine metabolites are present in biofluids from virtually all individuals due to environmental exposure to nicotine. Controls were prepared from plasma, saliva, and urine of non-smokers spiked with stock solutions of the analytes, or in the case of low-level urine controls, an "artificial urine" was used as the matrix [50], prepared from major components reported in human urine [51].

2.5. Method Variation 1: biofluids from non-smokers

2.5.1. Extraction procedure 1: plasma, saliva, and urine from non-smokers

To 1 mL of biofluid sample, standard, or control contained in 13 mm × 100 mm glass culture tube was added 100 μL of a solu-

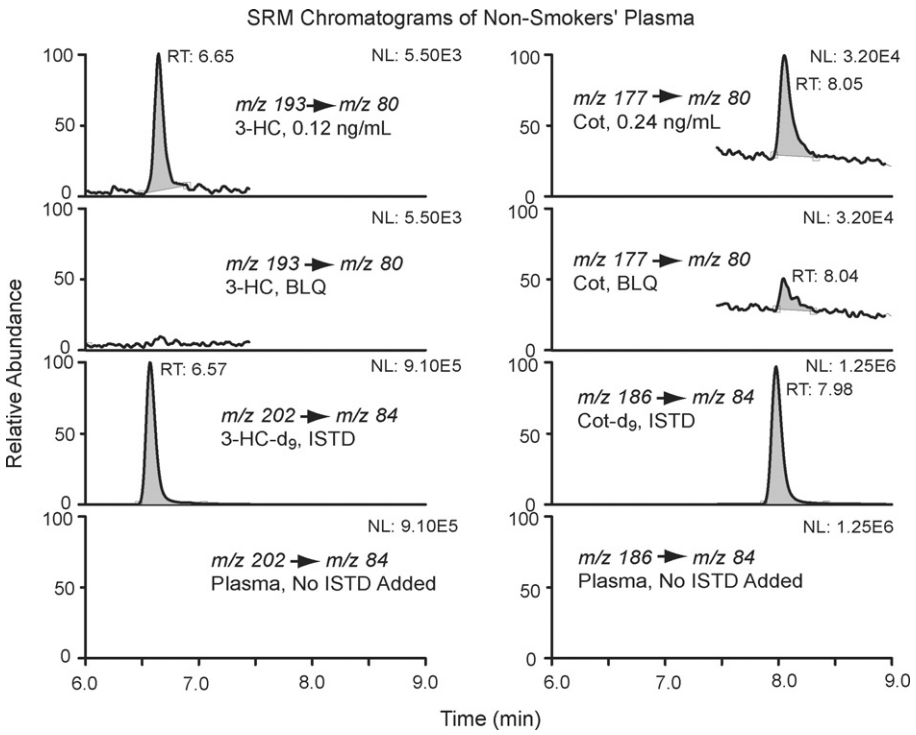

Fig. 3. SRM chromatograms of non-smokers' plasma.

tion of the internal standards in 0.01 M HCl. The internal standard solution contained 20 ng/mL of cotinine-d_9, and 20 ng/mL *trans*-3′-hydroxycotinine-d_9. The tube was briefly vortex-mixed, and 100 μL of 30% perchloric acid was added to precipitate protein. (Although urine should not contain protein, for some samples addition of perchloric acid resulted in less emulsion and cleaner separation of the organic layer in the subsequent extraction step, and therefore it was routinely added to urine samples as well as plasma and saliva.) After vortex-mixing and centrifugation, the supernate was decanted to a 16 mm × 100 mm culture tube. Tripotassium phosphate, 2 mL of 50% (w/v, pH ~14), and 6 mL of methylene chloride was added. The tube was vortex-mixed for 5 min, centrifuged, and placed in a dry ice-acetone mixture to freeze the aqueous layer. The organic (upper) layer was poured to a 13 mm × 100 mm culture tube, 100 μL of 10% HCl in methanol was added, and the extract was evaporated to dryness in a centrifugal vacuum evaporator. The dried extract was reconstituted in 150 μL of 100 mM ammonium acetate and 1% acetic acid in 80/20 water/methanol, and transferred to an autosampler vial for LC–MS/MS analysis. Twenty microliters was injected.

Extraction recovery was estimated by adding analytes to extracts of blank plasma, and comparing peak areas to those from spiked plasma carried through the extraction procedure.

2.5.2. Liquid chromatography system 1

The chromatography was carried out using a 4.6 mm × 150 mm Phenomenex Synergi Polar RP column (4 μm) fitted with a Phenomenex Polar-RP guard column, 4 mm LX 3.0 ID. A binary, linear gradient elution with 10 mM ammonium acetate/0.1% acetic acid in water (solvent A) and 10 mM ammonium acetate/0.1% acetic acid in methanol (solvent B) was used at a flow rate was 0.7 mL/min with the following program: the initial composition was 80% A, changing to 100% B over 6.5 min. 100% B was maintained from 6.5 to 8 min, then changed to 80% A at 8.1 min and maintained at this composition until the end of run at 13 min (Figs. 3–5).

2.5.3. Mass spectrometry parameters 1

The mass spectrometer was operated in the positive ion mode using atmospheric pressure chemical ionization (APCI). The ion source parameters were optimized by infusing an aqueous solution of cotinine *via* syringe pump. The vaporizer temperature was 450 °C, the heated capillary temperature was 250 °C, and the corona discharge current was set at 5 μA. Cotinine and 3HC solutions were infused to determine appropriate ion transitions and the optimum collision energies for CID. The collision gas (argon) pressure was 1.5 mTorr. The collision energy was set at 30 eV for cotinine and cotinine-d_9, and at 35 eV for 3HC and 3HC-d_9. The SRM transitions monitored were as follows: *m*/*z* 177 to *m*/*z* 80 for cotinine, *m*/*z* 193 to *m*/*z* 80 for *trans*-3′-hydroxycotinine and the transitions *m*/*z* 186 to *m*/*z* 84 and *m*/*z* 202 to *m*/*z* 84 for the respective internal standards. The resolution of the first quadrupole, FWHM, was set at 0.2 amu, the resolution of the third quadrupole was set at 0.7 amu FWHM.

2.6. Method Variation 2: biofluids from smokers

2.6.1. Extraction procedure 2: plasma, saliva, and urine from smokers

The procedure was identical to Extraction procedure A, except that 100 μL of biofluid was used, and it was diluted with 900 μL of HPLC grade water. For plasma and saliva samples, the evaporated extract was reconstituted in 150 μL of 100 mM aqueous ammonium formate; for urine the volume was 1 mL.

2.6.2. Liquid chromatography system 2

The chromatography was carried out using a 4.0 mm × 150 mm Supelco Discovery HSF5 column (5 μm) fitted with an HSF5 guard column, 4.0 mm × 20 mm. The mobile phase flow rate was 0.7 mL/min, and injection volume was 50 μL. A binary, linear gradient elution with 10 mM ammonium formate in water (solvent A) and 10 mM ammonium formate in methanol (solvent B), flow rate of 0.7 mL/min with the following program: the initial composition

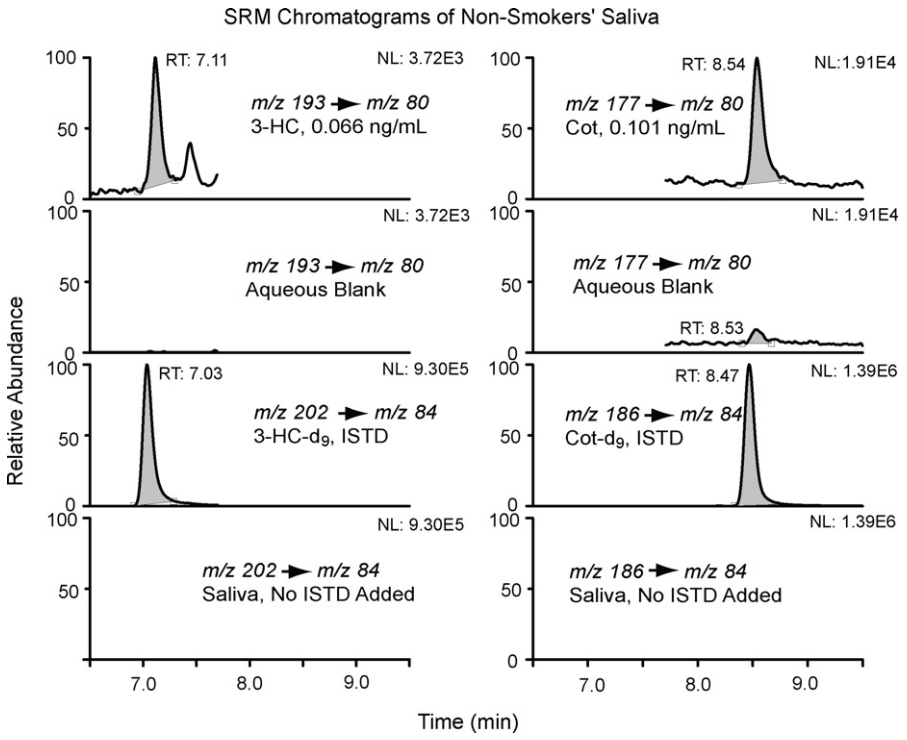

Fig. 4. SRM chromatograms of non-smokers' saliva.

was 80% A, changing to 100% B over 6.5 min. 100% B was maintained from 6.5 to 8 min, then changed to 80% A at 8.1 min and maintained at this composition until the end of run at 10 min. 3HC and cotinine eluted at approximately 5.3 and 6.2 min, respectively.

2.6.3. Mass spectrometry parameters 2

All parameters were the same as described for Method Variation 1, except that the resolution of both quadrupoles was set at 0.7 amu FWHM.

2.7. Data analysis

The Finnigan XCalibur/LC Quan software was used to generate calibration curves and calculate concentrations using peak area ratios of analyte/internal standard. Linear regression with $1/X$ weighting, "ignore origin" was used. Blanks were included in the standard curves and "ignore origin" was used to correct for the small amounts of cotinine present in solvents and reagents. (see Section 3.5). Standard curves were linear from 0.01 to 20 ng/mL

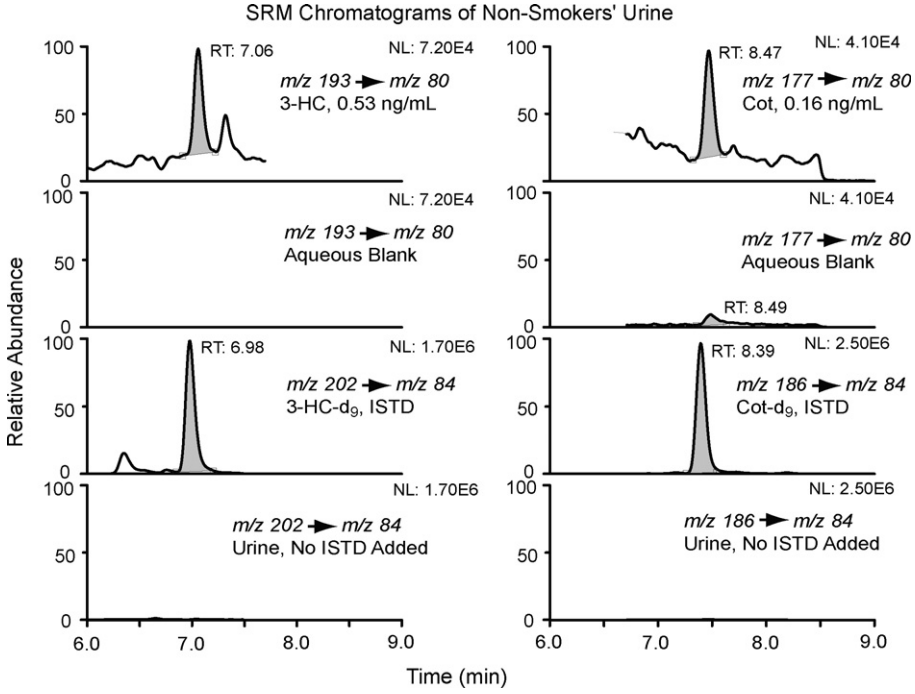

Fig. 5. SRM chromatograms of non-smokers' urine.

for SHS exposure studies, from 1 to 500 ng/mL analyses of smokers' plasma or saliva, and from 10 to 10,000 ng/mL for analyses of smokers' urine. Eight concentrations spanning each range were used, and standards were run in duplicate. Typically, one set of standards was injected at the beginning of the run, and one set following injection of the clinical study samples. Equations and correlation coefficients for representative standard curves are below.

- Cotinine, non-smokers' plasma or saliva (0.02–2 ng/mL): $Y = 0.00570 + 0.06318 \times X$ $r^2 = 0.9997$.
- 3HC, non-smokers' plasma or saliva (0.02–2 ng/mL): $Y = 0.000351 + 0.0445 \times X$ $r^2 = 0.9998$.
- Cotinine, non-smokers' urine (0.025–25 ng/mL): $Y = 0.00693 + 0.141 \times X$ $r^2 = 0.9998$.
- 3HC, non-smokers' urine (0.05–50 ng/mL): $Y = 0.000706 + 0.117 \times X$ $r^2 = 0.9993$.
- Cotinine, smokers' plasma or saliva (1–500 ng/mL): $Y = 0.00564X + 0.00724$ $r^2 = 0.9991$.
- 3HC, smokers' plasma (1–500 ng/mL): $Y = 0.00114 + 0.00557X$ $r^2 = 0.9996$.
- Cotinine, smokers' urine (10–10,000 ng/mL): $Y = 0.0119 + 0.00624 \times X$ $r^2 = 0.9995$.
- 3HC, smokers' urine (10–10,000 ng/mL): $Y = 0.00288 + 0.004053X$ $r^2 = 0.9989$.

2.8. Validation

Precision, accuracy, and limits of quantitation were determined by replicate analysis of spiked plasma, saliva, and urine samples, at concentrations spanning the expected concentration ranges (Tables 1–5) as described by Shah et al. [52]. and Viswanathan et al. [53]. In addition, 54 plasma samples obtained from smokers were analyzed for cotinine by GC using nitrogen–phosphorus detection [27] modified for use with a capillary column [29]. These results were compared with results obtained using the method described here (Fig. 6).

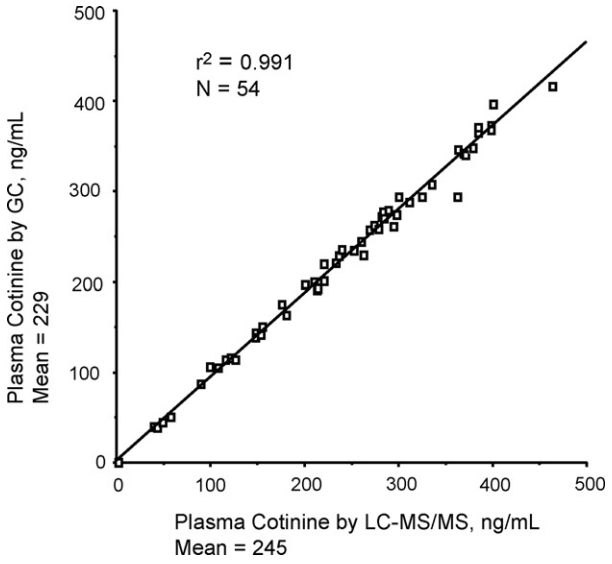

Fig. 6. Correlation of cotinine concentrations in smokers determined by GC [27,29] with concentrations determined by the method described in this paper. The analytical data were used for a published study [44].

3. Results and discussion

The goal of this study was to develop a versatile method with very high sensitivity (LLOQs less than 0.1 ng/mL) that would be practical for various large-scale clinical studies. Consequently, considerable effort was put into optimizing the chromatography, mass spectrometry, and extraction procedure.

Table 1
Within-run precision and accuracy for determination of cotinine and *trans*-3′-hydroxycotinine in plasma.

Analyte and Method Variation	Added amount (ng/mL)	Expected amount[a] (ng/mL)	Measured mean (ng/mL)	Accuracy (percent of expected)	Precision CV (%)
Cotinine Method Variation 1 $N = 6$	0.000	–	BLQ	–	–
	0.020	0.020	0.020	101	2.20
	0.100	0.100	0.100	100	1.80
	0.500	0.500	0.502	100	0.97
	2.00	2.00	2.06	103	0.96
	15.0	15.0	15.2	101	1.10
3HC Method Variation 1 $N = 6$	0.000	–		–	
	0.020	0.020	0.020	99.9	4.4
	0.100	0.100	0.105	105	3.6
	0.500	0.500	0.506	101	1.5
	2.00	2.00	2.00	100	0.79
	15.0	15.0	15.0	100	0.98
Cotinine Method Variation 2 $N = 6$	0.00	–	BLQ	–	–
	2.50	2.50	2.49	99.6	6.6
	5.00	5.00	5.00	100	4.3
	10.0	10.0	10.1	101	9.7
	50.0	50.0	50.4	101	3.7
	200	200	204	102	3.5
3HC Method Variation 2 $N = 6$	0.00	–	0.00	BLQ	–
	2.50	2.50	2.41	96.4	3.0
	5.00	5.00	4.93	98.7	2.5
	10.0	10.0	9.93	99.3	12.7
	50.0	50.0	50.5	101	1.7
	200	200	206	103	2.4

[a] Amount added + mean amount measured in blank plasma pool from non-smokers.

Table 2
Between-run precision and accuracy for determination of cotinine and *trans*-3′-hydroxycotinine in plasma.

Analyte and Method Variation	Added amount (ng/mL)	Expected amount[a] (ng/mL)	Measured mean (ng/mL)	Accuracy (percent of expected)	Precision CV (%)
Cotinine Method Variation 1	0	–	0.031	–	–
$N = 23$, 13 runs over a 5-month	0.02	0.051	0.054	106	27
period	0.05	0.081	0.078	96.3	12
	0.20	0.231	0.229	99.1	10
	2.00	2.03	2.07	102	6.4
	15.0	15.0	15.5	103	4.7
3HC Method Variation 1 $N = 23$,	0	–	0.009[b]	–	–
13 runs over a 5-month period	0.02	0.029	0.030	103	22
	0.05	0.059	0.058	98.3	9.6
	0.20	0.209	0.208	99.5	7.0
	2.00	2.01	2.08	104	6.2
	15.0	15.0	15.4	102	3.2
Cotinine Method Variation 2	1.00	1.00	1.05	105	7.5
$N = 28$, 14 runs over a 2-month	5.00	5.00	5.18	104	3.3
period	20.0	20.0	20.1	101	4.7
	200	200	196	98.0	2.7
3HC Method Variation 2 $N = 28$,	1.00	1.00	1.04	104	6.6
14 runs over a 2-month period	5.00	5.00	5.16	103	3.4
	20.0	20.0	20.3	101	4.9
	200	200	196	98.0	2.8

[a] Amount added + mean amount measured in blank plasma pool from non-smokers.
[b] Although below the lower limit of quantitation, the sum of this amount and the amount added was used to provide a better estimate of the expected amount.

3.1. Extraction

Cotinine and 3HC are hydrophilic substances that are relatively difficult to extract from aqueous matrices. Both solid-phase and liquid/liquid extraction procedures have been employed for cotinine and 3HC [11,31,39,42,54]. We found that the combination of high concentration (50%, w/v) tripotassium phosphate as a base and methylene chloride as solvent was convenient and efficient for extraction of the two analytes from biofluids. The high base concentration makes the density of the aqueous phase greater than that of methylene chloride. Therefore, the methylene chloride extract constitutes the upper layer, facilitating phase separation by the freeze and pour technique. Furthermore, the high base concentration improves recovery of the highly polar 3HC [39] by a "salting out" effect. Prior to evaporation of the methylene chloride, hydrochloric acid is added to convert the bases to non-volatile salts, since some losses occurred during vacuum evaporation if no acid was added. Extraction recovery from plasma was about 53% for 3HC and 65% for cotinine.

3.2. Chromatography

Several column and mobile phase combinations were evaluated. Extracts of plasma and urine from persons with little or no SHS exposure (do not live or work with a smoker) were used to test for interference derived from the matrix (Table 6). The column that provided the best separation of the analytes from traces of matrix-derived substances, particularly in urine, was a 4.6 mm × 150 mm Phenomenex Synergi Polar RP (embedded phenoxypropyl group) column using a water–methanol gradient with 10 mM ammonium acetate/0.1% acetic acid buffer. A 4.0 mm × 150 mm Supelco HSF5 (pentafluorophenylpropylsilane) column using a water–methanol gradient with 10 mM ammonium formate was satisfactory for plasma samples, and for urine concentrations above about 0.1 ng/mL.

3.3. Mass spectrometry

Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been utilized in mass spectrometric methods for determination of nicotine metabolites. Although ESI is generally more sensitive than APCI for detection of basic substances, it is also more prone to matrix suppression of ionization [55–57]. We found that sensitivity for an aqueous cotinine standard was about 10× higher using ESI than it was using APCI. However, when applied to urine extracts, suppression of ionization of 90% or more sometimes occurred with ESI. Therefore, APCI was evaluated, and it was found that significant matrix suppression of ionization rarely occurred. When it did occur, with some very concentrated urine samples, the stable isotope-labeled internal

Table 3
Within-run precision and accuracy for determination of cotinine and *trans*-3′-hydroxycotinine in saliva.

Analyte and Method Variation	Added amount (ng/mL)	Expected amount[a] (ng/mL)	Measured mean (ng/mL)	Accuracy (percent of expected)	Precision CV (%)
Cotinine Method	0	–	0.003[b]	–	–
Variation 1 $N = 6$	0.020	0.023	0.024	104	5.0
	0.050	0.053	0.054	102	4.4
	0.500	0.503	0.527	105	0.68
3HC Method Variation	0	–	0.006[b]	–	–
1 $N = 6$	0.020	0.026	0.025	96.2	12.7
	0.050	0.056	0.055	98.2	2.9
	0.500	0.506	0.497	98.2	0.88

[a] Amount added to pooled saliva + mean amount measured in blank saliva.
[b] Although below the lower limit of quantitation, the sum of this amount and the amount added was used to provide a better estimate of the expected amount.

Table 4
Within-run precision and accuracy for determination of cotinine and *trans*-3′-hydroxycotinine in urine.

Analyte, Method Variation, and number of replicates	Added amount (ng/mL)	Expected amount[a] (ng/mL)	Measured mean (ng/mL)	Accuracy (percent of expected)	Precision CV (%)
Cotinine Method Variation 1 $N = 6$	0[b]	–	BLQ	–	–
	0.050[b]	0.050	0.051	102	4.0
	0.100[b]	0.100	0.103	103	1.3
	0[c]	–	0.0998	–	7.4
	0.050[c]	0.150	0.152	101	1.4
	0.100[c]	0.200	0.203	102	2.3
	0.250[c]	0.350	0.356	102	0.7
	1.00[c]	1.10	1.11	101	0.8
	5.00[c]	5.10	5.16	101	0.7
3HC Method Variation 1 $N = 6$	0.100[b]	0.100	0.096	96	2.3
	0.200[b]	0.200	0.204	102	3.5
	0[c]	–	0.318	–	2.7
	0.100[c]	0.418	0.409	98	0.8
	0.200[c]	0.518	0.505	97	0.5
	0.500[c]	0.818	0.813	99	0.8
	2.00[c]	2.32	2.35	101	1.5
	10.0[c]	10.3	10.3	99	0.6
Cotinine Method Variation 2 $N = 6$	0	–	BLQ	–	–
	20[c]	20	21.1	106	4.9
	100[c]	100	107	107	8.8
	500[c]	500	505	101	3.5
	5000[c]	5000	5000	99.9	1.5
	20000[c]	20000	18600	92.8	2.7
3HC Method Variation 2 $N = 6$	0	–	BLQ	–	–
	20[c]	20	21.3	107	5.2
	100[c]	100	109	109	7.8
	500[c]	500	521	104	2.5
	5000[c]	5000	4970	99.4	2.0
	20000[c]	20000	22600	113	3.0

[a] Amount added to "artificial urine," or amount added + mean amount measured in pooled non-smokers' urine.
[b] Spiked "artificial urine."
[c] spiked non-smokers' urine.

standard corrected for ionization suppression. This was demonstrated by re-analysis of the sample, diluted 10-fold with HPLC grade water, in which case the internal standard peak area was similar to that of the standards, and the analytical result agreed within ±10% of the original analysis. Sensitivity using APCI was adequate for measuring low picogram per milliliter concentrations.

As reported for previous studies, we found that the most suitable SRM transitions for quantitation were m/z 177 to m/z 80 for cotinine and m/z 193 to m/z 80 for 3HC [22,31–35], as these produced the most abundant product ions. The corresponding transitions for the internal standards were used, m/z 186 to 84 for cotinine-d_9 and m/z 202 to 84 for 3HC-d_9. Neutral loss of the pyridine ring was also a

Table 5
Between-run precision and accuracy for determination of cotinine and *trans*-3′-hydroxycotinine in urine.

Analyte, Method Variation, and number of replicates	Added amount (ng/mL)	Expected amount[a] (ng/mL)	Measured mean (ng/mL)	Accuracy (percent of expected)	Precision CV (%)
Cotinine Method Variation 1 $N = 14$, 7 runs over a 1-month period	0	–	0.098	–	11.2
	0.050	0.148	0.148	100	8.4
	0.100	0.198	0.194	97.8	4.4
	0.250	0.348	0.348	100	4.0
	1.00	1.10	1.09	99.3	2.1
	5.00	5.10	5.06	99.2	1.5
3HC Method Variation 1 $N = 14$, 7 runs over a 1-month period	0	–	0.293	–	6.4
	0.100	0.393	0.385	98.1	5.0
	0.200	0.493	0.477	96.8	4.8
	0.500	0.793	0.787	99.3	2.3
	2.00	2.29	2.29	99.9	2.1
	10.0	10.3	10.2	99.3	1.9
Cotinine Method Variation 2 $N = 14$, 14 runs over a 4-month period	50.0	50.0	50.4	101	7.7
	500	500	533	107	7.2
	5000	5000	5340	107	4.8
3HC Method Variation 2 $N = 14$, 14 runs over a 4-month period	50.0	50.0	52.3	105	7.9
	500	500	543	109	9.5
	5000	5000	5320	107	6.4

[a] Amount added + mean amount measured in blank urine pool from non-smokers.

Table 6
Cotinine and 3HC concentrations in urine and saliva of non-smokers with little or no exposure to SHS.[a]

Subject	Cotinine urine ng/mL	3HC urine ng/mL	Cotinine saliva ng/mL	3HC saliva ng/mL
1	0.194	0.420	<LLOQ	<LLOQ
2	0.058	0.198	<LLOQ	<LLOQ
3	<LLOQ	0.142	<LLOQ	<LLOQ
4	0.057	0.227	<LLOQ	0.034
5	<LLOQ	0.315		
6	0.064	0.194		
7			<LLOQ	<LLOQ
8			0.102	0.066
LLOQ	0.05	0.10	0.02	0.02

[a] The spouse of subject 8 was an occasional smoker, typically one cigarette per day, smoked near an open window. The other subjects did not live or work with a smoker. All were residents of the San Francisco Bay Area, where smoking is banned in public buildings and indoor work places.

Table 7
Cotinine concentrations in a serum reference material determined by the method described in this paper and by the method of Bernert et al. [31].

Added amount (ng/mL)	Expected amount[a] (ng/mL)	Measured mean, $N = 20$	Measured described method
0	(0.048)	0.048	0.047[b]
0.461	0.509	0.491	0.506[b]
1.92	1.97	1.90	1.96[b]
46.1	46.2	46.2	42.5[c]
192	192	197	179[c]

[a] Amount added to + mean amount measured in a pooled non-smokers' serum.
[b] Method Variation 1 (1 mL aliquot), single analysis.
[c] Method Variation 2 (0.1 mL aliquot), mean of two analyses.

significant pathway for cotinine, resulting m/z 98. This ion has been used for confirmation in previous studies [22,31] but its abundance was too low to use for confirmation in our methods for low-level SHS exposure, which required maximum sensitivity. Likewise, the abundances of other product ions of m/z 193 for 3HC (e.g., m/z 86, 106, 114, and 134) were too low to be used for confirmation at low concentrations in samples from many non-smokers. At higher concentrations, these transitions would probably be suitable for confirmation. Chromatograms of plasma, saliva, and urine samples are shown in Figs. 3–5.

3.4. Calibration

Instrument response was found to be linear to at least 25 ng/mL for cotinine and to 50 ng/mL for 3HC for 1 mL sample extracts (Method Variation 1). For studies in active smokers, in which concentrations were higher than 50 ng/mL, a smaller sample size, 100 μL, was used, and linearity was demonstrated to 20,000 ng/mL (Method Variation 2). Calibration curves were generated using linear regression with $1/X$ weighting, using the peak area ratio of analyte/internal standard as response. Equations for typical standard curves are in Section 2.

Table 8
Concentrations of cotinine and 3HC in urine of non-smokers in two geographical areas.

Concentration (ng/mL)	Study 1[a] cotinine	Study 2[b] cotinine	Study 1[a] 3HC	Study 2[b] 3HC
Percent <0.20	19.8	72.5	3.0	36.7
Percent 0.2–1.0	45.5	26.1	14.9	36.5
Percent 1.0–3.0	21.8	10.6	36.6	19.4
Percent 3.0–10	7.9	4.8	33.7	11.3
Percent 10.0–30.0	4.0	1.3	4.0	5.2
Percent 30.0–100	1.0	0	7.9	1.3
Percent >100	0	0	0	0.3
Median	0.69	0.16	2.44	0.61
Mean	1.99	0.83	7.03	3.05
N	101	310	101	310

[a] Non-smokers in Mexico City [43].
[b] Non-smokers in San Francisco Bay Area [42].

3.5. Validation

We used the criteria of Shah et al. [52] and Viswanathan et al. [53] to validate the methods for precision, accuracy, and to determine the lower limits of quantitation (LLOQ). These are precision (CV) of ±15% and accuracy within ±15% of the expected amount, except at the lower limit of quantitation, for which ±20% is considered acceptable. LLOQs were determined using within-run precision and accuracy data. Between-run data also met the above criteria, with the exception of precision at the LLOQs for plasma, which were based on 13 analytical runs carried out over a 5-month period (Table 2). Within-run precision and accuracy was evaluated by analyzing six replicate samples at three or more concentrations spanning the expected range (Tables 1, 3 and 4). Between-run precision and accuracy was determined from QC specimens analyzed along with clinical study samples (Tables 2 and 5). For Method Variation 1, the LLOQs for cotinine are 0.02, 0.02, and 0.05 for plasma, saliva, and urine, respectively; the LLOQs for 3HC are 0.02, 0.02, and 0.10 for plasma, saliva, and urine, respectively. For Method Variation 2, the LLOQs for cotinine are 1 and 10 ng/mL for plasma and urine, respectively; the LLOQs for 3HC are 1 and 10 ng/mL for plasma and urine, respectively.

Determining specificity is complicated by nicotine metabolites being present in virtually all biofluid specimens, due to the presence of nicotine in the environment [27,58] and its ingestion, resulting in its metabolism and excretion of the metabolites. For example, in six people who reported little or no exposure to SHS, urine cotinine concentrations were above the LLOQ in four of the six, and 3HC concentrations were above the LLOQ in all six (Table 6). In six saliva samples from people who reported little or no exposure, only one had cotinine concentrations above the LLOQ, and two had 3HC concentrations above the LLOQ. We find peaks corresponding to cotinine, but not 3HC in our "blanks" (Figs. 4 and 5). It has been reported that nicotine can be converted to cotinine under typical environmental conditions, [59] which may explain its presence in solvents and reagents. Specificity was also evaluated by analyzing four serum samples, spiked with different concentrations of cotinine, that were being developed as serum reference materials by the US Centers for Disease Control (CDC). The results were in excellent agreement with the expected concentrations and with those determined [31] in the laboratories of the CDC (Table 7). For plasma samples with cotinine concentrations high enough to be measured by GC we compared analytical results determined by the described LC–MS/MS method with the results determined using GC. For 54 plasma samples with concentrations ranging from 2.4 to 514 ng/mL, there was excellent agreement, $r^2 = 0.991$ (Fig. 6).

4. Conclusions

Our method builds on the pioneering LC–MS/MS method developed by Bernert et al. at the CDC [31]. In our opinion, this was the first reported method with adequate sensitivity and specificity for determination of cotinine to evaluate low-level exposure to SHS. Application of this method has provided a large data base of serum cotinine levels in Americans who participated in the Third National Health and Nutrition Examination Survey (NHANES III) [60] and other studies. The two major applications

of our methods are (1) assessing exposure to secondhand smoke, and (2) using the 3HC/cotinine ratio, phenotyping individuals for CYP2A6 activity to optimize pharmacotherapies for tobacco dependence [25,26,45,61,62]. Metabolic activation of some carcinogenic nitrosamines, including some present in tobacco, is mediated by CYP2A6 [63,64], and that is another reason for interest in this phenotypic marker.

Exposure to SHS in the United States and many developed countries is declining, especially in persons living in areas where smoking bans are widespread and in people who are concerned about the health risks of SHS exposure. We found that many human subjects participating in studies of SHS exposure had cotinine concentrations below 0.1 ng/mL in plasma and urine, despite reporting some SHS exposure. Even at low exposure levels, there was a positive correlation between symptoms of chronic obstructive pulmonary disease (COPD) and urine cotinine concentrations, which were below 0.2 ng/mL for 73% of the samples [43]. In contrast, cotinine concentrations in urine of non-smokers in Mexico City, prior to SHS exposure in discotheques [44], were below 0.2 ng/mL for only 20% of the subjects (Table 8). Data from these studies also illustrates the generally higher concentrations of 3HC in urine, suggesting that it may be a more sensitive biomarker for low-level exposure (Table 8).

In summary, very sensitive and versatile methods for determination of the nicotine metabolites cotinine and 3HC in plasma, saliva, and urine has been developed and validated. Precision and accuracy are excellent, and the methods are suitable for large-scale studies. The methods have been used to analyze several thousand biofluid samples for studies of SHS exposure, and appear to be the most sensitive methods yet reported for these two important analytes.

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