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Measurement of nicotine, cotinine and trans-3′-hydroxycotinine in meconium by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Nicotine (NIC), cotinine (COT) and trans-3′-hydroxycotinine (OHCOT) are the most prevalent and abundant tobacco biomarkers in meconium. We have developed and validated an accurate and precise method for the measurement of these analytes in meconium in which potassium hydroxide is used to digest the meconium sample, followed by solid phase extraction from the liquified sample. The precision of OHCOT, COT and NIC measurements (intra-day and inter-day) were 4.8–10.6%, 3.4–11.6% and 9.3–15.8%, respectively. Evaluation of accuracy indicated bias of −4.0, 2.0 and 0.8% for OHCOT at concentrations of 0.5, 2.5 and 7.5 ng/g. The accuracy estimates for COT at concentrations of 0.5, 2.5 and 7.5 ng/g are 4.0, 4.0 and 5.7%, respectively. For NIC at 2, 10 and 30 ng/g the accuracy was calculated to be 3.0, 5.0 and 5.1%, respectively. The linear range of standard solutions was 0.125–37.5 ng/mL for OHCOT and COT, and 0.75–150 ng/mL for NIC. This method was applied to the analysis of 374 meconium samples from infants of both smoking and nonsmoking mothers. Positive correlations with $r^2 \geq 0.63$ were observed between NIC and COT, COT and OHCOT, NIC and OHCOT, and NIC and (OHCOT + COT) in these samples.

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1. Introduction

The 2004 Surgeon General's report states: "Smoking harms nearly every organ of the body, causing many diseases and reducing the health of smokers in general" [1]. Cancer, and especially lung cancer, is a concern for both active smokers and those who are repeatedly exposed to significant levels of second hand smoke (SHS) over time. Lung cancer alone accounts for 28% of all cancer deaths in the United States [2]. Numerous studies have identified additional cancer sites associated with tobacco smoke exposure including the upper aerodigestive tract, esophagus, stomach, liver, kidney and cervix uteri [3]. In 2006, 20.8% of U.S. adults, or 45.3 million people, were cigarette smokers [4]. Many of these smokers are women of childbearing age. In the U.S., the prevalence of smoking in the last 3 months of pregnancy ranged from 6.8% (Utah) to 25.3% (West Virginia) in 2002 [5].

The negative effects of tobacco smoking during pregnancy are well-established. Maternal smoking has been associated with increased risk of spontaneous abortion, intrauterine growth retardation, low birth weight, premature birth, perinatal mortality, sudden infant death syndrome, and early respiratory tract infections. Cognitive and neurodevelopmental disorders among newborns are associated with prenatal exposure to smoke also [6–10]. Nicotine (NIC) and its major metabolite cotinine (COT) have been used as markers for assessment of prenatal tobacco smoke exposure [11,12]. Generally, COT rather than NIC is preferred for assessing exposure because its half-life (about 15–20 h) [13–15] in blood or urine is substantially longer than that of NIC's (0.5–3 h) [15–17]. Another NIC metabolite, trans-3′-hydroxycotinine (OHCOT), is also abundant in urine [18,19], with an effective half-life similar to that of COT from which it is formed.

Meconium is the first intestinal discharge of a newborn infant, consisting mainly of epithelial cells, mucus and bile. For measuring prenatal exposure to smoke, meconium may offer an advantage over traditional matrices such as urine and hair; its collection is noninvasive and it can potentially provide evidence of a longer history of prenatal exposure to NIC and its metabolites [20–22]. Both of these advantages may offer a better index of fetal exposure to maternal smoking or SHS exposure during gestation than alternative matrices [23–25]. In this study, we developed a new liquid chromatography–tandem mass spectrometric (LC/MS/MS) method to measure OHCOT, COT and NIC in meconium simultaneously. This method may be valuable in the examination of NIC metabolites in meconium for studies that assess prenatal tobacco exposure.

2. Experimental

2.1. Reagent and standards

(−)-COT and (S)-(−) NIC were purchased from Aldrich (St. Louis, MO, USA). OHCOT was obtained from Toronto Research Chemi-

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cals (North York, Ontario, Canada). The corresponding isotopically labeled internal standards, cotinine-D_3 (COT-D_3), nicotine-methyl-D_3 (NIC-D_3), and (±)-3′-hydroxycotinine-methyl-D_3 (OHCOT-D_3) were acquired from Cambridge Isotope Laboratories (Andover, MA). Potassium hydroxide (KOH) was from Mallinckrodt Baker (Paris, KY). Acetic acid (99.9%; J.T. Baker, Phillipsburg, NJ) diluted to 1 N with HPLC-grade water, HPLC-grade hexane (Acros, Morris Plains, NJ) and isopropanol (Spectrum, Gardena, CA) were used in sample preparation. Formic acid (99%) and ammonium hydroxide (29.54%) were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade methanol, acetonitrile, ethyl acetate and methylene chloride were purchased from Burdick & Jackson (Muskgon, MI), and HPLC-grade water was obtained from TEDIA (Fairfield OH). Ammonium acetate (99.999%) was from Aldrich (Milwaukee, WI). All gases used were the highest purity available.

2.2. Instrumentation

2.2.1. Liquid chromatography

LC/MS/MS analysis was performed using an Agilent 1200 HPLC system interfaced to a PE Sciex API 5000 triple quadrupole mass spectrometer. The Agilent 1200 HPLC system consisted of a degasser, a binary pump, a HiP-ALT autosampler, a FC/ALS temperature controller and a TCC column heater. HPLC separation was achieved using a Gemini-NX 5μ C18 110A column (4.6 mm × 150 mm), purchased from Phenomenex (Torrance, CA). The column temperature was set to 40 °C and the eluant flow rate was 0.600 mL/min. The injection volume was 5 μL for both standards and samples. The mobile phase used in these separations consisted of A: 0.5 g/L ammonium acetate in water (6.5 mM), adjusted to ca. pH 9.3 using 25% ammonium hydroxide; and B: 100% acetonitrile. After equilibration with a mobile phase of 10% B and 90% A, samples were injected and the mobile phase remained 10% B and 90% A from 0 to 2 min. A gradient was initiated from 10 to 55% mobile phase B (acetonitrile) from 2 to 4 min. The mobile phase was then returned to 10% B from 4 to 6 min, and maintained until the end of the run at 10 min.

2.2.2. Mass spectrometry

The Turbo-ionspray ionization source of the mass spectrometer was operated at a source temperature of 600 °C and a spray voltage of 1500 V. All LC/MS/MS data were recorded in multiple reaction monitoring (MRM) mode, collecting data for the transition ions in two periods. In the first period of 8.3 min, we recorded transition ions at m/z 192.9 > 80.0 (OHCOT quantification), m/z 192.9 > 133.9 (OHCOT confirmation), m/z 195.9 > 80.0 (OHCOT ISTD), m/z 177.0 > 98.1 (COT quantification), m/z 177.0 > 80.1 (COT confirmation), and m/z 180.0 > 80.1 (COT ISTD). During the second period, beginning at 8.3 min, data were collected for transition ions at m/z 162.9 > 130.0 (NIC quantification), 162.9 > 117.0 (NIC confirmation), and 165.9 > 130.0 (NIC ISTD). The total run time was 10 min. The compound-related mass spectrometric parameters were optimized for each individual ion transition, as shown in Table 1. The collision gas and curtain gas were optimized for the m/z 177.0 > 80.0 (COT) transition, and the analytical quadrupoles were set to unit resolution.

Table 1
Tandem mass spectrometer parameters for OHCOT, COT and NIC.[a]

Analyte	Q1 mass	Q2 mass	DP	EP	CE	CXP
OHCOT	192.9	80.0	90	9	36	30
	192.9	133.9	90	9	28	30
	195.9	80.0	90	9	36	30
COT	177.0	98.1	90	9	52	30
	177.0	80.1	90	9	52	30
	180.0	80.1	90	9	52	30
NIC	162.9	130.0	50	10	52	13
	162.9	117.0	50	10	52	13
	165.9	130.0	50	10	52	13

[a] OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, cell exiting potential.

2.3. Preparation of standard solutions and control materials

One complete set of calibration standards in water was prepared consisting of 0, 0.125, 0.25, 0.5, 1.25, 2.5, 5, 12.5, 25, and 37.5 ng/mL of OHCOT and COT, and 0, 0.75, 1.5, 2.5, 5, 12.5, 25, 50, 75, and 150 ng/mL of NIC. OHCOT-D_3 and COT-D_3 were each 6.25 ng/mL, and NIC-D_3 was 25 ng/mL in the standard solutions. All standards were sealed in pre-cleaned amber ampoules and stored at 3–5 °C. The internal standard solution used to spike samples contained 12.5, 12.5 and 50 ng/mL of OHCOT-D_3, COT-D_3 and NIC-D_3, respectively. This solution was sealed in 3 mL aliquots and stored at 3–5 °C. 100 μL of this solution was spiked into each sample, blank or QC in the assay.

The two quality control (QC) materials, M1 and M2, were prepared from two separate pools of meconium that had been pretested and confirmed to have detectable and different levels of all three analytes present. Each pool was prepared by placing the meconium in 5 N KOH and digesting for 1.5 h. The samples were then dispensed in 3 mL aliquots into 200 screw cap tubes. After all the tubes were prepared, every twentieth tube was removed for homogeneity testing and the remaining tubes were stored at −70 °C until used. Samples from these two pools were used to estimate assay precision and sample stability.

2.4. Procedures

2.4.1. Meconium preparation

We accurately weighed 0.5 g ± 0.1 g meconium sample and recorded the weight to 10 μg. We then added 3 mL of 5 N KOH and 100 μL of ISTD to the weighed sample and vortexed the tube vigorously. The water blank was prepared by mixing 3 mL of 5 N KOH and 100 μL of ISTD. The prepared sample was placed on a rotator at 60 rpm to digest for 1.5 h. After digestion, the solution was loaded onto a 5 mL pre-washed Chem Elut column (Varian, Palo Alto, CA) and allowed to soak for 3 min. Analytes were eluted by using 5 mL of 100% methylene chloride followed by 2× 4 mL of 10% ethanol in methylene chloride. The combined eluents were taken to dryness in a vacuum evaporator and the dried sample was reconstituted in 2 mL of methylene chloride. We added 1 mL of 0.1 M HCl and mixed on a rotator for 5 min at 60 rpm, then centrifuged the sample for approximately 2 min at 3000 rpm to separate the layers. The top (HCl) layer was recovered and loaded onto a pre-conditioned CleanScreen CSDAU SPE cartridge (United Chemical Technologies, Bristol, PA).

2.4.2. Solid phase extraction

The CleanScreen SPE column was pre-washed with 2 mL of methylene chloride, 2 mL of methanol, and finally with 2 mL of water. After the acidified sample was slowly loaded onto the column (ca. 1 mL/min), the column was washed with 1 mL of 1 N acetic acid and dried under positive pressure N_2 flow at 15 psi for 5 min. The column was then washed with 1 mL of hexane and then 4 mL of hexane-ethyl acetate (1:1), followed by 1 mL of methanol. After washing, the analytes were slowly eluted with 2× 3 mL of freshly made methylene chloride/isopropanol/ammonium hydroxide (78:20:2) (ca. 1 mL/min). The final extract was dried in the vacuum evaporator without heat, and the residue was reconstituted in 100 μL of methanol with 0.1% formic acid for the analysis. The concentration of the sample was calculated in ng/mL based on

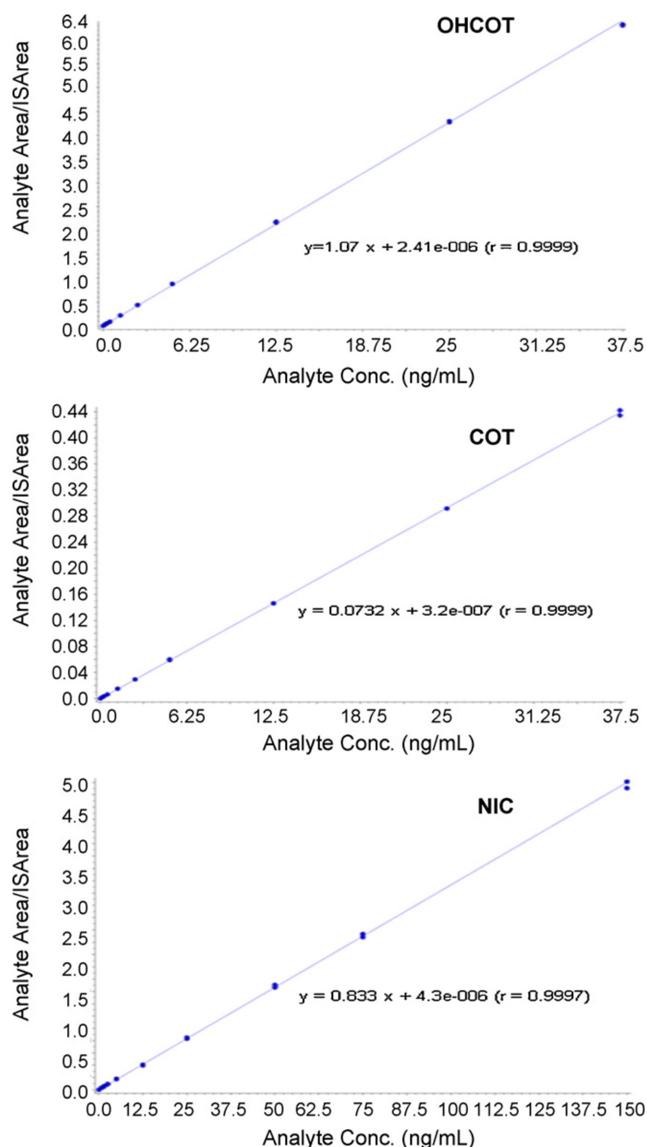

Fig. 1. Calibration curves for OHCOT, COT and NIC (OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine).

the calibration curve, and then converted to ng/g using the sample weight.

2.5. Data analysis

To generate a calibration curve, peak area ratios of the standards and respective ISTD were calculated and plotted against the concentration using weighted regression (weight = $1/X$). Fig. 1 shows the calibration curves observed over the range of standards. The ion transitions at m/z 192.9 > 80.0, m/z 177.0 > 98.1 and m/z 162.9 > 130.0 were used for OHCOT, COT and NIC quantification, respectively. The second ion transition served as confirmation. All calibrations and statistical analyses were conducted using the SAS Systems for Windows 9.1 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Precision and accuracy

Short-term precision was determined by the repetitive analysis of two pools of meconium samples. These two pools had different analyte concentrations and were assayed over a period of 4 weeks in ten runs. Each sample was assayed in duplicate in each run. Table 2 summarizes the mean calculated values, standard deviations (SDs) and coefficients of variations (CVs) for all three metabolites. The CVs for NIC in both pools were relatively larger than those for OHCOT and COT. This is probably due to the volatile and ubiquitous nature of NIC. Since the blank was positive in each run, the calculated values were blank-subtracted and the NIC values in blanks had a relatively larger variance than OHCOT and COT.

Table 2
Precision evaluation of OHCOT, COT and NIC.[a]

	Mean (ng/g)	Intra-day (N = 10)		Inter-day (N = 35)	
		SD	CV%	SD	CV%
Low QC M1					
OHCOT	1.98	0.17	8.5	0.23	10.6
COT	0.77	0.05	6.2	0.10	11.6
NIC	3.48	0.5	13.9	0.61	15.8
High QC M2					
OHCOT	3.51	0.17	4.8	0.22	6.1
COT	8.59	0.29	3.4	0.44	5.1
NIC	11.20	1.04	9.3	1.47	12.3

[a] OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine; QC, quality control; SD, standard deviation; CV, coefficient of variation.

Accuracy was examined by using OHCOT, and the salt forms COT perchlorate and NIC tartrate to make accurately prepared stock solutions. Because of the unavailability of certified blank meconium and the inherent lack of homogeneity of this matrix, the homogenized low QC pool was used as the matrix to prepare fortified samples. Three different levels of OHCOT, COT and NIC were spiked into the low QC pool, as shown in Table 3. The fortified samples were then treated as unknowns through the sample cleanup procedure and analyzed in two runs totaling five replicates per level, including five blanks in each run. The average of the blank and the mean QC value were subtracted from the results for accuracy determinations.

3.2. Linearity and limit of detection (LOD)

Calibration curve characterizations of OHCOT, COT and NIC are summarized in Table 4, along with the limit of detection (LOD) for each analyte. Because the blank was positive for all three, the LOD was calculated based on 3× SD of the blank (N = 25) in ng for each analyte. The positive blanks presumably resulted from the accumulation of trace amounts present in the solvents, reagents, columns and other possible sources encountered during the sample preparation process. This finding is consistent with our observation that these analyte concentrations increased when more solvents were

Table 3
Accuracy evaluation of OHCOT, COT and NIC.[a]

Target conc. (ng/g)	Calc. conc. (N = 5)	CV%	Bias%
OHCOT			
0.5	0.48	3.8	−4.00
2.5	2.55	3.1	2.00
7.5	7.56	1.2	0.80
COT			
0.5	0.52	1.6	4.00
2.5	2.60	1.6	4.00
7.5	7.93	1.1	5.73
NIC			
2	2.06	0.9	3.00
10	10.505	2.4	5.00
30	31.53	2.0	5.10

[a] OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine; CV, coefficient of variation.

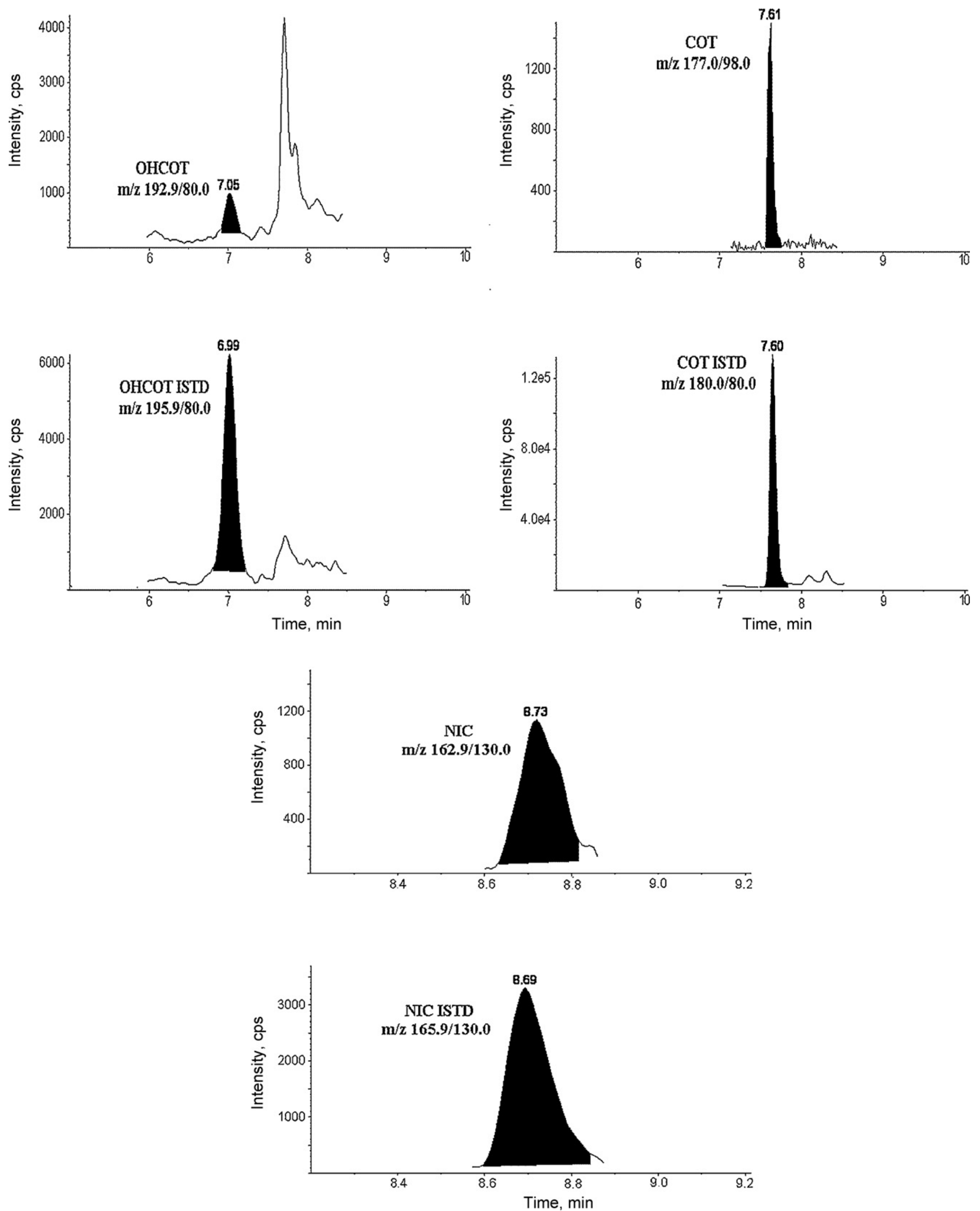

Fig. 2. Two-period chromatogram of OHCOT, COT and NIC quantitation ions in a meconium sample (OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine).

used, regardless of the weight of each meconium sample. The LODs for OHCOT, COT and NIC measured in this study were 0.092, 0.070 and 0.946 ng/g, respectively. These LODs are somewhat lower than those previously reported from analyses using a similar method [26]. Lower limit of quantitation (LLOQ) was estimated as 10× SD of the blank. The LLOQs for OHCOT, COT and NIC measured by this method were calculated to be 0.307, 0.233 and 3.15 ng/g, respectively. Fig. 2 displays the two-period chromatogram of OHCOT, COT and NIC quantitation and ISTD ion traces in a meconium sample at retention times of 7.05, 7.61 and 8.69, respectively. In this sam-

Table 4
LOD[a] and calibration results.

	LOD (ng/sample)	Slope ± SD (N = 20)	Intercept ± SD (N = 20)	R^2 ± SD (N = 20)	Linear range (ng/mL)	Retention time (min)
OHCOT	0.0458	1.10 ± 0.07	0.00044 ± 0.00192	0.9998 ± 0.0001	0–37.5	7.17
COT	0.0349	0.074 ± 0.001	0.000039 ± 0.000116	0.9998 ± 0.0001	0–37.5	7.62
NIC	0.473	0.895 ± 0.057	0.00064 ± .00275	0.9983 ± 0.0011	0–150	8.75

[a] LOD, limit of detection; OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine; SD, standard deviation.

ple, OHCOT, COT and NIC were calculated to be 0.097, 0.133 and 1.10 ng/g, in the vicinity of the respective LODs.

3.3. Sample stability and recovery

Meconium QC pools M1 and M2 were also used to study freeze–thaw stability. Samples were analyzed after 1–5 freeze–thaw cycles. For each cycle all samples were removed from the −70 °C freezer and allowed to remain at room temperature for 1 h before refreezing. The CVs for COT in M1 and M2 (N = 5) were 1.9 and 2.7%, respectively, whereas NIC in M1 and M2 (N = 5) had CVs of 9.9 and 1.4%, respectively. In these analyses, both NIC and COT appeared to be stable under these freeze–thaw conditions. The measurement of hydroxycotinine was not included in these stability analyses.

The sample recovery varies from sample to sample due to the complex nature of meconium and multiple extraction steps in sample preparation. The overall recovery ranged 20–40% for all three

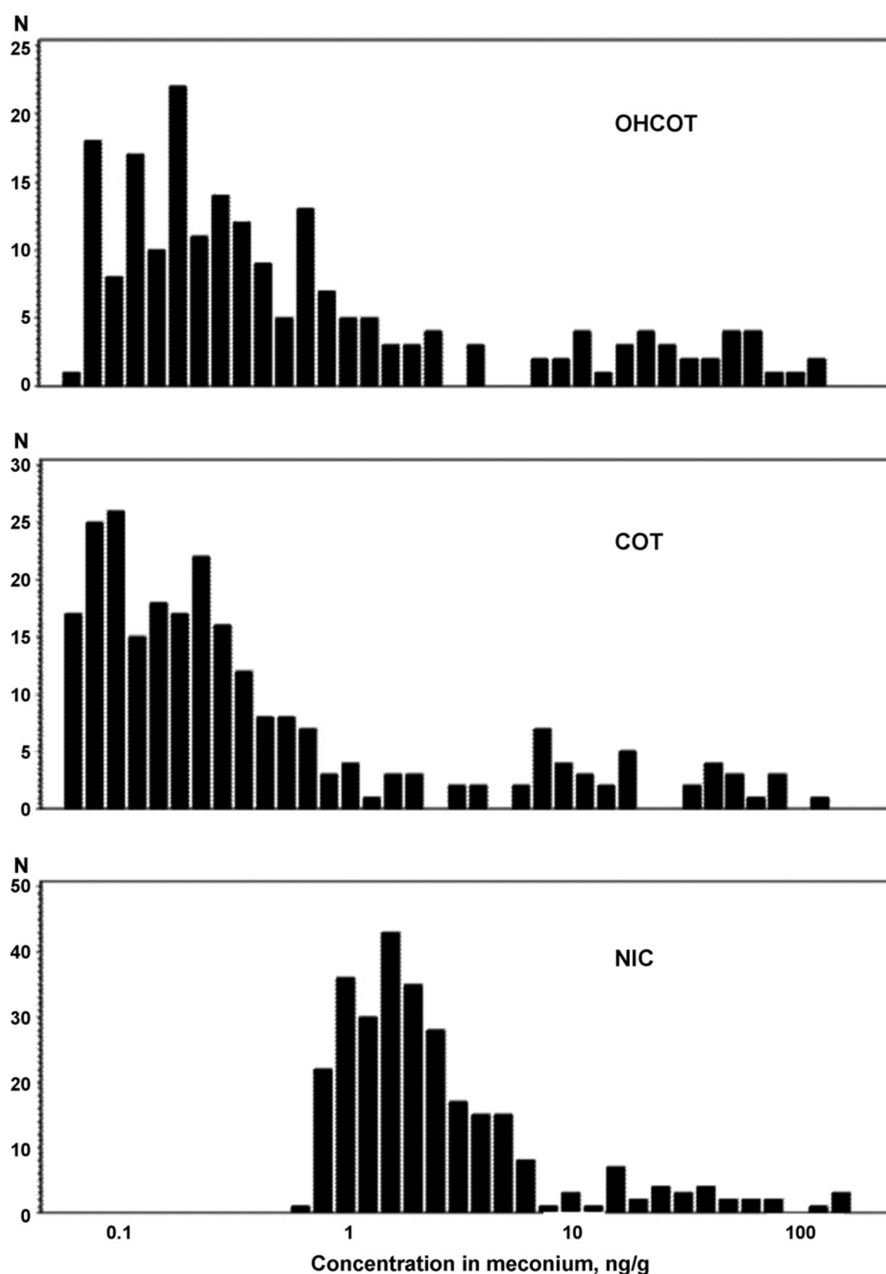

Fig. 3. Distributions of OHCOT, COT and NIC in meconium samples (OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine).

Table 5
OHCOT, COT and NIC[a] measured in meconium samples.

Analyte (ng/g)	N	Below LOD	Percentiles					Geometric mean
			5	25	50	75	95	
OHCOT (ng/g)	205	169	0.087	0.171	0.397	1.82	117.3	0.845
COT	246	128	0.070	0.110	0.260	0.832	77.6	0.501
NIC	285	89	1.05	1.53	2.51	5.19	56.9	3.53

[a] OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine; LOD, limit of detection.

metabolites based on the absolute ISTD area counts. OHCOT was found to decompose in KOH hydrolysis at first, and the hydrolysis time was then optimized to minimize the loss of OHCOT during digestion. Due to the volatile nature of NIC, there was larger variation in NIC recovery than the other two metabolites in meconium samples.

4. Application

This method was used for the analysis of these three NIC metabolites in 374 meconium samples collected from newborn infants whose mothers were from a population among whom the smoking status was mixed. According to self-reported exposure data [27], 36 women were known smokers, 48 women were nonsmokers but exposed to SHS, 232 women were nonsmokers with no exposure to SHS and some women have missing smoking status. Fig. 3 shows the distribution of OHCOT, COT and NIC concentrations in all detectable samples. COT and OHCOT concentrations were predominately less than 1 ng/g, whereas NIC concentrations were most prevalent in the 1–10 ng/g range. The distribution pattern of OHCOT was quite similar to that of COT. In these meconium samples, NIC had the highest geometric mean overall, and OHCOT had a mean

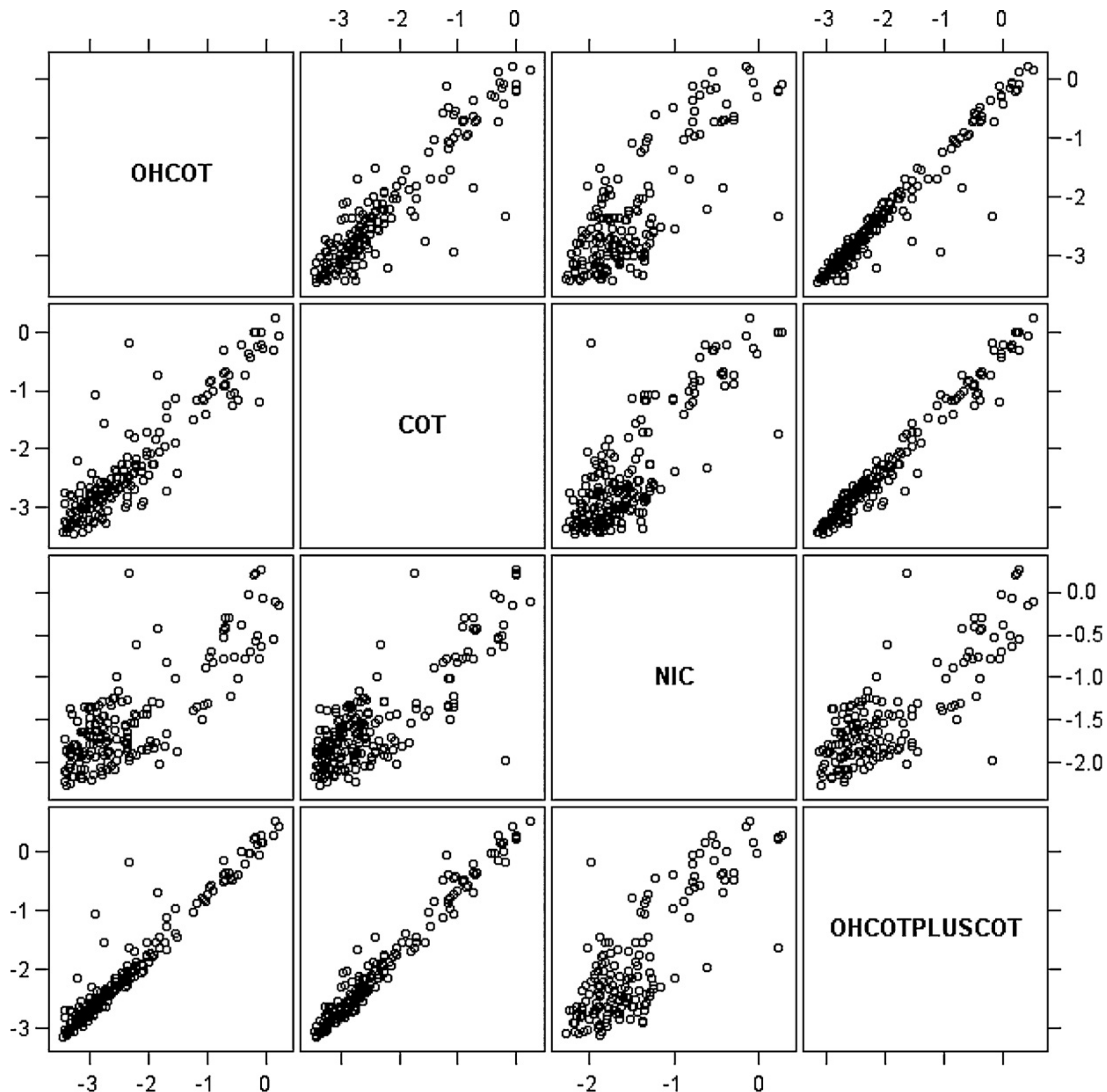

Fig. 4. Scatter matrix plot of OHCOT, COT, NIC and OHCOT + COT (in nmol/g log scale) (OHCOT, trans-3′-hydroxycotinine; COT, cotinine; NIC, nicotine).

higher than COT (Table 5). This observation is consistent with the tendency for OHCOT, followed by COT, to be the most abundant NIC metabolite present in the urine of smokers [28].

Positive correlations were observed between NIC and COT, NIC and OHCOT, and COT and OHCOT in these samples, as shown in Fig. 4. COT is the proximate metabolites of NIC, with 70–80% of NIC metabolized to COT in most smokers. COT, in turn, may be converted to other metabolites including OHCOT. There was also a positive correlation between NIC and the sum of OHCOT and COT (Fig. 4). The sum of (OHCOT + COT) has been found to be strongly associated with reported SHS exposure in young children [29]. In the current study, it was found that the geometric mean concentrations of all three metabolites OHCOT, COT and NIC were positively associated not only with serum cotinine, but also with self-reported tobacco exposures [27].

5. Discussion

The method we report here involves an initial digestion of the meconium sample in concentrated KOH before subsequent extraction and analysis of NIC metabolites. This preliminary treatment is a crucial aspect and differs from several published methods that have simply extracted intact meconium with acidified methanol [30,31] or other solvents. Under those conditions reported, the meconium is not always well-dispersed and tends to form solid particulate masses in the extracting solvent. Consequently, the recovery of metabolites in the sample may not be complete. Although externally spiked analyte has been reported to be routinely recovered in most methods, such spikes into the extraction mixture may not fully replicate true recoveries of endogenously bound analyte. Our use of strong base would also be expected to hydrolyze any glucuronides of the metabolites that might be present. The optimized hydrolysis time of 1.5 h at room temperature was adequate for KOH to completely digest the meconium samples. Analysis of spiked samples confirmed that the analytes were stable under these conditions, although the use of additional heating or longer digestion times was associated with a demonstrable degradation of OHCOT.

After the initial digestion, we conducted a preliminary liquid-liquid extraction on Chem-Elut columns followed by recovery of the extract for final processing using SPE essentially as described previously by Ostrea et al. [11] and Gray et al. [31]. Although this extraction and processing was somewhat more time-consuming, it provided consistent and reproducible results, including data compiled from "pooled" meconium samples that were mixed and stored before being analyzed in separate runs. Because meconium is an inherently inhomogeneous matrix, the latter approach provided a means to assess and report reliable inter-run reproducibility with these samples.

By using this method, OHCOT, COT and NIC were detected in a number of meconium samples from individual newborns (Table 5). These detection rates are higher than those reported in a previous study [32], which simply may reflect differences in the populations used, or differences in sensitivity or recoveries of the methods. With all three analytes, we found rather wide concentration ranges as indicated by the percentiles summarized in Table 5. This method has been used to assess prenatal exposure to tobacco smoke among newborns of both smoker and nonsmoker mothers [27]; NIC metabolite concentrations in meconium were found to be positively correlated with COT measurements in serum from the same newborns, and inversely associated with birthweight. Thus, meconium measurements of NIC metabolites may be of value in assessing the prenatal exposure to tobacco smoke among newborns, and potential adverse outcomes from such exposures.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the Centers for Disease Control and Prevention.

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