Rapid communication

Biomedical Chromatography

Received: 31 October 2013, Revised: 23 December 2013, Accepted: 16 January 2014 Published online in Wiley Online Library: 24 February 2014

(wileyonlinelibrary.com) DOI 10.1002/bmc.3159

Simultaneous determination of cotinine and *trans*-3-hydroxycotinine in urine by automated solid-phase extraction using gas chromatography–mass spectrometry

Fouad Chiadmi and Joël Schlatter*

ABSTRACT: A gas chromatography–mass spectrometry method was developed and validated for the simultaneous automated solid-phase extraction and quantification of cotinine and *trans*-3-hydroxycotinine in human urine. Good linearity was observed over the concentration ranges studied ($R^2 > 0.99$). The limit of quantification was 10 ng/mL for both analytes. The limits of detection were 0.06 ng/mL for cotinine (COT) and 0.02 ng/mL for *trans*-3-hydroxycotinine (OH-COT). Accuracy for COT ranged from 0.98 to 5.28% and the precision ranged from 1.24 to 8.78%. Accuracy for OH-COT ranged from −2.66 to 3.72% and the precision ranged from 3.15 to 7.07%. Mean recoveries for cotinine and *trans*-3-hydroxycotinine ranged from 77.7 to 89.1%, and from 75.4 to 90.2%, respectively. This analytical method for the simultaneous measurement of cotinine and *trans*-3-hydroxycotinine in urine will be used to monitor tobacco smoking in pregnant women and will permit the usefulness of *trans*-3-hydroxycotinine as a specific biomarker of tobacco exposure to be determined. © 2014 The Authors. *Biomedical Chromatography* published by John Wiley & Sons Ltd.

Keywords: cotinine; *trans*-3-hydroxycotinine; urine; GCMS; SPE

Introduction

Smoking is an unquestionable risk factor for disease and consequent death because there are many toxic chemical substances in cigarette smoke (Smith and Hansch, 2000; Hammond and O'Connor, 2008). Nicotine is the main active ingredient of tobacco and is the principal contributor that leads to smoking dependence. Because the blood levels of nicotine decrease rapidly, cotinine, a major degradation product of nicotine metabolism, is considered a specific biomarker for evaluating cigarette smoke exposure (Benowitz and Jacob, 2009). Its half-life is a much longer (16–20 h) than that of nicotine (2 h), is identical in biological fluids and presents higher concentrations than nicotine (Benowitz, 1996; Benowitz and Jacob, 2009). Cotinine is hydroxylated to give *trans*-3-hydroxycotinine which is eliminated predominantly as unchanged drug (Bao *et al.*, 2005; Benowitz *et al.*, 2009). The measurement of the ratio cotinine/*trans*-3-hydroxycotinine in smokers makes it possible to explore on a large scale the metabolism of nicotine. Such calculation is likely to facilitate links between the catabolism of nicotine and the criteria of assessment of dependency and consumption (St Helen *et al.*, 2013; Murphy *et al.*, 2013). However, urinary cotinine and *trans*-3-hydroxycotinine are the preferred markers because urine collection is noninvasive. Several methods are used to quantify nicotine and metabolites in human urine, including ultraperformance liquid chromatography, liquid chromatography–mass spectrometry, gas chromatography–mass spectrometry (GCMS), gas chromatography with nitrogen phosphorus detection and immunoassays (Shulgin *et al.*, 1992; Ji *et al.*, 1999; Meger *et al.*, 2002; Moyer *et al.*, 2002; Xu *et al.*, 2004; Chao *et al.*, 2005; Heavner *et al.*, 2005; Kim *et al.*, 2005; Song *et al.*, 2005; Hoofnagle *et al.*, 2006; Kataoka *et al.*, 2009; Shakleya and Huestis, 2009a and 2009b; Baumann *et al.*, 2010; Hu *et al.*, 2010; Malafatti *et al.*, 2010; Marclay and Saugy, 2010; Miller *et al.*, 2010; Dobrinas *et al.*, 2011; Jacob *et al.*, 2011; Rangiah *et al.*, 2011; Kuhn *et al.*, 2012; Scheidweiler *et al.*, 2012). Older methods include quantification of cotinine and *trans*-3-hydroxycotinine in urine by GCMS (Shulgin *et al.*, 1992; Ji *et al.*, 1999; Kim *et al.*, 2005). This paper reports an automated solid-phase extraction (SPE) procedure to determine simultaneous quantification of cotinine and *trans*-3-hydroxycotinine by GCMS across a wide range of concentrations in human urine.

Experimental

Materials

Cotinine (COT), *trans*-3-hydroxycotinine (OH-COT), cotinine-d3 and *trans*-3-hydroxycotinine-d3 were obtained from LGC Standards (Molsheim,

* Correspondence to: J. Schlatter, Laboratoire de toxicologie de médecine légale, Hôpital Jean Verdier – APHP, Avenue du 14 juillet, 93140 Bondy, France. E-mail: joel.schlatter@jvr.aphp.fr

Laboratoire de toxicologie de médecine légale, Hôpital Jean Verdier – APHP, Avenue du 14 juillet, 93140 Bondy, France

Abbreviations used: BSTFA, *bis*(trimethylsilyl)trifluoroacetamide; COT, cotinine; OH-COT, *trans*-3-hydroxycotinine; SPE, solid-phase extraction; TMS, trimethylchlorosilane

France). Caffeine, ibuprofen, methadone, morphine, pseudoephedrine, codeine, cocaine, clomipramine, propranolol and Δ-9-tetrahydrocannabinol analytical reference standards presented in methanol were obtained from Cerilliant (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Sigma-Aldrich (St Louis, MO, USA). Methanol, hexane, isopropanol were of analytical grade and were obtained from Merck (Darmstadt, Germany). Sterile water was obtained from Fresenius (Sevres, France). Ammonia solution 25% was purchased from VWR (Fontenay-sous-Bois, France). Hydrochloric acid 0.2 M was obtained from VWR (Fontenay-sous-Bois, France). Clean Screen SPE columns 200 mg were purchased from UCT (Bristol, PA, USA). The derivatizing agent utilized was *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMS) (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Blank, standard and quality controls

Urine samples from nonsmoker volunteers were collected, extracted and analyzed. Urine samples with nondetectable COT and OH-COT were pooled and used in the preparation of calibration and quality control samples (QCs). The commercial solutions containing 1 mg/mL of COT and OH-COT in methanol were used as stock solutions. Three working solutions (1, 10 and 100 μg/mL) were prepared from the stock solutions. A set of eight calibrators made up of 25, 50, 100, 500, 1000, 2000, 4000 and 6000 ng/mL COT and OH-COT in urine was prepared daily from working solutions. Three QCs (30, 2500 and 5000 ng/mL) of COT and OH-COT in urine were also prepared from a separate stock solution (1 mg/mL). The internal standards, cotinine-d3 and *trans*-3-hydroxycotinine-d3, were mixed in methanol to prepare the internal standards working solution (10 μg/mL). All solutions, blank and QCs were stored at −20°C prior to analysis.

Sample preparation

An aliquot of 2 mL of urine sample, QC sample or calibration standard was mixed with 2 mL of 0.1 M phosphate buffer (pH 6.0). Twenty microliters of internal standards working solution were added to each sample prior to extraction. The SPE columns were preconditioned in the following order, 3 mL of methanol, 3 mL of sterile water, and 1 mL of phosphate buffer (pH 6.0). Each sample was loaded onto SPE column at 1 mL/min and washed with 3 mL of sterile water, 2 mL of 0.2 M hydrochloric acid, 2 mL of hexane and 3 mL of methanol. Analytes were eluted with 1 mL dichloromethane–2-propanol–concentrated ammonium hydroxide (78:20:2). This SPE protocol was entirely automated using the Gilson GX-271 ASPEC system. Extracts were evaporated to dryness under a stream of nitrogen at 35°C using a Thermec Dri-Block® DB-3D Evaporator. Residues were reconstituted in 20 μL of ethyl acetate and 20 μL of BSTFA (with 1% TMS) and derivatized at 70°C for 30 min. The derivatized extract (1 μL) was injected onto the GCMS with selected ion monitoring mode.

Gas chromatography–mass spectrometry

The Thermo Focus DSQ II gas chromatograph/mass spectrometric system was used for GC separation and detection. Helium was used as a carrier gas at a constant flow of 1.20 mL/min. The system was equipped with an Uptibond® UB5 premium column (30 m × 0.25 mm × 0.25 μm). The instrument was programmed from 70 to 190°C at 30°C/min, to 230°C at 5°C/min, and to 290°C at 25°C/min, for a total analysis time of 15 min. The transfer line temperature was maintained at 290°C. One microliter of derivatized extract was injected. The injection port temperature was held at 250°C and operated in the pulsed splitless mode. The instrument utilized electron impact ionization and was operated in the selected ion monitoring mode. The ions for each analyte were monitored in the following elution order for the derivatized analytes: cotinine-d3, *m/z* (122), 179; cotinine, *m/z* (119), 176; *trans*-3-hydroxycotinine-d3, *m/z* (147), 252; and *trans*-3-hydroxycotinine, *m/z* (249), 144.

Data analysis

Data were collected and analyzed utilizing Thermo Electron GCMS Solution software (Xcalibur™, version 1.4.2; Thermo Electron Corporation, San Jose, CA, USA).

Selectivity

Five blank urine samples from different nonsmokers were extracted and analyzed for assessment of potential interferences that may be present owing to passive smoke exposure. Potential interferences from 10 commonly used drugs were evaluated by adding compounds at concentrations of 0.2, 0.5 and 10 μg/mL (caffeine, ibuprofen, methadone, morphine, pseudoephedrine, codeine, cocaine, clomipramine, propranolol and Δ-9-tetrahydrocannabinol).

Linearity and sensitivity

The linearity of the method was determined by linear regression of calibrator concentrations vs peak area ratio of either COT or OH-COT peak area divided by the peak area of the respective internal standard. The linearity of the curves was accepted when each of the calibrators achieved concentration not exceeding 20% of the nominal actual concentration. The sensitivity of the assay was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The LOD is the lowest concentration of an analyte determined with signal-to-noise of at least 3:1 by peak area. The LOQ is the lowest concentration of an analyte in a calibration curve and it may use the criteria of LOQ.

Precision and accuracy

Inter- and intra-assay precision and accuracy data for COT and OH-COT were calculated by using low (50 ng/mL), medium (600 ng/mL) and high (5000 ng/mL) QC samples. Intra-assay data were assessed by comparing data from within one run ($n = 9$). Inter-assay data were determined by comparing data between six runs. Precision was expressed as percentage relative standard deviation (RSD), and accuracy was expressed as the deviation (%) of the mean concentration from nominal concentration.

Recovery

Recovery was determined by adding analytes to a series of six replicates in urine with low (100 ng/mL), medium (1000 ng/mL) and high concentrations (6000 ng/mL) of each analyte that were extracted and compared nonextracted standards in methanol. Recovery was expressed as a percentage of the mean peak area of the extracted replicates divided by the mean peak area of nonextracted replicates.

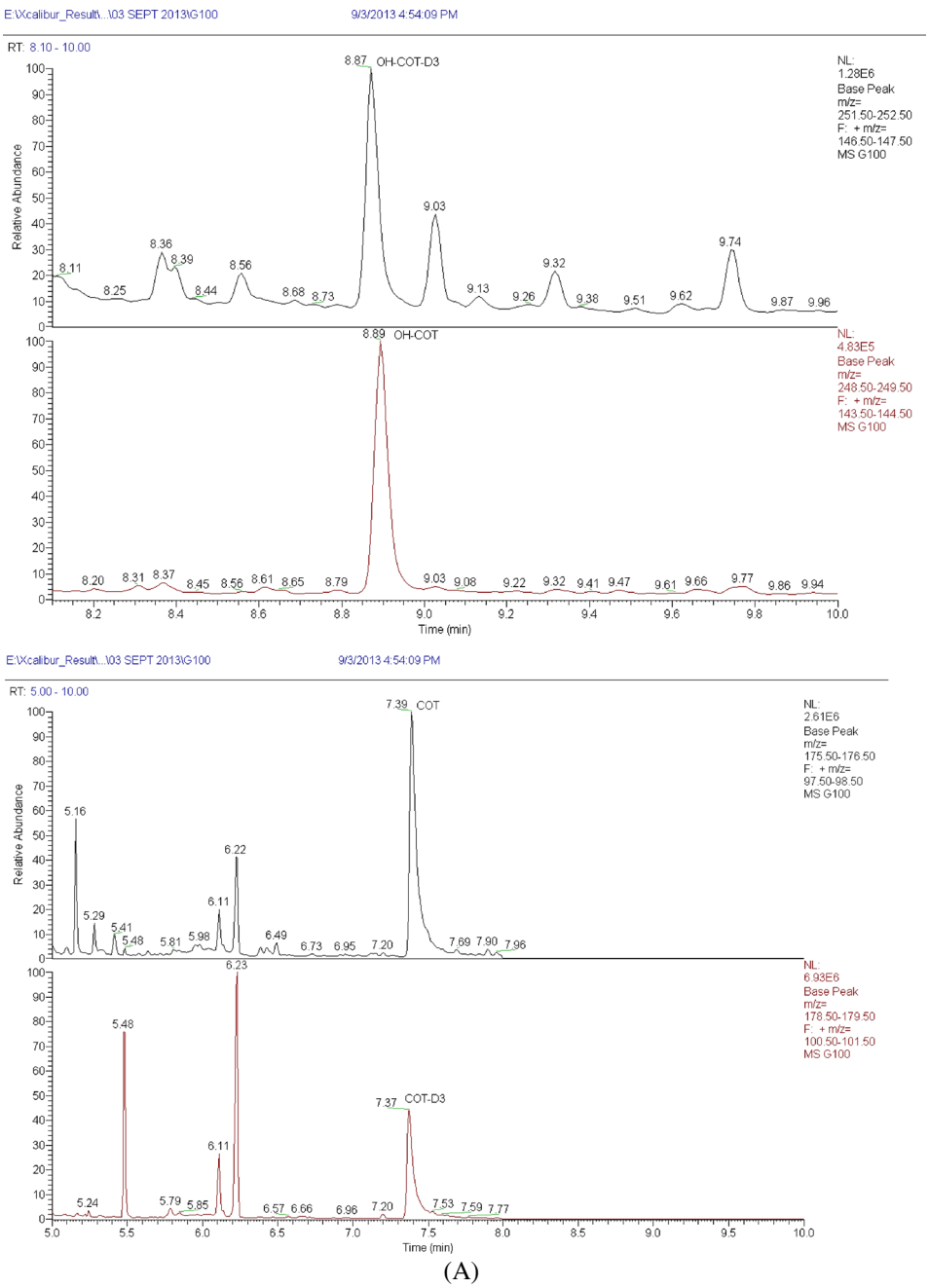
Stability

The stability of samples in the autosampler tray was tested using blank urine samples spiked with low (50 ng/mL), medium (600 ng/mL) and high concentrations (5000 ng/mL) of each analyte and internal standards. The spiked samples were by the procedures described above followed by evaporation, reconstitution with 20 μL ethyl acetate and 20 μL BSTFA with 1% TMS, transfer to vial insert, and placement in the autosampler tray. One microliter of the solution was injected every hour from the same sample. The response ratios of each analyte and its internal standard of successive injections were compared with that of the first injection.

Results

Method validation

A typical chromatogram for the two analytes and their internal standards can be found in Fig. 1. Figure 1 shows that first cotinine-d3 is



(A)

Figure 1. (A) Typical chromatogram showing the elution of cotinine (COT) 100 ng/mL and *trans*-3-hydroxycotinine (OH-COT) 100 ng/mL and their respective d3-labeled internal standards (COT-D3, OH-COT-D3). (B) A chromatogram of an average smoker sample with COT 507.3 ng/mL and OH-COT 447.7 ng/mL. (C) A chromatogram of a heavy smoker with COT 3534.5 ng/mL and OH-COT 6502.5 ng/mL.

eluting at 7.37 min, followed by cotinine at 7.39 min, *trans*-3-hydroxycotinine-d3 at 8.87 min, and *trans*-3-hydroxicotinine at 8.89 min.

Linearity and sensitivity

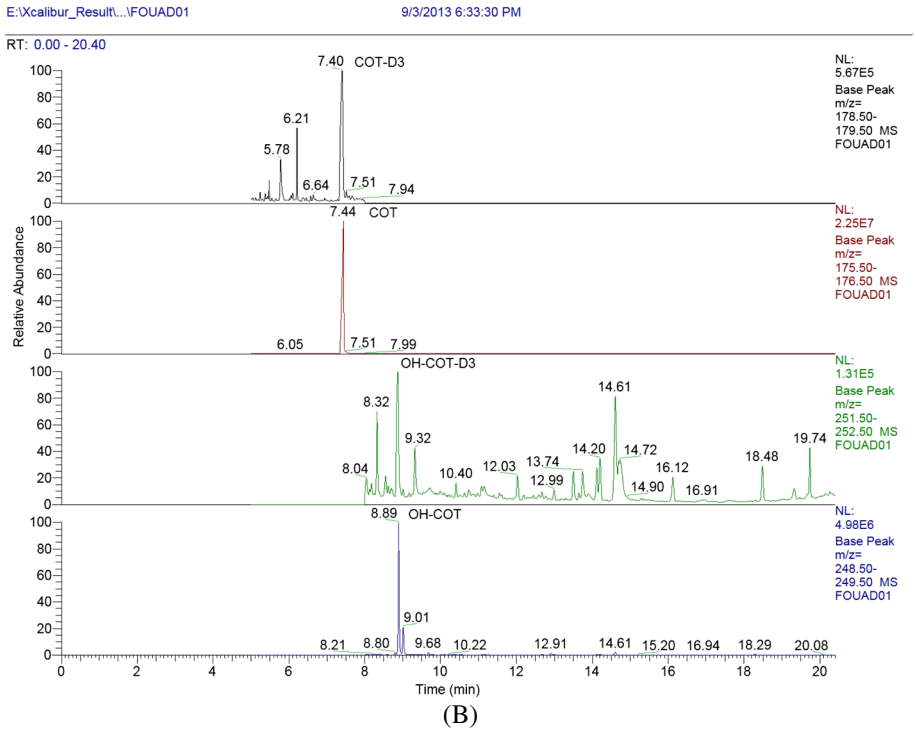
Cotinine and *trans*-3-hydroxycotinine were linear over the range 10–6000 ng/mL with $R^2 > 0.997$ and $R^2 > 0.999$, respectively. The LOD and LOQ of cotinine were 0.06 and 10 ng/mL, respectively. The LOD and LOQ of *trans*-3-hydroxycotinine were 0.02 and 10 ng/mL, respectively.

Precision and accuracy

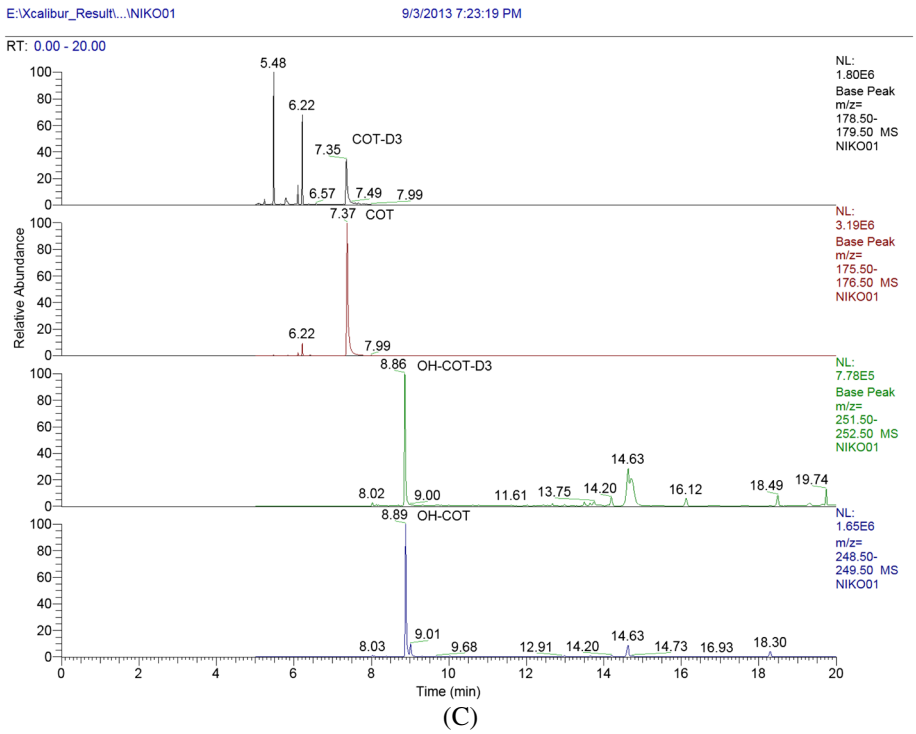
Precision (represented by RSD) and accuracy (represented by percentage deviation) of the method results are given in Table 1. Accuracy for COT ranged from 0.98 to 5.28% and the precision ranged from 1.24 to 8.78%. Accuracy for OH-COT ranged from −2.66 to 3.72% and the precision ranged from 3.15 to 7.07%.

Recovery

Recoveries for the analytes (n = 6) are shown in Table 2. Mean recoveries for cotinine and *trans*-3-hydroxycotinine ranged from



(B)



(C)

Figure 1. (Continued)

77.7 to 89.1%, and from 75.4 to 90.2%, respectively, for the three QC concentrations of 100, 1000 and 6000 ng/mL.

Stability

Analytes concentrations in capped GC autosampler vials were stable for up to 48 h.

Discussion

If some articles were published describing LC/MS methods regarding nicotine and its metabolites, only two publications described the quantification of cotinine and *trans*-3-hydroxycotinine in urine by GCMS method (Shulgin *et al*., 1992; Ji *et al*., 1999). The most recent article dated from 1999 and used liquid-liquid extraction procedure using volatile and

Table 1. Precision and accuracy for cotinine and *trans*-3-hydroxycotinine at three concentration levels in urine samples

	Intraday			Interday		
Nominal concentration (ng/mL)	Observed concentration (ng/mL)	Precision (%)	Accuracy (%)	Observed concentration (ng/mL)	Precision (%)	accuracy (%)
Cotinine						
50	51.1 ± 1.5	2.9	2.2	52.6 ± 4.5	8.6	5.3
600	605.9 ± 7.5	1.2	0.9	622.4 ± 54.6	8.8	3.7
5000	5128.3 ± 292.5	5.8	2.6	5155.1 ± 336.6	6.5	3.1
Trans-3-hydroxycotinine						
50	48.7 ± 1.9	4.1	-2.7	51.9 ± 1.7	3.4	3.7
600	618.1 ± 23.9	3.9	3.0	589.2 ± 37.5	6.4	-1.8
5000	5102.9 ± 160.7	3.2	2.1	5125.0 ± 362.5	7.1	2.5

Table 2. Recoveries of cotinine and *trans*-3-hydroxycotinine from urine (n = 6)

Analyte	Expected concentration (ng/mL)	Mean peak ratio of extracted samples	Mean peak ratio of nonextracted samples	Recovery (%)
Cotinine				
	100	0.9	1.0	89.1
	1000	8.8	11.4	77.7
	6000	46.3	52.5	88.2
Trans-3-hydroxycotinine.				
	100	0.6	0.6	90.2
	1000	4.4	5.1	87.8
	6000	24.1	32.0	75.4

toxic solvents as n-butyl acetate and dichloromethane (Ji *et al*., 1999). A recent GC method was developed for determination of urinary cotinine in active and passive smokers using liquid–liquid extraction (Malafatti *et al*., 2010). The sample volume of urine was high with 5.0 mL, and intra- and inter-day assay standard relative deviations were up to 14.2%. Our SPE method increased significantly the throughput and reduced the manual handling tasks associated with the liquid–liquid extraction method. However, capping SPE cartridges for use on the automated system is physically demanding and thus becomes a manual handling hazard. In Ji *et al*. (1999), the inter-assay CVs were 4.2–12% for OH-COT and 10–12% for COT. In our method, inter- and intra-assay were <9% for both analytes. This analytical method for the simultaneous measurement of cotinine and *trans*-3-hydroxycotinine in urine will be used to monitor tobacco smoking as pregnant women and will permit the usefulness of *trans*-3-hydroxycotinine as a specific biomarker of tobacco exposure to be determined.

Conclusion

Our report describes a rapid, sensitive, accurate and simple method for simultaneous quantification of urinary cotinine and *trans*-3-hydroxycotinine. SPE procedure was simplified by automating the extraction of the analytes, which are the critical steps of analysis methods. The applicability of the assay should be useful for routine monitoring of active smoking and exposure to environmental tobacco smoke. *Trans*-3-hydroxycotinine requires additional data to provide a useful indicator as a biomarker for tobacco smoke and to investigate differences in the metabolism of COT and OH-COT.

References

Bao Z, He XY, Ding X, Prabhu S and Hong JY. Metabolism of nicotine and cotinine by human cytochrome P450 2A13. *Drug Metabolism and Disposition* 2005; **33**: 258–261.

Baumann F, Regenthal R, Burgos-Guerrero IL, Hegerl U and Preiss R. Determination of nicotine and cotinine in human serum by means of LC-MS. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* 2010; **878**: 107–111.

Benowitz NL. Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiology Review* 1996; **18**: 188–204.

Benowitz NL and Jacob PJ. Nicotine chemistry metabolism, kinetics and biomarkers. *Handbook of Experimental Pharmacology*. Springer: Berlin, 2009; **192**: 29–60.

Benowitz NL, Hukkanen J and Jacob P. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handbook of Experimental Pharmacology*. Springer: Berlin, 2009; **192**: 29–60.

Chao MR, Wang CJ, Yang HH, Chang LW and Hu CW. Rapid and sensitive quantification of urinary N7-methylguanine by isotope-dilution liquid chromatography–electrospray ionization tandem mass spectrometry with on-line solid-phase extraction. *Rapid Communications in Mass Spectrometry* 2005; **19**: 2427–2432.

Dobrinas M, Choong E, Noetzli M, Cornuz J, Ansermot N and Eap CB. Quantification of nicotine, cotinine, *trans*-3-hydroxycotinine and varenicline in human plasma by a sensitive and specific UPLC-tandem mass spectrometry procedure for a clinical study on smoking cessation. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* 2011; **879**: 3574–3582.

Hammond D and O'Connor RJ. Constituents in tobacco and smoke emissions from Canadian cigarettes. *Tobacco Control* 2008; **17**(suppl. 1): i24–i31.

Heavner DL, Richardson JD, Morgan WT and Ogden MW. Validation and application of a method for the determination of nicotine and five major metabolites in smokers' urine by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Biomedical Chromatography* 2005; **19**:312–328.

Hoofnagle AN, Laha TJ, Rainey PM and Sadrzadeh SMH. Specific detection of anabasine, nicotine, and nicotine metabolites in urine by liquid chromatography–tandem mass spectrometry. *American Journal of Clinical Pathology* 2006; **126**: 880–887.

Hu CW, Chang YZ, Wang HW and Chao MR. High-throughput simultaneous analysis of five urinary metabolites of areca nut and tobacco alkaloids by isotope-dilution liquid chromatography–tandem mass spectrometry with online solid phase extraction. *Cancer Epidemiological Biomarkers Prevention* 2010; **19**: 2570–2581.

Jacob PJ, Yu L, Duan M, Ramos L, Yturralde O and Benowitz NL. Determination of the nicotine metabolites cotinine and *trans*-3-hydroxycotinine in biologic fluids of smokers and non-smokers using liquid chromatography–tandem massspectrometry: biomarkers for tobacco smoke exposure and for phenotyping cytochrome P450 2A6 activity. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* 2011; **879**: 267–276.

Ji AJ, Lawson GM, Anderson R, Dale LC, Croghan IT and Hurt RD. A new gas chromatography-mass spectrometry method for simultaneous determination of total and free *trans*-3′-hydroxycotinine and cotinine in the urine of subjects receiving transdermal nicotine. *Clinical Chemistry* 1999; **45**: 85–91.

Kataoka H, Inoue R, Yagi K and Saito K. Determination of nicotine, cotinine, and related alkaloids in human urine and saliva by automated in-tube solid phase microextraction coupled with liquid chromatography–mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2009; **49**: 108–114.

Kim I, Darwin WD and Huestis MA. Simultaneous determination of nicotine, cotinine, norcotinine, and *trans*-3′-hydroxycotinine in human oral fluid using solid phase extraction and gaschromatography–mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* 2005; **814**: 233–40.

Kuhn J, Vollmer T, Martin C, Hendig D and Knabbe C. Fast and sample cleanup-free measurement of nicotine and cotinine by stable isotope dilution ultra-performance liquid chromatography–tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2012; **67**: 137–143.

Malafatti L, Penido Maia P, Coutinho M, Martins G, Pereira Bastos de Siqueira ME and Martins I. Single gas chromatography method with nitrogen phosphorus detector for urinary cotinine determination in passive and active smokers. *Brazilian Journal of Pharmaceutical Sciences* 2010; **46**:769–776.

Marclay F and Saugy M. Determination of nicotine and nicotine metabolites in urine by hydrophilic interaction chromatography–tandem mass spectrometry: potential use of smokeless tobacco products by ice hockey players. *Journal of Chromatography A* 2010; **1217**: 7528–7538.

Meger M, Meger-Kossien I, Schuler-Metz A, Janket D and Scherer G. Simultaneous determination of nicotine and eight nicotine metabolites in urine of smokers using liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* 2002; **778**: 251–261.

Miller EI, Norris HRK, Rollins DE, Tiffany ST and Wilkins DG. A novel validated procedure for the determination of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine by solid-phase extraction coupled with liquid chromatography–electrospray ionization–tandem mass spectrometry. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* 2010; **878**: 725–737.

Moyer TP, Charlson JR, Enger RJ, Dale LC, Ebbert JO, Schroeder DR and Hurt RD. Simultaneous analysis of nicotine, nicotine metabolites and tobacco alkaloids in serum or urine by tandem mass spectrometry with clinically relevant metabolic profiles. *Clinical Chemistry* 2002; **48**: 1460–1471.

Murphy SE, Wickham KM, Lindgren BR, Spector LG and Joseph A. Cotinine and *trans*-3′-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. *Journal of Exposure Science and Environmental Epidemiology* 2013; **23**: 513–518.

Rangiah K, Hwang WT, Mesaros C, Vachani A and Blair IA. Nicotine exposure and metabolizer phenotypes from analysis of urinary nicotine and its 15 metabolites by LC-MS. *Bioanalysis* 2011; **3**: 745–761.

Scheidweiler KB, Shakleya DM and Huestis MA. Simultaneous quantification of nicotine, cotinine, *trans*-3-hydroxycotinine, norcotinine and mecamylamine in human urine by liquid chromatography–tandem mass spectrometry. *Clinical Chimica Acta* 2012; **413**: 978–984.

Shakleya DM and Huestis MA. 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. *Analytical and Bioanalytical Chemistry* 2009a; **395**: 2349–2357.

Shakleya DM and Huestis MA. Simultaneous and sensitive measurement of nicotine, cotinine, *trans*-3-hydroxycotinine and norcotinine in human plasma by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* 2009b; **877**: 3537–3542.

Shulgin AT, Yu L, Benowitz NL and Jacob P. Determination of the nicotine metabolite *trans*-3′-hydroxycotinine in urine of smokers using gas chromatography with nitrogen-selective detection or selected ion monitoring. *Journal of Chromatography* 1992; **583**: 145–154.

Smith CJ and Hansch C. The relative toxicity of compounds in mainstream cigarette smoke condensate. *Food and Chemical Toxicology* 2000; **38**: 637–646.

Song L, Davis W, Abrams SM, Hemiup J, Kazim AL, Cummings KM and Mahoney MC. Sensitive and rapid method for the determination of urinary cotinine in non-smokers: an application for studies assessing exposures to second hand smoke (SHS). *Analytical Chimica Acta* 2005; **545**: 200–208.

St Helen G, Jacob P and Benowitz NL. Stability of the nicotine metabolite ratio in smokers of progressively reduced nicotine content cigarettes. *Nicotine Tobacco Research* 2013; **15**: 1939–1942.

Xu X, Iba MM and Weisel CP. Simultaneous and sensitive measurement of anabasine, nicotine, and nicotine metabolites in human urine by liquid chromatography–tandem mass spectrometry. *Clinical Chemistry* 2004; **50**: 2323–2330.