Journal of Chromatography B, 878 (2010) 725–737

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Eleanor I. Miller[a,*], Hye-Ryun K. Norris[a], Douglas E. Rollins[a], Stephen T. Tiffany[b], Diana G. Wilkins[a]

[a] University of Utah, Center for Human Toxicology, Department of Pharmacology and Toxicology, Salt Lake City, UT 84108, USA
[b] University at Buffalo SUNY, Department of Psychology, 228 Park Hall, Buffalo, NY 14260, USA

ARTICLE INFO

Article history:
Received 17 September 2009
Accepted 10 December 2009
Available online 22 December 2009

Keywords:
Nicotine and nicotine metabolites
Plasma/urine
LC–ESI–MS/MS
7-mg transdermal nicotine patch

ABSTRACT

A novel validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) procedure was developed and fully validated for the simultaneous determination of nicotine-N-β-D-glucuronide, cotinine-N-oxide, *trans*-3-hydroxycotinine, norcotinine, *trans*-nicotine-1′-oxide, cotinine, nornicotine, nicotine, anatabine, anabasine and cotinine-N-β-D-glucuronide in human plasma or urine. Target analytes and corresponding deuterated internal standards were extracted by solid-phase extraction and analyzed by LC–MS/MS with electrospray ionization (ESI) using multiple reaction monitoring (MRM) data acquisition. Calibration curves were linear over the selected concentration ranges for each analyte, with calculated coefficients of determination (R^2) of greater than 0.99. The total extraction recovery (%) was concentration dependent and ranged between 52–88% in plasma and 51–118% in urine. The limits of quantification for all analytes in plasma and urine were 1.0 ng/mL and 2.5 ng/mL, respectively, with the exception of cotinine-N-β-D-glucuronide, which was 50 ng/mL. Intra-day and inter-day imprecision were ≤14% and ≤17%, respectively. Matrix effect (%) was sufficiently minimized to ≤19% for both matrices using the described sample preparation and extraction methods. The target analytes were stable in both matrices for at least 3 freeze–thaw cycles, 24 h at room temperature, 24 h in the refrigerator (4 °C) and 1 week in the freezer (−20 °C). Reconstituted plasma and urine extracts were stable for at least 72 h storage in the liquid chromatography autosampler at 4 °C. The plasma procedure has been successfully applied in the quantitative determination of selected analytes in samples collected from nicotine-abstinent human participants as part of a pharmacokinetic study investigating biomarkers of nicotine use in plasma following controlled low dose (7 mg) transdermal nicotine delivery. Nicotine, cotinine, *trans*-3-hydroxycotinine and trans-nicotine-1′-oxide were detected in the particular sample presented herein. The urine procedure has been used to facilitate the monitoring of unauthorized tobacco use by clinical study participants at the time of physical examination (before enrollment) and on the pharmacokinetic study day.

1. Introduction

According to the Centers for Disease Control and Prevention (CDC), cigarette smoking is the leading cause of preventable death in the US. The CDC estimated that at least 443,000 deaths were attributable to cigarette smoking or exposure to second hand smoke each year between the years 2000 and 2004 [1]. The findings of a report by the US Environmental Protection Agency in 1992 concluded that environmental tobacco smoke (ETS) was a human lung carcinogen and was the cause of death for approximately 3000 non-smokers per year in the US [2]. The results of a national survey by the Substance Abuse and Mental Health Services Administration in 2008 indicated that an estimated 59.8 million people or 23.9% of the population aged 12 or older had smoked cigarettes in the past month [3].

Nicotine is the major constituent of the *Nicotiana tabacum* (tobacco) plant and causes stimulation of the autonomic ganglia in the central nervous system. Specifically, nicotine acts on nicotinic cholinergic brain receptors as well as other parts of the nervous system primarily by releasing or facilitating the production of a variety of neurotransmitters including dopamine, norepinephrine, serotonin, acetylcholine, vasopressin and beta endorphin [4]. When smoked and inhaled into the lungs, nicotine is efficiently absorbed

* Corresponding author at: University of Utah, Center for Human Toxicology, 417 Wakara Way Suite 2111, Salt Lake City, UT 84108, USA.
E-mail address: Eleanor.Miller@utah.edu (E.I. Miller).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.12.018

into the bloodstream and delivered to the brain in less than 20 s, where it interacts with multiple neurotransmitter systems to produce psychoactive effects including reward. Both active smokers and passive smokers absorb nicotine through the skin, mucous membranes in the mouth, nose and by the lungs during smoke inhalation. The use of smokeless tobacco products, transdermal nicotine patches and nicotine gum produce a more gradual and steadier delivery of nicotine to the bloodstream, resulting in a longer time period for the development of tolerance as well as less prominent pharmacological effects.

The nicotine metabolic pathway is complex and involves the formation of a number of Phase I metabolites through oxidation, hydroxylation and *N*-demethylation, and, also Phase II metabolites through conjugation with glucuronic acid (Fig. 1). Furthermore, there are inter-individual variations in rate and pattern of nicotine metabolism. In general, more than 70% of nicotine undergoes C-oxidation by the hepatic enzyme cytochrome P450 2A6 (CYP 2A6) system to form cotinine, its primary metabolite. However, the percent conversion of nicotine to cotinine via this metabolic route has been demonstrated to range between 55% and 92% in healthy cigarette smokers and non-smokers [5]. The same enzyme system is responsible for hydroxylation of cotinine to produce *trans*-3-hydroxycotinine. Nicotine has a short half-life in plasma of 1–2 h [5] however cotinine and *trans*-3-hydroxycotinine have longer half-lives (6–22 h [6] and 4.6–8.3 h [7] respectively) and are therefore considered as potentially more useful biomarkers for the assessment of exposure to nicotine. Microsomal flavin-containing mono-oxygenase enzyme systems are responsible for the oxidation of nicotine to form predominantly the *trans*-nicotine-1′-oxide isomer in humans [8]. The identification of the enzyme system(s) responsible for the oxidation of cotinine to cotinine-N-oxide and also for the demethylation of nicotine in the formation of nornicotine have yet to be identified. Although nornicotine has been identified as a metabolite of nicotine in humans, it is not a unique metabolite as it is present in the tobacco plant itself [9].

Although the prototypical cigarette smoker is characterized as someone who consistently smokes at least 1 pack of cigarettes per day, a large number of current smokers neither smokes daily nor consumes 1 pack of cigarettes on days on which they do smoke. These “low-level” smokers pose an increased health risk as a consequence of the inhalation of tobacco smoke with increased rates of coronary heart disease, myocardial infarction and lung cancer compared with non-smokers [10,11]. Furthermore, for many, low-level smoking initiates a transition period which ultimately leads to the development of nicotine dependence.

The relationship between inhalation of tobacco smoke by both active and passive smokers and serious health conditions such as lung cancer and heart disease has resulted in the need for analytical methods for the determination of nicotine biomarkers in biological samples. These biomarkers of cigarette smoking are frequently used to assess tobacco exposure. In 2002, the Society for Research on Nicotine and Tobacco recommended that biomarkers of cigarette smoking be used in new product and harm-reduction studies and in studies of smoking cessation in selected populations such as adolescents, pregnant women and medical patients with smoking-related diseases [12]. In the context of our on-going clinical study, the identification and quantification of potential nicotine biomarkers will provide useful data on their disposition in plasma collected from nicotine-abstinent human study participants following controlled low dose transdermal nicotine exposure.

A number of quantitative gas chromatography–mass spectrometry (GC/MS) [13–16] and liquid chromatography–tandem mass

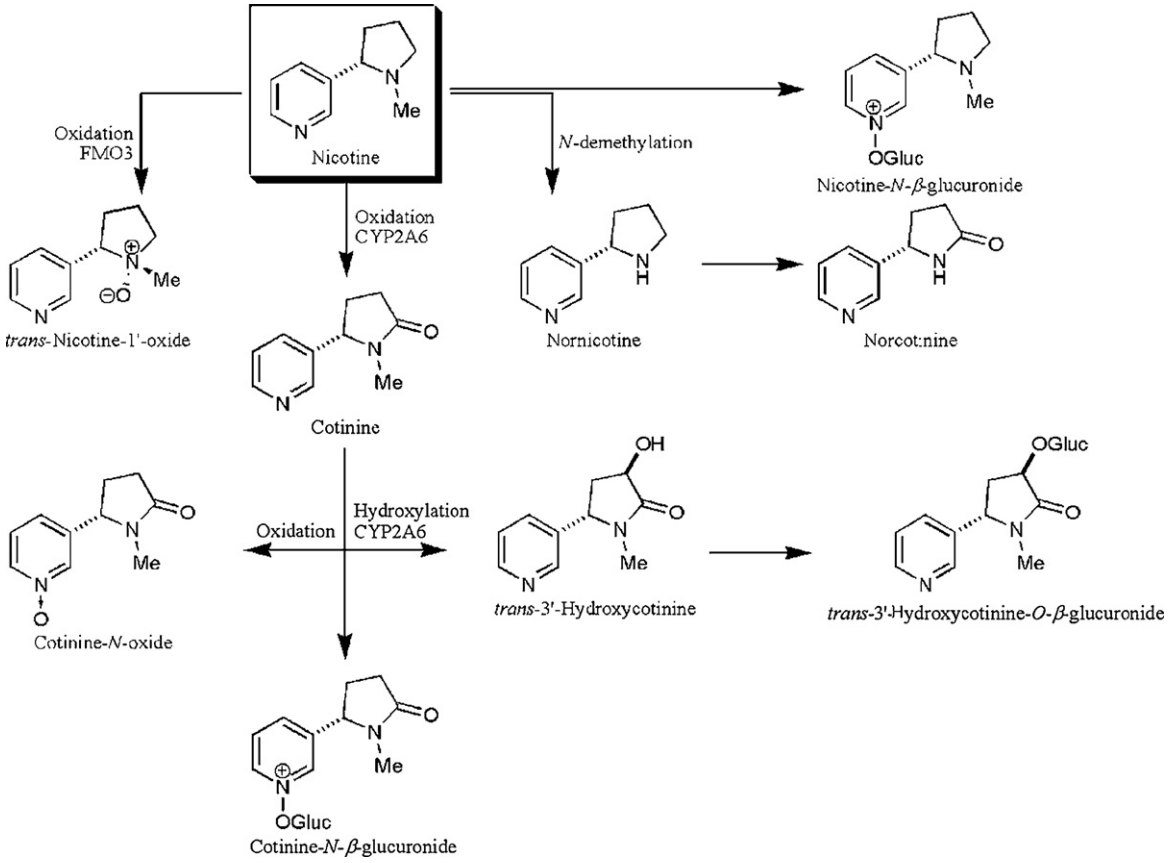

Fig. 1. Nicotine metabolic pathway [8].

spectrometry (LC–MS/MS) [17–23] procedures have been published in recent years involving the determination of nicotine and metabolites in human plasma or urine. Analysis of nicotine alone in biological samples can provide insufficient information due to its short half-life and the dependancy of the analytical result on the time of sampling [24]. It can therefore be more useful to test for nicotine metabolites which have longer half-lives and detection windows compared to nicotine. All of the previously published procedures include at least cotinine, as well as nicotine, in the analysis. The target analytes in these procedures were extracted by mixed mode solid-phase extraction or liquid–liquid extraction prior to analysis.

However, most of the reported procedures only target nicotine and nicotine metabolites produced by the CYP 2A6 enzyme system. A study by Yoshida et al. determined that nicotine metabolism can be impaired as a result of a genetic polymorphism at the CYP 2A6 gene and noted that homozygotes possessing CYP 2A6*4, CYP 2A6*7, CYP 2A6*10, or, heterozygotes possessing a combination of both, demonstrated a loss in CYP 2A6 enzymatic activity for 7% of the test population of 301 subjects [25]. Furthermore, a more recent study by the same group in 2006 found that a combined CYP 2A6 allele frequency either lacking or demonstrating reduced enzymatic activity varies between races, as least prevalent in Caucasian subjects and as most prevalent in Japanese subjects [26]. For those individuals who possess this polymorphism, it may be beneficial to monitor metabolites formed via other enzyme systems or mechanisms, such as nicotine-N-β-glucuronide, cotinine-N-oxide (potentially) and *trans*-nicotine-1′-oxide. Depending on whether or not an individual possesses the CYP 2A6 polymorphism, cotinine-N-β-glucuronide could also potentially be used as a nicotine biomarker, in addition to cotinine, *trans*-3-hydroxycotinine and norcotinine; therefore these analytes are included in our current study for evaluation.

Monitoring the presence of minor tobacco alkaloids, such as anatabine and anabasine, in biological matrices will enhance our interpretation of the extent of an individual's continued tobacco use during participation in smoking cessation programs. Such measurements will distinguish whether an individual's nicotine exposure has occured via active smoking (e.g. cigarette use) or from another route of administration (such as a transdermal nicotine patch).

A study by Jacob et al. determined that the mean anabasine concentration in 13 different commercially available cigarette brands in the US was approximately 11% of the mean anatabine concentration [16]. Furthermore, a relatively high standard deviation (SD 0.039) was calculated in comparison to the mean anabasine concentration determined in these cigarette brands (0.030 mg/g). The standard deviation calculated for anatabine (SD 0.034) was similar to that for anabasine however the mean anatabine concentration was almost 10 times higher (0.271 mg/g) than the mean anabasine concentration; therefore the variability between anatabine concentrations in the different cigarette brands was much lower than determined for anabasine. However, very few published reports have included these analytes in their sample testing procedures [16,17,20,23], and their practical use as biomarkers for determination of active smoking is unknown. Therefore, we included these minor tobacco alkaloid biomarkers in the development of our assay. The inclusion of nornicotine in our proposed plasma procedure will also provide a means of monitoring unauthorized tobacco use prior to transdermal nicotine application. Furthermore, in the context of our on-going clinical study, the identification and quantification of nornicotine will provide useful data on the disposition of nornicotine in biological samples from nicotine-abstinent human participants who have received nicotine via a transdermal nicotine patch (which does not contain nornicotine).

Xu et al. [20] presented a LC–MS/MS method for the quantification of nicotine, cotinine, *trans*-3-hydroxycotinine, cotinine-N-oxide, nicotine-N-oxide, nornicotine and anabasine in urine samples collected from smokers, however, this method did not include anatabine or conjugated cotinine or nicotine as we present in both our plasma and urine procedures. In addition, the authors did not specify which nicotine-N-oxide standard they used in the analysis and there is a single peak for this compound in their reported method. Our procedures include the use of a mixture of *trans*-nicotine-1′-oxide diastereomers, which we have been able to partially separate on the same LC column, and very similar mobile phase system to those used by these authors. Feng et al. [27] reported an LC–MS/MS procedure for the direct analysis of conjugated cotinine and nicotine, in addition to other CYP 2A6 nicotine metabolites, in smokers' urine. However the authors did not include any nicotine metabolites produced via other enzyme systems or mechanisms. Heavner et al. [22] proposed a LC–MS/MS method for the same range of analytes in smokers' urine samples as Feng et al.; however the concentrations of conjugated cotinine and nicotine were calculated indirectly, following cleavage of the conjugate with β-glucuronidase [22]. One major concern with the indirect analysis of conjugates includes the use of unconjugated calibrators and QCs with which to calculate the unconjugated drug released after hydrolysis and also the potential variability of enzyme activity in conjugated samples. The analytical procedures proposed in our current study accommodate the direct analysis of cotinine and nicotine glucuronide conjugates. Hoofnagle et al. [23] presented an LC–MS/MS method for the simultaneous quantification of nicotine, cotinine, nornicotine and anabasine using a very simple centrifugal urinary filtration procedure followed by direct injection [23]. The authors' selection of nicotine metabolites includes only cotinine, which is formed by the CYP 2A6 enzyme system. Although nornicotine is included in the method, with a sensitive limit of quantification (LOQ) (0.03 ng/mL), it is not a unique metabolite of nicotine, as mentioned previously. The observation of nornicotine levels as low as the LOQ could have been present from exposure to nornicotine found in the leaves of the tobacco plant, produced as a result of active smoking, or a combination of both.

The work presented herein describes a novel procedure for the simultaneous extraction and quantification of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine using solid-phase extraction coupled with liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS). These analytical procedures have been applied to the determination of nicotine and metabolites in plasma samples which were collected as part of a pharmacokinetic study investigating potential nicotine biomarkers in nicotine-abstinent human participants receiving a controlled low dose (7 mg) of transdermal nicotine, and in "baseline" urine samples for the purpose of monitoring unauthorized tobacco use by clinical study participants both before enrollment onto the study and on the pharmacokinetic during study day.

2. Experimental

2.1. Plasma/urine in method development and validation

Analyte-free human plasma lots used in method development and validation were obtained from BioChemed (Winchester, Virginia, USA). The lots were then extracted, analyzed and confirmed to be negative (<limit of detection (LOD)) before preparation of calibrator and quality control (QC) samples. Analyte-free urine used in method development and validation was collected from nicotine-abstinent members of laboratory personnel within the Center for Human Toxicology at the University of Utah.

2.2. Biological samples for clinical study

Clinical samples were collected as part of an Institutional Review Board-approved study (IRB #21414, University of Utah) investigating potential nicotine biomarkers in nicotine-abstinent participants following the application of a 7-mg transdermal nicotine patch (Nicotine Transdermal System®, Novartis®, Basel, Switzerland) for 4 h.

Blood samples collected during the 24 h pharmacokinetic study period were stored in 6 mL Vacutainer® heparin venous blood collection tubes containing 86 USP sodium heparin units (BD Diagnostics, Franklin Lakes, NJ, USA) and refrigerated at 4 °C at the Center for Clinical and Translational Science (CCTS) at the University of Utah hospital. On completion of the 24 h blood draw, the samples were transferred to the Center for Human Toxicology at the University of Utah where they were centrifuged, on arrival, for 10 min at 1100 × *g* to separate the plasma component of the blood. The plasma supernatants were transferred to clean silanized 16 mm × 100 mm glass test tubes and subsequently stored in the freezer at −20 °C. These samples were analyzed after less than 1 week of freezer storage.

Urine samples were collected from potential study participants on the day of the physical examination (before enrollment onto the study) and before application of the transdermal nicotine patch on the pharmacokinetic study day (maximum of 2 weeks after the physical examination). Urine collection cups (4 oz) were obtained from Medegen Medical Products (Gallaway, TN, USA).

2.3. Reference standards, chemicals and reagents

The following reference standards and deuterated internal standards were obtained from Toronto Research Chemicals (North York, Canada): cotinine N-β-D-glucuronide (COT GLUC) and cotinine-d3 N-β-D-glucuronide (COT GLUC-d3); nicotine-N-(4-deoxy-4,5-didehydro)-β-D-glucuronide (NIC GLUC) and nicotine-N-(4-deoxy-4,5-didehydro)-β-D-glucuronide-methyl-d3 (NIC GLUC-d3); (S)-cotinine N-oxide (CNO) and (R,S)-cotinine-N-oxide-methyl-d3 (CNO-d3); trans-3′-hydroxycotinine (3HC) and trans-3′-hydroxycotinine-d3 (3-HC-d3); (R,S)-norcotinine (NCOT) and (R,S)-norcotinine pyridyl-d4 (NCOT-d4); (1′S, 2′S)-nicotine 1′-oxide and (1′R, 2′S)-nicotine-1′-oxide mixture (NNO) and (±)-trans nicotine-1′-oxide-methyl-d3 (NNO-d3); (R,S)-nornicotine (NNIC) and (R,S)-nornicotine-d4 (NNIC-d4); (R,S)-anatabine (AT) and (R,S)-anatabine-2,4,5,6-d4 (AT-d4); (R,S)-anabasine (AB) and (R,S)-anabasine-2,4,5,6-d4 (AB-d4). (−)-Nicotine (NIC) hydrogen tartrate salt (≥98%) was obtained from Sigma (St Louis, MO). (−)-Cotinine (COT), (±)-cotinine-d3 (COT-d3) and (±)-nicotine-d3 (NIC-d3) were obtained from Cerilliant (Austin, TX). Solid-phase extraction cartridges (Oasis® HLB and Oasis® MCX (60 mg, 3 mL)) were obtained from Waters (Milford, MA). HPLC grade methanol was obtained from Honeywell Burdick & Jackson (Morristown, NJ). Ammonium acetate and glacial acetic acid were obtained from Spectrum (Gardena, CA). Trichloroacetic acid, concentrated formic acid and concentrated ammonium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA). All chemicals and reagents were HPLC grade (≥99% purity).

2.4. Calibrator and quality control solutions

A deuterated internal standard working solution was prepared in methanol at 1 μg/mL and contained COT GLUC-d3, NIC GLUC-d3, CNO-d3, 3-HC-d3, NCOT-d4, NNO-d3, NNIC-d4, NIC-d3, COT-d3, AT-d4 and AB-d4. The working deuterated internal standard solution was stored in the freezer at −20 °C until required for an analytical run.

Three calibrator working solutions were prepared in methanol at concentrations of 10 μg/mL, 1 μg/mL and 0.1 μg/mL for NIC GLUC, CNO, 3HC, NCOT, NNO, NIC hydrogen tartrate salt (weight corrected for nicotine), NNIC, COT, AT and AB (preparation of COT GLUC calibrators involved the two higher concentrations only). The NIC hydrogen tartrate salt was used as it was determined to be more stable in solution than nicotine free base. Separate methanolic working solutions were prepared for quality control samples at the same concentrations as the calibrator working solutions. Due to the unavailability of different sources and lot numbers of sufficient purity (>99%) for these compounds, the same lot numbers were used to prepare both calibrator and QC working solutions, however, they were prepared by two separate analysts. All working solutions were stored in the freezer at −20 °C until required for an analytical run.

Calibrator and QC samples were prepared in analyte-free plasma or urine on a batch to batch basis. Table 1 provides the calibrator and QC concentrations used for each analyte. In some cases, a higher concentration of analyte was used for the urine low and medium QC levels compared to the plasma. This was a result of the need for a higher LOQ in urine due to the absence of a second MRM transition with a signal to noise (S/N) ≥10 at the lower concentration plasma equivalents.

2.5. Sample preparation and extraction

An aliquot of plasma (1 mL) or urine (1 mL) sample, calibrator or QC was fortified with 50 μL of 1 μg/mL deuterated internal standard solution to produce a final concentration of 50 ng/mL. In the preparation of plasma samples, to aid in matrix clean-up, 1 mL of 10% aqueous trichloroacetic acid was added, the tube contents vortex mixed and centrifuged for 10 min at 1100 × *g*. Urine samples were prepared and extracted according to a previously published

Table 1
Calibrator and quality control concentrations.

Analyte	Plasma and urine calibrators (ng/mL)	Quality control concentrations (ng/mL)					
		Low plasma	Low urine	Medium plasma	Medium urine	High plasma	High urine
NIC GLUC	1.0*, 2.5, 5.0, 7.5, 10, 25, 50	5.0	5.0	25	25	45	45
CNO	1.0*, 2.5, 5.0, 7.5, 10, 25, 50	5.0	5.0	25	25	45	45
3-HC	1.0*, 2.5, 5, 7.5 10, 25, 50, 75, 100	1.0	10	10	50	100	100
NCOT	1.0*, 2.5, 5.0, 7.5, 10, 25, 50	5.0	5.0	25	25	45	45
NNO	1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100	1.0	1.0	10	10	100	100
COT	1.0*, 2.5, 5, 7.5, 10, 25, 50, 75, 100	1.0	10	10	50	100	100
NNIC	1.0*, 2.5, 5.0, 7.5, 10, 25, 50	5.0	5.0	25	25	45	45
NIC	1.0*, 2.5, 5.0, 7.5, 10, 25, 50	5.0	5.0	25	25	45	45
AT	1.0*, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100	1.0	10	10	50	100	100
AB	1.0*, 2.5, 5.0, 7.5, 10, 25, 50	5.0	5.0	25	25	45	45
COT GLUC	50, 75, 100, 200, 400, 500	75	75	200	200	400	400

* Plasma calibrator only.

method [27]. Specifically, urine was acidified with 1.5 mL of 5 mM aqueous ammonium formate (pH 2.5), followed by vortex mixing. The acidified plasma supernatant and urine were then subjected to solid-phase extraction (SPE) using a combination of Oasis® HLB and Oasis® MCX mixed mode cartridges (Waters® Corporation, Milford, MA). The SPE cartridges for both plasma and urine were conditioned with 2 mL methanol followed by 2 mL 10% aqueous trichloroacetic acid for plasma and 2 mL of 5 mM aqueous ammonium formate (pH 2.5) for urine. The samples were loaded onto the cartridges and the target analytes were subsequently eluted with 2 mL methanol containing 5% concentrated aqueous ammonium hydroxide (v/v). 100 μL of 1% concentrated aqueous hydrochloric acid in methanol (v/v) was added prior to evaporation of the eluant. Extracts were evaporated to dryness under a stream of air at 40 °C using a Zymark Turbovap® LV Evaporator. Extracted plasma and urine residues were reconstituted in 150 μL and 130 μL respectively of initial mobile phase conditions (10 mM ammonium acetate + 0.001% formic acid (A) (~pH 4.97): methanol (B) (85:15; v/v)).

2.6. Liquid chromatography–tandem mass spectrometry conditions

Liquid chromatography was conducted using an Acquity UPLC® system (Waters®, Milford, MA). Chromatographic separation was achieved using a Discovery® HS F5 HPLC column (100 mm × 4.6 mm, 3 μm, Supelco®, Bellefonte, PA) with a gradient system consisting of 10 mM ammonium acetate with 0.001% formic acid (pH 4.97) (A), and methanol (B) at a flow rate of 0.6 mL/min. The initial mobile phase condition was 15% B which was increased linearly to 76% after 11 min, then decreased back to the initial mobile phase condition of 15% B after 11.6 min and held for 3.4 min to re-equilibrate the LC column (total chromatographic run time was 15 min). The retention time (tR) for each analyte is given in Table 2.

Mass spectrometric analysis was conducted using a Quattro Premier XE™ triple quadrupole mass spectrometer (Waters ® Corporation, Milford, MA) with MassLynx™ v 4.1 software. The mass spectrometer was operated in electrospray positive mode using MRM data acquisition. Two MRM transitions were monitored for each analyte with the exception of COT GLUC (which produced only one fragment ion). The following ESI conditions were applied: capillary voltage 3.25 kV; source temperature 100 °C; desolvation temperature 350 °C; desolvation gas (nitrogen) 600 L/h; cone gas (nitrogen) 50 L/h; collision cell pressure (argon) $7.38e^{-3}$ mbar; and collision gas flow rate 0.35 mL/min. Analyte-specific cone voltages, collision energies and MRM transitions are provided in Table 2. Confirmation of analyte identification and reporting criteria for positive samples included: peak shape, tR within 2% of corresponding deuterated analogue and peak area ratios of analyte quantification ion to analyte confirmation ion within ±20% of the positive control.

Table 2
Liquid chromatography–electrospray ionization–tandem mass spectrometry parameters.

Analyte	Plasma tR (min)	Plasma tR SD (min)	Urine tR (min)	Urine tR SD (min)	Cone voltage (V)	Collision energy (AU)	MRM transitions[a]
COT GLUC	2.03	0.09	2.06	0.03	20	21	353.3 → 177.2*
COT GLUC-d3	2.03	0.09	2.06	0.02	20	21	356.3 → 180.2*
NIC GLUC	3.07	0.04	3.11	0.08	15	30	321.2 → 163.0
							321.2 → 83.9
NIC GLUC-d3	3.07	0.03	3.07	0.10	15	30	324.3 → 166.1
							324.3 → 86.9
CNO	3.62	0.03	3.65	0.04	25	30	193.2 → 96.0
							193.2 → 98.1
CNO-d3	3.60	0.03	3.62	0.11	25	30	196.4 → 96.0
							196.4 → 101.2
3-HC	4.83	0.02	4.83	0.04	25	32	193.1 → 79.8
							193.1 → 85.9
3-HC-d3	4.82	0.02	4.83	0.04	25	32	196.1 → 79.8
							196.1 → 88.9
NCOT	5.37	0.04	5.37	0.04	25	35	163.0 → 79.8
							163.0 → 83.8
NCOT-d4	5.33	0.04	5.34	0.03	25	35	167.0 → 83.9*
NNO[b]	6.16	0.21	6.38	0.08	30	27	179.0 → 129.9
							179.0 → 116.8
NNO-d3	6.19	0.13	6.42	0.07	30	27	182.0 → 129.9
							182.0 → 116.8
COT	6.46	0.06	6.44	0.04	25	36	177.2 → 79.9
							177.2 → 97.9
COT-d3	6.44	0.05	6.41	0.04	25	36	180.1 → 79.9
							180.1 → 100.9
NNIC	6.79	0.65	6.30	0.15	20	32	149.0 → 79.9
							149.0 → 129.9
NNIC-d4	6.81	0.64	6.28	0.14	20	32	153.0 → 83.9
							153.0 → 134.0
NIC	7.23	0.78	6.79	0.15	15	30	163.2 → 130.0
							163.2 → 116.9
NIC-d3	7.22	0.75	6.74	0.12	15	30	166.1 → 129.9
							166.1 → 116.9
AT	7.80	0.77	7.61	0.62	20	28	161.1 → 144.0
							161.1 → 116.9
AT-d4	7.78	0.76	7.59	0.62	20	28	165.1 → 148.0
							165.1 → 121.0
AB	8.72	0.93	8.09	0.20	20	40	163.1 → 130.0
							163.1 → 116.9
AB-d4	8.71	0.89	7.93	0.16	20	40	167.2 → 134.0
							167.2 → 122.0

* Only 1 product ion produced on fragmentation.
[a] The quantification ion MRM transition for each analyte is given in the upper row.
[b] tR calculated for the predominant diastereomer.

Table 3
LOD, LOQ, recovery and matrix effect for plasma.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Total extraction recovery (%)* (n = 5)		Matrix effect (%)* (n = 5)		
			Low	Medium	High	Low	High
NIC GLUC	0.25	1.0	65 (9.4)	58 (14)	59 (7.8)	84 (11)	99 (4.6)
CNO	0.25	1.0	74 (2.9)	73 (3.3)	66 (2.3)	105 (3.2)	91 (5.6)
3-HC	0.25	1.0	78 (2.5)	65 (6.8)	63 (8.0)	97 (4.3)	110 (8.8)
NCOT	0.75	1.0	67 (8.2)	67 (4.5)	60 (3.6)	114 (2.4)	101 (8.2)
NNO	0.25	1.0	88 (4.7)	67 (8.1)	71 (7.8)	108 (20)	98 (12)
COT	0.25	1.0	76 (3.6)	67 (8.8)	79 (9.4)	99 (2.9)	95 (10)
NNIC	0.25	1.0	84 (6.1)	76 (4.5)	69 (2.5)	99 (4.3)	104 (4.8)
NIC	0.75	1.0	79 (6.0)	85 (4.1)	84 (3.6)	119 (16)	103 (9.9)
AT	0.50	1.0	62 (7.5)	52 (6.7)	57 (7.7)	104 (8.6)	106 (11)
AB	0.75	1.0	68 (8.3)	64 (7.6)	54 (3.7)	112 (2.4)	99 (12)
COT GLUC	25	50	62 (3.8)	70 (6.1)	82 (6.9)	112 (13)	102 (12)

* The number provided in parentheses after the reported % total extraction recovery and % matrix effect is the % relative standard deviation (% RSD).

Table 4
LOD, LOQ, recovery and matrix effect for urine.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Total extraction recovery (%)* (n = 5)			Matrix effect (%)* (n = 5)	
			Low	Medium	High	Low	High
NIC GLUC	1.0	2.5	85 (11)	98 (4.5)	93 (4.9)	109 (9.9)	106 (4.6)
CNO	1.0	2.5	82 (12)	88 (3.9)	83 (6.8)	119 (4.1)	114 (3.1)
3-HC	1.0	2.5	107 (5.1)	93 (1.9)	89 (5.8)	93 (5.1)	101 (13)
NCOT	1.0	2.5	77 (2.5)	84 (4.6)	86 (6.7)	107 (5.2)	106 (5.3)
NNO	1.0	1.0	89 (3.0)	100 (6.6)	82 (4.1)	86 (12)	119 (7.4)
COT	1.0	2.5	87 (3.1)	88 (1.7)	107 (4.1)	119 (6.0)	105 (12)
NNIC	1.0	2.5	84 (3.4)	90 (2.7)	86 (4.4)	101 (5.1)	100 (7.8)
NIC	1.0	2.5	113 (8.3)	118 (2.5)	98 (2.8)	103 (8.9)	98 (5.0)
AT	1.0	2.5	107 (2.2)	88 (2.9)	92 (3.1)	82 (9.2)	100 (12.6)
AB	1.0	2.5	81 (8.9)	88 (2.0)	90 (4.6)	97 (4.3)	96 (2.1)
COT GLUC	25	50	51 (11)	59 (7.9)	63 (6.1)	112 (5.1)	113 (2.3)

* The number provided in parentheses after the reported % total extraction recovery and % matrix effect is the % relative standard deviation (% RSD).

Table 5
Stability in human plasma.

Analyte	Target concentration (ng/mL)	24 h at RT (%)*,[a]	24 h at 4 °C (% of target concentration)*	1 week in freezer at −20 °C (% of target concentration)*	Autosampler 72 h at 4 °C (% of target concentration)*	Freeze–thaw cycles (% of target concentration)*
NIC GLUC	5.0	118 (3.6)	109 (6.2)	90 (9.4)	84 (4.4)	92 (17.9)
	45	109 (6.3)	108 (6.4)	99 (1.2)	82 (7.0)	94 (9.6)
CNO	5.0	95 (4.8)	110 (8.7)	86 (5.0)	89 (2.5)	93 (8.3)
	45	83 (7.1)	87 (4.0)	82 (1.8)	80 (4.8)	81 (1.2)
3-HC	1.0	119 (0.20)	94 (6.1)	81 (6.0)	82 (2.6)	119 (4.5)
	100	109 (10)	83 (3.1)	95 (3.2)	80 (5.5)	94 (1.6)
NCOT	5.0	97 (3.7)	111 (7.0)	87 (6.0)	84 (4.1)	89 (7.3)
	45	95 (1.7)	91 (4.9)	87 (2.8)	80 (3.0)	89 (4.2)
NNO	1.0	119 (1.0)	81 (3.7)	111 (6.7)	83 (4.4)	106 (8.0)
	100	102 (5.2)	90 (2.7)	93 (4.2)	82 (3.9)	99 (9.8)
COT	1.0	90 (5.7)	107 (10)	84 (5.3)	102 (5.2)	100 (4.0)
	100	81 (4.1)	116 (3.4)	87 (5.0)	82 (3.9)	86 (5.3)
NNIC	5.0	81 (2.4)	92 (5.4)	80 (2.3)	81 (8.4)	102 (5.5)
	45	82 (3.0)	100 (3.5)	81 (4.4)	84 (4.1)	80 (2.9)
NIC	5.0	94 (6.5)	97 (6.4)	98 (11)	91 (3.2)	100 (3.2)
	45	94 (6.3)	100 (1.6)	94 (2.8)	89 (3.0)	86 (3.7)
AT	1.0	101 (6.3)	94 (5.3)	108 (12)	98 (12)	104 (1.1)
	100	102 (3.2)	93 (1.9)	93 (4.9)	89 (3.7)	82 (6.3)
AB	5.0	81 (2.2)	92 (4.0)	83 (13)	82 (3.8)	88 (4.8)
	45	84 (3.7)	89 (3.1)	92 (8.1)	86 (7.2)	83 (3.3)
COT GLUC	75	112 (5.1)	99 (11.2)	86 (9.8)	94 (11)	112 (1.5)
	400	112 (9.6)	107 (4.4)	110 (8.0)	113 (8.6)	104 (3.5)

[a] RT: room temperature.
* The number provided in parentheses after the reported % total extraction recovery and % matrix effect is the % relative standard deviation (% RSD).

Table 6
Stability in human urine.

Analyte	Target concentration (ng/mL)	24 h at RT (%)[*,a]	24 h at 4 °C (% of target concentration)[*]	1 week in freezer at −20 °C (% of target concentration)[*]	Autosampler 72 h at 4 °C (% of target concentration)[*]	Freeze–thaw cycles (% of target concentration)[*]
NIC GLUC	5.0	92(8.5)	81(8.3)	96(6.3)	96(4.5)	81(6.7)
	45	85(3.7)	97(4.7)	95(2.5)	104(4.7)	81(2.5)
CNO	5.0	117(6.5)	120(6.4)	91(11)	117(4.4)	80(3.9)
	45	103(7.3)	117(2.7)	83(2.7)	112(5.1)	120(5.1)
3-HC	10	88(6.4)	106(5.2)	97(2.9)	86(9.8)	104(4.5)
	100	86(2.9)	92(2.8)	88(4.1)	85(5.6)	90(5.2)
NCOT	5.0	81(3.8)	86(5.1)	90(2.6)	86(2.3)	84(4.9)
	45	93(4.7)	100(3.2)	89(4.6)	91(0.3)	80(5.5)
NNO	1.0	80(6.1)	93(8.3)	100(7.5)	90(2.6)	105(5.4)
	100	82(3.3)	98(4.8)	104(11)	93(5.9)	81(16)
COT	10	105(1.5)	115(5.3)	101(2.5)	95(3.1)	105(2.5)
	100	95(3.7)	101(5.9)	83(2.7)	96(1.5)	81(6.5)
NNIC	5.0	87(3.9)	87(4.2)	85(4.6)	92(6.7)	95(5.4)
	45	81(4.4)	89(1.7)	80(2.2)	84(7.2)	82(3.4)
NIC	5.0	111(4.9)	105(7.3)	98(4.9)	93(3.8)	82(17)
	45	105(3.0)	106(2.8)	101(2.3)	85(1.9)	89(9.9)
AT	10	96(6.4)	117(4.7)	100(4.0)	93(6.6)	90(7.1)
	100	85(2.6)	96(1.6)	85(3.2)	90(3.8)	80(5.7)
AB	5.0	84(5.6)	101(8.2)	82(4.5)	109(13)	102(7.1)
	45	87(3.0)	96(4.2)	82(5.8)	81(2.5)	86(2.2)
COT GLUC	75	92(5.9)	95(11)	96(3.7)	99(2.0)	113(15)
	400	80(6.5)	84(6.6)	97(3.3)	93(2.4)	97(13)

[a] RT: room temperature.
[*] The number provided in parentheses after the reported % total extraction recovery and % matrix effect is the % relative standard deviation (% RSD).

3. Method validation

The specificity of the method was assessed by the analysis of analyte-free human plasma and urine samples from six individuals that had been confirmed as negative (<LOD) for nicotine and metabolites. Each plasma and urine sample was extracted and analyzed in triplicate to determine the presence of any potential interference from endogenous plasma matrix components.

Potential carry-over between LC injections was investigated by analysis of an analyte-free plasma or urine sample fortified with deuterated internal standard after the analysis of the highest calibrator. Carry-over was determined based on the analyte iden-

Table 7
Intra-assay imprecision and accuracy for plasma and urine ($n = 5$).

Analyte	Target concentration (ng/mL)	Observed concentration ± standard deviation (ng/mL)		Imprecision (%RSD)		Accuracy (%)	
		Plasma	Urine	Plasma	Urine	Plasma	Urine
NIC GLUC	5.0	5.2 ± 0.30	5.2 ± 0.50	6.5	8.8	103	103
	25	25 ± 2.3	23 ± 0.80	9.2	3.6	100	91
	45	41 ± 2.6	46 ± 3.2	6.4	7.0	91	102
CNO	5.0	4.4 ± 0.40	4.1 ± 0.20	8.4	5.8	88	82
	25	21 ± 2.1	21 ± 0.50	10	2.4	84	83
	45	39 ± 3.9	37 ± 1.8	10	4.7	87	83
3-HC	1.0P, 10U[*]	1.0 ± 0.15	10 ± 0.60	15	5.9	100	100
	10P, 50U[*]	9.7 ± 0.70	51 ± 1.4	6.8	2.8	97	103
	100	92 ± 5.1	103 ± 4.1	5.5	4.0	92	103
NCOT	5.0	4.6 ± 0.60	4.3 ± 0.20	13	4.5	92	108
	25	23 ± 1.6	21 ± 0.60	6.9	2.6	90	84
	45	42 ± 2.0	38 ± 2.7	4.8	7.3	93	84
NNO	1.0	0.83 ± 0.070	0.90 ± 0.040	8.1	4.9	83	90
	10	11 ± 0.20	9.4 ± 0.60	2.7	6.0	106	94
	100	106 ± 2.5	99 ± 4.5	2.3	4.5	106	99
COT	1.0P, 10U[*]	1.1 ± 0.050	9.6 ± 0.20	5.2	2.6	106	96
	10P, 50U[*]	11 ± 0.26	56 ± 0.80	2.4	1.5	107	112
	100	96 ± 2.6	88 ± 4.0	2.7	4.5	96	88
NNIC	5.0	4.8 ± 0.40	4.4 ± 0.50	7.7	11	96	88
	25	25 ± 0.90	20 ± 1.0	3.7	4.7	98	81
	45	46 ± 1.5	37 ± 1.6	3.2	4.3	102	82
NIC	5.0	5.3 ± 0.30	5.4 ± 0.2	5.9	4.1	107	107
	25	23 ± 0.70	25 ± 1.0	2.9	4.1	93	100
	45	42 ± 0.70	46 ± 1.3	1.6	2.9	92	101
AT	1.0P, 10U[*]	0.97 ± 0.070	8.7 ± 0.50	7.4	5.6	97	87
	10P, 50U[*]	11 ± 1.0	58 ± 1.0	9.6	1.7	106	116
	100	107 ± 4.7	83 ± 2.8	4.4	3.3	107	83
AB	5.0	4.8 ± 0.20	4.0 ± 0.10	3.7	3.1	96	80
	25	21 ± 0.90	20 ± 0.60	4.2	3.2	86	81
	45	40 ± 1.0	37 ± 1.8	2.5	4.9	88	82
COT GLUC	75	74 ± 9.2	82 ± 7.2	12	8.8	99	109
	200	241 ± 14	195 ± 6.4	5.8	3.3	120	98
	400	434 ± 60	327 ± 21	14	6.5	108	82

[*] 1.0P = 1.0 ng/mL for plasma, 10P = 10 ng/mL in plasma, 10U = 10 ng/mL in urine, 50U = 50 ng/mL in urine.

tification and reporting criteria parameters for positive samples detailed in Section 2.6.

The expected plasma concentration ranges for NIC and COT were based on a previously published clinical study investigating nicotine and cotinine concentrations in human plasma following application of a 7-mg Nicotine Transdermal System [28]. Linearity of the developed method was calculated for each analyte by fitting a simple linear regression line to the calibrator data and also the calculation of the coefficient of determination (R^2). The linearity of the calibration curve for each analyte was comprised of a minimum of 6 calibrators. QCs were prepared at low, medium and high concentrations ($n = 2$ at each level). An analyte-free plasma or urine sample containing deuterated internal standards was also included. Calibrator and QC concentrations were calculated from the calibration curve and were required to be within 20% of the theoretical target concentration. At least 1 QC was required to pass at each concentration level in order to pass an analytical batch containing clinical samples.

Method sensitivity was determined by the LOD and the LOQ, which were calculated relative to peak height. The LOD was defined as the concentration of analyte that produced a S/N of 3 for selected MRM transitions. The LOQ was the lowest standard in the calibration graph that produced a S/N ratio of $\geq$10 for the selected MRM transitions with acceptable precision and accuracy (% difference compared to target concentration within ±20%). The LOD and LOQ parameters were determined empirically as the average concentration obtained on three separate days ($n = 3$) for a series of decreasing concentrations of analyte spiked into analyte-free human plasma and urine.

The total extraction recovery for each analyte was calculated at a low, medium and high concentration ($n = 5$ for each level). Analyte-free human plasma and urine samples were fortified with analyte before SPE concurrently with five unextracted samples, which were prepared at identical analyte concentrations. Deuterated internal standard solution was added to the SPE eluant before evaporation and also to the unextracted samples. Total extraction recovery (%) was calculated by comparing the average analyte peak area ratio of extracted standards with the average analyte peak area ratio of unextracted standards.

To evaluate the effect of matrix on the analyte ionization response, peak area ratios of target analyte quantification ion to their respective deuterated internal standards quantification ion were compared for extracted analyte-free plasma or urine samples from five individuals fortified at low and high concentrations with five unextracted standard prepared in initial mobile phase composition at the same concentrations. Matrix effect was calculated as a percentage of the mean peak area ratio of the unextracted samples. A mean % matrix effect of <100% is indicative of ion suppression whereas a mean % matrix effect of >100% is indicative of ion enhancement.

Stability was assessed using analyte-free human plasma and urine QC samples fortified at low and high concentrations over the linear dynamic range of the assay. The stability scenarios were designed to mimic the storage conditions of plasma and urine samples collected from the clinical study as well as the stability of the reconstituted extracts stored in the autosampler. The stored QC samples were compared to a freshly prepared calibrator curve and QCs. Short-term temperature stability was calculated for human plasma and urine samples stored for 24 h at room temperature and 24 h at 4 °C ($n = 5$). Stability of the analytes following 3 freeze–thaw cycles of fortified human plasma and urine was also determined ($n = 5$). Autosampler stability

Table 8
Inter-assay imprecision and accuracy for plasma and urine ($n = 20$).

Analyte	Target concentration (ng/mL)	Observed concentration ± standard deviation (ng/mL)		Imprecision (%RSD)		Accuracy (%)	
		Plasma	Urine	Plasma	Urine	Plasma	Urine
NIC GLUC	5.0	4.4 ± 0.60	4.7 ± 0.50	13	9.8	88	94
	25	22 ± 2.8	23 ± 2.1	13	9.0	87	91
	45	40 ± 7.0	44 ± 3.7	17	8.5	89	97
CNO	5.0	4.4 ± 0.50	4.3 ± 0.40	11	8.3	87	87
	25	21 ± 1.6	21 ± 1.2	7.8	5.6	83	84
	45	37.2 ± 2.6	39 ± 2.0	7.0	5.3	83	85
3-HC	1.0P, 10U*	1.0 ± 0.10	9.5 ± 0.90	15	9.6	98	96
	10P, 50U*	9.6 ± 1.0	51 ± 5.4	10	11	96	102
	100	93 ± 7.0	93 ± 9.4	7.6	10	93	93
NCOT	5.0	4.4 ± 0.40	4.2 ± 0.40	8.1	9.4	89	85
	25	21 ± 1.6	21 ± 1.4	7.5	6.7	86	85
	45	38 ± 3.4	41 ± 3.1	8.8	7.5	84	90
NNO	1.0	0.90 ± 0.11	0.96 ± 0.13	12	13	90	96
	10	9.8 ± 1.2	9.2 ± 0.60	13	6.7	98	92
	100	96 ± 12	92 ± 7.9	12	8.6	96	92
COT	1.0P, 10U*	1.1 ± 0.090	9.5 ± 0.50	8.5	5.6	107	95
	10P, 50U*	10 ± 1.6	52 ± 7.4	16	14	103	103
	100	89 ± 9.2	87 ± 9.3	10	11	89	87
NNIC	5.0	4.2 ± 0.50	4.4 ± 0.50	12	11	84	88
	25	22 ± 2.1	20 ± 1.0	9.4	5.0	90	81
	45	39 ± 5.1	38 ± 1.2	13	7.4	87	85
NIC	5.0	4.7 ± 0.60	5.1 ± 0.60	14	12	94	102
	25	23 ± 0.70	24 ± 2.1	2.9	8.8	93	96
	45	43 ± 2.7	45 ± 3.9	6.3	8.6	94	101
AT	1.0P, 10U*	0.90 ± 0.10	8.9 ± 0.70	11	8.1	90	89
	10P, 50U*	8.9 ± 1.2	52 ± 6.8	13	13	89	103
	100	91 ± 14	84 ± 7.3	15	8.7	91	84
AB	5.0	4.4 ± 0.4	4.4 ± 0.40	8.5	9.9	88	88
	25	21 ± 1.5	21 ± 1.6	7.0	7.7	85	85
	45	39 ± 5.3	40 ± 2.8	14	6.9	86	89
COT GLUC	75	77 ± 11	73 ± 11	14	15	102	97
	200	215 ± 27	193 ± 25	12	13	108	97
	400	423 ± 57	379 ± 59	14	16	106	95

* 1.0P = 1.0 ng/mL for plasma, 10P = 10 ng/mL in plasma, 10U = 10 ng/mL in urine, 50U = 50 ng/mL in urine.

was assessed for reconstituted extracts stored for 72 h at 4 °C ($n = 4$).

Intra- and inter-assay imprecision and accuracy was calculated for all analytes in analyte-free human plasma and urine samples fortified at low, medium and high concentrations (Table 1). Intra-assay imprecision data was calculated from the concentration variability of the replicate analysis of QCs ($n = 5$) within an analytical batch. Inter-assay imprecision data was calculated from the concentration variability of a total of 20 QC samples analyzed over 4 separate analytical batches. The imprecision is expressed as a percent relative standard deviation (% RSD) and accuracy as a percent of the target concentration.

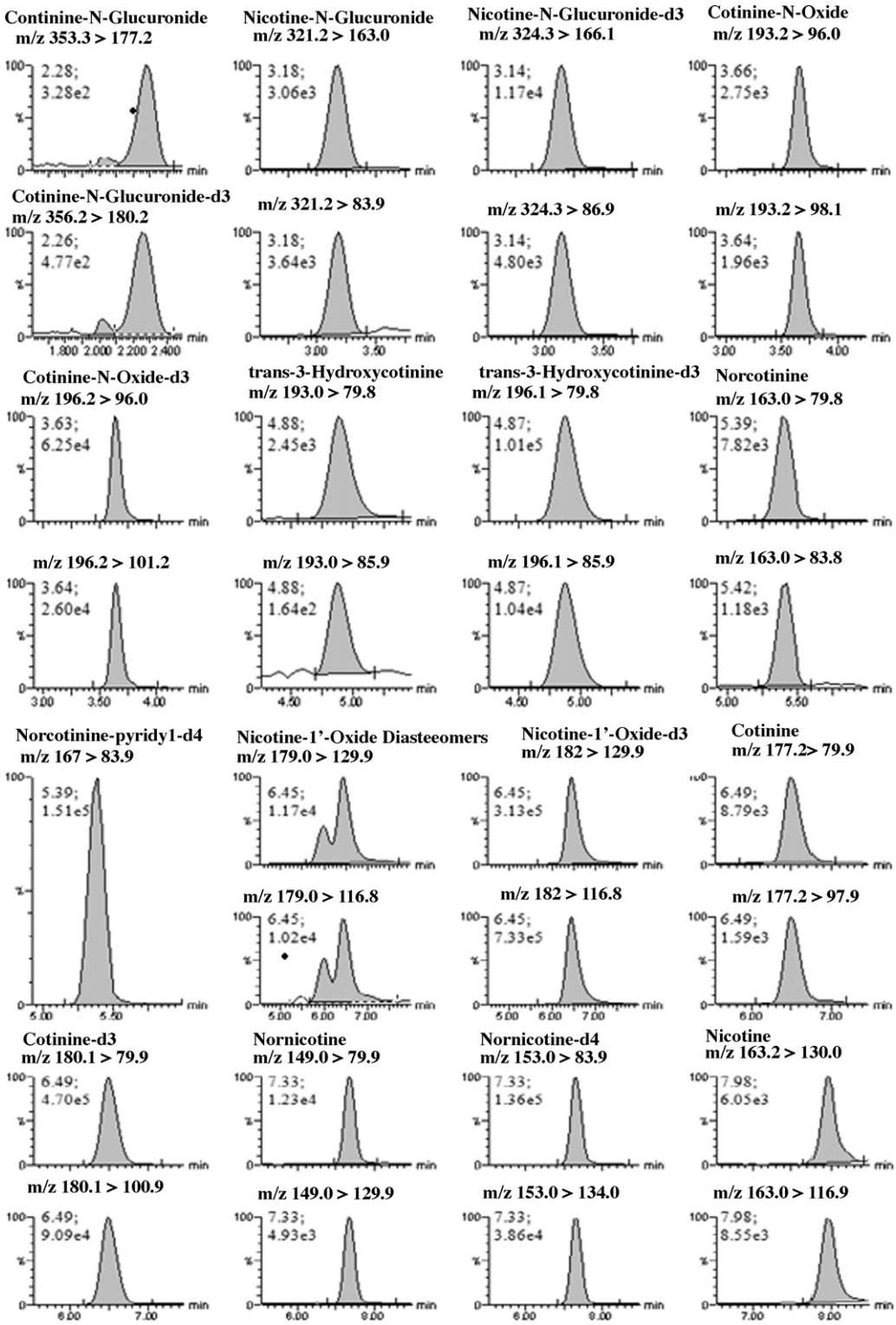

Fig. 2. (a) Chromatogram for a low concentration quality control sample in human plasma for nicotine, eight nicotine metabolites and two minor tobacco alkaloids with corresponding deuterated internal standards. (b) Chromatogram of analytes detected in participant's plasma sample 1 h after patch removal. (c) Chromatogram for analyte-free human plasma fortified with deuterated internal standard only (corresponding to analytes detected in participant sample in b).

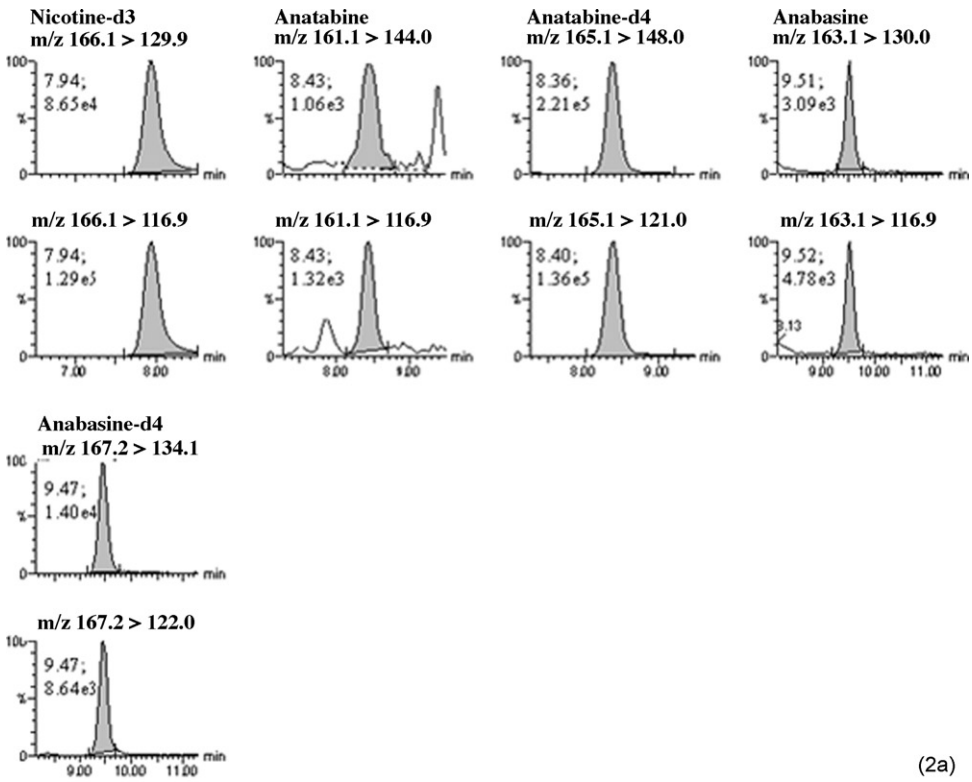

Fig. 2. (*Continued*)

4. Results and discussion

4.1. Method development

Optimization of tandem mass spectrometry parameters was conducted by the direct infusion of individual analyte solutions prepared in methanol at a concentration of 10 μg/mL with mobile phase composition and flow rate equivalent to those at the time of analyte elution from the LC column. Following the optimization of the general capillary voltage (kV), desolvation temperature (°C), desolvation gas and cone gas flow rates (L/h), the cone voltages (V) and collision energies (AU) were

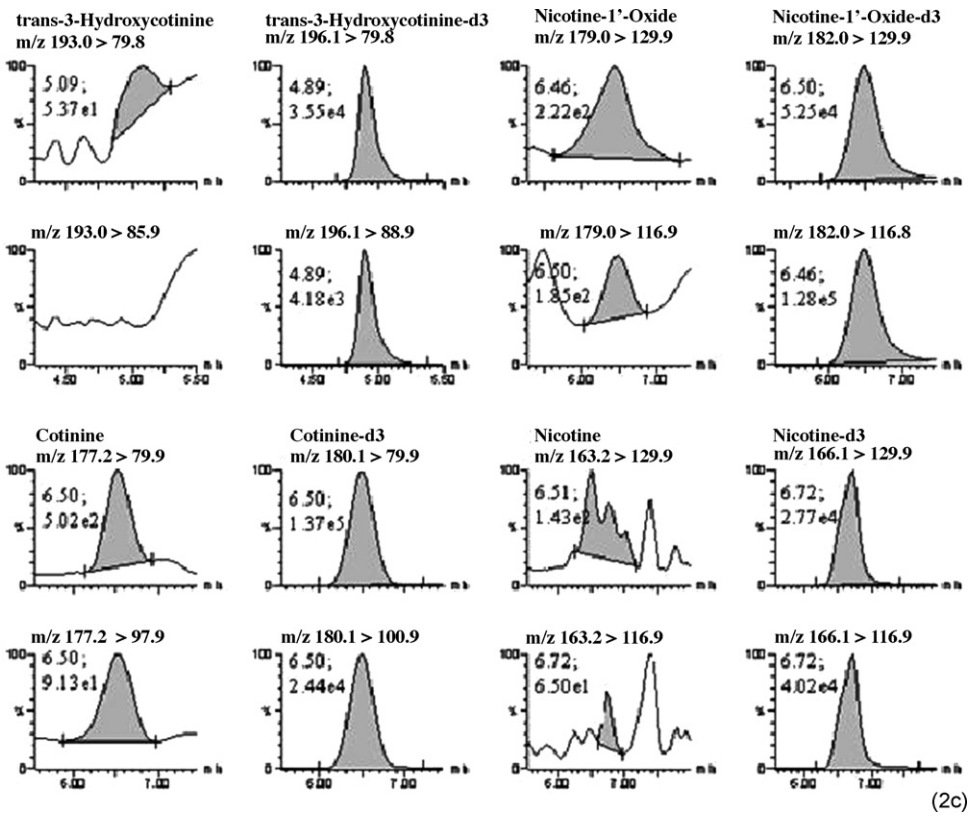

Fig. 2. (*Continued*).

selected for individual analytes through manual tuning such that the precursor ion response was <10% abundance for the purposes of maximum sensitivity towards the product fragment ions (Table 2).

The 11 target analytes and their respective deuterated standards were adequately separated within 10 min for both the plasma (Fig. 1a) and urine procedures. Retention time (tR) reproducibility was assessed by calculating tR variability in both plasma and urine (Table 2), over 4 inter-day imprecision batches performed over an approximate 6 month period (84–96 injections). The number of injections depended on the number of calibrators (Table 1) and QCs ($n = 5$ at low, medium and high concentrations). As shown in Fig. 1a, there are two peaks for the *trans*-nicotine-1-oxide QC, which represents the (1′S, 2′S)-nicotine 1′-oxide and (1′R, 2′S)-nicotine-1′-oxide diastereomers. According to the manufacturers, the (1′R, 2′S)-nicotine-1′-oxide diastereomer is present in higher proportions compared to the (1′S, 2′S)-nicotine 1′-oxide diastereomer (1.78:1) therefore the tR variability was calculated for this predominant diastereomer.

4.2. Method validation

The specificity of the method was assessed by the triplicate analysis of plasma and urine samples collected from six different nicotine-abstinent individuals. In plasma, there was no signal for the selected MRM transitions for COT GLUC, NIC GLUC, CNO, 3-HC, NCOT, AT or AB. Some of the plasma samples did contain a peak for the major product ion (quantification ion) transition for *trans*-nicotine-1′-oxide (179.0 → 129.9), cotinine (177.2 → 79.9), nornicotine (149.0 → 79.9) and nicotine (163.2 → 130.0) with good peak shape and a S/N of >5 with a tR within ±2% of the corresponding deuterated analogue. However the peak area ratios of analyte quantification ion to analyte confirmation ion did not meet ion ratio criteria based on the two selected MRM transitions for establishing the identity of these compounds. In urine, there was no signal for the selected MRM transitions for NIC GLUC, CNO, 3-HC, NCOT, NNO, NIC, AT or AB. Some of the urine samples produced a peak with a signal to noise ratio of >5 for some of the selected MRM transitions for COT GLUC (353.3 → 177.2), COT (177.2 → 79.9 and 177.2 → 97.9) and NNIC (149.0 → 79.9 and 149.0 → 129.9). However the peak area ratio of analyte quantification ion to analyte confirmation ion did not meet ion criteria for establishing the identity of COT or NNIC and the peak area ratio of analyte quantification ion to deuterated internal standard quantification ion for COT GLUC was outside the ±20% of the ratio for the LOQ calibrator.

The presence of these peaks in the plasma and urine samples provided by nicotine-abstinent individuals could be due to one or a combination of: passive exposure to environmental tobacco smoke (ETS) [24], nicotine intake through dietary sources [8] and endogenous "interfering" matrix component(s). The findings of The Third National Health and Nutrition Examination Survey between 1998 and 2001 determined that serum cotinine concentrations could be utilized to distinguish smokers from non-smokers but not non-smokers with ETS exposure from non-smokers without ETS exposure [29]. Similarly, a study by Wall et al. [30] found that although mean urine cotinine concentrations were higher in those individuals who were passively exposed to ETS compared to non-smokers, there was significant overlap between these two groups, even after adjustment for urine creatinine.

There was no carry-over observed in the analyte-free plasma or urine fortified with deuterated internal standard injected after the highest calibrator of either matrix.

Calibration curves were linear for each analyte over the selected concentration ranges (Table 1), with R^2 values >0.99. Calibration graphs were generated from peak area ratios of target analyte quantification ions and their corresponding deuterated internal

standard quantification ions over the concentration ranges shown in Table 1. Simple linear regression lines were fitted to the data because of the narrow concentration ranges used. The calibration graphs were generated using the TargetLynx™ feature of the MassLynx™ v 1.4 software and also using Microsoft® Office Excel 2007. Both weighted 1/x and non-weighted linear regression fits were evaluated with no significant difference in calculated concentration. Therefore non-weighted fits were chosen.

The LOD and LOQ values in plasma and urine (Tables 3 and 4) were derived from the criteria discussed in the Method Validation section. In plasma, most analytes were quantifiable down to 1 ng/mL whereas in urine this was 2.5 ng/mL. This is likely a result of the protein precipitation matrix clean-up step used in the plasma preparation. The percent total extraction recovery ($n = 5$) was calculated for the low, medium and high concentrations given in Table 1. This calculation compared the peak area ratio of quantification ion to deuterated internal standard quantification ion for extracted samples, fortified in either plasma or urine, with unextracted samples (Tables 3 and 4). In plasma, the mean percent recovery was >70% for COT GLUC, CNO, NNO, COT, NNIC and NIC; >60% for NIC GLUC, 3-HC, NCOT and AB; and 57% for AT. In urine, the mean percent recovery for all analytes was >82%, with the exception of COT GLUC, which was 57%.

Plasma pretreatment with a protein precipitation, followed by SPE, produced acceptable % mean ion suppression matrix effects (Table 3) of 8% for NIC GLUC, 3% for CNO, 3% for COT and acceptable % mean ion enhancement matrix effects of 7% for COT GLUC, 4% for 3-HC, 8% for NCOT, 3% for NNO, 2% for NNIC, 11% for NIC, 5% for AT and 6% for AB. For urine, SPE alone resulted in higher mean percent matrix effects (Table 4), however the % mean ion suppression matrix effects were sufficiently minimized to 3% for 3-HC, 9% for AT and 3% for AB and % mean ion enhancement matrix effects to 13% for COT GLUC, 8% for NIC GLUC, 16% for CNO, 6% for NCOT, 3% for NNO, 13% for COT, 1% for NNIC and 1% for NIC.

Analytes that had been fortified in plasma and urine at low and high concentrations (Tables 5 and 6) were calculated to be within ±20% of the target concentrations respectively under all five storage conditions, demonstrating matrix stability for 24 h at RT, 24 h in the refrigerator at 4 °C, 1 week in the freezer at −20 °C and 3 freeze–thaw cycles (−20 °C) and also reconstituted extract stability in the LC autosampler for 72 h at 4 °C (Tables 5 and 6).

Intra-assay imprecision and accuracy, which were calculated from replicate ($n = 5$) analysis of QC samples within a batch, fortified at low, medium and high concentrations (Table 1), ranged from 1.6–14% to 84–120% for plasma and 1.5–11% to 80–116% for urine over the linear dynamic range of the assay. Inter-assay imprecision and accuracy, which were calculated from 20 samples fortified at low, medium and high concentrations over 4 separate analytical batches, were calculated as <18% and ≥83% for plasma and ≤16% and >81% for urine (Tables 7 and 8).

5. Method application to clinical samples

The analytical procedure presented herein for plasma has been successfully applied in the determination of NIC, COT, 3-HC and NNO in pharmacokinetic samples collected from a healthy male clinical study participant after removal of a 7-mg transdermal nicotine patch which had been worn for 4 h. Selected chromatograms for this particular participant demonstrate results for an analyte-free plasma sample fortified with analytes and deuterated internal standard at the low QC concentration (2a), a plasma sample collected from the clinical study participant 1 h after nicotine patch

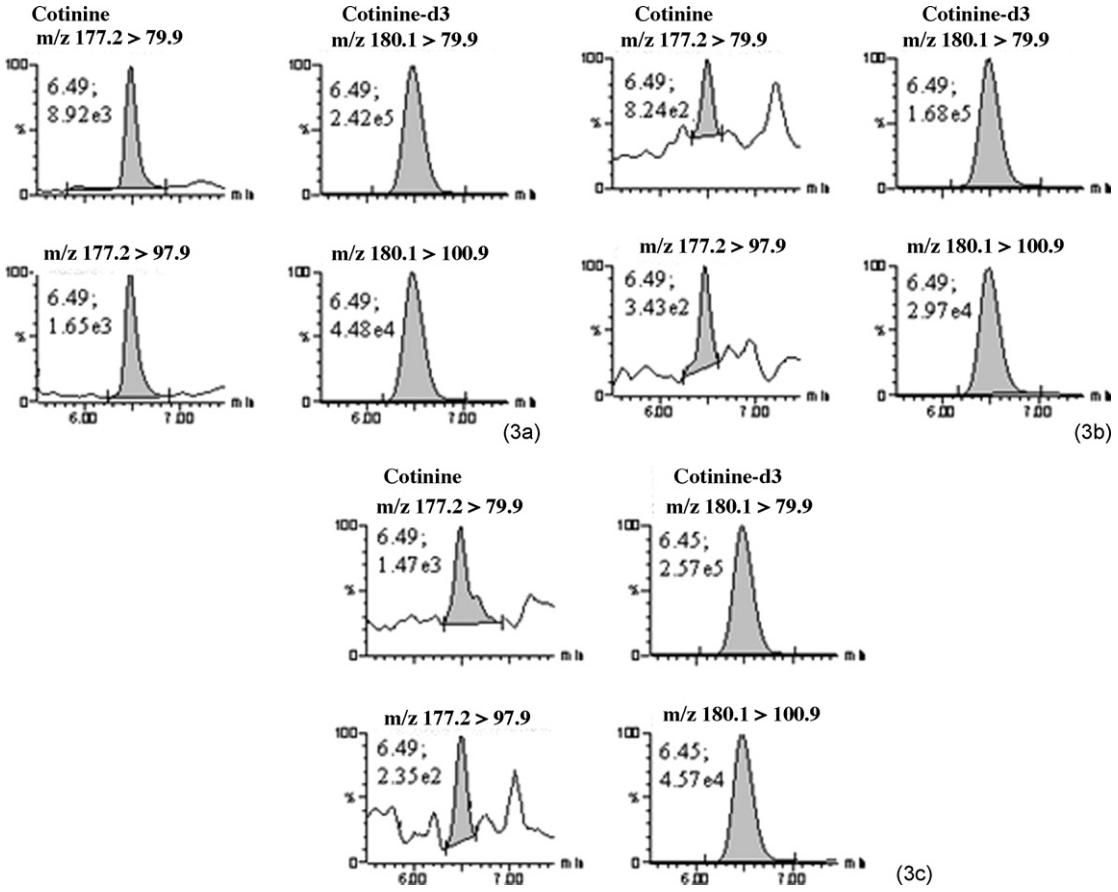

Fig. 3. (a) Chromatogram of lowest urine calibrator for cotinine (2.5 ng/mL). (b) Chromatogram of a participant's urine sample collected on day of physical examination before enrollment into the study. (c) Chromatogram of cotinine and cotinine d3 transitions for urine fortified with deuterated internal standard only.

removal (chromatograms are for positive analytes only) (2b), and an analyte-free plasma sample fortified with deuterated internal standard only (corresponding to the positive analytes detected in the participant sample) (2c). The NIC and COT plasma concentrations were 4.4 ng/mL and 12 ng/mL respectively. 3-HC and NNO were detected in (2b), however, the concentrations were <LOQ (1 ng/mL). Gorsline et al. [28] found an average steady-state minimal plasma concentration (C_{min}) and an average steady-state maximum plasma concentration (C_{max}) for NIC and COT of 3.9 ng/mL and 8.3 ng/mL respectively, in 24 healthy adult male smokers receiving a 7-mg Nicotine Transdermal System (NTS, Nicoderm®) manufactured by the ALZA Corporation in 1993. The NIC concentration detected in the particular sample shown in Fig. 2b, is within this reported range.

The urine procedure has been applied as a useful tool for determining recent nicotine exposure of potential study participants both at the time of the physical examination and, also prior to nicotine patch application on the pharmacokinetic study day. Fig. 3a–c shows the chromatograms obtained for COT for an analyte-free urine sample fortified with COT and deuterated internal standard at the LOQ (2.5 ng/mL) (3a), a urine sample collected from a participant prior to nicotine patch application on the pharmacokinetic study day (3b), and an analyte-free urine sample fortified with deuterated internal standard (3c). As seen in Fig. 3b, no COT was detected in the participant's "baseline" urine sample prior to application of the nicotine patch on the pharmacokinetic study day.

6. Conclusion

A novel LC–ESI–MS/MS method for the simultaneous extraction and quantification of nicotine, eight metabolites and two minor tobacco alkaloids from human plasma or urine has been successfully developed and validated. A combination of SPE and LC–MS/MS has proven to provide an accurate and precise approach for the quantification of target analytes. The plasma method has been applied in the sensitive and specific analysis of pharmacokinetic plasma samples collected after low dose transdermal nicotine delivery as part of a clinical study investigating the identity and concentration range of biomarkers of nicotine use in mimicked "low-level" smokers. The urine method has been applied in the analysis of "baseline" urine samples to monitor the unauthorized use of tobacco before participant enrollment onto the study and also on the pharmacokinetic study day.

Acknowledgements

This research was supported financially by the National Cancer Institute Grant Number: R01 CA12040412. The authors would like to thank the Center for Clinical and Translational Science (CCTS) at the University of Utah Health Sciences Center for financial support (#ULIRR025764) and the CCTS staff for valuable assistance with the clinical study. The authors would also like to thank Dr Matthew H Slawson at the Center for Human Toxicology, University of Utah for helpful advice during the preparation of this manuscript.

References

[1] US Center for Disease Control and Prevention, Morbidity and Mortality Weekly Report 57 (45) (2008) 1226.
[2] US Environmental Protection Agency, EPA/600/6-90/006F, 1992.
[3] Substance Abuse and Mental Health Services Administration, Results from the 2008 National Survey on Drug Use and Health: National Findings (Office of Applied Studies, NSDUH Series H-36, HHS Publication No. SMA 09-4434). Rockville, MD, 2009.
[4] P.B.S. Clarke, M. Quik, F. Adlkofer, K. Thurau (Eds.), Effects of Nicotine on Biological Systems II, Birkhäuser Verlag, Basel, Switzerland, 1995.
[5] N.L. Benowitz, P. Jacob III, Clin. Pharmacol. Ther. 56 (5) (1994) 483.
[6] S. Zevin, P. Jacob III, N. Benowitz, Clin. Pharmacol. Ther. 61 (1997) 649.
[7] N.L. Benowitz, P. Jacob III, Br. J. Clin. Pharmacol. 51 (2001) 53.
[8] J.W. Gorrod, P. Jacob III, Analytical Determination of Nicotine and Related Compounds and their Metabolites, Elservier Science B.V., Amsterdam, The Netherlands, 1999.
[9] D.W. Armstrong, X. Wang, J.-T. Lee, Y.-S. Liu, Chirality 11 (1) (1999) 82.
[10] A. Rosengren, L. Wilhelmsen, H. Wedel, J. Intern. Med. 231 (1992) 357–362.
[11] National Cancer Institute, Cigars: Health Effects and Trends. Smoking and Tobacco Control. Monograph No. 9, National Cancer Institute, 1998.
[12] Society for Research on Nicotine and Tobacco (SRNT) Subcommittee on Biochemical Verification, Nicotine Tob. Res. 4 (2002) 149.
[13] C.N. Man, L.-H. Gam, S. Ismail, R. Lajis, R. Awang, J. Chromatogr. B 844 (2) (2006) 322.
[14] H.-S. Shin, J.-G. Kim, Y.-L. Shin, S.H. Jee, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 769 (1) (2002) 177.
[15] E. Cognard, C. Staub, Clin. Chem. Lab. Med. 41 (2003) 1599.
[16] P. Jacob III, L. Yu, A.T. Shulgin, N.L. Benowitz, Am. J. Public Health 89 (5) (1999) 731.
[17] H. Kataoka, R. Inoue, K. Yagi, K. Saito, J. Pharmaceut. Biomed. (1) (2009) 108.
[18] I. Kim, M.A. Huestis, J. Mass Spectrom. 41 (2006) 815.
[19] P.J. Taylor, K.K. Forrest, P.G. Landsberg, C. Mitchell, P.L. Pillans, Ther. Drug Monit. 26 (5) (2004) 563.
[20] X. Xu, M.M. Iba, C.P. Weisel, Clin. Chem. 50 (2004) 2323.
[21] D.M. Shakleya, M.A. Huestis, J. Chromatogr. B 877 (29) (2009) 3537.
[22] D.L. Heavner, J.D. Richardson, W.T. Morgan, M.W. Ogden, Biomed. Chromatogr. 19 (4) (2005) 312.
[23] A.N. Hoofnagle, T.J. Laha, P.M. Rainey, S.M. Sadrzadeh, Am. J. Clin. Pathol. 126 (6) (2006) 880.
[24] N.L. Benowitz, Epidemiol. Rev. 18 (2) (1996) 188.
[25] R. Yoshida, M. Nakajima, Y. Watanabe, J.-T. Kwon, T. Yokoi, Br. J. Clin. Pharmacol. 54 (5) (2002) 511.
[26] M. Nakajima, T. Fukami, H. Yamanaka, E. Higashi, H. Sakai, R. Yoshida, J.T. Kwon, H.L. McLeod, T. Yokoi, Clin. Pharmacol. Ther. 80 (3) (2006) 282.
[27] S. Feng, S. Kapur, M. Sarkar, R. Muhammed, P. Mendes, K. Newland, H.J. Roethig, Toxicol. Lett. 173 (2) (2007) 101.
[28] J. Gorsline, S.K. Gupta, D. Dye, C.N. Rolf, J. Clin. Pharmacol. 33 (1993) 161.
[29] J.L. Pirkle, K.M. Flegal, J.T. Bernert, D.J. Brody, R.A. Etzel, K.R. Maurer, JAMA 275 (1996) 1233.
[30] M.A. Wall, J. Johnson, P. Jacob, N.L. Benowitz, Am. J. Public Health 78 (1988) 699.