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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☆

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ABSTRACT

A sensitive and specific ultra performance liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of nicotine, its metabolites cotinine and *trans*-3′-hydroxycotinine and varenicline in human plasma was developed and validated. Sample preparation was realized by solid phase extraction of the target compounds and of the internal standards (nicotine-d4, cotinine-d3, *trans*-3′-hydroxycotinine-d3 and CP-533,633, a structural analog of varenicline) from 0.5 mL of plasma, using a mixed-mode cation exchange support. Chromatographic separations were performed on a hydrophilic interaction liquid chromatography column (HILIC BEH 2.1 × 100 mm, 1.7 μm). A gradient program was used, with a 10 mM ammonium formate buffer pH 3/acetonitrile mobile phase at a flow of 0.4 mL/min. The compounds were detected on a triple quadrupole mass spectrometer, operated with an electrospray interface in positive ionization mode and quantification was performed using multiple reaction monitoring. Matrix effects were quantitatively evaluated with success, with coefficients of variation inferior to 8%. The procedure was fully validated according to Food and Drug Administration guidelines and to Société Française des Sciences et Techniques Pharmaceutiques. The concentration range was 2–500 ng/mL for nicotine, 1–1000 ng/mL for cotinine, 2–1000 ng/mL for *trans*-3′-hydroxycotinine and 1–500 ng/mL for varenicline, according to levels usually measured in plasma. Trueness (86.2–113.6%), repeatability (1.9–12.3%) and intermediate precision (4.4–15.9%) were found to be satisfactory, as well as stability in plasma. The procedure was successfully used to quantify nicotine, its metabolites and varenicline in more than 400 plasma samples from participants in a clinical study on smoking cessation.

1. Introduction

Smoking represents the major avoidable cause of premature deaths, which makes smoking cessation a substantial part of the strategy against tobacco use, providing clear health benefits [1]. However, few people succeed to stop smoking, because of several factors such as nicotine addiction, environmental, personal and social factors [2].

Nicotine is the psychoactive substance in tobacco and it is the major agent responsible for tobacco addiction [2]. Nicotine replacement therapy (NRT) is thus being used for persons who want to quit smoking, providing the user with a dosage of nicotine sufficient to reduce withdrawal symptoms without further reinforcing the behavior of cigarette smoking [3]. Nicotine is metabolized in the liver by the cytochrome P450 2A6 (CYP2A6) to its main metabolites cotinine and *trans*-3′-hydroxycotinine. The ratio of *trans*-3′-hydroxycotinine to cotinine can be used as a phenotypic marker for CYP2A6 activity and for the rate of nicotine metabolism [4], which in turn can reflect the addiction level and the chances to quit smoking [5]. Varenicline is a partial agonist of the δ4ß2 nicotinic acetylcholine receptor and is prescribed as an aid to smoking cessation for its effects of reducing the severity of nicotine withdrawal symptoms and nicotine craving [6].

Studying the pharmacogenetics of these drugs could improve the success rates of smoking cessation programs, allowing to better adapt them to the characteristics of an individual [7]. Thus,

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Table 1
MRM parameters and retention times of the analytes and IS.

	$[M+H]^+$	MRM transition (m/z)	Cone voltage (eV)	Collision energy (eV)	t_R (min)[a]
Nicotine	163	163→130	30	20	3.32
Nicotine-d4	167	167→134	30	20	3.33
Cotinine	177	177→80	40	25	1.70
Cotinine-d3	180	180→80	35	25	1.71
trans-3′-Hydroxycotinine	193	193→80	40	25	1.69
trans-3′-Hydroxycotinine-d3	196	196→80	35	25	1.70
Varenicline	212	212→169	45	25	3.31
CP-533,633	240	240→197	35	25	3.30

[a] t_R: Retention time.

determining blood levels of these drugs in smokers who are trying to quit allows studying their metabolism and elimination in relation with genetic markers, and could therefore lead to a better understanding of their efficacy and side effects.

Several analytical methods for the quantification of nicotine and/or its metabolites in different biological samples were described, including plasma [8–11], serum [12–14], urine [15–18], breast milk [19,20] and oral fluid/saliva [12,21]. The majority of methods used liquid chromatography (LC) systems coupled with single quadrupole mass spectrometric (MS) detection [10,14] or triple quadrupole mass spectrometry (MS/MS) [8,9,15,16,19]. Sample preparation was mostly performed by solid phase extraction (SPE) [8–10,12,15,16] or by liquid–liquid extraction (LLE) [13,19]. Several methods have been recently published for the quantification of nicotine and metabolites using hydrophilic interaction liquid chromatography (HILIC) [22–24], which has been demonstrated to be a useful technique for the retention of polar compounds [25]. To the best of our knowledge, only one method has been published for the quantification of varenicline in human plasma, using HPLC–MS/MS preceded by LLE [26], which has then been applied for the study of varenicline pharmacokinetics [27,28].

For the present work, high specificity was required to minimize the risk of interferences with compounds from the matrix (human plasma) and to discriminate between the chemically related analytes. High sensitivity was needed to ensure low limits of quantification (LLOQ), allowing to confirm abstinence from smoking, and wide calibration ranges, allowing to detect overdosage. Moreover, short analysis time and simultaneous quantification of the compounds are highly preferred. Therefore, the ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) allows to combine the advantages of the UPLC, such as increased resolution and sensitivity, decreased analysis time and solvent consumption [29] and the high detection specificity and sensitivity of the MS/MS. Thus, this methodology provides the most sensitive and specific method today available for the analysis of drugs and their metabolites.

The objective of the present work was to develop and validate a rapid and sensitive UPLC–MS/MS method for the simultaneous determination of smoking cessation drugs in human plasma and further apply it to samples of participants from a clinical study on smoking cessation.

2. Experimental

2.1. Chemicals and reagents

Varenicline, its internal standard (IS) (CP-533,633) and nicotine were kindly provided by their manufacturers: Pfizer (Groton, USA) and Siegfried (Zofingen, Switzerland), respectively. Cotinine, cotinine-d3 (1 mg/mL solution in methanol) and nicotine-d4 (100 μg/mL solution in methanol) were purchased from Sigma–Aldrich (Buchs, Switzerland), *trans*-3′-hydroxycotinine from Chemos (Regenstauf, Germany), and *trans*-3′-hydroxycotinine-d3 (1 mg/mL solution in methanol) from Toronto Research (Ontario, Canada). Biosolv® UPLC-grade acetonitrile, formic acid, methanol and isopropanol were bought from Chemie Brunchwig (Basel, Switzerland), Lichrosolv® 37% hydrochloric acid and 85% ortho-phosphoric acid from Merck (Darmstadt, Germany), 25% ammonium hydroxide solution, ammonium formate and ammonium acetate puriss p.a. for mass spectrometry from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was obtained from a Milli-Q® RG with a QPAQ2 column system (Millipore, MA, USA). All chemicals were of analytical grade. For the preparation of calibrators and quality control (QC) samples and the evaluation of matrix effects, more than 10 different batches of human plasma were obtained from the hospital's blood transfusion center (CHUV, Lausanne, Switzerland), that were tested for the presence of nicotine and/or metabolites prior to their use.

2.2. Equipment

The LC system consisted of a Waters Acquity UPLC instrument equipped with a binary pump and a 96-vial autosampler (Waters, Milford, MA, USA). Chromatographic separations were performed on a HILIC BEH column (2.1 × 100 mm, 1.7 μm) (Waters) equipped with a HILIC BEH cartridge (2.1 × 10 mm, 1.7 μm). Column temperature was set at 40 °C and the autosampler was kept at 8 °C. The chromatographic system was coupled to a triple quadrupole MS (TQD) (Waters) equipped with an ESI interface operated in positive ionization mode. Data acquisition handling and instrument control were performed by the Masslynx software version V4.1 (Waters).

2.3. Stock and working solutions

Standard stock solutions of all analytes each at 1 mg/mL in methanol were stored at −20 °C. Working solutions at 50 μg/mL were obtained by the dilution of the stock solutions with 0.01 N HCl. Calibrators and QC samples were prepared independently at different concentrations in analyte-free plasma, which was fortified with freshly prepared dilutions of the working solution in 0.01 N HCl at 0.05, 0.5 and 5 μg/mL. A single IS working solution at 1 μg/mL containing nicotine-d4, cotinine-d3, *trans*-3′-hydroxycotinine-d3 and CP-533,633 in 0.01 N HCl was prepared. All working solutions were stored at −20 °C until required for analysis.

2.4. Sample preparation

An aliquot of plasma sample (500 μL) was fortified with 50 μL of IS working solution. The mixture was then diluted with 500 μL of 4% H_3PO_4, followed by vortex mixing. The acidified plasma was then treated by SPE, using an Oasis MCX 10 mg 96-well extraction plate. The SPE plates were conditioned and equilibrated with 500 μL acetonitrile and water, respectively. 1000 μL of diluted sample was loaded onto the SPE plate and allowed to flow by gravity, after which a slow vacuum was applied. Plates were washed with

Table 2
Extraction recovery, matrix effect and process efficiency for nicotine, metabolites and varenicline extracted from human plasma.

Analyte	Concentration (ng/mL)		Extraction recovery (%, $n = 6$, CV%[a])		Matrix effect (%, $n = 6$, CV%[a])		Process efficiency (%, $n = 6$, CV%[a])	
	Low	High	Low	High	Low	High	Low	High
Nicotine	10	500	86 (8)	94 (4)	92 (6)	79 (6)	79 (3)	74 (4)
Nicotine-d4	100	100	91 (10)	91 (3)	92 (7)	84 (4)	83 (3)	76 (4)
Cotinine	10	500	87 (5)	70 (3)	103 (6)	106 (3)	89 (3)	84 (3)
Cotinine-d3	100	100	82 (5)	75 (3)	105 (4)	97 (3)	86 (3)	94 (3)
trans-3′-Hydroxycotinine	10	500	54 (10)	49 (13)	102 (5)	98 (6)	55 (12)	48 (15)
trans-3′-Hydroxycotinine-d3	100	100	52 (9)	51 (15)	108 (4)	105 (2)	56 (12)	53 (15)
Varenicline	10	250	92 (11)	83 (4)	136 (6)	120 (6)	125 (6)	99 (5)
CP-533,633	100	100	91 (7)	88 (4)	130 (8)	122 (5)	118 (3)	108 (4)
Nicotine/Nicotine-d4	10	500	96 (4)	104 (1)	102 (2)	94 (3)	97 (2)	98 (3)
Cotinine/Cotinine-d3	10	500	106 (3)	94 (3)	97 (4)	95 (3)	103 (3)	89 (4)
trans-3′-Hydroxycotinine/ *trans*-3′-hydroxycotinine-d3	10	500	104 (5)	107 (6)	95 (4)	93 (4)	98 (3)	99 (4)
Varenicline/CP-533,633	10	250	101 (5)	94 (4)	105 (3)	98 (2)	106 (6)	92 (2)

[a]CV%: Coefficient of variation.

1000 μL of 2% formic acid solution followed by 1000 μL acetonitile. The analytes were then eluted with 250 μL of acetonitrile/25% ammonium hydroxide (90:10, *v/v*) followed by 250 μL acetonitrile. After each step a slow vacuum was applied. The elution was vortex mixed and 5 μL were directly injected into the UPLC–MS/MS system.

2.5. UPLC–MS/MS conditions

The most suitable chromatographic conditions were achieved at a flow rate of 0.4 mL/min with a mobile phase composed of 10 mM ammonium formate buffer pH 3 (solution A) and acetonitrile with 0.1% formic acid (solution B), using the following gradient program: 95% of B maintained for 1.8 min, gradient to 70% of B from 1.8 to 2.0 min, hold at 70% of B from 2.0 to 3.4 min. The gradient was followed by recondition with 95% of B until 8.0 min, as requested for HILIC columns.

Detection was performed on a triple quadrupole MS detector, operated with an ESI interface in positive ionization mode. Quantification was achieved using multiple reaction monitoring (MRM) of the transitions *m/z* 163→130 (nicotine), 177→80 (cotinine), 193→80 (*trans*-3′-hydroxycotinine), 212→169 (varenicline) for the analytes and 167→134 (nicotine-d4), 180→80 (cotinine-d3), 196→80 (*trans*-3′-hydroxycotinine-d3) and 240→197 (CP-533,633) for the IS. These transitions were previously reported in other publications for the detection of the studied compounds [8,17,26]. Nitrogen was used as desolvation gas at a flow rate of 800 L/h and a temperature of 400 °C, and argon as collision gas at a flow rate of 0.2 mL/min. Source temperature was set at 150 °C and capillary voltage at 3 kV. Dwell time for each ion was 50 ms. Table 1 presents the optimized cone voltages and collision energies, together with the *m/z* ratios and the MRM transitions chosen for all the compounds.

2.6. Method validation

The method validation was based on the recommendations of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [30] and on the guidelines for Bioanalytical Method Validation published online by the Food and Drug Administration (FDA) [31].

2.6.1. Selectivity and carry-over

Method selectivity was tested by analyzing 10 blank plasma batches from different sources for interfering peaks. Possible carry-over effects were assessed by injecting solvent (acetonitrile) and plasma samples ($n = 3$) after the highest calibrator at 500 ng/mL for nicotine and varenicline and 1000 ng/mL for cotinine and *trans*-3′-hydroxycotinine.

2.6.2. Matrix effects, extraction recovery and process efficiency

Matrix effects were assessed quantitatively at low (10 ng/mL) and high concentration levels (500 ng/mL for nicotine and its metabolites, 250 ng/mL for varenicline) and at 100 ng/mL for the IS based on the approach of Matuszewski [32]. Three different series were processed: a neat standard solution of the analytes and IS in the SPE elution solvent (acetonitrile/25%ammonium hydroxide (90:10, *v/v*)) (A), duplicates of 6 analyte-free plasma extracted and fortified with the analytes and IS after extraction (B) and duplicates of 6 analyte-free plasma fortified with the analytes and IS before extraction (C). The matrix effect was evaluated for each analyte and IS by calculating the ratio of the mean peak area in the presence of the matrix (samples fortified after extraction) to the peak area in absence of the matrix (neat standard) and expressed in percentage (ME = B/A). The recovery of the extraction from the matrix was determined by comparing the mean peak areas of samples fortified before extraction (C) to samples fortified after extraction (B) (RE = C/B). The overall process efficiency, taking into account the ME and the RE, was assessed by calculating the ratio of the mean peak areas of samples fortified before extraction to the peak area in the neat standard (PE = C/A). For all three parameters, the variability between the different plasma batches was evaluated and expressed as coefficient of variation (CV%). A value inferior or equal to 15% was considered satisfactory. The same parameters and respective coefficients of variation were calculated considering the IS-normalized peak areas for each analyte.

2.6.3. Trueness and precision

Three validation series were performed on independent days to examine trueness and precision of the analytical procedure. Calibrators were initially prepared in duplicate at 8 levels (1, 2, 5, 25, 50, 100, 500, and 1000 ng/mL for nicotine and metabolites and 1, 2, 5, 25, 50, 100, 250, and 500 ng/mL for varenicline). QC samples were prepared in quadruplicate at 8 levels (same as calibrators) covering the expected range of concentrations, according to previously measured levels in human plasma [6,33–35]. Finally, 3 levels in duplicate for nicotine and 4 levels in duplicate for metabolites and varenicline were chosen to build the calibration curves, which were freshly prepared each day. Results were based on the peak area ratio between the drug and its respective IS. QC samples were analyzed against the calibration curve of the same series and the trueness of each concentration level was expressed as percentage of the theoretical value. Precision was estimated with variances of repeatability (within-run variance) and intermediate precision

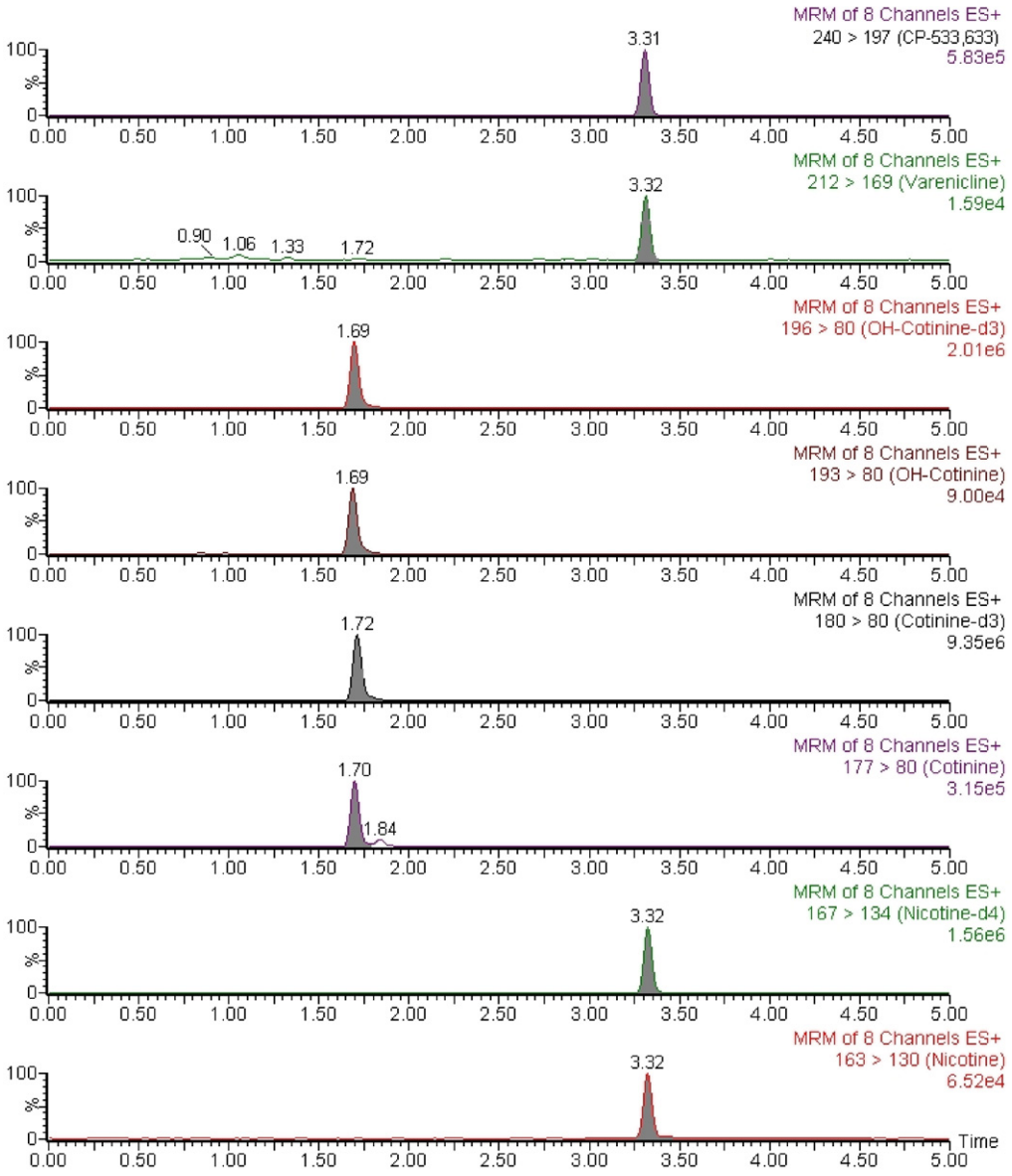

Fig. 1. Multiple reaction monitoring chromatogram of a QC plasma sample at 3 ng/mL (cotinine, varenicline), 6 ng/mL (*trans*-3′-hydroxycotinine, nicotine) and 100 ng/mL IS.

(sum of within-run and between-run variances), expressed as coefficient of variation (CV%) [30]. The LLOQ for each analyte was determined by the lowest QC concentration with a trueness and precision of ±20%, according to the above mentioned guidelines. Accuracy profiles within the acceptance limit ($\lambda = \pm 30\%$), and with confidence interval ($\alpha = 0.05$) were built for each analyte in the dosing range. The linearity of the method was equally assessed using a linear regression model which fitted the recalculated QC concentrations *vs.* theoretical concentrations [30].

2.6.4. Stability

The effect of different storage conditions on the stability of the compounds in plasma was assessed. Blank plasma from 5 different batches was fortified with the compounds of interest at low (5 ng/mL) and high (850 ng/mL for nicotine and metabolites, 450 ng/mL for varenicline) concentrations and quantified after storage at ambient temperature and at 4 °C up to 72 h, after one to three freeze–thaw cycles and after one, three and eight months at −20 °C. The postpreparative stability was assessed by leaving the processed samples for 24 h and 48 h at 8 °C before reanalysis. The variations in drug concentrations were expressed as percentage of the initial concentration found in the samples analyzed after preparation. The coefficients of variation (CV%) of the set of 5 plasma batches were also calculated for each compound and concentration level. A variation in plasma concentration inferior to 20% and CVs inferior to 15% were considered satisfactory.

3. Results and discussion

3.1. Solid-phase extraction

The sample preparation was based on a SPE procedure using a mixed-mode sorbent which combines hydrophobic interactions and cation-exchange, being thus suitable for the extraction of basic drugs from plasma. The use of different organic solvents as well as different washing steps was investigated. Satisfactory results regarding recovery, repeatability and selectivity were obtained by using ACN as organic solvent and 2% formic acid in water as washing solution. Recoveries were comprised between 70% and 94%, with the exception of *trans*-3′-hydroxycotinine and

Table 3
Assay validation parameters for nicotine, metabolites and varenicline.

Analyte	Concentration (ng/mL)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
Nicotine	2	107.4	6.3	7.4
	5	106.5	4.6	11.3
	25	86.2	3.2	12.7
	50	96.3	4.6	11.8
	100	98.0	5.5	11.4
	500	112.0	5.8	13.5
	1000	113.6	7.7	15.3
Cotinine	1	97.8	8.8	8.8
	2	102.7	6.1	6.4
	5	104.4	6.6	9.6
	25	109.3	4.5	6.4
	50	107.3	3.7	8.2
	100	108.2	4.8	5.8
	500	97.7	3.9	6.5
	1000	90.6	5.0	5.0
trans-3′-Hydroxycotinine	2	103.6	9.9	10.9
	5	96.2	10.2	10.2
	25	96.3	5.9	8.5
	50	99.2	7.2	9.9
	100	99.9	8.5	8.5
	500	104.5	4.3	9.1
	1000	107.0	7.1	7.7
Varenicline	1	108.2	12.3	15.9
	2	99.2	10.9	10.9
	5	103.5	8.7	9.7
	25	102.3	1.9	4.6
	50	103.4	7.0	8.4
	100	101.7	9.0	9.0
	250	101.2	4.2	4.4
	500	96.1	4.6	4.6

trans-3′-hydroxycotinine-d3 for which recoveries were of 49–54%, probably due to their polar nature and thus to a poor retention on the SPE cartridge. However, the repeatability of results was satisfactory in all cases (CVs equal or below 15% for analytes and equal or below 6% for the analyte/IS ratio) (Table 2). No evaporation and reconstitution step was required and 5 μL of the SPE elution were directly injected into the UPLC–MS/MS system.

3.2. Optimization of chromatographic and MS/MS conditions

Preliminary separation assays have been performed using reversed phase chromatography (Acquity BEH C_{18} columns obtained from Waters) and a satisfactory separation was obtained. However, given the polar character of the compounds, the retention was poor and the percentage of acetonitrile at the beginning of the gradient was too low to allow for a sufficient desolvatation [36]. This resulted in a poor sensitivity and in estimated LLOQs that were not satisfactory for the purpose of the method. Given that varenicline is prescribed in small dosage (0.5 or 1 mg twice a day) [6], plasma levels were expected to be low: between 0.1 and 28.3 ng/mL in a population pharmacokinetic analysis [6]. Moreover, we aimed to use the procedure also for confirming abstinence from smoking in the study population. Therefore LLOQs for the studied compounds should ideally be low.

The HILIC technology involves the partitioning of an analyte between an organic mobile phase mainly polar and a water-enriched layer of the mobile phase, partially immobilized on the stationary phase [37]. The main advantages are an increased retention of polar compounds, enhanced sensitivity due to the high organic content in the mobile phase and possibility of directly

Table 4
Stability of nicotine, metabolites and varenicline in human plasma ($n = 5$) and after extraction.

Nominal conc. (ng/mL)	Nicotine		Cotinine		*trans*-3′-Hydroxycotinine		Varenicline	
	5	850	5	850	5	850	5	450
	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])
Room temperature, 24 h	0 (5)	5 (6)	3 (9)	2 (5)	6 (4)	3 (9)	18 (7)	0 (5)
Room temperature, 72 h	7 (2)	1 (2)	0 (8)	−1 (4)	12 (7)	5 (4)	3 (2)	−3 (5)
4 °C, 72 h	0 (3)	3 (3)	−2 (6)	−3 (5)	9 (7)	7 (2)	−3 (7)	−4 (3)
Freeze/thaw, cycle 1	6 (4)	6 (1)	3 (9)	3 (5)	13 (5)	6 (3)	2 (4)	−3 (3)
Freeze/thaw, cycle 2	7 (5)	7 (3)	3 (7)	1 (5)	13 (14)	5 (3)	3 (6)	−4 (6)
Freeze/thaw, cycle 3	11 (3)	8 (4)	0 (5)	0 (4)	15 (6)	7 (2)	10 (8)	−4 (3)
Storage −20 °C, 1 month	5 (8)	7 (5)	−1 (3)	−1 (4)	7 (10)	2 (6)	−3 (3)	−6 (5)
Storage −20 °C, 3 months	−1 (8)	−5 (5)	3 (2)	0 (3)	2 (5)	3 (3)	9 (3)	−7 (4)
Storage −20 °C, 8 months	−3 (9)	−5 (2)	−3 (2)	−1 (2)	10 (8)	2 (1)	6 (6)	−3 (2)
Postpreparative 8 °C, 24 h	1 (4)	5 (2)	−2 (7)	5 (2)	−1 (6)	7 (3)	6 (9)	3 (6)
Postpreparative 8 °C, 48 h	−1 (3)	−1 (4)	3 (7)	4 (5)	4 (2)	4 (7)	12 (4)	0 (4)

[a] Var%: Variations in drug concentrations, expressed as percentage of the initial concentration found in the samples analyzed after preparation.
[b] CV%: Coefficient of variation.

Table 5
Plasma levels of nicotine, metabolites and varenicline measured in the study population.

	n	Calibrated range (ng/mL)	Quantified range (ng/mL) (6, 32–34)	Study samples (ng/mL) median (range)
Nicotine	406	2–500	1–100	17.5 (0–52)
Cotinine	406	1–1000	1–1000	251.5 (0–818)
trans-3′-Hydroxycotinine	406	2–1000	1–500	79 (0–314)
Varenicline	199	1–500	1–30	3 (0–20)

injecting the extraction organic elution onto the chromatographic column [25]. HILIC has been shown appropriate for hydrophilic bases at acid pH, using acetonitrile and ammonium formate buffers [25]. Therefore, to set up the chromatographic parameters a scouting gradient from 95% to 50% acetonitrile was used, testing buffers (ammonium formate, ammonium acetate) at different concentrations (5 mM, 10 mM) and pHs (from 2.8 to 5). The addition of different organic modifiers in the aqueous phase (3% of methanol or isopropanol) was also tested, in the attempt to obtain changes in retention and selectivity.

However, changes in pH or in buffer concentration did not significantly influence the selectivity of the separation, reflecting similar polarities and ionization between the compounds. Thus, cotinine and *trans*-3′-hydroxycotinine ($\log P = 0.34$ and -0.5; pKa = 4.5 and 4.5, respectively), coeluted, as well as nicotine and varenicline ($\log P = 1.09$ and 1.13; pKa = 8.2 and 8.8, respectively) [38,39]. The addition of 3% of methanol or isopropanol to the aqueous phase led to a complete separation of the compounds (data not shown). However, it was observed that organic modifiers interfered with the HILIC mechanism of retention, leading to a poor peak shape of nicotine. Thus, no organic modifier was used and instead, 0.1% of formic acid was added to acetonitrile to improve peak shapes, as previously described [25]. Overall, a significant increase in sensitivity was obtained with HILIC columns compared to reverse phase columns, which was in agreement with other published reports [40]. The most suitable chromatographic conditions were thus achieved with a mobile phase composed of 10 mM ammonium formate buffer pH 3 (solution A) and acetonitrile with 0.1% formic acid (solution B), using a gradient program from 95% of B until 70% of B and an overall time of 8.0 min. The average retention times of the analytes (t_R) are listed in Table 1 and a multiple reaction monitoring chromatogram of a QC plasma sample containing the compounds at 3 ng/mL (cotinine, varenicline), 6 ng/mL (*trans*-3′-hydroxycotinine, nicotine) and the IS at 100 ng/mL is shown in Fig. 1.

The high selectivity of an MS/MS system allows a successful quantification of the compounds, although their separation is not complete. This was further confirmed in our study by testing signal suppression in the two coeluting pairs, by keeping one substance at a fixed concentration (100 ng/mL) and increasing the other one up to 500 ng/mL (for nicotine and varenicline) and up to 1000 ng/mL (for cotinine and *trans*-3′-hydroxycotinine). No significant signal suppression was observed in the two pairs of coeluting compounds (CVs inferior to 5%) (data not shown). Moreover, no cross-talk was observed between the compounds when each analyte was injected separately and all MRM transitions were followed.

Deuterated internal standards, which allow to compensate for signal alterations due to matrix components and for variability in the extraction procedure [31,41], were used for nicotine and its metabolites. Varenicline is a newly developed drug and no deuterated standard was available during the development of the present procedure. Therefore, a structurally related compound (CP-533,633) which coeluted with varenicline was used, kindly supplied by the manufacturer together with varenicline.

Cone voltage and collision energy were optimized by directly infusing into the MS/MS detector a solution of each analyte at 1 μg/mL in 0.01 N HCl, at a flow of 10 μL/min and in combined mode with the mobile phase (30% of A and 70% of B). Cone voltage was tested in MS scan mode (values between 5 and 50 eV) and the value corresponding to the highest signal intensity was retained. The fragmentation spectrum was obtained in product scan mode and collision energies (between 5 and 50 eV) corresponding to the daughter ions with the highest abundance were retained.

3.3. Validation

3.3.1. Selectivity and carry-over

No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma extracts evaluated. However, in some blank plasma of non-smokers we observed traces of nicotine and cotinine, maybe due to passive exposure to environmental tobacco smoke (ETS) or to nicotine intake through dietary sources, as previously reported [8]. Nevertheless, the peak areas of nicotine and cotinine in blank plasma were not significant, corresponding to less than 20% of the concentration at LLOQ. However, it was difficult to evaluate the presence of carry-over by injecting blank plasma after the highest calibrator. Therefore, acetonitrile was injected after the highest calibrator ($n = 10$) and no carry-over of the studied compounds was observed. Moreover, no significant cross-talk was observed between the IS and the parent compounds.

3.3.2. Matrix effects, extraction recovery and process efficiency

A limitation of the MS ESI mode of detection is the presence of matrix effects, meaning a signal enhancement or suppression by endogenous compounds present in the biological matrix and potentially coeluting with the analytes [42].

The quantitative assessment of interfering endogenous plasma components revealed slight matrix effects ranging from 79% for nicotine to 136% for varenicline. However, when the ratios analyte/IS were considered, matrix effects were comprised between 93% and 105%, showing that the use of individual coeluting IS corrected for these effects. Moreover, more important than the absolute matrix effects, is a reduced variability of these effects between the different plasma batches. With the present extraction procedure this aim was achieved, with variabilities between the 6 different plasma sources inferior to 8% for the substances alone and inferior to 4% for the ratio analyte/IS. Process efficiencies, which consider the combined effects of extraction recovery and of matrix effects, were also found to be satisfactory, ranging between 89% and 106% (for the analyte/IS ratio), with CVs inferior to 6%. All results are presented in Table 2.

3.3.3. Trueness and precision

Three validation series were performed on three different days and 8 calibrators in duplicate were initially used for each compound, covering the range from 1 to 1000 ng/mL for nicotine and its metabolites and from 1 to 500 ng/mL for varenicline. Determination of the most suitable calibration was performed by testing different regression models. Calibration curves of 3 and 4 levels in duplicate were selected, taking into account the LLOQ obtained for each compound: 2, 25, and 500 ng/mL for nicotine; 1, 25, 500, and 1000 ng/mL for cotinine; 2, 25, 500, and 1000 ng/mL for *trans*-3′-hydroxycotinine and 1, 25, 250, and 500 ng/mL for varenicline. Calibration curves were fitted for all compounds by a linear regression with a weighting factor of $1/x^2$.

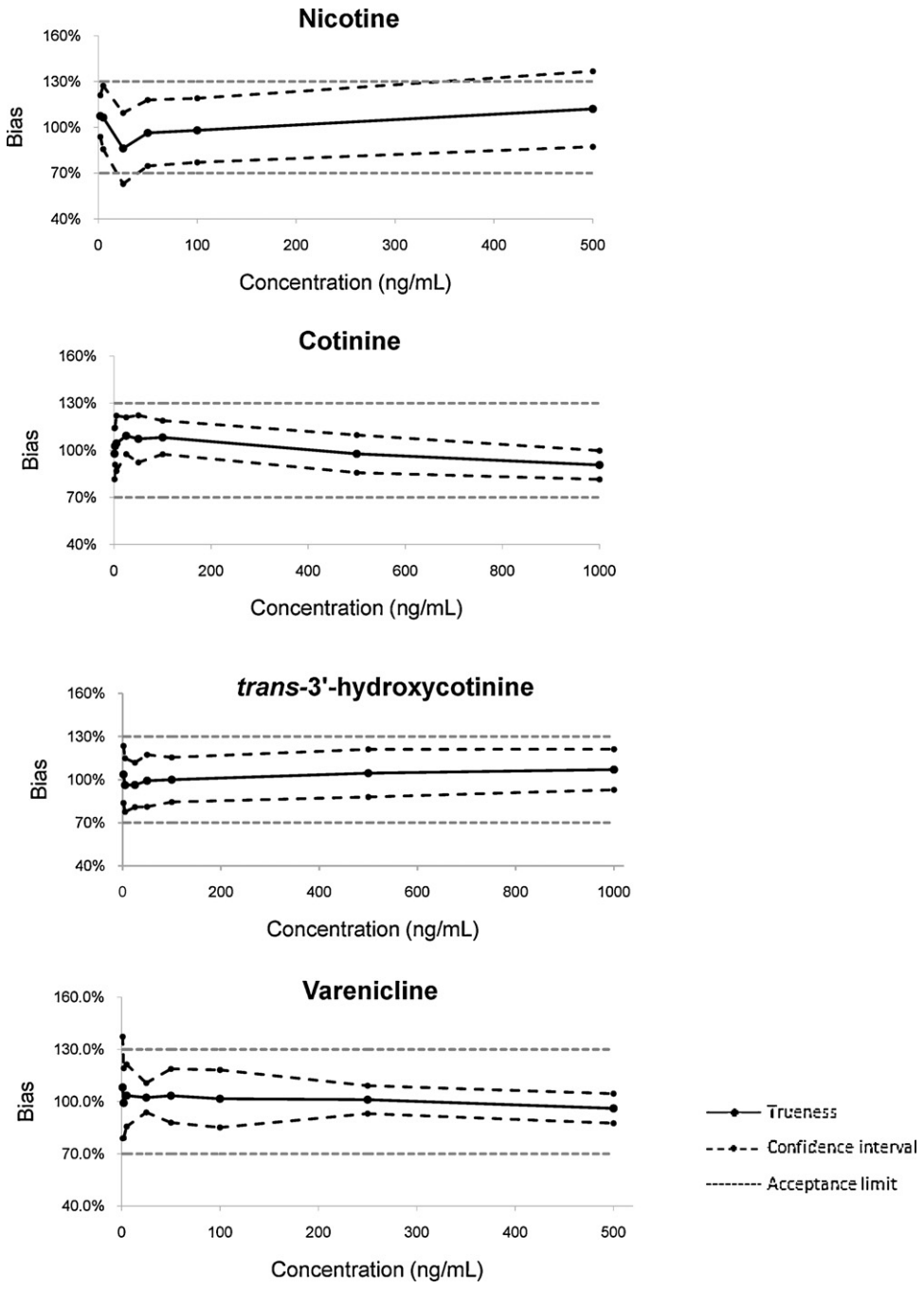

Fig. 2. Accuracy profiles for nicotine, metabolites and varenicline within the acceptance limit ($\lambda = \pm 30\%$), and with confidence interval ($\alpha = 0.05$).

The trueness, repeatability and intermediate precision of the back calculated QC samples are reported in Table 3. For all QC samples, the determined trueness met the acceptance criteria of ±15% (LLOQ ± 20%) with values between 86.2% and 113.6%. Moreover, repeatability and intermediate precision were within the required limits of ±15% (LLOQ ± 20%) with CVs within 1.9–12.3% and 4.4–15.9%, respectively. The accuracy profiles for each analyte in the dosing range are shown in Fig. 2. It has to be mentioned that the accuracy profile of nicotine at the concentration levels 25 and 500 ng/mL passed the ±30% limit, thus for this compound an acceptance limit of ±35% can be set, which is still suitable for the intended purpose of the analytical procedure [43].

To assess the linearity of the method, the following slopes 1.127, 1.069, 0.912 and 0.966, and intercepts −5.58, −3.694, 7.108 and 2.411 were obtained for nicotine, cotinine, *trans*-3′-hydroxycotinine and varenicline, respectively. The corresponding determination coefficients were 0.985, 0.993, 0.994 and 0.996, indicating that the procedure was linear for the tested compounds.

3.3.4. Stability

The stability of the analytes in plasma at room temperature and at 4 °C was ascertained up to 72 h. The variation over time of each analyte was within a range of ±15% of the initial concentration, with the exception of varenicline after 24 h at room temperature, which exceeded 15% but remained below 20% (18% of gain at low concentration). This indicates that the drugs are stable in plasma at room temperature and at 4 °C for at least three days. Furthermore, stability in plasma was equally demonstrated after three

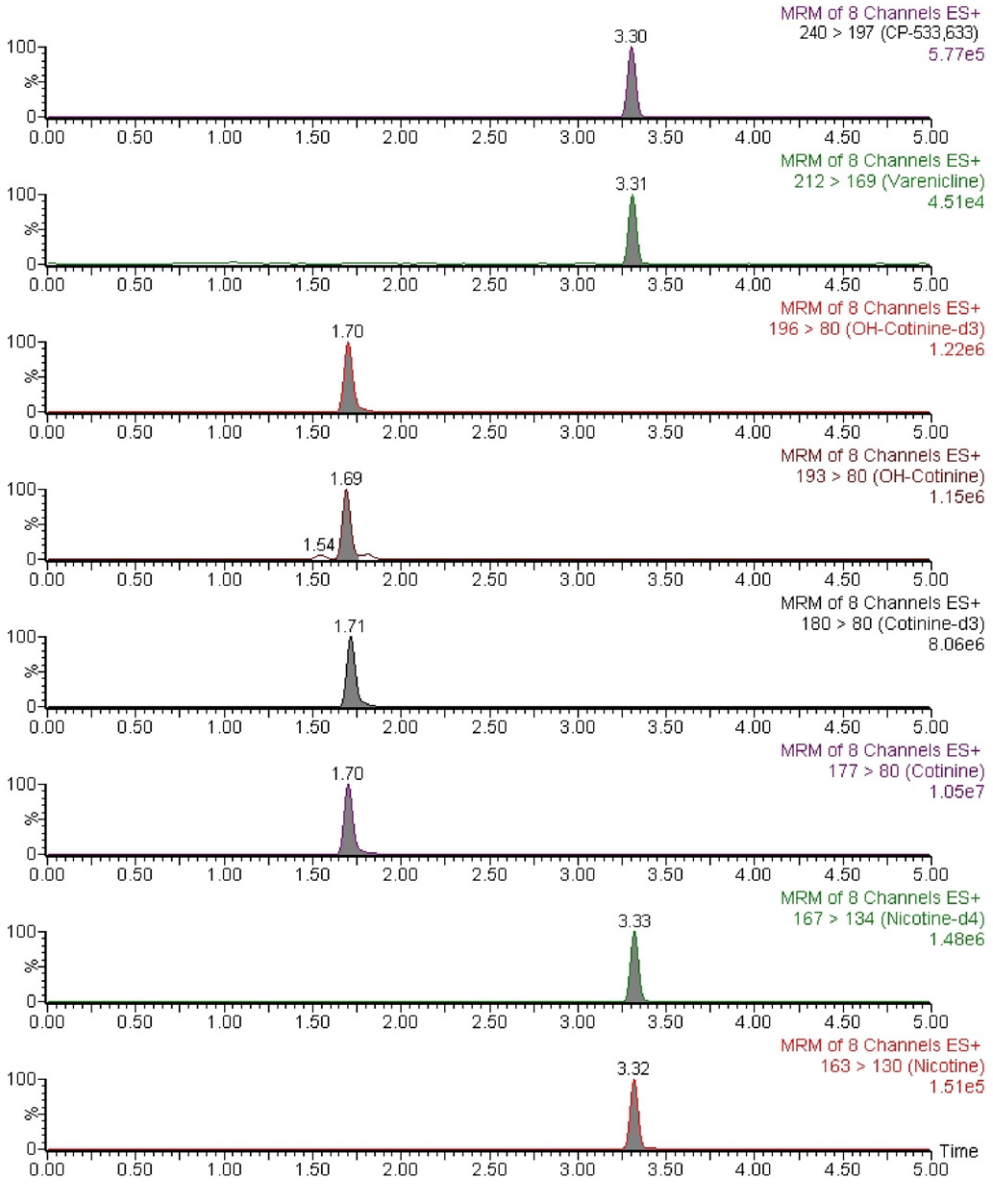

Fig. 3. Chromatogram of a participant's plasma at the beginning of the study, while smoking and having started varenicline treatment (nicotine 15 ng/mL, cotinine 185 ng/mL, *trans*-3′-hydroxycotinine 130 ng/mL and varenicline 9 ng/mL).

freeze and thaw cycles and long term storage stability at −20 °C was confirmed after one, three and eight months, with variations in drug levels comprised within ±15% of the initial concentrations. Additionally, the postpreparative stability of the compounds in the extracted samples, assessed by keeping the vials for 24 h and 48 h at 8 °C before reanalysis, yielded satisfactory results (below 12% of variation from the nominal value). Moreover, the CVs of all stability tests were below 14%. All results of the stability tests are presented in Table 4.

4. Clinical application

The analytical procedure was applied to determine plasma levels of the studied compounds in 194 subjects participating in a clinical study on smoking cessation [44]. The program consisted of a 3-month follow-up composed of smoking cessation counselling and pharmacological treatment prescription (nicotine replacement therapy or varenicline) and a 6-month conclusion visit. Blood sampling was performed at baseline when participants were still smoking, and served for the quantification of nicotine and its metabolites. One month after smoking cessation, blood sampling was used to quantify varenicline in subjects prescribed with this drug and to confirm abstinence in the same subjects. A last blood sample at the 6-month follow-up visit allowed confirmation of abstinence in all subjects that completed the study. Overall, 406 plasma samples were analyzed and results are presented in Table 5. All measured plasma concentrations were within the calibrated ranges and in accordance with plasma levels of these drugs previously reported in literature [6,33–35]. A representative chromatogram of a participant in the study, while smoking and who had already started varenicline treatment, is shown in Fig. 3 (nicotine 15 ng/mL, cotinine 185 ng/mL, *trans*-3′-hydroxycotinine 130 ng/mL and varenicline 9 ng/mL). These results are used to study the pharmacogenetics of varenicline and nicotine and to confirm abstinence

from smoking at the above mentioned time points in the clinical study.

5. Conclusion

A sensitive UPLC–MS/MS method using HILIC for the simultaneous quantification of nicotine, its metabolites cotinine and *trans*-3′-hydroxycotinine and varenicline was developed and validated according to the SFSTP and FDA guidelines. The drugs were extracted from plasma using a simple SPE procedure, which removed efficiently endogenous compounds from the matrix. The analytical procedure allows a fast quantification (in 3.4 min) of the four compounds over a usually measured concentration range, which was confirmed by applying the method to real samples of a smoking cessation study.

Conflict of interest

The authors declare no conflict of interest.

Role of the funding source

The funding sources have no role on the design, conduct, and reporting of the study or in the decision to submit the manuscript for publication.

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