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Journal of Chromatography B, 778 (2002) 251–261

JOURNAL OF CHROMATOGRAPHY B

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Simultaneous determination of nicotine and eight nicotine metabolites in urine of smokers using liquid chromatography–tandem mass spectrometry

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Abstract

A method based on liquid chromatography tandem mass spectrometry (LC–MSMS) applying atmospheric pressure chemical ionisation (APCI) in the positive ion mode was developed for the direct determination of nicotine, cotinine, *trans*-3′-hydroxycotinine, their corresponding glucuronide conjugates as well as cotinine-*N*-oxide, norcotinine, and nicotine-*N*′-oxide in the urine of smokers. The assay involves filtration of crude urine, fast liquid chromatography on a reversed-phase column and mass-specific detection using MSMS transitions. Deuterium-labeled nicotine, cotinine, and *trans*-3′-hydroxycotinine were used as internal standards. Glucuronides used as reference material were either chemically (cotinine-*N*-glucuronide) or enzymatically synthesized (nicotine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide). Precision for the major nicotine analytes at levels observable in urine of smokers was better than 10%. Accuracy expressed in recovery rates in urine matrix for nicotine, cotinine, *trans*-3′-hydroxycotinine, and cotinine-*N*-glucuronide ranged from 87 to 113%. Quantitative results for the three glucuronides in urine samples of 15 smokers were compared to an indirect method in which the aglycons were determined with gas chromatography and nitrogen-selective detection (GC–NPD) before and after enzymatic splitting of the conjugates. Good agreement was found for cotinine-*N*-glucuronide (coefficient of variation, CV: 9%) and *trans*-3′-hydroxycotinine-*O*-glucuronide (CV: 20%), whereas the accordance between both methods was moderate for nicotine-*N*-glucuronide (CV: 33%). The described LC–MSMS method allows the simultaneous determination of nicotine and eight of its major metabolites in urine of smokers with good precision and accuracy. Since the method requires a minimum of sample clean-up and a very short time for chromatography (3 min), it is suitable for determining the nicotine dose in large-scale human biomonitoring studies.
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Keywords: Nicotine; Cotinine; *trans*-3′-Hydroxycotinine; Nicotine-*N*′-oxide; Cotinine-*N*-oxide; Norcotinine

1. Introduction

Nicotine, the major alkaloid in tobacco, is rapidly and extensively metabolized in humans. The predominant pathway during first pass metabolism is *C*-oxidation of nicotine to yield cotinine, which is subsequently hydroxylated to *trans*-3′-hydroxycotinine [1,2] (Fig. 1). The occurrence of glucuronide conjugates of nicotine, cotinine, and *trans*-3′-hydroxycotinine in urine of smokers and nonsmokers exposed to environmental tobacco smoke (ETS)

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PII: S0378-4347(01)00451-0

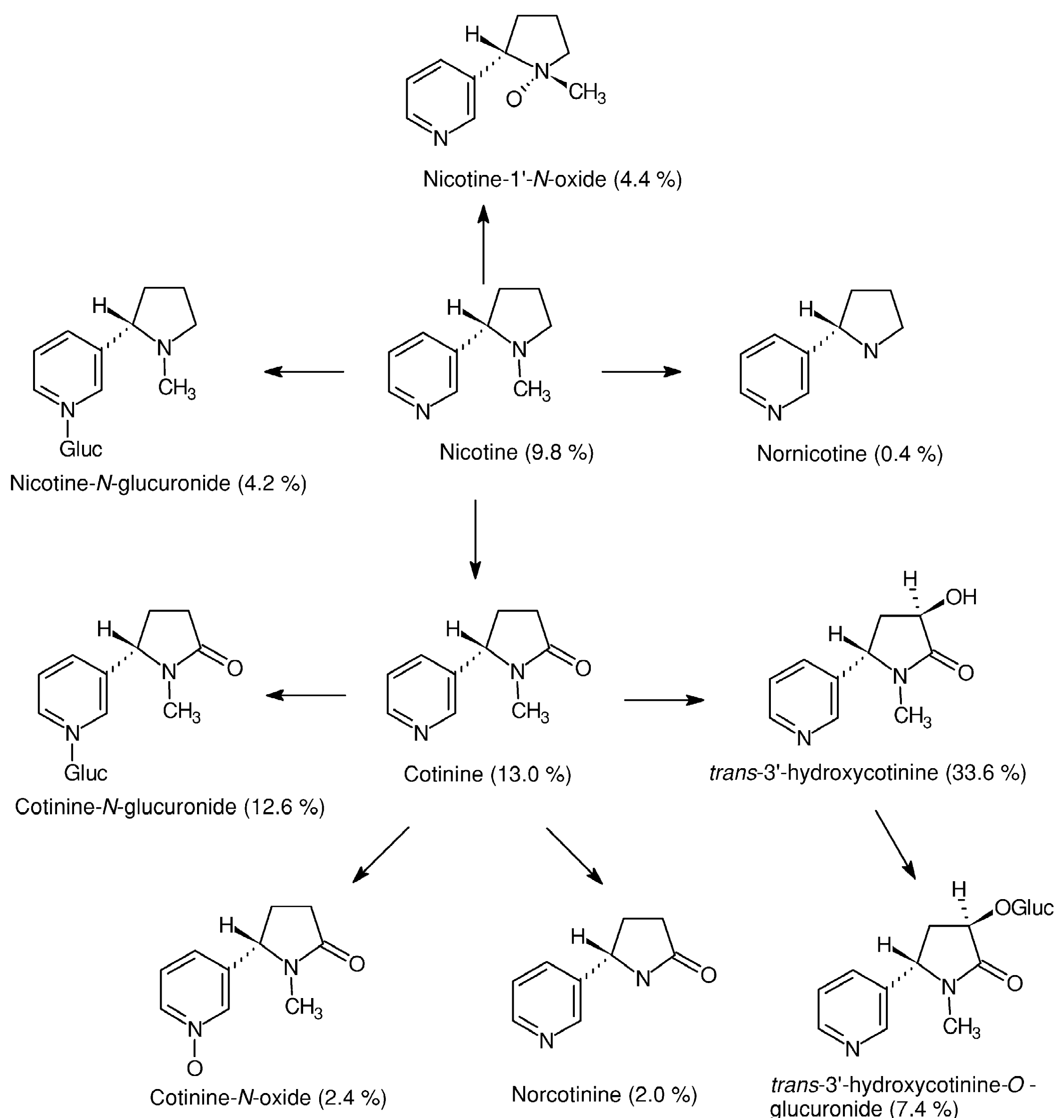

Fig. 1. Simplified scheme of the human nicotine metabolism according to Ref. [2], modified. Percentages in parentheses indicate the amount of excreted metabolite relative to the total amount of nicotine taken up.

reveals that phase II reactions are considerably involved in nicotine metabolism [3,4].

The determination of urinary nicotine, cotinine, *trans*-3′-hydroxycotinine, and their corresponding glucuronides accounts for approximately 80–85% of the total nicotine uptake [5,6]. As the extent of glucuronidation of nicotine and cotinine appears to be subject to genetic polymorphisms [7], the simultaneous analysis of nicotine-derived aglycons and their conjugates provides a detailed view on the pharmacokinetics of nicotine in humans, and the bioavailability of nicotine via various routes of administration.

Numerous papers on methods for the determination of nicotine and its metabolites in biological fluids have been published, including radioimmunoassays (RIA) (for review, see Ref. [8]), enzyme-linked immunosorbent assays (ELISA) [9], gas

chromatography (GC) (for review, see Ref. [10]), or high-performance liquid chromatography (HPLC) (for review see Ref. [11]). LC–MSMS methods for nicotine and nicotine metabolites in serum [12,13], saliva [14] and urine [15] have also been described. Nicotine-derived glucuronides in urine were commonly analysed by indirect methods comprising the quantitation of the aglycon before and after hydrolysis with β-glucuronidase [5,16–20]. While these methods provide acceptable estimates of the concentrations in urine, they are expensive, time and labour intensive, and error-prone due to incomplete hydrolysis of the conjugates. By the direct determination of the glucuronides, these disadvantages should be avoided. Byrd et al. [3] reported the direct determination of cotinine-*N*-glucuronide in urine using thermospray LC–MS.

We describe here a LC–MSMS method for the simultaneous determination of nicotine, cotinine, *trans*-3′-hydroxycotinine, and their glucuronides as well as norcotinine, cotinine-*N*-oxide, and nicotine-*N*′-oxide in urine of smokers. Deuterium-labeled analogues of nicotine, cotinine, and *trans*-3′-hydroxycotinine were used as internal standards. Urine samples were directly analyzed after filtration. The results were compared with those from an indirect method comprising the determination of the aglycon before and after splitting the conjugates with β-glucuronidase.

2. Experimental

2.1. Standards and reagents

Nicotine [(−)-nicotine, in methanol, 1 mg/ml], cotinine [(−)-cotinine, in methanol, 1 mg/ml] and (±)-nicotine-methyl-d_3 (deuterated internal standard, solid) were purchased from Sigma, Deisenhofen, Germany. *trans*-3′-Hydroxycotinine was purchased from Dr Georg Neurath (Contract Research, Hamburg, Germany). Cotinine-methyl-d_3, (±)-*trans*-3′-hydroxycotinine-methyl-d_3 (deuterated internal standards), norcotinine, nicotine-*N*′-oxide, cotinine-*N*-oxide, and cotinine-*N*-glucuronide were obtained from Toronto Research Chemicals, Ontario, Canada. The purity of all reference compounds commercially available was ≥98%. Ammonium acetate was supplied by Merck, Darmstadt, Germany. Methanol (HPLC grade) was purchased from Promochem, Wesel, Germany. Ultrapure water was prepared by a Seralpur Pro 90 C apparatus (Seral, Minden, Germany).

2.2. Enzymatic synthesis

Cotinine-*N*-glucuronide, nicotine-*N*-glucuronide, and *trans*-3′-hydroxycotinine-*O*-glucuronide were enzymatically synthesized using human liver microsomes. Briefly, enzymatic reactions were performed using 50 m*M* aglycon in 50 m*M* Tris/hydrochloric acid buffer pH 7.5, 1 m*M* cofactor UDPGA (uridine 5′-diphosphoglucuronic acid), 10 m*M* magnesium chloride, and 2% acetonitrile. The reaction mixture containing 100 μg of pooled human liver microsomes (NatuTec GmbH, Frankfurt/Main, Germany), activated with 2.5 μg of the pore-forming peptide alamethicin, was incubated for 3 h at 37°C. Enzymatic reactions were optimized using HFC (7-hydroxy-1-trifluoromethylcoumarin, NatuTec GmbH Frankfurt/Main) as substrate [21,22]. Ten incubation assays were pooled and microsomes were removed adding twice the volume of ice-cold methanol to precipitate proteins followed by 5-min centrifugation at 13 360 *g*. Methanol was evaporated in a stream of nitrogen (TurboVap, Zymark, Idstein, Germany). In the case of nicotine and cotinine glucuronide synthesis, the pH was adjusted to 10 by adding sodium hydroxide. After removal of the aglycon by extraction with dichloromethane (8×), the pH was adjusted to 7.5. Removal of *trans*-3′-hydroxycotinine was performed by extracting eight times with a mixture of dichloromethane and 2-propanol (3+1) at pH 7.5. Salts were removed by repeated precipitation with methanol. Glucuronides were purified by semi-preparative HPLC on a reversed-phase column with an octadecylsilyl-modified spherical silica carrier (Merck, Lichrospher RP 18, 250×8 mm, 100 Å pore size, 10 μm particle size). The average formation rate of the glucuronides was about 20 pmol/mg liver protein. Enzymatic glucuronidation of *trans*-3′-hydroxycotinine with liver microsomes yielded two different glucuronides which could be separated on the semi-preparative reversed-phase column. Only the more polar glucuronide revealed the same retention time and the same mass transition as the

glucuronide detected in the urine of smokers. This isomer has been identified as *trans*-3′-hydroxycotinine-*O*-glucuronide [23].

2.3. Primary and working stock solutions

The primary stock solutions for cotinine (1 mg/ml methanol) and nicotine (1 mg/ml methanol) were purchased from Sigma (Deisenhofen, Germany). *trans*-3′-Hydroxycotinine (200 μg/ml) was dissolved in 0.05 N hydrochloric acid; cotinine-*N*-glucuronide was prepared as aqueous solution (1 mg/ml). *trans*-3′-Hydroxycotinine-methyl-d_3, cotinine-methyl-d_3, and nicotine-methyl-d_3 (1 mg/ml) were prepared as aqueous solutions. Primary stock solutions were stored at −20°C and were found to be stable for more than a year.

The primary stock solutions of nicotine, cotinine, *trans*-3′-hydroxycotinine, and cotinine-*N*-glucuronide were diluted with water to working stock solutions with concentrations of 100, 10 and 1 μg/ml.

A mixed working stock solution of cotinine-methyl-d_3, nicotine-methyl-d_3, and *trans*-3′-hydroxycotinine-methyl-d_3 (50 μg/ml) was prepared in water.

All working stock solutions were stored refrigerated at 4°C. The enzymatically synthesized and purified glucuronides (only tiny amounts) were dissolved in water and stored at 4°C. Under these conditions, aqueous solutions of cotinine-*N*-glucuronide proved to be stable for more than 3 months.

2.4. Calibration standards

Quantitation was based on the internal standard method. Calibration standards were freshly prepared daily by spiking analyte-free nonsmoker urine (no detectable amounts of nicotine and cotinine, LOD (GC–NPD)≤2 ng/ml) with aliquots of working stock solutions of nicotine, cotinine, *trans*-3′-hydroxycotinine, and cotinine-*N*-glucuronide yielding a calibration range of 10–5000 ng/ml. The internal standard mix was added to each calibration level (1000 ng/ml urine).

2.5. Spiked urine samples for validation

Analyte-free human urines were spiked with three levels (100, 1000 and 5000 ng/ml) of nicotine, cotinine, *trans*-3′-hydroxycotinine, and cotinine-*N*-glucuronide as well as with the internal standards (1000 ng/ml). Samples were centrifuged and filtered as described in Section 2.6.

2.6. Sample preparation

Twenty-four hour urine samples from 15 smokers were stored at −20°C in polypropylene bottles. Urine samples were thawed overnight at 4°C and thoroughly mixed. Aliquots were transferred into plastic tubes and the internal standards were added to yield a final concentration of 1000 ng/ml. The tubes were mixed for 2 min using a VWR multi-tube vortexer (Pabisch GmbH, Munich, Germany). The samples were centrifuged at 13 360 *g* for 15 min (Hermle Z 230 M, Gosheim, Germany). The supernatants were filtered through a 0.22-μm Millex syringe driven filter unit (Millipore, Eschborn, Germany). The filtrates were transferred into amber glass vials (32×11 mm, Agilent Technologies, Waldbronn, Germany). Aliquots of 1 μl were injected into the LC–MSMS system by means of an autosampler.

2.7. LC–MSMS analysis

Liquid chromatography (LC) was performed using a HP 1100 system including a binary pump, an automatic sampler, and a column oven (Agilent Technologies, Waldbronn, Germany). Separation of analytes was performed on a Synergy MAX RP (dodecylsilyl modified silica, endcapped, 80 Å pore size, 4 μm particle size, 150×4.6 mm I.D.) analytical column with a guard column (MAX RP, 4 μm particle size, 4×3 mm I.D., Phenomenex, Aschaffenburg, Germany), operated at 45°C oven temperature. Analytes were eluted isocratically, using 10 m*M* aqueous ammonium acetate (pH 6.8)/methanol (binary solvent system, 20:80, v/v) at a flow-rate of 1 ml/min. The total time for chromatography was less than 3 min.

The LC was connected to a PE Sciex API 2000 Tandem mass spectrometer (Applied Biosystems,

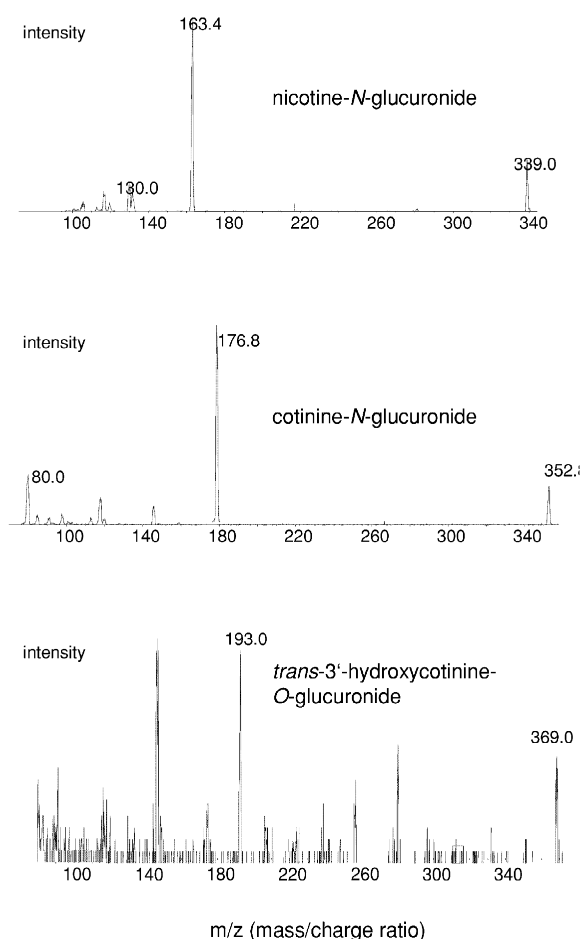

Fig. 2. Full scan ion-spray mass spectra of nicotine-derived glucuronide conjugates. Nicotine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide have been synthesized enzymatically as described in Section 2.2.

Langen, Germany) operated in the positive atmospheric pressure chemical ionisation mode (APCI, heated nebulizer source). The nebulizer heater was maintained at 450°C with the nebulizer current (corona discharge current) at 2 μA. Nitrogen was used as nebulizing, auxiliary, and curtain gas at 413, 103, and 379 kPa, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using nitrogen as collision gas. The collision energy ranged from 19 to 49 V. Ten mass/charge (m/z) ion transitions were recorded in multiple reaction monitoring (MRM) mode: m/z 193 $[M\text{-Gluc}+H]^+ \rightarrow m/z$ 80 (*trans*-3′-hydroxycotinine-*O*-glucuronide); m/z 193 $[M+H]^+ \rightarrow m/z$ 80 (*trans*-3′-hydroxycotinine); m/z 196 $[M+H]^+ \rightarrow m/z$ 80 (*trans*-3′-hydroxycotinine-methyl-d_3); m/z 177 $[M\text{-Gluc}+H]^+ \rightarrow m/z$ 80 (cotinine-*N*-glucuronide); m/z 177 $[M+H]^+ \rightarrow m/z$ 80 (cotinine); m/z 180 $[M+H]^+ \rightarrow m/z$ 80 (cotinine-methyl-d_3); m/z 193 $[M+H]^+ \rightarrow m/z$ 96 (cotinine-*N*-oxide); m/z 163 $[M+H]^+ \rightarrow m/z$ 80 (norcotinine); m/z 163 $[M\text{-Gluc}+H]^+ \rightarrow m/z$ 130 (nicotine-*N*-glucuronide); m/z 163 m/z $[M+H]^+ \rightarrow m/z$ 130 (nicotine); 166 $[M+H]^+ \rightarrow m/z$ 80 (nicotine-methyl-d_3); m/z 179 $[M+H]^+ \rightarrow m/z$ 132 (nicotine-*N*-oxide); m/z 149 $[M+H]^+ \rightarrow m/z$ 80 (nornicotine). The retention times (min) were as follows: 1.50 (*trans*-3′-hydroxycotinine-*O*-glucuronide), 1.75 (*trans*-3′-hydroxycotinine), 1.75 (*trans*-3′-hydroxycotinine-methyl-d_3), 1.58 (cotinine-*N*-glucuronide), 1.73 (cotinine-*N*-oxide), 1.79 (norcotinine), 1.87 (cotinine), 1.87 (cotinine-methyl-d_3), 1.66 (nicotine-*N*-glucuronide), 1.82 (nicotine-*N*-oxide), 2.08 (nornicotine), 2.49 (nicotine), and 2.47 (nicotine-methyl-d_3). The variation of the retention times was ≤0.02 min.

2.8. Indirect method for the determination of glucuronide conjugates

Urine samples were enzymatically hydrolysed with β-glucuronidase according to a modified method of Curvall et al. [5], using an incubation time of 18 h. Before and after enzyme hydrolysis, nicotine and cotinine were determined according to Feyerabend and Russell [24] and *trans*-3′-hydroxycotinine according to Jacob et al. [25]. Gas chromatography with nitrogen-selective detection (GC–NPD) was used for both methods.

3. Results

3.1. Chromatography and mass-selective detection

Urine aliquots of smokers were centrifuged and micro-filtered. The injection volume was 1 μl. More than 3000 samples were run on the same analytical column without any loss in resolution or sensitivity.

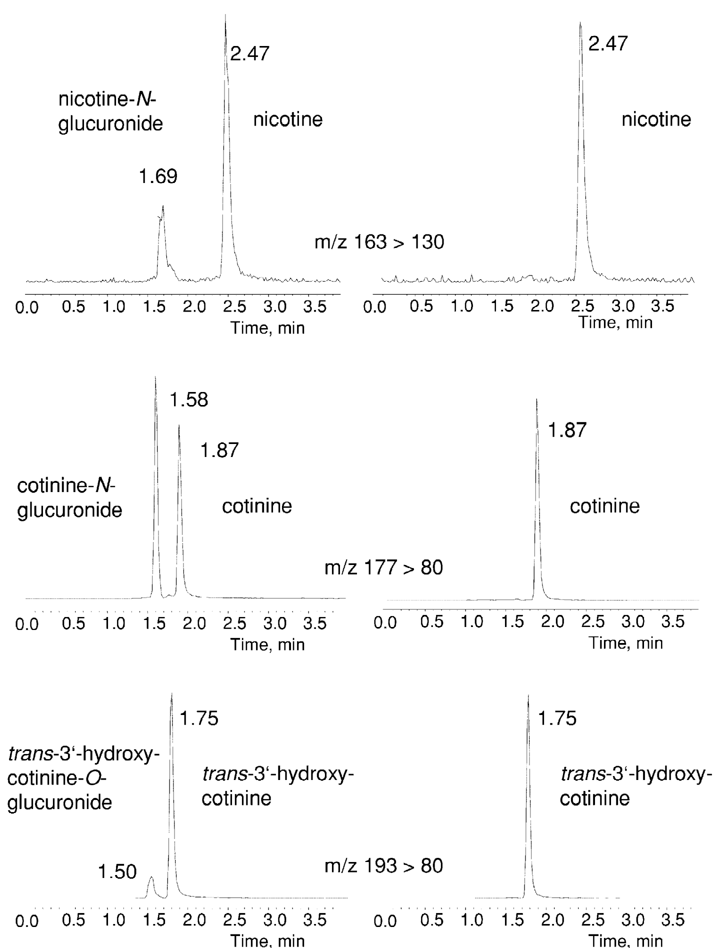

Fig. 3. Ion chromatograms of a urine sample from a smoker: Ion transitions for nicotine, cotinine, and *trans*-3′-hydroxycotinine before (left panel) and after (right panel) hydrolysis with β-glucuronidase.

The guard column was changed after approximately 300 runs.

Full scan ion-spray mass spectra of nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, and *trans*-3′-hydroxycotinine-*O*-glucuronide are shown in Fig. 2. These three conjugates were identified by their respective protonated molar ions $[M+H]^+$ m/z 339, 353, and 369. Depending on the parameters used during the ionisation process, all glucuronides formed adducts with sodium and potassium, $[M+Na]^+$, $[M+K]^+$, $[M-H+2Na]^+$, or $[M-H+Na+K]^+$ (data not shown), confirming the expected molecular mass of the analyte. However, even under moderate ionisation conditions, fragmentation occurred by neutral loss of m/z 176, resulting in the aglycon ions $[M\text{-}Gluc+H]^+$ m/z 163, 177, and 193, representing nicotine, cotinine and *trans*-3′-hydroxycotinine, respectively.

Under atmospheric pressure chemical ionisation (APCI), all three glucuronides proved unstable and completely decomposed to their corresponding protonated aglycons ions of nicotine (m/z 163), cotinine (m/z 177), and *trans*-3′-hydroxycotinine (m/z 193). Optimized chromatographic conditions led to both best signal-to-noise ratios and sufficient resolution on an endcapped dodecylated silica phase column (Synergy MAX RP). Conjugated and free form metabolites were eluted with baseline resolution

(Fig. 3). Matrix-dependent variations in extent of chemical ionisation were controlled for by the deuterated internal standards of nicotine, cotinine, and *trans*-3′-hydroxycotinine.

In order to investigate the interference with minor nicotine metabolites, we studied the mass transitions and retention times of norcotinine, cotinine-*N*-oxide, nornicotine, and nicotine-*N*′-oxide (Fig. 4). Norcotinine, which is isobaric to nicotine, shows two signals in urine samples at the ion transitions m/z 163→m/z 80 at 1.79 min (norcotinine) and 2.49 min (nicotine), whereas nicotine (m/z 163→m/z 130) reveals no interference at 1.79 min. Cotinine-*N*-oxide (isobaric to *trans*-3′-hydroxycotinine) was registered at 1.73 min by monitoring the specific reaction m/z 193→m/z 96. Partial decomposition during APCI (loss of oxygen, $[M\text{-}16+H]^+$) accounts for 4% and releases gas phase cotinine, indicated by the mass transition m/z 177→m/z 80. Due to different retention times, cotinine-*N*-oxide can be separated from cotinine (1.73 vs. 1.87 min). Nicotine-*N*′-oxide was eluted at 1.82 min (m/z 179→m/z 132). Nornicotine was not detected in the urine samples investigated.

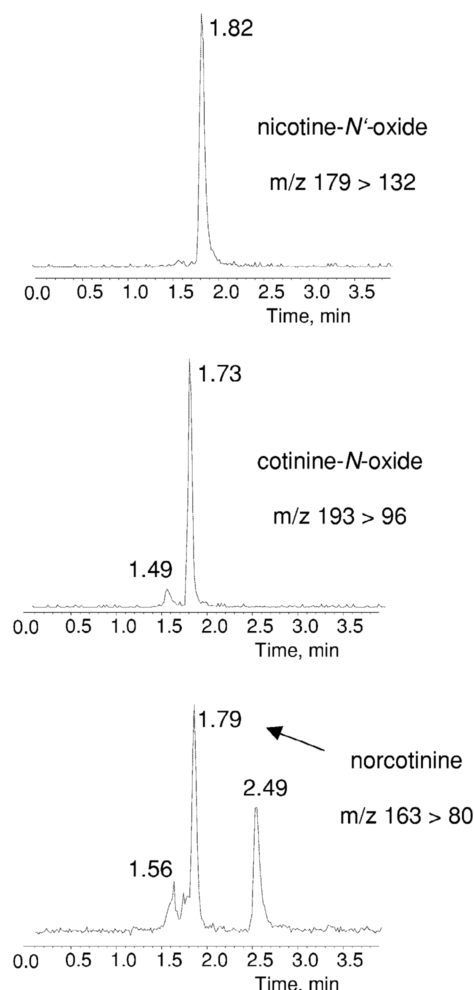

Fig. 4. Same urine sample as in Fig. 3. Ion transitions for nicotine-*N*′-oxide, cotinine-*N*-oxide, and norcotinine.

3.2. Characteristics of the method

Intra- and *inter*-day precision as well as the accuracy in spiked urine samples for the six major nicotine metabolites are shown in Table 1. Precision for all analytes was better than 10%, except for nicotine-*N*-glucuronide at the low level. Recovery rates for the three major aglycons and cotinine-*N*-glucuronide ranged from 87 to 113%. The recovery for the enzymatically synthesized glucuronides of nicotine and *trans*-3′-hydroxycotinine was not determined.

The linear through zero regression lines for peak area ratios (analyte/deuterated internal standard) plotted against the concentration fitted well ($r>0.999$) for nicotine, cotinine, *trans*-3′-hydroxycotinine, and cotinine-*N*-glucuronide (vs. cotinine-methyl-d_3) in the range of 10–5000 ng/ml. Calibration curves for nicotine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide were constructed by plotting directly measured glucuronide/aglycon-d_3 area ratios against concentration of glucuronide indirectly determined by LC–MSMS by measuring the aglycons before and after enzyme hydrolysis. Linear through zero regression lines for nicotine-*N*-glucuronide ($r=0.9301$) and *trans*-3′-hydroxycotinine-*O*-glucuronide ($r=0.9543$) fitted well.

For norcotinine, cotinine-*N*-oxide, nicotine-*N*′-oxide, and nornicotine, the peak areas of aqueous standard solutions were plotted against four calibration levels (10, 100, 500, and 1000 ng/ml) and showed an excellent linear fit ($r>0.999$).

The limit of detection (LOD, signal-to-noise ratio 3:1) in urine for nicotine and the eight nicotine-derived metabolites was about 0.06 nmol/ml.

Table 1
Precision (expressed as coefficient of variation, CV) and recovery rate for the determination of nicotine, cotinine, and *trans*-3′-hydroxycotinine and their glucuronides in urine

Analyte/level (nmol/ml)	*Intra*-day ($n=6$) CV%	*Inter*-day[a] ($n=6$) CV%	Recovery ($n=6$) (%)
Nicotine			
High (18.5)	3.9	4.0	92
Middle (12.3)	3.2	6.0	92
Low (0.3)	4.8	4.5	95
Cotinine			
High (11.4)	4.1	2.8	104
Middle (5.7)	6.9	5.6	104
Low (0.6)	4.0	5.2	109
trans-3′-Hydroxycotinine			
High (39.8)	1.8	3.9	87
Middle (28.4)	3.8	3.2	98
Low (2.8)	3.7	4.1	113
Nicotine-N-glucuronide			
High (6.0)	4.6	9.1	
Middle (4.1)	7.8	7.1	n.d.[b]
Low (0.1)	5.7	18.0	
Cotinine-N-glucuronide			
High (22.0)	4.7	1.7	n.d.
Middle (16.4)	5.3	7.1	89
Low (0.9)	3.6	6.4	n.d
trans-3′-Hydroxycotinine-O-glucuronide			
High (19.7)	2.7	5.4	
Middle (14.4)	2.0	8.3	n.d.
Low (0.4)	4.7	7.8	

[a] On five subsequent days.
[b] Not determined.

3.3. Application to urine samples of smokers

The described LC–MSMS method was applied to urine samples of 15 smokers. For cross validation, the results were compared to those obtained independently with a GC–NPD method (see Section 2.8). In this method, only cotinine has been fully validated. Intra-day ($n=9$) and inter-day ($n=15$) precision was 1.7 and 4.6% at a concentration level of 2.2 nmol/ml, and the recovery rate ($n=9$) was 93%. Similar data have been calculated for nicotine and *trans*-3′-hydroxycotinine. As for the three glucuronides determined indirectly, precision data ranged from 4.7 to 10.0% (cotinine-*N*-glucuronide), 3.9–12.5% (*trans*-3′-hydroxycotinine-*O*-glucuronide), and 13.5–27.5% (nicotine-*N*-glucuronide).

Data obtained with both methods correlated well for the aglycons nicotine ($r=0.994$), cotinine ($r=0.993$), and *trans*-3′-hydroxycotinine ($r=0.998$). Correlation plots (LC–MSMS vs. GC–NPD method) for the glucuronides are shown in Fig. 5. The correlation between both methods was good for cotinine-*N*-glucuronide ($r=0.972$) and *trans*-3′-hydroxycotinine-*O*-glucuronide ($r=0.952$), but only moderate for nicotine-*N*-glucuronide ($r=0.755$). The corresponding mean coefficients of variation were 9% (range 1.3–14.9), 20% (3.2–36.1), and 33% (1.3–95.1), respectively.

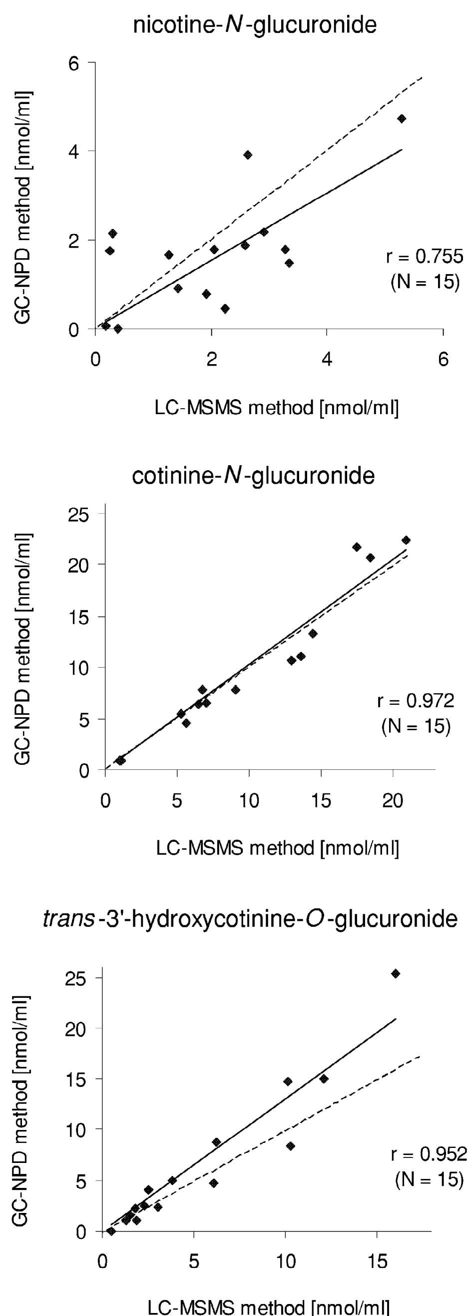

Fig. 5. Linear regression between the indirect (GC–NPD) and direct (LC–MSMS) method for the determination of nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, and *trans*-3′-hydroxy-cotinine-*O*-glucuronide. The solid lines are the regression through zero lines. The dashed lines represent the 1:1-lines (indicating ideal agreement between both methods).

Table 2 shows the absolute and relative concentrations of nicotine and eight nicotine metabolites in urine samples of five smokers. For comparison, the relative amounts of these urinary metabolites in smokers and snuffers as reported in six other studies [2,3,5,17,19,20] are also shown. Our results are in good agreement with those reported in the literature.

4. Discussion

Nicotine is extensively metabolised in the human body [26] (Fig. 1). In order to obtain reliable dosimetric data on the nicotine uptake by smoking or other routes of application, at least 80–90% of the nicotine dose taken up should be assessed in the form of the major urinary metabolites. Previous studies [2,3,5,17,19,20] have shown that the urinary metabolites nicotine, cotinine, and *trans*-3′-hydroxy-cotinine together with their corresponding glucuronides represent about 80–85% of the amount of nicotine taken up. Nicotine-*N*′-oxide, cotinine-*N*-oxide, and norcotinine represent another 8–12%. Thus nicotine and the eight nicotine metabolites included in our method make up 90% (or more) of the nicotine dose. The percentage of the glucuronide conjugates of nicotine, cotinine, and *trans*-3′-hydroxycotinine amounts to almost 30% and, therefore, represents a major part of the urinary nicotine metabolites. In earlier studies of the urinary nicotine metabolites profile [5,16–20], the glucuronides were determined indirectly by measuring the aglycons before and after hydrolysis with β-glucuronidase. Our goal was to determine the major nicotine metabolites including the glucuronides in urine in one HPLC run thus circumventing the major disadvantages of the indirect methods (time consuming, expensive and imprecise at low glucuronide levels).

Since no sample extraction is required, variations due to different physico-chemical properties of the analytes like polarity, solubility, and surface activity, are absent. The direct determination of glucuronides further reduces the methodological variation compared to the indirect method which includes two separate measurements for one glucuronide. In particular, the indirect determination of low urinary glucuronide levels in the presence of moderate to high levels of the aglycon is imprecise.

Table 2
Concentrations (nmol/ml) of nicotine and eight nicotine metabolites in urine of five smokers

	NIC	NIC-N-Gluc	NIC-N′-Ox	COT	COT-N-Gluc	COT-N-Ox	Nor-COT	THC	THC-O-Gluc	Sum
Subject 1	2.3	0.5	3.8	8.3	4.6	1.0	0.7	26.1	5.0	52.3
	(4.4)	(1.0)	(7.3)	(15.9)	(8.8)	(1.9)	(1.3)	(49.9)	(9.6)	(100)
Subject 2	4.0	3.1	4.2	5.1	6.3	0.6	0.3	3.2	0.6	27.4
	(14.6)	(11.3)	(15.3)	(18.6)	(23.0)	(2.2)	(1.1)	(11.7)	(2.2)	(100)
Subject 3	10.2	3.7	6.3	15.7	31.0	2.1	1.3	41.5	8.4	120.2
	(8.5)	(3.1)	(5.2)	(13.1)	(25.8)	(1.7)	(1.1)	(34.5)	(7.0)	(100)
Subject 4	6.0	1.0	2.6	7.6	6.3	0.9	0.6	16.1	2.3	43.4
	(13.8)	(2.3)	(6.0)	(17.5)	(14.5)	(2.1)	(1.4)	(37.1)	(5.3)	(100)
Subject 5	6.5	2.9	3.6	9.6	13	2.0	1.0	17.1	6.2	61.9
	(10.5)	(4.7)	(5.8)	(15.5)	(21.0)	(3.2)	(1.6)	(27.6)	(10.0)	(100)
Mean ($N=5$) (%)	9.5	3.7	6.7	15.2	20.1	2.2	1.3	34.1	7.4	
Studies from the literature (data are mean molar percentages)										
Curvall et al. [5] $N=15$	10–15[a]		5	20–25		3	n.d.	50–60		
Byrd et al. [17] $N=11$	10.0	3.0	7.0	13.0	17.0	4.0	2.0	35.0	9.0	
Byrd et al. [3] $N=4$	8.0	1.0	4.0	13.0	17.0	2.0	1.0	43.0	10.0	
Benowitz et al. [2] $N=12$	10.4±4.4	4.6±2.9	3.7±0.9	13.3±3.1	15.8±7.8	4.5±1.5	n.d.	39.1±12.5	7.8±5.9	
Byrd et al. [19] $N=33$	10.4–22.9		3.7–6.0	26.6–30.2		3.1–4.6	0.8–1.5	40–53.3		
Byrd et al. [20] $N=72$	14.4±6.8		12.2±8.5	25.4±7.1		3.8±1.4	1.1±0.7	43.1±12.2		

Molar percentages in parentheses. The mean relative molar amounts are compared with six reports from the literature. NIC, nicotine; NIC-*N*-Gluc, nicotine-*N*-glucuronide; NIC-*N′*-Ox, nicotine-*N′*-oxide; COT, cotinine; COT-*N*-Gluc, cotinine-*N*-glucuronide; COT-*N*-Ox, cotinine-*N*-oxide; Nor-COT, norcotinine; THC, *trans*-3′-hydroxycotinine; THC-*O*-Gluc, *trans*-3′-hydroxycotinine-*O*-glucuronide; n.d., not determined.

[a] Ranges or means±S.D. covering two cells indicate the sum of free and conjugated metabolite.

Our LC–MSMS method was validated for nicotine, cotinine, *trans*-3′-hydroxycotinine, and their glucuronides (Table 1), using the corresponding deuterated (methyl-d_3) free bases as internal standards for both the aglycon and the conjugate. Deuterated glucuronides would be preferable as internal standards for the conjugates in order to minimize matrix effects. (The enzymatic synthesis of the required deuterated glucuronides is in preparation in our laboratory.) Preliminary results indicate that the recovery of the glucuronides and aglycons is different in concentrated urine samples, leading to an underestimation of the glucuronide concentration. Adjustment for urinary density or creatinine concentration would resolve this problem. Precision and recovery rates of the method for the six major glucuronides are good. The LOD for these analytes (10–20 ng/ml) is sufficient for the determination of urine samples of tobacco users or users of nicotine replacement therapies. The quantitation of the nicotine dose in passive smokers requires a modified method which is presently being developed in our laboratory.

Levels of glucuronides in urine of smokers determined by the (direct) LC–MSMS method were in good (cotinine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide) or moderate agreement (nicotine-*N*-glucuronide) with the results of the (indirect) GC–NPD method (Fig. 5). We suggest that the less favourable results for nicotine-*N*-glucuronide are mainly due to the much lower urinary levels of this conjugate. Nevertheless, the validation data available so far show that LC–MSMS is the superior assay.

Nicotine-*N′*-oxide, cotinine-*N*-oxide, and norcotinine were quantified by an external standard method. Our results (Table 2) indicate that these three metabolites represent about 10% of the urinary nicotine metabolites. This finding corresponds to published data [2,3,5,17,19,20]. A deuterated internal standard for these minor nicotine metabolites may improve the analytical precision.

In conclusion, with the described LC–MSMS method, nicotine and eight nicotine-derived metabolites can simultaneously be determined in urine of smokers or users of other nicotine products. The assessment of about 90% of the total nicotine dose by measuring these urinary metabolites provides exposure dose information which is virtually independent of genetically-based *inter*-individual variations in the nicotine metabolism. Since the method is extremely fast, requiring minimum efforts for sample clean-up and only about 3 min for chromatography, it allows a high sample throughput. In addition, compared to the commonly used indirect method for glucuronide determination, the direct LC–MSMS method is less expensive (requiring no enzymes for conjugate splitting) and more precise.

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