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Determination of nicotine, cotinine, and related alkaloids in human urine and saliva by automated in-tube solid-phase microextraction coupled with liquid chromatography–mass spectrometry

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ABSTRACT

A simple, rapid and sensitive method for the determination of nicotine, cotinine, nornicotine, anabasine, and anatabine in human urine and saliva was developed. These compounds were analyzed by on-line in-tube solid-phase microextraction (SPME) coupled with liquid chromatography–mass spectrometry (LC–MS). Nicotine, cotinine and related alkaloids were separated within 7 min by high performance liquid chromatography (HPLC) using a Synergi 4u POLAR-RP 80A column and 5 mM ammonium formate/methanol (55/45, v/v) as a mobile phase at a flow-rate of 0.8 mL/min. Electrospray ionization conditions in the positive ion mode were optimized for MS detection of these compounds. The optimum in-tube SPME conditions were 25 draw/eject cycles with a sample size of 40 μL using a CP-Pora PLOT amine capillary column as the extraction device. The extracted compounds could be desorbed easily from the capillary by passage of the mobile phase, and no carryover was observed. Using the in-tube SPME LC–MS method, the calibration curves were linear in the concentration range of 0.5–20 ng/mL of nicotine, cotinine and related compounds in urine and saliva, and the detection limits (S/N = 3) were 15–40 pg/mL. The method described here showed 20–46-fold higher sensitivity than the direct injection method (5 μL injection). The within-run and between-day precision (relative standard deviations) were below 4.7% and 11.3% ($n = 5$), respectively. This method was applied successfully to analysis of urine and saliva samples without interference peaks. The recoveries of nicotine, cotinine and related compounds spiked into urine and saliva samples were above 83%, and the relative standard deviations were below 7.1%. This method was used to analyze urinary and salivary levels of these compounds in nicotine intake and smoking.

1. Introduction

The use of tobacco products may be most critical public health problem [1]. It is well known that tobacco smoke is a major cause of mortality and morbidity [2]. More than 4000 compounds have been identified in tobacco smoke, at least 50 of which have been shown to be carcinogenic [3]. Epidemiological studies in smokers have indicated a dose-response relationship between the number of cigarettes smoked per day and the risk of developing certain smoking-related diseases [4]. Tobacco alkaloids are the active principal components in all tobacco products. Among more than 20 different alkaloids found in tobacco, nicotine is the most abundant (98% of the total alkaloids) and accounts for widespread human use of tobacco products throughout the world because of its addictive properties. Furthermore, nicotine is the major pharmacologically active substance in tobacco [5] and is suspected to contribute to human diseases, such as cardiovascular and reproductive disorders [6,7]. There is good evidence than most smokers are dependent on nicotine and that the severity of tobacco dependence may be related to the level of nicotine intake. The minor alkaloids, including nornicotine, anabasine, and anatabine are also pharmacologically active. However, they are less potent than nicotine [8]. Nicotine is absorbed rapidly in humans through the skin and mucosal lining of the mouth and nose or by inhalation in the lungs, and exerts a number of physiological effects in both active and passive smokers, defined as cigarette smokers and non-smokers exposed to environmental tobacco smoke. It is estimated that an average of 70–80% of the nicotine absorbed by a smoker is metabolized to cotinine [9]. Nicotine and its metabolite cotinine can be measured in various biological fluids, including blood, saliva, and urine [10,11]. Therefore, these compounds have been widely used as biological markers to determine tobacco smoking status and estimate exposure to environmental tobacco smoke [10–14]. Serum nicotine and urinary/saliva cotinine have

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also been used to guide the dose of nicotine replacement therapy [15,16].

Nicotine, cotinine, and related alkaloids in various biological fluids have been determined by radioimmunoassay [17], enzyme immunoassay [18,19], gas chromatography (GC) [20,21], GC with mass spectrometry (GC–MS) [20–22], high performance liquid chromatography (HPLC) [20], and LC with tandem mass spectrometry (LC–MS–MS) [23–29]. The immunological methods are sensitive but have cross-reactivity with nicotine and related compounds, thus leading to overestimation. LC–MS–MS can provide a sensitive and selective means for comprehensive measurement of nicotine and its metabolites. However, most of the above methods generally require time-consuming sample preparation procedures, such as liquid–liquid extraction or solid-phase extraction, to remove coexisting substances in biological samples prior to analysis except for some LC–MS–MS methods [24,26].

In-tube solid-phase microextraction (SPME) [30], using an open tubular fused-silica capillary with an inner surface coating as the SPME device is simple and can be coupled easily on-line with HPLC and LC–MS. In-tube SPME allows convenient automation of the extraction process, which not only reduces the analysis time, but also provides better precision and sensitivity than manual off-line techniques. We recently developed an in-tube SPME method for the determination of urinary drugs [31,32] and salivary cortisol [33] by coupling with LC–MS. The details of the in-tube SPME technique and its applications have also been summarized in a number of reviews [34–36]. Here, we report an automated on-line in-tube SPME LC–MS method for simultaneous determination of nicotine, cotinine, and related alkaloids in urine and saliva samples. These samples can be obtained easily and salivary cotinine level has been reported to be an especially good indicator of plasma cotinine concentration [37]. Using this method, we also analyzed the changes in urinary and salivary levels of these compounds in nicotine intake and tobacco smoking.

2. Experimental

2.1. Materials

Nicotine, cotinine, nornicotine, and anabasine were purchased from Sigma–Aldrich Japan (Tokyo, Japan) and anatabine was purchased from Toronto Research Chemicals (North York, ON, Canada). Each compound was dissolved in methanol to make a stock solution at a concentration of 1 mg/mL. Each solution was stored at 4 °C and diluted to the required concentrations with pure water prior to use. LC–MS grade methanol and distilled water used as mobile phases were purchased from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Instrument and analytical conditions

The LC–MS system was a Model 1100 series LC coupled with an atmospheric pressure electrospray ionization (ESI) MS (Agilent Technologies, Boeblingen, Germany). A Synergi 4u POLAR-RP 80A column (150 mm × 4.6 mm i.d., particle size of 2.5 μm) from Phenomenex (Torrance, CA, USA) was used for LC separation. LC conditions were as follows: column temperature, 30 °C; mobile phase, 5 mM ammonium formate/methanol (55/45, v/v) and flow-rate, 0.8 mL/min (during the in-tube SPME treatment, the flow-rate was set to 0.2 mL/min to save mobile phase solution). ESI–MS conditions were as follows: nebulizer gas, N_2 (55 psi); drying gas, N_2 (12 L/min, 350 °C); fragmenter voltage, 110 V; capillary voltage, 2500 V; ionization mode, positive mode; mass scan range, 60–200 amu; selected ion monitoring (SIM), m/z 149 (nornicotine), m/z 161 (anatabine), m/z 163 (nicotine and anabasine), and m/z 177 (cotinine) and dwell times for the ions in SIM, 144 ms. LC–MS data were processed using an HP ChemStation (Hewlett–Packard, Palo Alto, CA, USA).

2.3. In-tube solid-phase microextraction

A CP-Pora PLOT amine capillary column (60 cm × 0.32 mm i.d., 10 μm film thickness; Varian Inc., Lake Forest, CA, USA) was used as the in-tube SPME device. The column was placed between the injection loop and injection needle of the autosampler, and the injection loop was retained in the system to avoid fouling of the metering pump. Capillary connections were facilitated by use of a 2.5-cm sleeve of 1/16-in polyetheretherketone (PEEK) tubing at each end of the capillary (1 in ≈2.54 cm). A PEEK tubing (330 μm i.d.) was found to be suitable to accommodate the capillary used. Normal 1/16-in stainless steel nuts, ferrules, and connectors were then used to complete the connections. The autosampler software was programmed to control the in-tube SPME extraction, desorption, and injection. Vials (2 mL) were filled with 1.0 mL of sample for extraction, and set into the autosampler programmed to control the SPME extraction and desorption technique. In addition, 1.5 mL aliquots of methanol and water in 2 mL autosampler vials with a septum were set on the autosampler. The capillary column was washed and conditioned by two repeated draw/eject cycles (40 μL each) of these solvents, and then a 50-μL air plug was drawn prior to the extraction step. The extraction of cortisol onto the capillary coating was performed by 25 repeated draw/eject cycles of 40 μL of sample at a flow-rate of 150 μL/min with the six-port valve in the LOAD position. After washing the tip of the injection needle by one draw/eject cycle of 2 μL of methanol, the extracted compounds were desorbed from the capillary coating with mobile phase flow. Then, the compounds were transported to the LC column by switching the six-port valve to the INJECT position, and detected by the MS system with SIM mode. During the analysis, the SPME capillary was washed and conditioned with mobile phase for the next extraction. Outline of the in-tube SPME/LC–MS system is shown in previous papers [33,36].

2.4. Sample preparation

Urine samples from healthy volunteers were collected in glass bottles and processed immediately or stored at −20 °C until use. Saliva samples were collected in Salisoft tubes containing a polypropylene–polyethylene sponge (Assist, Tokyo, Japan), and the tubes were centrifuged at 2500 × g for 5 min to elute the saliva. Urine or saliva solutions (0.1–0.2 mL) were added to 0.1 mL of 0.2 M acetate buffer (pH 5) and the total volume was made up to 1.0 mL with distilled water. The mixtures were used for the following in-tube SPME LC–MS analysis. Standard mixture was added to control urine and saliva samples (which did not include nicotine or related compounds) at concentrations of 0.5, 1.0, 2.0, 5.0, 10, 20, and 50 ng/mL of each compound, and calibration curves were constructed. Urinary creatinine concentrations were determined by the Jaffé method using a creatinine test kit (Wako Pure Chemicals, Osaka, Japan).

2.5. Nicotine intake and smoking

The aim of the experiment was explained to the subjects beforehand and consent was obtained after confirmation that they fully understood the experiment. The non-smoking subject consisted of 52 male volunteers who chewed Nicorette® (Pfizer Co. Ltd.,

Tokyo, Japan) gum containing 1 mg of nicotine for 30 min from 9:00 a.m. Urine and saliva were sampled just before nicotine intake and after 2, 4, 6, 9, 12, 15, and 21 h. The smoking subject consisted of 22 male volunteers who smoked a cigarette (including 0.3 mg nicotine) at 9:00 and 15:00 after stopping smoking the day before. Urine was sampled just before smoking and after 2, 4, 6, 8, 10, and 12 h. Urine samples were also collected from smokers (active smoking) and non-smokers (passive smoking). The collected samples were stored at −20 °C until assayed.

3. Results and discussion

3.1. LC–MS analysis of nicotine and related compounds

For MS operation, ESI positive ion mode was evaluated for the determination of nicotine and related alkaloids. To select the monitoring ion for these compounds, the ESI mass spectra were initially analyzed by LC–MS with direct liquid injection into the column. Nicotine and related compounds gave $[M+H]^+$ as a base ion in the mass scan range of 60–200 amu. The $[M+H-NH_3]^+$ was also observed in nornicotine (m/z = 132.2) and anatabine (m/z = 144.1). Parameters, including nebulizer gas pressure, drying gas flow-rate, fragmenter voltage, and capillary voltage were optimized by flow injection analysis.

LC separation of nicotine and related compounds was performed using a Synergi 4u POLAR-RP 80A column. As shown in Fig. 1, these compounds were eluted within 7 min using 5 mM ammonium formate/methanol (55/45, v/v) as a mobile phase, at a flow-rate of 0.8 mL/min. Nicotine and related compounds could be detected selectively in SIM mode.

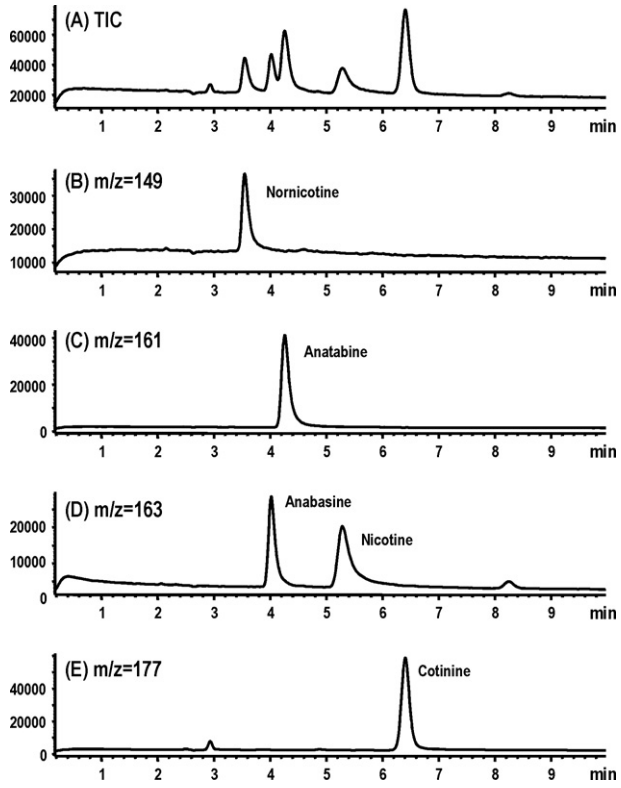

Fig. 1. Chromatograms obtained from 100 ng/mL standard compounds by direct injection. (A) Total ion chromatogram, (B)–(E) selected ion chromatograms. See Section 2 for LC–MS conditions.

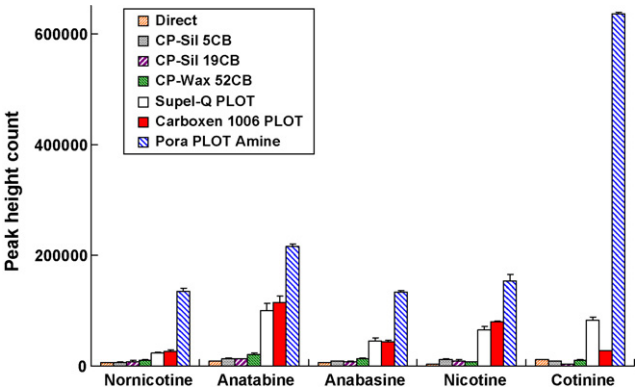

Fig. 2. Effects of capillary coatings on the in-tube SPME of nicotine and related compounds. Each compound was extracted by 20 draw/eject cycles of 40 μL of standard solution (20 ng/mL of each) at a flow-rate of 150 μL/min.

3.2. Optimization of in-tube solid-phase microextraction and desorption

To optimize the extraction of nicotine and related compounds by in-tube SPME, several parameters, such as the stationary phase of the in-tube SPME capillary column and number and volume of draw/eject cycles were investigated. Extraction efficiency in in-tube SPME was evaluated by comparison of peak height in each condition. Six different capillary columns, CP-Sil 5CB (Varian Inc., Lake Forest, CA, USA, 100% polydimethylsiloxane, 5 μm film thickness), CP-Sil 19CB (Varian, 14% Cyanopropyl phenyl methylsiloxane, 1.2 μm film thickness), CP-Wax 52CB (Varian, Polyethyleneglycol, 1.2 μm film thickness), and CP-Pora PLOT amine (Varian, basic modified stylene divinylbenzene polymer, 10 μm film thickness), Carboxen 1006 PLOT (Supelco, Bellefonte, PA, USA, carbon molecularsives, 15 μm film thickness) and Supel Q PLOT (Supelco, Divinylbenzene polymer, 17 μm film thickness) were tested as extraction device. In in-tube SPME, the amount of analyte extracted into the stationary phase of capillary column depends on factors such as the surface area, film thickness and polarity of the capillary coatings. As shown in Fig. 2, the extraction efficiency of the porous polymer-type capillary column was better than those of the other columns. As the PLOT column has a large adsorption surface area and thick film layer, the amount extracted was greater than that with liquid-phase-type columns. Among the PLOT column, a CP-Pora PLOT amine gave superior extraction efficiency because of its affinity to relatively polar compounds.

With in-tube SPME, the extraction time, flow-rate, and sample pH are related to the amounts of compounds extracted. To monitor the extraction time profile of nicotine and related compounds by in-tube SPME, the number of draw/eject cycles was varied from 0 to 25 using a CP-Pora PLOT amine capillary column. As shown in Fig. 3(A), the extraction equilibrium of these compounds was not reached with 25 draw/eject cycles of 40 μL of sample. Although extraction equilibrium is incomplete, it is possible to cease extraction before equilibrium to reduce the analysis time, because quantitative reproducibility is obtained by fixing SPME conditions using an autosampler. Draw/eject rate in in-tube SPME was tested at 50, 100, 150 and 200 μL/min. Extraction efficient is highest at 50 μL/min, and it decreased slowly with increase of draw/eject rate. In this method, a draw/eject rate of 150 μL/min was used as optimal flow-rate. Below this level, extraction requires an inconveniently long time, and above this level, bubbles tend to form inside the capillary column, reducing the extraction efficiency. The effect of the pH of the sample matrix on extraction of nicotine and related compounds was examined using several buffer solutions. As shown

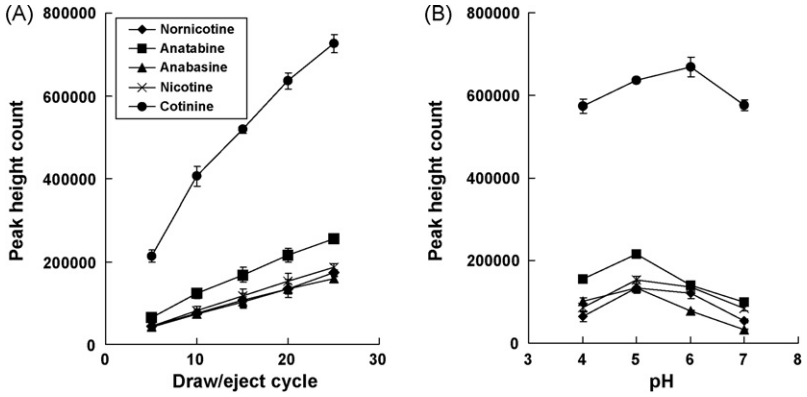

Fig. 3. Effects of (A) draw/eject cycle and (B) flow-rate on the in-tube SPME of nicotine and related compounds. Each compound was extracted by draw/eject of 40 μL of standard solution (20 ng/mL of each) using a CP-Pora PLOT amine capillary.

in Fig. 3(B), acetate buffer (pH 5 or 6) was more effective, and the optimal concentration of this buffer was 20 mM. The absolute amounts of these compounds extracted by the SPME capillary column were calculated by comparing peak area counts with the corresponding direct injection of the sample solution onto the LC column. At a sample concentration of 20 ng/mL, 5.1 ng (25.5%) of nicotine, 5.3 ng (26.5%) of cotinine, 2.4 ng (12.0%) of nornicotine, 2.7 ng (13.5%) of anatabine, and 2.6 ng (13.0%) of anabasine were extracted onto the CP-Pora PLOT amine column by in-tube SPME. Although the extraction yields of these compounds were relatively low, they showed good reproducibility (R.S.D. < 5%) due to the autosampler.

The mobile phase was found to be suitable for the desorption of nicotine and related compounds extracted into the stationary phase of the capillary column. Dynamic desorption of these compounds from the capillary could be achieved readily by switching the six-port valve of LC–MS instrument. The desorbed the compounds were transported to the LC column by mobile phase flow.

Air plugging before the extraction step was carried out to prevent not only sample mixing but also desorption of analyte from the capillary coating by the mobile phase during the ejection step. No carryover was observed because the capillary column was washed and conditioned by draw/eject cycles of methanol and mobile phase prior to extraction. The extraction and desorption of nicotine and related compounds by the in-tube SPME method were accomplished automatically within 30 min, and automated analysis of about 48 samples per day was possible by overnight operation.

3.3. Sensitivity, linearity, and precision

Nicotine and related compounds provided an excellent response in ESI–MS. As shown in Table 1, the detection limits of these compounds were 15–40 pg/mL with signal-to-noise ratios of 3:1 under our LC–MS conditions. The in-tube SPME method was 20–46-fold more sensitive than the direct injection method (5 μL injection), because these compounds were concentrated in the capillary column during draw/eject cycles. Sensitivity of this method was about 10 times higher than those of LC–MS–MS method reported previously [23–29]. The calibration curves for nicotine and related compounds were constructed from the peak height counts. As shown in Table 1, a linear relationship was obtained for each compound in the range 0.5–20 ng/mL urine (six-point calibration) and the correlation coefficients were above 0.9969. On the other hand, the within-run and between-day precision (relative standard deviations, R.S.D.) at the concentration of 2 ng/mL were below 4.7% and 11.3% ($n = 5$), respectively (Table 1).

3.4. Application to the analysis of urine and saliva samples

Saliva samples were collected easily using Salisoft tubes containing a polypropylene–polyethylene sponge. Urine and saliva samples could be analyzed directly by the dilution of the sample without any other pretreatment. The recovery rates of nicotine and related compounds added to urine and saliva samples by comparison with pure standard sample were 30–57% and 43–75%, respectively. The lower recoveries were corrected by using calibration curves of nicotine and related compounds spiked into the pooled urine and saliva as described in Section 2. As shown in Fig. 4, the urine and saliva samples were analyzed successfully without interference peaks by SIM mode detection. To confirm the validity of this method, known amounts of nicotine and related compounds were spiked into 0.1 mL of pooled urine and saliva samples, and their recoveries were calculated. As shown in Table 2, the recoveries of these compounds were above 83–98% and relative standard deviations were below 7.1%.

Table 1
Linear regression data, detection limits and within-run and between-day precisions of nicotine and related compounds by in-tube SPME/LC–MS.

Compound	SIM (m/z)	Regression line[a]		Correlation	Detection limit (ng/mL)[b]		D/I	Within-run	Between-day
		Slope	Intercept	Coefficient	Direct injection	In-tube SPME	Ratio[c]	R.S.D. (%)[d]	R.S.D. (%)[d]
Nornicotine	149	6898	−1776	0.9969	1.84	0.040	46.0	4.73	11.3
Anatabine	161	11032	195	0.9975	0.5	0.024	20.8	3.84	5.21
Anabasine	163	6807	−792	0.9982	0.82	0.035	23.4	3.47	7.15
Nicotine	163	7811	−1590	0.9969	1.24	0.030	41.3	3.85	7.06
Cotinine	177	31798	6802	0.9994	0.34	0.015	22.7	0.53	1.54

[a] Calibration range: 0.5–20 ng/mL, six-point ($n = 18$).
[b] S/N = 3.
[c] Sensitivity rate of direct injection method against in-tube SPME method.
[d] $n = 5$.

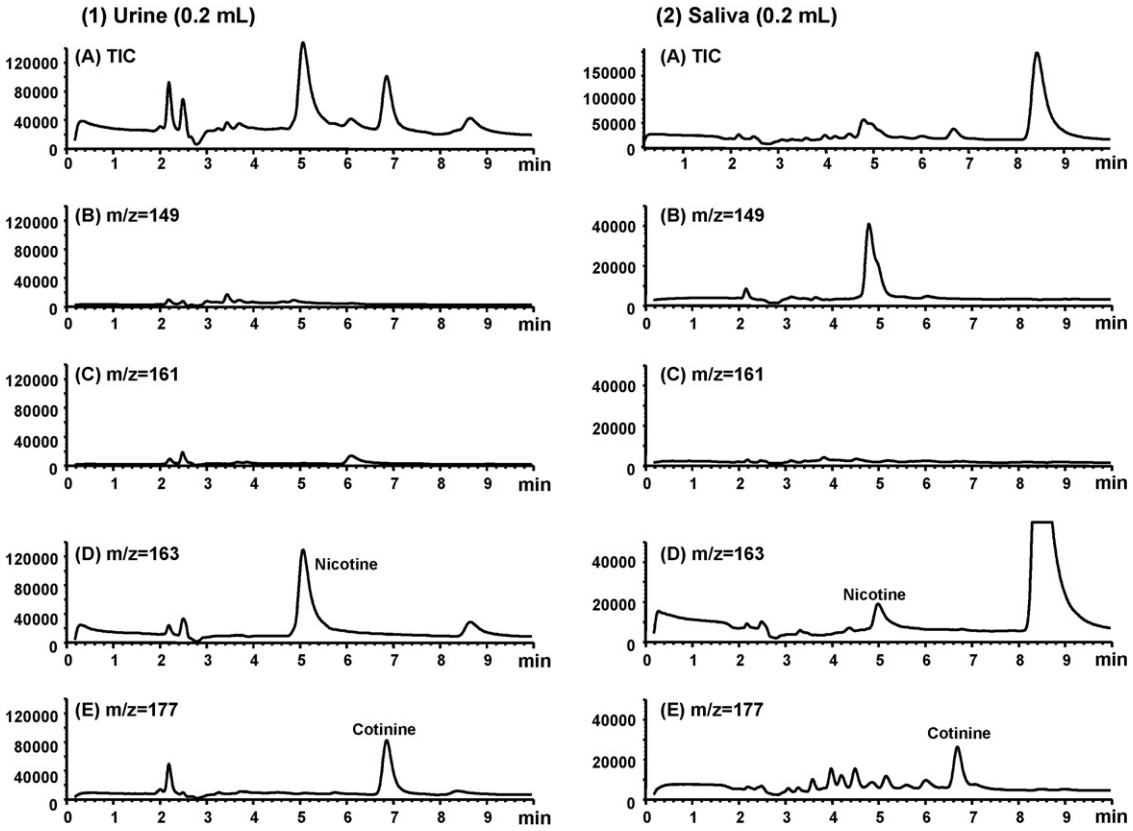

Fig. 4. Chromatograms obtained from urine and saliva samples after nicotine intake. See Section 2 for in-tube SPME/LC–MS conditions.

3.5. Excretion of nicotine and related compounds by nicotine intake and smoking

Urinary nicotine and cotinine contents are useful biomarkers to evaluate smoking [10–14]. To evaluate the utility of the developed method, we analyzed the influence of the intake of nicotine. The test involved chewing Nicorette® gum containing 1 mg of nicotine for 30 min from 9:00 a.m., and urine and saliva were sampled just before nicotine intake and after 2, 4, 6, 9, 12, 15, and 21 h. As shown in Fig. 4, nicotine and cotinine were detected in urine and saliva samples, but the other compounds tested in this study were not detected. As shown in Fig. 5(A), the urinary nicotine content reached a maximum level after 2 h and subsequently decreased by degrees. The urinary cotinine content reached a maximum level after 4 h. On the other hand, the salivary nicotine content increased transiently after 2 h, while the cotinine content hardly increased (Fig. 5(B)). To evaluate the urinary excretion of nicotine and cotinine with smoking, subject who was smoker smoked a cigarette containing 0.3 mg of nicotine at 9:00 and 15:00 after stopping smoking the day before. As shown in Fig. 6, the urinary nicotine and cotinine contents increased with smoking. As shown in Table 3, urinary excretion of nicotine and cotinine also increased depending on the number of cigarettes smoked in one session. Furthermore, urinary excretion of these compounds increased in non-smoker associated with passive smoking. These results suggest that urinary excretion and salivary secretion of nicotine and cotinine sufficiently reflect active and passive smoking of cigarettes, and it was confirmed that these compounds are useful biomarkers to evaluate smoking.

Table 2
Recoveries of nicotine and related compounds spiked into urine and saliva samples.

Compound	Spiked (ng/mL)	Recovery (%)/mean ± S.D. (n = 3)			
		Urine		Saliva	
		Average	R.S.D. (%)	Average	R.S.D. (%)
Nornicotine	2	83.5 ± 0.6	1.0	86.8 ± 6.1	7.1
	20	83.2 ± 2.6	3.1	90.6 ± 4.0	4.4
Anatabine	2	95.1 ± 0.5	0.5	90.6 ± 2.8	3.1
	20	84.3 ± 2.7	3.2	98.2 ± 2.5	2.6
Anabasine	2	97.4 ± 3.4	3.5	94.9 ± 2.8	2.9
	20	88.5 ± 0.9	1.0	96.3 ± 4.6	4.7
Nicotine	2	93.1 ± 1.6	1.7	83.0 ± 2.9	3.5
	20	88.2 ± 1.8	2.1	95.2 ± 2.0	2.1
Cotinine	2	86.3 ± 0.8	0.9	93.8 ± 1.1	1.2
	20	88.0 ± 1.7	1.9	96.5 ± 2.0	2.1

Table 3
Urinary excretion of nicotine and cotinine in active and passive smoking.

Smoking condition	Sampling time	Nicotine (ng/mg creatinine)[a]	Cotinine (ng/mg creatinine)[a]
Smoker (one cigarette)	Before	8.9 ± 0.3	125.0 ± 6.5
	After 2 h	140.8 ± 13.7	218.5 ± 2.2
Smoker (three cigarettes)	Before	5.1 ± 0.4	90.8 ± 2.1
	After 2 h	228.6 ± 25.7	188.0 ± 0.6
Non-smoker	Before	ND	ND
(passive smoking: 1 h)	After 1 h	27.2 ± 2.0	0.76 ± 0.02
	After 3 h	37.0 ± 0.9	1.76 ± 0.03

[a] $n = 3$.

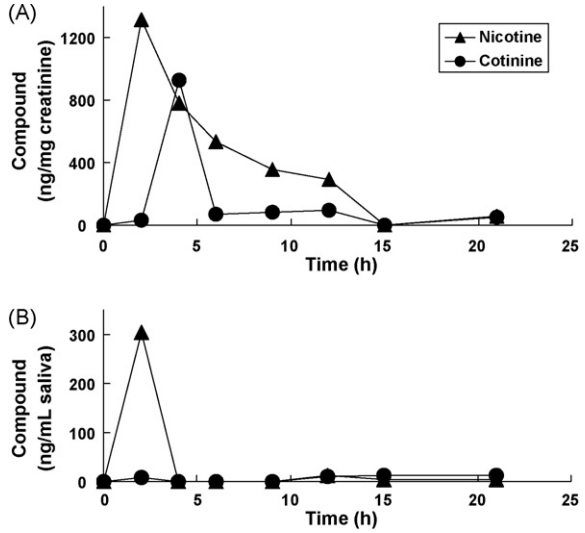

Fig. 5. Concentration of nicotine and cotinine in (A) urine and (B) saliva after nicotine intake.

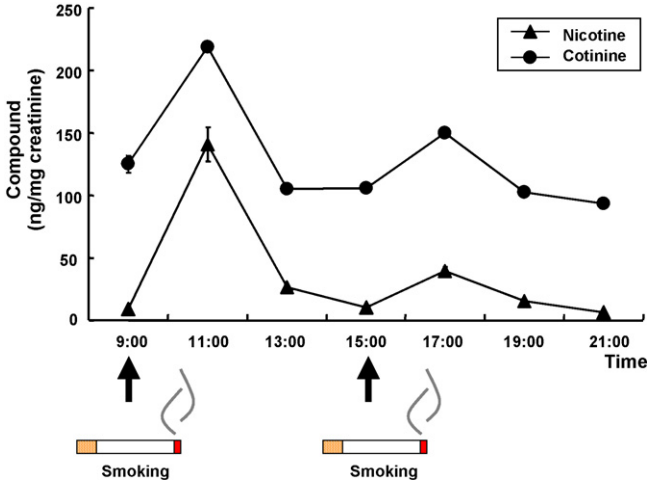

Fig. 6. Urinary excretion of nicotine and cotinine after smoking.

4. Conclusions

The on-line in-tube SPME/LC–MS method developed in the present study can continuously perform extraction and concentration of nicotine and related compounds from urine and saliva samples, and then allow analysis by LC–MS. This method is automated, simple, rapid, selective, and sensitive, and can be applied easily to the analysis of urine and saliva samples. This method is a very useful tool for monitoring of tobacco smoking, for estimating the uptake of nicotine and tobacco-related toxicants, for understanding the pharmacologic effects of nicotine and nicotine addiction, and for optimizing nicotine dependency treatment.

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