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Short communication

# Sensitive and simple method for the determination of nicotine and cotinine in human urine, plasma and saliva by gas chromatography–mass spectrometry

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**Abstract**

A method is proposed for the determination of nicotine and cotinine in human urine, plasma and saliva. Nicotine and cotinine were extracted from alkalinized sample with ethyl ether and concentrated to minimum volume with nitrogen stream. The volatility of nicotine was prevented by the addition of acetic acid to the organic solvent during evaporation. Peak shapes and quantitation of nicotine and cotinine are excellent, with linear calibration curves over a wide range of 1–10 000 ng/ml. The detection limits of nicotine and cotinine are 0.2 ng/ml in urine and 1.0 ng/ml in plasma and saliva. The intra-day precision of nicotine and cotinine in all samples was <5% relative standard deviation (RSD). Urine, plasma and saliva samples of 303 non-smoking and 41 smoking volunteers from a girl's high school in Korea were quantified by the described procedure. As a result, the concentrations of nicotine and cotinine in plasma ranged from 6 to 498 ng/ml and 4 to 96 ng/ml. Otherwise, those of nicotine and cotinine in saliva ranged from 0 to 207 ng/ml and 0 to 42 ng/ml, and those of nicotine and cotinine in urine ranged from 0 to 1590 ng/ml and 0 to 2986 ng/ml, respectively. We found that the concentration of cotinine in plasma was successfully predicted from the salivary cotinine concentration by the equation $y=2.31x+4.76$ ($x$=the concentration of cotinine in saliva, $y$=the concentration of cotinine in plasma). The results show that through the accurate determination of cotinine in saliva, the risk of ETS-exposed human can be predicted. 

*Keywords:* Nicotine; Cotinine

## 1. Introduction

Environmental tobacco smoke (ETS) is the material released into the ambient atmosphere by smoking tobacco products, which consist of a heterogeneous mixture of gases, uncondensed vapors, tar and particulate phase. Since the beginning of the 1980s, the health risks of environmental tobacco smoke (ETS) containing lung cancer and heart disease have been reported [1–6]. The U.S. Environmental Protection Agency (EPA) announced ETS to be a known

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human lung carcinogen [1] and the American Heart Association acknowledged passive smoking to be a cause of cardiovascular disease in nonsmokers [4].

The determination of nicotine and its metabolite, cotinine, in biological fluids has aroused particular interest. These biochemical markers have been used to estimate active smoking behavior, to validate abstinence smoking, and to evaluate the levels and significance of ETS exposure [7–12].

The true smoking status is based on cotinine level in body fluids, but cut-off points and distribution of metabolites in body fluids are dependent on sex, age, diet, racial and ethnic differences as well as many other factors [13,14].

In order to estimate of ETS exposure, a technique is needed for the rapid and simultaneous determination of nicotine and cotinine in biological samples.

Many methods have been proposed for the determination of nicotine and cotinine in human urine, plasma and saliva. These methods utilize radio immunoassay [15], high-performance liquid chromatography [16–19], and gas chromatography using electron-capture [20], or nitrogen–phosphorus [21–24] detectors. For the determination of ng/l concentrations of nicotine and cotinine in biological samples, the most frequently used method is gas chromatography–mass spectrometry (GC–MS) [25–30].

This paper describes a one-step liquid–liquid extraction (LLE) procedure of the trace nicotine and cotinine in human urine, plasma and saliva combined with analysis of the extract by gas chromatography–mass spectrometry-selected ion monitoring (GC–MS-SIM). A simple and reproducible procedure for the simultaneous analysis of nicotine and its metabolite and a quick turnaround time were targeted. The method was applied to the determination of nicotine and cotinine in urine, plasma and saliva from smokers or passive smokers.

## 2. Experimental

### *2.1. Materials and sample collection*

Nicotine, cotinine and diphenylamine (internal standard) were obtained from Sigma (St Louis, MO, USA). Potassium carbonate, sodium sulfate, sodium chloride, diethylether, and acetic acid (Merck, Darmstadt, Germany) were used as reagents and solvents.

Subjects were 303 non-smoking and 41 smoking volunteers from a girl's high school aged 16–18; 276 among total subjects provided urine samples, 140 saliva, and 96 plasma for the determination of nicotine and cotinine. The urine and saliva samples were collected in previously delivered pasteurized glass containers. The respective samples were collected in a sports center after the weekend on Monday morning. The collected samples were moved in the same morning as quickly as possible from the center to the laboratory. All samples were stored at −20 °C until analysis. Blood samples were collected in EDTA-containing tubes and centrifuged immediately at 1000 *g*. The plasma supernatant was then collected and frozen at −70 °C until analyzed.

### *2.2. Extraction of nicotine and cotinine from urine*

In a 20-ml glass test-tube, was placed 5 ml of urine. About 300 mg of $K_2CO_3$ and 50 μl of diphenylamine (10 μg/ml in methanol) as an internal standard were added to the solution, and the sample was extracted with 7 ml of ethyl ether by mechanical shaking for 10 min. The organic phase was transferred into a 20-ml test-tube containing 20 μl of acetic acid and dried with a nitrogen stream to about 50 μl volume. The solution was dried with about 100 mg of sodium sulfate and a 2-μl sample was automatically injected into the GC system.

### *2.3. Extraction of nicotine and cotinine from plasma or saliva*

For the analysis of nicotine and cotinine in plasma or saliva, 0.5 ml of plasma or saliva was placed in a 20-ml test-tube. About 100 mg of $K_2CO_3$, 0.5 ml of Milli-Pore water and 20 μl of diphenylamine (10 μg/ml in methanol) as an internal standard were added to the solution, and the sample was extracted twice with 3 ml of ethyl ether by mechanical shaking for 10 min. The total organic phase was transferred into a 20-ml test-tube containing 20 μl of acetic acid and dried with a nitrogen stream until about 50 μl volume. The solution was dried with about 100 mg of sodium sulfate and a 2-μl sample was automatically injected into the GC system.

### 2.4. Calibration and quantitation

Calibration curves of nicotine and cotinine were established by extraction after adding amounts in range of 1.0–5000 ng of standards and 0.5 μg (or 0.2 μg in the case of plasma or saliva sample) of internal standard in 5 ml urine, 0.5 ml plasma or saliva. The ratio of the peak area of standard to that of internal standard was used in the quantitation of the analytes.

### 2.5. Gas chromatography–mass spectrometry

All mass spectra were obtained with a Agilent 6890/5973 N instrument. The ion source was operated in the electron ionization mode (EI; 70 eV, 230 °C). Full-scan mass spectra ($m/z$ 40–800) were recorded for analyte identification. Separation was achieved with an HP fused-silica capillary column with cross-linked methylsilicone (HP 1), ~30 m length, 0.2 mm I.D., 0.33 μm film thickness. Samples were injected in the split mode with a splitting ratio of 1:8. The flow rate of the helium was 1.0 ml/min. The operating parameters were as follows: injector temperature, 280 °C; transfer line temperature, 300 °C; oven temperature, programmed from 80 °C at 20 °C/min to 300 °C (held for 5 min). The ions selected in this study were $m/z$ 84, 133 and 161 for nicotine, $m/z$ 168 and 169 for diphenylamine, $m/z$ 98 and 176 for cotinine.

### 2.6. Statistical analysis

The correlation between the cotinine concentrations in saliva and plasma was analyzed using SPSSWIN (ver 10.x) and SAS (ver 6.2).

## 3. Results and discussion

### 3.1. Chromatography

For the GC separation of the nicotine and cotinine, the use of the non-polar stationary phase was found to be efficient. Chromatograms are shown in Fig. 1. As can be seen from the figure, the peaks of nicotine, cotinine and internal standard are symmetrical and separation of the analytes from the background compounds in biological samples was very good. The retention times of nicotine, internal standard and cotinine were 4.21, 5.97 and 6.47 min. There were no extraneous peaks observed in chromatograms of biological samples of non-smoker at the retention times of 4.21, 5.97 and 6.47 min.

### 3.2. Mass spectrometry

The mass spectrum of nicotine illustrates molecular ion at $m/z$ 162 and the major peak at $m/z$ 84 and 98, which are both due to the loss of the pyridyl group from the molecular ion. That of cotinine shows molecular ion at $m/z$ 178 and the base peak at $m/z$ 98, and the diagnostic ions at $m/z$ 118, 119 and 147.

### 3.3. Linearity

Examination of typical standard curves by computing a regression line of peak area ratios of nicotine and cotinine to internal standard on concentration using a least-squares fit demonstrated a linear relationship with correlation coefficients being consistently greater than 0.998. Table 1 shows the lines of best fit for the analytes.

### 3.4. Recovery

Several biological samples of varying composition were prepared and the relative recovery was calculated by the percentage of analytes recovered. The recoveries were in the range of 88–98% at the concentration of 100 and 500 ng/ml nicotine and those were found to be in the range of 94–99% in case of cotinine (Table 2).

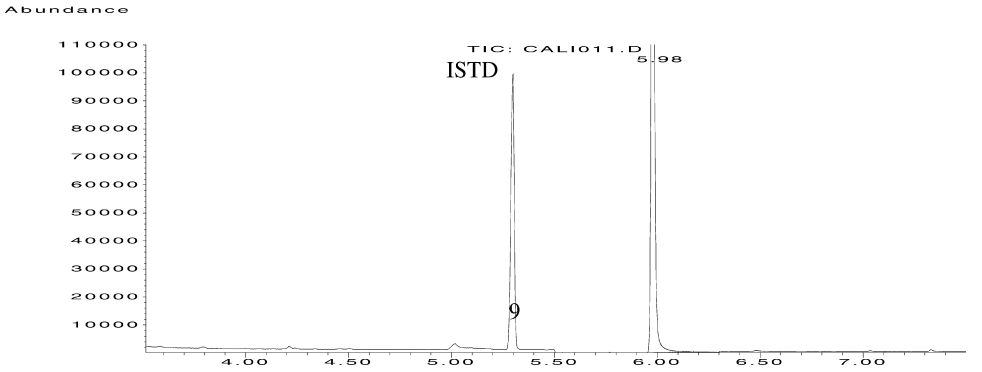
### 3.5. Precision and accuracy

The reproducibility of the assay was very good, as shown in Table 3. For five independent determinations at each concentration, the coefficient of variation was less than 5%.

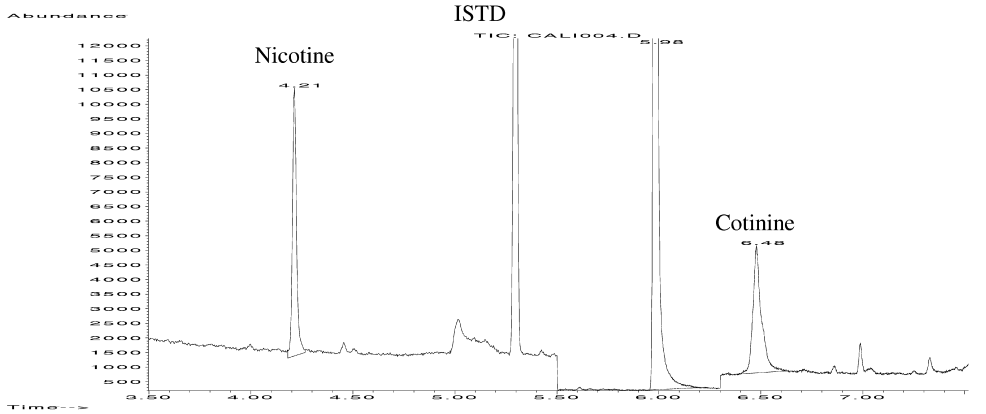
### 3.6. Sensitivity

The combination of low background, high extraction yield, and the high sensitivity of the analytes by the detector permit their determination in bio-

**Nonsmoker Sample**




**Smoker Sample**




**Nonsmoker Sample Spiked as 5 ng/mL**

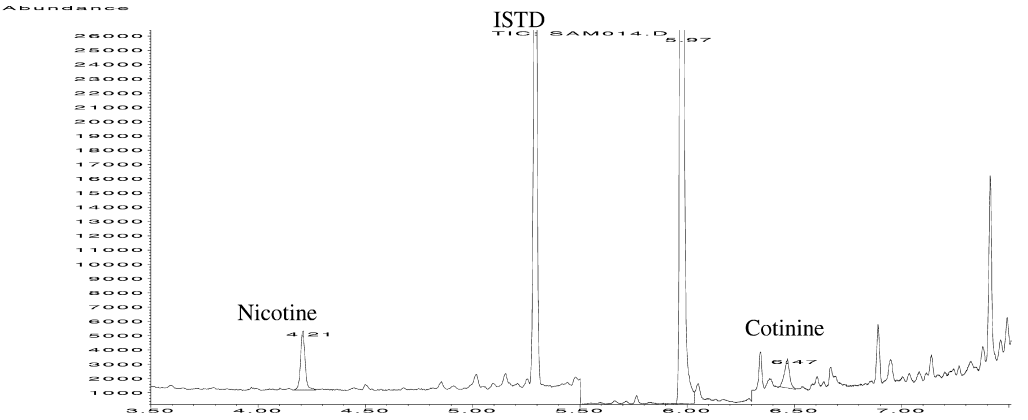



Fig. 1. Chromatograms of the extracts from saliva.

Table 1
The line of best fit and correlation coefficient of nicotine and cotinine in biological samples

| Sample | Line of best fit | | Correlation coefficient | |
|---|---|---|---|---|
| | Nicotine | Cotinine | Nicotine | Cotinine |
| Urine | $y=0.0491x-1.2258$ | $y=0.0515x+0.1439$ | 0.998 | 0.998 |
| Plasma | $y=0.0012x-0.0209$ | $y=0.0013x-0.0039$ | 0.999 | 0.998 |
| Saliva | $y=0.0065x-0.0467$ | $y=0.0072x-0.0265$ | 0.998 | 0.998 |

$y$, peak area ratio of the analyte to internal standard; $x$, analyte concentration (ng/ml).

Table 2
Recoveries of nicotine and cotinine from biological samples ($n=3$)

| Sample | Spiked conc. (ng/ml) | Recovery (%)±SD (RSD) | |
|---|---|---|---|
| | | Nicotine | Cotinine |
| Urine | 100 | 92.1±4.7 (5.1%) | 98.2±0.6 (0.6%) |
| | 500 | 97.7±1.2 (1.3%) | 96.3±2.1 (2.2%) |
| Plasma | 100 | 90.5±3.7 (4.1%) | 96.4±3.5 (3.6%) |
| | 500 | 88.1±3.6 (4.1%) | 94.8±3.3 (3.5%) |
| Saliva | 100 | 98.2±4.7 (4.8%) | 99.8±3.2 (3.2%) |
| | 500 | 89.1±3.9 (4.4%) | 97.1±1.6 (1.7%) |

logical samples at low concentrations. Detection limits of nicotine and cotinine were 0.2 ng/ml in urine and 1.0 ng/ml in plasma and saliva, respectively. Limits were defined by a minimum signal-to-noise ratio of 3 and coefficients of variation for replicate determinations ($n=5$) of 15% or less.

### *3.7. Analysis of biological samples of non-smoking and smoking volunteers*

Urine, plasma or saliva samples of 303 non-smoking and 41 smoking volunteers from a girl's high school in Korea were quantified by the described methods. As a results, the concentrations of nicotine and cotinine in plasma ranged from 6 to 498 ng/ml and 4 to 96 ng/ml. Otherwise, those of nicotine and cotinine in saliva ranged from 0 to 207 ng/ml and 0 to 42 ng/ml, and those of nicotine and cotinine in urine ranged from 0 to 1590 ng/ml and 0 to 2986 ng/ml, respectively. The mean and the median concentration of cotinine in plasma were 51 and 62 ng/ml and those of cotinine in saliva were 21 and 24 ng/ml, respectively.

The relationship of salivary-plasma cotinine concentrations was studied (Fig. 2). From the results, salivary cotinine was found to be significantly correlated with the concentration of cotinine in plasma. It appeared that cotinine is on average excreted in fixed relationships from plasma into saliva. We also found that the concentration of cotinine in plasma was successfully predicted from the salivary cotinine concentration by the regression equation $y=2.31x+4.76$ ($R=0.96$, $P=0.00$) where $x$ is the concentration of cotinine in saliva and $y$ the concentration of cotinine in plasma.

Otherwise, salivary nicotine showed no significant

Table 3
Within-run precision and accuracy of nicotine and cotinine in biological samples ($n=5$)

| Sample | Spiked conc. (ng/ml) | Recovery (%)±SD (RSD) | |
|---|---|---|---|
| | | Nicotine | Cotinine |
| Urine | 100 | 97.2±4.1 (4.2%) | 98.2±0.6 (0.6%) |
| | 250 | 243.2±8.5 (3.5%) | 96.3±2.1 (2.2%) |
| | 500 | 495.1±21.4 (4.3%) | 489.1±24.1 (4.9%) |
| Plasma | 10 | 9.0±0.4 (4.4%) | 10.5±0.5 (4.8%) |
| | 50 | 47.8±2.3 (4.8%) | 49.9±2.4 (4.8%) |
| Saliva | 10 | 10.5±0.5 (4.8%) | 9.9±0.3 (3.0%) |
| | 50 | 51.1±1.9 (3.7%) | 50.9±1.2 (2.4%) |

SD, standard deviation; RSD, relative standard deviation.

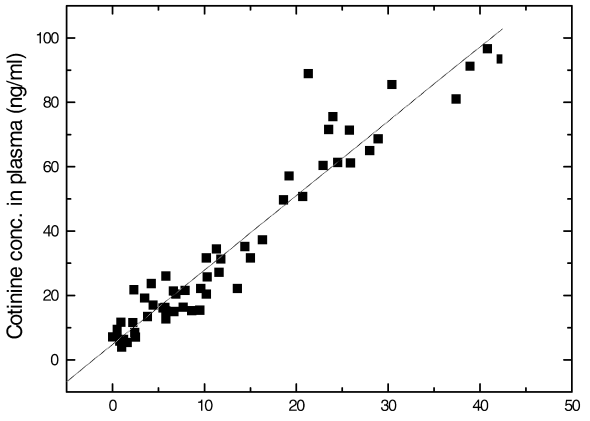



Fig. 2. Relationship of salivary-plasma cotinine concentrations.

correlation with the concentration of nicotine in plasma ($R = 0.090$, $P = 0.499$).

## 4. Conclusions

We have found the described methods to be quite satisfactory for the simultaneous analysis of nicotine and cotinine in various biological samples. The peaks have good chromatographic properties and offer a very sensitive response for the EI-MS (SIM). The one-step extraction of these compounds from biological samples also gave relatively high recoveries with small variations and a range of method detection limits of 0.2–1.0 ng/ml. The developed method is simple, convenient, and can be learned easily by relatively inexperienced personnel.

It was applied to urine, plasma and saliva from smokers or passive smokers. The results show that through the accurate determination of cotinine in saliva, the risk of lung cancer and heart disease can be predicted.

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