

SIMPLE AND RAPID ASSAY METHOD FOR SIMULTANEOUS QUANTIFICATION OF URINARY NICOTINE AND COTININE USING MICRO-EXTRACTION BY PACKED SORBENT AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A simple and rapid method for determination of nicotine and cotinine levels in urine was developed using samples prepared by micro-extraction by packed sorbent (MEPS) and subjected to gas chromatography-mass spectrometry (GC-MS) analysis. This method provided good reproducibility, as well as good linearity of calibration curves in the range of 1–100 and 50–1000 ng/mL for quality control samples spiked with nicotine and cotinine, respectively. The detection limit of nicotine and cotinine was as low as 0.25 and 20 ng/mL, respectively. An evaporation procedure is not suitable for nicotine determination, thus an advantage of the present MEPS assay method is direct testing with GC-MS without the need for evaporation to a dry solvent. Our findings show that it may be useful for determining nicotine levels in various types of research studies.

Key Words: micro-extraction by packed sorbent (MEPS),
gas chromatography-mass spectrometry (GC-MS), nicotine, cotinine

INTRODUCTION

Tobacco smoking is considered to increase the risk of a variety of cancers as well as cardiovascular disease in a dose-dependent manner.^{1, 2)} Nicotine is the most abundant alkaloid found in tobacco and responsible for the addictive properties of cigarettes,³⁾ while cotinine, one of the major metabolites of nicotine, has a relatively longer half-life (19–31 vs. 2.0–2.6 hours),⁴⁾ and can be easily detected in urine, plasma, and saliva. Urinary cotinine is widely used as a biomarker due to its higher concentration in the urine matrix as compared to other matrices, resulting in accurate detection in samples.⁵⁾

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Several different analytical methods have been described for determination of urinary nicotine and cotinine, including colorimetry, gas chromatography, high-performance liquid chromatography, and immunosorbent assays.⁶⁻¹³⁾ In addition, there are various methods for sample preparation procedures prior to analysis of organic compounds in complex matrices such as biological fluids. Commonly used preparations of biological fluids for analysis of nicotine and cotinine are based on solid-phase extraction (SPE) and liquid-liquid extraction (LLE) techniques, with the obtained extract evaporated to dryness or the analyte subjected to salting prior to chromatography analysis.¹⁴⁾ Micro-extraction by packed sorbent (MEPS) is a simple method to perform that has been developed following the miniaturization of conventional SPE devices.¹⁴⁻²¹⁾ The purpose of MEPS is to reduce the sorbent bed volume, making it suitable for a large sample volume range (from as low as 10–1000 μL), thus reducing the number of steps typically involved in conventional SPE procedures.¹⁸⁾ A typical MEPS assay is designed in a syringe format, in which a few mg of the sorbent is packed inside a syringe (100–250 μL) as a plug, or between the barrel and needle as a cartridge. Any sorbent material, such as silica-based (C2, C8, C18), strong cation exchange (SCX), or molecular imprinted polymers (MIPs), can be used as the packing bed or as a coating.¹⁸⁻²¹⁾ Furthermore, it was recently reported that an MEPS sample preparation technique significantly improved the matrix effect by significantly reducing the concentrations of phospholipids in the final elution, resulting in a more selective method. This method has been previously applied with biological fluids for determination of a wide variety of drugs in biological fluids such as urine, plasma, and blood.¹⁸⁻²¹⁾ Our objective in the present study was to evaluate MEPS as a sample preparation technique to determine nicotine and cotinine levels in human urine samples.

MATERIALS AND METHODS

Chemicals

Nicotine and cotinine were purchased from Sigma-Aldrich (St. Louis, USA), and 6-methyl nicotine (IS) came from Wako (Osaka, Japan). Laboratory distilled water (DW) was purified using a Synergy UV device (Millipore, Molsheim, France). Other common chemicals used were of the highest purity commercially available.

MEPS procedure

MEPS was performed using 250- μL gas-tight syringes obtained from SGE Analytical Science (Melbourne, Australia). The sorbent contained 4 mg of combined solid-phase silica based octyl (C8) and benzenesulfonic acid (SCX), and had a mean particle size of 45 μm and pore size of 60 \AA . The packed syringe was activated with 250 μL of MeOH and then conditioned with 250 μL of DW before use. Before the MEPS procedure, urine samples were prepared as follows: 250 ng of IS and 200 μL of 1M NaOH were added to 500 μL of the sample, then centrifuged at $2000 \times g$ for 5 minutes. This mixture was drawn up and down through the syringe once and discarded, which was repeated 3 times for each total sample mixture. The solid-phase portion was washed once with DW (250 μL) to remove interfering substances, then the analytes were eluted with 30 μL of MeOH. Finally, the eluate was subjected to gas chromatography-mass spectrometry (GC-MS) for analysis. After each extraction, cleaning of the sorbent was done with $3 \times 250 \mu\text{L}$ of 95% MeOH, followed by $3 \times 250 \mu\text{L}$ of 5% MeOH. This step decreased the memory effects and also acted as the conditioning step for the next extraction.

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Table 1 Major fragment ions obtained in EI mode

Compound	MW	m/z (% intensity)
Nicotine	162.26	84 (100), 133 (22), 42 (16), 162 (10)
Cotinine	176.22	98 (100), 176 (16)
6-Methyl nicotine (IS)	176.26	84 (100), 42 (33), 147 (16), 175 (10)

GC-MS analysis

GC-MS analysis was performed using a GCMS-2010Plus instrument (Shimadzu, Kyoto, Japan) equipped with a DB-5MS capillary column (30 m \times 0.25 mm i.d.; 0.25- μ m film thickness; Agilent Technologies, Santa Clara, USA). The mass spectrometer was operated in electron impact (EI) mode at an ionization energy of 70 eV. The temperature of the injection port was set at 290°C, while that of the interface was 280°C and the ion source was set at 250°C. The program consisted of an initial hold of 1 minute at 50°C, then an increase from 50–200°C at 15°C/minute and from 200–300°C at 20°C/minute. Helium was used as the carrier gas at a flow rate of 1.1 mL/minute. The samples were injected in splitless mode and the splitter was opened after 1 minute. Quantitative analysis was carried out in selected ion monitoring (SIM) mode. Major fragment ions of the drugs are listed in Table 1. The monitored ions for quantitation were m/z 84 for nicotine and 6-methyl nicotine (IS), and m/z 98 for cotinine.

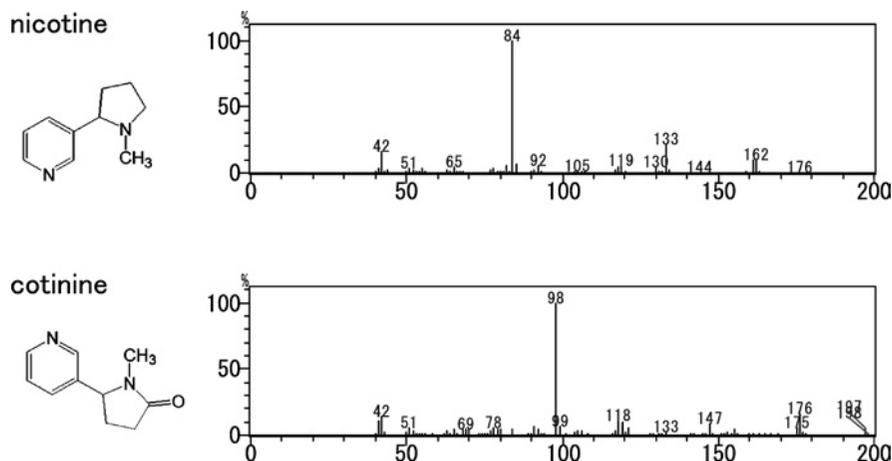
Human experiment

The chief scientist of this research project (healthy 49-year-old male) volunteered as a human subject. He smoked 1 cigarette within a 24-hour period and urine was sampled 1 hour later.

RESULTS AND DISCUSSION

Mass spectra and selected reaction monitoring chromatograms

The positive EI mass spectra of nicotine and cotinine are shown in Figure 1. A small molecular

**Fig. 1** Chemical structures and electron ionization mass spectra of nicotine and cotinine.

peak of nicotine appeared at m/z 162 and a base fragment peak at m/z 84, while those for cotinine appeared at m/z 176 and m/z 98, respectively. These values were used for quantitation (Table 1).

Reliability of the method

Figure 2b shows SIM chromatograms for nicotine, cotinine, and IS spiked into blank human urine at concentrations of 100, 1000, and 500 ng/mL, respectively. There were no impurity peaks for up to 10.0 minutes of retention time. The peaks for nicotine, IS, and cotinine appeared at retention times of 6.3, 7.0, and 8.7 minutes, respectively.

The calibration curve of nicotine showed good linearity from 1 to 100 ng/mL in urine with an equation of $y = 0.0314x + 0.0160$ and a coefficient of determination (r^2) of 0.999 (using 5 concentrations; 1, 5, 10, 50, 100 ng/mL). The calibration curve of cotinine showed good linearity from 50 to 1000 ng/mL in urine with an equation of $y = 0.000517x - 0.00582$ and an r^2 of 0.996 (using 5 concentrations; 50, 100, 200, 500, 1000 ng/mL) (Table 2). The detection limit (signal-to-noise ratio = 3) of nicotine and cotinine was 0.25 and 20 ng/mL, respectively (Table 2). The recovery rates of the compounds were calculated by comparing each chromatogram area obtained from the spiked urine sample with the corresponding area obtained from a sample obtained by adding standard compounds to the final sample extract of blank urine just prior to instrumental analysis. The recovery rate of nicotine from human urine was 25.4% at 1 ng/mL and 37.3% at 100 ng/mL ($n=6$ each), while that of cotinine was 5.4% at 50 ng/mL and 11.0% at 1000 ng/mL ($n=6$ each) (Table 3).

Table 4 shows accuracy and precision data for the present method. Accuracy values were not less than 84.6% and precision values were not greater than 13.0%.

Extraction of nicotine and cotinine using SPE has been reported by several authors.^{12, 13, 22-26} Conventional SPE assays use large volumes (2–15 mL) of organic solvents, which are then evaporated in order to recover the analytes. Nicotine is quite volatile as compared to cotinine, though both can be easily lost during evaporation. Thus, solvent selection and evaporation are

Table 2 Regression equations, quantitation ranges, and detection limits for nicotine and cotinine in human urine

Compound	$y = ax + b$			Quantitation range (ng/mL)	Detection limit (ng/mL)
	a	b	r^2		
Nicotine	3.14×10^{-2}	$+1.60 \times 10^{-2}$	0.999	1–100	0.25
Cotinine	5.17×10^{-4}	-5.82×10^{-3}	0.996	50–1000	20

y is the ratio of analyte peak area to that of IS and x is the concentration of nicotine and cotinine. The slope a and intercept b values were calculated by regression, and shown as the mean of 3 experiments. Each equation was obtained from plots at 5 or 7 concentrations.

Table 3 Recovery of nicotine and cotinine in human urine

Compound	Amount added (ng/mL)	Recovery ($n=6$) (%)
Nicotine	1	25.4
	100	37.3
Cotinine	50	5.4
	1000	11.0

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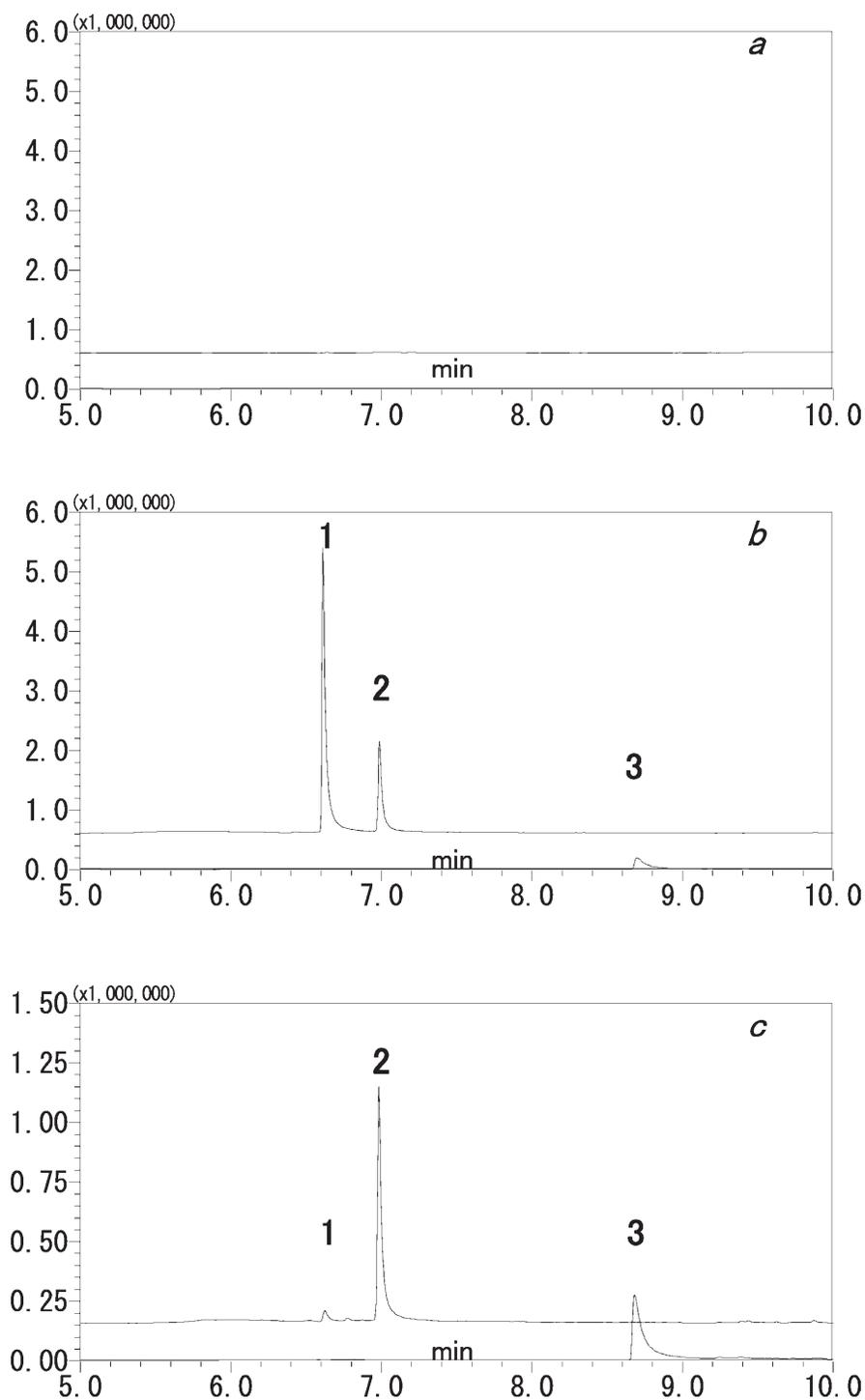


Fig. 2 Selected ion monitoring (SIM) chromatograms for nicotine (1), IS (2), and cotinine (3) extracted from human urine. In Fig. 2*b*, nicotine, IS, and cotinine were spiked into blank human urine samples at concentrations of 100, 500, and 1000 ng/mL, respectively.

Table 4 Intra- and inter-day precision and accuracy for nicotine and cotinine extracted from human urine

Compound	Amount added (ng/mL)	Intra-day (n=5)		Inter-day (n=5)	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Nicotine	1	100	4.1	93.0	10.8
	100	110	2.2	104	4.2
Cotinine	50	98.3	8.5	111	13.0
	1000	84.6	4.4	94.3	6.6

critical in nicotine-cotinine sample preparations,⁸⁾ as evaporation of the solvent should be done with extra caution to prevent over-drying, which can result in loss of the analytes. In order to reduce the volatility of nicotine during evaporation, agents such as hydrochloric acid, acetic acid, phosphoric acid, and sulfuric acid are used to form nicotine salts.⁷⁾ In the present MEPS assay method, the extraction is directly subjected to GC-MS, without evaporation. Therefore, no evaporation of solvent or salting is necessary, because of the extremely small volume of solvent utilized and use of a solvent mixture at the correct composition for GC-MS analysis.

The LOD value and recovery rate of cotinine with the present method may not be acceptable. Lafay *et al.*⁵⁾ analyzed cotinine in human urine using GC-MS with MEPS, and reported that the most effective type of MEPS adsorbent was C8, while the most effective elution solvent was acetonitrile. In the present study, we used C8+SCX and MeOH as the adsorbent and elution solvent, respectively, as we focused on nicotine extraction from urine with MEPS.

Actual analysis of nicotine and cotinine in human urine after smoking a single cigarette

A urine sample was obtained from a human volunteer at 1 hour after smoking a single cigarette within 24 hours. No peak appeared at 6.3 minutes on the SIM channel of m/z 84 or 7.0 minutes on the SIM channel of m/z 98 with the blank urine sample (Fig. 2a). The concentrations of nicotine and cotinine in human urine after smoking 1 cigarette were calculated with the present system to be 1.56 ng/mL and 1.07 µg/mL, respectively (Fig. 2c).

CONCLUSIONS

An advantage of the present MEPS assay method is that the extract is directly subjected to GC-MS analysis without evaporation. In addition, this method is simple, easy, and convenient, and can be learned quickly by relatively inexperienced personnel. We consider that our novel method based on MEPS is useful for determining nicotine levels in various types of research studies.

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