Scientific paper

Determination of Nicotine and Cotinine in Urine by Headspace Solid Phase Microextraction Gas Chromatography with Mass Spectrometric Detection

Irena Brčić Karačonji,* Ljiljana Skender, Višnja Karačić

Clinical Toxicological Chemistry Unit, Institute for Medical Research and Occupational Health, Ksaverska c. 2, Post Office Box 291, HR-10001 Zagreb, Croatia.

Received: 25-09-2006

Paper based on a presentation at the 12th International Symposium on Separation Sciences, Lipica, Slovenia, September 27–29, 2006.

Abstract

A simple and rapid method for determination of low concentrations of nicotine and cotinine in urine has been developed. The method is based on headspace solid phase microextraction (HS-SPME) followed by gas chromatographic determination and mass spectrometric detection (GC-MS). The calibration curves were linear ($r > 0.9998$) over the concentration range tested (1–500 μg L^{-1}), with detection limits of 1.1 μg L^{-1} and 0.9 μg L^{-1} for nicotine and cotinine, respectively. The repeatability for nicotine and cotinine (expressed as relative standard deviation) was < 9%. The accuracy of the method ranged from 90 to 99%.

The method was applied for the quantitative analysis of nicotine and cotinine in urine samples collected from 30 nonsmokers, 15 without any environmental tobacco smoke (ETS) exposure (group I) and 15 who reported exposure to ETS (group II). There were statistically significant differences between groups I and II for nicotine ($p < 10^{-4}$; ranges 2.1–28.0 and 10.1–499.7 μg L^{-1}) and cotinine ($p < 10^{-5}$; ranges < 0.9–15.0 and 14.5–200.9 μg L^{-1}).

Good sensitivity, short analysis time, small sample volume, no solvent use and sample preparation, make the method convenient for determination of nicotine and cotinine in nonsmokers' urine.

Keywords: cotinine, nicotine, nonsmokers, urine, HS-SPME, GC-MS.

1. Introduction

Environmental tobacco smoke (ETS) is a widespread pollutant despite the growing awareness of its adverse effects on the health of nonsmokers. Many epidemiological studies reported that ETS can cause lung cancer in adult nonsmokers and that children of parents who smoke have increased frequency of respiratory symptoms and acute lower respiratory tract infections, as well as reduced lung function.[1] Recent analyses of a few epidemiological and toxicological studies[2] suggested that ETS exposure might be a risk factor for cardiovascular disease. The primary components of ETS are sidestream smoke emitted from the smoldering tobacco between puffs and exhaled mainstream smoke from the smoker. ETS, although diluted compared with the smoke inhaled by active smoker, contains many of the same carcinogenic and toxic agents.[1] ETS has been classified as a class A carcinogen or human lung carcinogen by U. S. Environmental Protection Agency (US EPA)[1] and as carcinogenic to humans by International Agency for Research on Cancer (IARC).[3]

The biological monitoring of nonsmokers exposed to ETS is the first step toward evaluation of the toxicological effects of ETS. Nicotine and its main metabolite cotinine are widely used as biomarkers of ETS exposure and can be measured in blood,[4] saliva,[4–6] urine,[4,6–11] and hair.[6,12–14] Concentrations of biomarkers in biological fluids reflect recent exposure, while their concentrations in hair reflect long-term exposure. Only 17% of nicotine is eliminated as cotinine in urine.[15] Cotinine is mostly determined in urine, a biological medium easy to obtain. Because of the longer urinary half-life of cotinine compared with nicotine (20 vs. 2 h), cotinine in urine is currently considered to be the biomarker of choice for ETS exposure assessment.[16] The half-life of cotinine makes it a good indicator of ETS exposure over the previous day or two.

A number of analytical methods have been reported for the analysis of nicotine and cotinine in urine of nonsmokers such as gas chromatography using mass spectrometry (GC/MS)[4,6] and nitrogen-phosphorous detector (NPD),[10,17] high performance liquid chromatography (HPLC),[11,17] radioimmunoassay (RIA),[18] and enzyme lin-

ked immunosorbent assay (ELISA).[17,19] Chromatographic techniques are preferred over the other types of analyses because they are highly sensitive, specific, and they can analyse both nicotine and cotinine in a single assay.[4,6,11] These techniques are often complex and time consuming because they include liquid-liquid [4,6,10,11,17] or solid phase[7,20] extraction and preconcentration step prior to analysis. In order to minimize sample preparation and concentrate analytes in a solvent-free manner, simple and rapid headspace solid phase microextraction (HS-SPME) procedure followed by gas chromatographic determination and mass spectrometric detection was developed. SPME is a sampling technique developed by the Pawliszyn research group in the late 1980s,[21] in which a fused-silica fibre coated with a thin layer of a selective coating is used to trap and concentrate volatile and semi-volatile analytes directly from the headspace of samples. Although exposure to ETS quite often involves determination of nicotine and cotinine in urine, there are no data on using HS-SPME as sampling technique for assessment of exposure to ETS. The method was applied for the quantitative analysis of nicotine and cotinine in urine samples collected from 30 nonsmokers.

2. Experimental

2.1. Reagents

Nicotine, cotinine and diphenylamine (internal standard) were purchased from Sigma (St. Louis, MO, USA). Methanol (gradient grade for liquid chromatography) and potassium carbonate were products of Merck (Darmstadt, Germany). Ultra-pure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Standard Preparation

The stock standard solution for nicotine and cotinine (1 g L^{-1} of methanol) was prepared as aqueous solution and then diluted with ultra-pure water to working standards. These standards were stored at 4 °C and were stable up to one month.

Calibration standards were freshly prepared daily by spiking blank (analyte-free) urine of nonsmoker (no detectable amounts of nicotine and cotinine) with aliquots of working standard solutions of nicotine and cotinine. 10 µL of the internal standard solution of diphenylamine (10 mg L^{-1}) prepared in methanol was added to each calibration level.

2.3. Urine Sample Collection

The study was carried out in March 2006 and involved 30 nonsmokers, 15 without any ETS exposure and 15 who reported exposure to ETS because someone in their environment smoked. Median age of subjects was 33 years, 60% were females. All subjects completed a short questionnaire and exposure to ETS, in the 2-day period preceding the sampling, was recorded. The first urine samples were collected in the morning in clean 80-mL bottles and analysed on the day of collection.

2.4. Extraction Procedure

One mL of urine sample and 10 µL of diphenylamine (10 mg L^{-1} of methanol) as internal standard for nicotine and cotinine were added in the 6-mL clear glass vial containing 1.3 g of potassium carbonate. The addition of the salt improves extraction efficiency. The vial was sealed with PTFE septum and holed aluminum cap and heated at 80 °C for 60 min. Fused silica fiber 10 mm long with an 85-µm thick polyacrylate coating (PA) was purchased from Supelco (Bellefonte, USA). The SPME needle was pass through the septum, and the extraction fibre was exposed to the headspace above the urine at 80 °C for 15 min. The needle was removed from the vial after retracting the fiber, and inserted into the injection port of GC/MS where analytes were desorbed at 280 °C for 10 min.

2.5. Gas Chromatography-mass Spectrometry

The GC-MS analysis of nicotine and cotinine was carried out on Varian 3400 CX gas chromatograph equipped with Saturn ion trap mass spectrometer operating in the electron impact (EI) mode. Compounds were separated on Rtx-5 capillary column (5% diphenyl-95% dimethylpolysiloxane), 30 m x 0.25 mm I. D., 0.25 µm film thickness (Restek, Bellefonte, USA). The oven temperature was kept at 50 °C for 1 min, and then increased at 230 °C at rate of 20 °C min^{-1}. Late-eluating compounds were removed by increasing the temperature to 280 °C at rate of 50 °C min^{-1}. The transfer line temperature was set at 260 °C. The flow rate of the helium was 1 mL min^{-1}. Selected ion monitoring mode was used in the analysis. Monitored ions were m/z 162, 84, 133 (nicotine), m/z 176, 98, 118 (cotinine) and m/z 169, 168 (diphenylamine). Quantification was based on the peak area integration at m/z 162 (nicotine), 176 (cotinine) and 169 (I.S.).

2.6. Method Validation

To construct 5- point calibration curves, blank urine sample spiked with nicotine and cotinine at concentration ranging from 1 to 500 µg L^{-1} was prepared and analysed using the above procedure. Repeatability and accuracy were evaluated by analysing blank urine samples spiked with two different concentrations of nicotine and cotinine (20 and 100 µg L^{-1}) in six replicates. Detection limits (DL) were calculated by a signal-to-noise ratio of 3.

2.7. Statistics

Because the data did not follow a normal distribution, the results within groups were presented as median and range, and the significance of the difference between groups was calculated by using the Mann-Whitney U-test. The test was considered statistically significant when $p < 0.05$. Undetectable concentrations were set at 0.5×DL. We performed all statistical analyses using Statistica for Windows, release 5.5 (StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results and Discussion

The described method permits the separation, sensitive, fast and reliable determination of low μg L^{-1} nicotine and cotinine levels in urine. Calibration curves were linear over the concentration range 1–500 μg L^{-1} with correlation coefficients > 0.9998. Relative standard deviations (RSD) and accuracy of the method ranged between 5–9% and 90–99%, respectively. Analytical parameters of the method are presented in Table 1.

Table 1: Repeatability and accuracy of the method (N = 6).

Compound	Concentration (μg/L^{-1})	Repeatability (RSD %)	Accuracy (%)
Nicotine	20	8.5	97.5
	100	5.4	98.8
Cotinine	20	6.5	90.1
	100	5.9	97.2

They are comparable with repeatability and accuracy reported by authors using the liquid-liquid (LL) [4,6] or solid phase (SP) [20] extraction methods. Detection limits were 1.1 μg L^{-1} and 0.9 μg L^{-1} for nicotine and cotinine, respectively. Shin et al.[4], Torano et al.[6] and James et al.[20] reported lower detection limits for nicotine and cotinine in urine using LL and SP extraction methods. However, our results suggest that the proposed HS-SPME extraction method is sensitive enough to distinguish passive smokers from those who reported no ETS exposure.

Figure 1 shows total and selected ion chromatograms of nicotine and cotinine in the urine of nonsmoker exposed to ETS. No interfering peaks were observed in the urine samples studied.

Urine is usually a sample of choice for determining exposure to ETS since collection procedure is non-invasive. Our method was applied for determination of nicotine and cotinine in urine of 15 nonsmokers without any ETS exposure and 15 who reported exposure to ETS. Concentrations of nicotine and cotinine in the urine of these subjects are presented in Table 2.

Table2: Mass concentration of nicotine and cotinine in nonsmokers' urine.

	Mass concentration (μg/L^{-1})	
Compound	Nonsmokers without ETS exposure (N = 15)	Nonsmokers with ETS exposure (N = 15)
	Median (Range)	Median (Range)
Nicotine	9.9 (2.1–28.0)	30.1(10.1–499.7)
Cotinine	6.2 (<0.9–15.0)	34.5 (14.5–200.9)

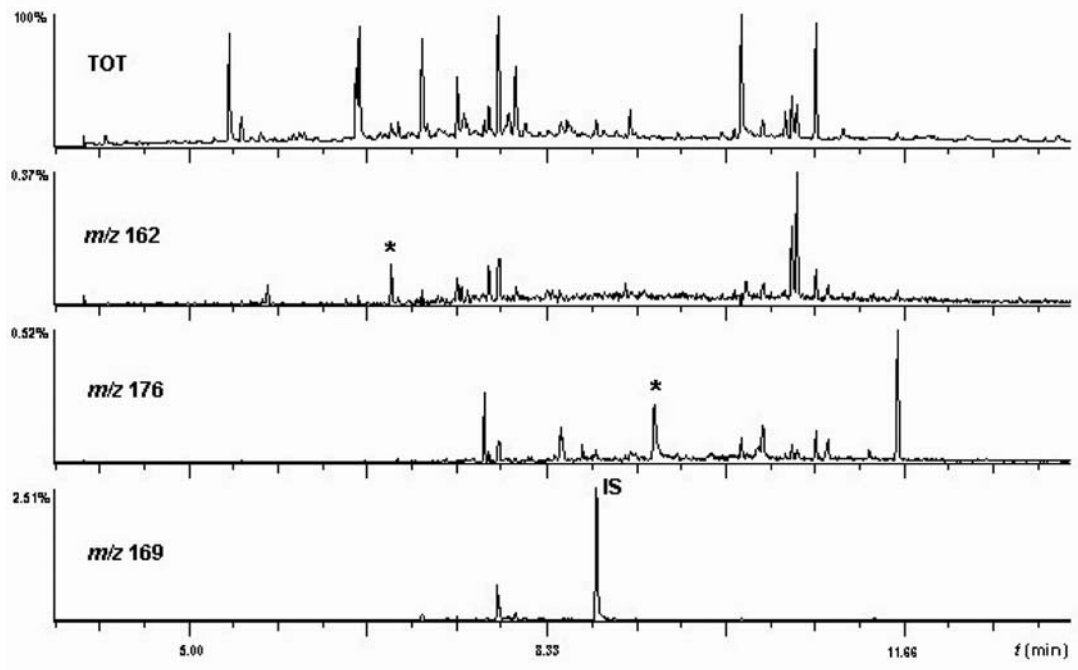

Figure 1. Total and selected ion chromatograms of nicotine (m/z 162), cotinine (m/z 176) and diphenylamine (IS, m/z 169) in nonsmoker's urine with 21.2 μg L^{-1} of nicotine and 28.4 μg L^{-1} of cotinine.

Among currently employed passive smokers, 73% reported exposure to tobacco smoke at home. All subjects had detectable nicotine concentration in urine. Cotinine concentrations were detectable in all urine samples but one in non-exposed nonsmoker. In that case the limit of detection was divided by two for further statistical analyses.[11] There were statistically significant differences in urinary concentrations between non-exposed and exposed group for nicotine ($p < 10^{-4}$) and cotinine ($p < 10^{-5}$). The median nicotine and cotinine concentrations were about 3 and 6 times higher in subjects who reported ETS exposure, than in those who did not report exposure to ETS. The highest urinary nicotine (499.7 μg L^{-1}) and cotinine (200.9 μg L^{-1}) concentrations were found in a subject who stated exposure to ETS during more than 10 h on previous day. The maximum urinary cotinine concentration determined in our study was within the suggested discriminatory values of 60–500 μg L^{-1} between smokers and nonsmokers.[22]

Nicotine and cotinine urinary concentrations in our study were comparable to those measured in 29 nonsmokers reported by Torano et al.[6] They were, however, higher than those reported by Thaqi et al.[11] for 770 children, aged 11–14 years, probably due to longer duration of exposure to ETS in our group. A review of literature data[7,8,10] showed similar concentrations of cotinine in urine of children without any ETS exposure compared with our results. However, urinary cotinine concentrations in subjects exposed to ETS found in these studies were lower than in our study and did not exceed value of 45 μg L^{-1}.

4. Conclusions

We developed a HS-SPME/GC-MS method for simultaneous determination of urinary nicotine and cotinine. The main advantage of described method over the previously published ones is that extraction and preconcentration of analytes are carried out in a single step without using a solvent. Additionally, the method is simple to perform and it requires short analysis time and minimal sample preparation.

The detection limits of 1.1 μg L^{-1} and 0.9 μg L^{-1} for nicotine and cotinine, respectively, make the method sufficiently sensitive to distinguish nonsmoker who stated exposure to ETS from those without any ETS exposure. Urinary nicotine and cotinine concentrations in ETS-exposed nonsmokers were significantly higher than those in nonsmokers reporting no ETS exposure.

Further investigations will be carried out using the same method on larger number of samples considering age and gender differences.

5. References

1. United States Environmental Protection Agency (US EPA), Off. Health Environ Assess, Respiratory Health Effects of Passive Smoking: Lung Cancer and Other Disorders, Washington DC 1992, EPA/600/6-90/006F.
2. National Institute for Occupational Safety and Health (NIOSH), Environmental tobacco smoke in the workplace: lung cancer and other health effects. Current Intelligence Bulletin 54, U.S. Department of Health and Human Services, NIOSH, Cincinnati, OH, USA 1991.
3. International Agency for Research on Cancer, IARC Monographs on the Evaluation of the Carcinogenic Risk of the Chemicals to Man. Tobacco Smoke and Involuntary Smoking, Vol 83, World Health Organization, Lyon, France, 2002.
4. H. S. Shin, J.G. Kim, Y. J. Shin, S. H. Jee, *J. Chrom. B* **2002**, *769*, 177–183.
5. K. Phillips, M. C. Bentley, M. Abrar, D. A. Howard, J. Cook, *Hum. Exp. Toxicol.* **1999**, *18,* 291–296.
6. J. S. Torano, H. J. M. van Kan, *Analyst* **2003,** *128*, 838–843.
7. Oddozze, J. C. Dubus, M. Badier, X. Thirion, A. M. Pauli, J. Pastor, B. Bruguerolle, *Clin. Chem*. **1999**, *45*, 505–509.
8. S. Willers, A. Axmon, C Feyerabend, J. Nielsen, G. Skarping, S. Skerfving, *J. Clin. Epidemiol.* **2000,** *53*, 715–721.
9. G. C. Wong, B. A. Berman, T. Hoang, C. Bernaards, C. Jones, J. T. Bernert, *Arch. Environ. Health* **2002,** *57,* 584–590.
10. R. Bono, M. Vincenti, T. Schiliro, D. Traversi, C. Pignata, E. Scursatone, G. Dotti, G. Gilli, *J. Expo. Anal. Environ. Epidemiol.* **2005**, *15*, 66–73.
11. Thaqi, K. Franke, G. Merkel, H. E. Wichmann, J. Heinrich, *Indoor Air* **2005,** *15*, 302–310.
12. K. Zahlsen, O. G. Nilsen, *Pharmacol. Toxicol*. **1994,** *75*, 143–149.
13. P. Nafstad, G. Botten, J. A. Hagen, K. Zahlsen, O. G. Nilsen, T. Silsand, J. Kongerud, *Int. J. Epidemiol.* **1995,** *24*, 88–94.
14. N. Kalinic, Lj. Skender, V. Karacic, I. Brcic, V. Vadjic, *Bull. Environ. Contam. Toxicol*. **2003,** *71*, 1–5.
15. T. P. Moyer, J. R. Charlson, R. J. Enger, L. C. Dale, J. O. Ebbert, D. R. Schroeder, R. D. Hurt, *Clin. Chem.* **2002,** *48*, 1460–1471.
16. P. Dhar, *J. Pharm. Biomed. Anal.* **2004**, *35*, 155–168.
17. H. W. Kuo, J. S. Yang, M. C. Chiu, *J. Chrom. B* **2002,** *768*, 297–303.
18. S. de Weerd, C. M. G. Thomas, J. E. T. G. Kuster, R. J. L. M. Cikot, E. A. P. Steegers, *Environ. Res.* **2002,** *90,* 119–124.
19. W. K. Al-Delaimy, J. Crane, A Woodward, *J. Epidemiol. Comm. Health* **2002**, *56*, 66–71.
20. H. James, Y. Tizabi, R. Taylor, *J. Chrom. B* **1998,** *708*, 87–93.
21. C. L. Arthur, J. Pawliszyn, *Anal. Chem.* **1990**, *62*, 2145–2148.
22. T. Lindgren, S. Willers, G. Skarping, D. Norbäck, *Int. Arch. Occup. Environ. Health* **1999,** *72*, 475–479.

Povzetek

Razvili smo enostavno in hitro metodo za določanje nikotina in kotinina v majhnih koncentracijah v urinu. Osnovana je na ekstrakciji plinske faze nad vzorcem z uporabo mikroekstrakcije na trdni fazi, ki ji sledi plinska kromatografska analiza z masnospektrometrično detekcijo. Umeritvene krivulje so linearne ($r > 0{,}9998$) v konsentracijskem intervalu, ki smo ga preverjali (1–500 μg L^{-1}) z mejo določljivosti 1,1 μg L^{-1} za nikotin in 0,9 μg L^{-1} za kotinin. Ponovljivost, izražena kot standardni odmik, je pod 9 %. Točnost je med 90 in 99 %.

Metodo smo uporabili za kvantitativno določitev nikotina in kotinina v vzorcih urina, ki smo jih zbrali pri 30 nekadilcih, od tega pri 15 takih, ki niso bili izpostavljeni okoljskemu tobačnemu dimu (skupina I), in 15 takih, ki so javili tako izpostavljenost (skupina II). Ugotovili smo statistično značilne razlike med skupinama I in II pri vsebnosti nikotina ($p < 10^{-4}$; obseg koncentracij 2,1–28,0 in 10,1–499,7 μg L^{-1}) in kotinina ($p < 10^{-5}$; obseg koncentracij <0,9–15,0 in 14,5–200,9 μg L^{-1}).

Občutljivost, kratek čas analize, majhen vzorčni volumen, odsotnost topil in kakršnekoli priprave vzorca so dobre lastnosti predlagane metode za določanje nikotina in kotinina v urinu nekadilcev.