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Chemical Analysis of Urinary Cotinine in Subjects Exposed to A Mixture of Contaminants

KEYWORDS: biomarker, cotinine, GC-MS analytical method

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ABSTRACT *Several analytical methods have been described in the literature for the analysis of cotinine in urine samples, such as gas chromatography using flame ionization detection, mass spectrometry or nitrogen-phosphorous detector, high performance liquid chromatography with ultraviolet or mass spectrometry detector, capillary electrophoresis, radioimmunoassay and enzyme linked immunosorbent assay. Chromatographic techniques are preferred over the other types of analytical methods because of their high sensitivity and specificity.*

In our study, we measured cotinine as a biomarker of exposure to environmental tobacco smoke. Spot urine samples were collected and kept frozen at -20°C until performing the analysis. Cotinine was measured in urine by gas chromatography - mass spectrometry (GC-MS) analytical method.

The sensitivity of the GC-MS procedure was estimated as the limit of detection calculated for cotinine, at 1.37 µg/L. The accuracy of the GC-MS method was 3.02%.

INTRODUCTION

Environmental tobacco smoke (ETS) is one of the most "popular" chemical mixtures to which different population groups are frequently exposed in occupational and home environment, as long as tobacco consumption remains the most important avoidable cancer risk. In the 20th century, approximately 100 million people died world-wide from tobacco-associated diseases (cancer, chronic lung disease, cardiovascular disease and stroke), half of regular smokers are killed by the habit and one quarter of smokers will die prematurely during middle age (35 to 69 years) (Stewart and Kleihues, 2003). Cigarette smoke contains more than 4000 chemicals, including volatile organic compounds, polycyclic aromatic hydrocarbons, heavy metals, tar, etc. Nicotine is the main pharmacologically active component of tobacco smoke. Cotinine is the major metabolite of nicotine and it is an important biomarker for both active and passive tobacco exposure. Cotinine is a more reliable indicator than nicotine or other nicotine metabolites, being more stable ($t_{½}$=7-20 hours) than nicotine ($t_{½}$=2 hours) and having lower interference during detection and analysis (Henrich-Ramm et al., 2002).

Several analytical methods have been described in the literature for the analysis of cotinine in urine samples, such as gas chromatography using flame ionization detection (GC-FID), mass spectrometry (GC-MS) (Shin et al., 2002), or nitrogen-phosphorous detector (GC-NPD) (Kuo et al., 2002), high performance liquid chromatography with UV (HPLC-UV) or MS detector (LC-MS-MS) (Kuo et al., 2002), capillary electrophoresis (Edward et al., 2003), radioimmunoassay (RIA) (Weerd et al., 2003) and enzyme linked immunosorbent assay (ELISA) (Kuo et al., 2002). Chromatographic techniques are preferred because of their high sensitivity and specificity (Shin et al., 2002).

MATERIAL AND METHODS

Objective - to validate a sensitive, fast and reliable GC-MS method for cotinine analysis in urine samples in order to evaluate the exposure to ETS using cotinine as a biomarker of exposure.

Urine sampling and sample preparation - Spot urine samples were collected in 50 mL polyethylene containers, previously demineralized with HNO_3 4% to avoid sample contamination. Immediately after collection, the samples were stored in a freezer at –20°C until analysis.

For cotinine extraction, 2 mL urine sample was mixed with 1.4 mL HCl (0.1 M), 2 mL NaOH (5 M) and 5 mL dichloromethane, then centrifuged for 5 min. (3000 rpm) (Müller, 2010). The aqueous component was removed, while the organic component was dried (3 g Na_2SO_4 anhydrous) and centrifuged (5 min) (1600 rpm). The supernatant was evaporated to dryness under nitrogen steam (15 mL/min, 45°C). The residue was dissolved in 1 mL toluene and 2 µL were injected into GC-MS. Blank samples (analyte-free urine) were prepared and analyzed following the same procedure.

Urinary creatinine determination - The determination of urinary creatinine is necessary to report cotinine concentration to urinary creatinine level. For creatinine analysis, 10 mL urine sample was centrifuged for 15 min.; then 1 mL of urine sample was diluted with distilled water to 100 mL. 2 mL of diluted urine were mixed with 6 mL picric acid (1.6%) and 0.4 mL NaOH (10%) and the absorbances were measured by UV-VIS spectrophotometry (λ=530 nm). Blank samples were prepared and analyzed following the same procedure.

All chemicals used were of high purity grade, provided by Sigma-Aldrich (Germany) and Merck (Germany). The water for analysis was purified using Millipore Direct-Q3UV Ultrapure Water System.

Applicability of GS-MS procedure for cotinine determination - The analyses were performed with a Shimadzu (GC-MS QP2010, Japan) gas chromatograph equipped with automatic split/splitless injector (AOC-20i) and MS detector. A fused silica capillary column (TG-5MS, 30m×0.25mm×0.25 µm, Thermo, USA) was used. The operating conditions were: 250°C injector port temperature; helium as carrier gas (1.20 mL/min); high pressure injection (350 kPa for 1 min) splitless mode. The column temperature was maintained at 95°C for 3 min., then programmed at 110°C (5°C/min.), followed by a final ramp to 250°C at a rate of 30 °C/min and held for 1.3 min. The ion source temperature was 230°C with interface temperature of 250°C. The MS detector was operated in electron impact ionization mode and analysis was performed with selected ion monitoring (SIM) using one target (m/z=98)

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and one qualifier ion (m/z=176). The target and qualifier ratios were determined by injecting individual cotinine standards under the same chromatographic conditions using full scan with the mass/charge ratio from 60 to 400 m/z.

Calibration curves - The cotinine calibration curve was generated based on the concentration range 50-250 µg/l cotinine (50, 100, 150, 200, 250 µg/l), prepared as follows: 2 mL urine mixed with different volumes (100, 200, 300, 400 and 500 µL) of 1 mg/L stock solution and HCl 0.1M (1300, 1200, 1100, 1000, 900 µL).

The creatinine calibration curve was generated based on the concentration range 0-10 mg/L (0, 1.5, 3, 4, 5, 6, 7, 8 and 10 mg/L) prepared by diluting specific volumes of 10 mg/L stock solution (0, 0.9, 1.8, 2.4, 3, 3.6, 4.2, 4.8 and 6 mL) with water (6, 5.1, 4.2, 3.6, 3, 2.4, 1.8, 1.2 and 0 mL). 2 mL from each standard solution were mixed with 6 mL picric acid (0.6%) and 0.4 mL NaOH (10%), and the absorbances were measured by UV-VIS spectrophotometry. Cotinine and creatinine concentrations were read directly on the calibration curve.

RESULTS

Experimental

Methods validation - In order to ensure the data quality, the GC-MS method was validated by assessing the following parameters: linearity, sensitivity, accuracy, precision and method uncertainty.

Method linearity was performed according to SR ISO 8466:99 recommendations. The calibration curve, shown in Figure 1, was plot using blank urine samples spiked with cotinine from 50 to 250µg/L. The linear range was determined by repeated analysis (10 times) of the lowest and the highest concentrations of standard solutions and by analyzing in duplicate, other standard solutions. The corresponding dispersion ratio (PG) was compared with F distribution value for α=0.99, the freedom degrees number being the number of repeated analysis for each concentration.

The performance parameters of the GC-MS method are shown in Table 1.

The residual standard deviation (S_y), one of the performance parameter, which estimates the accuracy of the calibration (dispersion of experimental data around the calculated regression line), the method standard deviation (S_{x0}) which is the ratio between residual standard deviation (S_y) and method sensitivity (calibration curve slope-b), were calculated as follows:

$$S_y = \sqrt{\frac{\sum (y_i - \overline{y_i})^2}{N-2}} \quad (1); \quad S_{x_0} = \frac{S_y}{b} \quad (2)$$

TABLE – 1 Performance parameters of GC-MS method

Performance parameters	Value		
Linearity			
equation	y=311.8x+978.7		
correlation coefficient	R=0.9988		
residual standard deviation	S_{y1}=1413.2449 µg/L		
method standard deviation	S_{x0}=4.5319 µg/L		
variances homogeneity test	PG=4.32; F=5.35; PG<F		
linearity test	S_{y1}=1413.2449 S_{y2}=1730.3262 PG=0.0012; PG<F		
Accuracy and precision			
C[a] (µg/L)	50	150	250
$\lvert\overline{C_0}\rvert$ (µg/L)±SD[c]	49.59 ±2.43	149.1 ±3.81	251.9 ±5.01
RDS[d]	4.90	2.55	1.98
$\lvert\overline{C_0} - C\rvert$ (µg/L)	0.41	0.90	1.90
$0.6 \cdot 2^{(1-0.5 \cdot \lg C)}$	5.30	4.80	4.20
Uncertainty			
measurement extended uncertainty (µg/L)	±12.74	±36.22	±60.66
method uncertainty (%)	±23.75%		

C[a] – known concentration; $\lvert\overline{C_0}\rvert$ [b] – average value of measured concentration for 10 replicates; SD[c] – standard deviation; RSD[d] – relative standard deviation;

According to variances homogeneity test, the PG was lower than F (F=5.35 in our case) indicating that the dispersion deviation is not significant (SR ISO 8466:99). The linearity test showed that calibration function does not provide adjustment (PG<F), being linear (SR ISO 8466:99). Thus, the examined concentration range met all the criteria to be used for calibration.

GC-MS sensitivity was estimated as the limit of detection (LOD), calculated by multiplying the Student t factor with the standard deviation (S_r) of 10 repeatedly analyzed standard solutions (8-10 µg/L). The best results were obtained for 10 µg/L standard solution (S_r=0.6064 µg/L and t=2.26 for 95% confidence level and 9 freedom degrees). The LOD calculated for cotinine was 1.37 µg/L.

The accuracy and precision (intra-day precision) of the GC-MS method were evaluated by analyzing ten replicates (same day, same person and same GC-MS) blank urine samples spiked with cotinine (50, 150 and 250 µg/L) (Table-1). The $\lvert\overline{C_0} - C\rvert$ ranged between 0.41 and 1.90 µg/L, being lower than 10%, indicating that the method has good accuracy. Intra-day precision was evaluated based on the relative standard deviation (RSD) of 10 replicates (Table-1). The RSD was lower than modified Horwitz value $0.6 \cdot 2^{(1-0.5 \cdot \lg C)}$, imposed by internal laboratory requirements, indicating that the GC-MS method has good precision.

Measurement uncertainty was evaluated by analyzing ten replicates blank urine samples spiked with cotinine (50, 150 and 250 µg/L). Method uncertainty was ±23.75%, lower than 30%, thus complying with the internal laboratory requirements.

Applicability of GC-MS method

Mass spectra in SIM mode and the chromatogram of cotinine in an analyzed urine sample are presented in Figure 1. We measured urinary cotinine concentrations between 0-395.41 µg/g creatinine. The level of cotinine in urine in smokers and nonsmokers are showed in Table 2 (higher average values in smokers as compared to nonsmokers).

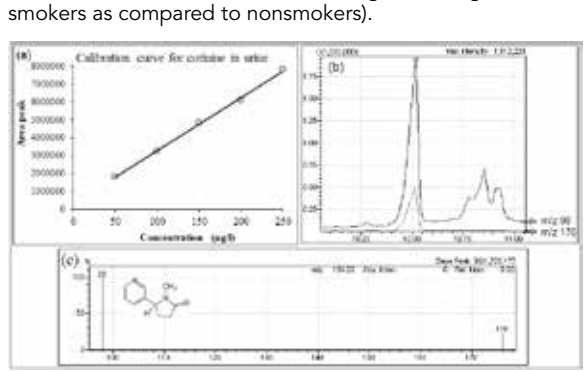

Figure 1: (a) calibration curve for urinary cotinine; (b) GC-MS chromatogram of cotinine in urine sample (m/z=98 for quantitation and m/z=176 for confirmation); (c) cotinine

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electron ionization mass spectra

TABLE – 2 Cotinine levels in urine in smokers and non-smokers

Smoking habits	Cotinine in urine (µg/g creatinine)	
	Average	Std. deviation
Nonsmoker	31.91	62.36
Smoker	152.23	164.96

CONCLUSIONS

Validation experiments confirmed that the proposed GC-MS method is sensitive, fast and reliable for urinary cotinine quantification. The method is suitable also, for complementary tests for pre- and peri-operative assessments of patients undergoing surgical procedures and patient monitoring during nicotine replacement therapy for smoking cessation.

Cotinine has shown to be a reliable biomarker for tobacco exposure, being considerably higher in smokers (average of 152.23 µg/g creatinine) as compared to nonsmokers (average of 31.91 µg/g creatinine).

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