



A simplified method for the analysis of urinary cotinine by GC-MS

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ABSTRACT

Cotinine is the major metabolite of nicotine and, being very stable and having a long biological half-life, it can be used as a biomarker for tobacco exposure. The aim of this study was to develop an analytical GC-MS technique to measure levels of cotinine in the urine of active and passive smokers and to compare the results with reference values. The extraction of cotinine to generate the calibration curve was performed by mixing urine (250 µL) with 50 µL of a cotinine standard, 50 µL of an internal standard of deuterated cotinine (15 µg·mL⁻¹) and 50 µL of 10% NH₄OH solution. Next, 2 mL of a mixture of MTBE:dichloromethane:ethyl acetate (30:30:40 by volume) was added and the whole was vortexed, then centrifuged at 3000 rpm. Finally, 1.6 mL of the organic layer was evaporated under a stream of dry air at 50 °C. The resulting extract was dissolved in methanol and injected into the GC-MS system. The LOQ and LOD for cotinine were 100 and 20 ng·mL⁻¹, respectively. The curve was linear over the whole tested range of 100 - 5000 ng·mL⁻¹ and the method achieved 50% recovery. The intra and inter-day precisions were 1.62 - 7.28% and 0.86 - 2.68%, respectively. Accuracy was determined at three concentrations (low, medium and high), with six replicates (95.24 - 97.67%). The validation of this cotinine assay by GC-MS showed that it exhibited satisfactory limits and the assay could be performed with a one-step liquid-liquid extraction. The technique presented here can thus be used for the quantitation of cotinine levels in the urine of passive and active smokers.

Keywords: GC-MS. Cotinine. Urine. Tobacco smokers.

INTRODUCTION

For many decades in the past and again recently, much research has been done on the adverse medical

consequences of smoking (Costa e Silva, 1990; Brasil, 1998; Sachar et al., 1998; Romero & Costa e Silva, 2011).

The Instituto Nacional do Câncer (INCA), associated with the Brazilian Ministry of Health, estimates that the prevalence of smoking in Brazil from 2006 to 2010, among adults (18 years and older) in 26 state capitals and the Federal capital, Brasília, is 15.1% being 17.9% for men (approximately 5.5 million people) and 12.7% for women (approximately 4 million people) (Brasil, 2011). Additionally, the majority of these smokers became addicted at a young age and the incidence of smoking is especially high among people between 20 and 49 years of age (Cabar et al., 2003).

Over 4000 substances are known to be present in cigarette smoke. Many of these substances are pharmacologically active, mutagenic or carcinogenic (Byrd, 1992; Odo et al., 2000), which makes it difficult to characterize cigarette smoke with respect to the chemical composition and toxicity of its components (Kim et al., 2005). However, nicotine is generally accepted as the principal pharmacologically active component of tobacco smoke. Nicotine itself is odorless and colorless and it can stimulate the release of biogenic amines and result in vasoconstriction and decreased tissue perfusion when inhaled or injected (Frick & Seals Jr, 1994). Nicotine spreads rapidly to the brain, but its levels fall abruptly (resulting in the need to smoke again) as it is distributed to other tissues (Benowitz, 1986).

Nicotine is metabolized by the liver, lungs and kidney and has a biological half-life of 2 hours. Cotinine and derivatives such as cotinine N-oxide are the main metabolites of nicotine and, while these compounds are not known to be pharmacologically active, they can be used as biomarkers of nicotine exposure (Tyrpien et al., 2000).

Other biomarkers for blood or plasma monitoring of cigarette exposure include carboxyhemoglobin, thiocyanate and nicotine. However, carboxyhemoglobin levels do not indicate daily variations in exposure, and the presence of thiocyanate is not specific to nicotine. The analytical investigation of plasma nicotine levels is hampered by the short half-life and low boiling point of nicotine (Kim et al., 2005; Tyrpien et al., 2000).

Cotinine is an important biomarker for exposure to standard tobacco smoke and is a more reliable

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indicator than nicotine itself or other nicotine metabolites. Owing to its long half-life (7-20 hours) and low level of interference during detection and analysis (with respect to contamination, matrix effects and extraction efficiency), cotinine can be detected for over a week after smoking. Cotinine levels in the blood and urine are proportional to the intensity of exposure, so that cotinine is an important biomarker for both active and passive exposure to tobacco (Henrich-Ramm et al., 2002). However, several factors (e.g., the presence of menthol, puff size, gender and race) contribute to a high variability in the pharmacokinetics of cotinine (Florescu et al., 2009).

Several methods have been proposed for the determination of nicotine and cotinine levels in biological matrices. Some of the most widely used include radio-immunoassay (RA), enzyme immunoassay (EIA), ELISA, high-performance liquid chromatography (HPLC) and gas chromatography with flame ionization detection (GC-FID), nitrogen-phosphorus detection (GC-NPD) or mass spectrometry (GC-MS) (Jacob et al., 2000; Shin et al., 2002; Jacob et al., 2005).

Chromatographic methods (in particular, GC-MS or LC-MS-MS) are expected to exhibit high degrees of selectivity and specificity, because of the sample preparation steps (extraction, purification and concentration) and chromatographic separation (resolution) that occur before the analyte reaches the detector. By contrast, other tests, such as immunoassays, may respond falsely to metabolites that are structurally similar to cotinine (Man et al., 2006), specially because of antibody crossreactivity with 3-hydroxycotinine (Jacob et al., 2005).

Toxicological analysis is an indispensable resource that can be used to confirm exposure to drugs of abuse and to monitor and prevent the licit or illicit use of drugs such as tobacco (Odo et al., 2000; Culea et al., 2005). It can also be used for occupational purposes, pre- and peri-surgery and to identify active or passive smokers. The results of such analysis can potentially be used to identify the development of diseases that may be correlated with tobacco consumption, such as duct ectasia, the onset of which has been associated with smoking (Rahal et al., 2005) and which involves the dilation of mammary ducts accompanied by the extravasation of serous fluid and occurs with a prevalence of 1.1 to 75% (Clegg-Lamprey et al., 2009).

The aim of this study was to validate a simplified GC-MS analytical technique, to monitor the amount of cotinine in urine samples from smokers (active and passive) and nonsmokers.

EXPERIMENTAL

Reagents and Standards

Methanol was purchased from JT Baker® (Xalostoc, Mexico), methyl *tert*-butyl ether (MTBE) and ethyl acetate from Tedia® (Fairfield, USA), dichloromethane from Isofar® (Duque de Caxias, Brazil), ammonium hydroxide from Cromato Produtos Químicos (São Paulo, Brazil);

cotinine and deuterated cotinine standards were from Cerilliant® (Texas, USA). Ultrapure water was produced with a Gehaka® Master P & D-TOC system (São Paulo, Brazil).

Preparation of stock solutions and calibration standards

Solutions of cotinine (100 µg·mL⁻¹) and deuterated cotinine (100 µg·mL⁻¹) were prepared in methanol and stored in the dark at -20 °C; these samples were considered stable for 6 months, in light of abundant evidence in the literature (Curvall et al., 1990; Niedbala et al., 2002; Behera et al., 2003), including a stability test in which cotinine samples in urine were stored in a freezer at -20 °C for over 10 years (Riboli et al., 1995).

To generate a calibration curve, cotinine and deuterated cotinine were added to blank human urine samples to final concentrations of 100, 250, 500, 1000, 2500 and 5000 ng·mL⁻¹. Samples at concentrations of 300, 2000 and 4000 ng·mL⁻¹ were also prepared for quality control purposes.

Ethical considerations

The research from which the data used for analytical validation in this article were derived, involved 105 outpatients, postpartum women and nursing mothers, with and without duct ectasia, and was approved by the Human and Animal Medical Research Ethics Committee at the Federal University of Goiás Teaching Hospital (HC-UFG) (CEPMHA, Protocol 035/06). However, for this article, only one urine sample was used to demonstrate the applicability of the method, as shown in the results.

Preparation of samples

Blank human urine was centrifuged at 3000 rpm for 5 min. A mixture containing 250 µL of this urine, 50 µL of a cotinine standard (100-5000 ng·mL⁻¹), 50 µL of an internal standard of deuterated cotinine (15 µg·mL⁻¹) and 50 µL of 10% NH₄OH was stirred for 5 s. Next, 2 mL of mixed MTBE/dichloromethane/ethyl acetate (30:30:40 by volume) was added and the whole stirred for an additional 60 s. After centrifugation for 4 min at 3000 rpm, 1.6 mL of the solution was transferred to a clean tube and the solvent was removed with a stream of dry air at 50 °C. The resulting samples were dissolved in 200 µL of methanol and stirred for 60 s, and a 6 µL aliquot was injected into the GC-MS.

The urine sample was collected in the morning in a 50-mL polyethylene bottle with a screw cap and delivered to the Clinical Analysis laboratory at HC-UFG. The sample was divided into two test tubes with screw caps, labeled and taken to the freezer at -20 °C until analysis by GC-MS.

Chromatographic conditions

Chromatography was performed in a gas chromatograph coupled to a mass spectrometer (GC-MS, GC-2010, Shimadzu Corporation, Japan), and GC-MS Solution software was used for data processing. An RTX-5

column (30 m x 0.25 mm id x 0.25 μm film) was used. The oven temperature started at 120 $^{\circ}\text{C}$, rising at a rate of 10 $^{\circ}\text{C}/\text{min}$ to 300 $^{\circ}\text{C}$, where it stayed for 3 min. The total analysis time was 22 min. The injector and detector temperatures were 280 and 300 $^{\circ}\text{C}$, respectively. The carrier gas was helium flowing at a rate of 40 cm^3s^{-1} . The ionization energy was 70 eV and the injection volume 6 μL . Detection was performed in the SIM mode, and cotinine and deuterated cotinine were identified by ions at m/z 176 and 179, respectively.

Validation of the bioanalytical method

The proposed method for the quantitation of cotinine was validated according to regulation 899 of the Brazilian Food, Drug and Sanitation Agency, ANVISA (Brasil, 2003). The limits of detection and quantitation were estimated to have peak heights three and ten times greater than the signal to noise ratio, respectively. The linearity of the method was assessed in duplicate by constructing calibration curves from six points covering concentrations of 100-5000 $\text{ng}\cdot\text{mL}^{-1}$. The accuracy, intra-day precision and inter-day precision were determined with six replicates at three concentration levels (300, 2000 and 4000 $\text{ng}\cdot\text{mL}^{-1}$). The average amount of cotinine recovered during the extraction was determined by comparing the areas of the corresponding peaks for the extracted samples with those that had not been extracted. The selectivity was assessed by comparing chromatograms of blank urine samples with those obtained after the addition of standards (cotinine and deuterated cotinine) to blank urine samples.

Application of the bioanalytical method

The new GC-MS method was developed to determine cotinine levels in the urine of active and passive smokers. The eventual goal was to correlate these levels with reference values obtained from the literature and the incidence of mammary duct ectasia in women smokers (data not shown). The method can also be used to detect levels of cotinine in active, passive (relatives, children and the elderly) and non-smokers. Potential applications include occupational purposes (Kolb et al., 2010), the prevention of abuse (Benowitz, 2008), complementary tests for pre- and peri-operative assessments of patients undergoing surgical procedures (Warner, 2006; Knobloch et al., 2008) and patient monitoring during nicotine replacement therapy for smoking cessation (Aubin et al., 2011).

RESULTS AND DISCUSSION

Method Development

To develop an analytical method for the quantitation of cotinine in human urine by GC-MS, several optimization steps were necessary. Several variables, such as the temperature gradient of the column oven, the injection volume, the injection and detection temperatures and the

mode of data acquisition (SCAN and SIM), were assessed, to optimize the chromatographic conditions.

The internal standard, deuterated cotinine, was chosen because it is structurally identical to cotinine. The same extraction and analysis conditions can therefore be used, significantly reducing the amount of random error.

Both precipitation and liquid-liquid extraction methods were tested, but the latter was chosen because interfering substances co-eluted with cotinine when the precipitation method was used. Various organic solvents were tested, including hexane, dichloromethane, ethyl acetate and MTBE. The most efficient solvent system was a mixture of MTBE:dichloromethane:ethyl acetate (30:30:40, v/v/v), which yielded a recovery of 50%.

In an attempt to minimize the influence of interfering substances during the detection phase, several ionic fragments of cotinine and deuterated cotinine were monitored. The best results were achieved by measuring the ions at m/z 176 for cotinine and m/z 179 for deuterated cotinine (Figure 1).

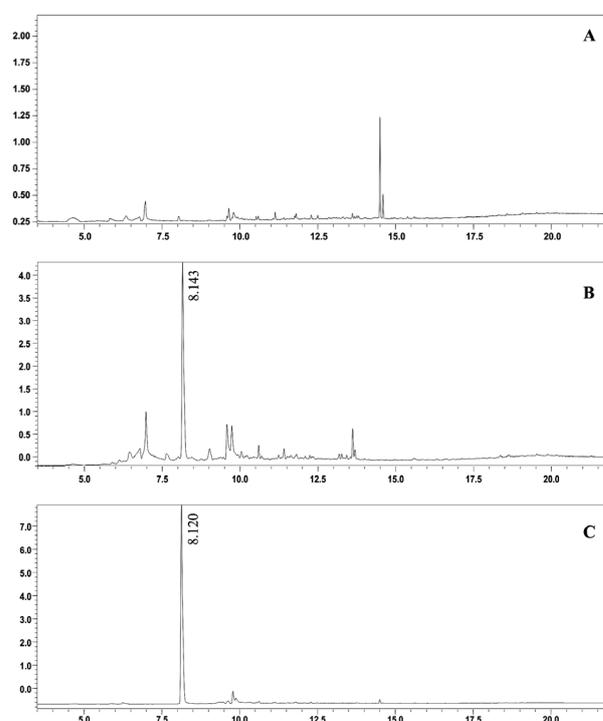


Figure 1. Chromatograms of urine samples extracted by the proposed methodology. (A) Blank. (B) 250 $\text{ng}\cdot\text{mL}^{-1}$ Cotinine (m/z 176). (C) 3500 $\text{ng}\cdot\text{mL}^{-1}$ Deuterated cotinine (m/z 179).

Method Validation

The validation experiments confirmed that this method is suitable for the quantitation of cotinine. The detection limit and lower limit of quantitation were 20 and 100 $\text{ng}\cdot\text{mL}^{-1}$, respectively. Levels of cotinine in urine have been established in the literature for the approximate classification of non-smokers and smokers: nonsmokers, < 20 $\text{ng}\cdot\text{mL}^{-1}$; passive smokers, 20-100 $\text{ng}\cdot\text{mL}^{-1}$; moderate smokers, 100-500 $\text{ng}\cdot\text{mL}^{-1}$; and heavy smokers, > 500 $\text{ng}\cdot\text{mL}^{-1}$ (Vine et al., 1993; Behera et al., 2003).

Table 1. Intra and inter-day precision and accuracy of the assay for cotinine in urine samples.

Level	Concentration (ng·mL ⁻¹)	Batch	Average ± s (ng·mL ⁻¹)	Intra-day		Inter-day	
				Precision (RSD)	Accuracy (%)	Precision (RSD)	Accuracy (%)
LOQ ^a	100	1 (n= 6)	95.75 ± 6.72	7.28	98.30	5.20	95.70
		2 (n= 6)	94.62 ± 3.23	3.74	94.62		
		3 (n= 6)	94.17 ± 4.07	4.57	94.17		
QCL ^b	300	1 (n= 6)	294.89 ± 4.37	1.62	98.30	3.39	98.01
		2 (n= 6)	299.78 ± 15.29	5.58	99.93		
		3 (n= 6)	287.40 ± 7.81	2.98	95.80		
QCM ^c	2000	1 (n= 6)	1931.44 ± 59.13	3.18	96.57	3.69	95.24
		2 (n= 6)	1892.36 ± 73.83	3.92	94.62		
		3 (n= 6)	1890.71 ± 75.98	3.97	94.54		
QCH ^d	4000	1 (n= 6)	3970.46 ± 95.32	2.67	99.26	2.65	97.30
		2 (n= 6)	3861.72 ± 93.77	2.71	96.54		
		3 (n= 6)	3844.35 ± 87.91	2.56	96.11		

^a Lower limit of quantitation; ^b Quality control, low concentration sample; ^c Quality control, medium concentration sample; ^d Quality control, high concentration sample.

The analytical method is linear in the 100-5000 ng·mL⁻¹ range. The linear equation was $y = 0.00018x + 0.0064$ (correlation coefficient $r = 0.9998$, $r^2 = 0.9996$). The inter- and intra-assay accuracy and precision were acceptable (Table 1), and the average extraction recovery of cotinine was 50% for the three concentration levels tested (300, 2000 and 4000 ng·mL⁻¹). This analytical method can thus be used for the quantitation of cotinine in active and passive smokers (Figure 2).

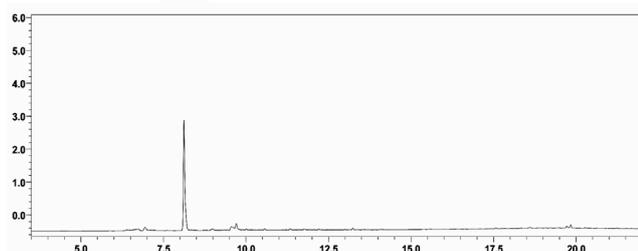


Figure 2. Chromatogram for a voluntary smoker sample analyzed by the developed methodology and validated for a cotinine concentration of 758 ng·mL⁻¹.

Thus, the measurement of cotinine levels by GC-MS showed satisfactory linearity, precision, accuracy, recovery, sensitivity, selectivity and robustness, and the assay can be performed with a one-step liquid-liquid extraction that simplifies the technique and does not require re-extraction or the use of various reagents in a multi-step analysis (Kuo et al., 2002; Heavner et al., 2005), or the hard and expensive procedures of solid phase extraction (SPE) (Heavner et al., 2005; Kim et al., 2005). Another advantage is the amount of urine used, only 250 μ L (liquid-liquid micro-extraction) against volumes up to 20 mL (Odoze et al., 1998; Tyrcien

et al., 2000), smaller volumes being less harmful to health at work and the environment and allowing savings in reagents, time and equipment.

This technique can be used for the quantitation of cotinine in both active and passive smokers, and can therefore be applied in various types of clinical and occupational study, including those investigating the correlation between mammary duct ectasia and smoking. Moreover, the speed and ease of routine analysis were improved, involving no derivatization and no long liquid-liquid extraction with several steps.

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RESUMO

Um método simplificado para análise de cotinina em urina usando CG-EM

A cotinina é o metabólito principal da nicotina, é muito estável e tem uma elevada meia-vida biológica e pode ser usado como biomarcador da exposição ao tabaco. O objetivo deste estudo foi desenvolver uma técnica analítica em CG-EM para medir os níveis de cotinina na urina de fumantes ativos e passivos e comparar os resultados com valores de referência. A extração da cotinina para construir a curva de calibração foi desenvolvida misturando 250 μ L de

padrão de cotinina em urina, 50 µL de padrão interno (cotinina deuterada 15 µg·mL⁻¹) e 50 µL de solução aquosa de NH₄OH 10%. Em seguida, 2 mL da mistura MTBE:diclorometano:acetato de etila (30:30:40 v/v) foi adicionada, agitada em vórtex e centrifugada a 3000 rpm. Finalmente, 1,6 mL da camada orgânica foi evaporada sob ar seco a 50 °C. O extrato resultante foi dissolvido em metanol e injetado no sistema CG-EM. Os limites de quantificação e de detecção foram 100 e 20 ng·mL⁻¹, respectivamente. A curva de calibração foi linear no intervalo de concentração testado (100 - 5000 ng·mL⁻¹), com 50% de recuperação. Os valores de precisão intra-dia e inter-dia foram 1,62 - 7,28% e 0,86 - 2,68%, respectivamente. A exatidão (95,24 - 97,67%) foi determinada sob 3 concentrações (baixa, média e alta), com 6 replicatas. A validação deste procedimento para análise de cotinina por CG-EM demonstrou valores satisfatórios, numa única etapa de extração líquido-líquido, podendo ser utilizada para a quantificação dos níveis de cotinina em amostras de urina de fumantes ativos e passivos.

Palavras-chave: CG-EM. Cotinina. Urina. Tabagismo.

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