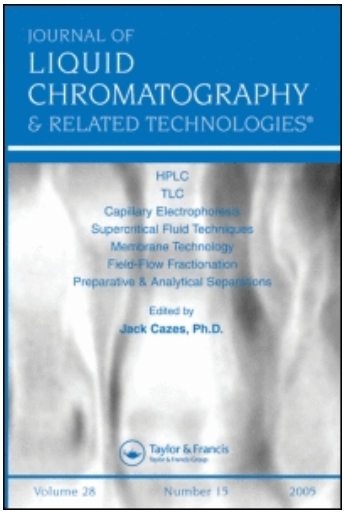

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Quantification of Cotinine in Plasma and Urine by HPLC-UV Detection

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ABSTRACT

Cotinine, the main metabolite of nicotine in man, is widely used as an abstention marker to *Nicotiana tabacum* smoke, as well as to evaluate passive inhalation of tobacco smoke by non-smokers. Development and validation of a reversed-phase high performance liquid chromatography (RP-HPLC) method with ultraviolet (UV) detection for identification and quantification of cotinine in human plasma and urine are described. After diluting plasma with distilled water (1/6), NaOH 5 M (1 : 1) was added to both matrices and cotinine extracted using a RP 18 solid phase extraction column (SPE). Extracts were resuspended in mobile phase phosphate buffer pH 6.8 : acetonitrile (90 : 10) and injected into a Lichrospher 100 RP-18 column (5 μm). The UV detector was set to

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260 nm. Linear calibration curves in the range of 10–1000 ng/mL of cotinine with correlation coefficients greater than 0.999 were obtained. Within-run and between-run accuracy was less than 5%. Cotinine detection limit was 20 ng/mL. The proposed chromatographic system allows cotinine to be separated from caffeine.

Key Words: Cotinine; Plasma; Urine; HPLC-UV detection.

INTRODUCTION

The main metabolite of nicotine, cotinine, is derived almost entirely from tobacco smoking by man.[1] Both substances are used as cigarette smoking markers.[2]

Cotinine has a half-life 10-fold greater than nicotine,[3] and for this reason it is widely used as a biological abstention marker for smokers and nonsmokers exposed passively to tobacco smoke.[4]

The aim of this study was to identify and to quantify cotinine in human plasma and urine by HPLC-ultraviolet (UV) detection. Since epidemiological studies have demonstrated that tobacco smoking and coffee intake correlate positively, interference of caffeine in the proposed method was analyzed.[5]

EXPERIMENTAL

Materials and Chemicals

(−)-Cotinine (C5923) and caffeine (C 8960) were purchased from Sigma Chemical (St. Louis, MO). Nicotine was from Fluka (72290). HPLC-grade acetonitrile and dichloromethane were from Merck Quimica (Argentina). Anhydrous monobasic potassium phosphate and anhydrous dibasic potassium phosphate were from Mallinckrodt.

Solid phase extraction (SPE) column: Light Load Octadecyl C_{18} Bakerbond SPE (J.T. Baker 7189-03).

Reagents

Phosphate buffer, pH 6.8, was prepared by weighing 1.940 g of anhydrous monobasic potassium phosphate and 2.481 g of anhydrous dibasic potassium phosphate, and dissolved in 700 mL water HPLC-grade. The pH was adjusted to 6.8 by addition of 1% formic acid solution and completed to 1000 mL with distilled water.

Cotinine solutions: 10 mg cotinine were dissolved in 10 mL water HPLC-grade. Working solutions ranging from 10 to 1000 ng/mL were obtained by dilution with mobile phase.

Caffeine solution: 10 mg caffeine were dissolved in 10 mL water HPLC-grade. Working solutions of 10 μg/mL was obtained by dilution with mobile phase.

Mobile phase was phosphate buffer pH 6.8 : acetonitrile (90 : 10).

SPE columns were conditioned with two volumes of methanol and two volumes of distilled water before using.

Biological Samples

Plasma samples: heparinized blood was collected from healthy non-smoker volunteers. Plasma was separated by centrifugation at 3000 rpm.

Urine samples: urine samples were obtained from non-smoker and smoker female volunteers. Mean smokers' age was 42 ± 3 years, and cigarette consumption 25–30 per day. Urine samples were collected before retiring for the night.

All samples were kept at -20°C until analysis.

Chromatographic Conditions and Apparatus

A JASCO PU980 liquid chromatograph equipped with an Intelligent JASCO AS-950 autosampler and a 5-μm Lichrospher RP-18 analytical column (12.5 × 4.6 mm ID) (Merck) was used. UV detection at 260 nm was performed with a JASCO UV 975 detector. Standard and sample volumes injected were 20 μL. A flow rate of 0.6 mL/min was maintained.

Procedure

Calibration instrument: calibration curves were prepared in the range of 10–1000 ng/mL of cotinine.

Preparation of spiked plasma and urine samples: 1 mL of either blank human plasma or urine was spiked with 5 and 10 μg of cotinine. Fortified plasma samples were diluted 1/6 with HPLC-grade water. A 1 mL of NaOH 5M was added to 1-mL aliquot of this dilution or 1-mL urine, and transferred to a SPE column.[6] Each column was washed with 600 μL of a mixture of ammonia–acetonitrile (9 : 1). Cotinine was eluted from the column twice with 4 mL dichloromethane each time.

The organic phase was evaporated under nitrogen stream and the residue dissolved in 1000 μL of mobile phase, while 20-μL aliquots were used for HPLC analysis.

RESULTS

Retention time (RT) of cotinine in the assayed chromatographic conditions was 6.93 min. Plots of peak height vs. cotinine concentration were linear up to 1000 ng/mL (Fig. 1). The typical regression equation was $y = 0.005x + 0.2394$ in mobile phase. Correlation coefficient of standard curves by least-squares linear regression analysis was 0.9992 ± 0.02. The *y*-intercepts were different from zero.

From the calibration chart made with nine different cotinine concentrations, the quantification limit was found to be 50 ng/mL and the detection limit 20 ng/mL.

Assay precision and accuracy were determined by assaying four concentrations of cotinine in mobile phase and plasma (20, 50, 100, and 400 ng/mL) and four concentrations of cotinine in urine (20, 50, 500, and 1000 ng/mL), repeated five times in each matrix.

Accuracy was determined by percentage of target value. This was within 90% for all concentrations. CV% was invariably better than 5% except for the lowest concentration assayed (20 ng/mL), with precision of 15%. These data suggest that this HPLC method is very consistent and reliable.

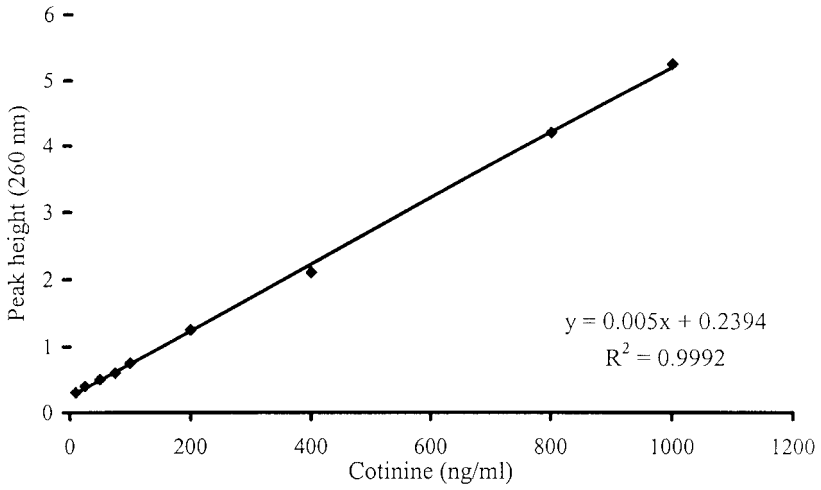

Figure 1. Representative standard calibration curve of cotinine in buffer solution.

In the described chromatographic conditions, cotinine (RT 6.93 min) was separated from caffeine, whose RT was 5.51 min [Fig. 2(A)].

Cotinine and caffeine recovery after extraction through the C_{18} columns was 88% and 54%, respectively [Fig. 2(B)].

Normal urine components failed to interfere in cotinine determination, as shown in Fig. 3.

Cotinine concentrations in urine from volunteer smokers were 420, 1670, and 2740 ng/mL.

DISCUSSION AND CONCLUSIONS

Nicotine metabolism is very complex, and at least 18 metabolites have been postulated.[6] Nevertheless, most studies on nicotine biotransformation focus almost exclusively on nicotine and/or its two principal metabolites, cotinine and nicotine–1′-N-oxide.[7]

Nicotine and cotinine,[8] expired carbon monoxide and thiocyanates, are the most widely used smoking biomarkers.[9] Among these substances, plasma and urine concentrations of cotinine are of special interest as a qualitative marker and quantitative indicator of tobacco smoke exposure[10,11] with regard to active or passive smoking.[1,10,11] Furthermore, according to Sepkovic and Haley,[12] protocols validating smoking cessation, plasma, or urinary cotinine determinations provide the most accurate indicators of compliance.

Several methods have been described to quantify cotinine or nicotine/cotinine jointly in biological fluids.[13–20] Gas chromatography (GC) and

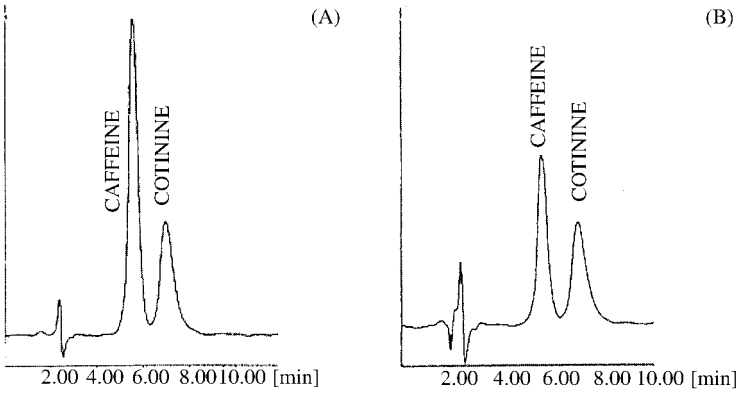

Figure 2. Chromatograms of caffeine and cotinine aqueous standards (10.0 and 5.0 μg/mL, respectively) before (A) and after C_{18} SPE (B).

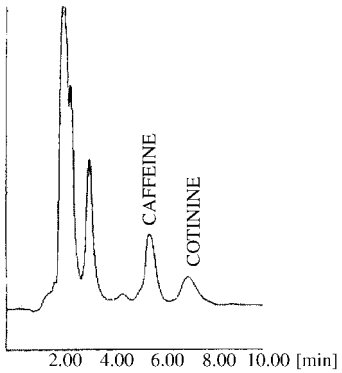

Figure 3. Chromatogram of urine obtained from woman smokers after C_{18} SPE. The level of both alkaloids were 7.2 μg/mL for caffeine and 2.74 μg/mL for cotinine.

high performance liquid chromatography (HPLC), coupled or not with mass spectrometry (MS), are the most frequently used techniques for their quantification.[21–25]

In this work, emphasis was laid on developing a technique using HPLC-UV detection that would be useful to identify and to quantify cotinine in plasma and urine samples.

The present HPLC method is relatively easy to perform and appears to be reliable in determining cotinine in plasma and urine at nanogram level (detection limit of 20 ng/mL). In addition, it is rapid and inexpensive.

Nakajima et al.[25] reported a quantification limit of cotinine in plasma of 1.0 ng/mL, but they injected four-fold sample volume compared to ours and used a noise cleaning unit that increased the signal considerably (15-fold). Furthermore, they reported a 2.4-fold RT of cotinine (16.66 vs. 6.93 min).

Unlike the method described by Sioufi et al.[7], the mobile phase described by us required no addition of triethylamine because the peaks obtained presented no tailing.

We found that the extraction procedure, as well as its linearity, precision, and accuracy were suitable to distinguish smokers from non-smokers. Reference urine blanks from three non-smokers had no detectable cotinine concentrations, whereas three urine samples from smokers (25–30 cigarettes per day), obtained before retiring for the night, had cotinine concentrations of 420, 1670, and 2740 ng/mL (mean value = 1610 ng/mL). Using a similar HPLC method in active smokers (>20 cigarettes per day), Sioufi et al.[7] documented an average cotinine concentration in urine of 2730 ng/mL.

Substitution of ammonium chloride (as recommended by column manufacturers) by ammonia in the extraction washing step yielded excellent

recovery of cotinine (88%), against 38% when manufacturers' instructions were followed.

It was also essential to determine caffeine as an interfering compound because this alkaloid is present in some medicines and soft drinks (cola drinks and "guarana"). In South America (mainly Argentina, Paraguay, and Uruguay) this alkaloid is ingested during "mate-drinking" or the habit of consuming the herbal infusion of Ilex paraguariensis. In a typical mate-round, 80–350 mg of caffeine may be ingested in 1–3 hr.[26] In Brazil, the use of the soft drink "guarana" is very common. Many people smoke cigarettes at the same time as they ingest some of the above mentioned drinks.

Based on these habits, the possibility of interference caused by caffeine was ruled out by concomitant injection of 10 μg/mL of this alkaloid. In the chromatographic conditions assayed, caffeine recovery was lower than cotinine (54% vs. 88%), and both substances had very different RT.

Nicotine interference was not observed because in the conditions of the proposed technique this alkaloid is not extracted.

Currently, HPLC with UV detection is the preferred technique in clinical laboratories because it is more accessible than GC and GC–MS or HPLC-MS.

Given its advantages, our method can be used in the analysis of a large series of plasma and urine samples under routine conditions.

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