A Simple, Sensitive, and Rapid Method for the Determination of Cotinine in Urine by High-Performance Liquid Chromatography with UV Detection

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Abstract

Cotinine levels in biological fluids are a reliable indicator of the presence of nicotine. In this paper, a simple and sensitive high-performance liquid chromatography (HPLC) procedure for the determination of cotinine in urine following liquid–liquid extraction with dichloromethane in an alkaline medium is described. Calibration curves show linearity over the 50 to 3000 ng/mL range with low intra- and interday variability as well as good selectivity and specificity. No solid-phase extraction is performed because the liquid dichloromethane extraction step yields excellent results. This method is a good alternative for routine analysis of urinary cotinine in laboratories where gas chromatography or HPLC–mass spectrometry is not available.

Introduction

During the past three decades, there has been an increasing focus on cigarette smoking and the adverse health consequences associated with it.

Nicotine is the primary causative agent in addiction to tobacco products. It is an amine composed of pyridine and pyrrolidine rings (1). One cigarette contains an average of 8.4 mg of nicotine.

In humans, nicotine is rapidly and extensively metabolized. It is mainly inactivated to cotinine, and cytochrome P450 2A6 mediates approximately 90% of this conversion (2). Cotinine is further metabolized to *trans*-3'-hydroxycotinine (3).

Biomarkers, such as carbon monoxide (CO) and cotinine, are used to assess smoking status. CO is easy to assess, relatively inexpensive, and provides immediate results. However, this marker has limited ability to identify smokers who have abstained for several hours because of the short half-life of CO (4).

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Urinary cotinine has been recommended as a quantitative measure of nicotine intake (5). Several analytical techniques for the determination of urinary cotinine are described in the literature. These include gas chromatography (GC), enzyme-linked immunosorbent assay, liquid chromatography (LC)–mass spectrometry (MS)–MS, and high-performance liquid chromatography (HPLC) with UV detection (6–9).

This paper describes a simple, sensitive, and rapid HPLC method for cotinine determination in urine. The liquid–liquid extraction used is easier to perform than the solid–liquid extraction described in the literature.

This method can be used to routinely assess cotinine concentrations in the urine of both active cigarette smokers and hubble-bubble (hookah or water pipe) active smokers.

Experimental

Reagents and standards

The following solvents and reagents were used: HPLC-grade water and acetonitrile (ROMIL, Cambridge, UK); methanol (super purity solvent) (ROMIL); analytical-reagent grade dichloromethane (LAB-SCAN, Dublin, Ireland); and cotinine [~ 98%, (*S*)-1-methyl-5-(3-Pyridyl)-2-pyrrolidinone, Lot No. 103K4014] (Sigma, St. Louis, Mo). The mother solution of cotinine was prepared by dissolving 64 mg in 100 mL methanol. Further dilutions with methanol were done. All stock solutions were protected from light and kept at –20°C. They were stable for at least six months.

Urine calibration samples were prepared using an appropriate dilution of cotinine stock solutions with drug-free urine. The internal standard was tadalafil (stock solution of 56 mg in 100 cc of a 50:50 mixture of acetonitrile and water).

The buffer for extraction was prepared by mixing 63 mL of solution A [boric acid (6.18 g)–potassium chloride (7.46 g) in 100 mL distilled water] with 37 mL of solution B (10.6 g

sodium carbonate in 100 mL distilled water). The pH of the final buffer solution was 11.

Instrumentation

The HPLC system consisted of an Agilent (Palo Alto, CA) 1100 series quaternary pump, degasser, automatic injector, thermostated column compartment, and diode array detector. A Vortex TecnoKartell TK3 (Kartell, Mi, Italy) and a shaker, BIOSAN Multi Bio RS-24 (BioSan, Riga, Latvia) set at the innovative mixing cycle were also used. The data were collected using the system software (Chemstation from Agilent).

HPLC conditions

The separation was performed on an Agilent LiChrospher 100, C18 column, 5-µm particle size, 250 × 4-mm i.d., with a 2-µm precolumn filter. The mobile phase consisted of phosphate buffer (pH 4.5) and acetonitrile (each in a separate vial). The following step gradient was used at each injection: from 0 to 10 min, the percentage of acetonitrile was 15% (85% buffer); after the first 10 min, the percentage of acetonitrile increased gradually until it reached 50% at 60 min.

The flow rate was set to 1 mL/min, with UV detection at 260 nm (this wavelength corresponds to the maximum peak absorption of cotinine, compared with 254, 230, and 280 nm). The injection volume was 25 µL and analysis was performed at 20°C.

Sample preparation and extraction

Single extraction step

Internal standard solution (50 µL) was mixed with 5 mL of urine and 4 mL buffer (pH 11), and the sample was vortexed for 10 s. Dichloromethane (5 mL) was added before vortex mixing for 1 min and agitating the sample for 15 min using an automatic shaker. After centrifugation for 5 min at 3000 rpm, the dichloromethane layer was evaporated under a gentle stream of nitrogen and the residue taken up with 30 µL of mobile phase.

Double extraction

The same procedure as described in the single extraction step section was followed until centrifugation. Then, a second extraction of the same urine sample was performed with another 5 mL of dichloromethane, and both organic layers obtained in the first and second extractions were combined and evaporated. The residue was taken up with 30 µL mobile phase.

Results

Assay validation

For assay validation, cotinine stock solutions were diluted with drug-free human urine to achieve concentration ranges of 50 to 3000 ng/mL, and each mixture was divided into several portions.

The double extraction procedure was used for validation. Intraday variability was conducted using three sample series, and interday variability was accomplished using four sample series on four separate days. The data in Tables I and II indicate the accuracy, precision, and linearity of the assay.

The calibration curves were linear over the concentration range studied (higher than 3000 ng/mL concentrations were not tested for linearity). Retention time was around 7 min for cotinine and 30 min for tadalafil.

Figure 1 shows typical chromatograms for a urine control (A), a 550 ng/mL patient urine sample (B), and a urine standard at a concentration of 1000 ng/mL (C).

Table I. Intraday Variability of the Assay for Cotinine in Urine (*n* = 3)

Cotinine added (ng/mL)	Average cotinine found (ng/mL)	SD*	Coefficient of variation (%)
50	48.22	0.82	3.67
150	149.90	8.09	4.00
300	300.11	7.54	2.00
500	511.42	3.55	2.34
1000	1032.53	15.58	3.00
1500	1420.62	11.71	5.00
2000	2038.56	22.08	2.00
2500	2484.23	26.34	1.34
3000	3014.40	2.57	0.34

* SD = standard deviation

Table II. Interday Variability of the Assay for Cotinine in Urine (*n* = 4)

Cotinine added (ng/mL)	Average cotinine found (ng/mL)	SD	Coefficient of variation (%)
50	49.70	1.41	2.50
150	148.44	5.11	2.75
300	291.16	11.74	4.25
500	516.32	1.87	3.50
1000	1027.90	11.04	2.75
1500	1425.50	9.87	5.00
2000	2060	31.90	3.25
2500	2467.32	42.51	1.75
3000	3012.98	25.63	0.50

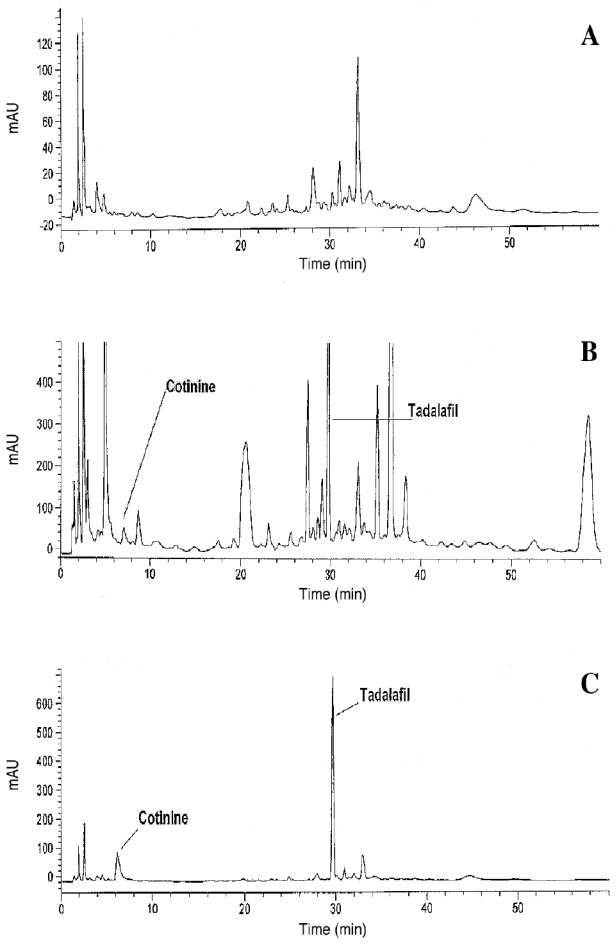

Figure 1. Urine control (A), 550 ng/mL patient urine sample (B), and urine standard at a concentration of 1000 ng/mL (C).

Table III. Specificity Data

Compound	Retention time (min)
Cotinine	7.3
Tadalafil	29.7
Nicotine	3.1
Nicotinic acid *N*-oxide	1.5
Caffeine	5.00
Hydrocortisone	24.6
Acetaminophen	NA*
Aspirin	NA
Benzodiazepines	NA
Glibenclamide	22.5
Glimepiride	23.0
Tricyclic antidepressants (Imipramine, clomipramine, and nortriptyline HCl)	NA

* NA = no detection of the compound up to 2 h after injection into the HPLC apparatus.

Extraction yield

Extraction recoveries were determined by comparing the peak heights obtained by direct injection of standard cotinine solution with those obtained after a single extraction with the organic solvent. Several organic solvents were tried: dichloromethane, chloroform, ether, and ethylacetate. Dichloromethane showed the highest cotinine recoveries, with no less than 65% over the concentration range used in the calibration curves.

When double liquid–liquid extraction with dichloromethane was used, the recovery increased to nearly 90%.

Detection limit

The detection limit was 30 ng/mL. However, concentrations less than 50 ng/mL showed a relative standard deviation of approximately 20% and were not included in the calibration curves.

Influence of buffer pH

Several pHs of the buffer used in the extraction procedure were tested: 8, 9, 10, 11, and 12. Optimum extraction recovery was realized with pH 11; therefore, pH 11 was used in all the validation procedures.

In addition, various pH values of the aqueous mobile phases were also examined. At a pH of less then 3 or higher than 8, cotinine peaks were not symmetrical. Better chromatographic results were obtained with pH values between 4 and 5.5. A pH of 4.5 was chosen for this procedure because it resulted in good selectivity and symmetry of the cotinine peak. No interferences were observed with nicotine or the nicotinic acid *N*-oxide. Some common substances available at our laboratory such as caffeine, hydrocortisone, acetaminophen, aspirin, benzodiazepines (bromazepam and alprazolam), oral hypoglycemic agents (glimepiride and glibenclamide), and tricyclic antidepressants (nortriptyline hydrochloride, clomipramine hydrochloride, and imipramine hydrochloride) were also assessed for interference with negative results (Table III).

Conclusion

The analytical technique described in this paper is currently being used in our laboratory to determine cotinine concentrations in the urine of active cigarette and hubble bubble (hookah or water pipe) smokers, and the results are entirely satisfactory.

In addition to being simple and sensitive, the cost of the analysis is significantly lower for samples compared with LC–MS–MS or GC. The lower cost may encourage a better follow up of smokers, especially when nicotine therapeutic substitution is considered.

We are currently investigating this method for cotinine determination in saliva and plasma.

References

1. D. Yildiz. Nicotine, its metabolism and an overview of its biological effects. *Toxicon.* **43(6):** 619–32 (2004).
2. V. Malaiyandi, E.M. Sellers, and R.F. Tyndale. Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. *Clin. Pharmacol. Ther.* **77(3):** 145–58 (2005).
3. Z. Bao, X.Y. He, X. Ding, S. Prabhu, and J.Y. Hong. Metabolism of nicotine and cotinine by human cytochrome P450 2A13. *Drug Metab. Dispos.* **33(2):** 258–61 (2005).
4. M. Acosta, A. Buchhalter, A. Breland, D. Hamilton, and T. Eissenberg. Urine cotinine as an index of smoking status in smokers during 96-hr abstinence: comparison between gas chromatography/mass spectrometry and immunoassay test strips. *Nicotine Tob. Res.* **6(4):** 615–20 (2004).
5. H. Kim, Y. Lim, S. Lee, S. Park, C. Kim, C. Hong, and D. Shin. Relationship between environmental tobacco smoke and urinary cotinine levels in passive smokers at their residence. *J. Expo. Anal. Environ. Epidemiol.* **14:** S65–70 (2004).
6. H.W. Kuo, J.S. Yang, and M.C. Chiu. Determination of urinary and salivary cotinine using gas and liquid chromatography and enzyme-linked immunosorbent assay. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **768(2):** 297–303 (2002).
7. X. Xu, M.M. Iba, and C.P. Weisel. Simultaneous and sensitive measurement of anabasine, nicotine, and nicotine metabolites in human urine by liquid chromatography–tandem mass spectrometry. *Clin. Chem.* **50(12):** 2323–30 (2004).
8. R.J. Bjercke, G. Cook, N. Rychlik, H.B. Gjika, H. Van Vunakis, and J.J. Langone. Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assays. *J. Immunol. Methods.* **90(2):** 203–13 (1986).
9. H. James, Y. Tizabi, and R. Taylor. Rapid method for the simultaneous measurement of nicotine and cotinine in urine and serum by gas chromatography-mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* **708(1-2):** 87–93 (1998).

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