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An high performance liquid chromatographic method for the quantification of cotinine in the urine of preschool children

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Tobacco smoke exposure is an important and preventable cause of morbidity among children. Enviromental tobacco smoke (ETS) increases respiratory symptoms and disease and also decreases lung function in children who live in a household with at least one smoker. We have developed a simple and reliable HPLC method with diode array dedection to determine the urine concentrations of cotinine in children aged 3 to 6 years, exposed to ETS. The assay involved a liquid-liquid extraction with chloroform. The HPLC method utilized a Chromasil C_{18} column (150 mm × 4.6 mm i.d.) and an isocratic mobile phase of phosphate buffer: acetonitrile (83 : 17 v/v, 0.02 M containing 0.1% triethylamine, adjusted to pH 6.72 with orthophosphoric acid), at a flow rate of 0.7 ml min^{-1}. The detection was performed at 260 nm and the total analysis time of analysis was less than 15 min. Linearity ranged from 0 to 80 μg L^{-1}; correlation coefficients (r^2) for calibration curves were greater than 0.99. With 2 mL of urine for extraction, the limit of detection was 0.1 μg L^{-1}. The mean extraction ratio of cotinine was 88.78%. This analytical method is suitable for the determination of cotinine levels in a large number of urine samples.

1. Introduction

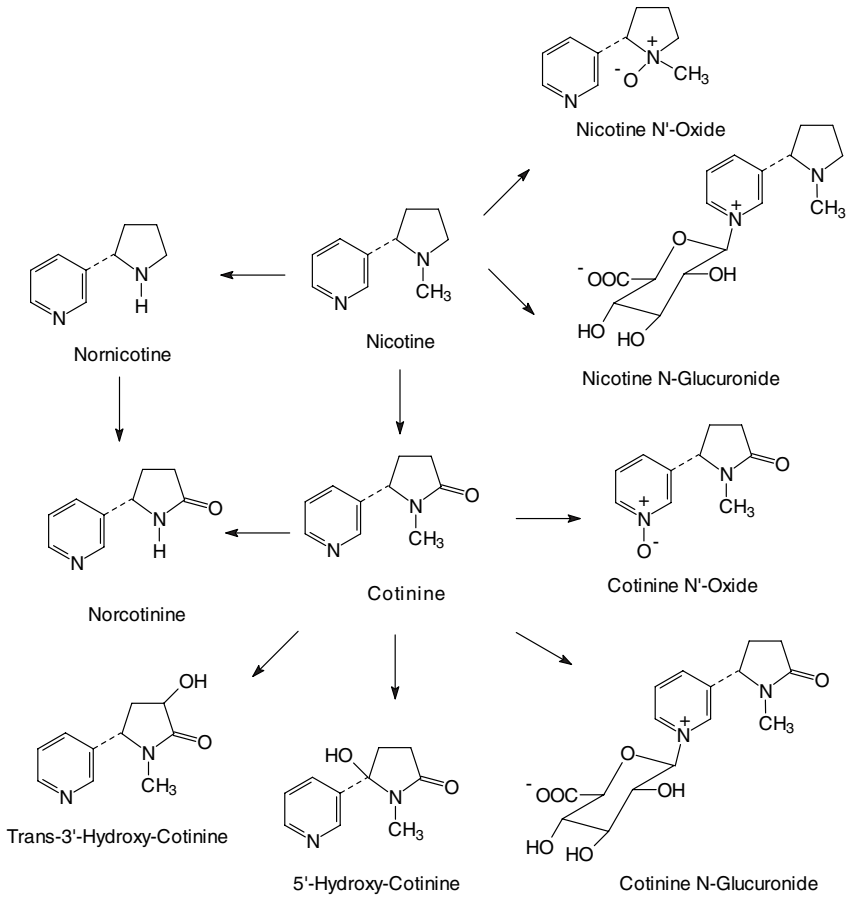
Addiction to the tobacco smoke is one of the world's most serious public health problems. Most studies that have examined the health effects of environmental tobacco smoke (ETS) on children have used reported ETS exposure or the presence of smokers in the child's household to define exposure (Knight et al. 1996; Matt et al. 1999; Mannino et al. 2001; Jarvis et al. 2001; Johansson et al. 2004; Kabesch et al. 2004). A cigarette contains 7–24 mg nicotine, which represents a lethal dose for pre-school children (Moriya and Hashimoto 2004).

ETS has previously been estimated by measuring nicotine and cotinine in body fluids (Davis 1988; Ghoshch et al. 2000). In this respect, it is now generally accepted that plasma, saliva and urine levels of nicotine alone may be poor indicators for the actual intake of nicotine during cigarette smoking because nicotine has a relatively short plasma half life (1–2 h) (Ghoshch et al. 2000; Tutka et al. 2005). Cotinine, (*S*) (−)-1-methyl-5-(3-pyridyl)-2-pyrrolidinone, is the major proximate metabolite of nicotine in a number of animal species (Nakayama 1988), including man (Rop et al. 1993) and its plasma half life (15–20 h) is considerably longer than that of nicotine (Ghoshch et al. 2000).

The liver is the main organ which metabolises nicotine. In humans, 70% of nicotine absorbed by a smoker is metabolised by C-oxidation to cotinine, 4% of nicotine is oxidised as N-oxidation, N-demethylation and is metabolised to its N-glucuronide conjugate. 9% is excreted unchanged in the urine and the metabolic outcome of the remaining 17% is still unknown (Scheme) (Tutka et al. 2005; Rop et al. 1993; Benowitz et al. 1994; Kyerematen and Vesell 1991; Kuehl and Murphy 2003; Dhar 2004). Metabolism of nicotine to cotinine is an NADPH-dependent process (Dhar 2004). The enzyme responsible for the metabolism of nicotine to cotinine is cytochrome P4502A6 (Nakajima et al. 1996; Messina et al. 1997; Nakajima et al. 2000). In the mammalian system, nicotine is metabolized, whereas the most important metabolic reaction of nicotine is the formation of cotinine by oxidation (Gorrod and Schepers 1999).

Cotinine is now widely used as a biomarker of enviromental tobacco smoke (ETS) exposure. Cotinine levels in plasma, urine and saliva of nonsmokers have been used in the assessment of ETS exposure and risk of ETS-related lung cancer (Sato et al. 1999). Cotinine levels provide the best biomarker for exposure to passive smoke. Cotinine in biological fluids can be quantitatively analysed by gas chromatography with nitrogen phosphorus dedection (GC-NPD) (Feyerabend and Russell 2005) or gas chromatography coupled to mass spectrometry (GC/MS) (Kim et al. 2005), radioimmunoassay (RIA) (Matsukura et al. 1975), HPLC (Moore et al. 1990; Perkins et al. 1991; Oddoze et al. 1998; Doctor et al. 2004) and enzyme-linked immunosorbent assay (ELISA) (Benowitz 1996; Kuo et al. 2002). RIA and ELISA have been used for small volume samples but cross-reactivity with closely related molecules

Scheme



often leads to over estimation of metabolite concentration. Currently, the most frequently used chromatographic method is GC with nitrogen phosphorus dedection, mass spectrometric or flame ionization dedection methods. The main disadvantage of the GC method is the difficulty of the indirectly detecting and quantifying the polar metabolites of nicotine. Sample preparation can be tedious, especially when using GC-MS. An additional problem is that some polar components decompose at the high temperatures utilized in the analysis. HPLC systems, connected to ultraviolet (UV), mass spectrometric, or electrochemical dedectors for quantifying major nicotine metabolites, are frequently used to overcome the disadvantages of the above methods. In HPLC, using a UV dedector is considered more practical than using other dedectors such as mass or electrochemical dedectors due to its simplicity and lower cost (Ghoshch et al. 2000).

The objective of the present study was to determine the cotinine concentrations in urine using HPLC. We describe a simple, rapid, sensitive and selective HPLC method, using a small amount of urine (2 mL), to measure the urinary cotinine concentration of children who grew up in a household with at least one smoker. Our liquid-liquid extraction protocol was simple and fast and did not require any clean up of sample.

2. Investigations, results and discussion

Cotinine was separated and quantified in urine samples taken from a group of 338 girls and boys of age between 3–6 years and with the mean age of 4.56 ± 0.06. The retention time of cotinine was 2.769 min. Nicotine and its other major metabolite *trans*-3′-hydroxycotinine and nornicotine were well separeted from the cotinine peak with the retention times 4.137, 2.769 and 2.062 min respectively (Fig). However nornicotine was an interfering urine component at 2.050 min in the spike sample. Cotinine was used as an external standard and a calibration curve was used for quantifying the samples. The calibration curves for each analyte were generated from at least eight points on each curve, with each point representing an average of four runs; concentrations ranged from 0–80 $\mu g\ L^{-1}$.

The results from method validation demonstrated an excellent precision and accuracy with acceptable specifity and chromatographic resolution. A good linearity in terms of peak area ratios as a function of analyte concentration coefficients observed for the reggession lines. The detection limit was 0.1 $\mu g\ L^{-1}$. The calibration curve equation was $y = 36.857 \times -1.905$. The correlation coefficient (r^2) was greater than 0.99 for all of the curves. The precision of the method was evaluated at three levels of concentration 1.25, 10 and 40 $\mu g\ L^{-1}$. The relative standard deviations for three different concentrations of in urine plasma in the same-day study were 3.66, 3.46 and 0.102%, respectively, whereas those in the day-to-day study were 2.96, 0.88 and 0.35% respectively. The analytical recovery was calculated by comparing the peak area of cotinine in the extracted sample to the area obtained from the samples to which the studied compound has been added after extraction. The mean extraction ratio of cotinine was

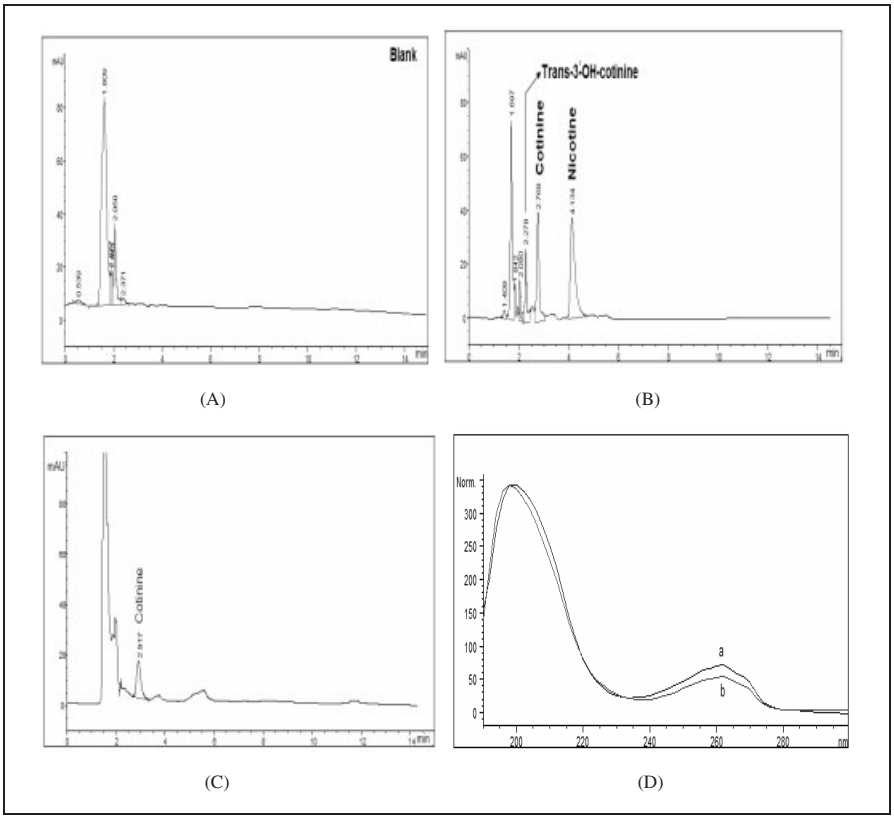


Fig.:
Four chromatograms illustrating HPLC analysis of: (A) Blank urine from a nonexposed children; (B) Blank urine spiked with the trans-3-hydroxycotinine, cotinine and nicotine; (C) urine sample of a ETS exposed children (6.85 μg L^{-1}) (D) Overlaid UV spectra of; (a) cotinine reference standard and (b) cotinine peak at 2.817 min obtained from chromatogram C

88.78%. By Ceppa et al. (2000), the mean extraction ration of cotinine was reported to be 92.1%.

HPLC data for 250 ETS exposed children afforded cotinine levels in the range 0.20–40.46 μg L^{-1} with a mean value of 4.979 ± 0.40 μg L^{-1}. The results were expressed according (Ceppa et al. 2000) and presented in the Table. Cotinine levels are < 20 μg L^{-1} for non smokers, between 20 and 50 μg L^{-1} for passive or occasional smokers and > 50 μg L^{-1} for regular smokers (Haddow et al. 1994). Undetectable levels of cotinine were found in 70 children. Cotinine peak interfered with other components in urine of 18 children. Because these children used some drugs such as analgesics, antibiotics and vitamins, these samples were not quantified. There were no significant differences between the groups regarding age, gender and health status.

The non-tabocco sources of cotinine ie. tomatoes, potatoes, eggplant, green pepper or green tea do not give positive results (Domino 1995). Our results were in agreement with a number of recent studies in ETS exposed children. Matt et al. (2004) have shown that the urine cotinine levels of children in the households were in the range of 2.47 to 20.43 μg L^{-1}. The same authors reported (Matt et al. 1999) urine cotinine levels of 0.1410 to 66.00 μg L^{-1} from ETS exposure children aged 3–6 years. In 2004, Johansson et al. (2004) measured urine cotinine levels at concentrations of 0.9 to 35.30 μg L^{-1} among 366 children.

Table: Cotinine levels and ratio of in ETS exposed children

Concentration (μg L^{-1})	Number	%
Cotinine > 20	11	3.25
Cotinine < 20	239	70.71
Cotinine ND	70	20.71
Cotinine NQ*	18	5.33
Total	338	100

ND: Not dedected; NQ: Not quantified;
* Cotinine peak interfered in urine

Urinary cotinine can be used to estimate daily nicotine intake. Benowitz (1996) showed that urinary cotinine concentrations of 7.7 and 1.6 μg L^{-1} corresponded to 100 μg and 20 μg for daily intake of nicotine by non smokers. Nelson et al. (1992) calculated an 8 hours exposure to ETS with a ventilation rate of 1 $m^3 h^{-1}$ and an urinary cotinine concentration of 0.1 to 0.3 μg L^{-1} which would produce a daily nicotine intake of 1.1–40 μg. Kuo et al. (2002) reported that urinary cotinine concentration is a more accurate biomarker for ETS and it is better suited for epidemiological studies.

For clinical and epidemiological research purposes the search for acceptable measures of ETS exposure has yet to be completed. Reliable and valid measures with at least interval-level scaling are needed to allow progress in determining minimum exposure levels for disease risk estimation. As a result, this study contributes the development of a simple and reliable HPLC method to measure the urine concentrations of cotinine in children aged 3 to 6 years, exposed to ETS.

3. Experimental

3.1. Subjects

All 338 children aged 3 to 6 years, lived in smoky environments in their houses. All parents were interviewed using a questionnaire and they were asked about their smoking habits. The urine samples provided by the parents and were recruited between May 25 and July 14, 2004. The urine samples were stored frozen (−20 °C) until analyzed. Cotinine analyses were performed by HPLC. The study protocol was approved by the Research Ethic Committees of the Marmara University School of Medicine.

3.2. Equipment

The chromatographic system consisted of a Hewlett Packard 1100 featuring a quaternary pump (G 1311 A), a manual injector (G 1328 B) and a diode array detector (DAD) (G 13115 B) which is set at 260 nm. Data

acquisition was performed using a chromatography software package (Agilent Chemstation version 9.01(1206).

3.3. Chemicals

The HPLC grade acetonitrile was supplied by Merck. Disodium hydrogene phosphate, potassium dihydrogene phosphate and cotinine were supplied by Fluka. Ortho phosphoric acid 85%, for analysis is purchased from JT Backer. The water was purified by the Milli-Q-Grade water system (Millipore) for use in HPLC.

3.4. Chromatographic conditions

The chromatographic separation was performed using a Chromasil C_{18} column (150 mm × 4.6 mm i.d.). The mobile phase consisted of 0.02 M phosphate buffer (83%), acetonitrile (17%). To prepare the buffer solution, disodium hydrogene phosphate (28.6 g) and potassium dihydrogene phosphate (5.4 g) were dissolved in HPLC grade water and 1 mL triethylamine was added to this mixture and than added to volume (1000 mL). The pH of the buffer was adjusted to 6.72 ± 0.05 with ortho phosphoric acid. Both the acetonitrile and the buffer solution were filtered though a 0.45 μm GH-membrane filter. The mobile phase was pumped through the chromatographic system at a flow rate of 0.7 mL min^{-1}. The diode array dedector was operated at 260 nm. A volume of 20 μL was injected into the chromatographic system.

3.5. Preparation of standard solution and calibration samples

Stock solution of cotinine was prepared by dissolving accurately weighed quantities in methanol to give a concentration of 1000 μg L^{-1} and stored at −20 °C until used. This stock solution was used to spike the drug free urine to cover various calibration ranges 0–80 μg L^{-1}. Cotinine was used as standard for quantitative analysis and external standard method was used.

3.6. Validation study

The calibration standards were prepared daily. The regression equations were obtained by the least-squares method using a regression analysis. Unweighed least-squares linear regression of the peak area ratio as a function of the theoretical concentrations was applied to each standard curve ($y = ax + b$, where x = concentration (μg L^{-1}), y = peak area ratio, a = slope, and b = intercept).

The method was checked to ensure that there was no interference with cotinine from matrix co-extractives. Chromatograms showed that cotinine peaks were resolved sufficiently from other peaks to enable reliable quantification.

Precision and accuracy of this LC method were established by repetitive analysis of samples in urine against a calibration curve. The accuracy and precision of the method were determined at concentrations of 1.25, 10 and 40 μg L^{-1}. Each sample at three concentration levels was analyzed four times consecutively within one day ($n = 4$) to determine within-run precision and accuracy, and once a day for four successive days ($n = 3$) at three concentration levels to determine between-run precision and accuracy. Accuracy was expressed as the recovery (100 × measured concentration/theoretical concentration), while the precision was given by the between- and within-run relative standard deviations (R.S.D.s). The limit of dedection (LOD) was defined as the lowest concentration of the analyte in the sample which can be dedected with acceptable accuracy and precision under the stated experimental conditions. LOD was derived from multiple measurements in the low concentration range and was determined based on the signal-to-noise approach. The level of approximately three times the noise level was used as the LOD (FDA/CDER guidance, Bioanalytical Method Validation, May 2001).

3.7. Preparation of urine samples

Nitric acid (65%, 750 μl) was added to 2 mL of urine, heated at 60 °C for 30 min and centrifuged at 6000 g for 5 min. Methanol (1 mL), chloroform (4 mL) and 5 M NaOH (1 mL) were added to supernatant (1mL) and centrifuged at 6000 g for 10 min. The organic layer was evaporated under a stream of nitrogen and the residue was reconstituted with methanol (0.5 mL) (Kuo et al. 2002). A 20 μL aliquot was injected onto the HPLC system. Each samples analysed in duplicate.

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ERRATUM

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1-N-(Arylmaleamoyl)-3,5-bis(phenylmethylene)-4-piperidones: a novel class of antimycobacterial agents

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By technical reasons, the Table in the above mentioned paper was published incorrectly. The Table should be read as follows. Editors apologize for the mistake.

Table: Evaluation of the compounds in series 2–4 for growth inhibiting properties of *Mycobacterium tuberculosis* $H_{37}Rv$

	R^1/R^{2a}	Percentage inhibition at 6.25 μg/mL		
		2	**3**	**4**
a	H	24	0	8
b	4-Cl	0	0	0
c	3,4-Cl_2	2	74	0
d	4-NO_2	0	98	6
e	4-CH_3	8	4	0
f	4-OCH_3	7	100	0

[a] R^1 and R^2 are the aryl substituents in ring C of the structure indicated in Figure 1