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Determination of urinary and salivary cotinine using gas and liquid chromatography and enzyme-linked immunosorbent assay

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Abstract

The objective of this study was to compare cotinine concentrations in urine and saliva using gas chromatography (GC), high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA). Ninety-four subjects were selected (27 smokers and 67 non-smokers) and interviewed using questionnaire. Of the non-smokers, 39 had been exposed to environmental tobacco smoke (ETS) and 28 had not been exposed to ETS. Cotinine levels among smokers were highest using all three measurements, followed by ETS exposed subjects and non-smokers. Cotinine levels in urine, using HPLC, correlated significantly with levels measured using ELISA ($r=0.92$) and GC–nitrogen–phosphorus detection (NPD) ($r=0.92$). Salivary cotinine levels measured using ELISA did not correlate significantly with either HPLC ($r=0.37$) or GC–NPD ($r=0.33$) measurements. Multiple regression models were used to adjust for age, gender, drug use and health status, and it was found that cotinine levels in urine and saliva were significantly correlated with smoking pack-year. The authors conclude that urinary cotinine concentration is a more accurate biomarker for ETS than salivary cotinine concentration.

Keywords: Cotinine

1. Introduction

Cotinine is the major proximate metabolite of nicotine and has been widely used as a biomarker of environmental tobacco smoke (ETS) exposure. Cotinine levels in plasma, urine and saliva of non-smokers have been used in the assessment of ETS exposure and risk of ETS-related lung cancer [1]. Another biomarker for ETS exposure is COHb (blood carboxyhemoglobin) but this best represents acute exposure and cannot show daily variations in ETS exposure. Thiocynate has been used as a biomarker for ETS exposure, however it displays a lack of specificity and sensitivity [2]. CO, thiocynate and plasma nicotine concentrations have been shown to be unrelated to ETS exposure. Cotinine levels provide the best biomarker for exposure to passive smoke [3]. Of all the biomarkers for ETS exposure, nicotine and cotinine have been shown to be the most specific and most sensitive, however, the former has a short (6 h) half-life. The quantitative analysis of cotinine in physiological fluids can be achieved using gas chromatography with nitrogen–phosphorus detection (GC–NPD), radioimmunoassay

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(RIA), high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) [4]. Monoclonal antibodies were used to develop non-isotopic and RIA for quantitative determination of cotinine levels and results showed a strong correlation with values obtained by RIA or by GC [5]. ELISA gives a reliable quantitative measure of cotinine as an indicator of active and passive exposure to tobacco smoke [6]. GC–NPD is well known for such sensitive and simultaneous measurements of both nicotine and cotinine using a well maintained capillary column [7]. However, HPLC values for nicotine and cotinine in urine samples from passive smokers compare quite well with those of the more sensitive and simpler GC method [8]. Salivary cotinine levels over 0.4 ng/ml corresponded to an increased risk of lung cancer and heart disease due to ETS exposure by 1/1000 and 1/100, respectively [9]. There are many factors which could affect the condition of saliva which makes it difficult to collect standard specimens of saliva to accurately represent ETS exposure. Also, factors such as diet, time and duration of smoking can affect salivary cotinine. There are few studies in the literature which have compared salivary and urinary cotinine using different analytical methods. In 1997, the Taiwan government introduced the Tobacco Control Act which aims to reduce tobacco consumption and thereby reduce the population's ETS exposure. There is no available data in Taiwan to investigate the relative reliability of biomarkers of ETS exposure using physiological fluids, such as serum, urine and saliva. Urine and saliva have been more widely investigated since they can be obtained non-invasively. The objective of this study was to compare the cotinine concentrations in urine and saliva using GC, HPLC and ELISA.

2. Materials and methods

2.1. Subjects

All 94 subjects were volunteers selected from college staff, college students and service industry workers. Subjects were interviewed using a questionnaire and subjects with renal dysfunction were excluded from the study. Subjects were classified into three groups (smokers, ETS exposed and non-smokers) according to the answers given in the questionnaire. Each subject monitored his/her own ETS exposure every 30 min for a period of 24 h by filling in a time activity table. ETS exposure was measured by counting the number of cigarette butts and people smoking within 30 m of the subject. Smokers were defined as subjects who smoked at least one cigarette per day.

2.2. Determination of urinary cotinine using GC–NPD, HPLC and ELISA

2.2.1. Pretreatment for GC–NPD

A 1.7-g amount of NaCl, 3 ml chloroform and 1 ml 5 *M* NaOH were added to 5 ml of urine, stirred for 5 min and centrifuged at 3000 *g* for 10 min. Nitrogen was used to purge the chloroform layer and 1 ml methanol was added to dissolve the precipitate before measurement using GC–NPD (HP6890). A DB-WAX column (30 m×0.25 mm I.D.) was used for the analyses and temperatures used started at 110°C for 2 min increasing by 20 °C/min to 150 °C for 6 min then increasing by 30 °C/min to 240 °C for 1 min. The injection temperature was 250 °C, and NPD temperature was 300 °C.

2.2.2. Pretreatment for HPLC

HNO_3 was added to 2 ml of urine, heated at 60 °C for 30 min and centrifuged at 3000 *g* for 5 min. A 1-ml volume of 100% methanol, 4 ml chloroform and 1 ml 5 *M* NaOH were added to 1 ml of supernatant and centrifuged at 3000 *g* for 10 min. Nitrogen was used to purge the chloroform layer and 0.5 ml methanol was added to dissolve the precipitate before measurement using HPLC. The column used was a TSK-gel ODS-80 (150 mm×4.6 mm I.D.). Flow-rate was 0.6 ml/min and the UV detector was set at 254 nm. The mobile phase was water–methanol buffer–acetate–acetronitrile–acetic acid (50:27:20:2:1, v/v). The pH of mobile phase was adjusted to 4.28 using diethylamine. The duration of each analysis was 30 min.

2.2.3. Pretreatment using ELISA (for both saliva and urine)

A 10-μl volume each of urine, standard and control were added to separate wells. A 100-μl

volume of cotinine enzyme was added into each well and left to stand at room temperature for 30 min. A 350-μl volume of buffer was used to wash the plates four times. A 100-μl volume of substrate solution was added to each well and left to stand for 30 min. A 100-μl volume of stop solution was then added to each well. After 30 min, an ELISA reader with a wavelength of 450 nm was used to measure absorbency. For saliva, the same procedure was followed, except that 50 μl of saliva, standard and control were used.

Quality control of measurements of urinary and salivary cotinine concentrations was performed. Table 1 shows the detection limits and calibration curves for each of the three measurements of urinary and salivary cotinine concentrations. For measurement of salivary cotinine levels, using ELISA, the correlation coefficient of the calibration curve was slightly lower than for the other measurements. The relative prediction deviation (RPD) percentage of the calibration curves showed that there was a higher level of variation using ELISA compared to the others. The recovery efficiency for urine using GC–NPD was higher (104.3%) than for HPLC (84.0%). Reproducibility for GC–NPD and HPLC was high (<4%). Fig. 1 shows the stability of the urinary cotinine at 4 °C and −20 °C using GC–NPD and HPLC. Fig. 1a shows that urinary cotinine was stable over 28 days at both concentrations (37.6 and 109.3 μg/ml) using GC–NPD. Overall, recovery efficiency was over 95%. However, for HPLC (Fig. 1b), urinary cotinine was unstable over 14 days at both concentrations (14.3 and 45.7 μg/ml). At 4 °C, recovery efficiencies of cotinine were 58.2 and 73.4%, and at −20 °C, recovery efficiencies were 62.3 and 90.2%, for low and high cotinine concentrations, respectively. The differences between recovery efficiencies using GC–NPD and HPLC may be due to the different pretreatments required for each method.

All data were analyzed using SAS/PC+6.12 [10]. Pearson's coefficient was used to calculate the correlation between urinary and salivary cotinine levels for GC–NPD, HPLC and ELISA. One-way analysis of variance (ANOVA) was used to compare urinary and salivary cotinine levels among active, passive and non-smokers for each of the three types of measurements. Multiple linear regression was used to determine the factors affecting urinary and salivary cotinine levels for GC–NPD, HPLC and ELISA.

3. Results and discussion

3.1. Correlation with urinary and salivary cotinine levels

Twenty-seven subjects were smokers and each smoked an average of 11.14 cigarettes per day (average 6 pack years). The most common location of smoking in the home was the living room (44%), followed by the dining room (30%) and balcony (22%). ETS-exposed subjects (39 subjects) were defined as non-smokers exposed to smoke either at home or in the workplace. Non-smokers (28 subjects) did not smoke and were not exposed to ETS. There were no significant differences between the groups with regard to age, gender, education level and health status. Among smokers, 93% were male and among ETS-exposed subjects 51% were male. In previous studies [8,11], the quantitative analysis of cotinine in physiological fluids was achieved using GC, RIA and HPLC. There have been few studies which have compared the inter-correlation between the methods used to determine urinary cotinine

Table 1
Calibration curves and detection limits for urinary and salivary cotinine levels using the three measurements

Specimen	Method	Calibration curve	*R*	RPD (%)	Detection limit (ng)
Urine	HPLC	$y=0.2613x+0.6346$	0.9985	0.758~8.422	0.078
	GC–NPD	$y=0.0004x+0.0092$	0.9997	0.335~4.678	0.200
	ELISA	$y=-1.8919x+4.5341$	0.9966	4.8~41.74	0.464
Saliva	ELISA	$y=-1.6862x+2.0319$	0.9889	2.252~3.403	0.386

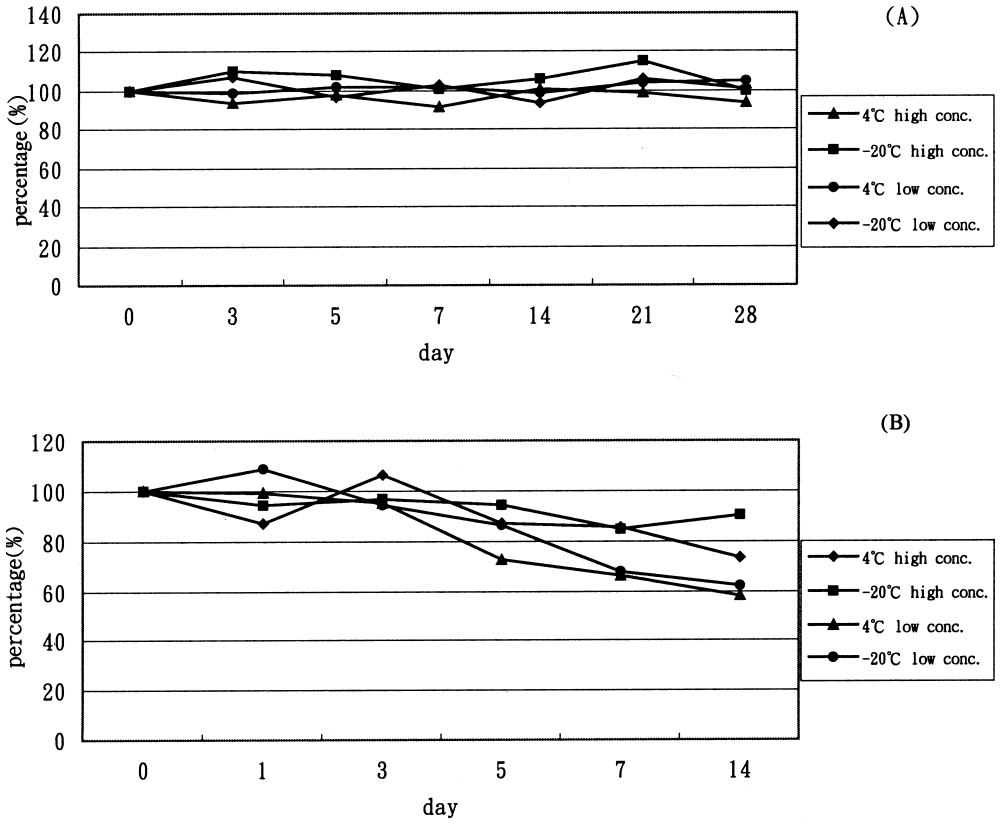

Fig. 1. Stability of urinary cotinine levels using GC–NPD (A) (low concentration: 37.6 μg/ml; high concentration: 109.3 μg/ml) and HPLC (B) (low concentration: 14.3 μg/ml; high concentration: 45.7 μg/ml).

levels. The current study shows that there was a high correlation between HPLC-urine and GC–NPD-urine ($r=0.92$) (Table 2). HPLC-urine and GC–NPD-urine both correlated strongly with ELISA-urine ($r=0.92$ and $r=0.94$). The correlations between ELISA-saliva and HPLC-urine and GC–NPD-urine were weaker than for the other correlations ($r=0.37$ and $r=0.33$). The correlation between ELISA-saliva and ELISA-urine was 0.45. Salivary cotinine was less useful as a biomarker of ETS exposure than urinary cotinine due to the impracticality of collecting sufficient volumes of saliva, and the lack of published methods for the determination of salivary cotinine using GC and HPLC. Salivary cotinine levels may also be readily

Table 2
Correlation between urinary and salivary cotinine concentrations among the three measurements ($n=94$)

	HPLC-urine	ELISA-urine	ELISA-saliva	GC–NPD-urine
HPLC-urine		0.92**	0.37	0.92**
ELISA-urine			0.45*	0.94**
ELISA-saliva				0.33
GC–NPD-urine				

**$P<0.01$; *$P<0.05$.

influenced by other factors such as smoking and diet. It is unclear how salivary nicotine metabolizes to cotinine in the mouth, or the exact metabolic pathway in the liver to the salivary glands. Urine samples are more convenient to collect and urinary cotinine is a well-known biomarker of ETS. Hariharan and Van Noord reported that there was a high correlation between GC and HPLC methods when determining nicotine and cotinine concentrations. GC–NPD was found to be more practical and had a lower detection limit than GC–MS [8]. However, urinary cotinine levels measured using GC–NPD were affected by the presence of theophylline, methotrexate and prednisone which are commonly taken drugs. Bjercke et al. [5] and Langone et al. [6] reported the ELISA and RIA lack sensitivity and are very expensive. Moreover, these assays are limited by persistent interference when concentrated fluids such as saliva and urine are measured and often are not sufficiently sensitive to detect passive exposure to ETS. Godin and Hellier [12] have used pre-column derivation with diethylthiovarbituric acid to determine cotinine by HPLC. However, these are not suitable for routine assays because the colored complexes are unstable. A solid-phase extraction (Extrelut-1 glass columns) was applied to determine cotinine and its metabolite *trans*-3′-hydroxycotinine by HPLC [13]. Oddoze et al. [14] developed a simple reversed-phased HPLC method with paired-ion and UV detection for determination of urinary nicotine and cotinine. The present method improved a reliable procedure for determination of cotinine levels for smokers and non-smokers exposed to ETS, in terms of its speed and facility of routine analysis, involving no derivatization, and no long liquid–liquid extraction with several steps.

3.2. Comparison of urinary and salivary cotinine levels among active, passive and non-smokers

Nicotine values may be an inaccurate biomarker in the case of unusual smokers who smoke only on the days when they drink alcohol or in the case of non-smokers who are exposed to ETS only in public areas. Nicotine is also highly volatile, particularly during extraction. Its value is a reflection of recent exposure because of its short half-life [13]. Urinary nicotine was not used in the current study as a biological marker of ETS exposure. Cotinine offers several advantages over biochemical markers as an objective indicator of nicotine intake or confirmation of non-smoker status. It is a specific indicator of nicotine intake. Its concentrations are not influenced by confounding factors such as diet or environment and its concentrations within a given individual varies by only 15 to 20% over 24 h [14]. The authors felt that it would have been unfeasible to take blood samples to measure blood cotinine levels, as this method is invasive. Also, non-invasive methods such as measuring urinary and salivary cotinine have been shown to be just as accurate. Table 3 compares salivary and urinary cotinine levels among active, passive and non-smokers using the three measurements. Urinary cotinine levels were higher for all three measurements among active smokers and lowest among non-smokers. Cotinine levels in urine using HPLC and GC–NPD were both higher than for ELISA. Using ELISA, urinary cotinine levels were higher than salivary cotinine levels. However, cotinine was not detected in urine by HPLC because the instrument used (Shimadzu integrator, C-R6A Chromatopac) cannot provide values at very low concentrations. The ND values were therefore re-

Table 3
Comparison of urinary and salivary cotinine levels (ng/ml) among active, passive and non-smokers using the three measurements

Specimen	Method	Smoker (n=27)	Non-smoker (n=67)		P value**
			ETS	No ETS	
Urine	HPLC	3055.17±2092.85*	ND	ND	NA
	GC–NPD	3054.61±2407.24	46.03±45.76	27.90±17.25	<0.01
	ELISA	2784.65±2779.84	27.93±33.19	16.16±15.78	<0.01
Saliva	ELISA	19.63±16.89	5.68±9.22	1.96±1.09	<0.01

*Mean±S.D.; **one-way ANOVA test; ND: detection limit; NA: non-available.

placed by 1/2 ND values to correlate GC, HPLC and ELISA data. Previous studies which have measured salivary cotinine levels using GC [3] and RIA [15] methods showed that salivary cotinine levels were lowest among non-smokers. Jarvis et al. [17] also reported that average salivary cotinine level was 310 ng/ml among 94 smokers and corresponded to urinary cotinine level of 1390 ng/ml. Salivary cotinine was higher than in the current study but urinary cotinine was lower, perhaps due to the different analytical methods used. Also, there may have been differences in sampling methodology for saliva. For example, the time of sampling after smoking may have been different which could affect the amount of cotinine retained in the saliva. As such, further research is needed to investigate the accuracies of GC and ELISA for determining salivary cotinine. Urinary pH may be highly dependent on microbial content and may vary with source and handling procedures [16]. Obviously, urine samples exposed to high temperatures for cumulatively greater time periods will be at the most risk for misleadingly high cotinine levels. It is also possible that adding acid to store urine samples would retard hydrolysis of the glucuronide, since quaternary *N*-glucuronides are resistant to acid catalyzed hydrolysis. The different pretreatments used for HPLC and GC may have affected the stability of the samples. Benowitz [4] compared the sensitivity, specificity and cost of five analytical methods for the measurement of cotinine in non-smokers and found that LC–MS was the most sensitive and showed greatest specificity, but the cost was extremely high. GC and HPLC showed good specificity and the cost was ''moderate''. Urinary or salivary cotinine can be used to estimate daily nicotine intake. Benowitz showed that urinary cotinine concentrations of 7.7 and 1.6 ng/ml corresponded to 100 μg and 20 μg for daily intake of nicotine by non-smokers. Jarvis et al. [17] reported the median saliva cotinine concentration was 7.95 ng/ml for 42 non-smoking bar staff in London and Birmingham, UK, with a range from 2.2 to 31.3 ng/ml. The median nicotine intake was estimated to be 630 μg/ml. The maximal nicotine intake, corresponding to a saliva cotinine concentration of 31.3 ng/ml, was found to be 2.5 mg/day. There is a strong correlation between ambient nicotine and urinary cotinine (Marbury et al. [18]: $r=0.81$; Coultas et al. [19]: $r=0.60$). Nelson et al. [20] calculated that an 8-h exposure to ETS with a ventilation rate of 1 m^3/h and nicotine concentration of 0.2–0.7 μg/m^3, would produce a daily nicotine intake of 1.1–4.0 μg, which would result in a urinary cotinine concentration of 0.1–0.3 ng/ml. Urinary cotinine has been shown to be a very useful indicator for estimating ambient nicotine and daily nicotine intake.

3.3. Factors affecting levels of urinary and salivary cotinine

Four multiple linear regressions were used to determine the factors affecting urinary and salivary cotinine levels for GC–NPD, HPLC and ELISA shown in Table 4. After adjustment for age, gender, whether or not there was a family member smoking at home, long-term medication and diagnosed disease, the data showed that there was a high correlation between urinary and salivary cotinine levels and smoking pack years. The lowest determination of the coefficient was obtained using ELISA-saliva (0.34), and partial coefficient for smoking (pack-year) was markedly lower for ELISA-saliva than for the urine samples and did not correspond to the trends of the urine samples. Also, there was a high fluctuation between samples using this sampling method. The authors suggest that salivary cotinine is not as accurate a biomarker for ETS exposure as urinary cotinine. Few previous studies have compared the various biomarkers for ETS. It is necessary to conduct further study to find an accurate biomarker for ETS. Our findings are consistent with Yoshioka et al.'s [21] study which used ELISA to assess cotinine levels in urine. They found that the number of cigarettes smoked per day was significantly correlated with urinary cotinine. Active smokers were found to have average cotinine levels of 1568, compared to 61 for passive smokers, and 27 for non-smokers. The concentration of urinary cotinine will depend on the original dose of nicotine, rate of conversion to cotinine, and competing metabolic transformation. Cotinine is just one of 10 pyridine alkaloids present in, and derived from cigarette smoke. Cotinine in urine accounted for less than 15% of total systemic dose of nicotine, while 3′-hydroxycotinine accounts for 34%, and nicotine

Table 4
Multiple linear regression models to show factors affecting concentrations of urinary and salivary cotinine among the three measurements

Variable	HPLC-urine β (S.E.)	ELISA-urine β (S.E.)	ELISA-saliva β (S.E.)	GC–NPD-urine β (S.E.)
Gender (female=0)	171.1(300.0)	185.0(351.2)	0.1(2.4)	162.7(325.7)
Age (years)	−12.3(14.1)	−21.2(16.5)	−0.2(0.1)	−18.4(15.3)
Smoking (pack-years)				
0~1 (non-smoker=0)	1183.9(423.4)*	1494.8(495.7)*	12.4(4.1)*	1119.1(459.6)*
1~5 (non-smoker=0)	2603.2(383.9)*	2822.7(449.5)*	9.2(3.0)*	3318.5(416.8)*
>5 (non-smoker=0)	2059.4(527.1)*	2058.3(517.1)*	14.7(3.3)*	2650.2(572.2)*
Smoking at home (No=0)	184.9(255.2)	294.2(298.8)	0.3(2.0)	78.4(277.0)
Drug usage (No=0)	−15.3(497.0)	8.5(581.9)	−1.2(3.9)	126.1(539.6)
Disease history (No=0)	−204.4(348.6)	−347.6(408.1)	−2.4(2.7)	−179.1(378.5)
R^2	0.49	0.46	0.34	0.55
P-value	<0.01	<0.01	<0.01	<0.01

*$P<0.01$.

itself for 10% [22]. Cross-reactivity of ELISA may increase the extent to which other metabolites of nicotine will confound the results. Therefore, salivary cotinine levels measured by ELISA may not accurately reflect ETS exposure.

In conclusion, salivary cotinine concentrations measured using ELISA were non-significantly correlated with HPLC ($r=0.37$) and GC–NPD ($r=0.33$) measurements. However, for urinary cotinine levels there was a strong inter-correlation between all three measurements ($r>0.92$). After adjusting for age, gender, use of medication and incidence of disease, the data showed that urinary and salivary cotinine levels were significantly correlated with smoking pack year. Salivary cotinine levels tend to fluctuate considerably and are susceptible to many factors. It is also impractical to collect sufficient volumes of representative saliva, whereas collection of urine samples is quick and convenient. Urinary cotinine concentration is a more accurate biomarker for ETS than salivary cotinine concentration and is better suited for epidemiological studies.

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