行政院國家科學委員會補助專題研究計畫成果報告

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計畫主持人：郭憲文　教授

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行政院國家科學委員會專題研究計畫成果報告
台灣地區民眾二手菸暴露之評估

Exposure assessment of environmental tobacco smoking(ETS) in Taiwan population

計畫編號：NSC 89-2314-B-039-028-

執行期限：89 年 8 月 1 日至 90 年 7 月 31 日

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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). 94 subjects were selected (27 smokers and 67 non-smokers) and interviewed using questionnaire. Of the non-smokers, 39 had been exposed to ETS (environmental tobacco smoke) 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-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: Nicotine concentration
Environmental tobacco smoke (ETS)

1. Introduction

Cotinine is the major proximate metabolite of nicotine and has been widely used as a biomarker of 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 were measured and found that they were unrelated to ETS exposure. The data indicated that cotinine levels provided the best biomarker for exposure to passive smoke.[3] Various biomarkers were compared for ETS exposure and found that nicotine and cotinine were the most specific and most sensitive, however the former had a short (six hours) half-life. The quantitative analysis of cotinine in physiological fluids can be achieved using gas chromatography with nitrogen-phosphorus detector (GC-NPD), radioimmunoassay (RIA), liquid chromatography and enzyme-linked immunosorbent assay (ELISA).[4] Monoclonalantibodies were used to develop nonisotopic and RIA for quantitative determination of the cotinine 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 over0.4ng/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

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 ETSexposure every 30 minutes for a period of 24 hours by filling in a time activity table. ETS exposure was measured by counting the number of cigarette butts and people smoking within thirty meters of the subject. Smokers were defined as subjects who consumed at least one cigarette per day. 27 subjects were smokers and each smoked an average of 11.14 cigarettes per day (Ave. 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 was no significant difference between the groups with regard to age, gender, educational level and health status. Among

smokers, 93% were male and among ETS-exposed subjects 51% were male.

Determination of urinary cotinine using GC-NPD, HPLC and ELISA

Pretreatment for GC-NPD

NaCl, chloroform and NaOH were added to 5 ml of urine, stirred for five minutes and centrifuged at 3000rpm for ten minutes. Nitrogen was used to purge the chloroform layer and 1ml methanol was added to dissolve the precipitate before measurement using GC-NPD.

Pretreatment for HPLC

HNO_3 was added to 2ml of urine, heated at 60 ℃ for 30 minutes and centrifuged at 3000rpm for five minutes. Methanol, chloroform and NaOH were added to 1 ml of supernatant and centrifuged at 3000rpm for ten minutes. Nitrogen was used to purge the chloroform layer and 0.5ml methanol was added to dissolve the precipitate before measurement using HPLC.

Pretreatment using ELISA (for both saliva and urine)

10μl each of urine, standard and control were added into separate wells. 100μl cotinine enzyme was added into each well and left to stand at room temperature for 30 minutes. 350μl of buffer was used to wash the plates four times. 100μl of substrate solution was added into each well and left to stand for 30 minutes. 100μl of stop solution was then added into each well. After 30 minutes ELISA reader with wavelength of 450nm was used to measure absorbency. For saliva, the same procedure was followed, except that 50μl of saliva, standard and control were used.

Quality control of measurements of urinary and salivary cotinine concentrations

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 rate for

urine using GC-NPD was higher (104.3%) than for HPLC (84.0%). Reproducibility for GC-NPD and HPLC was low (4%). Fig.1 shows the stability of the urinary cotinine at 4℃ and –20℃ using GC-NPD and HPLC. Fig 1(a) shows that urinary cotinine was stable over 28 days at both concentrations (37.6μg/ml and 109.3μg/ml) using GC-NPD. However, for HPLC (Fig.1 (b)), urinary cotinine was unstable over 14 days at both concentrations (14.3 μg/ml and 45.7 μg /ml).

All data was 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 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

In previous studies[8,11] the quantitative analysis of cotinine in physiological fluids were achieved using gas-liquid chromatography, radioimmunoassay (RIA) and liquid chromatography. There have been few studies which have compared the inter-correlation between the methods used to determine urinary cotinine levels. The current study shows that there was a high correlation between HPLC-urine and GC-NPD-urine (r = 0.92) in 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. 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. 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.[5,6] Precolum derivation with diethylthiovarbituric acid was used to determine cotinine by HPLC. However, these are not suitable for routine assays because the coloured complexes are unstable.[11] A solid-phase extraction (Extrelut-1 glass columns) was applied to determine cotinine and its metabolite trans-3'-hydroxycotinine by HPLC.[12] A simple reversed-phased HPLC method with paired-ion and UV detection was developed for determination of urinary nicotine and cotinine.[13] The present method improved a reliable procedure for determination of cotinine levels for smokers and nonsmokers exposed to ETS, in terms of its speed and facility of routine analysis, involving no derivitization, and no long liquid-liquid extraction with several steps.

Because nicotine values may be an inaccurate biomarker in 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 nonsmoker 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 hours.[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. Previous studies which have measured salivary cotinine levels using GC and RIA methods showed that salivary cotinine levels were lowest among nonsmokers.[3,15] Jarvis also reported that average salivary cotinine level was 310 ng/ml among 94 smokers and corresponded to urinary cotinine level of 1390 ng/ml.[3] Salivary cotinine was higher than in the current study but urinary cotinine was lower. This may be explained by the different analytical methods used. Therefore, further research is needed to investigate the accuracies of GC and ELISA for determining salivary cotinine. 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. 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 sensitivity, specificity and cost of five analytical methods were compared for the measurement of cotinine in nonsmokers 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'.[4] Urinary or salivary cotinine can be used to estimate daily nicotine intake. Benowitz showed that urine concentrations of 7.7 and 1.6 ng/ml corresponded to 100ug and 20 ug for daily intake of nicotine by nonsmokers. The median saliva cotinine concentration was 7.95 ng/ml for 42

nonsmoking bar staff in London and Birmingham, 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. [16] There is a strong correlation between ambient nicotine and urinary cotinine (Marbury, 1993: r = 0.81; Coultas, 1990: r = 0.60).[17-18] Nelson (1991) calculated that an eight-hour exposure to ETS with a ventilation rate of 1 m^3/hour 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 urine 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. [19]

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 pack years of smoking. Our findings are consistent with Yoshioka's (1998) study which used ELISA method to assess cotinine levels in urine.[20] He 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 itself for 10%.[21] Cross-reactivity of ELISA may increase the extent to which other metabolites of nicotine will be inaccurately assessed.

4. Conclusion

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. The authors conclude that urinary cotinine concentration is a more accurate biomarker for ETS than salivary cotinine concentration and is better suited for epidemiological studies.

References

[1] M.S. Jaakkola, J.M. Samet, Environ. Health Perspect. 107 (1999) 823-827.

[2] N.L. Benowitz, Enviorn. Health Perspect. (Suppl) 107 (1999) 349-355.

[3] M.J. Jarvis, M.A.H. Russell, C. Feyerabend, J.R. Eiser, M. Morgan, P. Gammage, E.M. Gray, Br. Med. J. 29 (1985) 927-929.

[4] N.L. Benowitz, Epidemiol. Review 18 (1996) 186-204.

[5] R.J. Bjercke, G. Cook, J.J. Langone, J. Immuno. Methods 96 (1987) 239-246.

[6] J.J. Langone, G. Cook, R.J. Bjercke, M.H. Lifschitz, J. Immuno. Methods 114 (1998) 73-78.

[7] R.A. Davis, J. Chromatogr. Sci. 24 (1986) 134-141.

[8] M. Hariharan, T. Van Noord, Clin. Chem. 37 (1991) 1276-1280.

[9] J.L. Repace, J. Jinot, S. Bayard, K. Emmons, S.K. Hammond, Risk Analysis 18 (1998) 71-83.

[10] D.A. Machacek, N.S. Jiang, Clin. Chem. 32 (1986) 979-982.

[11] SAS/STAT (1986) User's guide, release 6.04. SAS, Cary, North Carolina.

[12] J, Godin, J, Hellier, J. Chromatogr. 488 (1989) 487-491.

[13] P, Zuccaro, I. Altieri, M. Rosa, A.R. Passa, S. Pichini, R. Pacifici. S, J. Chromatogr. 668 (1995) 187-188

[14] C. Oddoze, A.A.M. Pauli, J. Pastor, J. Chromatogr. B 708 (1998) 95-100.

[15] N.L. Benowitz, F. Kuyt, P. 3 rd. Jacob, R.T. Jones, A.L. Osman, Cli Pharmaco Ther. 34 (1983) 604-11.

[16] R.L. Hangan, J.M. Ramos, P.M. Jacob, J. Pharm. Biomed. Analysis 16 (1997) 191-197.

[17] M.J. Jarvis, M. Phil, D.P. Strachan, C. Feyerabend, Am. J. Public Health 82 (1992) 1225-1229.

[18] M.C. Marbury, S.K. Hammong, N.J. Haley, Am. J. Epidemiol. 137 (1993) 1089-97.

[19] D.B. Coultas, J.M. Samet, J.F. McCarthy, J.D. Spengler, Am. J. Public Health 80 (1990) 988-90.

[20] N. Yoshioka, K.Yonemasu, Y. Dohi et al, Environ. Health Prev. Med. 3 (1998) 83-88.

[21] G. Cope, P. Nayyar, R. Holder, J. Gibbons, R. Bunce, Clinica Chimica Acta 256 (1996) 135-49.

Table 1 Calibration curve and detection limit 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

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

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)	Nonsmoker (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±SD

** One-way ANOVA test

ND：Detection limit

NA：non-available

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

Variables	HPLC-urine	ELISA-urine	ELISA-saliva	GC-NPD-urine
	β (SE)	β (SE)	β (SE)	β (SE)
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-square	0.49*	0.46*	0.34*	0.55*

*：P<0.01

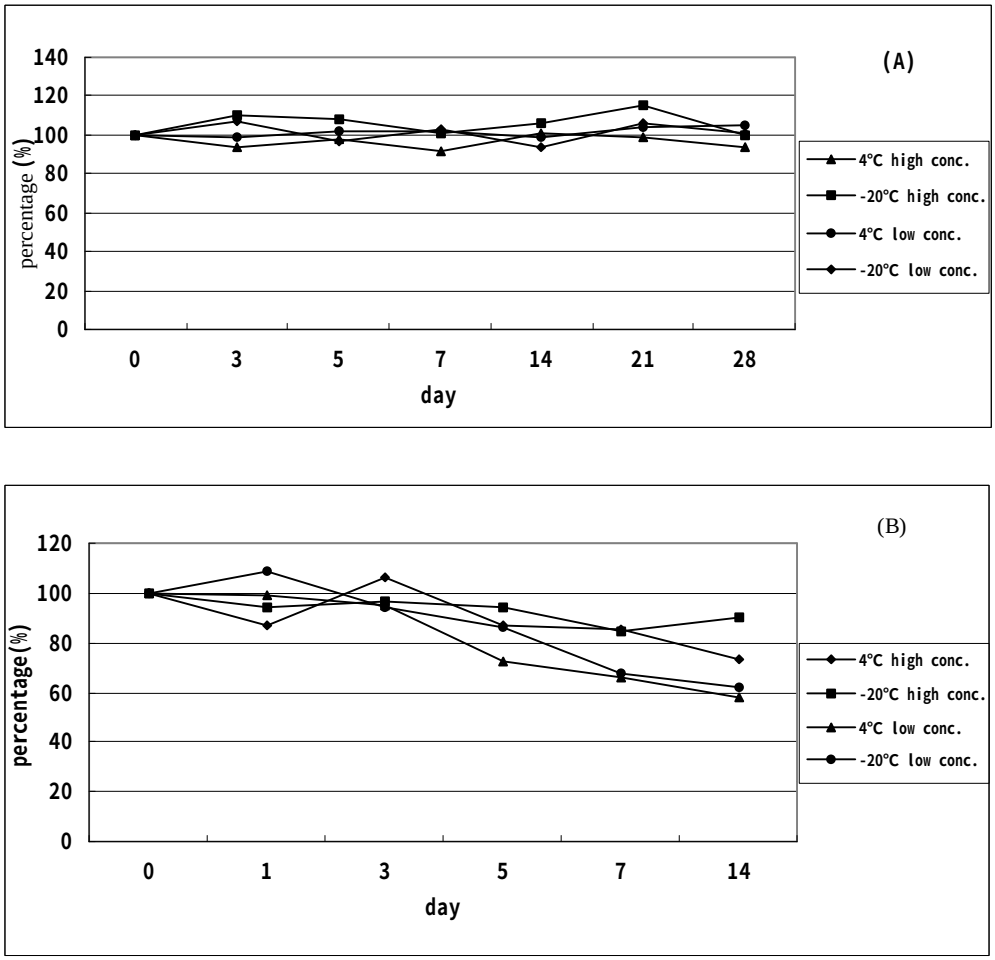

Figure 1. Stability of urinary cotinine levels using GC-NPD (A) (low conc.： 37.6 «g/ml ; high conc.：109.3 «g/ml) and HPLC (B) (low conc.：14.3 «g/ml ; high conc.：45.7 «g /ml).

行政院國家科學委員會專題研究計畫成果報告
台灣地區民眾二手菸暴露之評估

Exposure assessment of environmental tobacco smoking(ETS) in Taiwan population

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摘要

本研究之目的是探討暴露尼古丁之體內代謝物可丁寧濃度作為二手菸暴露生物指標之適用性。任意選擇 27 **位抽煙者、**39 **位非抽菸但有暴露二手菸者及** 28 **位非抽菸亦無暴露者，分析尿中可丁寧的濃度，分別比較高效率液相層析儀**(HPLC-UV)**、氣相層析儀-**氮磷偵測器(GC-NPD)**及競爭性酵素聯結免疫吸附分析法**(ELISA)**。同時分析尿中可丁寧濃度之再現性、回收率及偵測極限等品保及品管措施，以瞭解各種可丁寧分析方法的適用性。結果顯示以氣相層析儀-氮磷偵測器分析尿中可丁寧的濃度，在抽煙者、非抽菸但有暴露二手菸者及非抽菸亦無暴露者分別為** 3054.61**、**46.03 **及** 27.90 ng/ml**，以競爭性酵素聯結免疫吸附分析法所得的結果則為** 2784.65**、**27.93 **及** 16.16 ng/ml**，用高效率液相層析儀分析抽煙者尿中可丁寧濃度為** 3055.17 ng/ml**，其他非抽煙者的濃度則低於此分析方法之偵測極限。且以此三種分析方法同時分析尿中可丁寧濃度其相關係數均達** 0.92 **以上。若以** ELISA **分析唾液可丁寧的濃度與** HPLC-UV **及** GC-NPD **之結果，其相關係數則只有** r=0.37 **及** r=0.33**。利用多變量迴歸分析得知當控制性別、年齡、用藥及健康狀態等變項後，尿中可丁寧濃度隨抽煙支數之增加有劑量與反應之關係。因此，作者結論認為尿中可丁寧濃度可以當做暴露空氣中尼古丁或二手菸的生物指標。**

關鍵字：尼古丁、可丁寧、二手菸、尿液