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Determination of Cotinine in Human Urine by High-Performance Liquid Chromatography

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ABSTRACT

A reversed-phase liquid chromatography with ultraviolet detection is described for the determination of cotinine. A mobile phase consisted of an acetate buffer with sodium octanesulfonate as an ion pair reagent and methanol was used to resolve cotinine from theobromine, theophylline, arecoline and nicotine. Using an octadecyl-type column, a good separation was achieved in 12 min. The wavelength of UV detector was set at 260 nm. With *p*-nitroaniline as internal standard, human urine samples were cleaned up by liquid-liquid extraction. The regression equations obtained from both the standard and the biological samples were linear between 0.1 - 10.0 $\mu\text{g/mL}$ ($r > 0.999$). The detection limit for cotinine is 30.0 ng/mL. The R.S.D. and R.E. of intraday and interday are less than 3.93% and 3.07%, respectively ($n = 6$). The recoveries are all greater than 96.44%. This method has been successfully applied to the determination of urinary cotinine content in several heavy smokers.

Key words: cotinine, HPLC, human urine

INTRODUCTION

The tobacco is one of the most commonly used recreational substance in the world⁽¹⁾. Cigarette smoking increases the risk of malignant lung cancers, heart and other lung diseases⁽²⁻⁷⁾. In 1999 the epidemiologists of World Health Organization (WHO) predicted that approximately 8.4 million people would be killed annually due to their tobacco usage by late 2020s⁽⁸⁾.

Cotinine is a major metabolite of nicotine found in the urine of smokers⁽⁹⁾. Cotinine is used to evaluate secondhand exposure to environmental tobacco smoke⁽¹⁰⁾ and to validate smoking abstinence⁽¹¹⁾. It had been proposed that genetic polymorphisms in nicotine metabolism may serve as a major determinant of an individual's health risk to smoking and their addicting behavior. Hepatic cytochrome P4502A6 (CYP2A6) isozymes are responsible for catalyzing the major route of nicotine metabolism as well as C-oxidation of cotinine, followed by hydroxylation to *trans*-3'-hydroxycotinine⁽¹²⁾. Recently, the *trans*-3'-hydroxycotinine/cotinine ratio has been suggested as an useful index for determination of the rate of nicotine metabolism, and as a mean to assess this CYP2A6 biomarker activity⁽¹³⁾.

The most frequently used analytical methods for the determination of cotinine levels are high-performance liquid chromatography (HPLC)⁽¹⁴⁻¹⁷⁾ and gas chromatography⁽¹⁸⁻²⁰⁾. Other techniques such as capillary electrophoresis⁽²¹⁾ and enzyme-linked immunosorbent assay⁽²²⁾ have also been described. HPLC with ultraviolet (UV) detection is the most widely used analytical method, because it is more simple, economical, and widely available than other techniques.

We are interested in developing a simple analytical method for determination of urinary cotinine levels because it can be easily adopted as a practical tool for assessing health risks of both smokers and secondhand smokers in Taiwan. Furthermore, this method can also be used to simultaneously determine cotinine and *trans*-3'-hydroxycotinine levels in urine and serves as an useful tool for the assessment of the *trans*-3'-hydroxycotinine/cotinine ratio.

According to a previously published procedure, it was not easy to achieve a complete resolution of cotinine from caffeine by HPLC within a short period of 10 min⁽²³⁾. Caffeine is an ingredient in tea, coffee and many over-the counter medicines. In addition, many people also have a habit of chewing betel nuts in Taiwan. Therefore, an analytical method which can separate those active ingredients of betel nut, tea and coffee, such as

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arecoline, theobromine, theophylline and caffeine, will be a useful tool to determine urinary cotinine for these people in Taiwan.

In the present study, we describe a simple HPLC method using an octadecyl-type column coupled with UV detection for determination of cotinine in human urine with liquid-liquid extraction. This method can easily resolve cotinine from theobromine, theophylline, caffeine, arecoline and nicotine in a short period of 12 min. The method has also been successfully applied to determine urinary cotinine from several smokers.

MATERIALS AND METHODS

I. Reagents and Standards

All reagents used were of analytical grade and the organic solvents were of HPLC grade. Cotinine and (S)-(-)-Nicotine were purchased from Lancaster (UK). Arecoline hydrobromide was purchased from MP Biomedicals Inc. (Eschwege, Germany). *p*-Nitroaniline (internal standard; I.S.) was obtained from Merck (Darmstadt, Germany). Water, used for the preparation of mobile phase and related aqueous solution, was purified by Milli-Q from Millipore (Billerica, MA USA). Methanol (MeOH) and triethylamine (TEA) were from Tedia (Fairfield, OH USA). Caffeine, sodium *l*-octanesulfonate, theobromine and theophylline were from TCI (Tokyo, Japan).

II. Apparatus and Chromatographic Conditions

The HPLC analysis was carried out on an Agilent series 1100 LC system, (Foster City, CA USA) equipped with Soma S-3702 UV-Vis detector and DataApex Clarity™ software for LC system.

Analysis was performed on an Astec octadecyl-type (250 × 4.6 mm, 5 μm) column at ambient temperature. Mobile phase consisted of an acetate buffer (containing 30.0 mM sodium acetate, 100 mM acetic acid and 10.0 mM sodium octanesulfonate as ion pair reagent) and methanol (70: 30, v/v). The flow rate was 1.0 mL/min. Injection volume was 20 μL. The eluate was monitored by absorbance at 260 nm.

III. Preparation of Stock and Working Solutions

Stock solutions of cotinine were prepared at 1 mg/mL in 30% methanolic aqueous solution for human urine analysis. *p*-Nitroaniline was prepared at 0.2 mg/mL in 30% methanolic aqueous solution. Solutions were kept at 4°C.

Working solutions of cotinine were diluted from stock solutions with 30% methanolic aqueous solution to 0.5 μg/mL, 1.0 μg/mL, 2.0 μg/mL, 5.0 μg/mL and 10.0 μg/mL for calibration curve and to 0.8 μg/mL, 4.0 μg/mL and 8.0 μg/mL, as QC samples used for urine

analysis. *p*-Nitroaniline solutions were diluted with 30% methanolic aqueous solution to 1 μg/mL as internal standard for biological analysis.

IV. Extraction Method

Urine samples were obtained from test individuals by collecting the first urine in the morning and were kept frozen at -20°C until assay. Each of 500 μL urine samples was added with internal standard solution (1 μg/mL). After the addition of 100 μL 10 N potassium hydroxide and 2 mL dichloromethane/*n*-butanol solution (9: 1, v/v), the urine sample was shaken for 10 min at ambient temperature. One milliliter of inferior organic phase was evaporated to residue and was re-dissolved in 250 μL water/methanol (70: 30, v/v) solution and 20 μL was injected into HPLC for analysis.

V. Precision and Accuracy

For standard analysis, the intraday and interday assays in precision and accuracy were performed with six replicates for each of low (0.79 μg/mL), middle (3.98 μg/mL) and high (7.96 μg/mL) quality control samples. For biological analysis, the intraday and interday assays in precision and accuracy were run with six replicates for each of low (0.80 μg/mL), middle (4.00 μg/mL) and high (8.00 μg/mL) quality control samples.

VI. Recovery

The recovery for cotinine in human urine analysis was calculated at three concentrations (0.5 μg/mL, 5.0 μg/mL, 10.0 μg/mL). The absolute recovery of cotinine was determined by the peak area ratio of urine sample to standard solution and the relative recovery was calculated at three different concentrations (0.5 μg/mL, 5.0 μg/mL, 10.0 μg/mL) based on daily standard curve.

VII. Stability

Stability of cotinine in working solutions and in urine samples (previously stored in -20°C) at room temperature were determined by the solutions and samples stayed at room temperature for 72 hours. Furthermore, stability of cotinine in urine samples at room temperature was determined by the samples stayed at room temperature for 120 hours. Stability of cotinine in spiked urine samples through freeze-thaw procedure was tested at low (2.0 μg/mL) and high (8.0 μg/mL) concentrations after three freeze-thaw cycles.

VIII. Selectivity

A 30% methanolic aqueous solution containing seven different chemicals (i.e. theobromine, theophylline, cotinine, caffeine, arecoline, *p*-nitroaniline and nicotine)

was used to validate the selectivity of the developed analytical method.

XI. Application

The samples of the first urine in the morning of six smokers were separately collected for 5 consecutive days and the urinary cotinine levels were determined. Three of these smokers have agreed to undergo another 5 days cycle and the urinary cotinine contents were analyzed for comparative purpose. The levels of cotinine were determined based on the daily standard curve.

RESULTS AND DISCUSSION

I. High Performance Liquid Chromatography

The chromatograms of the extracts of urine blank and urine blank spiked with cotinine and *p*-nitroaniline are shown in Figure 1. The retention times of cotinine and *p*-nitroaniline are 6.5 and 10.9 min, respectively. An acceptable separation for cotinine and caffeine (r.t. 7.3 min) is achieved in a period of 8 min, which is faster than the method of Baranowski *et al.*⁽²³⁾, and no interference from caffeine and *p*-nitroaniline with cotinine was observed.

II. Linearity and Limit of Quantitation

In standard analysis based on six replicates, regression equations of intraday and interday analysis are $Y = (1.1096 \pm 0.0210) X + (0.0085 \pm 0.0336)$ and $Y = (1.1099 \pm 0.0212) X + (-0.0027 \pm 0.0243)$, respectively. The correlation coefficient of regression equation of intraday analysis is 1.00 as the same as that of analysis interday equation. The limit of quantitation (LOQ) was determined to be 100 ng/mL (S/N = 10, injection 20 μ L).

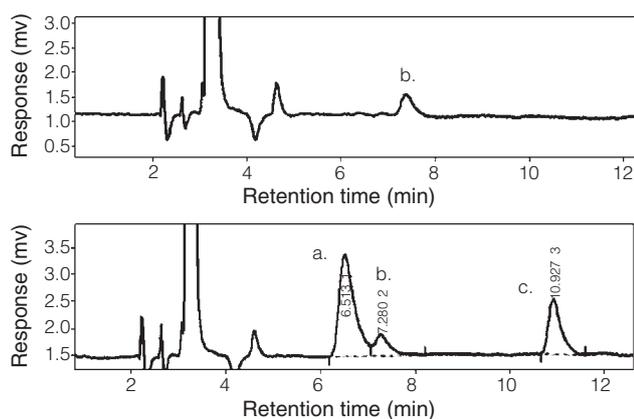


Figure 1. The chromatograms of cotinine and *p*-nitroaniline. (A) The chromatogram of urine blank, (B) The chromatogram of urine blank spiked with cotinine and *p*-nitroaniline. Peaks: a. cotinine 2 μ g/mL (11.3 μ M), b. caffeine, c. *p*-nitroaniline (I.S.) 1 μ g/mL (7.2 μ M).

In human urine analysis based on six replicate analyses, regression equations of intraday and interday analysis are $Y = (1.0118 \pm 0.0064) X + (0.0630 \pm 0.0071)$ and $Y = (1.0075 \pm 0.0140) X + (0.0662 \pm 0.0221)$, respectively. Both correlation coefficients of regression equations are over 0.999. The LOQ was also determined to be 100 ng/mL (S/N = 10, injection 20 μ L).

III. Accuracy and Precision

In standard analysis, the relative standard deviation (R.S.D.) and relative error (R.E.) of intraday and interday analysis were below 2.20% and 1.63% respectively (n = 6). In biological analysis, the R.S.D. and R.E. of intraday and interday were below 3.57% and 3.70% respectively (n = 6) (Table 1).

IV. Recovery

The recovery of cotinine from urine sample was measured under the extraction procedures as described above. The absolute recovery for a cotinine-spiked human urine samples was in the range of 96.44-97.42%. The relative recovery for cotinine spiked in human urine ranges from 97.06 to 100.67% (Table 2). The recovery from urine sample of this method by using a simple liquid-liquid extraction is better than the method of Doctor⁽¹⁵⁾, which the average recovery from urine sample was 88% for cotinine by a solid-phase extraction method.

V. Stability

The stability of cotinine in water/methanol (70/30, v/v) solution was assessed by re-analyzing samples which were left in the autosampler at room temperature for 72 hours. Analyte concentration is stable in standard at

Table 1. Precision and accuracy for the determination of cotinine spiked in human urine

Concentration known (μ g/mL)	Concentration measured (μ g/mL) ^a	R.S.D. (%)	R.E. (%)
Intraday analysis ^b			
0.8	0.82 \pm 0.02	3.57	3.02
4.0	3.91 \pm 0.11	2.89	-2.03
8.0	7.70 \pm 0.10	1.31	-3.70
Interday analysis ^c			
0.8	0.82 \pm 0.02	2.85	3.06
4.0	4.03 \pm 0.15	3.39	0.77
8.0	7.83 \pm 0.21	2.78	-2.02

^a Mean \pm S.D. of six replicate analyses.

^b Intraday data was based on six replicate analyses.

^c Interday data was based on six replicate analyses on six different days.

room temperature for 72 hours. The stability of cotinine in human urine at room temperature was also estimated by urine sample of cotinine stored in a room at ambient temperature for 120 hours. No degradation was observed in the analytic samples until 120 hours.

The results of stability determination of the urinary cotinine at low (2.0 µg/mL) and high (8.0 µg/mL) concentrations after three freeze-thaw cycles are listed in Table 3. No degradation was observed in neither of the two concentrations studies.

VI. Selectivity

In Taiwan, people are commonly drinking a lot of caffeinated beverages, teas and chewing betel nuts. The method was designed to determine the urinary cotinine contents of these people. Selectivity of cotinine among those substances, such as caffeine, theobromine, theophylline and arecoline must be met in this method. Figure 2 clearly demonstrated a good separation of cotinine peak with no interference from theobromine, theophylline, caffeine, arecoline and nicotine in the chromatogram of a short period of time (13.5 min). In previous studies^(14-17,23), theobromine, theophylline, caffeine, arecoline and nicotine were not included to examine the selectivity of HPLC method for cotinine. With this selectivity, the method will be feasibly applied in Taiwan and other Southeast Asian countries.

VII. Application

This method was applied for the determination of cotinine concentrations in urine of six male smokers for five consecutive days (Figure 3). The results are

Table 2. Relative recovery of cotinine in human urine

Concentration spiked (µg/mL)	Concentration measured (µg/mL)	Recovery (%) ^a
0.5	0.48 ± 0.01	97.06
5.0	4.90 ± 0.05	98.18
10.0	10.06 ± 0.10	100.67

^a Mean ± S.D. of five replicate analyses.

Table 3. The stability of urinary cotinine in three freeze-thaw cycles

Sample (µg/mL)	N	Cycle 1		Cycle 2		Cycle 3	
		Measured ^a (µg/mL)	R.E.(%)	Measured ^a (µg/mL)	R.E. (%)	Measured ^a (µg/mL)	R.E. (%)
2.0	3	2.05 ± 0.01	2.95	2.09 ± 0.01	4.85	2.08 ± 0.02	4.10
8.0	3	8.38 ± 0.08	4.80	8.28 ± 0.07	3.57	8.34 ± 0.09	4.28

^a Mean ± S.D. of triplicate analyses.

shown in Table 4. These smokers are classified as moderate smokers with urinary cotinine concentrations at 500-2500 ng/mL according to the literature⁽²⁴⁾, which defined urinary cotinine concentrations (ng/mL) at 150-500 as a light smoker, 500-2500 as a moderate smoker, and above 2500 as a heavy smoker. Without changing their life styles and habits, urine samples of three of six voluntary male smokers were analyzed once again for another 5-day period. The results indicated that mean cotinine concentrations of the urine samples taken from the same individual between the two test run differed within a small range of 47-100 ng/mL (Table 4).

CONCLUSIONS

A method using HPLC with UV detector for the

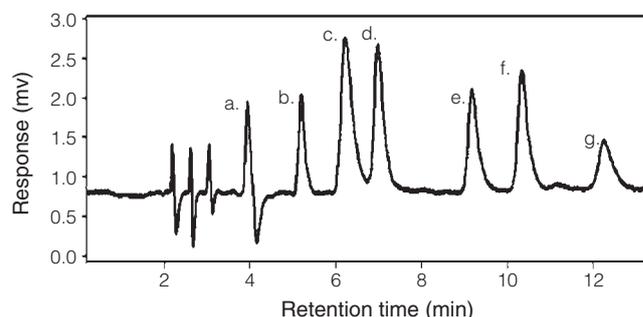


Figure 2. The chromatogram of some chemicals. Peaks: a. theobromine, b. theophylline, c. cotinine, d. caffeine, e. arecoline, f. *p*-nitroaniline, g. nicotine.

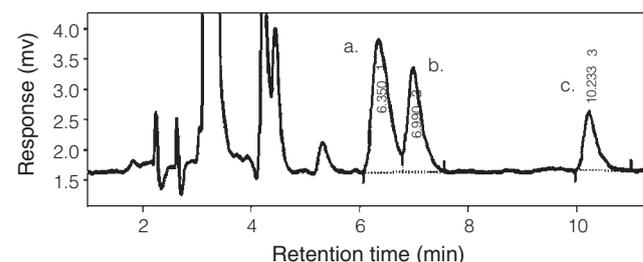


Figure 3. The Chromatogram of a smoker's urine. Peaks: a. cotinine, b. caffeine, c. *p*-nitroaniline.

Table 4. Assay results for smokers' urine

Smoker's number	Cotinine concentration (ng/mL)					Mean \pm SD
	Day 1	Day 2	Day 3	Day 4	Day 5	
1	847	857	719	551	630	720 \pm 133
2	1546	1805	1389	1455	1341	1507 \pm 183
2 [#]	2201	1854	836	848	1296	1407 \pm 608 [#]
3	1011	1414	1421	1833	1786	1493 \pm 333
4	1812	2570	2491	2631	1823	2265 \pm 411
4 [#]	2630	1879	2504	2602	1945	2312 \pm 368 [#]
5	1160	1822	1225	903	1077	1237 \pm 348
6	2087	1947	1943	1689	1617	1856 \pm 196
6 [#]	1867	1770	1333	1667	2163	1760 \pm 302 [#]

[#]: The results of second test run.

analysis of cotinine with good selectivity has been developed. The method is simple, precise and accurate with a sample prepared by liquid-liquid extraction. Because the method can well separate cotinine from theobromine, theophylline, caffeine, arecoline and nicotine, it will be suitable for determining urinary cotinine levels of the smokers and second-hand smokers in Taiwan and other Southeast Asian countries. The method has been successfully applied to determine the urinary cotinine concentrations of six smokers.

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