A Sensitive HPLC-ESI–MS–MS Method for the Determination of Cotinine in Urine

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Abstract

A simple, sensitive, selective, and reproducible method based on high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry was developed for the determination of nicotine and its major metabolite cotinine in human urine. The internal standard (acetaminophen) was separated from cotinine on a Hypersil Gold C_{18} column with retention times of 9.3 and 13.0 min, respectively. The mobile phase consisted of a mixture of 10 mM acetate buffer (pH 5.4) and methanol (45:55, v/v), running through the column at a flow rate of 0.6 mL/min. The chromatographic analysis was operated at 25°C. Sample preparation was prepared by liquid–liquid extraction with a mixture of methyl-*t*-butyl ether in dichloromethane (1:1, v/v). The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 15% (% coefficient of variation). Good accuracy was observed for both the intra-day or inter-day assays. Limit of quantification was accepted as 0.02 ng using 100 mL samples. The mean recoveries for cotinine and the internal standard were greater than 90%. The method has been applied to the investigation of a 2-h urinary excretion of cotinine in 154 healthy non-smoking Thai volunteers (aged 18–45 years) following the administration of a half-piece (2 mg) of nicotine gum.

Introduction

Tobacco smoking is one of the major causes of human death, especially by lung cancer. Nicotine, a major component in tobacco, is the major addictive agent in cigarette smoke. It is absorbed by inhalation in the lungs and through the skin and mucosal lining of the mouth and nose. Smokers adjust their nicotine intake to maintain plasma nicotine levels. Nicotine is extensively (approximately 70%) metabolized mainly via cytochrome P450 2A6 (CYP2A6) to cotinine, which is subsequently oxidized to *trans*-hydroxycotinine (1). The plasma half-life of cotinine is 18–20 h, which is much longer than that of nicotine itself (1–2 h). Cotinine concentration reliably measured in plasma, serum, saliva, and urine is therefore routinely used as a specific biomarker for nicotine exposure in active and passive smokers.

A number of analytical methods have been reported for the determination of cotinine and/or nicotine in human and animal biological fluids (plasma, serum, saliva, meconium, and urine). These methods involve radioimmunoassay (2), enzyme-linked immunoassay (3), gas chromatography (GC) (4–7), gas chromatography mass–spectrometry (GC–MS) (8–11), high performance liquid chromatography (HPLC) (12–15), and liquid chromatography–tandem mass spectrometry (LC–MS–MS) (16–26). Radioimmunoassay and enzyme-linked immunosorbent assay are sensitive, but cross-reactivities against metabolite(s) presented in the nicotine-dosed biological samples are likely. For GC, GC–MS, HPLC, LC–MS–MS methods, the limit of quantification (LOQ) ranges from 0.05 to 20 ng/mL. The main disadvantages of the GC methods with nitrogen phosphorous or flame ionization detection include tedious sample preparation and decomposition of the polar components at the high temperatures utilized in the analysis. HPLC with UV, mass spectrometric, or electrochemical detectors are used to overcome the disadvantages of the GC methods. Most of the LC–MS methods (16–26) are based on electrospray tandem mass spectrometry for determination of nicotine alone or simultaneously with its metabolites, cotinine, and/or 3-hydroxycotinine in human serum, saliva, urine, breast milk, or meconium. The sample cleanup and concentration procedures include either liquid–liquid extraction or solid-phase extraction. Due to serious health consequences from environmental tobacco smoke, particularly for passive smokers or non-smokers exposed to low levels of nicotine in the environment, a highly sensitive, specific, and simple assay method for measurement of cotinine is required. Although the currently used methods can simultaneously measure several metabolites of nicotine, the sensitivity and selectivity of some of these methods are not high enough to reliably measure urine cotinine in non-smoker populations who are exposed to low levels of nicotine (below 1 ng/mL) in the environment.

In the present study, we propose an alternative simple and sensitive LC–MS–MS method with electrospray ionization (ESI) for determination of solely cotinine based on a single step and

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straightforward liquid–liquid extraction with improved sensitivity down to 0.02 ng/100 µL urine sample. High specificity is obtained through focusing the development of the assay method on only cotinine because nicotine is rapidly metabolized to the undetectable level within 1–2 h. In addition, the assay method using urine as biological fluid rather than plasma or serum (12–14,16,19,22,24) is considered non-invasive and practical for monitoring of nicotine exposure/consumption in the populations. The method was applied for the determination of cotinine in urine samples collected at 2 h from a total of 134 Thai nonsmokers (27).

Experimental

Chemicals

Cotinine (98% pure, C5923) was purchased from Sigma Chemical (St. Louis, MO). The following chemicals and solvents were obtained in the highest purity available: dichloromethane, methyl-*t*-butyl ether, and methanol (LAB-Scan, Analytical Sciences, Bangkok, Thailand), acetic acid (BDH Laboratory Supplies, Poole, U.K.), ammonium hydroxide, ammonium acetate, and hydrochloric acid (Sigma Aldrich), and acetaminophen [internal standard (IS)] (Sigma Aldrich). Deionized double distilled water was used for the preparation of working nicotine standard solutions.

Preparation of standards

Stock solution of cotinine (1,000 ng/mL) was prepared by dissolving 5 mg of the compound in 5,000 mL methanol and stored at –20°C until use. Working cotinine standard solutions was prepared by diluting the stock standard solutions with methanol. Standard solution was stored at –20°C until analysis. Seven aliquots of blank control urine were spiked with cotinine stock solution in a serial dilution to obtain the standard calibration at concentrations of 10, 20, 50, 150, 250, and 500 ng/mL with 25 ng IS (5 ng/mL acetaminophen).

Chromatography

Cotinine and the IS were separated on a Hypersil Gold C_{18} column (4.6 × 150 mm, 5 mm particle size) (Thermo, Waltham, MA). The HPLC system was operated under gradient elution at a flow rate of 0.6 mL/min. The mobile phase was a mixture of 10 mM acetate buffer (pH 5.4) and methanol (55:45, v/v) with gradient elution. The HPLC system consisted of a solvent delivery system (SpectraSystem P4000 pump), and a vacuum membrane degasser (SpectraSystem SCM1000) was used to minimize gasses from the eluent flow prior to the introduction of a chromatographic sample into the mobile phase. For creating ions from analyses in solution, ESI was used to produce ions from solution into the gas phase and monitored the selected ions reaching the detector by measuring the ion current for one particular mass. The mass spectrometer consisted of a Finnigan LCQ Deca XP Max plus ion trap detector equipped with the positive ESI interface. The heated nebulizer was set at 300°C and pressure at 551 kPa; the flow rates of auxiliary nitrogen gas and curtain gas were set at 70 and 15 arb, respectively. Mass results were plotted and processed by the LcQuanTM 2.0 (Thermo). Ions monitored in the simple reaction monitoring (SRM) mode were *m/z* 177.2 (parent ion) to *m/z* 98.0 (daughter ion) for cotinine; *m/z* 152.2 (parent ion) to *m/z* 110 (daughter ion) for acetaminophen. Argon was used as the collision gas, and electron multiplier was set at 5,000 V. To increase the signal-to-noise ratio of the compounds, MS–MS was applied. A precursor ion selected by one mass separator was broken into fragments by collisions with an inert gas (nitrogen) at a pressure of approximately 0.1 Pa. Three fragments, two fragments of cotinine and IS (acetaminophen), respectively, were then selected for monitoring by a second mass separator.

Sample preparation

One hundred microliters of unknown urine samples, quality control samples, or blank urine were transferred to polypropylene tubes. These samples were alkalinized with 500 µL of 0.5 mol/L ammonium hydroxide to each sample and thoroughly mixed by vortex mixer for 30 s. Thereafter, 25 ng of acetaminophen (5 ng/mL) was added as an IS. The mixture was then extracted with 5 mL of the mixture methyl-*t*-butyl ether in dichloromethane (1:1, v/v) by horizontal shaking at 2,000 × *g* (speed 6) for 10 min. The upper organic phase was separated through centrifugation at 2,500 × *g* for 10 min. The clear supernatant was transferred into a second set of 10-mL screw-capped Teflon tubes. Methanolic hydrochloric acid (100 mL) was added and mixed gently before evaporated to dryness under a stream of nitrogen at 37°C. The residue was redissolved in 100 µL of the mobile phase, and 20 µL portion was injected into HPLC.

Calibration curves

The linearity of the method was observed in the expected concentration range, demonstrating its suitability for analysis. This LC–MS–MS method was linear over the concentrations range of 10–1,000 ng/mL.

Calibration curves of cotinine (10, 20, 50, 150, 250, and 500 ng/mL) were prepared in the same day as sample analyses with varying concentrations of cotinine and a fixed concentration of acetaminophen (25 ng). Samples were analyzed as described earlier.

Data analysis

Concentrations of cotinine were determined from the peak-height ratios (peak areas of cotinine/peak areas of IS), which corresponded to the known cotinine concentrations in a calibration curve as described earlier. Peak detection, peak-height integration, and peak-height ratio calculation were performed by the Millennium 2000 Chromatographâ software.

Method validation

Precision

The precision of the method based on within-day repeatability was determined by replicate analysis of six sets of samples spiked

at three different concentrations of cotinine (10, 50, and 500 ng/mL urine). The reproducibility (day-to-day variation) of the method was validated using the same concentration range of plasma as described earlier, but only a single determination of each concentration was made on six different days. Coefficients of variation (CV) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentages.

Accuracy

Accuracy of the method was determined by replicate analysis of six sets of samples spiked at three different levels of cotinine (10, 50, and 500 ng/mL urine) and comparing the difference between the spiked value and that actually found (theoretical value).

Recovery

The analytical recovery of sample preparation procedure for cotinine was estimated by comparing the peak heights obtained from samples (urine) prepared as described earlier with those measured with equivalent amounts of cotinine in methanol. Triplicate analysis was performed at cotinine concentrations of 10, 50, and 500 ng/mL urine.

Selectivity

The selectivity of the assay was demonstrated by checking for the absence of (*i*) endogenous interferences at the retention times of cotinine in human blank urine obtained from six different lots and (*ii*) the interference by commonly used drugs (i.e., most antibacterials and dimenhydrinate) after subjecting them to sample preparation procedures.

Limit of quantification

The LOQ of the assay procedure was determined from the lowest concentration of cotinine (in spiked urine sample) that produced a peak height ten times the baseline noise at a sensitivity of 0.005 a.u.f.s. (absorbance unit full scale) in a 100-mL sample, which also produced acceptable accuracy (< 20% of the nominal values) and precision (CV < 20%).

Stability

The stability of cotinine was determined by storing spiked urine samples at concentrations of 10, 50, and 100 ng/mL urine (triplicate analysis for each concentration) in a –20°C freezer (Sanyo, Osaka, Japan) for six months. Concentrations were measured periodically (1 week and 6 months). For freeze-and-thaw stability, samples were frozen at –20°C for at least 24 h and thawed unassisted at room temperature (25°C). When completely thawed, the samples were transferred back to the original freezer and refrozen for at least 24 h. The process was repeated for three cycles.

Quality control

Quality control samples for cotinine were made up in urine using a stock solution separated from that used to prepare the calibration curve at concentrations of 10, 50, and 500 ng/mL urine. Samples were aliquoted into cryovials and stored frozen at –20°C for use with each analytical run. The results of the quality control samples provided the basis of accepting or rejecting the run. At least four of the six quality control samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value but not at the same concentration.

Application of the method to biological samples

The method was applied to the investigation of a 2-h urinary excretion of cotinine in 154 healthy non-smoker Thai volunteers (80 males, 74 females, aged 18–45 years) who had no history of tobacco smoking, following the administration of half-piece (2 mg) of nicotine gum (Nicorette; 4 mg nicotine gum). This is part of a study to investigate the association between CYP2A6 genotypes and phenotypes (metabolic status) in a Thai population using nicotine as a probe substrate (27). Participants were residents of the three main areas of Thailand: Pathutanee Province (central; n = 58), Khon Kaen Province (northeastern; n = 39), and Mae Sot District, Tak Province (northern; n = 57). The study protocol was approved by the Ethics Committees of the Faculty of Medicine, Khon Kaen University, and the Ministry of Public Health of Thailand. Written informed consents for study participation were obtained from all subjects who had been informed of the study protocol. All subjects were healthy as verified by clinical and laboratory assessments. Exclusion criteria included those who had used medication within two days prior to the start of the experimental period. The ethnic background, full medication history, history of pregnancy, smoking and alcohol consumption, specific food ingredient consumption, and any history of drug use were ascertained by means of a questionnaire interview. All subjects received nicotine gum for 30 min with 300 mL drinking water at the rate of chewing for 15 cycles per min. Thereafter, urine samples were collected during 2-h interval after the intake of nicotine. Total volume of urine was recorded, and an aliquot of urine (5 mL) was separated and kept at –20°C until analysis.

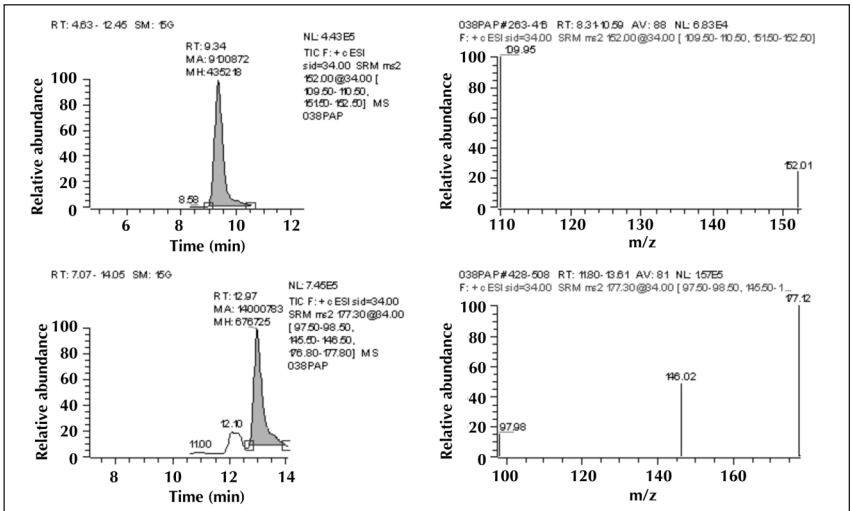

Figure 1. Chromatograms of urine spiked with 10 ng/mL cotinine and 25 ng/mL acetaminophen. The retention times for acetaminophen and cotinine were 9.34 and 12.97 min, respectively.

Results and Discussion

Chromatographic separation

Under the chromatographic condition previously described, the chromatograms of cotinine and IS (acetaminophen) were free from any interference peak and had good resolution and sharp peaks. Blank urine samples showed little noise fluctuation. The retention times of acetaminophen and cotininine were 9.3 and 13.0 min, respectively (Figure 1). Cotinine concentrations in unknown samples were determined by interpolating the peak-height ratio of cotinine and IS obtained with the calibration curves plotted.

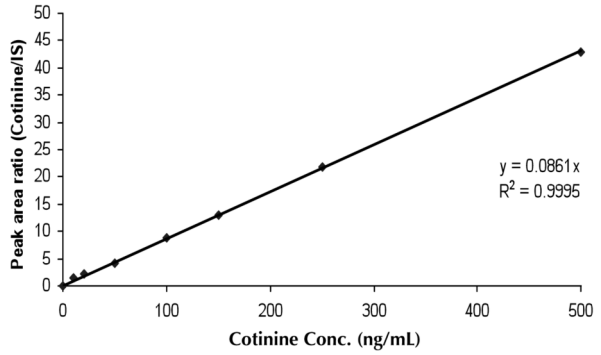

Figure 2. Mean calibration curve of cotinine in urine (n = 6).

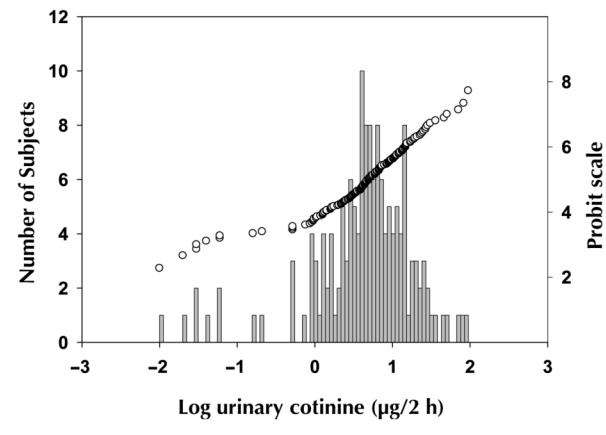

Figure 3. Frequency distribution of total amounts of cotinine (logarithmically transformed) excreted in urine over 2 h (µg/2 h) in a total of 134 Thai healthy subjects who were non-smokers.

Sample preparation

Chromatograms of blank urine and urine spiked with cotinine at concentrations of 10 and 25 ng/mL (with a fixed concentration of acetaminophen 25 ng/100 mL urine) are shown in Figure 1.

Calibration curves

Urine analysis was calibrated using the concentration range of 10–500 ng/mL urine. All calibration ranges yielded linear relationships with correlation coefficients (r^2 > 0.9995) or better (Figure 2). The linear regression equation obtained from the mean of the six calibration curves was $y = 0.0861x$, where y is the peak-height ratio and x is the analyte concentration in ng.

Method validation

Precision

Little variation of cotinine assays was observed: CV for six analyses at the concentration range observed were all below 15%. The intra-assay (within-day) and inter-assay (day-to-day) variation for cotinine assay at the concentration range of 10–500 ng/100 mL urine are summarized in Table I. Intra- and inter-day assay variation varied between 1.1–5.6% and 1.4–6.3% (% CV), respectively.

Accuracy

Good accuracy was observed for both the intra-day or inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added). The intra-assay (within-day) and inter-assay (day-to-day) accuracy for cotinine assay at the concentration range of 10–500 ng/100 mL urine are summarized in Table I. Intra- and inter-day assay accuracy, expressed as the mean deviation from the theoretical values varied between –1.1–5.9% and –0.98–6.8%, respectively (Table I).

Recovery

The mean recoveries for cotinine in urine at concentrations of 10, 50, and 500 ng/mL urine were greater than 95% (Table II). The results reflect essentially 100% recovery from the spiked urine and indicate lack of interference from the sample preparation procedure. The high recovery of exceeding 100% (129.2 + 21.1%) at the lowest cotinine concentration of 10 ng/mL may indicate interference from endogenous substances during sample preparation step. It was noted, however, that this recovery was determined based on a peak height 10 times the baseline noise. Repeat analysis using 20 urine samples from different sources at lower sensitivity ensures the selectivity of the assay procedure. The recovery of the IS from urine was 98.6 % at a concentration of 25 ng/mL.

Table I. Inter-Day (Between-Day) and Intra-Day (Within-Day) Validation of Cotinine Concentrations

	Intra-day precision (n = 6)			Inter-day precision (n = 6)		
Conc. added (ng/mL)	Conc. measured (mean ± SD; ng/mL)	% CV*	Accuracy (%DMV)*	Conc. measured (mean ± SD; ng/mL)	%CV	Accuracy (%DMV)
10.0	10.6 ± 0.6	5.6	5.9	10.8 ± 0.7	6.3	6.8
50.0	51.7 ± 2.4	4.6	4.7	51.6 ± 1.4	2.7	2.8
500.0	503.8 ± 5.7	1.1	1.1	504.4 ± 4.5	1.4	0.98

* %CV = coefficient of variation; %DMV = deviation of mean value from the theoretical value.

Selectivity

Selectivity of the chromatographic separation was demonstrated by the absence of interferences from endogenous peaks in urine at the

retention times of the IS and cotinine (9.3 and 13.0 min, respectively). Figure 1 illustrates typical chromatograms for blank urine and urine spiked with cotinine and IS.

Limit of quantification

The LOQ in human urine for cotinine was accepted as 0.02 ng using 100 mL urine.

Stability

Freeze-and-thaw stability of cotinine in urine, expressed as the deviation of the mean from the values before three freeze-and-thaw cycles procedure, were determined by repeated analysis of three aliquots of samples at three different concentrations (10, 50, 500 ng/mL). Aliquots of each sample were stored at the intended storage temperature for 24 h and thawed at room temperature. When completely thawed, they were again refrozen for 24 h under the same conditions. The freeze-thaw cycle was being repeated three times and then analyzed after third cycle. Cotinine assay in urine was found to be stable without decomposition of the drug after being subjected to long-term freezing (stored in a –20°C freezer for a minimum of 6 months) and thawing procedure. It was noted, however, that a significant change was observed at 500 ng/mL following three freeze-thaw cycles. The mean change at the observed periods (1, 3, and 6 months) is shown in Table III.

Table II. Recovery of Cotinine and IS From Urine

Cotinine conc. added (ng/mL)	Conc. measured (mean ± SD; ng/mL)	Conc. measured (mean ± SD; ng/mL)	Recovery (%)
10.0	10.7 ± 1.8	15.4 ± 2.7	129.2 ± 21.1
50.0	49.3 ± 2.5	50.6 ± 3.4	100.2 ± 11.0
500.0	501.3 ± 8.1	497.8 ± 6.8	98.0 ± 2.8
IS conc. added (ng/mL)	**Conc. measured (mean ± SD; ng/mL)**	**Conc. measured (mean ± SD; ng/mL)**	**Recovery (%)**
25.0	25.0 ± 5.7	26.0 ± 1.9	98.6 ± 4.3

Table III. Storage Stability for Cotinine in Spiked Urine*

Storage condition	Target conc. (ng/mL)	Measured conc. (ng/mL) (mean + SD; *n* = 3)
Freshly prepared	10.0	11.3 ± 1.6
	50.0	50.4 ± 1.9
	500.0	494.6 ± 5.3
6 hours at room temp. (25°C)	10.0	15.5 ± 3.2
	50.0	51.3 ± 2.8
	500.0	500.0 ± 2.7
7 days at –20°C	10.0	11.6 ± 3.1
	50.0	49.9 ± 3.4
	500.0	504.2 ± 14.3
	50.0	51.2 ± 6.8
	500.0	503.1 ± 9.7
6 months at –20°C	10.0	11.1 ± 5.1
	50.0	49.0 ± 6.1
	500.0	501.4 ± 5.6
1 freeze-thaw cycle	10.0	10.0 ± 9.6
	50.0	49.1 ± 6.4
	500.0	496.7 ± 7.0
	50.0	48.8 ± 6.4
	500.0	501.1 ± 6.0
3 freeze-thaw cycles	10.0	9.5 ± 5.9
	50.0	53.5 ± 3.2
	500.0	405.9 ± 8.1

* At concentrations of 10, 50, and 500 ng/mL at 6 months following –20°C storage and after freeze-thaw cycles.

Quality control

Three validated analysts conducted the urine analysis. A standard curve and quality control specimens were included with each analysis. Control samples with nominal concentrations of 10, 50, and 500 ng/mL urine cotinine were analyzed at the beginning and the end of the analytical run. All results were within the acceptable limit (± 20% of their respective nominal values).

Application of assay and analysis of specimens

The method appears to be robust and has been applied to the investigation of a 2-h urinary excretion of cotinine in 154 healthy non-smoker Thai volunteers following the administration of a half-piece (2 mg) of nicotine gum (Nicorette; 4 mg nicotine gum). Figure 3 shows the frequency distribution of the amounts of cotinine (logarithmically transformed) excreted in urine over 2 h in the sample population. Individuals with 2 h urinary cotinine excretion amounts in the ranges of 0.01–0.21 (5.8%) and 0.52–94.99 (93.5%) mg^2/h were classified as poor metabolizers (PMs) and extensive metabolizers (EMs), respectively. The full analysis and conclusion on the association between CYP2A6 genotypes and phenotypes in the Thai population is described elsewhere (27).

Conclusion

We have developed and validated a simple (a single-step sample preparation with isocratic mode of separation), sensitive, selective, accurate, robust, and inexpensive assay for measuring cotinine in urine using reversed-phase HPLC–ESI-MS–MS by utilizing a technique that has reduced the ion suppression effect. The procedure can be applied for measuring of cotinine in urine collected from a total of 154 healthy non-smoker Thai population with exposure to nicotine at low levels. The advantage features of the developed method over the previously reported method for analysis of urine samples (18) include the higher sensitivity (LOQ 0.2 ng/100 µL vs × 1 ng/mL) and less complexity and thus shorter analysis time (single-step liquid–liquid extraction vs. solid-phase extraction).

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References

1. J. Hukkanen, P. Jacob, and N.L. Benowitz. Metabolism and disposition kinetics of nicotine. *Pharmacol. Rev.* **57:** 79–115 (2005).
2. G.J. Knight, P. Wylie, M.S. Holman, and J.E. Haddow. Improved 125I radioimmunoassay for cotinine by selective removal of bridge antibodies. *Clin. Chem.* **31:** 118–121 (1985).
3. 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:** 203–213 (1985).
4. C. Dumas, R. Badré, A. Viala, J.P. Cano, and R. Guillerm. A micromethod for determination of nicotine and cotinine in blood and urine by gas chromatography. Results obtained from various smokers. *Eur. J. Toxicol. Environ. Hyg.* **8:** 280–286 (1975).
5. P. Jacob, M. Wilson, and N.L. Benowitz. Improved gas chromatographic method for the determination of nicotine and cotinine in biologic fluids. *J. Chromatogr.* **222:** 61–70 (1981).
6. C. Feyerabend and M.A. Russell. A rapid gas-liquid chromatographic method for the determination of cotinine and nicotine in biological fluids. *J. Pharm. Pharmacol.* **42:** 450–452 (1990).
7. 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:** 87–93 (1998).
8. A.J. Ji, G.M. Lawson, R. Anderson, L.C. Dale, I.T. Croghan, and R.D. Hurt. A new gas chromatography-mass spectrometry method for simultaneous determination of total and free trans-3'-hydroxycotinine and cotinine in the urine of subjects receiving transdermal nicotine. *Clin. Chem.* **45:** 85–91 (1999).
9. H.S. Shin, J.G. Kim, Y.J. Shin, and S.H. Jee. Sensitive and simple method for the determination of nicotine and cotinine in human urine, plasma and saliva by gas chromatography-mass spectrometry. *J. Chromatogr. B Analyst. Technol. Biomed. Life Sci.* **769:** 177–183 (2002).
10. E. Cognard and C. Staub. Determination of nicotine and its major metabolite cotinine in plasma or serum by gas chromatography-mass spectrometry using ion-trap detection. *Clin. Chem. Lab. Med.* **41:** 1599–1607 (2003).
11. J.S. Toraño and H.J. van Kan. Simultaneous determination of the tobacco smoke uptake parameters nicotine, cotinine and thiocyanate in urine, saliva and hair, using gas chromatography-mass spectrometry for characterisation of smoking status of recently exposed subjects. *Analyst* **128:** 838–843 (2003).
12. A. Sioufi, C. Parisot, N. Sandrenan, and J.P. Dubois. High performance liquid chromatographic determination of nicotine and cotinine in plasma and nicotine and cotinine, simultaneously, in urine. *Methods Find. Exp. Clin. Pharmacol.* **11:** 179–185 (1989).
13. O.A. Ghosheh, D. Browne, T. Rogers, J. de Leon, L.P. Dwoskin, and P.A. Crooks. A simple high performance liquid chromatographic method for the quantification of total cotinine, total 3'-hydroxycotinine and caffeine in the plasma of smokers. *J. Pharm. Biomed. Anal.* **23:** 543–549 (2000).
14. M. Nakajima, T. Yamamoto, Y. Kuroiwa, and T. Yokoi. Improved highly sensitive method for determination of nicotine and cotinine in human plasma by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **742:** 211–215 (2000).
15. M. Page-Sharp, T.W. Hale, L.P. Hackett, J.H. Kristensen, and K.F. Ilett. Measurement of nicotine and cotinine in human milk by high-performance liquid chromatography with ultraviolet absorbance detection. *J. Chromatogr. B Analyst. Technol. Biomed. Life Sci.* **796:** 173–180 (2003).
16. J.T. Bernert Jr, W.E. Turner, J.L. Pirkle, C.S. Sosnoff, J.R. Akins, M.K. Waldrep, Q. Ann, T.R. Covey, W.E. Whitfield, E.W. Gunter, B.B. Miller, D.G. Patterson Jr, L.L. Needham, W.H. Hannon, and E.J. Sampson. Development and validation of sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography/atmospheric pressure ionization tandem mass spectrometry. *Clin. Chem.* **43:** 2281–2291 (1997).
17. M.C. Bentley, M. Abrar, M. Kelk, J. Cook, and K. Phillips. Validation of an assay for the determination of cotinine and 3-hydroxycotinine in human saliva using automated solid-phase extraction and liquid chromatography with tandem mass spectrometric detection. *J. Chromatogr. B Biomed. Sci. Appl.* **723:** 185–194 (1999).
18. T. Tuomi , T. Johnsson, and K. Reijula. Analysis of nicotine, 3-hydroxycotinine, cotinine, and caffeine in urine of passive smokers by HPLC-tandem mass spectrometry. *Clin. Chem.* **45:** 2164–2172 (1999).
19. T.P. Moyer, J.R. Charlson, R.J. Enger, L.C. Dale, J.O. Ebbert, D.R. Schroeder, R.D. Hurt. Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. *Clin. Chem.* **48:** 1460–1471 (2002).
20. W.Z. Shou and W. Naidong. Simple means to alleviate sensitivity loss by trifluoroacetic acid (TFA) mobile phases in the hydrophilic interaction chromatography-electrospray tandem mass spectrometric (HILIC-ESI/MS/MS) bioanalysis of basic compounds. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **825:** 186–192 (2005).
21. N.L. Benowitz, C.N. Lessov-Schlaggar, G.E. Swan, and P. Jacob. Female sex and oral contraceptive use accelerate nicotine metabolism. *Clin. Pharmacol. Ther.* **79:** 480–488 (2006).
22. I. Kim and M.A. Huestis. A validated method for the determination of nicotine, cotinine, trans-3′-hydroxycotinine, and norcotinine in human plasma using solid-phase extraction and liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. *J. Mass Spectrom.* **41:** 815–821 (2006).
23. C.A. Chadwick and B. Keevil. Measurement of cotinine in urine by liquid chromatography tandem mass spectrometry. *Ann. Clin. Biochem.* **44:** 455–462 (2007).
24. S.E. Murphy, P. Vilalta, S.W. Ho, and L.B. Weymarn. Analysis of [3', 3'-d_2]-nicotine and [3', 3'-d_2]-cotinine by capillary liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. B* **857:** 1–8 (2007).
25. M. Pellegrini, E. Marchei, S. Rossi, F. Vagnarelli, A. Durgbanshi, O. Garcia-Algar, O. Vall, and S. Pichini. Liquid chromatography/electrospray inonization tandem mass spectrometry assay for determination of nicotine and metabolites, caffeine and arecoline in breast milk. *Rapid Commun. Mass Spectrom.* **21:** 2693–2703 (2007).
26. T.R. Gray, D.M. Shakleya, and M.A. Huestis. Quantification of nicotine, cotinine, trans-3'-hydroxycotinine, nornicotine and norcotinine in human meconium by liquid chromatography/tandem mass spectrometry. *J. Chromatogr. B* **863:** 107–114 (2008).
27. R. Apinan, W. Tassaneeyakul, S. Satarug, S. Kajanawart, S. Vannaprasaht, R. Ruenweerayut, and K. Na-Bangchang. Influence of genetic and environmental factors on cytochrome P450-mediated nicotine metabolism in Thai population. (submitted for publication)

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