Asian Journal of Chemistry; Vol. 27, No. 4 (2015), 1403-1407

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.18048

Simple, Sensitive and Rapid Ultra-High Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry for the Determination of Nicotine and its Nine Metabolites in Human Urine

ZHONG FAN[1], HUIMIN LIU[2], FUWEI XIE[2], QIAOLING XIA[2], SHENG WANG[2], ZHIYAN CHEN[1] and XIAOLAN LI[1,*]

[1]Techonology Centre, China Tobacco Guangxi Industrial Co. Ltd., Nanning 530001, P.R. China
[2]Chemistry Department, Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou 450001, P.R. China

*Corresponding author: Tel/Fax: +86 771 8098516; 8098519; E-mail: xiaolan-dg@163.com

Received: 20 May 2014; *Accepted*: 30 July 2014; *Published online*: 4 February 2015; AJC-16801

A simple, sensitive and rapid UHPLC-MS/MS method was developed for the quantication of nicotine and its nine metabolites in human urine. The assay only involves centrifugation and filtration of four times diluted urine, analysis was performed on an Agilent RRHD Eclipse XDB-C_{18} column (50 mm × 2.1 mm i.d., 1.8 µm, Agilent) using a gradient of 10 mM ammonium acetate, pH 6.8 and methanol as a mobile phase at a flow rate of 200 µL min^{-1}. Separated compounds were determined by atmospheric pressure chemical ionization (APCI) tandem mass spectrometry in the positive ion mode using MRM. The results showed that: recoveries for nicotine and nine of its major metabolites ranged from 93.2-103.8 %; RSD for all the compounds were between 0.9 and 9.5 %. The described method was suitable for determining the nicotine dose in large-scale human bio-monitoring studies.

Keywords: UHPLC-MS/MS, Nicotine, Metabolites, Human urine.

INTRODUCTION

Nicotine is a naturally occurring alkaloid found in many plants. However, the principal sources of nicotine expo-sure is through the use of tobacco, nicotine replacement therapy and exposure to environmental tobacco smoke. The absorbed nicotine is extensively metabolized. Nicotine is metabolized primarily by C-oxidation to cotinine (COT) and to a lesser extent by N-oxidation to nicotine-N'-oxide (NNO), nornicotine (NNIC) and nicotine-N-glucuronide (NIC-G). Cotinine is further metabolized by hydroxylation to *trans*-3'-hydroxy-cotinine (OHCOT), N-oxidation to cotinine-N-oxide (CNO), norcotinine (NCOT) and cotinine-N-glucuronide (COT-G). The compound *trans*-3'-hydroxycotinine is further metabolized by O-glucuronidation to *trans*-3'-hydroxy-cotinine-O-glucuronide (OHCOT-G)[1,2].

Nicotine and nicotine-derived metabolites in biological fluids as a biomarker of tobacco smoke and nicotine exposure is becoming more and more important in epidemiology and study of smoking and health. The determination of urinary nicotine and its nine metabolites accounts for more than 90 % of total nicotine metabolism[2,3], which provides a detailed view on the pharmacologic effects of nicotine exposure, smoking status in clinical research and the bioavailability of nicotine *via* various routes of administration.

Numerous methods include colorimetry[4], radioimmuno-assay[5,6], enzyme-linked immunoassay (ELISA)[5,7], GC[8,9] or GC-MS[10,11], GLC[12], HPLC[13,14] or LC-MS/MS[15-21] have been developed for analysis of nicotine and its metabolites in human fluids. Liquid chromatography coupled with tandem mass spectro-metry (LC-MS/MS) provides more comprehensive measurement of nicotine and its metabolites than other methods. However, each LC-MS/MS method may possesses some drawbacks, such as low through-put[15,16], instrument contamination from urine salts,[17] low sensitivity[17,19] or time-consuming procedure[18-21].

The present paper describes a rapid and very sensitive UHPLC-MS/MS method for the simultaneous analysis nicotine and its major metabolites (COT, OHCOT, NIC-G, COT-G and OHCOT-G) and four minor metabolites, NNO, CNO, NNIC and NCOT. Deuterium-labeled analogues of nicotine, COT and OHCOT were used as internal standards. The urine sample was four times diluted and centrifuged prior to UHPLC-MS/MS, which reduce matrix effect and instrument contamination from urine salts[22].

EXPERIMENTAL

Nicotine (NIC) and cotinine (COT) were purchased from Sigma-Aldrich (Poole, UK). OHCOT, NNIC, NCOT, NNO, CNO, NIC-G, COT-G, OHCOT-G, NIC-d_3 (nicotine-methyl-

d_3), COT-d_3 (cotinine-methyl-d_3) and OHCOT-d_3 (*trans*-3'-hydroxycotinine-methyl-d_3) were obtained from Toronto Research Chemicals, Ontario, Canada. The purity of all reference compounds commercially available wase ≥ 98 %. Ammonium acetate was supplied by Tedia Company Inc. (Fairfield, America). Methanol (HPLC grade) was purchased from J&T Baker (Phillipsburg, NJ, USA).

Preparation of stock solutions, calibration standard and quality control (QC) samples: Individual primary stock solutions of NIC (1 g/L), COT (1 g/L), OHCOT (1 g/L), NNO (1 g/L), CNO (1 g/L), NNIC (1 g/L), NCOT (1 g/L), NIC-G (1 g/L), COT-G (1 g/L) and OHCOT-G (0.1 g/L) were prepared in methanol, from which two mixed working solutions (1 and 10 μg/mL) were prepared in water. NIC-d_3 (1 g/L), COT-d_3 (1 g/L) and OHCOT-d_3 (0.1 g/L) were prepared in methanol, from which the mixed working solution of the internal standards (10 μg/mL) was prepared in water.

Calibrators (2.0, 5.0, 10.0, 50.0, 100.0, 500.0, 1000.0, 5000.0 and 8000.0 ng/mL) were freshly prepared by the addition of different aliquots of the working stock solution of the compounds to blank human urine. And blank urine was obtained from people not exposed to nicotine.

Quality control (QC) samples at three different concentrations (10.0, 150.0 and 1000.0 ng/mL for NIC, COT, OHCOT, NIC-G, COT-G and OHCOT-G and 10.0, 150.0 and 500.0 ng/mL for NNIC, NCOT, NNO and CNO) were also prepared with blank human urine.

Sample collection and preparation: Twenty-four hour urine samples from volunteers were stored at -40 ºC in glass tubes. Urine samples were thawed overnight at 4 ºC and thoroughly mixed. An aliquot of 250 μL urine sample was pipetted into separate polypropylene tubes, then 25 μL of the internal standards solution (10 μg/mL) and 725 μL water were added. The mixed samples were vortex-mixed for 2 min. After centrifugation at 14500 rpm for 10 min (Sigma, Germany), the supernatant was transferred into auto-sampler vials after filtering through a 0.22-μm syringe filter.

UHPLC-MS/MS conditions: Liquid chromatography (LC) was carried out using an Agilent 1290 infinity UHPLC system. UHPLC separations were performed with an Agilent RRHD Eclipse XDB-C_{18} column (50 mm × 2.1 mm i.d., 1.8 μm, Agilent) and the column temperature was designed to be 40 ºC. The elution was made in gradient mode with a mixture of (A) 10 mM ammonium acetate, pH 6.8 and (B) methanol programmed as follows: initial 88 % A maintained for 0.8 min, then decreased to 2 % A in 1.5 min, maintained at 2 % A for 1.5 min. The flow rate was 200 μL min^{-1}. The total time for chromatography was less than 3 min.

The UHPLC was connected to an API 4000 triple-stage quadrupole MS/MS system (Applied Biosystems, USA) operated in the positive APCI mode. The nebulizer heater was maintained at 600 ºC with the nebulizer current at 4 μA. Nitrogen was used as nebulizer, auxiliary and curtain gas at 50, 50 and 10 psi, respectively. Entrance potential (EP) and collision cell exit potential (CXP) were 10 and 9 V, respectively. Dwell time for each ion was 100 ms. The MRM parameters of the compounds are shown in Table-1.

RESULTS AND DISCUSSION

Mass spectrometry: The ionization of all compounds were studied in APCI positive and negative mode[22]. The positive ion mode was chosen because it appeared more selective and more sensitive. Each standard compound (including internal standards) was infused into the mass spectrometer in order to obtain a full scan of the compound. Under atmospheric pressure chemical ionization (APCI), all three glucuronides (NIC-G, COT-G and OHCOT-G), proved unstable even under moderate ionization conditions and fragmentation occurred by neutral loss of m/z 176, resulting in the aglycon ions $[M\text{-}Gluc+H]^+$ m/z 163.2, 176.9 and 193.2, respectively (Fig. 1). Full scan ion-spray mass spectra of other compound consisted of $[M + H]^+$ and $[M + Na]^+$ ions (data not shown), confirming the expected molecular mass of the analyte. The mass spectrometer parameters were adjusted to maximize the intensity of the $[M + H]^+$ or $[M\text{-}Gluc+H]^+$ ion, the declustering potential and collision energy were adjusted to optimize the signal for the most abundant product ions to obtain MRM transitions as sensitive as possible. The results of the optimized conditions for each MRM transition are summarized in Table-1. Two MRM transitions were chosen for each compound. The transition "precursor ion-product ion (P_1)" was used for quantification and the transition "precursor ion–product ion (P_2)" for confirmation (Table-1).

TABLE-1
MRM PARAMETERS AND RETENTION TIMES OF THE COMPOUNDS

Compounds	Precursor ion (m/z)	Product ion, P_1/P_2 (m/z)	Declustering potential (V)	Collision energy, E_1/E_2 (V)	RT (min)
NIC	163.2	130.1/131.9	30.0	27.0/27.0	2.76
NIC-G	163.2	130.1/131.9	30.0	27.0/27.0	0.92
COT	176.9	80.0/98.0	30.0	30.0/30.0	2.62
COT-G	176.9	80.0/98.0	30.0	30.0/30.0	0.51
OHCOT	193.2	79.8/134.1	40.0	32.0/32.0	2.02
OHCOT-G	193.2	79.8/134.1	40.0	32.0/32.0	0.83
NNIC	149.1	80.1/131.9	30.0	27.0/27.0	1.27
NCOT	163.0	80.0/118.1	32.0	32.0/33.0	2.53
NNO	178.9	119.9/131.8	30.0	25.0/27.0	1.21
CNO	192.9	96.0/98.1	30.0	28.0/30.0	0.86
NIC-d_3	165.9	86.9/80.0	35.0	28.0/28.0	2.73
COT-d_3	180.1	79.9/101.0	30.0	33.0/33.0	2.61
OHCOT-d_3	196.0	80.1/133.0	40.0	33.0/33.0	1.99

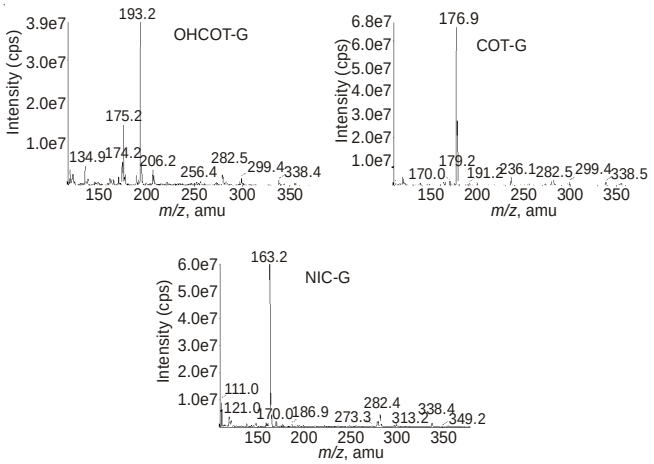

Fig. 1. Full scan mass spectras of NIC-G, COT-G and OHCOT-G

Chromatography: The chromatographic conditions for UHPLC method were optimized through several trials to achieve good resolution and symmetric peak shapes of compounds. The parameters that directly affect chromatographic separation such as mobile phase composition, gradients and flow rate were studied and optimized. The better chromatographic separation was achieved using a gradient of 10 mM ammonium acetate and methanol as the mobile phase. All compounds were eluted with a flow rate of 200 µL min^{-1} and a run time of 3 min per injection with good peak shapes. However, all compounds were not completely separated. We could separate and detect all compounds in the extracted ion chromatograms

Acording to the study, NCOT, which is isobaric to NIC and NIC-G, shows three signals at the ion transitions m/z 163.0 → m/z 80.0 at 0.92 min (NIC-G), 2.53 min (NCOT) and 2.76 min (NIC), where as NIC and NIC-G (m/z 163.2 → m/z 130.1) reveal no interference at 2.53 min (Fig. 2).

Recovery, matrix effects and precision: Recovery was calculated by comparing the absolute peak area for each analyte when spiked into urine before extraction (A) with the absolute peak area for each analyte spiked into the reconstitution solution following urine extraction (B). Matrix effects were also

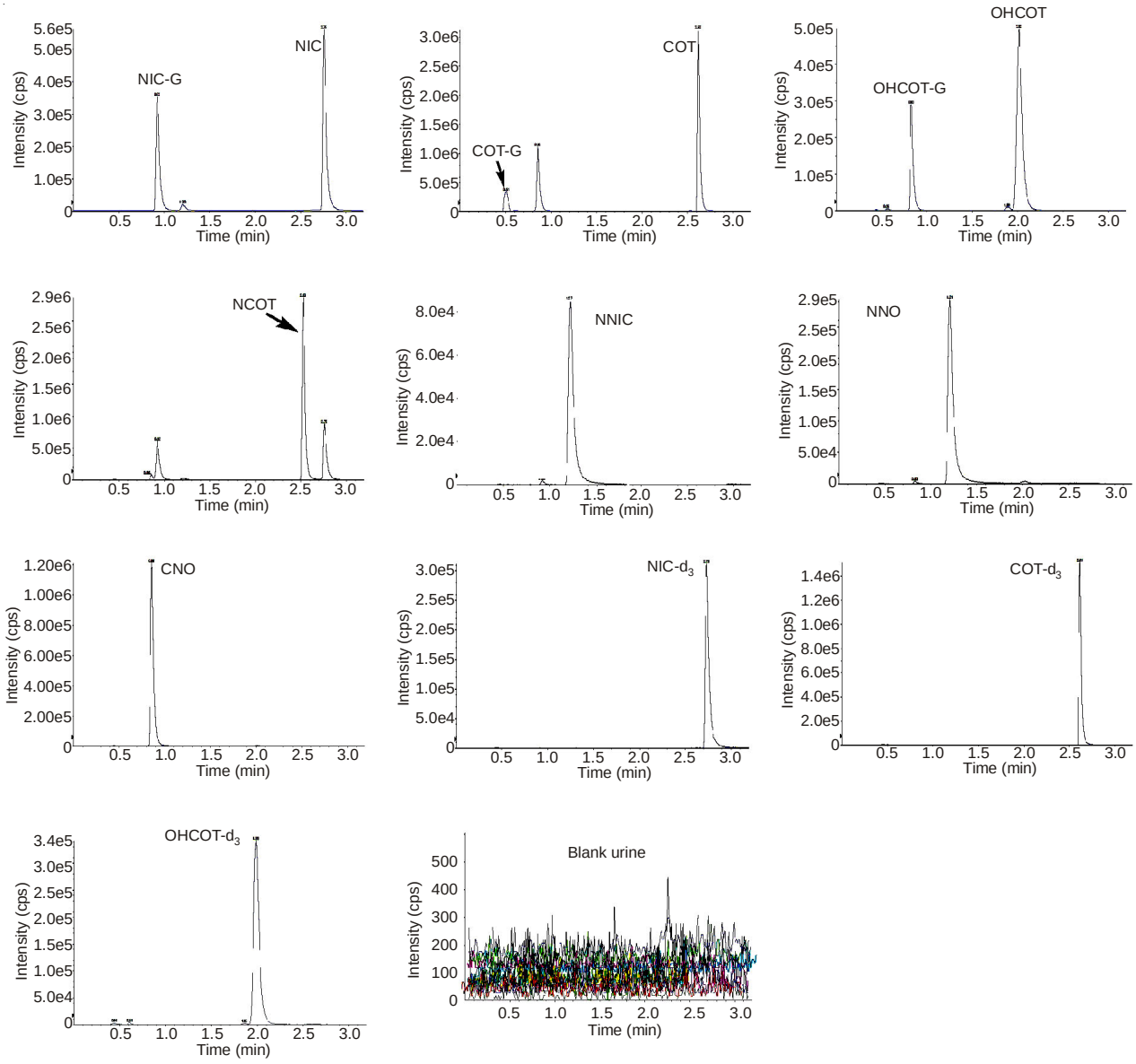

Fig. 2. Extract ion chromatograms of blank urine spiked with nicotine, nine nicotine metabolites and the internal standards (500 ng/mL)

examined by comparing the absolute peak area for compounds in neat solution (C) with compounds spiked after extraction (B) at the same concentrations. The calculation is as follows: recovery (RE) = A/B × 100 (%); matrix effect (ME) = B/C × 100 (%). Three levels of analyte concentrations were used to determine recovery and to study matrix effects. Recoveries for nicotine and nine of its major metabolites at three concentrations ranged from 93.2-103.8 %. The matrix effect value was less than 10.0 % at three concentrations for all compounds, which are listed in Table-2. Which indicate that a simple dilution of sample prior to analysis provided a better recovery than direct injection (urine filtration-only)[17,22].

Intra- and inter-day variations were chosen to determine the precision of the method. To determine intra-day precision of the assays, six replicates at three different quality control levels were analyzed. Inter-day precision were evaluated by analyzing six replicates at three different quality control levels on five different days. The overall intra- and inter-day precision (RSD) for the method ranged from 0.9-8.7 % and from 1.2-9.5 %, respectively (Table-2).

Calibration curve, linearity and limit of quantitation (LOQ): The calibration curves for ten compounds were analyzed by least-squares linear regression with no weighting. For NIC, COT, OHCOT and their glucuronides, we use the corresponding deuterated (methyl-d_3) free bases as internal standards for both the aglycon and the conjugate. Because isotope labeled NNO, CNO, NNIC and NOCT are not commercially available, we used COT-d_3 as the internal standard for these compounds. Correlation coefcients (r) of the calibration curves were greater than 0.998 for NNIC, CNO, NCOT and NNO in the range 2.0-4000.0 ng/mL, for COT, NIC-G, COT-G , NIC and OHCOT in the range 5.0-8000.0 ng/mL.

For all compounds, the limit of detection (LOD) defined as a signal-to-noise ratio of 3:1 was generally below 1.0 ng/mL. The limit of quantitation (LOQ) was defined as a signal-to-noise ratio of 10:1 and the calculated LOQs (ng/mL) for the compounds were as follows: NIC, 1.8; COT, 0.7; OHCOT, 2.2; NIC-G, 2.2; COT-G, 2.3; OHCOT-G, 3.4; NNIC, 0.3; NCOT, 1.6; NNO,1.6; CNO,1.3.

Stability: The stability of the compounds were verified by subjecting samples storage for 2 days at ambient temperature, 4 and -40 ºC prior to analysis. Quality control samples at three concentrations were utilized for this stability test. Analyte concentrations at three levels were within ± 8.0 % of expected values under three storage conditions, indicating stability for 48 h at room temperature (RT), 4 and -40 ºC. Quality control samples at three concentrations were also stable within a week at -40 ºC (RE within ± 7.1 % of expected values).

Application to urine samples: The described UHPLC-MS/MS method was applied to 24 h urine samples of ten smokers and five passive smokers. Smoking participants were instructed to continue smoking the same number of identical cigarettes within a week. Passive smokers were those exposed to ETS at home and at work. As shown in Table-3, nicotine uptake is determined mainly by individual smoking patterns and nicotine concentrations in body fluids vary widely among

TABLE-2
PRECISION, RECOVERY AND MATRIX EFFECT FOR THE COMPOUNDS AT DIFFERENT CONCENTRATIONS (n = 6)

Compounds	Concentrations (ng/mL)	Intra-day (RSD, %)	Inter-day (RSD, %)	RE (%)	ME (%)
NIC	1000.0	1.2	3.7	100.5	101.8
	150.0	2.6	2.0	101.2	100.5
	10.0	8.7	8.9	102.0	99.4
COT	1000.0	2.1	4.3	98.2	98.1
	150.0	3.2	5.8	98.7	96.7
	10.0	4.5	7.6	98.1	97.1
OHCOT	1000.0	1.4	2.0	101.8	100.5
	150.0	0.9	1.7	103.3	93.9
	10.0	5.4	5.2	94.3	95.3
NIC-G	1000.0	2.7	5.0	95.2	94.3
	150.0	1.8	2.8	95.7	96.1
	10.0	1.9	3.9	93.6	92.9
COT-G	1000.0	2.0	3.7	97.8	98.5
	150.0	3.6	5.2	99.1	97.8
	10.0	7.3	9.3	93.2	95.2
OHCOT-G	1000.0	1.8	1.4	103.8	99.9
	150.0	2.1	2.2	98.9	98.1
	10.0	5.1	3.8	95.2	97.4
NNIC	500.0	1.9	1.2	93.5	100.6
	150.0	1.8	2.2	94.2	103.6
	10.0	7.8	8.3	101.1	100.2
NCOT	500.0	2.8	2.1	97.2	97.3
	150.0	3.9	3.4	98.6	95.4
	10.0	6.9	9.5	100.7	95.7
CNO	500.0	2.3	7.2	96.7	94.4
	150.0	2.2	6.5	99.6	96.5
	10.0	6.5	5.5	96.5	97.7
NNO	500.0	3.4	3.1	95.3	94.8
	150.0	0.9	2.2	94.4	95.3
	10.0	3.7	6.1	101.5	96.2

TABLE-3
CONCENTRATIONS (ng/mL) OF NICOTINE AND NINE NICOTINE METABOLITES IN URINE OF TEN SMOKERS AND FIVE PASSIVE SMOKERS

Samples	NIC	COT	OHCOT	NIC-G	COT-G	OHCOT-G	NNIC	NCOT	NNO	CNO
1	238.0	300.1	452.0	58.8	47.2	30.1	14.4	28.2	41.2	30.6
2	324.8	910.2	3040.0	380.0	1648.0	664.0	45.6	102.8	552.0	332.6
3	736.0	701.5	1072.0	488.0	920.0	304.4	41.1	80.8	612.0	190.6
4	544.0	1092.2	1036.0	343.2	632.0	181.2	42.2	76.4	1076.0	114.6
5	228.0	114.9	164.0	62.0	101.6	70.4	16.2	23.1	59.6	42.3
6	464.0	839.2	2348.0	37.4	15.0	1020.0	36.4	75.6	288.0	229.2
7	836.0	1491.8	5840.0	420.0	1944.0	1776.0	50.0	102.4	420.0	500.0
8	1176.0	595.0	1428.0	254.8	784.0	338.8	48.6	53.6	375.6	159.5
9	480.0	1274.3	880.0	636.0	904.0	184.0	56.6	17.2	972.0	179.5
10	177.2	131.3	366.4	54.0	46.0	54.7	4.9	18.3	18.0	31.7
11	10.0	21.6	39.2	ND[a]	5.1	ND	3.4	ND	2.6	ND
12	28.0	19.2	40.8	ND	8.4	ND	2.5	ND	1.6	ND
13	18.4	18.7	55.2	ND	7.2	ND	9.3	ND	5.6	ND
14	7.4	10.3	20.6	ND	ND	ND	4.7	ND	ND	ND
15	21.2	11.1	34.3	ND	ND	ND	ND	ND	2.0	ND

[a]ND: Not detected.

individuals[23], wide inter-individual variation is observed even when smoking the same number of identical cigarettes. Among the passive smokers, NIC, COT and OHCOT were detected in all participant's urine sample, COT-G, NNIC and NNO were detected in some participant's urine sample.

Conclusion

The new developed UHPLC-MS/MS method allows the simultaneous determination of nicotine and its nine metabolites in human urine. The main advantages of this method are direct injection of diluted sample without preparative procedures, high sample throughput, low limits of detection, reliable linearity, precision and accuracy. The increased efficiency and ease of sample processivity make this method a key tool for its application to urine samples from smokers, passive smokers and people being treated with NRT, thereby providing a better estimate of nicotine exposure.

ACKNOWLEDGEMENTS

This research work was financially supported by the National Nature Science Foundation of China (21007094), the project supported by National Science and Technology (2012BAK01B03) and the Key Scientific and Technological Project of Guangxi Province (11194006).

REFERENCES

1. D. Yildiz, *Toxicon*, **43**, 619 (2004).
2. A.R. Triker, *Beitr. Tabakforsch Int.*, **22**, 147 (2006).
3. C. Boswell, M. Curvall, R.K. Elswick Jr and D. Leyden, *Biomarkers*, **5**, 341 (2000).
4. E. Bruckert, N. Jacob, L. Lamaire, J. Truffert, F. Percheron and J.L. de Gennes, *Clin. Chem.*, **38**, 1705 (1992).
5. J.J. Langone, H.B. Gjika and H. Van Vunakis, Use of Immunoassay Techniques for the Determination of Nicotine and its Metabolites, in eds.: J.W. Gorrod and P. Jacob III, Analytical Determination of Nicotine and Related Compounds and Their Metabolites, Elsevier, Amsterdam, pp. 265-283 (1999).
6. R. Heinrich-Ramm, R. Wegner, A.H. Garde and X. Baur, *Int. J. Hyg. Environ. Health*, **205**, 493 (2002).
7. R.J. Bjercke, G. Cook, N. Rychlik, H.B. Gjika, H. Van Vunakis and J.J. Langone, *J. Immunol. Methods*, **90**, 203 (1986).
8. P. Jacob III, M. Wilson and N.L. Benowitz, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **222**, 61 (1981).
9. M.S. Jaakkola, J. Ma, G. Yang, M.F. Chin, N.L. Benowitz, M. Ceraso and J.M. Samet, *Prev. Med.*, **36**, 282 (2003).
10. H. Chen, L. Yang, W. Yu and X. Yan, *Anal. Methods*, **4**, 863 (2012).
11. D.C.M. Bordin, M.N.R. Alves, O.G. Cabrices, E.G. de Campos and B.S. De Martinis, *J. Anal. Toxicol.*, **38**, 31 (2014).
12. C. Feyerabend and M.A.H. Russell, *J. Pharm. Pharmacol.*, **42**, 450 (1990).
13. O.A. Ghosheh, D. Browne, T. Rogers, J. de Leon, L.P. Dwoskin and P.A. Crooks, *J. Pharm. Biomed. Anal.*, **23**, 543 (2000).
14. M. Page-Sharp, T.W. Hale, L.P. Hackett, J.H. Kristensen and K.F. Ilett, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **796**, 173 (2003).
15. M.C. Bentley, M. Abrar, M. Kelk, J. Cook and K. Phillips, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **723**, 194 (1999).
16. T. Tuomi, T. Johnsson and K. Reijula, *Clin. Chem.*, **45**, 2164 (1999).
17. M. Meger, I. Meger-Kossien, A. Schuler-Metz, D. Janket and G. Scherer, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **778**, 251 (2002).
18. X. Xu, M.M. Iba and C.P. Weisel, *Clin. Chem.*, **50**, 2323 (2004).
19. D.L. Heavner, J.D. Richardson, W.T. Morgan and M.W. Ogden, *Biomed. Chromatogr.*, **19**, 312 (2005).
20. J. Kuhn, T. Vollmer, C. Martin, D. Hendig and C. Knabbe, *J. Pharm. Biomed. Anal.*, **67**, 137 (2012).
21. M. Piller, G. Gilch, G. Scherer and M. Scherer, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **951**, 7 (2014).
22. R. Dams, M.A. Huestis, W.E. Lambert and C.M. Murphy, *J. Am. Soc. Mass Spectrom.*, **14**, 1290 (2003).
23. F. Adlkofer, G. Scherer, A. Biber, W.D. Heller, P.N. Lee and H. Schievelbein, in eds.: N. Wald and P. Froggart, Consistency of Nicotine Intake in Smokers of Cigarettes with Varying Nicotine Yields, In: Nicotine, Smoking and the Low Tar Programme, Oxford University Press, Oxford, p. 130 (1989).