Medicine
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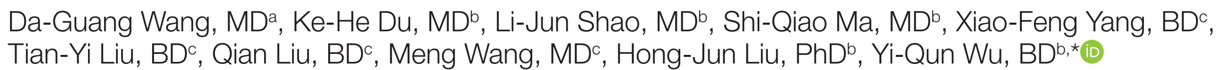
Determination of cotinine and 3-hydroxynicotinine in human serum by liquid chromatography-tandem mass spectrometry and its application



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Abstract

In this study, we developed a method for determining cotinine and 3-hydroxycotinine in human serum and established a methodology for an in-depth study of tobacco exposure and health. After the proteins in the human serum samples were precipitated with acetonitrile, they were separated on a ZORBAX SB-Phenyl column with a mobile phase of methanol encompassing 0.3% formic acid-water encompassing 0.15% formic acid. The measurement was performed on an API5500 triple quadrupole mass spectrometer in the multiple reaction monitoring mode. Cotinine, 3-hydroxycotinine, and cotinine-d_3 isotope internal standards were held for 2.56 minutes, 1.58 minutes, and 2.56 minutes, respectively. In serum, the linear range was 0.05 to 500 ng·mL^{-1} for cotinine and 0.50 to 1250 ng·mL^{-1} for 3-hydroxycotinine. The lower limit of quantification (LLOQ) was 0.05 ng·mL^{-1} and 0.5 ng·mL^{-1} for cotinine and 3-hydroxycotinine, respectively. The intra-day and inter-day relative standard deviations were <11%, and the relative errors were within ± 7%. Moreover, the mean extraction recoveries of cotinine and 3-hydroxycotinine were 98.54% and 100.24%, respectively. This method is suitable for the rapid determination of cotinine and 3-hydroxycotinine in human serum because of its rapidity, sensitivity, strong specificity, and high reproducibility. The detection of cotinine levels in human serum allows for the identification of the cutoff value, providing a basis for differentiation between smoking and nonsmoking populations.

Abbreviations: ESI = electrospray ionization, GS = Gas, LC-MS/MS = liquid chromatography-mass spectrometry/mass spectrometry, LLOQ = lower limit of quantification, QC = quality control, ROC = receiver operating characteristic curve.

Keywords: 3-hydroxycotinine, cotinine, cutoff value, human serum, liquid chromatography-tandem mass spectrometry

1. Introduction

The tobacco epidemic is one of the world greatest public health threats, causing more than 7 million deaths annually, among them more than 6 million are the result of direct tobacco use, while approximately 890,000 are nonsmokers who are exposed to secondhand smoke.[1] As a result, it is critical to accurately and reliably evaluate environmental tobacco smoke exposure. Due to its longer half-life and better stability than nicotine, cotinine, the main metabolite of nicotine in tobacco, is one of the best biomarkers for evaluating smoking exposure.[2,3] Currently, the methods for determination of cotinine and 3-hydroxycotinine in biological samples in China and overseas include gas chromatography,[4] high-performance liquid chromatography,[5] and liquid chromatography-tandem mass spectrometry (LC-MS/MS).[6–9] Unfortunately, these methods have certain limitations, such as the complicated sample treatment process,[6–8] the larger amount of serum,[7,8] the relatively long detection time, and low sensitivity. The goal of this study was to develop a highly efficient, sensitive, accurate, and specific LC-MS/MS method for the detection of cotinine and 3-hydroxycotinine levels in human serum. Additionally, due to the rapid metabolic rate, short half-life, difficult in vivo detection, and strict time limits of nicotine, the metabolites of nicotine, cotinine, and 3-hydroxycotinine, were chosen as the study objects, which are not only representative of the unique components in tobacco but also meet the detection requirements to ensure the detection rate of test results. Similarly, the component ratio in these 2 metabolites is a useful reference for analyzing addiction in smokers.

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The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

The clinical protocols used in this study were approved by the ethics committee of Beijing Hospital in Dec, 2013 (Approval number: 201302008), and the principles outlined in the Declaration of Helsinki were complied with. Informed consent was obtained from all study participants.

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Smoking is a major risk factor for various cardiovascular and respiratory diseases.[10] However, most smoking statuses are collected from questionnaires, and smokers conceal their actual smoking habits for various reasons,[11] leading to inaccuracies in assessment and underestimation of the actual prevalence. Therefore, using metabolites of tobacco-specific components as biomarkers to assess smoking status is a more accurate assessment method.

2. Instruments and materials

2.1. Instruments

An API5500 triple quadrupole mass spectrometer was purchased from Applied Biosystems (Carlsbad, CA), which was equipped with electrospray ionization (ESI) and Analyst 1.6 software. A Nexera XR Liquid Chromatograph was obtained from Shimadzu (Osaka, Japan), including an LC-20AD$_{XR}$ binary pump, a DGU-20A$_{3R}$ model degasser, a SIL-20AC$_{XR}$ autosampler, and a CBM-20A system controller. A high-speed VX-2400 Multi-tube Vortexer was purchased from VWR Scientific (Pittsburgh, PA). A high-speed GPR centrifuge was obtained from Beckman (Fullerton, CA).

2.2. Materials

Cotinine (99.8% purity) and cotinine-d$_3$ (99.3% purity) were purchased from Cerilliant (Round Rock, TX; FN051110-04 and FN06101501, respectively), and 3-Hydroxycotinine (98.0% purity) from Toronto Research Chemicals (Toronto, Ontario, Canada; H924500). Methanol and acetonitrile (Burdick & Jackson, Muskegon, MI) were chromatographically pure. The water was ultrapure (prepared by a Milli-Q water purification system). All other reagents were analytically pure. The human serum used as a blank control was obtained from healthy volunteers without a history of smoking.

3. Methods

3.1. Chromatographic and mass spectrometric conditions

A Zorbax SB-C$_{18}$-Phenyl Rapid Resolution column (75 mm × 4.6 mm, 3.5 μm, Agilent, Palo Alto, CA) was used as the chromatographic column. The mobile phase contained methanol (containing 0.3% formic acid) (B)-water (containing 0.15% formic acid) (A). The elution method was gradient elution. The gradient parameters were as follows: 0.01 to 1.00 minutes, 15% B; 1.00 to 1.01 minutes, 75% B; 1.01 to 3.00 minutes, 98% B; 3.00 to 4.01 minutes, 15% B. The flow rate was 0.30 mL·min^{-1}. The injection volume was 10 μL and the column temperature was room temperature.

The mass spectrometric conditions were as follows: ion source—ESI; ion injection voltage—5500 V; temperature—550°C; pressure of gas 1 (GS1, N_2) in the source—65 psi; pressure of gas 2 (GS2, N_2)—65 psi; pressure of curtain gas (high-purity N_2)—20 psi; detection—positive ionization method; scanning—multiple reaction monitoring mode; ion pairs used for quantitative analysis—*m/z* 177.2 → *m/z* 80.1 (cotinine), *m/z* 193.1 → *m/z* 80.1 (3-hydroxycotinine), and *m/z* 180.1 → *m/z* 80.1 (cotinine-d$_3$, internal standards); declustering potential voltage—64 V; collision energy—25 eV, 30 eV, and 30 eV; collision gas (collision-activated dissociation, N_2) pressure—5 psi; Q1 and Q3—UNIT.

3.2. Preparation of standard solutions, working solutions for the standard curve, and quality control (QC) samples

Standard stock solutions were prepared as follows: 0.50 mL primary cotinine stock solutions were precisely obtained and 50% methanol-water was added to prepare secondary cotinine stock solutions with a concentration of 100 μg·mL^{-1}, which were stored in a −20°C refrigerator for further use.

To prepare primary standard stock solutions, the 3-hydroxycotinine standards (5.23 mg) were precisely collected in a 5 mL volumetric flask and diluted with 50% methanol-water to the scale. Then, using 50% methanol-water, an appropriate amount of primary stock solution was converted to secondary standard stock solution with a concentration of 100 μg·mL^{-1} and stored in a −20°C refrigerator for subsequent use.

Preparation of QC solutions was as follows: For preparing the QC samples of the serum matrix, an appropriate amount of cotinine stock solution was collected, and cotinine was diluted with 50% methanol-water into the QC standard working solutions with concentrations of 20, 1000, and 25000 ng·mL^{-1}.

The following procedures were used to prepare the internal standard solutions: 0.05 mL cotinine -d$_3$ standard stock solution was accurately collected and prepared with 50% acetonitrile aqueous solution to 50 ng·mL^{-1} internal standard working solution, which was stored in a 4°C refrigerator for subsequent use.

Working solutions for the standard curves were prepared. For the standard curve, appropriate amounts of cotinine or 3-hydroxycotinine stock solutions were diluted with 50% methanol-water into 0.5, 5, 10, 50, 100, 250, 1250, 2500, and 5000 ng·mL^{-1} or 5, 10, 50, 100, 250, 1250, 6250, and 12500 ng·mL^{-1}, respectively.

QC samples were prepared. In detail, QC samples containing cotinine or 3-hydroxycotinine at concentrations of 0.20, 10.0, and 250 ng·mL^{-1} or 10, 125, and 625 ng·mL^{-1}, respectively, were prepared using a blank serum.

3.3. Serum treatment

Serum (100 μL) was added with 100 μL internal standard solutions and 500 μL acetonitrile, vortexed and shaken for 1 minute, and centrifuged at 13000 rpm for 3 minutes. Following that, 600 μL supernatant was transferred to a 2.0 mL plastic centrifuge tube and dried with nitrogen in a water bath at 80°C. The residues were re-dissolved with a 150 μL mobile phase and centrifuged at 13000 rpm for 3 minutes. Then, 10 μL samples were used for LC-MS/MS analysis.

4. Results

4.1. Standard curve and lower limit of quantification (LLOQ)

Blank serum (100 μL) was mixed with 10 μL serial standard solutions of cotinine and 3-hydroxycotinine, 100 μL internal standard solutions, and 490 μL acetonitrile (precipitant). Additionally, the rest of the procedures were performed according to the procedures explained in section "2.3 Serum treatment." The working standard curve was obtained by regression using the method of weighted ($W = 1/x^2$) least squares with the concentration of the analyte in the serum as the horizontal coordinate and the ratio of the analyte peak area to that of the internal standard as the vertical coordinate. A working standard curve was established for each analytical batch, and 5 analytical batches were measured consecutively at 3-day intervals. The results are listed in Tables 1 and 2, and the working curves are shown in Figures 1 and 2. The linear ranges for cotinine and 3-hydroxycotinine in the serum were 0.05 to 500 ng·mL^{-1} and 0.50 to 1250 ng·mL^{-1}, respectively.

The LLOQ samples with 0.05 ng·mL^{-1} and 0.50 ng·mL^{-1} cotinine and 3-hydroxycotinine, respectively, were processed as per the procedures explained in section "2.3 Serum treatment." The concentrations of the LLOQ samples were calculated using the working curve of the same analytical batch, from which the relative error of the LLOQ samples was calculated. The LLOQ for cotinine was 0.05 ng·mL^{-1} and 0.50 ng·mL^{-1}, both of which were within ± 15%, thus meeting the requirements of quantitative detection.

Table 1

Standard curve results of cotinine detection in human serum.

Batch number	Cotinine concentrations for standard curves (ng/mL)								
1	0.049	0.561	1.005	4.884	9.565	25.064	130.324	235.333	482.317
2	0.050	0.514	1.108	4.976	9.266	24.003	128.350	247.390	487.056
3	0.050	0.552	0.958	4.927	10.187	25.057	117.414	257.068	486.356
4	0.051	0.462	0.867	4.821	11.160	26.018	120.330	270.050	515.420
5	0.050	0.495	0.957	4.377	10.224	26.256	129.883	253.556	503.536
Mean	0.050	0.517	0.979	4.797	10.080	25.280	125.260	252.679	494.937
SD	0.001	0.041	0.088	0.242	0.729	0.898	5.967	12.754	14.040
RSD%	1.21	7.88	8.97	5.04	7.23	3.55	4.76	5.05	2.84
Mean %RE	–0.12	3.37	–2.11	–4.06	0.80	1.12	0.21	1.07	–1.01
N	5	5	5	5	5	5	5	5	5

RSD = relative standard deviation.

Table 2

Standard curve results of 3-hydroxynicotinine detection in human serum.

	3-hydroxynicotinine concentrations for standard curves (ng/mL)							
Batch number	0.5	1	5	10	25	125	625	1250
1	0.542	0.857	4.576	9.246	24.683	136.320	650.300	1376.693
2	0.530	0.897	4.904	9.201	22.925	126.603	686.601	1393.812
3	0.532	0.881	4.861	9.759	24.265	117.814	696.118	1349.997
4	0.481	1.086	4.823	10.204	23.038	110.690	689.970	1320.500
5	0.506	1.004	4.480	9.542	23.391	122.583	667.729	1436.411
Mean	0.518	0.945	4.729	9.590	23.660	122.802	678.144	1375.483
SD	0.025	0.097	0.189	0.412	0.777	9.602	18.830	43.938
RSD%	4.78	10.26	3.99	4.29	3.28	7.82	2.78	3.19
Mean%RE	3.63	–5.49	–5.42	–4.10	–5.36	–1.76	8.50	10.04
N	5	5	5	5	5	5	5	5

RSD = relative standard deviation, SD = standard deviation.

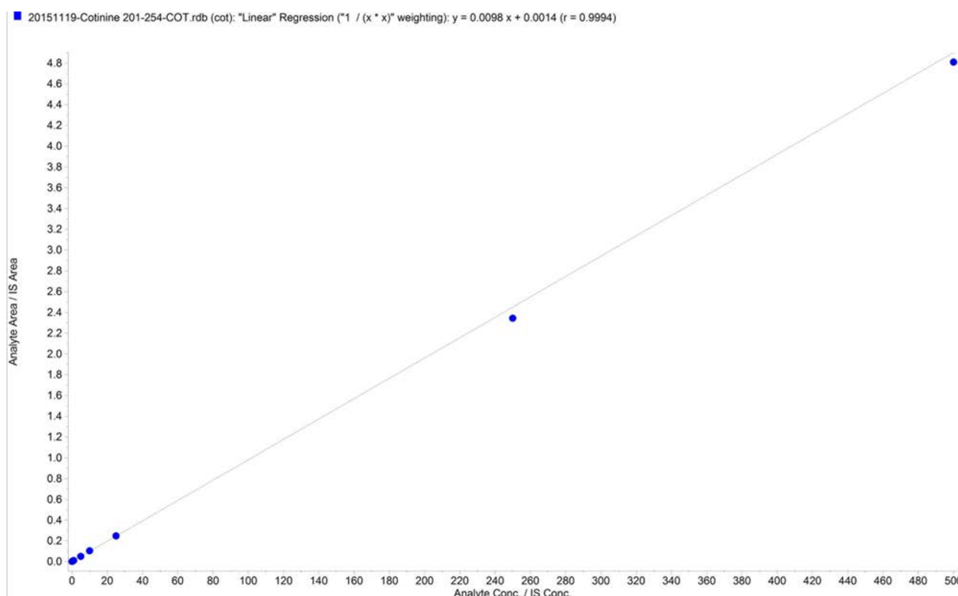

Figure 1. Standard curve of cotinine content in human serum.

4.2. Accuracy and precision

The QC samples with low, medium, and high concentrations of cotinine and 3-hydroxycotinine were treated as per the procedures explained in section "2.3 Serum treatment," and their concentrations were calculated using the working curve of the same analytical batch. The results are displayed in Tables 3 and 4. The REs were all within ± 15%, and the intra-batch and inter-batch relative standard deviations were all <15%.

4.3. Extraction recoveries

The QC samples with low, medium, and high concentrations of cotinine and 3-hydroxycotinine were processed as per the procedures explained in section "2.3 Serum treatment." After centrifugation, 600 μL supernatant was transferred into a 2.0 mL clean plastic centrifuge tube, supplemented with 20 μL of 50% methanol-water, 100 μL of 50% acetonitrile-water, and 30 μL of mobile phase, and vortexed. After that, 10

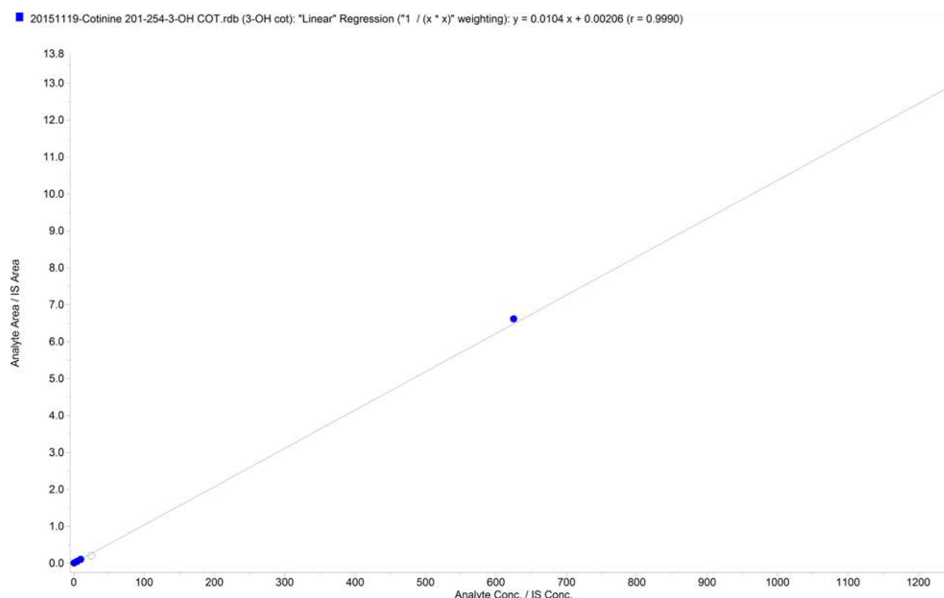

Figure 2. Standard curve of 3-hydroxycotinine content in human serum.

Table 3

Accuracy and precision results of the LC-MS/MS method in the determination of cotinine in human serum.

Theoretical concentration/ ($ng \cdot mL^{-1}$)	Measurement concentration/ ($ng \cdot mL^{-1}$)	RE/%	Intra-day RSD/%	Inter-day RSD/%
0.20	0.20 ± 0.01	−0.23	10.94	7.35
10.0	10.15 ± 0.30	1.51	2.33	2.99
250	252.49 ± 6.91	1.00	2.30	2.73

RSD = relative standard deviation.

Table 4

Accuracy and precision results of the LC-MS/MS method in the determination of 3-hydroxynicotinine in human serum.

Theoretical concentration/ ($ng \cdot mL^{-1}$)	Measurement concentration/ ($ng \cdot mL^{-1}$)	RE/%	Intra-day RSD/%	Inter-day RSD/%
10.0	10.17 ± 0.41	1.74	3.46	4.04
125.0	132.74 ± 7.22	6.19	4.87	5.44
625.0	656.34 ± 19.29	5.01	2.80	2.94

RSD = relative standard deviation.

μL samples were collected in an injection vial for LC-MS/MS analysis, and the peak areas of cotinine and 3-hydroxycotinine were calculated. At the same time, 100 μL of blank serum was obtained and treated as per the procedures explained in section "2.3 Serum treatment," and the internal standard solution was replaced by an equal volume of 50% acetonitrile-water. After centrifugation, 600 μL supernatant was collected in another 2.0 mL clean plastic centrifuge tube and mixed with 10 μL cotinine and 3-hydroxycotinine at corresponding concentrations, 100 μL internal standard solutions, and 30 μL mobile phase. After vortexing, 5 μL samples were transferred into injection vials for LC-MS/MS analysis, and cotinine and 3-hydroxycotinine peak areas were obtained. The extraction recoveries were calculated by dividing the average peak areas of cotinine and 3-hydroxycotinine obtained from the former treatment method by the average peak areas obtained from the latter treatment method for each concentration. Cotinine extraction recoveries were 102.10%, 91.82%, and 101.71% for low, medium, and high concentrations of serum samples, respectively, with the mean extraction recoveries of 98.54%. Furthermore, the extraction recoveries of 3-hydroxycotinine were 97.09%, 106.35%, and 97.29%, respectively, with a mean extraction recovery of 100.24%.

4.4. Specificity

Blank serum (100 μL) was obtained, and the internal standard solutions were replaced by an equal volume of 50% acetonitrile-water. Then, Figures 3 and 4 were obtained according to the procedures described in section "2.3 Serum treatment." Cotinine, 3-hydroxycotinine, and cotinine-d_3 internal standards were added to the blank serum samples, and Figures 5 to 8 were acquired following the same procedures. Subsequently, Figures 9 and 10 were obtained with the actual serum samples, following the same procedures. Results revealed that the endogenous substances in the blank serum did not interfere with the determination of cotinine, 3-hydroxycotinine, and internal standards, indicating that this method has high specificity.

5. Application of the detection method to the measurement of clinical samples

5.1. Participant information

The participants who met the following criteria were included: individuals aged 60 years or older, regardless of gender; individuals without ketosis or other stressful conditions in the last 6 months; individuals not participating in drug or clinical trials in the last 6 months.

Those fulfilling the following criteria were excluded: individuals with poor liver and kidney function (blood creatinine levels > 1.2 times the upper limit of normal and alanine transaminase levels > 2 times the upper limit of normal); pregnant or lactating women; individuals with progressive fatal diseases; individuals with a history of alcohol or drug abuse; individuals being treated for psychiatric disease; patients being treated with chemotherapy or radiotherapy for tumors; patients suffering from acute and chronic infectious diseases.

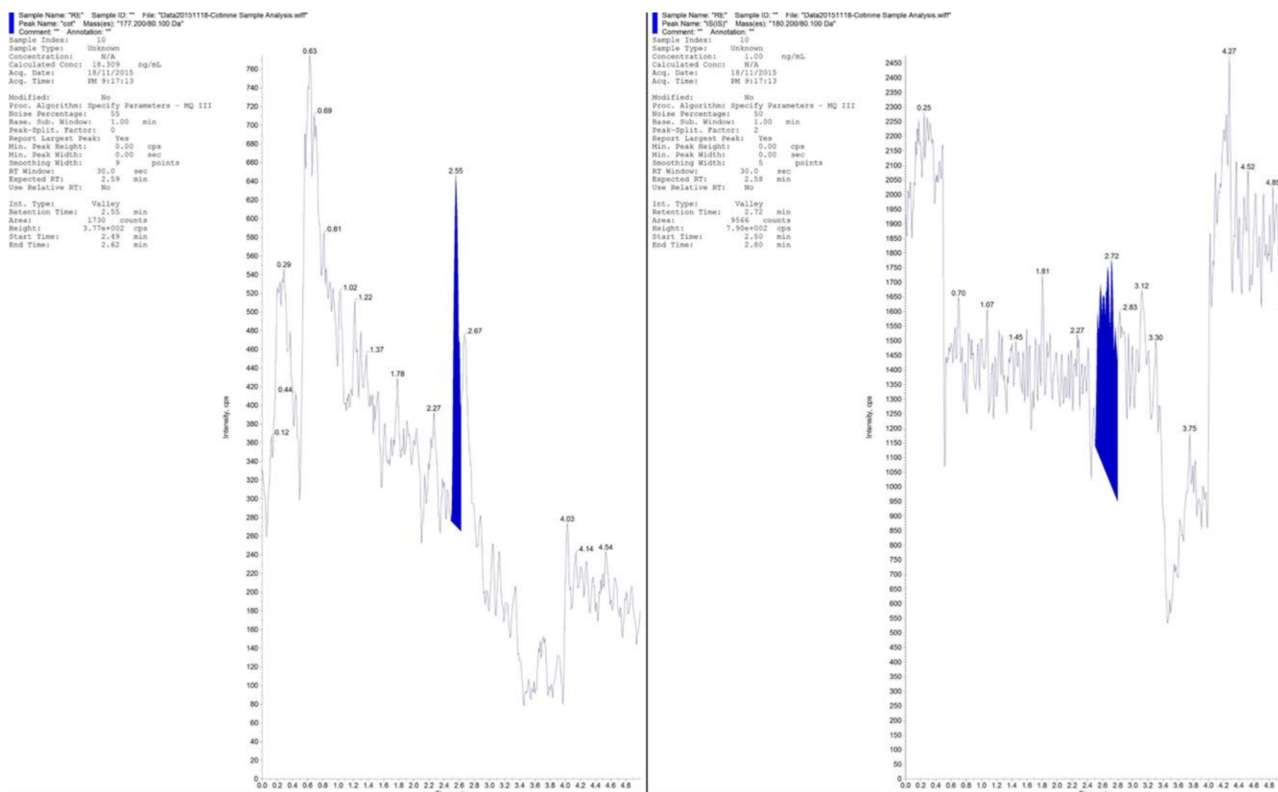

Figure 3. Chromatogram mass spectra of cotinine and internal standards in blank human serum.

A total of 785 individuals participated in the matching questionnaire, including 140 smokers and 645 nonsmokers, with a smoking rate of 17.8%. Furthermore, there were 433 males, among which 133 were smokers and 300 were nonsmokers, with a smoking rate of 30.7%, and 352 females, of whom 7 were smokers and 345 were nonsmokers, with a smoking rate of 1.99%.

5.2. Collection of specimens and relevant information

Fasting venous blood was collected from each participant twice at the start and end of the study, for a total of 20 mL each time. Following that, the serum was separated from the blood, frozen at −80°C, and transported on dry ice. Meanwhile, physical examinations such as height, weight, blood pressure, and abdominal circumference, as well as lifestyle questionnaires, were performed following a uniform method.

5.3. Determination of cotinine and 3-hydroxynicotinine content

The developed LC-MS/MS method was used to assess the clinical samples, and the results were analyzed. Cotinine and 3-hydroxycotinine levels were measured in 785 random clinical serum samples provided by the biological sample bank of Beijing Hospital (Table 5).

5.4. Calculation of cotinine content and cutoff values

As cotinine is an optimal biological indicator for assessing smoking exposure levels, measuring its levels can provide a more accurate assessment of current smoking status and smoking-related health hazards. The smoking status was used to create the receiver-operating characteristic curves (Fig. 11). Furthermore, Youden index analysis revealed that the optimal cutoff value for all participants was 2.170 ng/mL (sensitivity, 88.6%; specificity, 92.1%), 2.125 ng/mL for men (sensitivity, 89.5%; specificity, 88.3%), and 52.655 ng/mL for women (sensitivity, 71.4%; specificity, 98.3%).

With the optimal cutoff value as a criterion for determining whether or not the participants smoke, the smoking rate was 22.3% for all participants (610 participants with cotinine levels below 2.170 ng/mL), 35.6% for men (279 men with cotinine levels below 2.125 ng/mL), and 3.31% for women (340 women with cotinine levels below 52.655 ng/mL), which was higher than that calculated based on the questionnaire, which may be due to underreporting or prolonged exposure to secondhand smoke.

Additionally, the number of smoking women who participated in this study was relatively small but was statistically significant (n ≥ 5). Furthermore, the statistical results revealed certain issues, indicating that the statistical method used in this study is feasible. However, since more systematic research requirements are needed, the sample size needs to be expanded in future studies to obtain more objective and comprehensive statistical results.

6. Discussion

In this study, an LC-MS/MS method with high selectivity, sensitivity, and throughput for the determination of cotinine in human serum was developed. The treatment method prior to protein precipitation is simple and fast, and the optimized chromatographic conditions significantly reduce the detection time, with only 5 minutes of analysis time per sample, improving the detection efficiency. Furthermore, this method uses only 100 μL serum, and the lowest LLOQ of cotinine is 0.05 ng/mL, which is a significant improvement compared with previously reported detection methods. Notably, the increased sensitivity greatly elevates the detection rate of cotinine in clinical serum samples, allowing cotinine and 3-hydroxycotinine to be more effectively used as effective markers for determining tobacco exposure.

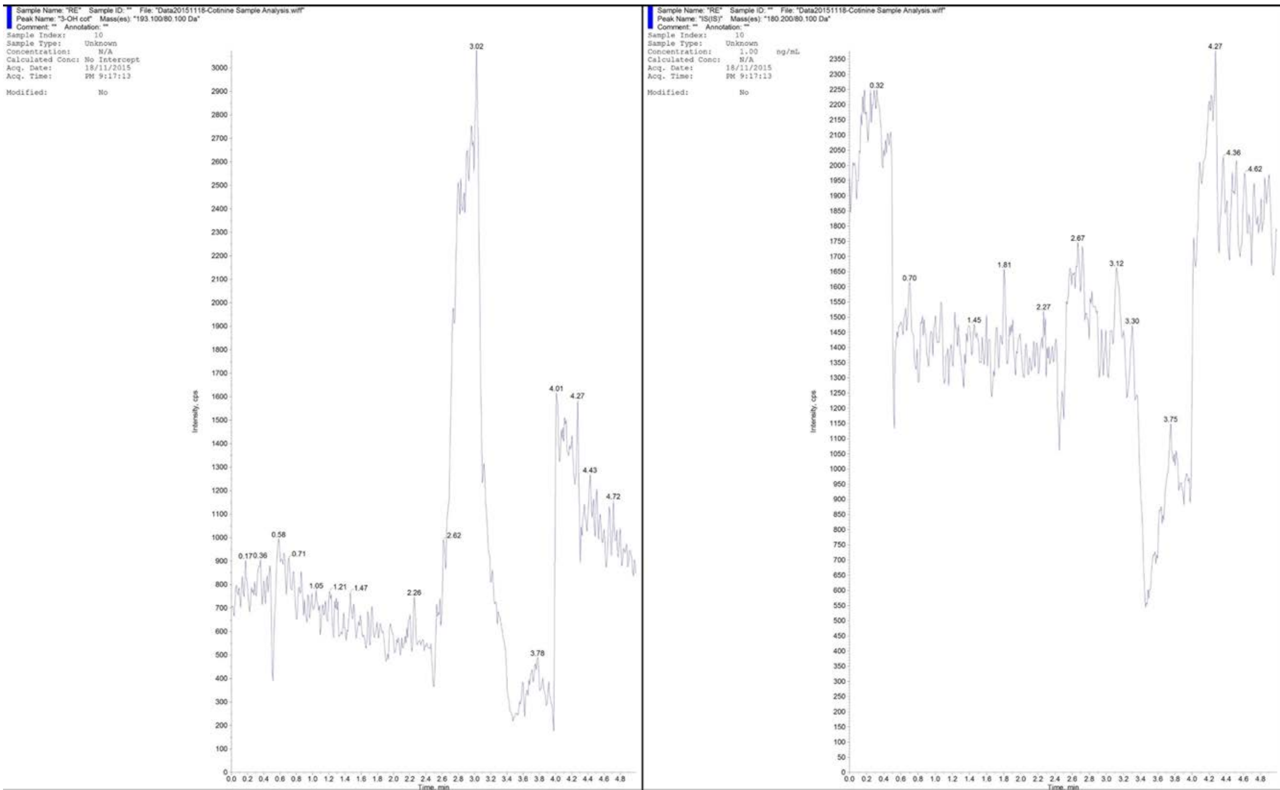

Figure 4. Chromatogram mass spectra of 3-hydroxycotinine and internal standards in blank human serum.

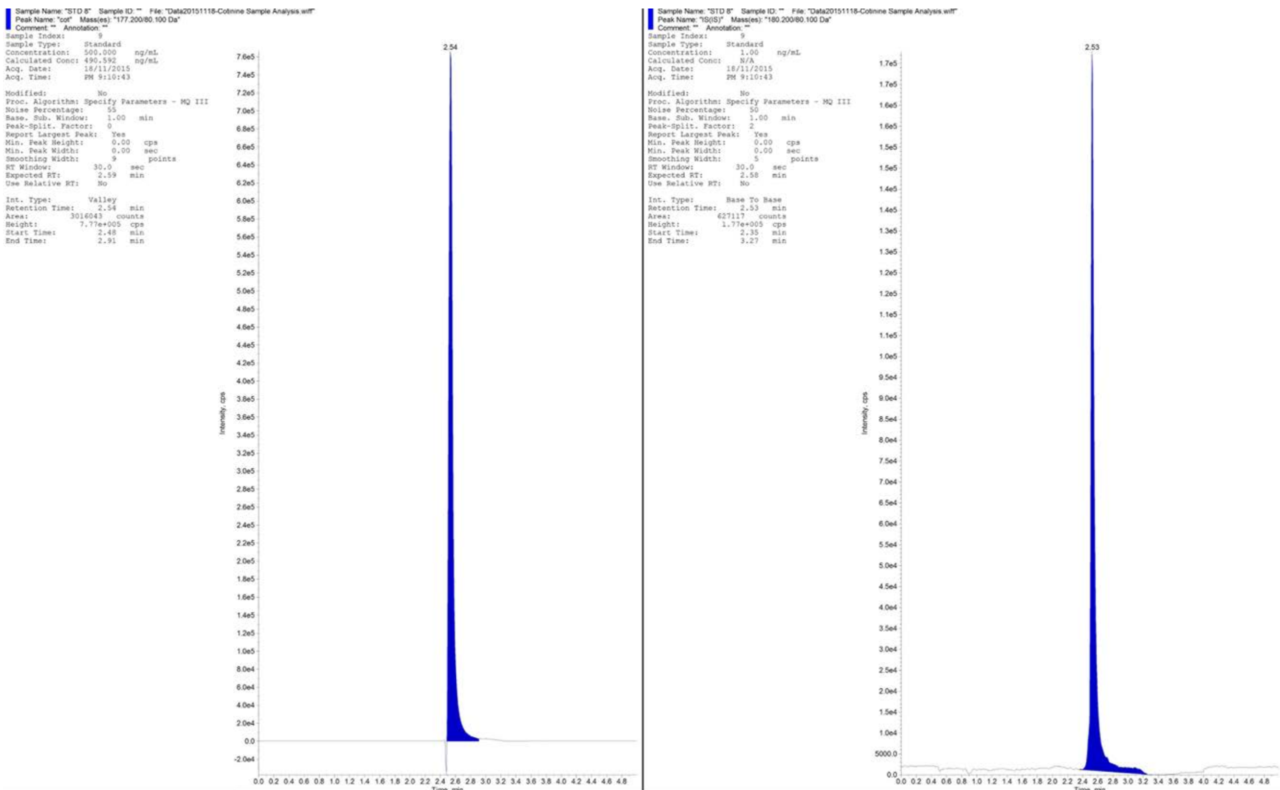

Figure 5. Chromatographic mass spectra of blank serum samples added with cotinine standard solutions (highest concentration on the marker) and internal standard solutions.

Cotinine and 3-hydroxycotinine are secondary metabolites of nicotine, a unique component of tobacco, in the human body and have advantages over nicotine in terms of favorable specificity, long half-life, and good stability. International studies researching exposure to smoking have also used cotinine or 3-hydroxycotinine content in serum samples to

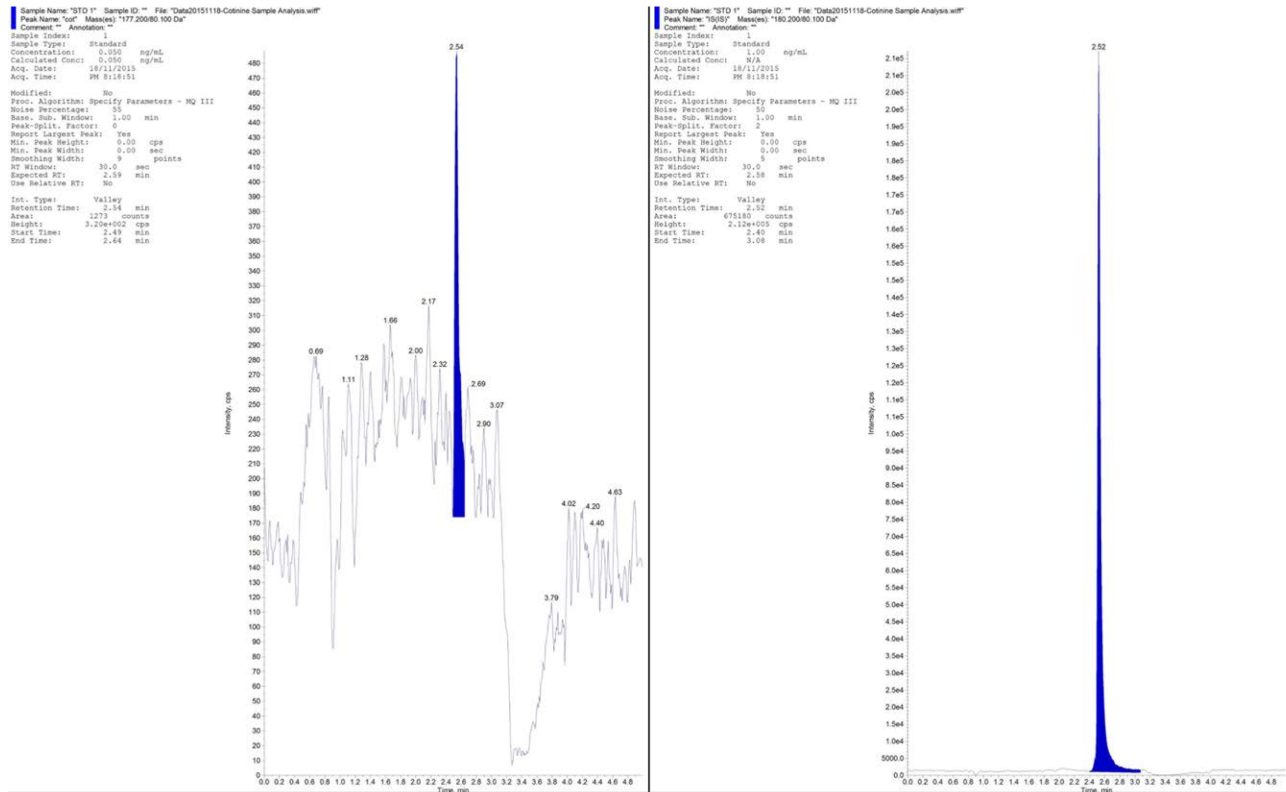

Figure 6. Chromatographic mass spectra of blank serum samples added with cotinine standard solutions (lower limit of quantification concentration) and internal standard solutions.

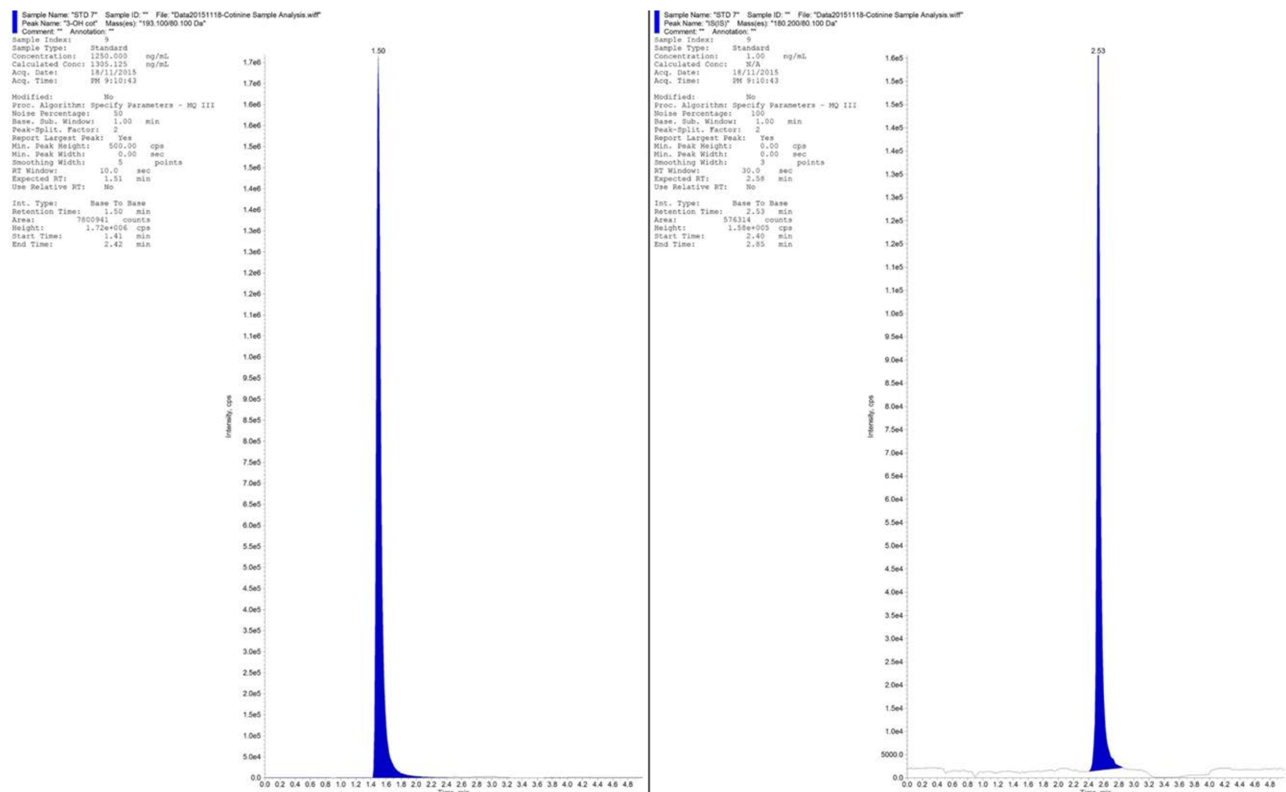

Figure 7. Chromatographic mass spectra of blank serum samples added with 3-hydroxycotinine standard solutions (highest concentration on the marker) and internal standard solutions.

determine the cutoff values for smokers and nonsmokers. In this study, smokers and nonsmokers among the 785 participants were examined, and the results of the questionnaire and the detection cutoff values for the overall, male, and female respondents were analyzed to plot ROC curves and calculate sensitivity coefficients and specificity, which revealed

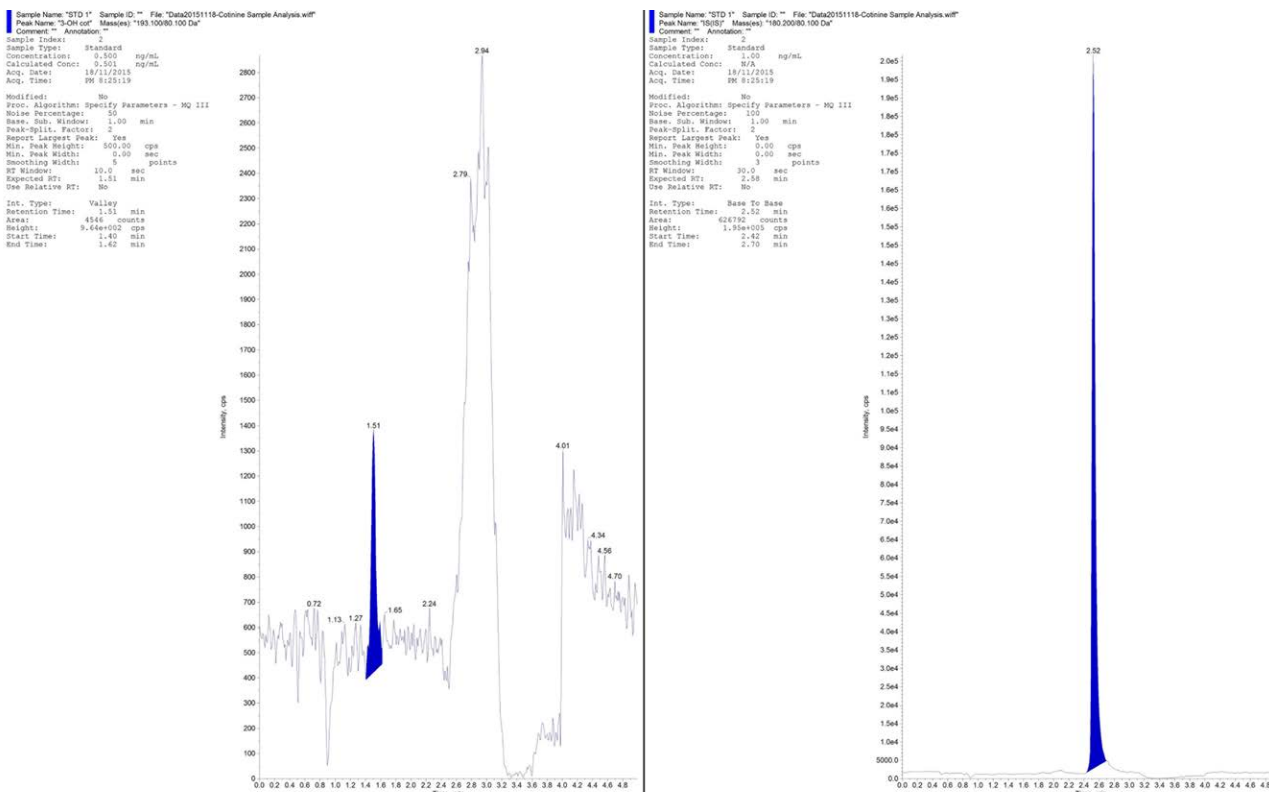

Figure 8. Chromatographic mass spectra of blank serum samples added with 3-hydroxycotinine standard solutions (lower limit of quantification concentration) and internal standard solutions.

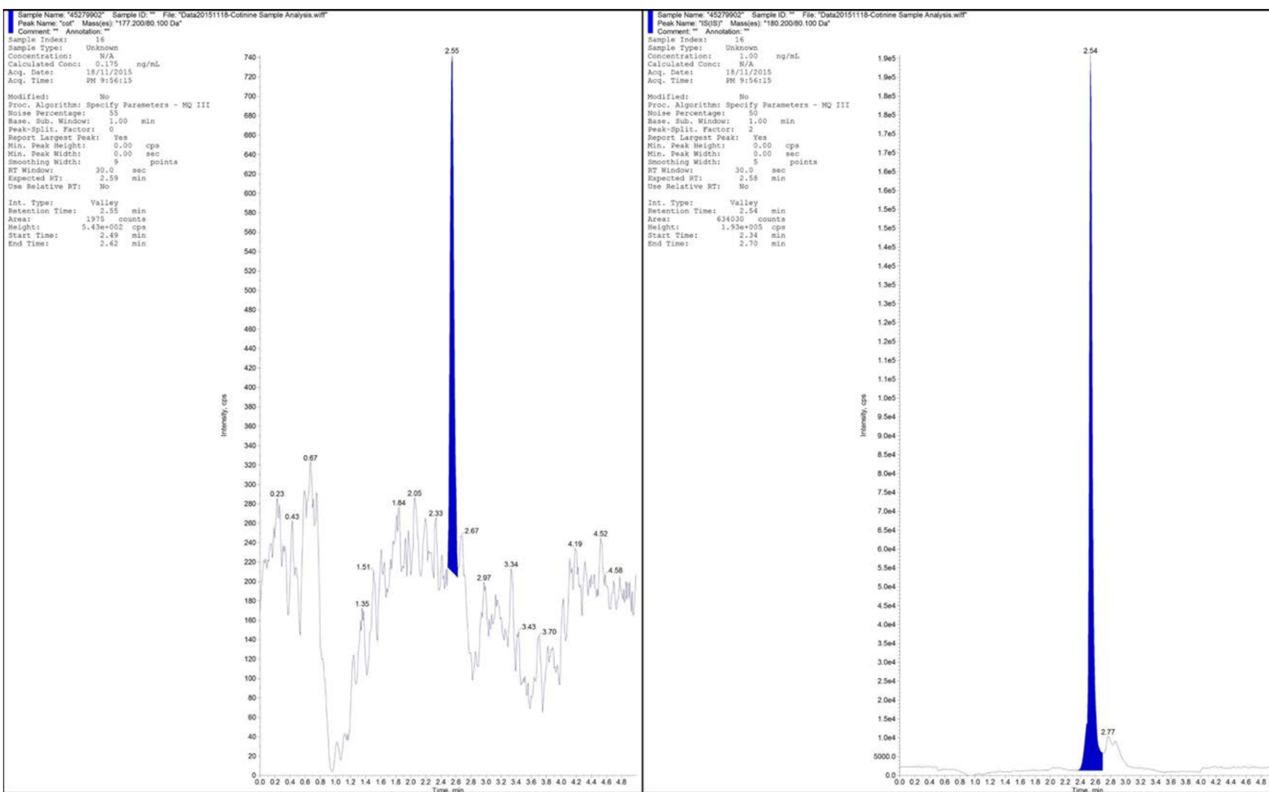

Figure 9. Chromatographic mass spectra of cotinine and internal standards in serum samples.

relatively consistent analytical results and identified the optimal cutoff values for smokers and nonsmokers, providing a basis for accurate determination of smoking status and prediction of smoking-related diseases. However, with respect to detailed values of sensitivity and specificity, female data had the lowest sensitivity (71.4%) and the highest specificity

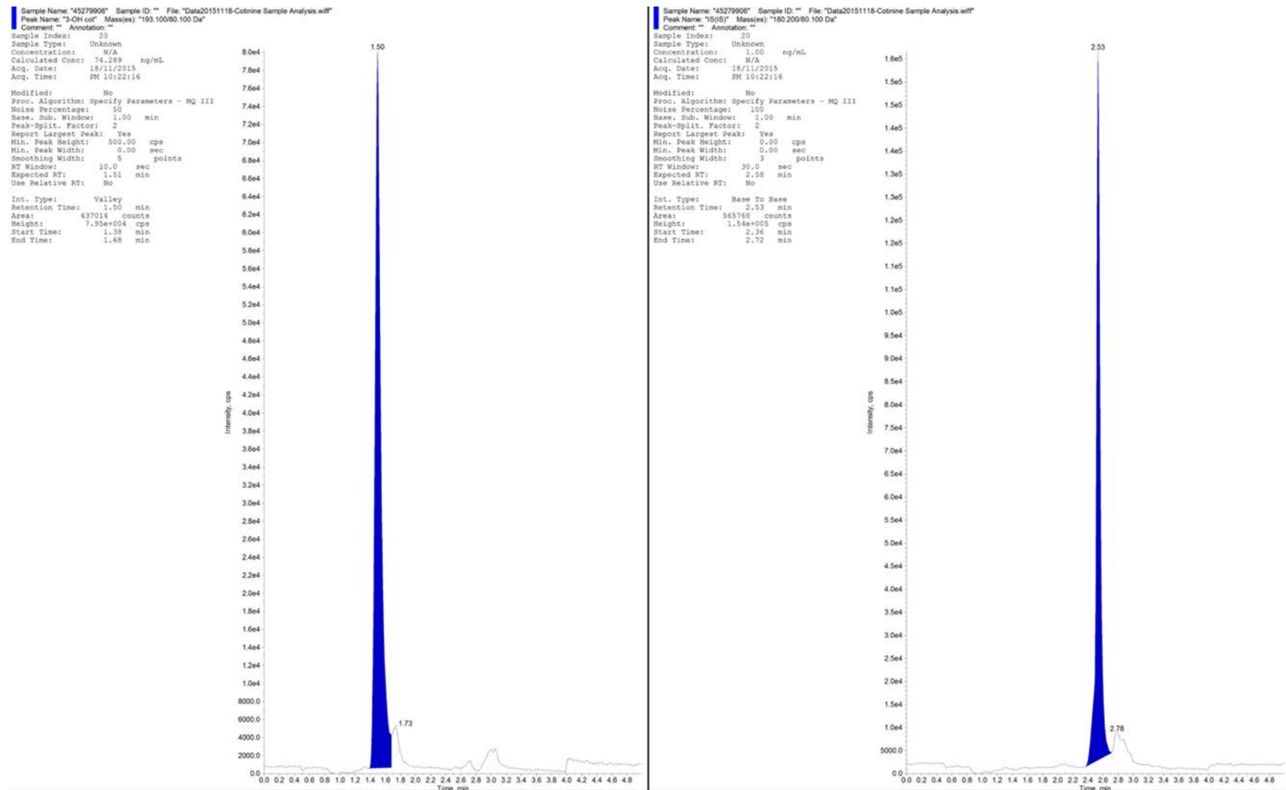

Figure 10. Chromatographic mass spectra of 3-hydroxycotinine and internal standards in serum samples.

Table 5

Statistics of cotinine and 3-hydroxycotinine detection results.

Cotinine	Concentration range ng/mL	(0,0.05)	(0.05,2.5]	(2.5,5]	(5,50]	(50,100]	(100,1000]
	Number of participants	9	605	14	47	22	88
3-hydroxycotinine	Concentration range ng/mL	(0,0.5)	(0.5,1)	(2.5,5)	(5,10)	(50,100)	(100,1000)
	Number of participants	560	80	10	78	34	23

(reaching 98.3%). These statistics suggest a certain percentage of underreporting or concealment among female smokers and the highest veracity for female smokers. This phenomenon may be related to the traditional views in China, where some female smokers do not want to be exposed as smokers to others.

Furthermore, more research is required in the future. The ratio of 3-hydroxycotinine to cotinine can be used as a basis for determining the addiction of smokers. If the health status and disease occurrence of the participants in this study, as well as the disease status of smokers can be followed up and analyzed retrospectively, addiction analysis based on the ratio of the measurement results can be conducted, further investigating the correlation between the number of cigarettes smoked and the development of diseases.

7. Conclusion

The LC-MS/MS method established in this study for the determination of cotinine and 3-hydroxycotinine in human serum has the advantages of high sensitivity, specificity, as well as a wide linearity range of standard curves. It can provide accurate and reliable detection data and lay a solid foundation for the studies of tobacco exposure and related hazards. This method is extremely promising for in-depth study and the assessment of tobacco hazards in different populations, as well as the study of tobacco-related diseases.

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Author contributions

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Funding acquisition: Hong-Jun Liu, Yi-Qun Wu.
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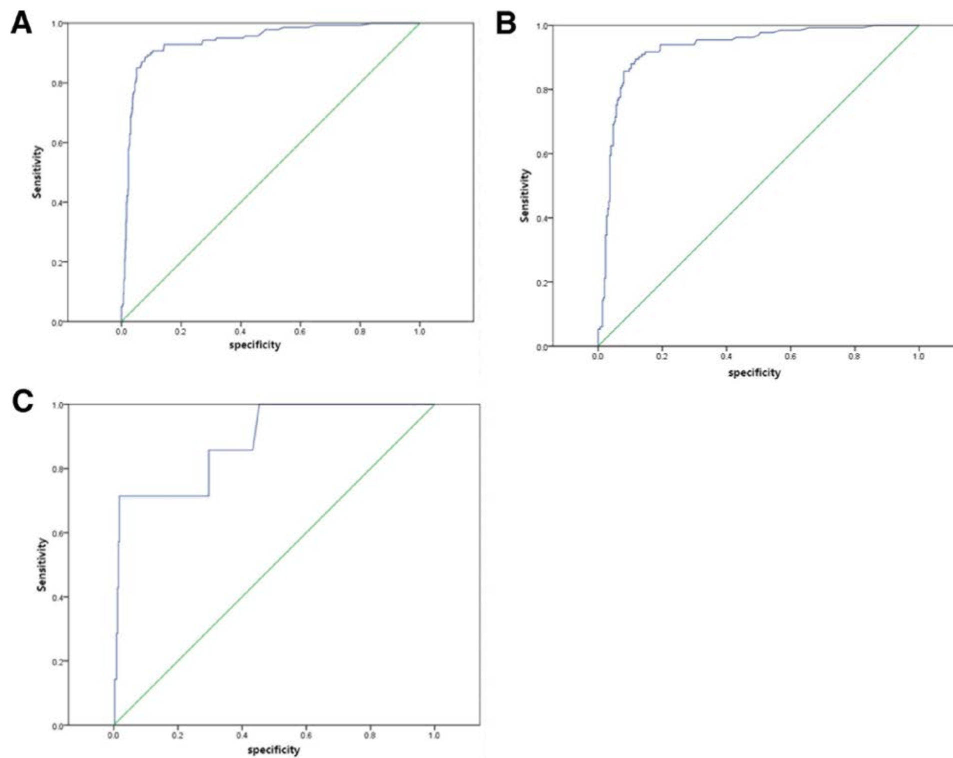

Figure 11. (A) ROC curve for the total number of participants. (B) ROC curve for men. (C) ROC curve for women. ROC = receiver operating characteristic curve.

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