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Short communication

Determination of nicotine and cotinine in human serum by means of LC/MS

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ABSTRACT

As part of a joint clinical research project to study the effects of nicotine on the brain, a HPLC electrospray ionisation mass spectrometry method with a solid-phase extraction sample preparation was developed for the quantitative determination of nicotine and cotinine in human serum in volunteers. The measured concentrations of nicotine and cotinine were used as control for smoking behaviour. A X-Bridge-column from Waters, and a SSQ 7000 single quadropole mass spectrometer with a TSP liquid chromatographic system were used. The method includes a simple and robust sample preparation and this assay has been shown to be of a sufficient sensitivity for this application. The limits of quantification were 5 and 2 ng/ml for cotinine and nicotine, respectively. A simultaneous study was conducted to measure nicotine receptor availability and the vigilance in the same group of volunteers.

1. Introduction

Nicotine is the main addictive agent in tobacco [1,2]. When cigarette smoke is inhaled, nicotine is absorbed through the lungs and undergoes extensive metabolism in the liver. Typically, 70–80% of nicotine is metabolized into cotinine [3].

In both the study measuring central nicotine receptor availability and the vigilance of volunteers and our measuring concentration of nicotine and cotinine, samples were taken during normal smoking behaviour and after 24 h of nicotine abstinence.

Many analytical methods have been used for the analysis of nicotine and cotinine: GC/MS [4–10], LC/MS [11–15] and LC/UV [16–19]. These methods utilize liquid-liquid extraction (LLE) or solid-phase extraction (SPE) for sample preparation. The SPE technique provides cleaner blood extracts than the LLE and the influence on sensitivity is considerably smaller because the ion suppression is lower apparent.

We have developed a LC electrospray ionisation MS method for the determination of nicotine and its major metabolite cotinine by means of simple solid-phase extraction with a strong cation exchanger. This assay is simple and robust, as well as sufficiently sensitive for above described application.

2. Materials and methods

2.1. Chemicals and reagents

Methanol solutions of cotinine and nicotine in acetonitrile were purchased from Sigma–Aldrich (Steinheim, Germany). The internal standards d_4-nicotine (in acetonitrile) and d_3-cotinine (in methanol) were supplied by Cerilliant (Round Rock, TX). Dichloromethane, isopropanol, toluene were acquired from J.T. Baker (Phillipsburg, NJ, US). Acetonitrile, methanol, water, and concentrated ammonia solution (extra pure) were obtained from VWR (Darmstadt, Germany). Trichloroacetic acid ACS reagent and hydrochloric acid 37% ACS reagent were got from Sigma–Aldrich. The Oasis MCX cartridges were purchased from Waters (Milford, MA, US) and the PRS (Isolute) cartridges were obtained from Separtis GmbH (Grenzach-Wyhlen, Germany).

The concentrated ammonia solution was extracted three times with the same sample volume of toluene. The Dichloromethane and isopropanol were cleaned with a strong cation exchanger PRS (Isolute). The first 3 ml were discarded and the next 4–8 ml per cartridge were collected. These solvents were used for the eluent.

2.2. Instruments and chromatographic conditions

The LC/MS analyses were performed on a SSQ 7000 single quadropole mass spectrometer with a TSP (Egelsbach, Germany) liquid chromatographic system. We used an α-DEC station 3000 with the following programs: ICIS 8.3.0 and ICL version 8.3.2. A X-Bridge (Waters) C18 column (3.5 μm, 2.1 mm × 150 mm) with a 10 mm long guard column was used for the chromatographic separation. The mobile phase was 20% acetonitrile. The flow rate was 0.2 ml/min and the injection volume was 25 μl. The mass spectrom-

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eter was adjusted to the following parameters: 20 V CID, 220 °C capillary temperature, 4.5 kV ionisation voltage in positive mode, 60 psi sheath gas, and 10 psi nitrogen auxiliary gas. The single ion mode was used with the following *m*/*z*-values: 163, 167, 177 and 180 at 0.6 mass units and 2 s, where *m* is the molar mass and *z* is the charge of ionised molecule.

2.3. Sample preparation

0.5 ml serum of calibrators, controls, or volunteer samples, 0.1 ml of working internal standard solution and 0.25 ml trichloroacetic acid (10 g/100 ml) were mixed and then centrifuged at 12,000 rpm for 3 min. Extraction columns were prepared by washing with 1 ml methanol and 1 ml 0.5 M HCl, taking care not to dry the columns. The supernatants of centrifuged samples were transferred into the Oasis MCX cartridges. The columns were washed with 1 ml deionised water, followed by 1 ml 0.5 M HCl and 1 ml methanol. Analytes were eluted with 1 ml of a mixture of dichloromethane/isopropanol/concentrated ammonia solution (78/20/2, v/v/v). Before using the eluent, this mixture was cleaned as described above. Solutions having undergone this cleaning process showed less contaminants on chromatogram. The collected organic phase after the solid-phase extraction over the MCX cartridges was evaporated by means of nitrogen at 45 °C. The residue was dissolved in 200 μl of 20% acetonitrile with concentrated ammonia solution (pH 11).

2.4. Contamination

Cognard and Staub [20] reported that nicotine in the atmosphere of their laboratories was a major source of contamination. Therefore smokers were not allowed to enter our laboratory. It was also necessary to clean all glassware and solvents thoroughly. Additionally all solvents, such the concentrated ammonia solution, dichloromethane and isopropanol, were cleaned as described above. Methanol and bidistilled water were replaced daily in beakers.

Environmental intake is common and therefore it is not easy to get plasma or serum free of nicotine and its metabolites. Therefore Kim and Huestis [21] described a validation method for bovine serum. In our work we used blank serum from non-smokers. The concentrations of nicotine and cotinine were determined in these sera. Only serum samples with values below the detection limits were used for the preparation of controls and calibrators.

2.5. Study design

Six volunteers took part in a clinical study of measurement of central nicotine receptor availability by means of positron emission tomography (PET) and the vigilance by means of electroencephalography during regular smoking and after 24 h nicotine withdrawal. The concentrations of nicotine and cotinine in human serum were determined during this study period. These measured results served as control for the smoking behaviour and in future will be compared with the PET data.

The study was carried out in accordance with the Helsinki Declaration and an ethical vote was obtained from the local ethics commission.

3. Results and discussion

3.1. Precision, accuracy, recovery and ion suppression

Fig. 1 shows the selectivity of the method. No noticeable interferences are seen in the chromatograms. Cotinine is still sufficiently separated from the front.

The within-run (intra-assay) and between-run (inter-assay) standard variations were determined using blank serum samples spiked with aliquots of solution of nicotine and cotinine at three various concentrations. Five samples of each concentration were prepared. These solutions were measured on the same day for the intra-assay (within-run). The inter-assay was determined by measuring the calibration curves on three different days. We used a mixture with 2 μg/ml d_3-cotinine and 200 ng/ml d_4-nicotine as working solution for internal standards. The peak height ratios between nicotine and d_4-nicotine as well as cotinine and d_3-cotinine were used for the analysis. The standard variations for the ratios are shown in Table 1 .

The recovery of each compound was measured under the extraction conditions described above. The peak heights of the spiked samples were compared with those of standard directly injected solutions. Additionally an extracted solution from blank serum materials was spiked after extraction. The differences between the peak heights of the last two mixtures correspond to effect conditioning through the ion suppression. These results are also presented in Table 1.

The ion suppression ranged from 3.8 to 7.9%. This amount is relatively small, so a further sample preparation, for example liquid-liquid extraction, only reduces the recovery. Murphy et al. [14] described such a procedure. Unfortunately a recovery rate was not described. Therefore only a simple SPE-procedure without liquid-liquid extraction was used in this work.

The standard variations of within-run are lower than those of the between-run, so we used daily calibration curves for the calculation of concentrations and consequently also for the determination of accuracy. The three daily calibration curves of between-run procedure were used for the calculation of accuracy. Table 2 shows the respective results.

In our opinion this method is sufficiently precise on the basis of a simple sample preparation for our application. The quantification limits were 5 and 2 ng/ml for cotinine and nicotine, respectively (6× noise). The standard curve for nicotine was linear over the concentration range from 2 to 100 ng/ml ($r^2 > 0.99$, with *r* as regression constant), and that for cotinine was linear from 5 to 500 ng/ml ($r^2 > 0.99$). In a review Heavner et al. [22] described various methods with limits of quantification ranging from 1 to 10 ng/ml for nicotine and from 0.1 to 10 ng/ml for cotinine.

3.2. Stability

Foulds et al. [23] have shown nicotine and cotinine to be stable in unfrozen plasma samples for up to 12 days. Cognard and Staub [20] have presented the stability of nicotine and cotinine over 6 months in frozen samples. Other authors have also verified the stability of these both substances [21,24,25].

The stability was tested under various conditions (repeated thaw procedure, room temperature, storage from frozen samples at −20 °C) in this investigation.

3.2.1. Repeated freeze–thaw procedure

Three samples containing 50 ng/ml cotinine and 10 ng/ml nicotine were thawed and refrozen three times at −20 °C. The samples were prepared as previously described and the results were compared with samples that were thawed only once. The peak height ratios were used for this comparison. The ratios were 102.7 and 106.3% for nicotine and cotinine, respectively.

3.2.2. Storage conditions in autosampler

Three samples of spiked sera were prepared. After evaporating to the same concentration they were combined. One half of this solution was put into an autosampler at ambient tempera-

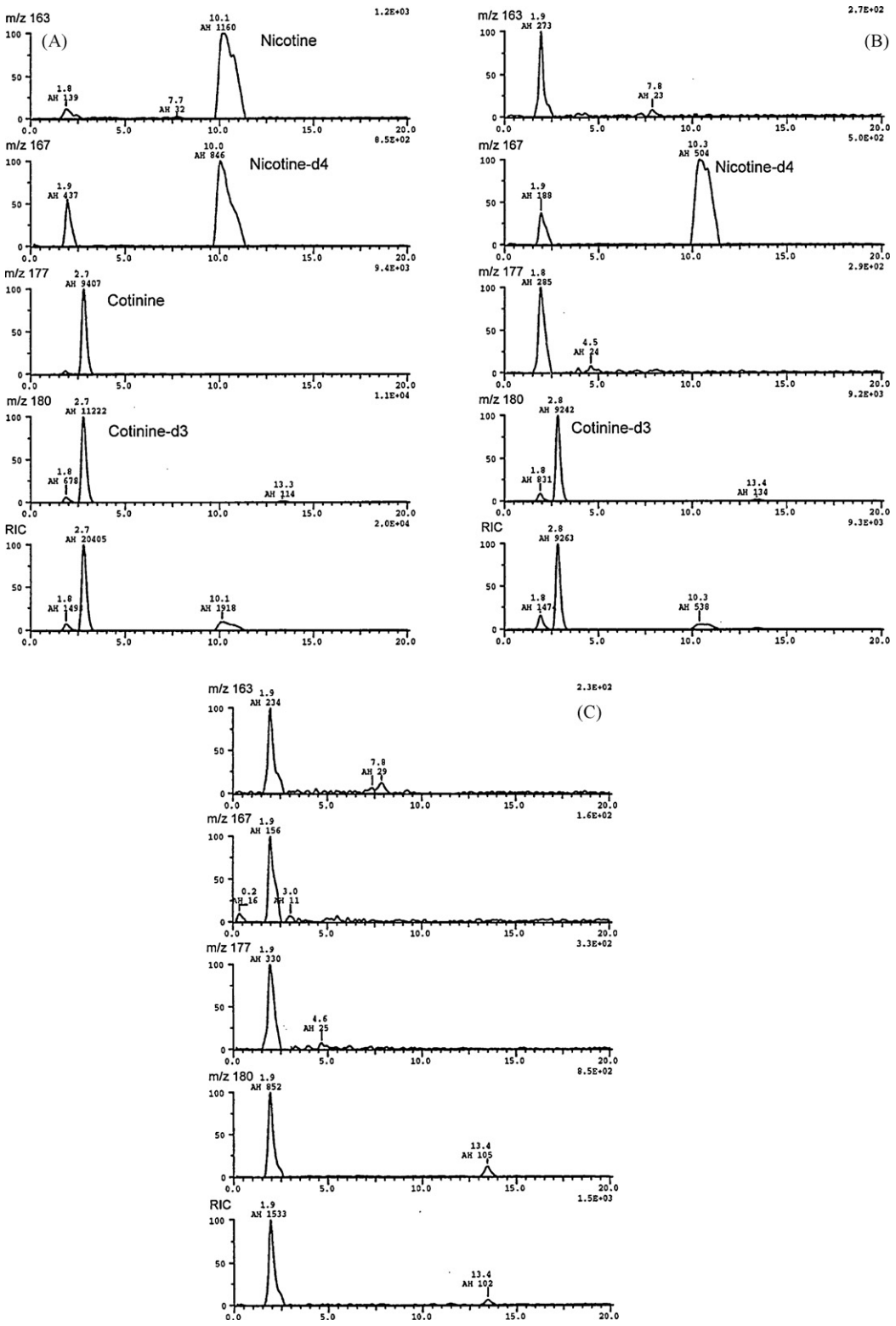

Fig. 1. Chromatograms of (A) spiked serum sample with internal standards and 100 ng/ml cotinine and 20 ng/ml nicotine, (B) blank serum with internal standards and (C) blank serum without internal standards. RIC reconstructed ion chromatogram (total ion intensity).

ture. The other half was divided in 60 μl portions and then frozen at −80 °C. These samples were thawed shortly before being measured. The peak heights were compared. The results of two different concentrations are represented in Table 3.

Nicotine, cotinine and the internal standards were stable under these conditions over 24 h at ambient temperature.

3.2.3. Storage of frozen samples at −20 °C over 3 months

Spiked serum samples were produced and prepared on the same day. The peak height ratios were compared with the results of spiked serum samples that had been stored at −20 °C for over 3 months. The quotients between mean peak height ratios (each $n = 3$) were calculated for two different concentrations. The calcu-

Table 1
Standard variations of between-run and within-run procedure, recovery and effect of ion suppression.

Concentration, ng/ml	Standard variation of peak height ratios		Recovery, %	Quotient[a] (%) (ion suppression, %)
	Between-run % $n=3$	Within-run % $n=5$		
Cotinine				
500	6.9	11.4	72.4	96.2 (3.8)
250	9.3	5.6	66.3	95.1 (4.9)
50	16.2	3.4	70.2	92.1 (7.9)
Nicotine				
100	15.5	5.8	85.5	94.0 (6.0)
50	21.3	8.2	87.8	95.3 (4.7)
10	12.0	10.0	96.5	93.4 (6.6)

[a] Peak height ratios between a spiked solution of prepared blank serum and a solution without sample preparation at the same concentrations.

Table 2
Accuracy calculated from daily calibration curves ($n=3$) of between-run procedure.

Cotinine			Nicotine		
Spiked concentration, ng/ml	Calculated concentration, ng/ml	Accuracy, %	Spiked concentration, ng/ml	Calculated concentration, ng/ml	Accuracy, %
500	494.1 ± 5.6	98.8	100	100.1 ± 5.4	100.1
250	265.8 ± 14.8	106.3	50	50.0 ± 4.5	100.0
100	101.4 ± 3.1	101.4	20	20.1 ± 2.7	100.5
50	46.9 ± 6.4	93.8	10	9.7 ± 1.1	97.0
25	25.5 ± 0.8	102.0	5	5.2 ± 0.6	104.0

lations for the first level (100 ng/ml cotinine; 20 ng/ml nicotine) were 112.9% for cotinine and 95.6% for nicotine and for the second level (25 ng/ml cotinine; 5 ng/ml nicotine) 99.4% for cotinine and 101.0% for nicotine.

Under such conditions cotinine and nicotine were stable over 3 months.

3.3. Application

The concentrations of nicotine and cotinine were determined in a clinical study of the investigation of nicotine receptor availability by means of positron emission tomography. Two blood samples were taken from each smoker. Then after having not smoked for 24 h another sample was taken and in a later period a control measurement was executed. In the first two samples the mean serum concentrations of cotinine and nicotine were determined to be 299.6 ± 149.4 and 15.2 ± 7.0 ng/ml ($n=12$), respectively. After a 24 h withdrawal period these values were 10.8 ± 2.6 and 2.8 ± 0.8 ng/ml ($n=2$, values under limit of quantification were not taken into consideration). A second control measurement performed later resulted in 100.2 ± 110.7 and 7.0 ± 4.3 ng/ml ($n=4$), respectively. In Fig. 2 the relationship between both concentrations is presented. The results demonstrate that the concentrations of cotinine and nicotine in human serum show a strong correlation ($r=0.926$). The best adjustment was given by a potency function ($C_{\text{cotinine}} = 5.03 \times C_{\text{nicotine}}^{1.47}$). C_{cotinine} and C_{nicotine} are the concentrations of cotinine and nicotine in human serum. Tutka et al. [26] also described a strong relationship between both concentrations in man after transdermal administration and the authors [26] have proposed that the cotinine concentrations could be a good marker of nicotine clearance.

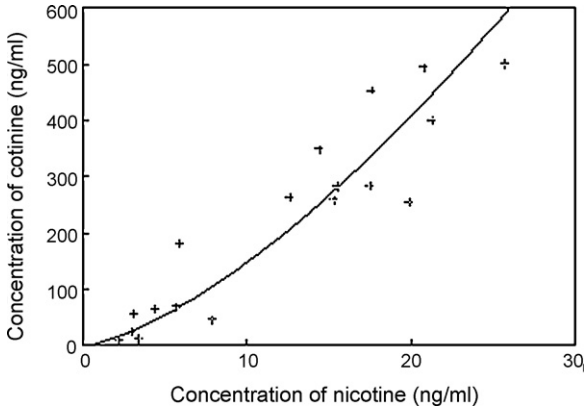

Fig. 2. Relationship between serum concentrations of cotinine and nicotine in human serum.

Table 3
Percentage of peak heights between samples that were storaged for 24 h in autosampler and at −80 °C, respectively ($n=3$).

Substances/samples	N1	N2
Cotinine	98.8 ± 6.7%	99.8 ± 2.4%
d_3-Cotinine	98.8 ± 4.3%	97.7 ± 5.9%
Nicotine	103.0 ± 4.3%	102.1 ± 6.1%
d_4-Nicotine	101.0 ± 3.2%	97.6 ± 3.9%

N1, 100 ng/ml cotinine, 20 ng/ml nicotine; N2, 25 ng/ml cotinine, 5 ng/ml nicotine. Addition of internal standards as described in article (200 ng d_3-cotinine and 20 ng d_4-nicotine).

4. Conclusions

A sensitive and robust LC/MS method with a simple SPE-sample preparation was developed. The limits of quantification were 5 and 2 ng/ml for cotinine and nicotine, respectively. It is very important, when determining lower concentrations of nicotine and cotinine in human serum to consider the contamination of ambient air and the solvents. This assay was proved in a clinical research project parallel to the investigation of nicotine receptor availability of smoker during normal behaviour and after a 24 h non-smoking phase.

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