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Nicotine exposure can be detected in cerebrospinal fluid of active and passive smokers

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ABSTRACT

A simple, rapid and sensitive liquid chromatography/mass spectrometry (LC/MS) method has been utilized for the quantitative determination of nicotine and its major metabolite cotinine (COT) in human cerebrospinal fluid (CSF) of active and passive smokers. CSF samples from 18 smokers, 15 non-smokers, 15 children, 15 infants, and 9 neonatal were analyzed for nicotine (NIC) and cotinine content. Cotinine levels in the CSF of smokers ranged from 27.3 to 457.1 ng/ml, whereas nicotine levels were considerably lower (6.0–215.1 ng/ml). Cotinine could be detected in 4 of the 15 CSF samples from non-smokers (3.5–30.4 ng/ml), and a few other passive smokers, including neonates from smoking mothers (15.6–81.1 ng/ml). The concentrations of cotinine in CSF samples suggests that nicotine easily passes into the CSF, which makes it an excellent CSF marker for tobacco-smoke exposure.

1. Introduction

Nicotine (NIC), the principal active constituent in tobacco, is one of the most widely consumed natural products and contributes to diseases that are related to tobacco use. Cotinine (COT) is the major oxidative metabolite of NIC (Fig. 1), which accounts for 70–80% of NIC metabolites in serum [1]. COT has a relatively long half-life of 10–40 h in humans [2], which makes it a suitable marker for assessing exposure to cigarette smoke.

When inhaled, tobacco smoke is efficiently absorbed from the lung, where 82–92% of NIC is absorbed into the pulmonary circulation followed by rapid permeation through the blood/brain barrier [3]. NIC reaches the central nervous system (CNS) within 20 s of tobacco smoke inhalation; it has a plasma half-life of 2–4 h and a brain half-life of 52 min [4]. Because of the health risks associated with tobacco exposure, the search for suitable biomarkers of tobacco smoke exposure has increased [5,6]. Tobacco usage is one of the main causes of newborn premature death in the country and has been shown to increase the incidence of various types of cancers [7]. Smokers generally have plasma cotinine levels greater than 15 ng/ml, while heavy smokers (>20 cigarettes/day) usually have levels higher than 300 ng/ml. Second-hand smoke, or environmental tobacco smoke (ETS), has been an area of active study in the health sciences for several decades. The National Health and Nutrition Examination Survey data from 1988 to 1991 found that 88% of non-smokers in U.S. had detectable concentrations of serum COT [8]. Despite the increasing awareness, environmental tobacco smoke (ETS) is considered to be a major public health concern in the United States, and has been classified as a Group A (known human) carcinogen by the Environmental Protection Agency [9].

There are numerous reports on tobacco-related health risks for women, children, and neonates. Women are at risk for coronary heart disease, chronic obstructive pulmonary disease, and cancer [10]. For the fetus, maternal smoking is associated with premature birth and intrauterine growth restriction [11,12]. Neonates and young children who are exposed to second-hand smoke in their homes are at increased risk for the development of asthma, and other respiratory disorders later in childhood, and death from sudden infant death syndrome [13].

Cerebrospinal fluid (CSF) is a clear bodily fluid that occupies the subarachnoid space around and inside the brain. It acts as a buffer for the cortex, providing also a basic mechanical and immunological protection for the brain inside the skull [14]. The cerebrospinal fluid is produced at a rate of 500 ml/day [15] and contains approximately 0.3% plasma protein [16]. Analysis of drugs in CSF is important for centrally acting drugs such as NIC, since it provides concentrations of the drug and its metabolites in the vicinity of the receptor active sites that are responsible for the drug's pharmacological

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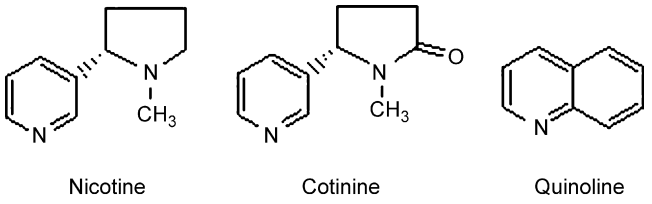

Fig. 1. Chemical structure of S-(−)-nicotine, S-(−)-cotinine, and quinoline (internal standard).

properties. Since NIC exerts its pharmacological actions on the central nervous system, plasma concentrations of NIC and COT do not necessarily reflect their actual levels in the brain after NIC exposure. Thus, based on a rat model of NIC exposure at a particular time point after NIC exposure [17], concentrations of NIC in the plasma of smokers may not match the concentrations of this tobacco alkaloid in the brain. Accordingly, measurement of NIC and its metabolites in the CSF is of importance to correctly account for the total levels of these xenobiotics in the human body. A study designed to determine brain concentrations of NIC and its metabolites after acute and chronic peripheral administration of NIC suggests that NIC and its metabolites accumulate in the central nervous system after intermittent and continuous exposure to NIC [18].

A variety of methods has been reported in literature for the determination and quantification of NIC and its metabolites in biological fluids, i.e. serum, urine, saliva, and whole blood, including HPLC [19–21] and GC–MS [22–24] methodologies. Several LC/MS/MS methods have been previously reported. However, some of these methods require troublesome extraction procedures and/or multiple sample preparation before assay [25,26]. Some other methods require large sample volume (1 ml) of plasma [27]. In general, liquid chromatography/mass spectrometry (LC/MS) methods are considered more accessible and less expensive to run when compared to LC/MS/MS methodologies. A thermospray LC/MS method [28] was reported for the simultaneous detection of NIC and COT in biological fluids. However, the lower end of the linearity range was 80 and 20 ng/ml for NIC and COT, respectively.

To the best of our knowledge, there are no LC/MS methods reported for the quantification of NIC and COT in CSF. Also, due to the difficulty in obtaining human CSF samples, very little information is available on the concentrations of NIC and its major metabolite COT, in the CSF of smokers.

The identification of COT in the CSF of smokers has been reported by one group of researchers, who utilized an immunoassay procedure [29]. However, possible cross-reactivity with closely related molecules often leads to over estimation of metabolite concentrations using this method of analysis [30]. A gas chromatography-flame ionization detection analysis has also been used to quantify COT in smokers' CSF in the range of 2–220 ng/ml, but no details of the assay were given in this report [31].

To our knowledge, there have been no reports that have specifically focused on the determination of NIC and COT levels in the CSF of active and passive smokers. Accordingly, this study was conducted to utilize a simple and inexpensive, yet rapid and sensitive LC/MS method for the determination NIC and COT in human CSF. The study focuses on analyzing CSF samples from several groups of active and passive smokers, including children and neonates from smoking mothers. Due to the difficulty in obtaining large amounts of authentic blank human CSF samples, the study also determines whether artificial CSF can be used as an alternative to authentic human CSF for the construction of calibration curves for the quantitation of NIC and COT in CSF.

2. Experimental

2.1. Chemicals and reagents

S-(−)-NIC free base was purchased from Eastmen Kodak Company, (Rochester, NY), S-(−)-COT free base and the Internal Standard (IS), quinoline, (Fig. 1) were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade ethyl acetate, methanol, and acetonitrile were all obtained from Fisher Scientific (Pittsburg, PA). Ultrapure water was generated from a Mili-Q system from Milipore (Bedford, MA). Working solutions of compounds were prepared daily by appropriate dilution with methanol. All standards and stock solutions were stored at 4 °C until use.

2.2. Stock solutions and sample preparation

Artificial CSF was prepared according to the method of Oka et al. [32] as follows: sodium chloride (7.592 g/l), potassium chloride (0.146 g/l), magnesium chloride hexahydrate (0.24 g/l), calcium chloride dihydrate (0.174 g/l), potassium dihydrogen phosphate (0.154 g/l), sodium DL-lactate (1.69 g/l), anhydrous D-(+)-glucose (0.719 g/l), and sodium hydrogen carbonate (0.924 g/l) were dissolved in ultrapure water. The resulting solution was stored at 4 °C until use.

Stock solutions of NIC, COT, and the IS were prepared to afford an approximately 1 μg/ml concentration of each compound by separately dissolving each compound in methanol. Working solutions were obtained by diluting the appropriate stock solution with methanol to afford concentrations in the range 1–500 ng/ml.

All frozen CSF samples were thawed at room temperature. Individual CSF samples (250 μl) were each transferred to 2 ml polypropylene test tubes; ethyl acetate (250 μl), 25 μl of 0.1 N NaOH and 25 μl of the IS were added. The samples were vortex-mixed for 1 min and centrifuged at 600 × *g*-force (3000 rpm) for 10 min. Aliquot-parts of the resulting supernatant were directly transferred to clean 2 ml vials, evaporated to dryness and reconstituted in 100 μl of mobile phase, from which 20 μl was then directly injected onto the LC/MS system for analysis.

2.3. Chromatography

Chromatography was performed on a Waters® Suplico C_{18} (4.6 mm × 50 mm, 5 μm) column with a mobile phase consisting of methanol, acetonitrile and 0.01% formic acid (35:35:30, v/v/v). The flow-rate was set at 0.3 ml/min. Quantitative analysis was performed with an LC–MS system consisting of a Waters 2690 HPLC pump, a Waters 2695 autosampler, and a Micromass ZQ detector which utilized electrospray ionization (ESI) detection. MassLynxTM software, version 3.5 (Waters) was used for system control, data acquisition, and data processing. Selected ion monitoring (SIM) was performed in the positive mode. The theoretical *m*/*z* values of the $[M+H]^+$ ions were set at 63.23, 177.22, and 130.16 for NIC, COT, and IS, respectively. The capillary voltage was 3 kV and the cone voltage was 30 V with a dwell time of 0.5 s. The source block and desolvation temperatures were 110 and 350 °C, respectively. Nitrogen was used as the nebulization and drying gas at flow rates of 75 and 400 l/h, respectively.

The method was validated to determine specificity, linearity, accuracy, and precision. Calibration curves were generated from the analysis of seven different concentrations (1, 5, 25, 50, 100, 250, and 500 ng/ml) of NIC or COT in drug free artificial CSF matrix containing a fixed concentration of IS. The calibration curves were constructed by plotting the peak area ratio (y) of analyte to IS versus analyte concentration (x). Slope and coefficient of determination (r^2) were calculated as regression parameters by weighted ($1/x$) lin-

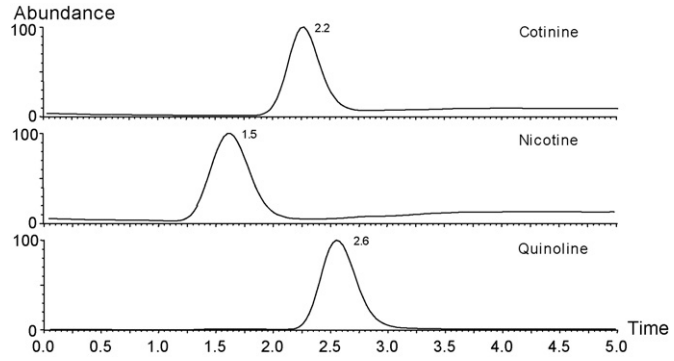

Fig. 2. Representative mass chromatograms in SIM mode of a blank human CSF sample containing 100 ng/ml of nicotine, cotinine, and IS.

ear regression. Quality control (QC) samples of NIC, COT, and the IS (10, 250, and 500 ng/ml) were prepared for both analytes in human CSF and artificial CSF to evaluate the selectivity of the method, and to check for possible matrix effects in the two media. Precision and accuracy were evaluated by determining the concentration in three replicates of each QC sample at three different concentrations on 3 separate days. The specificity of the method was investigated by analyzing three blank CSF samples. Recovery and matrix interference were investigated by analyzing three CSF samples at three different concentrations.

2.4. CSF sample collection

CSF samples were collected at the University of Kentucky Hospital-Chandler Medical Center following approved standard clinical protocols and procedures. The samples were collected for clinical reasons, and the back-up samples, with no identifiers, were utilized for this study, after approval from the Institutional Review Board. Patterns of smoking were not known or controlled in the tested population, but all smokers were current users of cigarettes, according to their medical records.

3. Results and discussion

3.1. Mass spectrometric analysis

The optimization of MS parameters was performed via direct infusion of a methanolic solution of the analytes into the ESI chamber; the optimized parameters are reported in Section 2.3. Representative mass chromatograms of an artificial CSF sample spiked with each of the analytes NIC, COT, and the IS, are shown in Fig. 2. The retention times for NIC, COT, and the IS were 1.5, 2.2 and 2.6 min, respectively.

The method requires a simple liquid–liquid extraction of a small amount of CSF sample (250 μl) without any extensive sample work-up. The simple extraction into ethyl acetate and the 5-min assay time allows a large number of samples to be analyzed in unit time, and makes this method an ideal assay for use in large research studies where multiple analytes and limited sample volumes are common.

3.2. Matrix effect and recovery

Matrix effects were determined by analyzing QC samples of NIC, COT, and the IS (5, 100, and 500 ng/ml) prepared in human CSF and artificial CSF to check for possible matrix effects in the two media. No interfering peaks caused by endogenous components in either of the CSF samples were observed that overlapped with the retention time for NIC, COT, or the IS. Therefore, the present study establishes that artificial CSF can be utilized in place of authentic blank CSF samples for the preparation of a standard calibration curve.

The extraction efficiency was determined by analyzing three quality control samples of both NIC and COT at different concentrations, i.e. 5, 250, and 500 ng/ml. The recoveries were calculated by comparing peak areas obtained from CSF samples with those obtained by direct injection of a standard solution at the same concentration and under the same conditions. Mean recoveries of NIC and COT were found to be 81.3% and 86.5%, respectively, and were independent of analyte concentration.

3.3. Linearity, precision, and accuracy

The calibration curves for both NIC and COT were linear over the tested concentration range of 1–500 ng/ml with a correlation coefficient of 0.99 or more. Limit of detection (LOD) and limit of quantification (LOQ) were determined at the concentration that produced a signal-to-noise ratio of 10:1 and 3:1, respectively. LOD was 1 ng/ml for both NIC and COT. However, LOQ was determined at 5 and 3 ng/ml for NIC and COT, respectively.

Inter-day and intra-day precision and accuracy were assessed by extracting and analyzing three replicates of the three QC concentrations (5, 250, and 500 ng/ml). The inter-day and intra-day assay precision (expressed as %R.S.D.) did not exceed 12%, while the inter-day and intra-day accuracy (expressed as nominal values) did not exceed ±12%.

3.4. Determination of NIC and COT in human CSF

CSF samples from 18 smokers, 15 non-smokers, 15 children, 15 infants, and 9 neonates were analyzed for the presence of NIC and COT. COT was detected in most (15/18) of the CSF samples from smokers and in a few (12/62) CSF samples from non-smokers, children, and infants. Furthermore, COT was detected in significant concentrations (15.6–81.1 ng/ml) in 3 of the 5 neonatal CSF samples from newborn babies of smoking mothers, indicating that the fetus is exposed to nicotine as a result of maternal smoking. Table 1 shows a summary of the analytical results. Fig. 3 illustrates a representative chromatogram obtained from the analysis of a CSF sample from a smoker.

Table 1
Summary of NIC and COT analytical testing of CSF samples.

No. of subjects	Nicotine		Cotinine	
	Subjects with quantitatively detectable levels	Avg. Conc., ng/ml ± S.D. (Conc. range, ng/ml)	Subjects with quantitatively detectable levels	Avg. Conc., ng/ml ± S.D. (Conc. range, ng/ml)
Smokers (18)	11	38.5 ± 75.6 (6.0–215.1)	15	101.2 ± 105.0 (27.3–457.13)
Non-smokers (15)	1	18.1	4	19.7 ± 14.5 (3. 5–30.4)
Children (15)	0	–	3	11.8 ± 11.7 (4.4–25.3)
Infant (15)	1	6.9	2	10.5 (7.1–13.9)
Neonatal (9)	0	–	3	44.2 ± 33.8 (15.6–81.1)

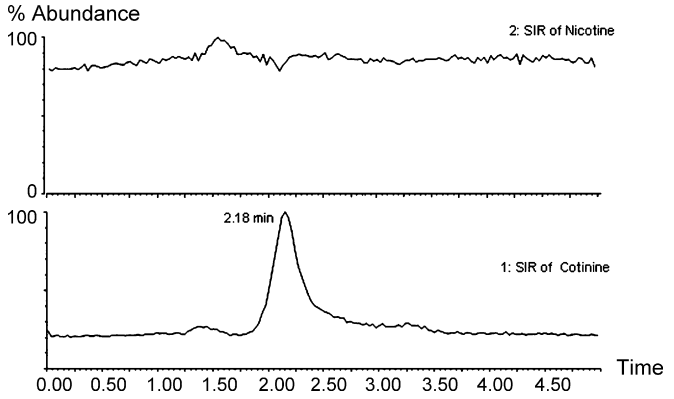

Fig. 3. Representative chromatogram of cotinine detection in CSF sample of a smoker.

This study shows that active and passive exposure to tobacco smoke can be monitored by measuring COT levels in CSF. The positive detection of NIC and COT in the CSF of 11 passive smokers out of a test population of 54 individuals suggests that exposure to second-hand smoke may afford CNS-levels of NIC that have pharmacological and toxicological consequences. The detected COT in children and infants indicates that many of these individuals are passively exposed to NIC.

Due to the low levels of enzymes in the biological matrix, virtually no metabolism of NIC occurs in the CSF; thus, we can conclude that the high concentrations of COT detected in the CSF of smokers and passive smokers indicate that this NIC metabolite readily passes from the blood to the CNS.

Although this study revealed that NIC exposure was generally associated with the presence of measurable amounts of COT, it did not explain the high variation of COT levels in active and passive smokers. Since there was insufficient information (i.e. details of gender, age, time since last exposure, diet, etc.) to ascertain conclusively whether individuals had been exposed to second-hand smoke or not, the source of the detected levels of NIC and COT in non-smokers, as well as other potential passive smokers, could not be determined.

4. Conclusion

NIC and COT can be detected and quantified in human CSF samples utilizing LC/MS analysis. This study has successfully demonstrated that COT is present in the CSF of active and passive smokers. Low concentrations of COT were also observed in CSF samples of neonates from smoking mothers, as well as potential passive smokers.

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