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# Simultaneous determination of nicotine and its metabolite, cotinine, in rat blood and brain tissue using microdialysis coupled with liquid chromatography: Pharmacokinetic application

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**Abstract**

To elucidate the disposition of nicotine in the brain is important because the neuropharmacological effects from nicotine exposure are centrally predominated. The aim of the present study was to develop a rapid and simple method for the simultaneous determination of unbound nicotine and its main metabolite, cotinine, in rat blood and brain tissue. We coupled a multiple sites microdialysis sampling technique with HPLC–UV system to characterize the pharmacokinetics of both nicotine and cotinine. Microdialysis probes were inserted into the jugular vein/right atrium and brain striatum of Sprague–Dawley rats, and nicotine (2 mg/kg, i.v.) was administered via the femoral vein. Dialysates were collected every 10 min and injected directly into a HPLC system. Both nicotine and cotinine were separated by a phenyl-hexyl column (150 mm × 4.6 mm) from dialysates within 12 min. The mobile phase consisted of an acetonitrile–methanol–20 mM monosodium phosphate buffer (55:45:900, v/v/v, pH adjusted to 5.1) with a flow-rate of 1 ml/min. The wavelength of the UV detector was set at 260 nm. The limit of quantification for nicotine and cotinine were 0.25 μg/ml and 0.05 μg/ml, respectively. Intra- and inter-day precision and accuracy of both measurements fell well within the predefined limits of acceptability. The blood and brain concentration–time profile of nicotine and cotinine suggests that nicotine is easily to get into the central nervous system and cotinine exhibits a long retention time and accumulates in blood.


*Keywords:* Cotinine; Microdialysis; Nicotine; Pharmacokinetics

## 1. Introduction

Nicotine is responsible for tobacco addiction and is the most specific component of cigarette smoke. This cigarette ingredient is absorbed and measurable in both active and passive smokers. Nicotine can be metabolized to more than 20 different derivatives. Cotinine is a major oxidized metabolite of nicotine and with similar structure to nicotine (Fig. 1). With long elimination half-life of cotinine (about 20 h compare to 2 h for nicotine) in blood which can be used as a reliable measure of nicotine intake. The pharmacokinetics of nicotine has been widely studied in humans [1] and experimental animals [2,3]. Although peripheral metabolism of nicotine has been studied extensively [4], nicotine disposition in the brain has been given little attention until recently. The presence of nicotine and its metabolites in the brain is an important factor to consider because nicotine biotransformation products have been shown to be pharmacologically active [5–7], and their presence in the brain constitutes a potential contribution to the neuropharmacological effects resulting from nicotine exposure.

Lately, it has been suggested that nicotine may have therapeutic applications in a variety of disorders including Alzheimer's disease, Parkinson's disease, obesity, depression, anxiety, ulcerative colitis, and attention deficit disor-

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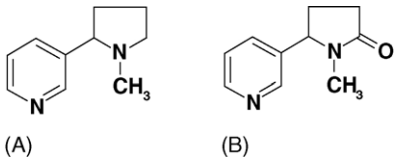


Fig. 1. Chemical structure of nicotine (A) and cotinine (B).

der. Indeed, clinical trials of nicotine in a number of these disorders with positive outcomes have been reported [8,9]. Consequently, a simple, rapid, and sensitive assay for the measurement of nicotine and cotinine is fundamental in the effective development and evaluation of nicotine effects both in research and clinical settings. A large number of publications deal with the quantitative determination of nicotine and/or cotinine in biological fluids, among the methods used are radioimmunoassay, HPLC, and gas chromatography [10–13]. Over the past several years, microdialysis has been increasingly used for in vivo sampling of unbound endogenous or exogenous compounds in the blood, brain, bile or liver in various animal experiments [14–16]. The samples of microdialysate are protein-free, making it possible for direct coupling of the microdialysis to the liquid chromatographic analysis with no sample cleanup required. In this paper, we describe a rapid, simple and cost-saving method for the simultaneous determination of nicotine and cotinine in rat blood and brain tissue utilizing microdialysis sampling technique coupled with HPLC–UV for a pharmacokinetic study.

## 2. Experimental

### 2.1. Chemicals and reagents

Nicotine and cotinine were purchased from Sigma (St. Louis, MO, USA). Liquid chromatographic grade solvents and reagents were obtained from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

### 2.2. Animals

All experimental protocols involving animals were reviewed and approved by the Institutional Animal Experimentation Committee of the National Research Institute of Chinese Medicine. Male specific pathogen-free Sprague–Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei, Taiwan. Before experimentation, animals were allowed 1-week acclimation period at the animal quarters with air conditioning and an automatically controlled photoperiod of 12 h of light daily. Animals had free access to food (Laboratory rodent diet no. 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to experimentation, at which time only food was removed. The rats were initially anesthetized with urethane 1 g/ml and α-chloralose 0.1 g/ml (1 ml/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. During the experiment, rat body temperature was maintained at 37 °C using a heating pad.

### 2.3. Chromatography

The chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), an on-line injector (CMA/160, Stockholm, Sweden) equipped with a 20-μl sample loop and a UV–Vis detector (Varian, Walnut Creek, CA, USA). Samples obtained were separated using a reversed-phase Luna phenylhexyl column (150 mm × 4.6 mm). The mobile phase comprised acetonitrile–methanol–20 mM monosodium phosphate buffer (55:45:900, v/v/v, pH adjusted to 5.1 by orthophosphoric acid), 0.5% 1 M citric acid, 0.1% diethylamine, 1 mM octanesulfonic acid, and the flow-rate was 1 ml/min. The UV wavelength was set at 260 nm. Chromatography was performed at ambient temperature. The output signal from the HPLC UV–Vis detector was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

### 2.4. Microdialysis experiment

Both blood and brain microdialysis systems consisted of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and the appropriate microdialysis probes. The dialysis probes for blood (10 mm in length), and brain (3 mm in length) were made of silica glass capillary tubing arranged in a concentric design [17]. Their tips were covered by a dialysis membrane (150 mm outer diameter with a nominal molecular mass cut-off of 13 000, Spectrum, Laguna Hills, CA, USA) and all unions were cemented with epoxy. To allow adequate time for the epoxy to dry, the probes were made at least 24 h prior to use.

The blood microdialysis probes were positioned within the jugular vein/right atrium and then perfused with anticoagulant dextrose (ACD) solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow-rate of 3 μl/min using the CMA microinjection pump. For brain microdialysis, the rat was mounted on a stereotaxic frame and perfused with Ringer's solution (147 mM $Na^+$; 2.2 mM $Ca^{2+}$; 4 mM $K^+$; pH 7.0). After being washed with Ringer's solution at a flow-rate of 3 μl/min, the microdialysis probe was implanted in the right striatum (coordinates: AP 0.4 mm, ML −3.0 mm, DV −7.0 mm) according to the Paxinos and Watson atlas [18]. The positions of the probes were verified by standard histological procedure at the end of experiments.

### 2.5. Drug administration

After a 2 h post-surgical stabilization period subsequent to the implantation of probes, nicotine (2 mg/kg, i.v.) was

administered via the femoral vein. The volume of each injection was 1 ml/kg. The dialysates from the blood and brain were connected into a fraction collector (CMA/140). The sampling interval was 10 min for each probe. The dialysates were immediately measured by a validated HPLC system.

### 2.6. Recovery of microdialysate

For in vivo recovery, the blood and brain microdialysis probes were inserted into the jugular vein and striatum under anesthesia. Perfusate solution containing nicotine or cotinine were prepared respectively and passed through the microdialysis probe at a constant flow-rate (3 μl/min) using an infusion pump (CMA/100). Two hours after probe implantation, the perfusate ($C_{perf}$) and dialysate ($C_{dial}$) concentrations of both drugs were determined by HPLC. The relative recovery in vivo ($R_{dial}$) across the microdialysis probes inserted in rat blood and brain were calculated according to the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$.

### 2.7. Pharmacokinetic application

Both nicotine and cotinine microdialysate concentrations ($C_m$) were converted to unbound concentration ($C_u$) as follows: $C_u = C_m/R_{dial}$. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA). The areas under the curve (AUC) from time zero to time infinity were calculated by the trapezoidal rule and extrapolated to time infinity by the addition of $AUC_{t-inf}$. The AUC values were thus given by adding the product of the measured concentrations and the collection time interval to the residual area, that is: $AUC = AUC_{0-t} + AUC_{t-inf}$.

## 3. Results and discussion

Typical chromatograms of nicotine and cotinine sampled from the biological fluid are shown in Figs. 2 and 3. Separation of both drugs from endogenous chemicals in biological dialysates was achieved in an optimal mobile phase containing 90% of 20 mM monosodium phosphate buffer (pH 5.1) containing 5.5% acetonitrile and 4.5% methanol. Retention time of nicotine and cotinine were about 11.0 and 9.5 min, respectively. The calibration curves of both drugs were linear over the investigated concentration ranges of 0.25–100 μg/ml for nicotine and 0.05–100 μg/ml for cotinine. The correlation coefficient was higher than 0.999, indicating good linearity. The intra- and inter-assay accuracy and precision were found to be acceptable for the analysis of a dialysis sample in support of pharmacokinetic studies [19]. As shown in Table 1, the overall mean precision (RSD) of both drugs ranged from 0.04 to 9.1%. Analytical accuracy expressed as the percent difference of the mean observed values compared with known concentration varied from 0.07 to 8.0%. In vivo recovery of nicotine in blood (0.5–5 μg/ml) was 27.2 ± 2.2%, in brain (0.5–5 μg/ml) was 3.2 ± 1.2%. In vivo recovery of cotinine in blood (0.1–1 μg/ml) was 32.6 ± 3.6%, in brain (0.1–1 μg/ml) was 3.4 ± 1.9% (Table 2). The limits of quantification (LOQs) for assay of nicotine and cotinine were 0.25 and 0.05 μg/ml, respectively. The limits of detection (LODs) of nicotine and continine were 0.1 and 0.01 μg/ml, respectively.

Fig. 2 shows the chromatogram of a blood dialysate sample containing nicotine (0.45 μg/ml) and cotinine (0.10 μg/ml) collected 30 min after nicotine administration (2 mg/kg, i.v.). Fig. 3 shows the chromatogram of a brain sample containing nicotine 0.34 μg/ml collected 10 min after nicotine administration. None of the observed peaks interfered with the analytes in the chromatograms of blood and

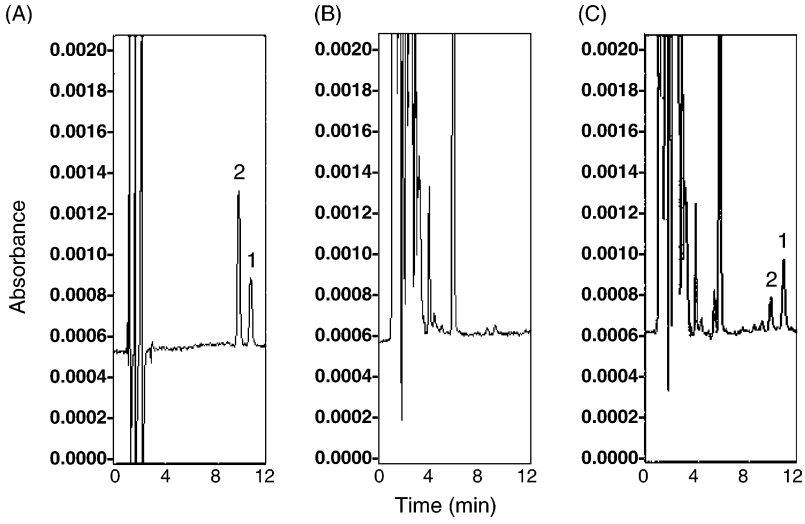


Fig. 2. Typical chromatograms of (A) standard nicotine (0.44 μg/ml) and cotinine (0.51 μg/ml); (B) a blank blood dialysate from the microdialysis probe prior to drug administration; and (C) a blood dialysate sample containing nicotine (0.45 μg/ml) and cotinine (0.10 μg/ml) collected from a rat blood microdialysate 30 min post nicotine administration (2 mg/kg, i.v.). Peaks: 1 = nicotine; 2 = cotinine.

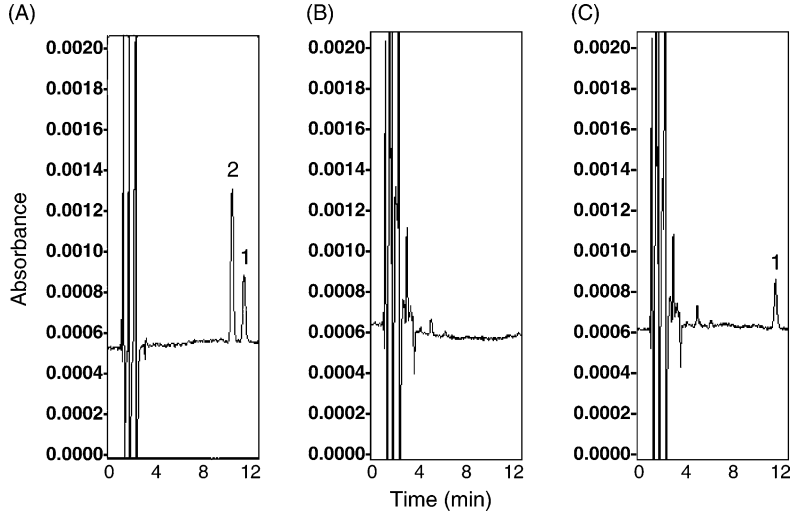


Fig. 3. Typical chromatograms of (A) standard nicotine (0.44 μg/ml) and cotinine (0.51 μg/ml); (B) a blank brain dialysate from the microdialysis probe prior to drug administration; and (C) a brain dialysate sample containing nicotine (0.34 μg/ml) collected from a rat blood microdialysate 10 min post nicotine administration (2 mg/kg, i.v.). Peaks: 1 = nicotine; 2 = cotinine.

Table 1
Intra- and inter-assay precision (RSD) and accuracy (bias) of the HPLC method for the determination of nicotine and cotinine

| Nominal conc. (μg/ml) | Observed conc. (μg/ml)[a] | RSD (%) | Bias (%) |
|---|---|---|---|
| Intra-assay of nicotine | | | |
| 0.25 | 0.264 ± 0.024 | 9.1 | −5.6 |
| 0.50 | 0.483 ± 0.020 | 4.1 | 3.4 |
| 1.0 | 1.004 ± 0.028 | 2.8 | −0.4 |
| 5.0 | 5.020 ± 0.046 | 0.9 | −0.4 |
| 10.0 | 10.06 ± 0.23 | 2.3 | −0.6 |
| 50.0 | 49.71 ± 0.33 | 0.7 | 0.6 |
| 100 | 100.1 ± 0.14 | 0.1 | −0.1 |
| Inter-assay of nicotine | | | |
| 0.25 | 0.249 ± 0.008 | 3.2 | 0.4 |
| 0.50 | 0.498 ± 0.009 | 1.8 | 0.4 |
| 1.0 | 1.004 ± 0.017 | 1.7 | −0.4 |
| 5.0 | 4.999 ± 0.002 | 0.04 | 0.02 |
| 10.0 | 10.08 ± 0.07 | 0.7 | −0.8 |
| 50.0 | 49.58 ± 0.33 | 0.7 | 0.8 |
| 100 | 100.0 ± 0.09 | 0.1 | −0.1 |
| Intra-assay of cotinine | | | |
| 0.05 | 0.048 ± 0.004 | 8.3 | 4.0 |
| 0.10 | 0.101 ± 0.002 | 2.0 | −1.0 |
| 0.50 | 0.503 ± 0.007 | 1.4 | −0.6 |
| 1.0 | 1.002 ± 0.007 | 0.7 | −0.2 |
| 5.0 | 4.988 ± 0.062 | 1.2 | 0.2 |
| 10.0 | 10.04 ± 0.06 | 0.6 | −0.4 |
| 50.0 | 50.12 ± 0.31 | 0.6 | −0.2 |
| 100 | 99.93 ± 0.15 | 0.15 | 0.07 |
| Inter-assay of cotinine | | | |
| 0.05 | 0.046 ± 0.002 | 4.3 | 8.0 |
| 0.10 | 0.101 ± 0.003 | 3.0 | −1.0 |
| 0.50 | 0.506 ± 0.004 | 0.8 | −1.2 |
| 1.0 | 0.997 ± 0.002 | 0.2 | 0.3 |
| 5.0 | 4.993 ± 0.027 | 0.5 | 0.1 |
| 10.0 | 10.05 ± 0.04 | 0.4 | −0.5 |
| 50.0 | 50.28 ± 0.12 | 0.2 | −0.6 |
| 100 | 99.86 ± 0.04 | 0.04 | 0.1 |

[a] Data are expressed as mean ± SD ($n$ = 6).

Table 2
In vivo microdialysates recoveries from rat blood and brain

| Concentration (μg/ml) | Recovery[a] |
|---|---|
| Nicotine | |
| Blood (0.5–5.0) | 27.24 ± 2.24 |
| Brain (0.5–5.0) | 3.22 ± 1.15 |
| Cotinine | |
| Blood (0.1–1.0) | 32.61 ± 3.56 |
| Brain (0.1–1.0) | 3.40 ± 1.87 |

[a] Data are expressed as mean ± SD ($n$ = 3).

brain samples. The concentrations versus time curves of mean unbound levels of nicotine and cotinine in rat blood and brain was shown in Fig. 4. The pharmacokinetic profiles of unbound nicotine and cotinine in rat blood and brain are presented in Table 3. The results indicated that both blood and brain levels of nicotine decline rapidly after drug adminis-

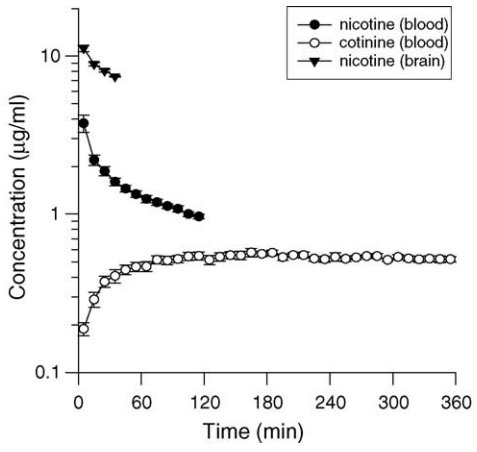


Fig. 4. Mean unbound levels of nicotine and cotinine in rat blood and brain after nicotine administration (2 mg/kg, i.v.); each point is a mean ± SEM from six values.

Table 3
Estimated pharmacokinetic parameters for nicotine and cotinine in rat blood and brain following nicotine administration (2 mg/kg, i.v.)[a]

| Parameters | Nicotine | | Cotinine blood |
|---|---|---|---|
| | Blood | Brain[b] | |
| $t_{max}$ (min) | – | – | 233 ± 35 |
| $C_{max}$ (μg/ml) | – | – | 0.62 ± 0.02 |
| AUC (min μg ml) | 414 ± 23 | 1204 ± 91 | 179 ± 7 |
| MRT (min) | 180 ± 12 | 108 ± 14 | 192 ± 3 |
| $t_{1/2}$ (min) | 143 ± 8 | 76.5 ± 9.6 | – |
| $AUC_{brain}/AUC_{blood}$ | – | 2.91 | – |

[a] Data are expressed as mean ± SEM ($n = 6$).
[b] Data are derived form four concentration–time points.

tered and nicotine had easy access to brain tissues. While cotinine remained at a consistent level in blood, it did not appear in brain dialysates.

The presence of nicotine metabolites in brain is an important factor to consider because nicotine biotransformation products have been shown to be pharmacologically active [5–7], and their presence in the brain constitutes a potential contribution to the neuropharmacological effects resulting from nicotine exposure. Unfortunately, the amount of cotinine might be too low to be detected in brain tissue or the measured concentrations were below the detection limit of the present analytical method. On the other hand, the brain penetration of nicotine, defined as the brain-to-blood coefficient of distribution ($k$ value), was calculated as the AUC of nicotine in brain divided by the AUC of nicotine in blood ($k = AUC_{brain}/AUC_{blood}$) [20]. The $k$ value of unbound nicotine in brain following nicotine administration was 2.91. It indicates that nicotine was easily and relatively predominately diffused into striatum areas of rat brain and rapidly equilibrated between the peripheral compartment and brain tissue (Fig. 4).

Plasma cotinine concentration is of special interest as a qualitative and quantitative indicator of nicotine intake. It is present in the blood of smokers at a much higher concentration than nicotine [21]. Because of its long half-life, there is much less fluctuation in the cotinine than the nicotine concentration throughout the day. In the present study, the cotinine concentration in the plasma was maintained without steep fluctuation for 6 h (Fig. 4), supporting the previous findings both in human and animals [21,22]. Hence, cotinine is the analyte of choice as it fulfills the prerequisites of specificity and retention time (18–20 h) and is found at detectable levels [23,24].

The simultaneous determination of nicotine and cotinine has been reported in the literature. Some of these methods require a large sample volume, utilize many extraction steps and reagents, while others are unable to measure both nicotine and cotinine simultaneously [25,26]. In the present study, we describe a rapid, simple and cost-saving method for the simultaneous determination of nicotine and cotinine in rat blood and brain tissue utilizing microdialysis sampling technique coupled with HPLC–UV. Because there are no loss of biological fluids, microdialysis offers many advantages such as continuous monitoring of analyte concentrations in the extracellular compartment of the same animal, less biological fluid loss and, therefore, minimal stress on hemodynamics [20]. It also provides protein-free samples that can be directly injected into a liquid chromatographic system for continuous in-vivo monitoring of unbound drugs in blood, other biological fluids and tissues. Compared to the microdialysis techniques, other assay methods which extract drugs from biological samples may have to go through a relatively complicated process of biological sample cleanup before they can be analyzed. However, the present HPLC–UV system for determining nicotine and cotinine is less sensitive than some GC–MS or LC–MS systems [27–29]. Further researches utilizing more sensitive chromatographic system coupled with microdialysis technique may provide more efficient way to perform pharmacokinetic studies.

In conclusion, we have developed a specific and rapid HPLC method for the simultaneous determination of protein-unbound nicotine and cotinine in blood and brain tissue. Current data obtained from rats suggest that nicotine has easy access to the central nervous system, and cotinine exhibits a long retention time and accumulates in blood.

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