Quantification of Dopamine in Brain Microdialysates with High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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The dopamine level in the brain and the mesolimbic dopaminergic system are responsible for nicotine addiction. In the present study, extracellular dopamine in nucleus accumbens was collected by a brain microdialysis technique. Also a sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method with a gradient elution procedure was developed for a precise determination of dopamine in brain dialysate. The retention time of dopamine was about 11.32 min. The linear range was 20 – 1000 pg/mL. The limit of detection (LOD) and the limit of qualification (LOQ) were 5 and 20 pg/mL, respectively. The recovery ranged from 98.2 to 109.0%, and both the intra-RSD and inter-RSD were below <9%, respectively. The probe recovery for dopamine in this brain microdialysis experiment was about 25%. Finally, the dopamine concentrations in the rat brain microdialysates were determined, and the pharmacokinetics of extracellular dopamine in the brain nucleus accumbens after an intravenous injection of nicotine was successfully evaluated.

Keywords Dopamine, microdialysis, brain microdialysate, high-performance liquid chromatography-tandem mass spectrometry

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Introduction

Dopamine (DA) is an important neurotransmitter in the brain, and the level of DA in the central nervous system is responsible for attention, sleep, mood, some diseases as well as drug addiction, and so on.¹ Previous studies have demonstrated that the concentration of DA in the brain would increase after tobacco use, and that DA is also implicated in tobacco addiction.² Tobacco addiction is generally related to the mesolimbic dopaminergic system (MLDS), which consists of ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala and so forth.³ After tobacco use, nicotine (NIC), the most primary alkaloid in tobacco, was delivered into the brain and bonded to the acetylcholine receptor (AChR), while raising the extracellular DA level in NAc. Then the mesolimbic dopaminergic reward system was activated to produce pleasure feelings, which was the main mechanism of tobacco addiction.^{4–6} Tobacco has been used throughout the world for several centuries. Owing to vigorous propaganda from the World Health Organization (WHO) and most governmental agencies, increasing focus has been put on the harm of cigarettes, and the development of smokeless tobacco products.⁷ Currently, moist snuff, snus, chewing tobacco, nicotine patches and other types of smokeless tobacco products have been listed. Since different tobacco

products have different nicotine delivery systems, pharmacokinetics and bioavailability,⁸ the concentration of nicotine and/or its relative metabolites in brain tissue⁹ and many body fluids were determined after the treatment of tobacco products.^{10–13} However, fewer studies have paid attention to determining the DA neurotransmitter after NIC exposure, while DA was the main substance responsible for the physiological and psychological effects of tobacco use and tobacco addiction.

The DA concentration and its changes can be real-time monitored by a microdialysis technique, which was developed in the 1980s. It is an emerging, but widely used, technology for sampling extracellular fluid.^{14,15} For microdialysis experiments, a semi-permeable membrane was implanted into a sampling region, followed by the perfusion of a suitable perfusate. With the perfusion process, some extracellular small molecules are transported into the probe interior perfusate by passive diffusion, while biomacromolecules are excluded, so the collected microdialysis samples can be analyzed directly without complex sample processes. Due to the features of minimal invasion and real-time sampling, microdialysis has generally been used in the collection of intracerebral neurotransmitters samples in targeted brain areas.^{16–18} In the present study, a brain microdialysis probe was placed into a dopaminergic reward system-related area, NAc, where the DA level can reflect the intake of NIC in the brain after a tobacco products treatment.

For brain microdialysis, the concentrations of DA in samples are so low (picomole concentrations) that high sensitivity and accuracy of the analysis method are highly required.¹⁹ So far, different analytical techniques have been used to determine the

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DA concentration in biological samples, such as a fluorimetric assay method,²⁰ high-pressure liquid chromatography coupled with electrochemical detection (HPLC-ECD),^{21–23} high-pressure liquid chromatography with fluorescence detection (HPLC-FL),²⁴ high-pressure liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) and so on.^{25,26} Due to the property of DA's electrochemistry, it has been widely analyzed by HPLC-ECD, but this method requires lengthy equilibration, and the retention time is the only identification signal.²⁷ Compared with other analysis methods, HPLC-MS/MS is more sensitive and specific, and now many types of biological samples have been determined by HPLC-MS/MS.^{12,28} Therefore, HPLC-MS/MS was optional to be used to determine the concentration of DA in the brain microdialysate.

In the present study, the microdialysis technology and HPLC-MS/MS were combined to develop a simple, convenient and sensitive method for the real-time quantification of extracellular DA in the brain NAc of freely moving rats after NIC injection. Then, the pharmacokinetics of DA in brain NAc was determined and evaluated.

Experimental

Materials and animals

The standard substances of DA were obtained from Sigma-Aldrich (USA). Mass spectrometry methanol and acetic acid were purchased from Merck (Germany). Ringer's solution was kindly donated by Zhongshan Hospital (Shanghai, China). CMA 20 Elite microdialysis probes (membrane length, 10 mm), CMA 12 microdialysis probes (membrane length, 4 mm) and CMA 12 Guide Cannula were obtained from CMA (Sweden). Double distilled water was purified using a Millipore Simplicity System (Millipore, Bedford, MA, USA).

Male Wistar rats weighing 250–300 g were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The animals used for the experiment were treated according to protocols that had been evaluated and approved by the ethical committee of Fudan University.

In vivo microdialysis in free moving rats

The microdialysis system consisted of a CMA 400 syringe pump, a CMA 130 *in vitro* stand, a CMA 120 system for freely moving animals, and a CMA 470 refrigerated fraction collector. The Ringer's solution was used as the microdialysis medium.

Wistar Rats were anaesthetized with a 10% chloral hydrate solution (300 mg/kg, intraperitoneally) and placed in a stereotaxic apparatus (Stoelting, USA) on a flat-skull position. An intracerebral guide cannula was implanted, while aiming at the accumbens nucleus (NAc) at the following coordinates relative to bregma: Anteroposterior (AP), +1.85 mm; Mediolateral (ML), –1.4 mm; Dorsoventral (DV), –7.8 mm (from dura); according to the rat brain atlas of Paxinos and Watson.²⁹ It was then fixed to the skull with three stainless-steel screws and dental cement. After surgery, the rats were housed individually in Macrolon cages, and allowed to recover for 2 days before performing the *in vivo* microdialysis procedure.

Before microdialysate sampling, brain microdialysis probes were inserted into the guide cannulas in rats. The probes were perfused with Ringer's solution at a uniform flow rate of 2 μ L/min, controlled by a CMA 400 syringe pump until the experiment was over. After 1-h of equilibration, three blank dialysates were collected every 20 min, followed by the intravenous (i.v.) administration of a nicotine solution at a dose of 0.5 mg/kg. Dialysis samples were collected every 10 min in

Table 1 HPLC gradient elution procedure

Time/ min	Eluent A, %	Eluent B, %	Flow rate/ mL min ^{–1}
0	98	2	0.2
6	98	2	0.2
14	60	40	0.2
16	60	40	0.2
24	98	2	0.2
30	98	2	0.2

Table 2 MRM parameters of DA

Compound	MRM (<i>m/z</i>)	Dwell time/ ms	Declustering potential/ V	Collision potential/ V	Collision cell exit potential/V
DA 1	154.1→137.1	100	60	15	13
DA 2	154.1→91.0	100	60	32	13

the first 2 h, and then every 20 min in the next 2 h after nicotine administration. Before sampling, to each collection tube was added 20 or 40 μ L of 0.2 M HCL containing 0.2 μ M Vitamin C (Vc) as a DA stabilizer in advance. The refrigerated collector was set at 4°C during the experiment. After each sampling, the sample was directly subjected to HPLC-MS/MS. After the experiment was finished, the correct localization of the brain probes was verified, and only rats with appropriate probe placement were included in the experiments.

HPLC-MS/MS method

The HPLC-MS/MS system consists of an Agilent 1290 HPLC system (America, Agilent Corp.) and a QTRAP 5500 tandem mass spectrometry (America, AB SCIEX Corp.), controlled by an Analyst workstation. A Sigma HS-F5 (2.1 $\times$ 150 mm, 3 μ m, US) was used to conduct chromatographic separation. The HPLC eluent contained a 0.5% acetic acid water solution (Eluent A) and methanol (Eluent B), and the gradient elution procedure was performed according to Table 1. The column temperature was maintained at 40°C, and a 10- μ L of sample solution was injected for each run with the autosampler temperature at 4°C. To limit the salts to enter the MS/MS system, the sample was eluted into waste for the first 5 min, and then switched to the MS/MS system for each run. Each chromatogram was recorded from 6 to 16 min.

Mass spectrometric data were acquired in the positive ion mode with electrospray ionization (ESI) and multiple reactions monitoring (MRM) mode. Analyte-specific declustering potential, collision energies and a MRM transitions are provided in Table 2. The spray voltage was 4500 V and the source temperature was 600°C. The nebulizer gas pressure was set at 60 psi, the auxiliary gas pressure was set at 65 psi and the curtain gas pressure was 20 psi.

Method validation

The stock solution of DA (1 mg/mL) was freshly prepared in methanol, stored at under –20°C and equilibrated to room temperature before use. Calibrators and quality-control (QC) working solutions were prepared in a Ringer's solution containing 0.1 M HCl and 0.1 μ M Vc.

A specificity evaluation was performed to confirm that the analyzed signals belong to the expected analyte, and that they were not interfered by other substances by this HPLC-MS/MS

method. The calibration solution with 40 pg/mL of DA, a blank Ringer's solution containing 0.1 M HCl and 0.1 μ M Vc, brain microdialysis samples after NIC administration were determined by the above HPLC-MS/MS method. All samples were analyzed by the HPLC-MS/MS system controlled by the Analyst workstation. The software generated the analyte characteristics, including the concentration, quantifying ion peak height, quantifying ion peak area, and retention time.

The working solution for calibration curve were prepared at concentrations of 20, 50, 100, 200, 500, 1000 pg/mL, and each concentration level was analyzed for 6 replicates over 5 days. A calibration curve was generated by the ions quantification peak area (y) versus the DA concentration levels (x), and the weighing factor was $1/x$ to obtain a smaller fitting error at low concentration. A correlation coefficient (r) above 0.990 and $F > F_{\alpha}$ were required for determining the linearity.³⁰ The lowest concentration of the calibration curve was gradually diluted and analyzed for determining the LOD and LOQ. The LOD was defined as the concentration level at which the signal-to-noise ratio (S/N) equals to or is greater than 3 for selected ion transitions. Similarly, the LOQ was defined as the concentration level at which the signal-to-noise ratio (S/N) equals to or is greater than 10.

The recovery, representing accuracy, of an analyte is the ratio of the measured amounts to the true amounts of the analyte, which should be conducted at three concentration levels (low, medium, and high). Generally, the recovery should be in the range of 85 – 115%. The precision represents the reproducibility of an analytical method, and good precision is the premise of good assay results. In a general way, the target QC concentrations included three levels: the low QC concentration is lower than three-times LOQ; the medium QC concentration is the median concentration value of the calibrate curve; and the high QC concentration is around 80% of the upper limit of quantification. In the present study, 40, 200 and 800 pg/mL concentration levels were selected as quality-control (QC) concentrations for evaluating accuracy and precision. The accuracy and precision of the analytical method were determined by quantitating three replicates of QC samples, and were represented by the recovery and RSD values, respectively.

DA was easy to be oxidized, and should be kept in acid solvent under low temperature. In the present study, a mixture of HCL and Vc (0.1 M HCl and 0.1 μ M Vc) was selected as the protective agent for DA, and the protective effects at three DA concentration levels (20, 100 and 400 ng/mL) over 6 h at 4°C were evaluated.

Recovery of microdialysis probe

Many factors can affect the efficiency of dialysis (probe recovery, PR), such as the temperature, perfusion rate, and the nature and dimensions of the probe.³¹ Thus, the calibration of probes is needed and a stable physiological state of the experimental animals should be maintained as much as possible. A microdialysis probe was placed into a Ringer's solution containing the analyte, which was maintained at 37°C and perfused with an analyte-free Ringer's solution at a speed of 2 μ L/min. Then, the analyte concentration was determined by the HPLC-MS/MS method, as described above. The determination was conducted with the concentrations of the analyte at low, medium and high QC concentrations, and each concentration level was determined three times. The probe recovery was calculated according to a previous paper.³²

Pharmacokinetics of extracellular DA in NAc

DA concentrations in brain dialysis samples were determined

Fig. 1 Representative chromatograms of monitored transition ions of DA obtained from (A) a blank Ringer's solution containing 0.1 M HCl and 0.1 μ M Vc, (B) calibration solution with 40 pg/mL of DA, and (C) brain microdialysis samples after NIC administration.

by the validated HPLC-MS/MS method, as described above. The real concentration of DA *in vivo* was calculated by the concentration of DA in microdialysate divided by PR. The concentrations of extracellular DA in NAc were plotted versus the time to draw the pharmacokinetics curves.

Results and Discussion

In this study, all biologic samples were obtained by brain microdialysis. Therefore, in the methodological validation section the biological matrix should be brain microdialysates without NIC exposure. However, abundant brain microdialysates

were not easy to achieve, and there were DA in brain microdialysates, so it was replaced by the common perfusate Ringer's reagent.

In the brain microdialysis experiment, a Ringer's solution was perfused into the microdialysis system; thus, many salts existed in collected dialysate samples. Because DA is a polar compound, it is weakly retained in the common C_{18} chromatographic column. The pentafluorophenylpropyl stationary phase can produce a strong dipole interaction with polar molecules, which was suitable for separation of polar molecules from the inorganic salts in dialysate samples.^{27,33} Therefore, a Sigma HS-F5 column with pentafluorophenylpropyl stationary phase was used to conduct chromatographic separation in the present study. To limit the effect of the salts on the MS/MS signal of DA, the salts were excluded in the MS/MS system by eluting the sample into waste for the first 5 min of each run.

A calibration solution with 40 pg/mL of DA, a blank Ringer's solution containing 0.1 M HCl and 0.1 μ M Vc, brain microdialysis samples after NIC administration were analyzed by the above-mentioned HPLC-MS/MS method. As shown in

Fig. 1, for calibration solution, the ion peak of DA (m/z 154.1 $\rightarrow$ 137.1, 154.1 $\rightarrow$ 91.0) appeared at about 11.32 min (Fig. 1B). For collected brain microdialysis samples after NIC administration, the ion peak of DA (m/z 154.1 $\rightarrow$ 137.1, 154.1 $\rightarrow$ 91.0) (Fig. 1C) appeared at about 11.26 min. The difference of the retention times for these two samples was within the normal instrument fluctuation. Both the blank Ringer's solution and the biological matrix in brain microdialysis showed no interference in any DA determination.

The linearity was evaluated by the analysis of six levels of calibrator solutions, as mentioned above. As presented in Table 3, all of the regression coefficients met the laboratory's criteria ($r > 0.99$) and $F > F_{\alpha}$. In many reports linearity was simply evaluated by the correlation coefficient, r , but this might be not sufficient and F-test is also needed.³⁰ The linear range for DA quantification was from 40 to 1000 pg/mL.

As shown in Table 3, the LOD and LOQ values of DA were 5 and 20 pg/mL, respectively. Representative chromatograms of the monitored transition ions of DA at 20 pg/mL (LOQ) are shown in Fig. 2. The S/N values were 13.5 and 10.9 for m/z 154.1 $\rightarrow$ 137.1 and m/z 154.1 $\rightarrow$ 91.0, respectively. Our HPLC-MS/MS method gave a much lower LOD than those obtained by the ultra-fast liquid chromatography coupled to electrochemical detection (UHPLC-ECD) method (0.8 nM, equivalently 122.5 pg/mL)³⁴ and other traditional coulometric detection methods.³⁵ In addition, our LOQ was also far better than those determined by the HPLC-MS/MS method (26.8 and 122.5 pg/mL, respectively),^{27,28} and was lower than the concentrations measured in tissue extracts from most brain areas in previous publications.^{27,28,32}

The recovery and precision of the methodology were performed with the low, medium and high concentration levels

Table 3 Linearity, LOD and LOQ of DA (6 concentration levels)

MRM	Regression equation	Weight	LOD/ pg mL ⁻¹	LOQ/ pg mL ⁻¹
154.1 $\rightarrow$ 137.1	$y = 191.22x + 16444$ ($r = 0.9995$)	1/c	5	20
154.1 $\rightarrow$ 91.0	$y = 132.78x + 10693$ ($r = 0.9992$)	1/c	5	20

Fig. 2 Representative chromatograms of monitored transition ions of DA at 5 pg/mL (A, B) and 20 pg/mL (C, D). (A) m/z 154.1 $\rightarrow$ 91.0, $S/N = 4.2$; (B) m/z 154.1 $\rightarrow$ 137.1, $S/N = 4.6$; (C) m/z 154.1 $\rightarrow$ 91.0, $S/N = 10.9$; (D) m/z 154.1 $\rightarrow$ 137.1, $S/N = 13.5$.

Table 4 Recovery and precision of each analyte ($n = 3$)

MRM	Concentration/ ng mL ⁻¹	Recovery, %	Intra-RSD, %	Inter-RSD, %
154.1→137.1	40	109.0	4.8	8.6
	200	98.2	2.9	6.7
	800	99.3	2.3	4.9
154.1→ 91.0	40	106.1	4.1	8.9
	200	98.6	3.3	6.6
	800	101.2	3.0	5.9

Table 5 DA stability over 6 h at 4°C

DA concentration/ pg mL ⁻¹	2 h, %	4 h, %	6 h, %
40	99.5 ± 1.3	99.3 ± 1.9	97.8 ± 2.3
200	99.3 ± 1.7	99.2 ± 1.5	99.6 ± 1.2
800	99.7 ± 2.2	99.8 ± 2.1	99.6 ± 1.8

Values were means ± SD ($n = 4$).

given in Table 4. The recovery of the three concentration levels ranged from 98.2 to 109.0%, and the RSD values were all lower than 9%. The recovery and precision met the laboratory's criteria to quantitatively determine the concentration of DA in brain microdialysates.

DA is easy to be oxidized, and an acidic solution is a good candidate to act as a protective agent. In order to choose an ideal DA protective agent, aqueous solutions of 0.1 M HCl, 0.1 μM Vc, 0.1 M HCl containing 0.1 μM Vc, and 0.1 M HClO₄ containing 0.1 mM sodium pyrosulfite were investigated as candidates of DA protective agents. The results indicated that the protection effect of 0.1 M HCl containing 0.1 μM Vc was the best among those four protective agents (data not shown). Therefore, in the present study, 10 μL of 0.1 M HCl containing 0.1 μM Vc was added in the sample tube to stabilize DA in advance before sampling. In order to evaluate the stability of DA in 0.1 M HCl containing 0.1 μM Vc, three concentration levels of calibrator solutions were monitored over 6 h at 4°C. As shown in Table 5, DA was rather stable for over 6 h at 4°C.

The microdialysis probes were characterized *in vitro* before implantation, and used in our experiments. In the present study, the probe recovery of DA at low, medium and high concentrations were 26.2 ± 1.5, 25.6 ± 2.6 and 24.3 ± 2.3%, respectively, and the DA concentration did not affect the PR, which agreed well with the product manual. *In-vitro* testing of 4 mm microdialysis probes after 6–8 h of use in the NAc showed no significant decrease in the probe recovery (data not shown). According to the probe recovery, the baseline value of extracellular DA in NAc was 335 ± 21 pg/ml ($n = 6$).

As shown in Fig. 3, the DA concentration, expressed as percentage of baseline, was plotted with time. After intravenous NIC injection, the extracellular DA concentration in NAc increased sharply and the peak appeared at 20 min, namely, $T_{\max}$ was 20 min. Compared with the acute subcutaneous injection of NIC ($T_{\max} = 180$ min),³⁶ intravenous NIC injection caused DA to release much faster. The $C_{\max}$ of DA was 1.6-fold that of the baseline value before nicotine injection, which was comparable to that induced by subcutaneous injection of 0.3 mg/kg NIC.³⁹ Interestingly, 20 min post injection, the DA concentration slowly decreased with time, which was similar to the results observed in another report.³⁷

Fig. 3 Pharmacokinetics curve of extracellular DA in NAc. The rats were intravenously injected with 0.5 mg/kg NIC and the extracellular levels of DA in NAc were monitored. The control was injected with the same volume of saline. The values are expressed as a percentage of the preinjection baseline DA levels. Data were expressed as means ± standard error of the mean (SEM) ($n = 6$).

In this study, Ringer's solution was selected as perfusate, and the collected microdialysis samples were analyzed by HPLC-MS/MS directly without any complicate processing. Since abundant salt would induce significant ion suppression and sensitivity reduction, and the DA concentration level *in vivo* is very low, it is necessary that the chromatogram column should be able to separate DA from other salts so as to enhance the detection sensitivity. Given that, a HS-F5 column, having good retention ability for polar compounds, was selected. The results showed that it achieved good separation for DA and interferential salts. Besides, in order to reduce the effects of salts from Ringer's solution as much as possible, the salts were excluded in the MSMS system for the first 5 min of each run. Moreover, the analytical instrument was timely cleaned so as to remove the ion suppression effect of salts on the MSMS signal of DA.

Conclusions

In the present study, a sensitive and selective HPLC-MS/MS method for DA analysis was successfully developed and validated. This method has been applied to determine the real-time pharmacokinetics of extracellular DA in the brain NAc of freely moving rats after NIC administration with the microdialysis technique. The study provided a solid foundation for assessing tobacco exposure and developing novel tobacco products.

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