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# Determination of the penetration of 9-fluoropropyl-(+)-dihydrotetrabenazine across the blood–brain barrier in rats by microdialysis combined with liquid chromatography–tandem mass spectrometry

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ABSTRACT

To evaluate the penetration of the blood–brain barrier by 9-fluoropropyl-(+)-dihydrotetrabenazine (AV-133), microdialysis probes were implanted simultaneously into rat blood and brain, and a liquid chromatography–tandem mass spectrometric method was developed and validated to monitor the AV-133 concentration in the microdialysates. The chromatographic separation was performed on an XTerra $C_{18}$ column (150 mm × 2.1 mm i.d., 5 μm particles) with gradient elution. The mass spectrometer was operated in positive mode using electrospray ionization. The analytes were measured using the multiple-reaction-monitoring mode. The calibration curves were linear over the range of 5.00–1000 ng/mL AV-133, with a coefficient of determination >0.995. The accuracies ranged from 99.5% to 105.0% and the precisions were <10% for AV-133. This method was used to determine the concentrations of AV-133 and its pharmacokinetics in the brains and blood of rats. The blood and brain concentration–time profiles for AV-133 were obtained, and the blood–brain barrier penetration was evaluated.

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## 1. Introduction

Positron emission tomography (PET) is an advanced imaging technology used to assess biological, physiological, and pharmacological functions using imaging agents containing short-lived $\beta^+$-emitting radionuclides, such as $^{11}C$, $^{13}N$, $^{15}O$, and $^{18}F$. An ideal PET imaging agent should have enhanced selectivity, optimal affinity, and appropriate pharmacokinetics and metabolic fate [1–3]. Generally, the determination of drug tissue penetration and the drug pharmacokinetics in the target tissues are important in PET drug development. A better understanding of the disposition of PET drugs in the target tissues early in drug development facilitates the appropriate channeling of new imaging agents into clinical disease diagnosis.

The pharmacokinetics of PET imaging agents can be evaluated in a living animal model using several time points or with small-animal PET imaging. The method is very sensitive, but the PET signal comes from the total radioactivity in a given tissue. It can neither provide chemical information about this radioactivity nor identify the radioprobe and its radioactive metabolites [4].

Microdialysis sampling can monitor regional chemical information when a probe is implanted into a tissue's extracellular fluid. It is possible to continuously and simultaneously sample from multiple sites by implanting more than one probe in the same animal [5]. Microdialysis sampling coupled to an appropriate analytical technique can be used to study the target site pharmacokinetics and metabolism [6,7]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a sensitive analytical technique that can be used to detect microdialysis samples.

9-[$^{18}$F]fluoropropyl-(+)-dihydrotetrabenazine ([$^{18}$F]AV-133) is a potential imaging agent for mapping the vesicular monoamine transporter type 2 sites in the rat brain. The excellent binding affinities of [$^{18}$F]AV-133 have been observed in the rat striatum and hypothalamus. It is currently in phase II clinical trials to establish its usefulness in the diagnosis of neurodegenerative diseases, including dementia with Lewy bodies and Parkinson's disease (PD) [8–10].

The pharmacokinetics in the monkey brain has been reported using PET scans based on radioactivity [8]. In this study, microdialysis probes were simultaneously inserted into the jugular veins and striata of anesthetized rats. A liquid chromatography–tandem mass spectrometric method was developed and validated to determine the unradiolabeled AV-133 in the microdialysis samples from the rat brains and plasma after the administration of a single i.v. dose of AV-133. The pharmacokinetic parameters in the brain and blood were determined and the penetration of the blood–brain barrier (BBB) by AV-133 was evaluated. This study reports a simultaneous and useful method of microdialysis combined with LC–MS/MS

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that is suitable for evaluating BBB penetration by compounds in brain-targeting research.

## 2. Experimental

### 2.1. Chemicals and reagents

AV-133 was synthesized with previously developed methods [11]. HPLC-grade ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was purchased from Merck (Damstadt, Germany). Distilled deionized water (18.2 MΩ) was made with an Elga Classic UVF Purification System (Buckinghamshire, UK). All other chemicals and reagents were of analytical grade or better.

### 2.2. Animals

Male Sprague Dawley rats, 6–7 weeks old and 180–220 g, were obtained from the Vital River Laboratory Animal Co. Ltd. (Beijing, China). The rats were specific-pathogen free and were allowed to acclimate to their environmentally controlled quarters (temperature, 20–23 °C; humidity, 50 ± 5%; 12 h light/dark cycle) for 1 week before the experiments. Food and water were available ad libitum. All the animals were maintained according as the Chinese Government guidelines for the care and use of laboratory animals. The rats were fasted overnight before the day of the experiment.

### 2.3. Instruments and conditions

The HPLC system consisted of a Waters high-pressure gradient 1525μ high-pressure binary gradient pump (Waters, Milford, MA, USA), a model 7725i manual injection valve (Rheodyne, Cotati, CA, USA) equipped with a 10 μL sample loop and an on-line degasser. Mass spectra were detected with a Quattro micro triple quadrupole mass spectrometer (Waters). The data were processed with MassLynx 4.1 software.

Separation was achieved with an XTerra $C_{18}$ column (150 mm × 2.1 mm i.d., 5 μm particles; Waters); the mobile phases consisted of 1 mM ammonium acetate in water (A) and 1 mM ammonium acetate in acetonitrile (B). The gradient program was set at 10% B for the first 2.0 min, linearly increasing from 10% B to 80% B in the next 8.0 min, and then returning to 10% B by 12 min. The flow rate was 0.2 mL/min. The end time of the program was set at 20 min. The injection volume was 5 μL.

The mass spectrometer with an electrospray ionization (ESI) source was operated in positive mode. The desolvation temperature was maintained at 300 °C and the source temperature was 110 °C. The capillary voltage was set at 3.0 kV. Nitrogen was used as the cone gas and desolvation gas, and the flow rates were 30 L/h and 350 L/h, respectively. Argon was used as the collision gas. Quantification was performed using multiple reaction monitoring (MRM) of the transitions $m/z$ 366 → 348 and $m/z$ 366 → 320 for AV-133, at a cone voltage of 30 V and a collision energy of 20 eV. The dwell time was 0.1 s for each transition.

### 2.4. Preparation of stock solutions, calibration standards, and quality control (QC) samples

Ringer's solution, which was used as the perfusate for the microdialysis probes, consisted of 149 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 0.8 mM $MgCl_2$. The standard stock solution of AV-133 at a concentration of 1.00 mg/mL was prepared in ethanol and stored at −20 °C. The calibration standards were prepared by the serial dilution of the stock solution with Ringer's solution to obtain AV-133 concentrations of 5.00, 10.0, 20.0, 50.0, 100, 200, 500, and 1000 ng/mL. The QC solutions were similarly prepared at concentrations of 10.0, 100, and 750 ng/mL for AV-133 by weighing the reference substances separately.

The samples were directly analyzed by LC–MS/MS without prior sample purification, except for centrifugation at 3000 × $g$.

### 2.5. Assay validation

The method was validated for selectivity, linearity, precision, accuracy, recovery, matrix effect, and stability [12,13].

#### 2.5.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing blank blood and brain dialysates, blood and brain dialysates spiked with a standard solution of AV-133 (5.00 ng/mL), and blood and brain microdialysis samples after the intravenous administration of AV-133. The chromatograms were examined to determine the presence of any endogenous constituents that might potentially interfere with the analysis of AV-133.

#### 2.5.2. Linearity

The calibration curves for AV-133 were constructed using standard solutions containing AV-133 at eight concentrations in the range of 5.00–1000 ng/mL with weighted (1/$x$) least squares linear regression. Because the microdialysis samples were directly detected using LC–MS–MS without prior sample purification, except for centrifugation at 3000 × $g$, and the stability of analytical system was good, an external standard method was used to establish the calibration plots. The LLOQ was defined as the lowest concentration on the calibration curve at which acceptable accuracy (relative error [RE] within ±15%) and a precision (relative standard deviation [RSD] below 15%) could be obtained.

#### 2.5.3. Accuracy and precision

Precision and accuracy were assessed with the QC samples using five replicates containing AV-133 at three concentrations (10.0, 100, and 750 ng/mL) in Ringer's solution on 5 different days. Precision was calculated as the RSD within a single run and between different runs. Accuracy was expressed as the RE = ([determined concentration − nominal concentration]/nominal concentration) × 100%. The intra- and inter-day precision should not exceed 15% and the accuracy should be within ±15%.

#### 2.5.4. Stability

Three concentrations (10.0, 100, and 750 ng/mL) of QC samples in Ringer's solution were subjected to the conditions described below. Bench top stability was assessed by analyzing the QC samples left at the ambient temperature for 4 h. Short-term stability was determined by analyzing the QC samples kept in a refrigerator at 4 °C for 24 h. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (ambient temperature) cycles. Long-term stability was investigated by analyzing the QC samples after storage at −20 °C for 7 days.

#### 2.5.5. Matrix effect assessment

Because the microdialysate contained a reasonable amount of non-volatile salts, which generate high background noise and suppress the ionization of analytes, matrix effects should be carefully assessed when the microdialysis samples were analyzed directly by LC–MS/MS without prior sample purification. To exclude interferences from the inorganic salts in the samples, the mobile phase containing ammonium acetate was used and the chromatographic separation was accomplished by using gradient elution. Ammonium acetate can generate beneficial LC-electrolyte effects, including high sensitivity and negligible matrix interference [14].

In this study, the matrix effect was assessed by the comparison of three concentrations (10.0, 100, and 750 ng/mL) of QC standards in Ringer's solution and in the mobile phase. When the ratio value was 1, the responses in the mobile phase and in the dialysates were the same and there was no matrix effect [15].

*2.5.6. Recovery of the microdialysate*

The relative recovery of AV-133 with the microdialysis probe was estimated in delivery experiments. This approach assumes that the same relationship exists between the probe's delivery and the probe's recovery [15]. The delivery experiment was performed *in vivo* for each microdialysis probe. Perfusion solutions containing AV-133 (100 ng/mL) were passed through the microdialysis probes and separately into the rat blood and brain at a constant flow rate of 1 μL/min. One hour after probe implantation, the perfusate ($C_{perf}$) and dialysate ($C_{dial}$) concentrations of AV-133 were determined by LC–MS/MS. The *in vivo* relative recovery ($R_{dial}$) of AV-133 across the microdialysis probe was calculated with the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$.

### 2.6. Implantation of the microdialysis probe

The simultaneous collection of the dialysate from the blood and brain offers a useful approach to monitoring drug concentrations centrally and peripherally during drug administration. The procedure used for this study involved the implantation of two microdialysis probes: one into a specific brain region and the other into the jugular vein.

The rats were anesthetized with urethane (1.25 g/kg, i.p.) throughout the experiments and a temperature-controlled heating pad was used to maintain their body temperature at 37–38 °C. The fur in the abdominal region of each rat and the hair on the top of its skull were shaved and disinfected by swabbing the areas with ethanol.

The femoral vein was cannulated to allow i.v. injection and a sterile blood withdrawal catheter (O.D. 0.5 mm, length 24 mm; FEP i.v. catheter) containing heparinized saline (15 units/mL; sterile) was inserted into the vessel and secured in place with a suture.

The CMA 20 Elite blood microdialysis probe (10 mm membrane length; CMA Microdialysis, Solna, Sweden) was implanted into the jugular vein toward the rat's right atrium. The rat was then mounted on a stereotaxic frame in a flat-skull position. An intracerebral guide cannula was implanted into the striatum at the following coordinates relative to the bregma: anterior–posterior (AP) 0.2 mm, medial–lateral (ML) −3.2 mm, and dorsal–ventral (DV) −7.5 mm, according to the Paxinos and Watson atlas [16]. A hole was drilled for the placement of the guide cannula, which was fixed to the skull with two stainless-steel screws and dental cement. A CMA 12 brain microdialysis probe (4 mm membrane length; CMA Microdialysis, Solna, Sweden) was inserted into the guide cannula.

### 2.7. Dosing and sampling

After the implantation of the microdialysis probes, both probes were connected to a microinjection pump (CMA Microdialysis) and perfused with Ringer's solution at a flow rate of 1 μL/min. After an equilibration period of 1 h, the *in vivo* recovery experiment was performed. After the sample residue was washed out, AV-133 (5 mg/kg) was intravenously administered *via* the femoral vein in normal saline solution. The perfused samples were then collected for 4 h at 10 min intervals, and 5 μL of each dialysate was injected into the LC–MS/MS for analysis.

### 2.8. Pharmacokinetics and data analysis

The microdialysate concentrations of AV-133 were converted to unbound concentrations based on the relative recovery. Pharmacokinetic calculations were made for each individual set of animal data using the pharmacokinetic calculation software 3p87 (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

### 2.9. Histological localization of the microdialysis probe in rats

At the end of the experiment or several days after, animals were sacrificed by decapitation. The brain was quickly removed and frozen using liquid nitrogen to maintain the original volume and shape of the brain. Coronal sections of 20 μm thick were removed until the probe track placement was macroscopically identified. Slices corresponding to the photomicrographs were taken and stained using hematoxylin-eosin. Ten to fifteen photomicrographs and corresponding slices of the region of interest were taken for each brain [17].

## 3. Results and discussion

### 3.1. Optimization of the LC–MS/MS conditions

To determine the concentrations of AV-133 in the rat blood and brain, the LC–MS/MS conditions were optimized. Various mixture(s) of solvents (such as acetonitrile and methanol), and different buffers (such as ammonium acetate, acetic acid, and formic acid), together with varying gradient programs were tested to obtain high sensitivity and a low matrix effect. A mobile phase consisting of 1 mM ammonium acetate in water (A) and 1 mM ammonium acetate in acetonitrile (B) with the gradient program described above was found to be suitable. The chromatographic resolution, selectivity, and sensitivity of the method were good.

The protonated molecular ion of AV-133 was $m/z$ 366. Fig. 1 shows the product ion mass spectrum for AV-133 and its chemical structure. Following detailed optimization of the MS conditions, quantification was performed with MRM of the transitions $m/z$ 366 → 348 and $m/z$ 366 → 320.

### 3.2. Method validation

*3.2.1. Specificity and selectivity*

Under the given conditions, AV-133 was eluted at 8.62 min. The LC–MS/MS chromatograms of the blank blood and brain dialysates are shown in Fig. 2(A) and (B), respectively. Fig. 2(C) and (D) shows the chromatograms for the blood and brain dialysates spiked with 5.00 ng/mL AV-133, respectively. The LC–MS/MS chromatograms of AV-133 in the blood and brain dialysates after AV-133 administration (5 mg/kg, i.v.) are shown in Fig. 2(E) and (F), respectively. No detectable interfering peak with a retention time close to that of AV-133 was found in blood and brain dialysates, confirming good method selectivity.

*3.2.2. Linearity*

The calibration curves of AV-133 were linear over the concentration range of 5.00–1000 ng/mL. The typical calibration curve equation was $y = 152.6624x - 64.3462$ ($r^2 = 0.995$), where $y$ represents the peak area of each analyte and $x$ represents the plasma concentration of the analyte. The LLOQ was 5.00 ng/mL.

*3.2.3. Precision and accuracy*

The data describing the intra- and inter-day precision and accuracy for AV-133 determined from the QC samples are summarized

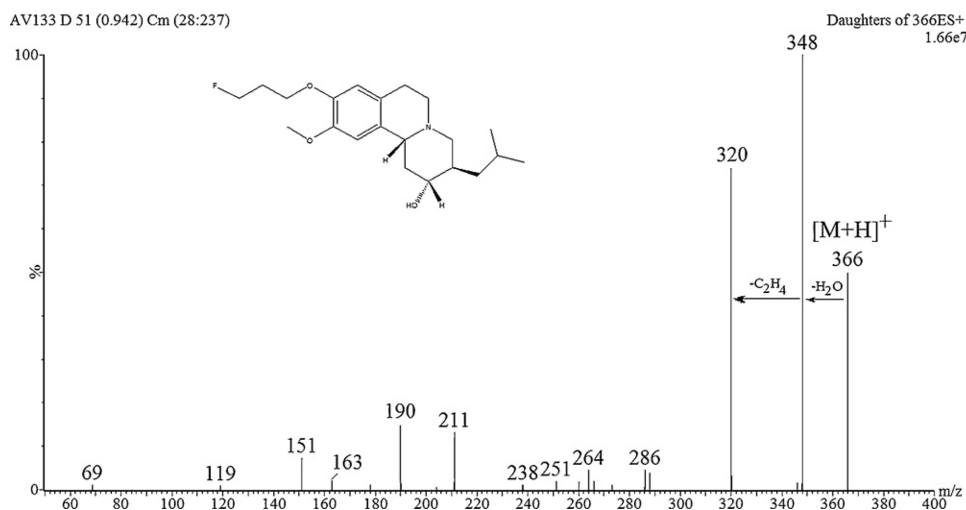


**Fig. 1.** The product ion mass spectra of [M+H]+ for AV-133.

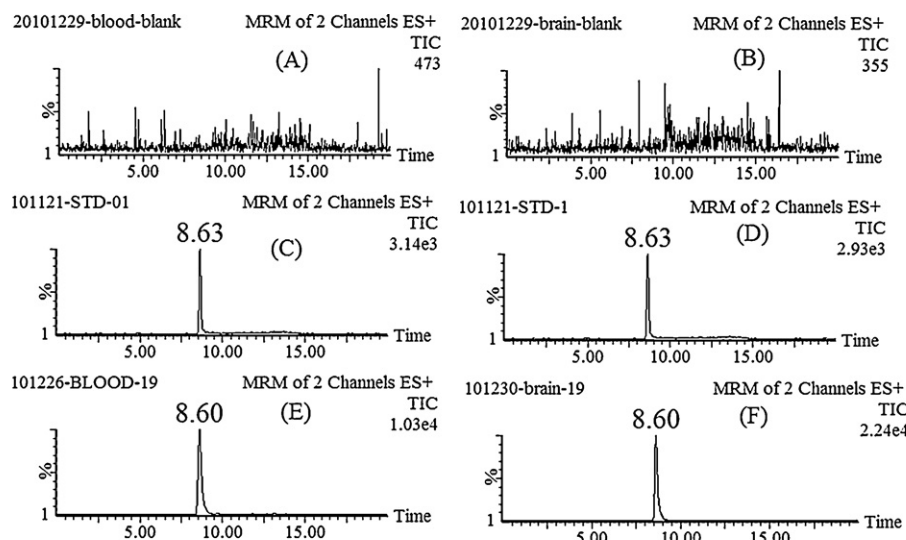


**Fig. 2.** Typical chromatograms of (A) blank blood dialysate and (B) blank brain dialysate from the microdialysis probe before drug administration; (C) blood dialysate and (D) brain dialysate spiked with AV-133 (5.00 ng/mL); (E) blood dialysate sample and (F) brain dialysate sample collected from the rat blood 100 min after AV-133 administration (5 mg/kg, i.v.).

in Table 1. The intra- and inter-day precisions were <10%, and the accuracies ranged from 99.5% to 105.0% for AV-133.

### 3.2.4. Stability

The stability of AV-133 in Ringer's solution was investigated under several storage and processing conditions. AV-133 was stable when stored at −20 °C for 20 days and after three freeze–thaw cycles in rat plasma (RE < 15%). AV-133 was also stable in Ringer's solution at the ambient temperature for 4 h (RE < 10%) and after its reconstitution at 4 °C for 24 h (RE < 10%). The data for AV-133 stability are given in Table 2.

### 3.2.5. Relative recoveries of the microdialysis probes

Based on the delivery experiments, the relative recoveries were determined to be 49.6 ± 2.3% for the plasma vascular probe and 33.4 ± 2.6% for the brain probe. The concentrations of AV-133 determined in the physiological samples were corrected for the relative recovery of the probe used.

### 3.2.6. Matrix effects

The matrix effects were calculated by comparing the peak areas of the standards at three concentrations in Ringer's solution and in the mobile phase. The results showed that the mean matrix effects

**Table 1**
Precision and accuracy of the LC–MS/MS method to determine AV-133 in Ringer's solution ($n = 5$ days, five replicates per day).

| Spiked concentration (ng/mL) | Intra-day ($n = 5$) | | | Inter-day ($n = 5$) | | |
|---|---|---|---|---|---|---|
| | Detected concentration mean ± SD (ng/mL) | RSD (%) | RE (%) | Detected concentration mean ± SD (ng/mL) | RSD (%) | RE (%) |
| 10.0 | 10.15 ± 0.47 | 4.7 | 1.5 | 10.5 ± 0.22 | 2.2 | 5.0 |
| 100 | 101 ± 7.8 | 7.8 | 1.0 | 102.6 ± 4.0 | 4.0 | 2.6 |
| 750 | 765 ± 60.75 | 8.1 | 2.0 | 746.25 ± 29.25 | 3.9 | −0.5 |

for AV-133 were 0.96 ± 0.02, 1.02 ± 0.08, and 0.98 ± 0.05, respectively. The values were all close to 1, so the matrix effects were low, and could be ignored in the quantitative analysis.

### 3.3. Pharmacokinetics and data analysis

The concentration versus time curves for AV-133 in anesthetized rat blood and brain after the administration of AV-133 (5 mg/kg, i.v.) are shown in Fig. 3. Each point represents an average of five determinations and the error bars are the standard deviations of the means. The pharmacokinetic parameters of AV-133 in the rat brain and blood are shown in Table 3. The half-life ($t_{1/2}$) of AV-133 in the blood was 51.35 min, which is consistent with a previous result (49.10 min) for the plasma pharmacokinetics of AV-133 [18]. The extra time taken here might be attributable to the dead volume between the sample site and the point of dialysate collection. The peak concentration ($C_0$) of AV-133 in the blood was 655.53 ng/mL, whereas AV-133 reached a peak concentration of 1062.95 ng/mL in the brain at 15 min, which suggests that AV-133

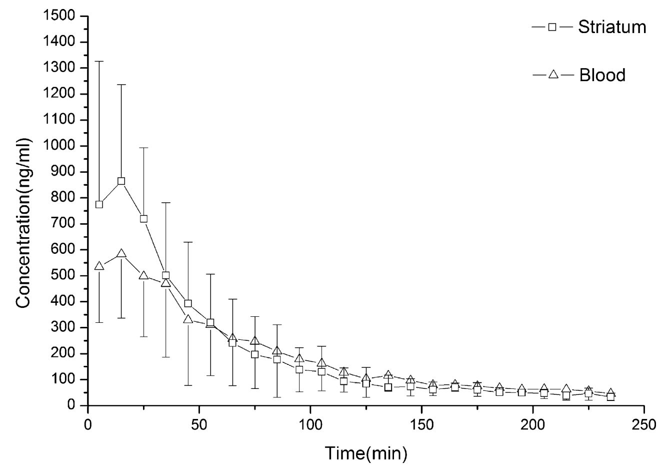


**Fig. 3.** Plasma concentration–time curves for AV-133 in rat blood (—△—) and in the rat striatum (—□—) after the i.v. administration of AV-133 (5 mg/kg).

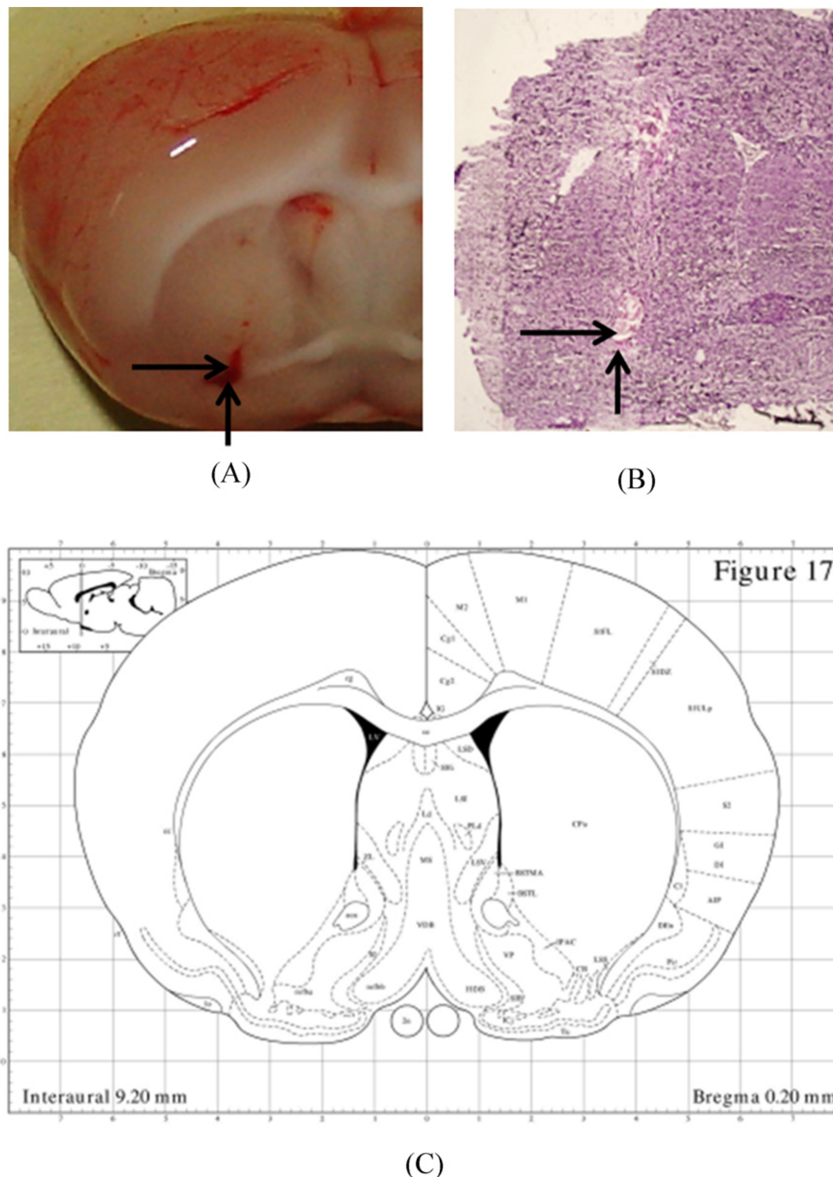


**Fig. 4.** The photomicrograph of (A) a coronal section of a whole rat brain; (B) a coronal stained slice of a rat brain; (C) the corresponding coronal diagram of Paxinos and Watson atlas. The arrows indicate the tip of the dialysis probe.

**Table 2**
Stability of AV-133 in Ringer's solution ($n = 5$).

| Spiked concentration (ng/mL) | Bench-top stability storage at ambient temperature for 4 h (%) | Short-term stability storage at 4 °C for 24 h (%) | Long-term stability storage at −20 °C for 7 days (%) | Freeze–thaw stability (%) |
|---|---|---|---|---|
| 10.0 | 1.2 | 5.6 | 13.5 | 3.7 |
| 100 | 4.2 | 7.1 | 1.7 | 0.7 |
| 750 | 5.2 | 3.8 | 3.0 | 3.3 |

**Table 3**
Pharmacokinetic parameters of AV-133 in rat blood and striatum microdialysis samples after i.v. administration of single AV-133 (5 mg/mL).

| Parameter | Blood | Striatum |
|---|---|---|
| $C_0$ (ng/mL) | 655.53 ± 293.35 | 1062.95 ± 545.36 |
| $t_{1/2}$(min) | 51.35 ± 11.73 | 34.10 ± 4.98 |
| *Vd* [(mg/kg)/(ng/mL)] | 0.009 ± 0.003 | 0.006 ± 0.002 |
| *Cl*(*s*) [mg/kg/min/(ng/mL)] | 0.00013 ± 0.00007 | 0.00012 ± 0.00006 |
| AUC (min ng/mL) | 50958.17 ± 2981.295 | 52370.26 ± 2719.080 |
| *Ke* (1/min) | 0.0141 ± 0.0036 | 0.0207 ± 0.0031 |

Values are mean ± SD of five animals.

is quickly distributed to the brain. This result is similar to that of a previous study in which the highest radioactivity uptake in the human brain was at 10 min after injection [19]. The blood-to-brain distribution ratio was defined by dividing the area under the concentration versus time curve (AUC) for the brain by that for the blood ($AUC_{brain}/AUC_{blood}$) [20]. The value was 1.03, which indicates that AV-133 readily crossed the BBB, and the degree of drug penetration into the brain was high.

The appropriate half-life and excellent BBB penetration of AV-133 are highly favorable characteristics for a PET imaging agent. These data provide additional chemical information for a clinical study of AV-133 for use in the diagnosis and monitoring PD and related movement disorders.

### 3.4. Histological detection

As shown in Fig. 4, the track of the microdialysis probe is easily visible on the photomicrograph. Fig. 4(A) is the photomicrograph of a coronal section of a whole rat brain, Fig. 4(B) is the photomicrograph of a coronal stained slice of a rat brain, and Fig. 4(C) was the corresponding coronal diagram of Paxinos and Watson atlas [16]. Comparing with the appropriate coronal section of Paxinos and Watson atlas, it can be found that the track of the dialysis probe could be located in the rat striatum with a high precision.and Fig. 4(C) was the corresponding coronal diagram of Paxinos and Watson atlas [16]

## 4. Conclusion

An LC–MS/MS method for the quantitative determination of unradiolabeled AV-133 from *in vivo* microdialysis samples of rat blood and brain was developed and validated. This method has the advantages of less tissue damage, fewer animals, no biological fluid loss, and no need radiolabeling the compound. This method will be useful for pharmaceutical studies of AV-133 at multiple sites in one animal. The concentration–time profiles and pharmacokinetic data for AV-133 in the target tissues were determined, and were used to evaluate the BBB penetration of AV-133. These data also extend our understanding of the kinetic relationships between the brain and blood after a single i.v. dose of AV-133.

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