

In Vivo Measurement of Blood-Brain Barrier Permeability

UNIT 7.19

There are many approaches to quantitate the blood-brain barrier (BBB) permeability of various test compounds (Pardridge, 1998). Various *in vivo* techniques include pharmacokinetic analysis, intracerebral microdialysis, positron emission tomography (PET), magnetic resonance imaging (MRI), and histochemistry. *In vitro* techniques typically use tissue culture models of cerebrovascular endothelial cells either alone or co-cultured with astroglia. This unit focuses on *in vivo* BBB permeability techniques that involve some form of pharmacokinetic analysis: the *in situ* brain perfusion method, the intravenous injection/multiple time point approach, and the brain efflux index (BEI) procedure. These techniques are suitable for a broad array of xenobiotics—from conventionally synthesized new chemical entities to biotechnology-derived peptides and proteins.

The BBB influx and efflux protocols described herein are readily applied to rodents. A guinea pig influx procedure has been published (Zlokovic et al., 1986) but is not described here. There are several influx procedures in this unit, but only one efflux procedure. The *in situ* brain influx perfusion protocols (see Basic Protocols 1 and 2; also see Alternate Protocol) involve terminal animal surgery on a small group of rodents and can be completed in a short time period. Basic Protocols 1 and 2 are for rats and mice, respectively; the Alternate Protocol is a simplified procedure for rats. While the intravenous injection/multiple time point procedure (see Basic Protocol 3) is technically less demanding, it requires additional surgical care for animal survival, a substantially larger number of rodents (because only one data time point is obtained per animal and multiple time points are needed), and much more time to complete the study in its entirety. The BEI protocol (see Basic Protocol 4) entails stereotaxic microinjections performed on a small number of rodents and can be completed in a short time period. *In vivo* BBB permeability measurements are readily mastered following some familiarity and practice with the technical surgical procedures.

Support Protocols describe construction of equipment used in the Basic Protocols, such as stainless steel connectors for PE50 tubing (see Support Protocol 1), *in situ* perfusion cannulas (see Support Protocol 2), jugular-vein catheters (see Support Protocol 6), and cerebrospinal fluid (CSF) collection units (see Support Protocol 8). Additionally, Support Protocols are provided for determining intravascular capillary volume (see Support Protocol 3) and cerebral perfusion flow (see Support Protocol 4). An *in situ* perfusion technique is also provided for assessing whether transport of a test compound occurs by carrier-mediated or saturable transport (see Support Protocol 5). Capillary depletion methods (see Support Protocol 7) may be performed, if necessary, to determine whether the test compound is trapped within the cerebroendothelium or penetrates into brain parenchyma.

Not discussed in this unit, but also essential, is the requirement for a sensitive and specific analytical assay. Radiochemical analysis, gas chromatography–mass spectrometry or high performance liquid chromatography–mass spectrometry are analytical methods that can detect small masses of substances that have penetrated into brain parenchyma. It is important to have an appropriate, validated analytical method established before undertaking BBB permeability studies.

CAUTION: If the test compound is radiolabeled, obtain appropriate permissions from an institutional radiation safety committee for use of isotopes in animals. Take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out

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7.19.1

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the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals. Sterile surgical procedures must be employed for all rodent surgical preparations.

BLOOD-BRAIN BARRIER INFLUX MEASUREMENT: IN SITU BRAIN PERFUSION PROCEDURE FOR RATS

The rodent in situ brain perfusion method is suitable for compounds suspected of (1) having reasonable or good BBB permeability or (2) using endogenous transport systems. The essence of the procedure is to perfuse a solution of defined composition through the cerebrovasculature of an anesthetized rodent for a short time period (no more than ~3 min). The brain is then harvested and assayed for accumulated test compound.

The surgical procedure requires some practice to master, as the external carotid artery is cannulated for retrograde brain perfusion via the internal carotid artery. While this procedure is highly recommended for rats because of its time-honored use and acceptance (Takasato et al., 1984), a newer, simplified surgical procedure for rats (see Alternate Protocol; Allen et al., 1997) or mice (see Basic Protocol 2; Dagenais et al., 2000) may be implemented should this protocol prove to be problematic for technical reasons.

Materials

- Krebs-bicarbonate buffer (see recipe), 37°C
- Perfusate solution: test compound(s) dissolved in Krebs-bicarbonate buffer, 37°C
- Laboratory rats, either gender, 200 to 350 g
- Anesthetic (as specified by the approved animal care protocol; *APPENDIX 4B*)
- Saline: 0.9% (w/v) NaCl (sterile)
- Tissue solubilizer (e.g., Soluene 350; Packard) or 2 N NaOH, for radiolabeled test compounds only
- Vials (scintillation vials, 7 to 20 ml, with caps, can be used when working with radiolabeled compounds)
- 1-, 10-, 20-, and 50-ml syringes
- 22-G hypodermic needles with the beveled end cut off (see Support Protocol 1)
- Warming pads (e.g., Deltaphase Isothermal Pad; Braintree Scientific), preheated to 37°C
- Syringe infusion pump (e.g., Pump 22; Harvard Apparatus)
- Animal fur shaver
- Surgical instruments (e.g., Harvard Apparatus), including:
 - 4.5-in. (~11.4-cm) operating scissors, sharp or blunt
 - Disposable scalpels
 - 4-in. microdissecting forceps, full or strongly curved
 - 5.5-in. Olsen-Hegar needle holder
 - 3.5-in. Hartman hemostatic forceps
 - 6-in. probe with eye (eye probe)
 - 3-in. Vannas spring scissors, straight
 - Tissue forceps (straight) or bone rongeurs
 - Inox no. 7 sharp-point forceps
- Suture, USP size 4.0
- Electrocautery (e.g., Change-A-Tip low-temperature power handle and disposable sterile tips; Aaron Medical Industries), 1100° to 1300°F

Microvessel arterial clamp (Kleinert-Kutz microvessel clip; blades 6 mm × 1 mm, curved; 11 mm in length; Pilling Weck Surgical)

Perfusion cannula (see Support Protocol 2)

PE50 polyethylene tubing, 0.58 mm (0.023 in.) i.d., 0.965 mm (0.038 in.) o.d. (e.g., Intramedic Clay Adams; Becton Dickinson Labware)

22-G stainless steel connectors (see Support Protocol 1)

Rodent guillotine

Filter paper

50°C water bath, optional

Additional reagents and equipment for analyzing nonradiolabeled test compounds (see Critical Parameters and Troubleshooting, discussion of sensitive analytical method), optional

Set up experiment

1. Label and record the individual weights of an appropriate number of empty vials for collecting brain tissue.

One vial is needed for every discrete brain region per rodent; six vials are needed for each rodent if this protocol is followed exactly.

2. Fill a 1-ml syringe with prewarmed Krebs-bicarbonate buffer and attach a 22-G hypodermic needle with the beveled end cut off. Keep the syringe and its contents warm by wrapping it in a preheated warming pad.

This syringe is used to fill the perfusion cannula.

3. Fill a 10- or 20-ml syringe with prewarmed perfusate solution (containing the test compound) and attach a 22-G hypodermic needle with the beveled end cut off. Keep the syringe and its contents warm by wrapping it in the preheated warming pad.

This syringe is used for brain perfusion.

4. Set up a syringe infusion pump within easy reach of the surgical area. Program the pump for the desired infusion rate (step 22).

Prepare rat for surgery

5. Anesthetize a 200- to 350-g laboratory rat with an appropriate anesthetic as approved by the IACUC. Administer sufficient anesthetic (with or without supplemental doses) to maintain proper anesthesia for a surgical procedure of ≤1 hr.

APPENDIX 4B describes anesthetics that are typically used in rat and mouse surgical procedures.

6. Place anesthetized rat upside down on its back on another warming pad with its head toward the surgeon and its tail away from the surgeon. Shave fur from its neck and shoulder areas using an animal fur shaver.

Perform surgery

7. Midway between the trachea and esophagus and the shoulder, make an ~2-cm-long skin incision with the operating scissors or disposable scalpel, running parallel to the trachea and esophagus (Fig. 7.19.1).
8. Using 4-in. curved microdissecting forceps, gently tease away the subcutaneous fat and muscle to expose the right common carotid artery (CCA; Fig. 7.19.1).

The left CCA can be exposed for left-handed investigators.

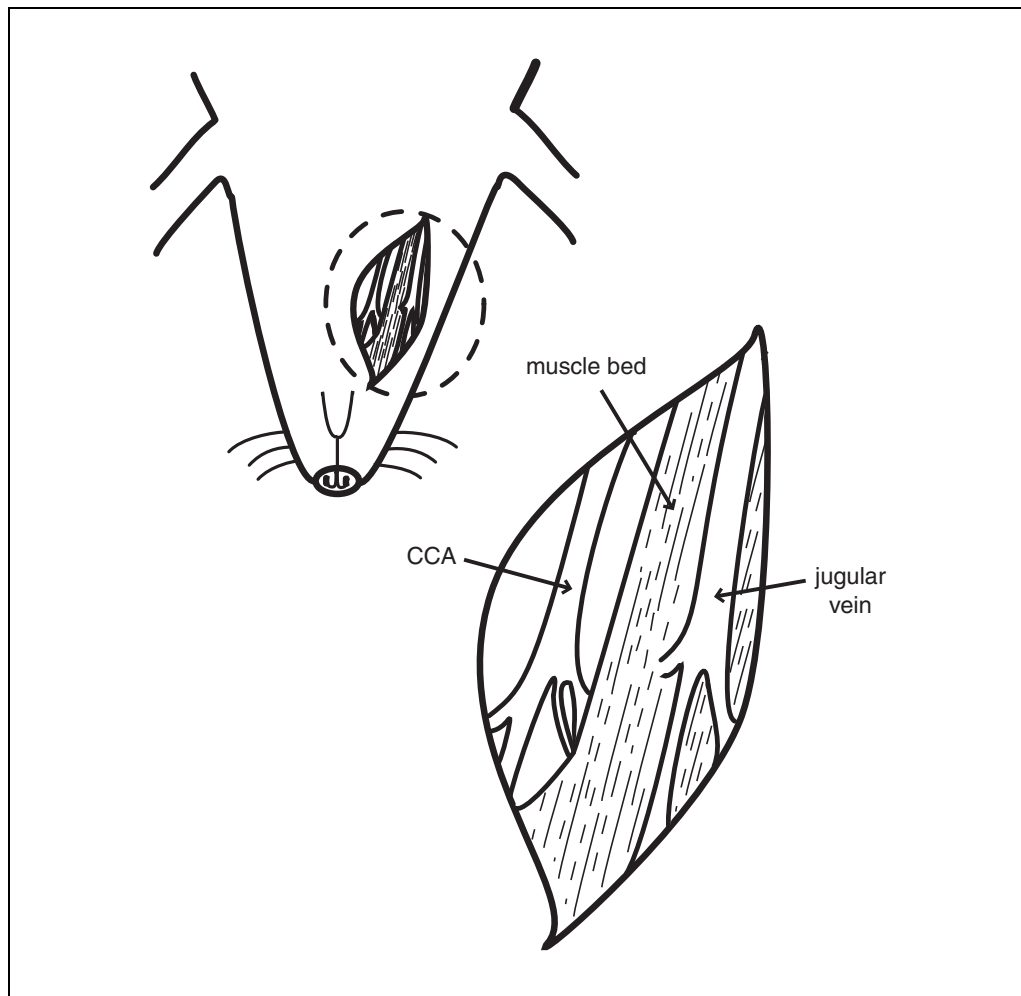


Figure 7.19.1 Relative location of the incision to expose the right common carotid artery (CCA) and jugular vein of a rat. The ventral view of a rat's head and shoulder area is shown with an enlarged view of the incision area.

9. Using the forceps, gently tease away the white nerve running along the length of the CCA. Expose the CCA to reveal the bifurcation of the external and internal carotid arteries (ECA and ICA; Fig. 7.19.2).

The hyoid bone may need to be removed using surgical shears to expose the ECA.

10. Using 4.0 suture, ligate shut the ECA rostrally (towards the snout) at the point where the ECA undergoes additional bifurcation (Fig. 7.19.3).

The Olsen-Hegar needle holder and Hartman hemostatic forceps may be used to aid in the ligation of the ECA.

11. Use an electrocautery to cauterize small arterial branches (superior thyroid and occipital arteries) that originate from the ICA and ECA (Figs. 7.19.2 and 7.19.3).

Direct application of the cautery tip is usually sufficient to coagulate and cauterize the superior thyroid artery. Sometimes the occipital artery may be located at the bifurcation point of the ECA and ICA (Fig. 7.19.2). This artery is smaller relative to the ECA and ICA. The curvature of the forceps can be gently inserted under the occipital artery to lift it upwards slightly for cauterization.

12. Locate the bifurcation of the ICA where the pterygopalatine artery branches from the ICA (Fig. 7.19.3). Carefully cauterize the pterygopalatine artery.

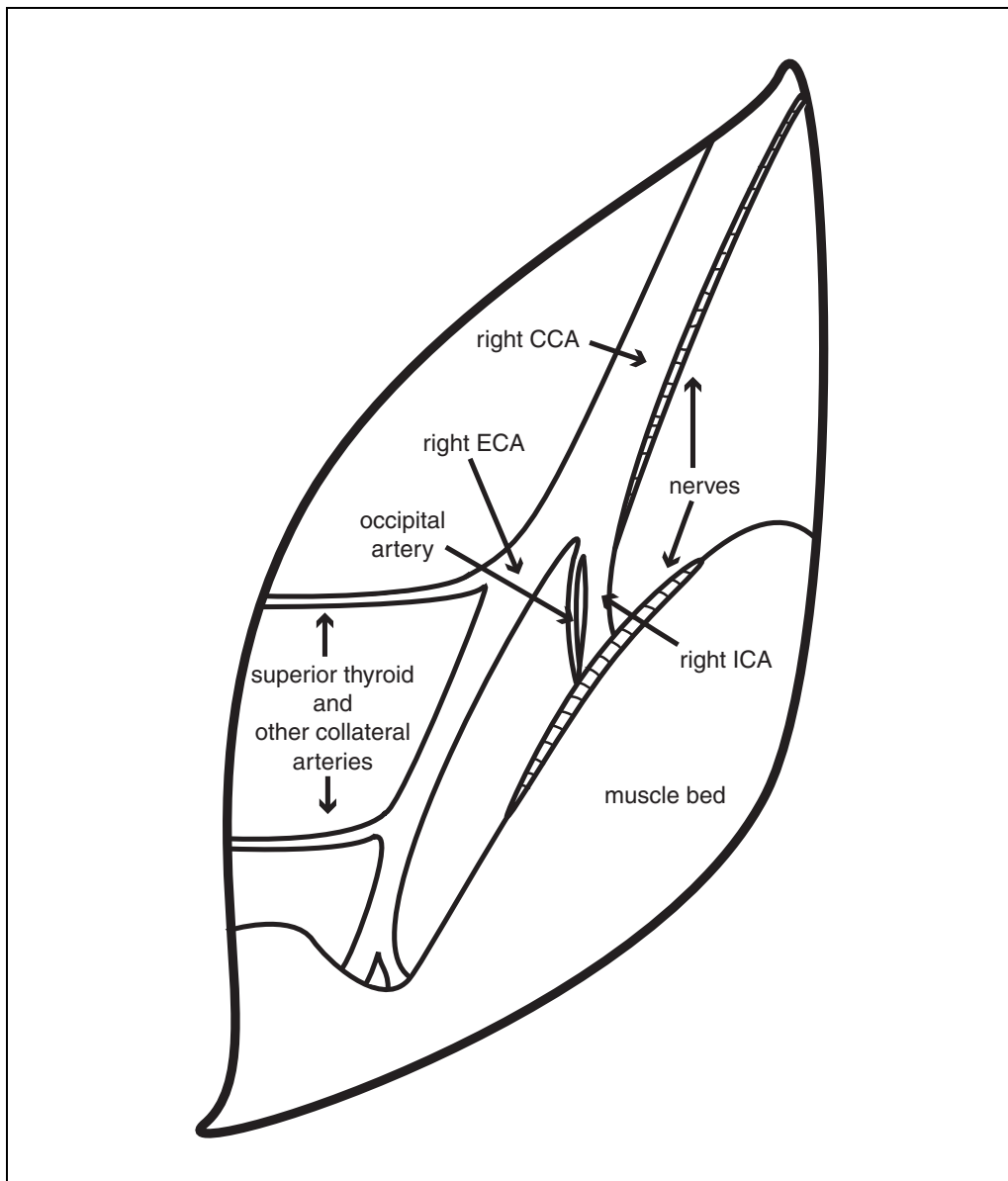


Figure 7.19.2 Detailed anatomy of the branches of the right CCA as viewed through the incision depicted in Figure 7.19.1. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery.

Incomplete cauterization of the pterygopalatine is the major cause of surgical failure. Typically, the cautery tip is not sufficiently hot and premature removal of the cautery will tear the pterygopalatine, leading to massive hemorrhaging into the incision site. For successful cauterization of the pterygopalatine, fresh batteries should be used in the cautery to ensure that the tip is at its thermal maximum. Also, a microspatula or eye probe can be placed between the bifurcation point to shield the ICA from the cautery tip.

13. Using 4.0 suture, place a loose ligature around the CCA caudally (towards the tail) before the bifurcation into the right ECA and ICA. Place the ligature at the point of the arrow identifying the right CCA (Fig. 7.19.3). Do not tighten the ligature yet.
14. Place a microvessel arterial clamp around the ECA at the point where it branches from the CCA. Be certain that the clamp does not impede blood flow through the CCA.

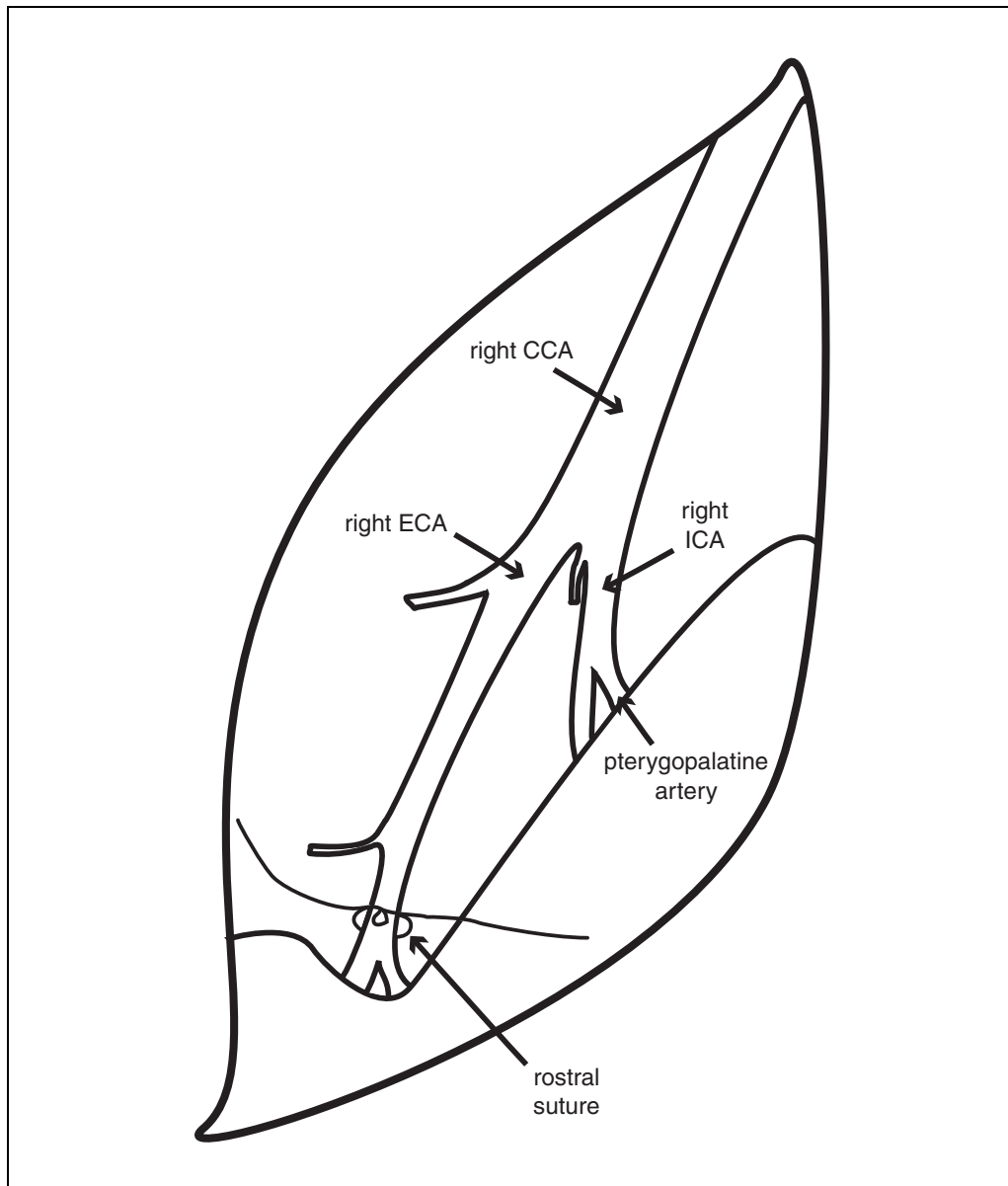


Figure 7.19.3 Detailed anatomy of the branches of the right CCA after cauterization as viewed through the incision depicted in Figure 7.19.1. The rostral suture of the ECA is also indicated. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery.

15. Using the 1-ml syringe (step 2), fill a perfusion cannula with Krebs-bicarbonate buffer.

The PE50 tubing of the cannula is connected to the syringe using the cut 22-G needle.

16. Catheterize the ECA with the buffer-filled perfusion cannula. Insert the cannula such that the tip points towards the caudal end. Carefully release the microvessel arterial clamp. Advance the tip of the cannula up to, but not past, the bifurcation point where the ECA branches off the CCA.

If the beveled cannula (cannula configuration 1) is used, a 25-G needle should be used to puncture and create a hole in the ECA. If the 25-G needle epoxied to PE50 tubing (cannula configuration 2) is used, no prior needle puncture is needed. Alternatively, the Vannas spring scissors can be used to make a small cut in the ECA.

Advancing past the bifurcation point may disturb cerebral blood flow.

17. Using 4.0 suture, tie one or two ligatures around the cannula to anchor it in place. Do not tie the ligatures so tightly as to occlude the cannula.

Perform brain perfusion

18. Place the 10- or 20-ml syringe containing the perfusate solution (step 3) in the infusion pump. Connect the syringe to a length of PE50 polyethylene tubing that can extend to the implanted perfusion cannula. Attach a 22-G stainless steel connector and fill the PE50 tubing with perfusate solution from the syringe.

19. Connect the free end of the fluid-filled PE50 tubing to the perfusion cannula.

20. Tighten the caudal ligature around the CCA (step 13).

At this point, it is critical to work quickly because the cerebral blood supply is interrupted. Prolonged blood flow interruption will provoke cerebral ischemia and lead to artifactual permeability measurements.

21. Quickly open the chest cavity and sever the cardiac ventricles with a pair of 4.5-in. (~11.4-cm) operating scissors. Immediately turn on the pump to start the perfusion.

22. Perfuse the cerebral hemisphere for 30 to 180 sec at a rate of 5 ml/min. Retain a 0.25-ml aliquot of the perfusate for test compound concentration analysis.

Pilot studies are needed to determine the most appropriate length of time for perfusion. This time should be sufficient for detectable penetration of test compound, yet not so long as to cause severe physiological disturbances. The perfusion time is determined from the linear phase of the influx clearance versus time graph (step 32). Perfusion times >5 min are not recommended because of increased potential for cerebral hypoxia and altered BBB permeability.

The aliquot may be stored up to 1 month at -20°C.

23. Stop the perfusion and quickly decapitate the rat using a rodent guillotine.

Harvest tissue

24. Using a pair of tissue forceps or bone rongeurs, remove the entire brain (forebrain and cerebellum) from the skull.

25. Place the brain in a disposable petri plate containing a piece of filter paper moistened with sterile saline. Place the petri plate on ice so the brain dissection is performed under cold conditions.

26. Using a pair of Inox no. 7 sharp-point forceps, remove meningeal membranes and associated surface blood vessels. Grossly dissect the cerebellum and the left (unperfused) hemisphere from the right (perfused) hemisphere. Set aside the cerebellum and left hemisphere for proper disposal.

The perfused hemisphere should appear blanched or whitish, in contrast to the pinkish coloring of the unperfused hemisphere.

27. Grossly dissect the right hemisphere into anatomically defined brain regions: frontal cortex, occipital cortex, posterior cortex, striatum, hypothalamus and thalamus, and hippocampus.

Ohno et al. (1978) describes the dissection procedure in detail.

28. Use a lint-free tissue to wick away any excess adherent moisture from the brain regions.

29. Place each piece of brain tissue in a preweighed vial (step 1) and determine the weight of the tissue.

Brain tissue can be frozen and stored up to 1 month at -20°C.

Quantitate test compound

30. Analyze the perfusate aliquot (step 22) as defined by the appropriate test compound analysis protocol (see Critical Parameters and Troubleshooting, discussion of sensitive analytical method).

31a. *For radiolabeled compounds in tissue:* Digest brain tissue with an appropriate amount of a tissue solubilizer as specified by the manufacturer's instructions. Alternatively, digest tissue with 1 to 2.5 ml (5 vol per gram of tissue) of 2 N NaOH and incubate 3 hr in a water bath at 50°C. Vortex vigorously. Add an appropriate volume of scintillation fluid that is chemically compatible with the tissue solubilizer so the sample forms a clear solution (with no emulsion). Quantify the radioactivity using an appropriate counting protocol in a scintillation counter.

If NaOH is used, a scintillation fluid (e.g., Hionic Fluor; Packard Instruments) that can neutralize the high concentration of aqueous base must be used.

31b. *For nonradiolabeled compounds in tissue:* Follow the tissue extraction and analysis procedures as specified by the test compound analysis protocol.

Analyze data

32. Calculate influx clearances, corrected for test compound present in the cerebrovascular blood volume, using the equation:

$$Cl_{in} = \frac{q_{tot} - (V_{vasc} \times C_{pf})}{T \times C_{pf}}$$

Equation 7.19.1

where Cl_{in} represents the influx transfer clearance (ml/min/g wet brain weight), q_{tot} is the observed total amount of test compound in the brain region (g compound/g wet brain weight) and represents the observed test compound entrapped within the parenchyma and cerebrovasculature, V_{vasc} represents the vascular blood volume (ml/g wet brain weight), C_{pf} is the compound perfusate concentration (g compound/ml), and T is the time (sec or min) of perfusion.

Influx clearance is a parameter that measures test compound penetration across the BBB into brain parenchyma (Takasato et al., 1984; Smith et al., 1992; Adkison and Shen, 1996). Equation 7.19.1 assumes negligible efflux (which may occur by passive diffusion or efflux transporters) and no metabolism during the short duration of the perfusion. Literature values of V_{vasc} may be used (e.g., see Smith et al., 1988, for comprehensive tabular data on inulin regional distribution volumes in rat brain). Alternatively, V_{vasc} is determined by perfusion with an intravascularly retained marker (such as [3H]inulin; see Support Protocol 3).

If performing pilot studies to determine the appropriate length of time for perfusion, graph the Cl_{in} versus time of perfusion. Select the duration of perfusion from the linear portion of this graph, which typically occurs between 0 and 180 sec of perfusion.

33. Calculate the apparent cerebrovascular permeability–surface area product (PA) using the equation:

$$PA = -F \left[\ln \left(1 - \frac{Cl_{in}}{F} \right) \right]$$

Equation 7.19.2

where F is cerebral perfusion fluid flow.

PA is a parameter that takes into account the effects of cerebral perfusion fluid flow. F is an important factor for highly lipophilic compounds where BBB permeability is rate limited by perfusion fluid flow (see Smith, 1989, for an excellent theoretical and practical overview). It is calculated from the brain uptake of [³H]diazepam and is $\sim 5 \times 10^{-2}$ ml/sec/g or 3 ml/min/g (Takasato et al., 1984; Smith et al., 1992) for an externally determined perfusion rate of 5 ml/min. Alternatively, F may be experimentally determined (see Support Protocol 4).

Equation 7.19.2 assumes that the test compound does not bind to proteins contained in the perfusion fluid. If the perfusion fluid contains albumin or other compound-binding protein components, then PA should be corrected for the unbound fraction of test compound (f_u) that is available for BBB permeation. In this case, the correction requires multiplying the right-hand side of Equation 7.19.2 by $1/f_u$ to obtain PA', as shown in the equation below. In the case of an albumin- or protein-free perfusate, $f_u = 1$ and $PA = PA'$.

$$PA' = -(F/f_u) \left[\ln \left(1 - \frac{Cl_{in}}{F} \right) \right]$$

Equation 7.19.3

BLOOD-BRAIN BARRIER INFLUX MEASUREMENT: SIMPLIFIED IN SITU BRAIN PERFUSION FOR RATS

In contrast to Basic Protocol 1, this protocol is a simplified in situ brain perfusion procedure for rats, where the common carotid artery is catheterized for brain perfusion instead of the external carotid artery (Allen et al., 1997). This surgical procedure is technically easier to perform, and does not require the technically challenging task of cauterization of the pterygopalatine artery. However, this protocol requires that one work quickly to sever the cardiac ventricles and initiate the perfusion following cannulation of the CCA. Any delay may lead to significant cerebral ischemia, with potential alterations in BBB integrity. The perfusion rate must also be increased to compensate for perfusate flux through the pterygopalatine artery.

1. Perform the experimental setup and animal preparation as described above (see Basic Protocol 1, steps 1 to 6), but use a 10-, 20-, or 50-ml syringe for the perfusate solution in step 3.
2. Perform the surgical procedure as in steps 7 to 11, 13, and 15 to 17 of Basic Protocol 1, but in step 16 cannulate the CCA (instead of the external carotid artery) for perfusion. Insert the cannula such that the tip points rostrally (towards the head). Do not advance the tip past the bifurcation point where the ECA branches off the CCA.

Do not cauterize the pterygopalatine artery.

3. Perfuse the cerebral hemisphere (steps 18 to 23), but perfuse for 20 to 180 sec at a rate of 10 ml/min using a 10-, 20-, or 50-ml syringe for the perfusate.
4. Harvest tissue and analyze the tissue and perfusate for the test compound (steps 24 to 33).

BLOOD-BRAIN BARRIER INFLUX MEASUREMENT: IN SITU BRAIN PERFUSION PROCEDURE FOR MICE

This protocol is a simplified in situ brain perfusion procedure for mice (Dagenais et al., 2000). In this protocol, the common carotid artery is cannulated for perfusion, in lieu of the external carotid artery cannulation described in Basic Protocol 1. Moreover, this protocol does not require cauterization of the pterygopalatine artery. Thus, this surgical procedure is technically easier to perform, but does have its caveats. Catheterization of

ALTERNATE PROTOCOL

BASIC PROTOCOL 2

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the CCA impairs cerebral blood flow, thereby mandating that one work quickly to sever the cardiac ventricles and initiate the in situ brain perfusion. Undue delay will cause cerebral ischemic changes in the BBB, leading to artifactual permeability data.

Materials

Krebs-bicarbonate buffer (see recipe), 37°C
Perfusate solution: test compound(s) dissolved in Krebs-bicarbonate buffer, 37°C
Laboratory mice (adult weight, either gender)
Anesthetic (as specified by the approved animal care protocol; *APPENDIX 4B*)
Vials (scintillation vials, 7 to 20 ml, with caps, can be used when working with radiolabeled compounds)
1- and 10-ml syringes
22-G hypodermic needles with the beveled end cut off (see Support Protocol 1)
Warming pads (e.g., Deltaphase Isothermal Pad; Braintree Scientific), preheated to 37°C
Syringe infusion pump (e.g., Pump 22; Harvard Apparatus)
Animal fur shaver
Surgical instruments (e.g., Harvard Apparatus; see Basic Protocol 1)
4.5-in. (~11.4-cm) operating scissors, sharp or blunt
Disposable scalpels
4-in. microdissecting forceps, full or strongly curved
5.5-in. Olsen-Hegar needle holder
3.5-in. Hartman hemostatic forceps
Tissue forceps (straight) or bone rongeurs
Inox no. 7 sharp-point forceps
Suture, USP size 4.0
Perfusion cannula configuration 1 or 2 (see Support Protocol 2), but made with a 26-G (not 25-G) hypodermic needle
PE50 polyethylene tubing, 0.58 mm (0.023 in.) i.d., 0.965 mm (0.038 in.) o.d. (Intramedic Clay Adams; Becton Dickinson Labware)
22-G stainless steel connectors (see Support Protocol 1)
Sharp surgical shears or rodent guillotine
Additional reagents and equipment for harvesting and analyzing tissue (see Basic Protocol 1)

Set up experiment

1. Label and record the individual weights of an appropriate number of empty scintillation vials for collecting brain tissue.

One vial is needed for every discrete brain region per rodent; six vials are needed for each rodent if this protocol is followed exactly.

2. Fill a 1-ml syringe with prewarmed Krebs-bicarbonate buffer and attach a 22-G hypodermic needle with the beveled end cut off. Keep the syringe and its contents warm by wrapping it in a preheated warming pad.

This syringe is used to fill the perfusion cannula.

3. Fill a 10-ml syringe with prewarmed perfusate solution and attach a 22-G hypodermic needle with the beveled end cut off. Keep the syringe and its contents warm by wrapping it in the preheated warming pad.

This syringe is used for brain perfusion.

4. Set up a syringe infusion pump within easy reach of the surgical area. Program the pump for an infusion rate of 2.5 ml/min.

Prepare mouse for surgery

5. Anesthetize a laboratory mouse with an appropriate anesthetic as approved by the IACUC. Administer sufficient anesthetic (with or without supplemental doses) to maintain proper anesthesia for a surgical procedure of ≤ 1 hr.
6. Place anesthetized mouse upside down on its back on another warming pad with its head towards the surgeon and its tail away from the surgeon. Shave fur from its neck and shoulder areas using an animal fur shaver.

Perform surgery

7. Midway between the trachea and esophagus and the shoulder, make an ~1- to 2-cm-long skin incision with the operating scissors or disposable scalpel, running parallel to the trachea and esophagus (Fig. 7.19.1).
8. Using a pair of 4-in. curved microdissecting forceps, gently tease away subcutaneous fat and muscle to expose the right CCA and the bifurcation of the right ECA and ICA (Fig. 7.19.2).

The left CCA can be exposed for left-handed investigators.

9. Using a 4.0 suture, ligate shut the ECA rostrally (towards the snout) after the bifurcation of the CCA into the right ECA and ICA (see Fig. 7.19.3 for anatomical reference).

The placement of the ligature in the mouse is similar to that of the rat. The Olsen-Hegar needle holder and Hartman hemostatic forceps may be used to aid in the ligation of the ECA.

It is not necessary to ligate the pterygopalatine artery in the mouse. Moreover, to do so is quite technically demanding.

10. Using 4.0 suture, place a loose ligature around the CCA caudally (towards the tail) before the bifurcation of the right ECA and ICA (at the point of the arrow identifying the right CCA; Fig. 7.19.3). Do not tighten the ligature yet.
11. Using the 1-ml syringe (step 2), fill a perfusion cannula with Krebs-bicarbonate buffer.

The PE50 tubing of the cannula is connected to the syringe using the cut 22-G needle.

12. Place the 10-ml syringe containing the perfusate solution (step 3) in the infusion pump. Connect the syringe to a length of PE50 polyethylene tubing that can extend to the implanted perfusion cannula. Attach a 22-G stainless steel connector and fill the PE50 tubing with perfusate solution from the syringe.
13. Connect the free end of the fluid-filled PE50 tubing to the perfusion cannula.
14. Catheterize the CCA caudally (before the bifurcation) with the buffer-filled perfusion cannula (with the tip of the cannula pointing towards the head). Using 4.0 suture, tie one or two ligatures around the cannula to anchor it in place. Do not tie the ligatures so tightly as to occlude the cannula.

At this point, it is critical to work quickly because the cerebral blood supply is interrupted. Prolonged blood flow interruption will provoke cerebral ischemia and lead to artifactual permeability measurements.

Perform brain perfusion

15. Quickly open the chest cavity and sever the cardiac ventricles with a pair of 4.5-in. operating scissors. Immediately turn on the pump to start the perfusion.
16. Perfuse the cerebral hemisphere for 30 to 180 sec at a rate of 2.5 ml/min. Retain a 0.25-ml aliquot of the perfusate for test compound concentration analysis.

Pilot studies are needed to determine the most appropriate length of time for perfusion. The duration should be sufficient for detectable penetration of the test compound, yet not so long as to cause severe physiological disturbances. Perfusion times >5 min are not recommended because of increased potential for cerebral hypoxia and altered BBB permeability. The perfusion time is determined from the linear phase of the influx clearance versus time graph (step 32 of Basic Protocol 1).

The aliquot may be stored up to 1 month at –20°C.

17. Stop the perfusion and quickly decapitate the mouse using a pair of sharp surgical shears or a rodent guillotine.

Harvest tissue

18. Harvest tissue and analyze as described for the rat (see Basic Protocol 1, steps 24 to 33).

SUPPORT PROTOCOL 1

CONSTRUCTION OF PE50–STAINLESS STEEL CONNECTORS AND CUT 22-G HYPODERMIC NEEDLES

The 22-G stainless steel connectors consist of a 1- to 1.5-cm piece of 22-G stainless steel tubing. These connectors are used to connect two lengths of PE50 tubing together. Cut 22-G hypodermic needles are useful in connecting a syringe to the free end of a PE50 catheter.

Materials

Metal file
22-G stainless steel disposable hypodermic needles
5.5-in. (14-cm) Olsen-Hegar needle holder (e.g., Harvard Apparatus)
Hartman hemostatic forceps, 3.5 in.

1. Using a metal file, score the stainless steel tubing near the beveled point of a 22-G stainless steel disposable hypodermic needle.

Be careful not to file all the way through the tubing because this may crimp the opening of the tubing.

2. Hold the luer-lock end of the needle with a pair of 5.5-in. Olsen-Hegar needle holders and snap the needle into two pieces along the score mark.

Hartman hemostatic forceps may be used to hold the other end of the needle.

The point should snap off cleanly and without bending the tubing if the score is adequate.

3. *Optional:* Construct a PE50–stainless steel connector by repeating the process at the end near the plastic luer-lock hub.

4. If necessary, file off any burrs left on the cut end(s) of the tubing.

SUPPORT PROTOCOL 2

CONSTRUCTION OF IN SITU PERFUSION CANNULAS

These catheters are constructed for use in Basic Protocols 1 and 2 and the Alternate Protocol. It is advisable to prepare several catheters in advance of the experimental study. The catheters may be reused if they are not damaged or contaminated by prior use. There are two different cannula configurations: configuration 1, a beveled length of PE50 tubing, is simple to construct, but may be more difficult to use for catheterization; configuration 2, a 25- or 26-G hypodermic needle affixed to PE50 tubing, requires a more complex construction, but may make the catheterization process easier. The cannula configuration is a matter of personal choice.

Configuration 1 Cannulas

Materials

PE50 polyethylene tubing, 0.58 mm (0.023 in.) i.d., 0.965 mm (0.038 in.) o.d.
(e.g., Intramedic Clay Adams; Becton Dickinson Labware)
Forceps and scissors
Boiling water bath

- 1a. Cut a 15-cm length of PE50 polyethylene tubing.
- 2a. Using a pair of forceps, immerse one end of the PE50 tubing into a beaker of boiling water for 10 to 30 sec until tubing is pliable.
- 3a. Remove the immersed end and quickly stretch the tubing to elongate it. Allow tubing to cool.

This stretching will create a narrower bore for easier catheterization.

- 4a. Using a pair of scissors, cut the elongated length of the PE50 tubing at an $\sim 30^\circ$ angle to give the tubing a sharp bevel.

Too sharp of a bevel will cause the tubing to puncture through the artery during the catheterization procedure.

Configuration 2 Cannulas

Materials

5.5-in. (14-cm) Olsen-Hegar needle holder (e.g., Harvard Apparatus)
Metal file
20- and 25- (for rats) or 26-G (for mice), 1-in. (2.5-cm) stainless steel hypodermic needles
PE50 polyethylene tubing, 0.58 mm (0.023 in.) i.d., 0.965 mm (0.038 in.) o.d.
(e.g., Intramedic Clay Adams; Becton Dickinson Labware)
Epoxy adhesive

- 1b. Using a 5.5-in. Olsen-Hegar needle holder and a metal file, cut a 25- or 26-G 1-in. stainless steel hypodermic needle from the plastic luer-lock syringe hub (see Support Protocol 1). Discard the plastic hub.
- 2b. Using the needle holder and metal file, cut a 20-G hypodermic needle from the plastic luer-lock syringe hub. Retain the plastic hub.

Although it takes patience and brute physical force, one can alternatively remove the epoxy cementing the needle to the hub using a razor blade, and then extricate the entire needle from the plastic, luer-lock syringe hub.

The 20-G needle should be discarded using proper safety techniques.

- 3b. Cut a 15-cm length of PE50 polyethylene tubing.
- 4b. Insert the cut end of the 25- or 26-G needle into the PE50 tubing, leaving 1.5 cm of the needle exposed.
- 5b. Glue the needle to the tubing using epoxy adhesive.
- 6b. Slide the 20-G plastic luer-lock syringe hub over the needle-tubing connection. Use epoxy adhesive to affix the plastic hub to the tubing and allow the epoxy to cure overnight.

The plastic hub permits one to comfortably grasp the cannula with gloved hands during the carotid artery catheterization procedure.

Table 7.19.1 Representative Inulin Regional Distribution Volumes for Assessment of Intravascular Capillary Bed Volume^a

Representative brain region	Mean brain regional vascular distribution volume (percent of ml/g brain tissue)	
	Inulin	Inulin and dextrans
Caudate-putamen	0.85%	0.93%
Cerebellum	1.32%	1.27%
Frontal cortex	1.13%	1.23%
Hippocampus	0.98%	1.11%
Inferior colliculus	1.62%	1.47%
Medulla	1.33%	1.43%
Midbrain	0.90%	1.07%
Occipital cortex	1.06%	1.18%
Olfactory bulb	2.06%	2.19%
Parietal cortex	1.09%	1.14%
Pons	1.15%	1.21%
Superior colliculus	1.20%	1.44%
Thalamus-hypothalamus	0.90%	0.97%

^aData are from Smith et al. (1988). Comparative volumes are given for inulin (mol. wt. 5 kDa) and inulin and various dextrans (5 to 18 kDa).

EXPERIMENTAL DETERMINATION OF INTRAVASCULAR CAPILLARY VOLUME USING RADIOLABELED INULIN

The in situ brain perfusion technique requires the analysis of a test compound present in perfused brain regions. Analytical techniques typically extract and quantitate the total amount of test compound in the brain region (g compound/g wet brain weight). This represents the observed test compound entrapped within the parenchyma and cerebrovasculature capillary bed. Subtraction of intravascularly localized test compound from the total amount of test compound yields the amount present in brain parenchyma. A correction for test compound resident in the cerebrovasculature provides an accurate determination of penetration of that compound across the BBB.

Calculation of the mass of the test compound in the cerebrovasculature is based on a knowledge of the concentration of test compound in the perfusate and the volume of the cerebrovascular bed, V_{vasc} . The perfusate concentration is a known controlled quantity that is confirmed by test compound analysis. V_{vasc} can be determined either from appropriate literature estimates or through experimental determination. Comprehensive tabular data on inulin regional distribution volumes in the rat brain have been published (e.g., Smith et al., 1988; also see Table 7.19.1 for representative data). However, V_{vasc} may need to be experimentally determined for animal models other than healthy, adult male rats. In this case, an in situ brain perfusion using the nonpenetrable BBB tracers [³H]inulin or [¹⁴C]methoxyinulin will provide good measurements of cerebrovasculature capillary bed volume.

Additional Materials (also see Basic Protocols 1 and 2)

Perfusate solution: 0.5 $\mu\text{Ci/ml}$ [³H]inulin (American Radiolabeled Chemicals) or 0.3 $\mu\text{Ci/ml}$ [¹⁴C]methoxyinulin (American Radiolabeled Chemicals) dissolved in Krebs-bicarbonate buffer (see recipe), 37°C

1. Perform in situ brain perfusion of rats (see Basic Protocol 1, steps 1 to 31b) or mice (see Basic Protocol 2, steps 1 to 18), using a perfusate solution containing [³H]inulin or [¹⁴C]methoxyinulin.
2. Calculate the vascular blood volume (the cerebrovascular capillary bed volume) using the following equation:

$$V_{\text{vasc}} = q_{\text{tot}} / C_{\text{pf}}$$

Equation 7.19.4

where V_{vasc} is the vascular blood volume (ml/g wet brain weight); q_{tot} is the observed total test compound amount in the brain region (g compound/g wet brain weight), representing the observed compound entrapped within the lumen of the cerebrovasculature; and C_{pf} is the [³H]inulin or [¹⁴C]methoxyinulin perfusate concentration (g/ml).

EXPERIMENTAL DETERMINATION OF CEREBRAL PERFUSION FLUID FLOW USING RADIOLABELED DIAZEPAM

SUPPORT PROTOCOL 4

Cerebral perfusion fluid flow (F) is an important factor for highly lipophilic compounds where BBB penetration is often limited by the rate of perfusion fluid flow (see Smith, 1989, for an excellent theoretical and practical overview). F is determined experimentally by in situ perfusion with radiolabeled diazepam or from literature estimates. For adult male rats and an externally controlled perfusion rate of 5 ml/min, F is $\sim 5 \times 10^{-2}$ ml/sec/g or 3 ml/min/g (Takasato et al., 1984; Smith et al., 1992).

Additional Materials (also see Basic Protocols 1 and 2)

Perfusate solution: 0.5 $\mu\text{Ci/ml}$ [³H]diazepam (American Radiolabeled Chemicals) or 0.3 $\mu\text{Ci/ml}$ [¹⁴C]diazepam (American Radiolabeled Chemicals) dissolved in Krebs-bicarbonate buffer (see recipe), 37°C

1. Perform an in situ brain perfusion and tissue harvest for rats (see Basic Protocol 1, steps 1 to 31b) or mice (see Basic Protocol 2, steps 1 to 18), using a perfusate solution containing radiolabeled diazepam.
2. Calculate cerebral perfusion flow using the following equation:

$$F = \frac{q_{\text{tot}}}{T \times C_{\text{pf}}}$$

Equation 7.19.5

where F is the cerebral perfusion flow rate in ml/min/g wet brain weight, q_{tot} is the observed total test compound amount in the brain region (g compound/g wet brain weight), T is the total time of the externally controlled perfusion, and C_{pf} is the concentration of radiolabeled diazepam in the perfusate (g/ml).

F is a parameter that measures the intracerebral rate of perfusion, which differs from the externally controlled perfusion rate (Takasato et al., 1984; Smith et al., 1992; Adkison and Shen, 1996). q_{tot} represents the observed compound entrapped within the parenchyma.

Diazepam is highly lipophilic and penetrates across the BBB quite rapidly. Therefore, it is assumed that all observed brain tissue diazepam is localized in the brain parenchyma. No correction for intravascularly entrapped tracer is required.

**USE OF THE IN SITU PERFUSION TECHNIQUE FOR MECHANISTIC
ASSESSMENT OF CARRIER-MEDIATED OR SATURABLE TRANSPORT**

The transport of essential nutrients, e.g., neurotransmitters and neurotransmitter precursors, requires specific transport proteins for facilitative penetration across the BBB. Compounds may also penetrate the BBB by carrier-mediated transport systems provided that the compound's molecular structural features permit binding and subsequent transport across the cerebroendothelial membrane.

The in situ brain perfusion technique readily lends itself to mechanistic studies of BBB transport (Smith et al., 1987; Takada et al., 1991; Stoll et al., 1993). Variations in test compound perfusate concentration will facilitate a discernment of saturable transport. Adding postulated competitive transport inhibitors to the perfusion fluid will assist in understanding the substrate specificity of the transport system.

Additional Materials (also see Basic Protocols 1 and 2)

Perfusate solution: variable concentrations of a test compound with or without a competitive inhibitor dissolved in Krebs-bicarbonate buffer (see recipe), 37°C

1. Perform an in situ brain perfusion for rats (see Basic Protocol 1, steps 1 to 31b) or mice (see Basic Protocol 2, steps 1 to 18), using a perfusate solution containing variable concentrations of a test compound with or without a competitive inhibitor.

Several different perfusate solutions may be prepared; however, only one defined perfusate solution is used per animal. Hence, multiple treatment groups are needed for each defined perfusate composition.

It is necessary to initially perfuse the brain with Krebs-bicarbonate buffer (without test compound) for 15 sec to flush the cerebrovasculature of blood-borne endogenous competitive substrates. After 15 sec, quickly switch over to the perfusate containing the test compound (with or without exogenously added competing inhibitors).

2. Determine Cl_{in} , the influx transfer clearance (ml/min/g wet brain weight), using Equation 7.19.1. Then transform the observed Cl_{in} data to the mass transport influx parameter, J_{in} (g compound/min/g wet weight) using the equation:

$$J_{in} = Cl_{in} \times C_{pf}$$

Equation 7.19.6

where C_{pf} is the test compound perfusate concentration (g/ml).

This equation is used when the BBB permeability mechanism is carrier-mediated with saturable transport.

When the BBB permeability mechanism is subject to competitive inhibition, the observed Cl_{in} data will decrease significantly compared to control (perfusate containing only the test compound).

3. Fit the resulting J_{in} data to the Michaelis-Menten equation:

$$J_{in} = \frac{V_{max} \times C_{pf}}{K_M + C_{pf}} + J_{in(ns)}$$

Equation 7.19.7

where V_{\max} is the maximal rate of saturable transport (g compound/min/g wet brain weight), K_M is the dissociation constant of the transporter–test compound complex (g/ml), and $J_{\text{in(ns)}}$ represents mass transport influx due to diffusion.

If saturable transport is observed, a graph of J_{in} versus C_{pf} will be parabolic. If no saturable transport is observed, the graph will be linear.

BLOOD-BRAIN BARRIER INFLUX MEASUREMENT: INTRAVENOUS ADMINISTRATION/MULTIPLE TIME POINT PROCEDURE FOR RATS

BASIC PROTOCOL 3

This protocol employs a traditional pharmacokinetic approach to quantitation of test compound influx across the BBB. Groups of animals are dosed with the compound of interest, blood samples are obtained, and animals are sacrificed at predetermined times for brain tissue analysis. The data analysis involves simplified pharmacokinetic formulas to calculate an influx rate constant. Unlike the in situ brain method (see Basic Protocols 1 and 2 and the Alternate Protocol), the intravenous administration/multiple time point procedure does not readily lend itself to the study of specific influx transport mechanisms.

NOTE: This procedure involves rodent survival surgery. Sterile surgical procedures must be employed, as specified by prevailing animal care regulations. All surgical instruments and consumable items used for surgery must be sterile.

Materials

Laboratory rats, either gender, 200 to 350 g
Anesthetic, antibiotic, and other required medications for rodent survival surgery and rapid sacrifice (as specified by the approved animal care protocol; *APPENDIX 4B*)
Betadine (povidone/iodine solution)
Saline: 0.9% (w/v) NaCl (sterile), 37° and 4°C
10 U/ml heparinized saline, sterile
Test compound(s) dissolved in Krebs-bicarbonate buffer (see recipe), 37°C
Tissue solubilizer (e.g., Soluene 350; Packard) or 2 N NaOH, for radiolabeled test compounds only
Hydrogen peroxide, 30% (e.g., Sigma)
Warming pads (e.g., Deltaphase Isothermal Pad; Braintree Scientific), preheated to 37°C
Animal fur shaver
Surgical instruments (e.g., Harvard Apparatus), including:
4.5-in. (~11.4-cm) operating scissors, sharp or blunt
Scalpel
4-in. (10-cm) microdissecting forceps, full or strongly curved
3.5-in. Hartman hemostatic forceps
6-in. (15-cm) eye probe
3-in. (7.5-cm) Vannas spring scissors, straight
Dumont no. 7 microdissecting forceps
5.5-in. Olsen-Hegar needle holder
Inox no. 7 sharp-point forceps
Sterile drapes
Sterile sutures with and without needle, USP size 2.0 and 4.0
Jugular vein catheter (see Support Protocol 6)
22-G hypodermic needles with the beveled end cut off (see Support Protocol 1)
1-, 3-, and 5-ml syringes
22-G stainless steel wire, sterile

Vials (scintillation vials, 7 to 20 ml, with caps, can be used when working with radiolabeled compounds)

Rodent guillotine

PE50 polyethylene tubing, 0.58 mm (0.023 in.) i.d., 0.965 mm (0.038 in.) o.d.
(Intramedic Clay Adams; Becton Dickinson Labware)

22-G stainless steel connectors (see Support Protocol 1)

Tissue forceps or bone rongeurs

Filter paper

50°C water bath, optional

1.5-ml microcentrifuge tubes with caps

Additional reagents and equipment for analyzing nonradiolabeled test compounds
(see Critical Parameters and Troubleshooting, discussion of sensitive analytical method), optional

Prepare rat for surgery

1. Anesthetize a 200- to 350-g laboratory rat with an appropriate anesthetic as approved by the IACUC. Administer sufficient anesthetic (with or without supplemental doses) to maintain proper anesthesia for a surgical procedure of ≤ 1 hr.
2. Administer appropriate antibiotic to the animal, as approved by the IACUC.
3. Place anesthetized rat right side up (lying on its belly) on a warming pad. Shave the fur from its back, between the shoulder blades, using an animal fur shaver. Wash the surgical area with Betadine solution. Using a pair of 4.5-in. operating scissors, make a small incision (0.3 cm long) between or slightly ahead of the shoulder blades.
4. Reposition the rat so that it is upside down (lying on its back) on the warming pad with its head located towards the surgeon and its tail away from the surgeon.
5. Shave the fur from its neck and shoulder areas. Wash surgical area with Betadine solution and cover area with sterile drapes, leaving only the surgical site exposed.

Perform surgery

6. Using a scalpel, make a 2- to 3-cm-long incision (Fig. 7.19.1) over the skin site where pulsation of the right jugular vein can be observed.

The left jugular vein can be exposed for left-handed investigators.

7. Using a pair of 4-in. curved microdissecting forceps, bluntly separate the subcutaneous and muscle tissues to expose a section of the jugular vein.

The vein is superficially located under the skin, is purple-bluish in color, and will disappear under the chest muscle towards the heart.

8. Using the forceps, carefully clean the vessel of connective tissue from the point of the chest muscle penetration (towards the heart) to a point where it bifurcates into two smaller vessels.
9. Using a sterile 4.0 suture without needle, ligate the vein shut at the point of the bifurcation (towards the snout).

The Hartman hemostatic forceps may be used to aid in the ligation of the vein.

10. Subcutaneously tunnel a 6-in. eye probe from the incision in the back (between the shoulder blades) to the incision in the neck and shoulder area. Insert a jugular vein catheter into the eye of the probe (Fig. 7.19.4A). Pull the probe out so that the catheter is subcutaneously tunneled under the skin, such that the catheter end extends out from the incision on the back and the silastic tubing end extends from the jugular vein incision (Fig. 7.19.4B).

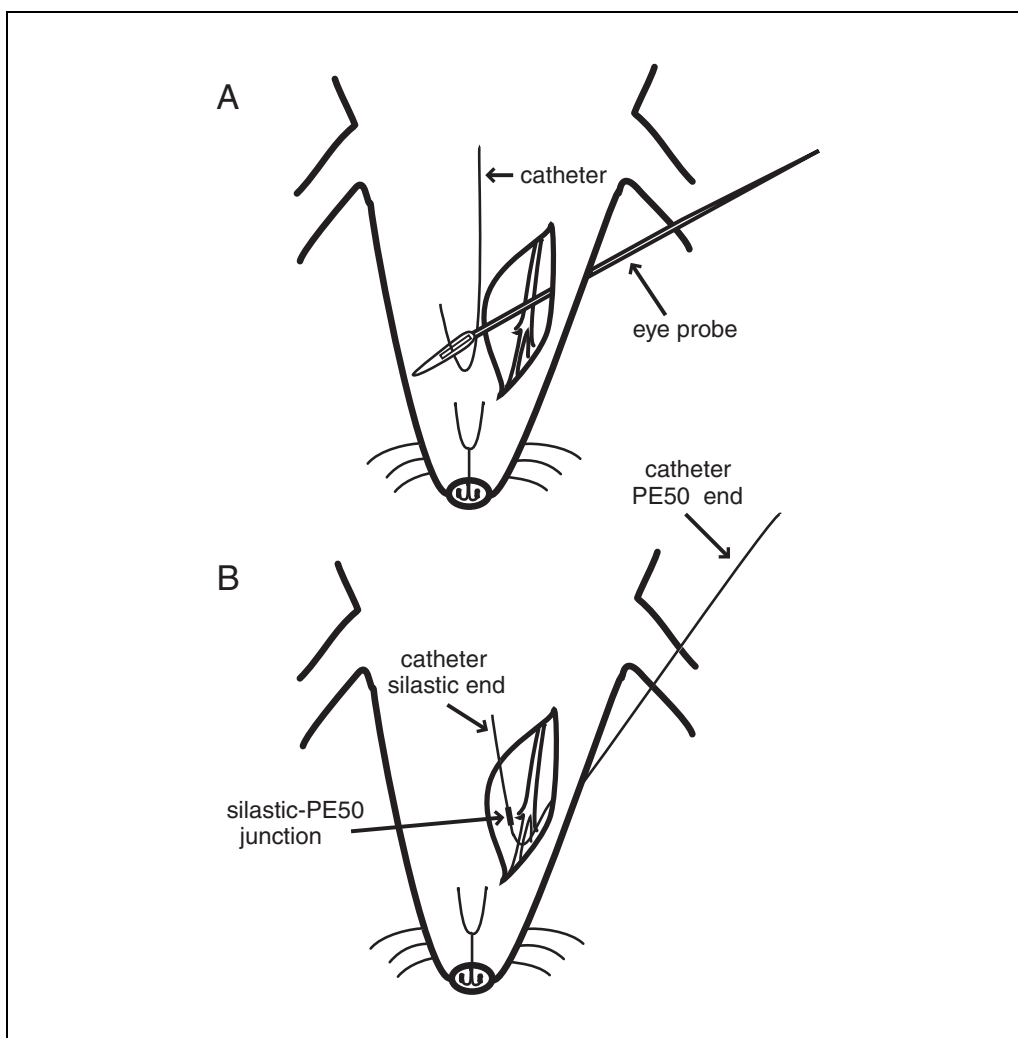


Figure 7.19.4 (A) Placement of the subcutaneously tunneled eye probe with the catheter threaded through the probe slit. (B) Orientation of the catheter in the jugular-vein incision for determination of the appropriate length for the silastic tubing end. In the final orientation of the catheter, the silastic tubing end is inserted into the jugular vein up to the point of the silastic-PE50 junction. The curved “U” of the catheter will lie comfortably within the incision site.

11. Position the catheter so that it lies comfortably in the jugular vein incision site. Cut the silastic tubing such that the free end is ~0.5 cm short of the midline of the front paws and the silastic-PE50 junction rests over the jugular vein (Fig. 7.19.4B). Make a beveled cut at the end of the silastic tubing.

The silastic tubing is typically no more than 2 to 3 cm in length after the sizing process.

12. Connect the PE50 tubing of the catheter to a 22-G hypodermic needle with the beveled end cut off, affixed to a 3-ml syringe containing prewarmed sterile saline. Fill the catheter with sterile saline.

An air-filled catheter will cause an air embolism, which can lead to seizures, cardiovascular problems, and/or death.

13. Place two or three more pieces of 4.0 suture under the jugular vein, towards the heart.

These pieces will be used to secure the catheter in the vein once the catheter is properly positioned.

14. Using a pair of 3-in. Vannas spring scissors, make a small, nicking incision in the jugular vein. Be careful not to cut through the vein.
15. Insert one point of a pair of Dumont no. 7 microdissecting forceps into the jugular vein incision. Slide the beveled end of the catheter along the probe into the incision. Continue inserting and guiding the catheter into the vein until the cut end of the vein covers the juncture of the silastic and PE50 tubing.
16. To test for catheter patency, inject 0.3 ml sterile saline and then slowly pull back on the syringe plunger to withdraw blood.
If no blood is forthcoming, then slightly pull the catheter back out from the vein and test for catheter patency again. If still no blood is forthcoming, remove the catheter, trim down the length of the silastic tubing and reimplant.
17. Clear the catheter of blood by flushing with 0.5 ml sterile saline.
Residual blood in the catheter will clot and cause catheter failure.
18. Anchor the catheter to the vein by tying the 4.0 sutures (step 13) securely (but not too tightly) around the vein and the PE50 tubing.
Sutures should not be tied around the silastic tubing as the thin and flexible silastic will be clamped shut. Tying the suture too tightly around the PE50 tubing can also clamp the catheter shut, resulting in catheter failure.
19. Suture the catheter to the muscle bed using an Olsen-Hegar needle holder and sterile 4.0 suture with needle.
This helps prevent the catheter from being pulled out by a conscious animal.
20. Close the internal incision by suturing the muscle and subcutaneous tissues with 4.0 sutures.
21. Close the skin incision using a sterile 2.0 suture with needle.
Alternatively, wound clips or staples can be used to close the incision.
22. Place anesthetized animal right side up (on its belly). Suture the exteriorized catheter to the skin and then close the incision on the back using 2.0 suture.
23. Trim the exteriorized PE50 tubing so that no more than 2.5 to 3 cm is exposed. Plug the open end of the catheter with a sterile piece of 22-G stainless steel wire.
24. Apply topical antibiotics to all incision sites as directed by the approved animal care protocol and allow animal to recover from the anesthetic using approved postoperative care protocols.
25. Slowly flush the jugular vein catheter with 0.5 to 1.0 ml sterile 10 U/ml heparinized saline each day to maintain patency.
At least 24 hr should elapse between the surgery and experimental study.

Prepare experimental equipment

26. Label and record the individual weights of an appropriate number of empty vials for collecting brain tissue.
One vial is needed for every discrete brain region per rodent; six to eight vials are needed for each rodent if this protocol is followed exactly.
27. Label an appropriate number of 1.5-ml microcentrifuge tubes with caps for the number of desired blood samples per rat.

28. Fill a 1-, 3-, or 5-ml syringe (depending on the dosing volume) with prewarmed test compound solution for each animal to be studied. To each syringe attach a 22-G hypodermic needle with the beveled end cut off. Keep the syringe and its contents warm by wrapping it in a preheated warming pad.

29. Prepare an appropriate number of 1-ml syringes filled with 1 ml prewarmed sterile saline.

For example, for each rat, prepare one syringe for every time point of blood collection plus one syringe to be used after compound dosing (to flush the catheter) and one extra syringe (for extra measure).

30. Prepare an appropriate number of empty 1-ml syringes for blood collection.

The number of syringes depends on the number of blood samples obtained per rat and the number of rats in the experimental group. If plasma is desired, the syringes can be rinsed with sterile heparin solution (1000 to 5000 U/ml) and allowed to dry.

31. Prepare sufficient medications for rapid sacrifice of the rat (as approved by the IACUC). Set up a rodent guillotine for brain harvesting.

One preferred expeditious method is to rapidly anesthetize the rodent with an intravenous bolus dose of a fast acting barbiturate, followed by intravenous administration of isotonic potassium chloride.

Perform experimental study

32. Transfer the catheterized rat to an appropriate animal holding cage.

33. Connect one end of a 20-cm-long piece of PE50 tubing to a 1-ml saline-filled syringe using a 22-G hypodermic needle with the beveled end cut off. Fill tubing with sterile saline and connect the free end of the saline-filled PE50 tubing to the exteriorized jugular vein catheter using a 22-G stainless steel connector. Gently flush catheter with 0.3 ml saline.

34. Withdraw a predose blood sample (blank; time zero) by gently pulling back the syringe plunger so that the luer-lock needle hub is filled with blood. Attach a fresh syringe (step 30) to the luer-lock hub and collect a 0.1- to 0.15-ml blood sample.

Sample volumes larger than this will cause a reduction in hematocrit with potential alterations in BBB permeability pharmacokinetics.

35. Remove the sample syringe, transfer sample to the prepared vial (step 27), and reattach the first syringe (filled with saline and possibly mixed with blood) to the needle hub. Flush the PE50 line and refill it with 0.3 to 0.5 ml sterile saline. Replace the initial syringe with a fresh syringe containing sterile saline.

It is important to replace the blood sample volume with an equivalent volume of sterile saline to prevent volume depletion.

36. Attach the test compound dosing syringe (step 28) to the PE50 tubing. Administer the dose intravenously over 30 to 60 sec. Remove the dosing syringe and attach a saline-filled syringe to the PE50 line. Flush the residual dose intravenously with saline and refill the tubing with sterile saline. Retain a 0.25-ml aliquot of the test compound solution for later analysis.

The aliquot may be stored up to 1 month at -20°C .

37. Withdraw blood samples at predetermined time intervals.

Suggested blood sampling times include 0 (predose, blank), 1, 2.5, 5, 10, 15, 20 min, and every 10 min thereafter for the duration of the study. It is important to capture influx of the

compound into the brain during early times where insignificant efflux occurs. Test compound influx data will be unreliable if pharmacokinetic distributional equilibrium is attained during a prolonged study period. The length of the study period is typically determined by the compound's preliminary pharmacokinetics. Test compounds with a rapid distribution phase (monoexponential time course or one-compartment pharmacokinetics) should be studied in <30 min; compounds with a more prolonged distribution phase (multiexponential time course or multiple-compartment pharmacokinetics) can be studied for longer time periods (≤ 60 min). If the pharmacokinetics of the test compound are unknown, a suggested time interval would encompass a 0- to 20-min (no longer than 60-min) study interval.

38. Withdraw the last blood sample ~30 to 60 sec before sacrifice, quickly anesthetize the rat, and decapitate it.

Harvest tissue

39. Using a pair of tissue forceps or bone rongeurs, remove the entire brain (forebrain and cerebellum, and optionally brainstem) from the skull.

Even if only forebrain is to be analyzed, forebrain and cerebellum should be removed for dissection purposes.

40. Place the brain in a disposable petri plate containing a piece of filter paper moistened with sterile saline. Place the petri plate on ice so the brain dissection is performed under cold conditions.
41. Using a pair of Inox no. 7 sharp-point forceps, remove meningeal membranes and associated surface blood vessels. Grossly dissect the forebrain, cerebellum, and separate the left hemisphere from the right hemisphere.
42. Grossly dissect both hemispheres into anatomically defined brain regions: frontal cortex, occipital cortex, posterior cortex, striatum, hypothalamus and thalamus, and hippocampus.

Ohno et al. (1978) describes the dissection procedure in detail.

43. Use a lint-free tissue to wick away any excess, adherent moisture from the brain regions.
44. Place each piece of brain tissue (forebrain regions plus cerebellum and brainstem) in a preweighed vial (step 26) and determine the weight of the brain tissue.

Brain tissue can be frozen and stored up to 1 month at -20°C .

Quantitate test compound

45. Analyze each blood sample, tissue sample, and dose aliquot (step 36) for the test compound as defined by the appropriate analysis protocol.
- 46a. *For radiolabeled test compounds in tissue:* Digest each tissue sample with an appropriate amount of a tissue solubilizer as specified by the manufacturer's instructions. Alternatively, digest tissue with 1 to 2.5 ml (5 vol per gram of tissue) of 2 N NaOH and incubate 3 hr at 50°C . Vortex vigorously. Add an appropriate volume of chemically compatible scintillation fluid. Sufficient scintillation fluid is added when the sample forms a clear solution (with no emulsion formation). Quantify the radioactivity using an appropriate counting protocol in a scintillation counter.

If NaOH is used, a scintillation fluid (e.g., Hionic Fluor; Packard Instruments) that can neutralize the high concentration of aqueous base must be used.

- 46b. *For nonradiolabeled test compounds in tissue:* Follow the tissue extraction and analysis procedures as specified by the test compound analysis protocol.
- 46c. *For radiolabeled test compounds in blood:* Transfer an appropriate volume of blood sample to a 20-ml scintillation vial with cap. For every 100 μl of whole blood, add 300 μl of 30% H_2O_2 to decolorize the sample for liquid scintillation counting. Cap the samples and incubate the sample overnight at room temperature, followed by an additional 2 hr in a 45°C water bath. Vortex vigorously. Add an appropriate volume of chemically compatible scintillation fluid so the sample forms a clear solution (with no emulsion).

The second incubation step at elevated temperatures is necessary to destroy any residual H_2O_2 prior to liquid scintillation counting.

- 46d. *For nonradiolabeled test compounds in blood:* Follow the blood extraction and analysis procedures as specified by the test compound analysis protocol.

Analyze data

47. Calculate the area under the curve (AUC) for the concentration of test compound in the blood versus time from time 0 to the time of sacrifice, T. The AUC is calculated by the linear trapezoidal rule:

$$\text{AUC} = \sum_0^n \frac{1}{2} (C_n + C_{n+1}) (t_{n+1} - t_n)$$

Equation 7.19.8

where C_n is the n th blood sample test compound concentration at time t_n , C_{n+1} is the next successive concentration at time t_{n+1} , and t is the observed time of collection for the blood sample.

48. Using the brain and blood data, calculate an apparent unidirectional (influx) transfer coefficient, $K_{\text{in(app)}}$, for BBB penetration into brain parenchyma by linear regression analysis using the equation:

$$\frac{q_{\text{tot}}}{C_T} = K_{\text{in(app)}} \left[\frac{\int_0^T C dt}{C_T} \right] + V_{\text{vasc}}$$

Equation 7.19.9

where q_{tot} is the observed total test compound amount in the brain region (g compound/g wet brain weight) and represents the observed compound entrapped within the parenchyma and cerebrovasculature, C_T is the test compound blood concentration at the time of sacrifice (T), $\int_0^T C dt$ represents the AUC from time 0 to T (from step 47), and V_{vasc} represents the calculated vascular blood volume (ml/g wet brain weight).

Equation 7.19.9 is in the form of a straight line with a slope of $K_{\text{in(app)}}$ and a y intercept of V_{vasc} (Ohno et al., 1978; Rapoport et al., 1980; Smith et al., 1988; Smith, 1989). This mathematical analysis assumes that: (1) the test compound is not metabolized by either peripheral or brain tissues; (2) the series of studied rats have similar values of $K_{\text{in(app)}}$ and V_{vasc} ; (3) the pharmacokinetic influx rate microconstant (k_{in}) is substantially greater than the pharmacokinetic efflux rate microconstant (k_{out}); and (4) radiotracer efflux from brain

**SUPPORT
PROTOCOL 6**

tissue is negligible at early times after dosing (i.e., there is unidirectional transfer of tracer from the cerebrovascular capillary bed into brain tissue). The validity of the unidirectional transfer assumption can be assessed by inspecting a plot of data derived from Equation 7.19.9 for curvilinearity; significant radiotracer efflux from brain tissue will produce data that systematically fall below those values predicted by the initial linear regression line (Fig. 7.19.8).

For comparative purposes for V_{vasc} , Smith et al. (1988) contains comprehensive tabular data on inulin regional brain distribution volumes.

CONSTRUCTION OF JUGULAR VEIN CATHETERS

The jugular vein catheter, once surgically implanted, permits intravenous dosing as well as blood sampling. This protocol describes how to fabricate an inexpensive, single-use jugular vein catheter.

Materials

Chloroform

PE50 polyethylene tubing, 0.58 mm (0.023 in.) i.d., 0.965 mm (0.038 in.) o.d.

(e.g., Intramedic Clay Adams; Becton Dickinson Labware)

Silastic tubing, 0.50 mm (0.020 in.) i.d., 0.965 mm (0.038 in.) o.d. (e.g., Silastic

Medical Grade; Dow Corning)

CAUTION: Chloroform is a toxic irritant and mild carcinogen; take suitable precautions and work in a fume hood.

1. Cut a 15-cm length of PE50 polyethylene tubing and a 5-cm length of silastic tubing.
2. Immerse one end of the silastic tubing into a beaker of chloroform for ~20 to 60 sec. Quickly remove from the chloroform and slip over one end of the PE50 tubing. Allow chloroform to evaporate.

The chloroform softens the silastic tubing and makes it sufficiently sticky so that it will adhere strongly to the PE50 tubing when dry.

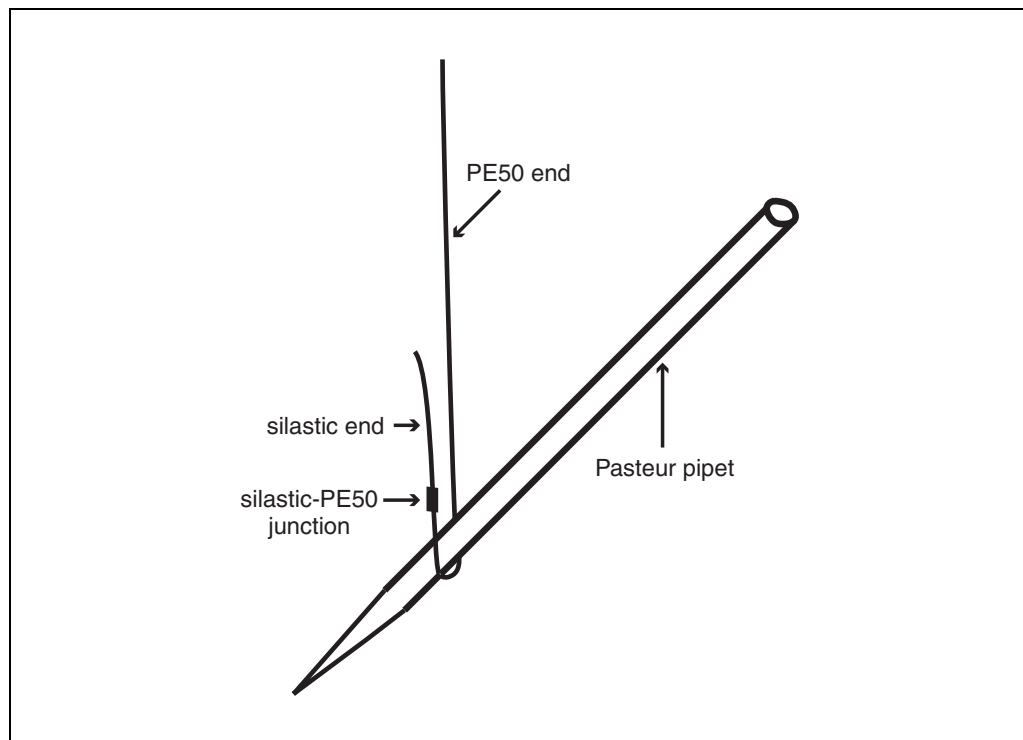


Figure 7.19.5 Orientation of the jugular-vein catheter wrapped around a glass Pasteur pipet prior to immersion into a beaker of boiling water.

3. Wrap the PE50 tubing in a U shape around a glass Pasteur pipet so the U of the PE50 tubing is ~1 to 1.5 cm from the juncture of the silastic and PE50 tubing. Using one pair of forceps to hold the silastic end and another pair (or the experimenter's hand) for the PE50 end, immerse the Pasteur pipet into a beaker of boiling water to cover the U shape for 10 to 30 sec (Fig. 7.19.5).

When the catheter is removed from the boiling water, the PE50 tubing should be reshaped in the form of a U. The catheter should look like a shepherd's crook. The U bend in the catheter helps to secure the catheter in the jugular vein.

CAPILLARY DEPLETION METHOD

Sometimes test compounds or macromolecules will bind to and/or be endocytosed by cerebroendothelial cells. The protocols in this unit assume that these complications are negligible. However, certain xenobiotic physiochemical properties (cationic molecules, macromolecules, or xenobiotics designed to bind to endothelial receptors or transporters) may predispose the xenobiotic to cerebrocapillary binding and/or endocytosis.

Additional experimental methods may be performed to discriminate mechanistically between actual penetration across the BBB into brain parenchyma versus capillary binding or endocytosis. A capillary depletion method isolates cerebrovascular capillary beds from brain parenchyma (Triguero et al., 1990). This method is performed on harvested brain tissue using any of the BBB influx protocols described in this unit (see Basic Protocols 1, 2, or 3 or the Alternate Protocol).

Additional Materials (also see Basic Protocols 1, 2, and 3)

Krebs-bicarbonate buffer (see recipe), 4°C

26% (w/v) dextran solution (average MW 74,000), 4°C

Glass homogenizer with glass pestle, prechilled

Perform BBB influx experiment

1. Follow the procedures as described (see Basic Protocol 1, steps 1 to 29; see Alternate Protocol; see Basic Protocol 2, steps 1 to 18; or see Basic Protocol 3, steps 1 to 44), but keep all brain tissues cold after harvesting and for the remainder of the protocol by placing all items on ice. For Basic Protocol 3, collect one blood sample immediately prior to sacrifice.

Quantitate test compound

2. Transfer brain tissue to a prechilled glass homogenizer.
3. Add 2 vol (based on tissue weight) ice-cold Krebs-bicarbonate buffer.
4. Homogenize the tissue with eight to ten up-and-down strokes of the pestle.
5. Add an equal volume of ice-cold 26% dextran solution.

The volume of dextran solution should be equivalent to the total volume of the homogenate (brain tissue with buffer); thus, the dextran solution concentration will be 13% (w/v) after addition of the dextran solution to the homogenate.

6. Homogenize the tissue with three additional strokes.

The homogenization procedure should be performed as rapidly as possible (within a minute or so).

7. Retain a 1-ml aliquot of the homogenate for test compound analysis and transfer remaining homogenate to a preweighed 50-ml tube.

The aliquot may be stored up to 1 month at -20°C.

SUPPORT PROTOCOL 7

8. Centrifuge homogenate 15 min at $5400 \times g$, 4°C , in a swinging-bucket rotor.
9. Carefully separate the supernatant (containing the test compound liberated from brain parenchyma and the cerebrovascular lumen) from the pellet (containing red blood cells, brain nuclei, and brain microvessels containing bound or endocytosed test compound) by pipetting.
10. Record the weight of the pellet fraction.
11. Determine the concentration of test compound in the whole homogenate, supernatant, pellet, and perfusate aliquot as defined by the appropriate test compound analysis protocol.

Analyze data

12. Calculate the volume of distribution, V_d , a measure of test compound distribution in each of the homogenate, supernatant, and pellet fractions using the equation:

$$V_d = \frac{M_F}{C}$$

Equation 7.19.10

where M_F , the test compound mass in the measured fraction, represents the compound amount per gram of brain tissue in the aliquot of the homogenate, pellet, or supernatant fractions, and C is the compound concentration in the perfusate (g/ml) for Basic Protocols 1 and 2, or the compound concentration in the blood sample immediately prior to sacrifice for Basic Protocol 3.

The mass of brain tissue present in the supernatant is calculated based on a knowledge of the initial brain tissue weight, the total volume of the homogenate, the volume of the homogenate aliquot taken for analysis, and the pellet weight.

The V_d of the homogenate represents the total test compound in the brain region. The V_d of the pellet fraction represents the compound bound and/or endocytosed in the brain cerebrovessels. The V_d of the supernatant is a composite value consisting of the V_d for test compound localized in brain tissue and the V_d for test compound in the cerebrovascular lumen. The fraction of the supernatant V_d attributable to luminally entrapped test compound can be determined by in situ perfusion using an intravascular marker, such as radiolabeled inulin (see Support Protocol 3) coupled with the capillary depletion technique. The supernatant V_d for inulin is entirely derived from tracer resident in the cerebrovasculature.

BASIC PROTOCOL 4

BLOOD-BRAIN BARRIER EFFLUX MEASUREMENT: THE BRAIN EFFLUX INDEX METHOD

The previous protocols in this unit are designed to quantitate influx of compounds across the BBB. However, there is an emerging interest in the structure and function of efflux transporters at the BBB (Kakee et al., 1996; Kusuhara et al., 1997; Hosoya et al., 1999). P-glycoprotein, a well-known efflux transporter expressed at the BBB and other organ sites, exports lipophilic molecules from brain endothelial cells. There are circumstances in which a compound has sufficient lipophilicity for BBB penetration, yet fails to accumulate in brain tissue in pharmacologically sufficient amounts. Should this be the case, one likely cause may be the active export of the test compound by BBB efflux transporters.

This brain efflux protocol requires microinjection of test and reference compounds into brain tissue, followed by analysis of brain tissue for residual compounds. Calculation of

the brain efflux index (BEI), a mathematical ratio of the amount of test compound relative to the reference compound, allows a determination of whether the test compound is a substrate for efflux transporters. Subsequent characterization of the efflux transporter is accomplished through inhibition studies.

This protocol employs a traditional pharmacokinetic approach to quantitation of blood-brain barrier drug efflux. Groups of animals are microinjected with the drug of interest and at predetermined times, animals are sacrificed for CSF brain tissue. The data analysis involves simplified pharmacokinetic formulae to calculate an efflux rate constant.

Materials

Laboratory rats (200 to 350 g, either gender)
Anesthetic (as specified by the approved animal care protocol; *APPENDIX 4B*)
2% (w/v) lidocaine/0.001% (w/v) epinephrine (commercially available as Xylocaine 2% with epinephrine 1:100,000; J.A. Webster Co.)
Injectate solution: test and reference compounds dissolved in ECF buffer (see recipe), 37°C
Saline: 0.9% (w/v) NaCl (sterile)
Tissue solubilizer (e.g., Soluene 350; Packard) or 2 N NaOH
Scintillation vials, 7 to 20 ml, with caps
Stereotaxic atlas (e.g., Paxinos and Watson, 1986, for rats)
5- μ l syringe (e.g., Hamilton Gas-Tight removable needle microliter syringe; Hamilton Company), with 28-G, 2-in. (5-cm), point-style no. 4 (12° bevel) needle, 0.18 mm (0.007 in.) nominal i.d., 0.36 mm (0.014 in.) nominal o.d. (may need to be custom fabricated by Hamilton Company)
Rodent stereotaxic frame
Animal fur shavers
Warming pads (Deltaphase Isothermal Pad; Braintree Scientific), preheated to 37°C
Scalpel
Drill and small drill bit, e.g., 1/32-in. (0.8-mm) bit inserted in a Dremel unit or affixed to a manual handle
Surgical shears
Cerebrospinal fluid (CSF) collection unit (see Support Protocol 8)
Olsen-Hegar needle holder (e.g., Harvard Apparatus)
Rodent guillotine, optional
Tissue forceps or bone rongeurs
Filter paper
Inox no. 7 sharp-point forceps (e.g., Harvard Apparatus)
50°C water bath, optional

NOTE: Because the amounts of injected test compounds are small, a highly sensitive analytical detection method is required. Radiolabeled test compounds are frequently used. Suggested reference compounds include ³H- or ¹⁴C-labeled inulin or carboxyinulin. If the radiolabeled form of a test compound is to be used, an isotopic form different from the inulin reference must be selected.

Prepare equipment

1. Label and record the individual weights of an appropriate number of empty scintillation vials for collecting brain tissue.

One vial is needed for every discrete brain region per rodent; three vials are needed for each rodent if this protocol is followed exactly.

2. Using a stereotaxic atlas, identify the target brain region for microinjection.

For basic efflux studies, the Par 2 region of the frontal cortex is recommended as the target because it is sufficiently isolated from the brain ventricular fluid system. In addition, published studies (Kusuhara et al., 1997; Hosoya et al., 1999) have demonstrated reproducible and reliable efflux data from this region.

3. Mount a 5- μ l syringe on the arm of a rodent stereotaxic frame.

Prepare animal for surgery

4. Weigh a laboratory rat and anesthetize it with an appropriate injectable anesthetic.
5. Shave its scalp using an animal fur shaver.
6. Place animal in the rodent stereotaxic frame, with the animal's trunk on top of a preheated warming pad to maintain normothermia.

Perform surgery

7. Using a scalpel, make an ~1-cm incision along the anterior posterior axis to expose the skull.
8. Blot the skull dry using a lint-free tissue, gauze, or a cotton-tipped applicator. Apply ~0.5 ml of 2% lidocaine/0.001% epinephrine to the exposed skull and skin. After a minute or so, blot the skull dry again.

Lidocaine serves to control topical bleeding as well as provide additional topical anesthesia.

9. Identify the bregma (Fig. 3.10.1; a reference point where the coronal and sagittal sutures intersect) and mark it with the tip of a sharp pencil.
10. Align the needle tip of the microsyringe with the pencil mark defining the bregma. Use the micromanipulator of the stereotaxic frame to lower the needle tip so that it just touches the pencil mark on the skull. Record the anterior and lateral stereotaxic coordinates.
11. Based on the published coordinates of the brain target region (obtained from the stereotaxic atlas), calculate the new micrometer coordinates.

Based on the rat brain atlas of Paxinos and Watson (1986), the Par 2 region is 0.2 mm anterior, 5.5 mm lateral with respect to the bregma, and 3.1 mm deep from the dura mater.

12. Raise the microliter syringe and manipulate the micromanipulator so that the needle is over the target brain region.
13. Lower the needle tip so that it nearly touches the skull over the target brain region. Mark the needle spot on the skull with a pencil mark.
14. Raise the microliter syringe and swing the micromanipulator arm to the side.
15. Use a drill and small drill bit to gently drill a small hole through the skull, taking care not to puncture the dura mater.

A change in resistance will occur as the drill penetrates the skull. Puncturing the dura mater will damage the brain tissue, cause excessive bleeding, and potentially lead to artifactual data.

Perform microinjection

16. Load the syringe with 1 μ l injectate solution without disturbing the mounted position of the syringe. Retain a 10- μ l aliquot of the injectate for later analysis.

The aliquot may be stored up to 1 month at -20°C .

17. Swing the micromanipulator arm back into place over the target brain region. Lower the needle until it nearly touches the dura mater. Record the vertical coordinate.
18. Based on the published vertical coordinate of the brain target region, calculate the new vertical micrometer coordinate.
19. Slowly lower the microliter syringe to the new vertical coordinate. Gently wick away minor bleeding with a lint-free tissue or cotton-tipped applicator, but be sure not to disturb the needle.
20. Slowly administer 0.5 to 0.6 μl injectate into the brain tissue by depressing the microliter syringe plunger over a period of 2 sec. Let the needle remain in the injection site for 10 sec.
21. Slowly withdraw the needle by carefully raising the syringe using the micromanipulator.
22. Allow the animal to remain positioned in the stereotaxic unit for a period of time (as specified by the experimental design) before harvesting CSF and brain tissue.

Substrate inhibition studies are procedurally more demanding as they require the preadministration of a competitor solution. This procedure requires the use of two different syringes, with both syringe needles aligned for the same brain region target site. A 50- μl volume of injectate (containing the competitor substance in isotonic ECF buffer) is preadministered over a period of 30 sec using a 50- μl gas-tight microliter syringe (Hamilton Company). Traumatic brain tissue damage can occur if the competitor solution is administered too quickly. After an appropriate period of time (as determined by experimentation; may range from 5 to 30 min), 0.5 to 0.6 μl test compound is administered.

Pharmacokinetic analysis of the apparent efflux rate constant of the test compound of the brain, K_{eff} , requires the sacrifice of multiple rats at different time intervals following the microinjection (a minimum of three rats per time point). It is suggested the experimental design include a minimum of four to five time points after injection, e.g., 0, 5, 10, 20, 30 min, or longer, if necessary.

Collect CSF

23. Use a pair of surgical shears to carefully cut the skin over the back of the skull and outstretched neck of the anesthetized rat.

Steps 23 to 26 should be performed as quickly and reliably as possible because test compound efflux is time dependent.

24. Use a scalpel to cut the neck muscle anchored at the base of the skull. Using the scalpel, gently scrape the cut neck muscle downwards (caudally) to expose the white alanto-occipital membrane that covers the cisterna magna (Fig. 7.19.6).

Excessive bleeding can be controlled by infiltrating the muscle and skin tissue with 2% lidocaine/0.001% epinephrine. This is done using a 25-G hypodermic needle and 1-ml hypodermic syringe.

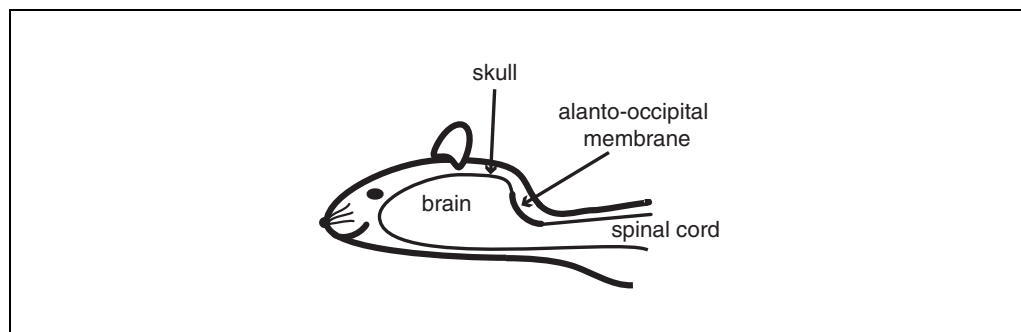


Figure 7.19.6 Side view of the head and neck region of a mouse or rat showing the location of the alanto-occipital membrane for collection of cerebrospinal fluid (CSF).

25. Hold a CSF collection unit with an Olsen-Hegar needle holder. Advance the needle through the membrane to a depth where the needle bevel just punctures through the membrane.

A deeper puncture may result in penetration of the cerebellar tissue, which causes bleeding and subsequent artifactual data.

26. Hold the CSF collection unit steadily in place with one hand and pull back on the syringe plunger with the other hand. Be careful to aspirate CSF uncontaminated with blood.

Blood-contaminated CSF may yield artifactual data.

Approximately 100 to 150 μ l CSF can be collected by this method.

27. Transfer CSF to a 1.5-ml microcentrifuge tube with cap and store up to 1 month at -20°C for analysis.

Collect brain tissue

28. Remove the animal from the stereotaxic frame and quickly decapitate it using sharp surgical shears or a rodent guillotine.
29. Using a pair of tissue forceps or bone rongeurs, remove the entire brain (forebrain and cerebellum) from the skull.
30. Place the entire brain in a disposable petri plate containing a piece of filter paper moistened with saline. Place the petri plate on ice so that the brain dissection is performed under cold conditions.
31. Using a pair of Inox no. 7 sharp-point forceps, remove meningeal membranes and associated surface blood vessels.
32. Harvest the cerebellum and both hemispheres (ipsilateral and contralateral).
33. Use a lint-free tissue to wick away any excess, adherent moisture from the brain region tissue. Place tissue in a preweighed vial (step 1) and determine the weight of the tissue.

Brain tissue can be frozen and stored up to 1 month at -20°C .

Quantitate test and reference compounds

- 34a. *For radiolabeled test and reference compounds in tissue:* Digest brain tissue with appropriate amount of a tissue solubilizer as specified by the manufacturer's instructions. Alternatively, digest tissue with 1 to 2.5 ml (5 vol per gram of tissue) of 2 N NaOH and incubate 3 hr at 50°C . Vortex vigorously. Add an appropriate volume of chemically compatible scintillation cocktail until the sample forms a clear solution (with no emulsion). Quantify the radioactivity using a dual-label counting protocol (for concurrent ^3H - and ^{14}C -labeled test and reference compounds) in a scintillation counter.

If NaOH is used, a scintillation fluid (e.g., Hionic Fluor; Packard Instruments) that can neutralize the high concentration of aqueous base must be used.

- 34b. *For radiolabeled test and reference compounds in CSF:* Pipet 100 μ l of CSF into a 7- or 20-ml scintillation vial with cap. Add an appropriate volume of chemically compatible scintillation cocktail until the sample forms a clear solution with no emulsion. Quantify the radioactivity using a dual-label counting protocol (for concurrent ^3H - and ^{14}C -labeled test and reference compounds) in a scintillation counter.

Analyze data

35. Calculate the brain efflux index (BEI) using the equation:

$$\text{BEI} = 1 - \left(\frac{M_{\text{TB}}}{M_{\text{RB}}} \right) \left(\frac{M_{\text{RI}}}{M_{\text{TI}}} \right)$$

Equation 7.19.11

where M_{TB} is the mass of the test compound in the brain, M_{RB} is the mass of the reference compound in the brain, M_{RI} is the mass of the reference compound injected, and M_{TI} is the mass of the test compound injected.

36. Multiply the BEI by 100 to convert the fraction into the percentage of efflux.
37. Calculate the percentage retained in the brain as $100 - \% \text{BEI}$.
38. Determine the apparent efflux rate constant of the test compound from the brain, K_{eff} , as the slope of the plot of $\ln(100 - \% \text{BEI})$ versus time. Alternatively, calculate K_{eff} as the slope $\times 2.303$ of the plot of $\log_{10}(100 - \% \text{BEI})$ versus time.

CONSTRUCTION OF CEREBROSPINAL FLUID COLLECTION UNITS

This protocol describes how to fabricate a single-use, inexpensive device to collect CSF from the cisterna magna.

Materials

5.5-in. (14-cm) Olsen-Hegar needle holder (e.g., Harvard Apparatus)
Metal file
22- and 25-G stainless steel disposable hypodermic needles
Silastic tubing, 0.50 mm (0.020 in.) i.d., 0.965 mm (0.038 in.) o.d. (e.g., Silastic Medical Grade; Dow Corning)
Suture, USP size 4.0
1-ml syringe

1. Using a 5.5-in. Olsen-Hegar needle holder and a metal file, cut a 25-G stainless steel disposable hypodermic needle from its plastic luer-lock hub. Save the needle and discard the hub.
2. Cut a 10-cm length of silastic tubing and slide one end over the cut end of the 25-G needle.
3. Securely tie the tubing onto the needle using 4.0 suture.
4. Using the needle holder and metal file, cut a 22-G stainless steel hypodermic needle in half. Save the end still connected to the plastic luer-lock hub. Properly discard the cut sharp needle.
5. Insert the cut end of the 22-G needle (with hub) into the free end of the silastic tubing.
6. Insert a 1-ml syringe into the plastic luer-lock hub.

SUPPORT PROTOCOL 8

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Extracellular fluid (ECF) buffer

122 mM NaCl

25 mM NaHCO₃

3 mM KCl

1.4 mM CaCl₂

1.2 mM MgSO₄

10 mM glucose

0.4 mM K₂HPO₄

10 mM HEPES

Adjust pH to 7.4 with concentrated HCl or NaOH

Store up to ~1 month at 4°C

Just prior to use, bubble for ~20 min with 95% (v/v) O₂/5% (v/v) CO₂ to attain pH 7.4 and filter through a 0.2-μm syringe filter unit. Add test and reference compounds to achieve the desired concentration (optional) and warm to 37°C.

Krebs-bicarbonate buffer

128 mM NaCl

24 mM NaHCO₃

4.2 mM KCl

1.5 mM CaCl₂

0.9 mM MgCl₂

2.4 mM NaH₂PO₄

9 mM glucose

10 U/ml heparin

Store up to ~1 month at 4°C

Just prior to use, bubble for ~20 min with 95% (v/v) O₂/5% (v/v) CO₂ to attain pH 7.4 and filter through a 0.2-μm syringe filter unit. Add test compound to achieve the desired concentration (optional) and warm to 37°C (or cool to 4°C; see Support Protocol 7).

To study the effects of test compound–protein binding effects on BBB permeability, 4% (w/v) albumin may be added to the buffer.

COMMENTARY

Background Information

The BBB is an anatomical feature that protects the internal milieu of the brain through regulation of substance permeability. The BBB is composed of cerebrovascular endothelial cells that are closely juxtaposed by astrocytic foot processes. These features restrict the penetration of xenobiotics to those compounds that are highly lipophilic and hence can freely diffuse across the barrier. Hydrophilic compounds are typically excluded, unless these substances possess an affinity for specialized, inwardly directed transport systems. Moreover, the expression of specialized efflux pumps at the BBB results in the active efflux of many lipophilic compounds.

A knowledge of a prototype compound's BBB permeability characteristics is important for successful discovery and development of new neuropharmacological entities. Lack of in vivo pharmacological efficacy despite in vitro activity may be due to poor BBB influx or active and rapid efflux. Alternatively, the lead compound may be superficially bound to or sequestered within the cerebrovasculature. Delineation of specific reasons for in vivo inactivity may provide insight into the development of improved analogs.

The influx and efflux protocols described in this unit are useful for preclinical assessment of test compound BBB permeability in rodents. The information gained from these studies yields predictive insight into the extent of com-

pound penetration into brain parenchyma (or lack thereof), as well as an understanding of the effective transport mechanisms.

Each influx method (in situ brain perfusion or intravenous injection/multiple time point procedure) permits a quantitative analysis of test compound penetration across the BBB. If the goal is to study xenobiotic influx, it is suggested to use the in situ brain perfusion techniques (see Basic Protocols 1 and 2; also see Alternate Protocol) first; however, if non-reproducible BBB permeability data or low permeability data are obtained, then it is advisable to use the intravenous injection/multiple time point procedure (see Basic Protocol 3). A capillary depletion method (see Support Protocol 7) may be used in conjunction with the basic influx protocols.

Currently, the in situ method is the preferred procedure. Compared to the intravenous injection procedure, the greater versatility of the in situ method permits a definitive mechanistic assessment of the operative transport mechanism(s) (e.g., passive diffusion, facilitative or active transport). Another advantage of the in situ procedure is the economy of animals: fewer animals are needed to obtain meaningful mechanistic data. The primary drawback to the in situ approach is that the short perfusion times (20 to 180 sec) yield unreliable numerical influx estimates for poorly penetrating substances. Moreover, the in situ method provides influx data under optimal, controlled conditions. If the goal is to obtain estimates under actual physiologic conditions in conscious animals, then the intravenous administration/multiple time point approach is the preferred experimental method.

The efflux method (see Basic Protocol 4) is of value when poor brain accumulation of lipophilic compounds is observed. Lipophilic compounds may diffuse into the cerebrovascular endothelium only to be exported by an efflux mechanism. Poor in vivo pharmacologic activity, in spite of good in vitro predictions, may be attributable to efflux mechanisms. Carefully designed studies will permit a pharmacologic characterization of the efflux transporter.

Critical Parameters and Troubleshooting

For all protocols in this unit

Purity of test compound. The test compound must be as chemically pure as possible because impurities may artifactually alter influx or efflux. This concern is of great importance if the

compound utilizes a carrier-mediated transport process and the impurities possess sufficient structural homology for competitive binding and/or transport. The purity issue is highly relevant if the compound is radiolabeled. The high sensitivity of radiochemical analytical methods will indiscriminately detect trace radiochemical impurities, ultimately causing inaccurate numerical permeability estimates. Commercially available radioisotopes may not have sufficient purity to use as received. It is good practice to purify the radiochemical tracer just prior to experimental use, striving for >99% radiochemical purity. Contact the radiochemical vendor for an appropriate purification method.

Sensitive analytical method. Although not discussed in this unit, a sensitive and specific analytical assay is essential to the collection of quality data. Radiochemical analysis, gas chromatography–mass spectrometry (GC/MS), or high performance liquid chromatography–mass spectrometry (HPLC/MS) are analytical methods that can detect small masses of substances that have penetrated into brain parenchyma. It is important to have an appropriate analytical method in place before undertaking BBB permeability studies.

In situ brain perfusion protocols

Initiation of the in situ perfusion. During the in situ brain perfusion procedure, it is important to initiate the perfusion as quickly as possible after ligating the CCA. Cerebral ischemia may confound estimation of barrier permeability if there is prolonged interruption of cerebral blood flow.

Perfusate time lag. It is also helpful to determine the time delay between initiation of the start of perfusion and the time of perfusate entry into the brain. The time delay, which is based on the perfusion rate and the tubing length, may be insignificant (1 to 2 sec) if the length of tubing (2 to 3 cm, not filled with test compound-containing perfusate) is short. However, substantial delays (5 sec) may occur with longer tubing lengths (≥ 10 cm). The actual length of the perfusion period should correct for the time lag.

Thoroughness of perfusion. An incomplete brain perfusion often is due to a poor surgical preparation whereby the perfusate flows through unintended pathways. The perfused hemisphere should appear blanched; in contrast, the unperfused hemisphere should appear pinkish. When learning the in situ perfusion technique, one may perfuse with 1% (w/v)

Table 7.19.2 Representative Permeability Data for Transport Via Passive Diffusion Obtained from the In Situ Brain Perfusion Technique^a

Representative substance	PA (ml/g brain tissue/min)	Reference
Anthralic acid	0.073	Fukui et al. (1991)
Antipyrine	0.78	Takasato et al. (1984)
Ethylene glycol	0.023	Takasato et al. (1984)
Glycerol	0.0021	Takasato et al. (1984)
Mannitol	0.0009	Takasato et al. (1984)
Quinolinic acid	0.003	Fukui et al. (1991)
Sucrose	0.0003	Takasato et al. (1984)
Thiourea	0.013	Takasato et al. (1984)
Trimethylene glycol	0.033	Takasato et al. (1984)
Urea	0.0038	Takasato et al. (1984)

^aEstimated average of forebrain regions. BBB permeability by passive diffusion is positively correlated to lipophilicity (Smith, 1989).

Table 7.19.3 Representative Permeability Data for Carrier-Mediated Transport Obtained from the In Situ Brain Perfusion Technique^a

Representative substance ^b	V_{\max} ($\mu\text{mol/g}$ brain tissue/min)	K_M (mM)	Reference
1-Aminocyclohexane-carboxylic acid	0.058	0.054	Aoyagi et al. (1988)
L-Kynurenine	0.027	0.16	Fukui et al. (1991)
Neutral amino acids ^c	0.042-0.096	<0.1	Smith et al. (1987)
β -N-Methylamino-L-alanine	0.096	2.3	Smith et al. (1992)
Valproic acid	4.8	13.8	Adkison and Shen (1996)

^aEstimated average of forebrain regions.

^bEndogenous nutrients or derivatives are typically substrates for carrier-mediated transport.

^cL-Leucine, L-phenylalanine, L-tyrosine, L-glutamine, L-valine.

Evans blue dye, in lieu of solution containing the test compound. The perfused hemisphere, if properly perfused, will be stained blue with the dye.

Composition of perfusate and rate of perfusion. The protocols described in this unit call for a physiologic, heparinized Krebs-bicarbonate buffer. Other perfusate compositions may be used, depending on the experimental hypothesis and goals. Various perfusate compositions include the addition of rat plasma, 1% to 4% (w/v) bovine serum albumin, or washed rat erythrocytes (hematocrit of ~40% to 45%) to the Krebs-bicarbonate buffer (Takasato et al., 1984). In these instances, the external perfusion rate for rats should be decreased to 2.5 to 3.0 ml/min to compensate for the increased perfusate viscosity. Too high of a perfusion pressure (≥ 160 to 190 mm Hg) will cause disruption of the BBB (Takasato et al., 1984). In addition, inclusion of plasma, albumin, or erythrocytes

in the perfusion buffer may result in binding of the test compound to protein (or red blood cells), which decreases the amount of free (unbound) compound available for barrier influx.

Intravenous injection/multiple time point method

Early sampling times. Regression of the data using Equation 7.19.9 yields estimates of the apparent influx rate constant, $K_{\text{in(app)}}$, and vascular capillary bed volume, V_{vasc} . Under most circumstances, V_{vasc} is invariant, and thus data estimates should be comparable to published literature estimates. If there is a discordance, one should place little confidence in the $K_{\text{in(app)}}$ (and V_{vasc}) parameter estimates, as a methodological problem confounds the experiment. One source of error may lie with the experimental design—namely, too few sampling points were obtained soon after dosing. Good estimates of V_{vasc} are often obtained when three or

more replicates are generated at initial sampling time points (e.g., 1, 2.5, 5 min). Another source of error may be related to a chemically or radioisotopically impure test compound.

Too long of a sampling period. Brain pharmacokinetic data collected over lengthy time periods will reflect both influx and efflux of the test compound. When the data are analyzed graphically (using Equation 7.19.9), a parabolic pattern will be evident if the sampling period is too long (see Anticipated Results for a detailed illustration). Influx occurs almost exclusively during the early time phase after intravenous administration, usually within the first 20 to 60 min. During this time, the concentration of the test compound in the brain is small and it can be assumed that there is a negligible brain concentration gradient for passive efflux and elimination.

Rapidity of sacrifice. It is important to follow the collection of the last blood sample with rapid anesthetization, sacrifice, and harvesting of brain tissues. Undue delay between the blood sample and sacrifice will significantly bias $K_{in(app)}$ estimates.

Brain efflux index method

Injection technique. Slow and careful insertion of the needle, slow administration of the injectate, and slow withdrawal of the needle are crucial to a successful experiment. Rapid or careless actions will result in tissue damage and/or leakage of the injectate back up through the needle track. Tissue bleeding after the microinjection is an indicator of an unreliable experimental preparation. Inclusion of the reference compound radiolabeled inulin assists in judging the quality of the experiment and the data generated therein. The inulin serves two important functions: it is an internal standard for variability in the delivered microinjection volume, and it is a reference marker for elimination of the test compound by bulk interstitial fluid flow. Reproducible injections usually yield reproducible inulin data, thus imparting confidence in the data obtained for the test compound.

Anticipated Results

***In situ* brain perfusion protocols**

Typical results for the *in situ* brain perfusion procedure are expressed as either absolute or relative numbers. Permeability data for lipophilic compounds that undergo passive diffusion are expressed as absolute Cl_{in} or PA values (e.g., Table 7.19.2). Usually, Cl_{in} or PA data are

positively correlated with test compound lipophilicity. For highly lipophilic compounds, the rate of cerebral blood flow may be the rate limiting step for barrier permeability. In this case, Cl_{in} data are corrected to reflect cerebral perfusion and the data are subsequently reported as PA values.

Cl_{in} (or PA) data are independent of the concentration of the test compound for a diffusional process; however, observation of a concentration dependence is diagnostic for a carrier-mediated transport process. In this case, Cl_{in} data are converted to J_{in} data (which account for perfusate concentrations), followed by the application of the Michaelis-Menten function to obtain V_{max} and K_M transport estimates (e.g., Table 7.19.3).

Lastly, Cl_{in} permeability data may be expressed relative to a control value. This approach is quite useful for transport inhibition studies where the control condition is a test compound alone at a fixed concentration and test conditions utilize a competitive transport inhibitor in combination with the test compound.

Intravenous injection/multiple time point method

Data for the intravenous injection/multiple time point influx technique is graphically depicted as traditional pharmacokinetic concentration–time course data (Fig. 7.19.7). These data are then analyzed by Equation 7.19.9, which is graphically presented in Figure 7.19.8. The data in Figure 7.19.8A appear to follow a parabolic curve, which indicates that passive efflux of the compound is significant at later sampling times. Regression analysis of all the data may lead to grossly biased estimates of $K_{in(app)}$ (Fig. 7.19.8A, dashed line). Restriction of the data analysis to earlier sampling times (Fig. 7.19.8B) reveals a better regression fit, yielding better estimates of $K_{in(app)}$ under conditions where influx of the compound is the predominate pharmacokinetic process.

Brain efflux index method

Similar to the *in situ* brain perfusion procedure, typical results for the BEI method are expressed as either absolute or relative numbers. A semilog plot of brain efflux–time course data enables the calculation of K_{eff} , an absolute value (e.g., Table 7.19.4).

The data may also be expressed in relative terms, which is often done for transport inhibition studies. The BEI of a test compound is calculated based on a single time point and

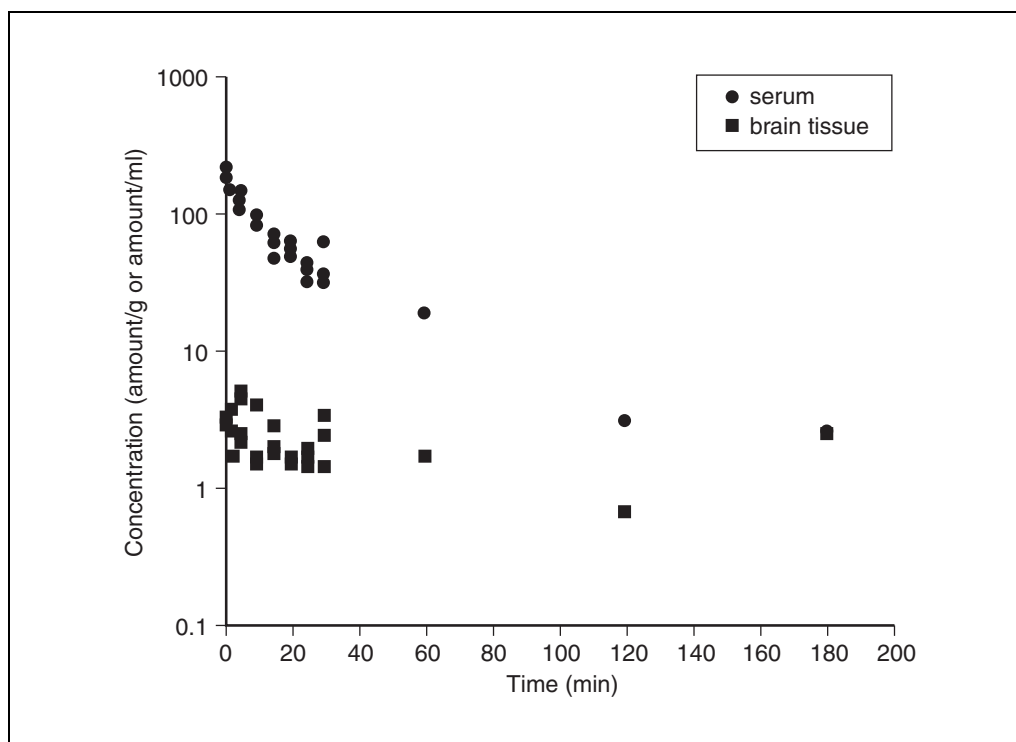


Figure 7.19.7 Typical ^{14}C -sucrose concentration time course data for hippocampal serum and brain tissue, following intravenous administration of $4.5 \mu\text{Ci}$ of ^{14}C -sucrose in 0.5 ml saline. Each point represents a single animal. The concentration is measured as either the amount of test compound/g wet brain weight or the amount of test compound/ml serum. (Data are previously unpublished from the author's laboratory.)

compound concentration; this BEI estimate is the control condition for comparison to BEI estimates obtained in the presence of added competitive efflux inhibitors.

On occasion, the observed BEI values may be greater than 100%, suggesting that the test compound is not selectively effluxed or eliminated, but is sequestered into brain tissue. This can occur for substrates that may have high affinity to brain proteins or neuronal or glial membrane transporters.

Time Considerations

In situ brain perfusion procedures

The experimental setup for terminal surgery takes ~1 hr. In experienced hands, the surgical procedures can be accomplished within 30 to 60 min per animal. The brain perfusion and tissue harvesting can take 20 to 30 min. If radiolabeled test compounds are used, the cleanup time can take as much as an hour to comply with appropriate radiation safety protocols.

Intravenous administration/multiple time point procedure

The experimental setup for survival surgery takes ~1 hr. In experienced hands, the surgical procedures can be accomplished within 30 to 60 min per animal. One or more days are required to permit the animal to recover from the minor surgical procedure. The experimental setup for the pharmacokinetic study can require 1 hr, with the study itself consuming ≥ 1 hr, depending on the pharmacokinetic sampling protocol. Tissue harvesting and cleanup may require ≥ 1 hr, depending on whether radiolabeled substrates are used.

BEI method

The experimental setup for terminal surgery takes ~1 hr. In experienced hands, the stereotaxic surgery and microinjection can be accomplished within 30 to 60 min per animal, with the study itself consuming ≥ 5 min, depending on the duration of time needed to detect sufficient test compound efflux (should it occur). The tissue harvesting and cleanup can take ≥ 30 min.

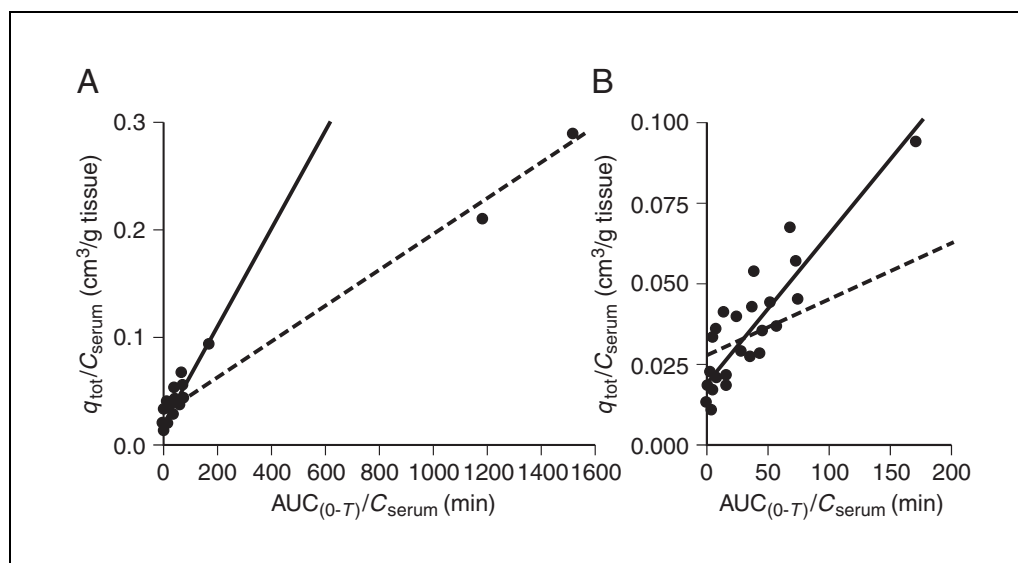


Figure 7.19.8 Graphical representation of $K_{in(app)}$ and V_{vasc} from an intravenous injection/multiple time point analysis. The data of Figure 7.19.7 were analyzed per Equation 7.19.9. Each point represents a single animal. The lines represent linear regression analyses of the data. **(A)** Data obtained from 0 to 180 min. The data appear to follow a parabolic curve, which is indicative of significant test compound efflux occurring at later sampling times. **(B)** The same data over a 0- to 30-min interval. The data appear to cluster around a straight line, suggesting that test compound influx predominates during the early sampling times. In both panels, the dashed lines are the regression analyses on the 0- to 180-min time intervals; the solid lines are the corresponding regression analyses for the 0- to 30-min time intervals. Note that the dashed lines show systematic deviations from the actual data at early times. (Data are previously unpublished from the author's laboratory. **NOTE:** For this experiment, C_{serum} represents the serum concentration at the time of sacrifice. If one measures blood concentrations, then one would use C_T .)

Table 7.19.4 Representative Data Obtained from the Brain Efflux Index Technique

Representative substance	K_{eff} (min ⁻¹) ^a	Reference
<i>p</i> -Aminohippuric acid	0.059	Kakee et al. (1997)
L-Aspartic acid ^b	0.207	Hosoya et al. (1999)
3'-Azido-3'-deoxythymidine (AZT) ^b	0.032	Takasawa et al. (1997)
2',3'-Dideoxyinosine (DDI) ^b	0.025	Takasawa et al. (1997)
L-Glutamic acid ^b	0.0346	Hosoya et al. (1999)
3- <i>O</i> -Methyl-D-glucose	0.13	Kakee et al. (1996)
Quinidine	0.066	Kusuhara et al. (1997)

^a K_{eff} data were obtained from a semilogarithmic graph of $(100 - \%BEI)$ versus time for injection of one concentration of the test compound. If efflux is saturable, the K_{eff} data may be fit to a Michaelis-Menten function.

^b K_{eff} data were determined at low concentrations. Saturation of the efflux mechanism was observed at higher concentrations.

Analyte analysis

If quantifying radiolabeled analytes, the automated liquid scintillation counting instrumentation should be able to analyze all samples within an 18-hr (or less) period (e.g., overnight). If quantifying non-radiolabeled analytes, the time to analyze the samples is highly

dependent on the particular analysis protocol, which is specific for the compound under study.

Use of the *in situ* brain perfusion procedure for mechanistic assessment

The time considerations are the same as that described for the *in situ* brain perfusion procedure.

Capillary depletion method

The time considerations are the same as that for the in situ brain perfusion procedure or the intravenous administration/multiple time point procedure, plus an additional hour for homogenization, centrifugation and sample collection.

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This paper illustrates the application of the intravenous injection/multiple time point procedure to studies of altered blood-brain barrier permeability during disease.

Kakee et al., 1996. See above.

This seminal paper introduces and validates the brain efflux index method.

Smith, 1989. See above.

This comprehensive review provides a theoretical and practical overview of the influx techniques presented in this unit.

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This excellent review critically evaluates peptide transport across the blood-brain barrier.

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