



Section Four

*Current In Vivo and In Vitro
Models of the Blood-CSF Barrier*





TF1719_C22.fm Page 540 Friday, January 21, 2005 1:38 PM





22 In Situ Perfusion Techniques Used in Blood–CSF Barrier Research

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CONTENTS

22.1	Introduction	541
22.2	In Situ Perfused Choroid Plexus Model	543
22.2.1	Technique Background	543
22.2.2	Animals and Pretreatment	543
22.2.3	Preparation of In Situ Perfused Sheep Choroid Plexus	544
22.2.4	Paired Tracer Method for Studying Blood to Plexus Transport ..	544
22.2.5	Kinetics Calculation	545
22.3	In Situ Brain Perfusion Technique	547
22.3.1	Technical Background	547
22.3.2	Animals	549
22.3.3	Perfusion Procedure	549
22.3.4	Calculations	550
22.4	Summary	550
	References	551

22.1 INTRODUCTION

The choroid plexus is a relatively independent tissue in the brain: independent blood supply, independent structural support, and independent functional connection to brain parenchyma. It is therefore convenient to distinguish the choroid plexus from the rest of brain structures and to illustrate the tissue, both anatomically and



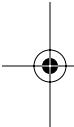
morphologically. Accordingly, techniques that are pertinent to elucidating the distribution of substances at the blood–cerebrospinal fluid (CSF) barrier or to studying the morphological changes of the tissue are most frequently used in choroid plexus research. For example, to see if *in vivo* metal exposure results in an accumulation of metal in the choroid plexus, the metal can be administered systemically, followed by sampling of the tissue at specified times. By quantifying the amount of the metal in the tissue, either by atomic absorption spectrophotometry or by measuring radioactivity if the radioisotope is used, one can estimate the tissue sequestration of the metal in comparison to other brain regions or other organs. In many cases, a high accumulation of the chemical in the choroid plexus may suggest a route for the chemical to enter or be removed from the CSF compartment.

Alternatively, autoradiography of a radiolabeled chemical in the choroid plexus is another useful tool to locate the transport site of this chemical at the blood–CSF barrier. In this kind of study, radiolabeled compounds can be given according to an acute or chronic dose regimen. At the end of dose administration, the whole brain can be frozen and sectioned for autoradiography. A strong signal from the brain ventricles usually implies a high accumulation of the studied substances in the choroid plexus.

In vivo investigation of the morphological changes of the choroidal epithelia can be accomplished by systemic administration of the study compound, followed by a sequence of procedures to dissect the choroid plexus tissue, to stain or prepare tissue section, and finally to examine by light or electron microscopes.

No great difficulty should be expected for these experiments as long as the investigator understands the location of the choroid plexus. One issue, however, should not be ignored—blood contamination in the choroid plexus tissue. As mentioned in previous chapters, the choroid plexus is rich in blood supply; the flow rate to the choroid plexus is nearly three times faster than that to the rest of the brain. Because of the richness of the blood component, the chemicals present in the blood, if they are not washed out prior to the tissue sampling, could complicate the results. Thus, for the aforementioned studies a rapid brain perfusion procedure is recommended. In an experiment performed in rats, the chest is cut open to expose the heart. An 18 gauge needle attached to a 10 mL syringe is inserted into the left ventricle. Immediately after cutting off the superior vena cava above the heart, 10 mL of ice-cold phosphate-buffered saline (PBS) is injected into the left ventricle. The PBS then circulates through the brain along the blood vessel path; drainage is achieved via the superior vena cava. The method is effective and easily judged for success by eye examination, as the perfused brain is pale in general, without visible blood contamination.

While a location-type study of the choroid plexus is relatively easy to perform, the functional study of the blood–CSF barrier, not at all an easy task. Several factors may contribute to the difficulties of the functional investigation. First, the size or mass of the choroid plexus tissue is rather small. In rats, the wet weight of the choroid plexus is only several milligrams, rendering it nearly impossible for cannulation study. Second, the choroid plexus contacts three fluid compartments: the CSF compartment, the blood compartment, and the choroidal intracellular fluid compartment. Unlike





study in the blood–brain barrier, where the transport property can be readily estimated by the appearance of the test compound in brain parenchyma following injection into the bloodstream, the ideal situation to estimate transport at the blood–CSF barrier would be to simultaneously determine the concentration of the test compound in both blood and CSF. Currently no such procedure has been satisfactorily established. The last, but not the least, factor is that sampling of CSF from animals is not straightforward, requiring considerable training and much practice.

Despite all these difficulties, several methods have been developed since the 1960s to study the function of the choroid plexus, particularly regarding the kinetic aspect of material transport by the blood–CSF barrier. This chapter will discuss the *in situ* techniques used in the perfusion of choroid plexus in sheep and perfusion of the whole brain in rodents.

22.2 IN SITU PERFUSED CHOROID PLEXUS MODEL

22.2.1 TECHNIQUE BACKGROUND

In situ perfusion of the choroid plexus in sheep was originally described by Pollay and Kaplan (1972) to study the transport of thiocyanate. The model was further described by Segal's group in King's College London (Blount et al., 1973) and later for investigation of sugar transport via the blood–CSF barrier (Deane and Segal, 1985). Most recently, the sheep model has been used to address the transport kinetics of lipton (Thomas et al., 2001), hypoxanthine (Redzic et al., 2002), and thyroxine (Zheng et al., 2003) in the choroid plexus.

The *in situ* perfused sheep choroid plexus model offers several advantages as an ideal tool for studying transport of molecules from the blood to the CSF. Unlike the *in vivo* CSF sampling method, where the CSF concentrations of substances reflect the dynamic balance of transport between the blood–brain barrier and blood–CSF barrier, the *in situ* perfused choroid plexus excludes interference from the blood–brain barrier and enables the characterization of the kinetic behavior of a given drug at the blood–CSF barrier. Since the tissue under investigation remains intact in the brain and the molecule investigated enters the plexus directly via the blood interface, this preparation provides better similarity to real-life choroid plexus than *in vitro* incubation of dissected plexus tissues or using cultured choroidal epithelial cells. In addition, application of a dual tracer technique enables one to correct the nonspecific loss of study molecules, making it possible to calculate the unique uptake (or removal) kinetics of the test compound by the choroid plexus.

22.2.2 ANIMALS AND PRETREATMENT

Sheep of either sex can be used for this study. The body weight is usually between 20 and 25 kg. The animals are allowed to have free access to water and food and are quarantined for a period of three to five days prior to experimentation. If *in vivo* treatment (or exposure) becomes necessary, sheep can be readily i.v. (or otherwise) dosed.



22.2.3 PREPARATION OF IN SITU PERFUSED SHEEP CHOROID PLEXUS

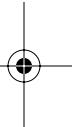
Prior to the experiment, each sheep is anesthetized with sodium thiopental (20 mg/kg, i.v.) and heparinized (10,000 units, i.v.). CSF samples (\approx 2 to 5 mL) can be obtained at this time through a butterfly needle (14 gauge) attached to polyethylene tubing. The needle is inserted between the protruberance and the spine of the atlas. CSF samples free of blood can be used for biochemical analysis. Upon CSF sampling, sheep are decapitated, the brains removed rapidly from the skull, and internal carotid arteries cannulated immediately.

The perfusion system can be started at a flow rate of 0.5 to 1.5 mL/min, with the perfusate being directed toward the anterior choroidal arteries by ligation of the other branches on the circle of Willis. The cerebral ventricles are cut open, and the exposed choroid plexus in the lateral ventricle can be readily seen. The ventricles are superperfused with artificial CSF (Preston and Segal, 1990). The perfusion fluid draining from the choroid plexuses is collected by a cannula inserted into the great vein of Galen (Deane and Segal, 1985). A simplified diagram of this *in situ* model is shown in Figure 22.1.

The perfusate is a modified mammalian Ringer solution (Preston and Segal, 1990) containing 4 g/dL dextran 70 (Gentran) as the colloid osmotic agent and 0.05 g/dL bovine serum albumin to maintain the integrity of the capillary wall. The artificial CSF is protein- and dextran-free. Both solutions are saturated with 95% O₂ and 5% CO₂ at pH 7.4 and warmed to 37°C, with the blood perfusate being filtered and debubbled prior to entering the choroid plexuses. The preparation should be kept warm with a water jacket and by an external heat source; temperature and pressure should be tightly monitored. A preparation like this can stay viable for two to three hours (Preston and Segal, 1990).

22.2.4 PAIRED TRACER METHOD FOR STUDYING BLOOD TO PLEXUS TRANSPORT

Cellular uptake of radiolabeled chemicals by the choroid plexus from the blood can be determined from the ratio of the recovery of radiolabeled chemicals to the recovery of [¹⁴C]mannitol, a nontransported extracellular marker, in choroidal venous outflow. For better understanding of the principle, we use [¹²⁵I]T₄ (thyroxine) as an example. A calibrated “side loop” of the perfusion system is filled with a 100 μ ML bolus Ringer containing 0.5 μ MCi [¹²⁵I]T₄ (4.3 fmol), various concentrations of unlabeled T₄, and 2 μ MCi of [¹⁴C]mannitol (0.1 nmol). By a system of taps, the flow of perfusate can be directed into this side loop and the bolus driven by the flow of perfusate toward either side of the choroid plexus without a rise in perfusion pressure. Approximately 20 seconds later, to enable the bolus to reach the plexus, a run of 20 sequential one-drop samples of venous effluent is collected in about 60 seconds followed by a continuous four-minute collection. Each brain preparation (both sides of the cannulated choroid plexus) can be used for 10 to 15 such runs. The final four-minute collection is usually used to calculate the flow rate. An aliquot (3 mL) of scintillation fluid can be added to each collected drop, 50 μ ML of final



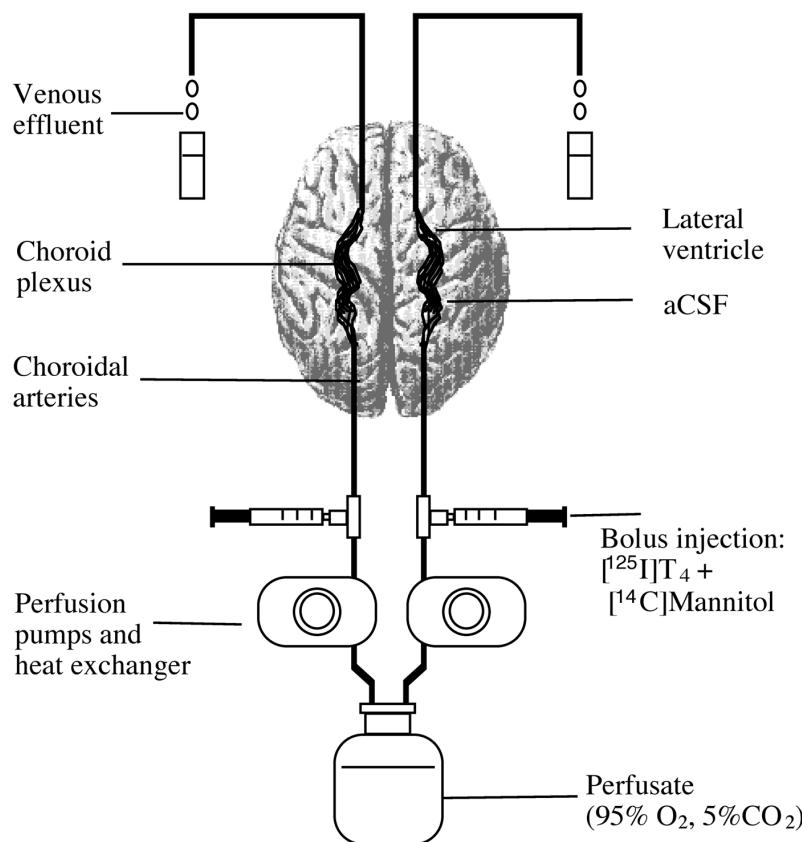


FIGURE 22.1 Illustration of in situ sheep choroid plexus perfusion model. The perfusion system is maintained at a flow rate of 0.5 to 1.5 mL/min at 37°C, with the perfusate being directed toward the anterior choroidal arteries. The cerebral ventricles are exposed, and the choroid plexus in the lateral ventricle is superperfused with an artificial CSF. The perfusion fluid draining from choroid plexuses is collected by a cannula inserted into the great vein of Galen.

four-min effluent, and 10 µML of the remaining injection bolus. Radioactivities will then be counted and expressed as dpm properly established quench curves.

22.2.5 KINETICS CALCULATION

The recovered [¹⁴C]mannitol and [¹²⁵I]T₄ in each venous drop is expressed as a percentage of the [¹⁴C]mannitol and [¹²⁵I]T₄ injected in the 100 µML bolus (% recovered). For any given drop, the recovery of [¹²⁵I]T₄ from the choroid plexus is less than the recovery of [¹⁴C]mannitol, but peak recovery of both isotopes should be simultaneous. The percentage uptake of [¹²⁵I]T₄ relative to mannitol, i.e., U%, can be calculated for each drop:



$$U\%([^{125}\text{I}]T_4) = \frac{\%[^{14}\text{C}]mannitol\ recovered - \%[^{125}\text{I}]T_4\ recovered}{\%[^{14}\text{C}]mannitol\ recovered} \times 100 \quad (22.1)$$

The U% values for those samples containing the highest levels of recovered radioactivity are averaged to give the maximal cellular uptake (U_{max}) of $[^{125}\text{I}]T_4$ for that “run.”

In addition, the net uptake (U_{net}) over the whole “run” and final four-minute sample can be calculated:

$$U_{net}\%([^{125}\text{I}]T_4) = \frac{\sum [^{14}\text{C}]mannitol\ received - \sum [^{125}\text{I}]T_4\ recovered}{\sum [^{14}\text{C}]mannitol\ recovered} \times 100 \quad (22.2)$$

where Σ is the sum of tracer recoveries for the whole “run,” plus the final four-minute sample.

The kinetic study of $[^{125}\text{I}]T_4$ uptake can be performed under conditions in which the plexuses are perfused with different concentrations of unlabeled T_4 . The concentration of unlabeled T_4 in perfusate varies from 0.015 to 20 μM in 100- μL bolus. Under these conditions the 100 μL injected bolus should contain both labeled and unlabeled T_4 and tracer amount of $[^{14}\text{C}]mannitol$. The bolus injection mixes with the main perfusion fluid before reaching the plexuses, so that the final concentration of T_4 can be estimated by calculating a dilution factor (usually 5 to 7) based on the passage of $[^{14}\text{C}]mannitol$ through the plexuses, as described by Segal et al. (1990). For example, a 100 μL injected bolus containing 0.15 μM T_4 would reach the plexuses at an actual concentration of 0.023 μM with an expected dilution factor of 6.5.

The unidirectional flux of $[^{125}\text{I}]T_4$ ($\mu\text{mol}/\text{min}/\text{g}$) can be calculated, using the U_{max} values, from the following equation:

$$\text{Flux} = -F \times \ln(1 - U_{max}) \times S \quad (22.3)$$

where F equals the perfusate flow rate ($\text{mL}/\text{min}/\text{g}$) and S equals unlabeled T_4 concentration (μM). The average weight of sheep choroid plexus is $0.195 \pm \text{SD } 0.08$ g ($n = 100$), which is taken from many studies previously published (Segal et al., 1990).

To calculate the V_{max} and K_m , the flux values in each run of all experiments are plotted against the actual concentration of the chemical studied, in this case T_4 , in each corresponding run. The data sets can be evaluated by any kinetic analysis software package. A concentration-effect math model that simulates the Michaelis-Menten relationship can be used to fit the observed data from which to estimate V_{max} and K_m .

Let us use the paired-tracer method with $[^{125}\text{I}]T_4$ as an example to illustrate the steady-state extraction of $[^{125}\text{I}]T_4$ relative to $[^{14}\text{C}]mannitol$ at the basolateral face of the perfused choroid plexus. A typical example of the $[^{125}\text{I}]T_4$ fractional extraction





measured during one run (a single drop per sample collected for 20 samples) is shown in Figure 22.2. Upon entering the choroidal vessels, the recovery of [^{14}C]mannitol arises quickly to reach the maximum values, suggesting that mannitol diffuses across the fenestrated capillaries into the extracellular space of the choroid plexus. The remainder diffuses back into the circulation and is recovered in the collected drops (see Figure 22.2A). [^{125}I]T₄ injected in the same bolus that accompanies the mannitol is also recovered in the collected drops, but the percentage of recovery of T₄ is smaller than extracellular marker mannitol, although T₄ has access to the same compartments as does the mannitol. This extra loss of T₄ represents the cellular uptake of T₄ by the choroidal epithelial cells. Figure 22.2B shows the uptake of [^{125}I]T₄ into the choroid plexus expressed as the percentage of recovery of [^{125}I]T₄ relative to that of mannitol; this corrects for nonspecific loss of T₄ via the extracellular distribution. The peak values of the uptake, i.e., the region where the greatest recovery occurs, are then averaged (as shown by points joined by a line in Figure 22.2B) to give rise to the value of U_{max}.

One limitation of this technique is that it is difficult to measure how much of a molecule has crossed from the blood into the choroid plexus and then entered the CSF. By collecting newly formed CSF from the surface of the choroid plexus, it is possible to check that the molecule crossing into the CSF is still intact, but it remains difficult to accurately quantify the magnitude of this transport.

22.3 IN SITU BRAIN PERfusion TECHNIQUE

22.3.1 TECHNICAL BACKGROUND

The technique of brain perfusion via the intracarotid injection of testing compounds has been used since the 1960s (Andjus et al., 1967; Thompson et al., 1968; Takasato et al., 1984; Bradbury et al., 1984). By directly introducing the drug molecules to the brain, one avoids a major problem associated with systemic injection: the hepatic or renal clearance of parent drug molecules. Such clearance (including biotransformation, redistribution, and elimination processes) in many cases makes it difficult to estimate blood-brain transport of substances, since the drug concentration at the site of transport is essentially unknown. Brain perfusion thus enables accurate delivery of a known amount of testing materials to the brain. Based on analysis of drug concentrations in brain parenchyma, which by definition is the brain tissue fractions free of capillaries, the unidirectional influx of drug can then be obtained. A refined method has been described by Takasato et al. (1984) in rat and by Zlokovic et al. (1986) in guinea pig. The same technique can also be applied to a smaller animal such as mouse (Bradbury et al., 1984). Although the method is most frequently used in blood-brain barrier studies, the choroid plexus can also be sampled and thus used for comparison to the brain uptake. The following discussion uses the rat for the purpose of technical description.

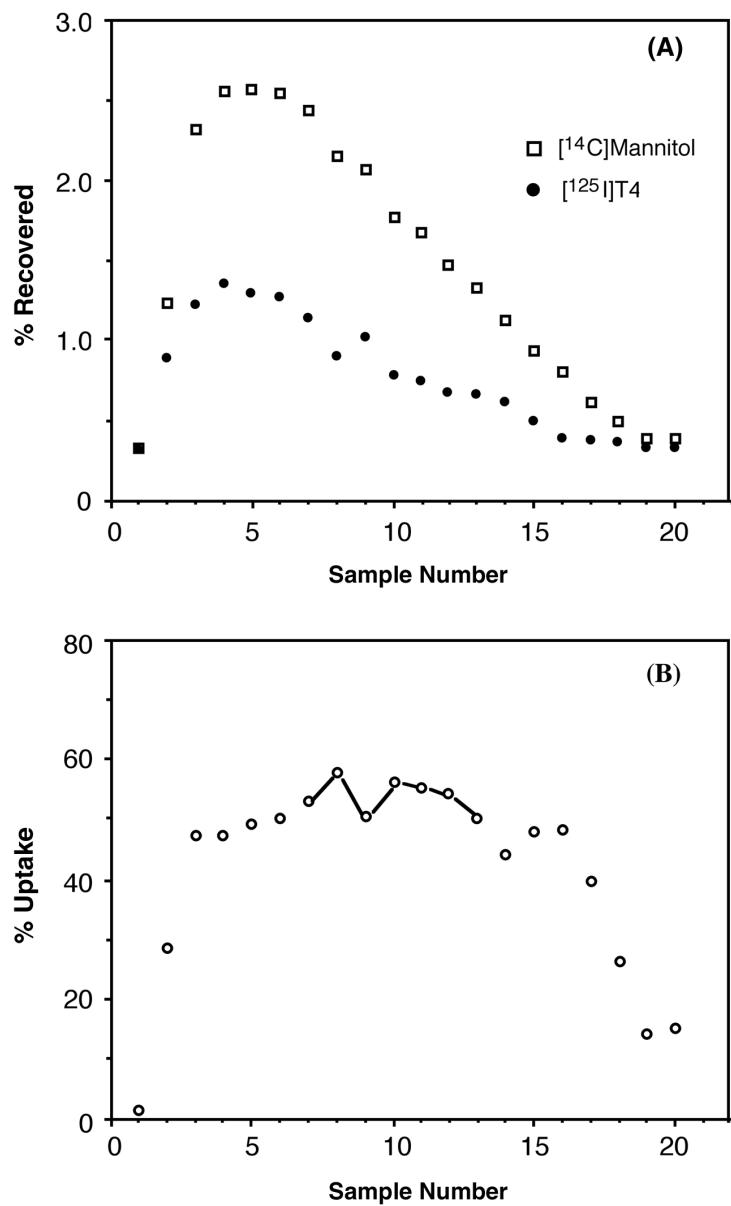


FIGURE 22.2 A typical run of paired-tracer study of T₄ transport by sheep choroid plexus. (A) Recovery of [¹⁴C]mannitol and [¹²⁵I]T₄ in one run of 20 venous samples (as % of radioactivity injected). The low recovery of [¹²⁵I]T₄ in comparison to [¹⁴C]mannitol indicates T₄ uptake at the basolateral face of the choroid plexus. (B) Uptake (%) of [¹²⁵I]T₄ in each venous sample relative to [¹⁴C]mannitol. Samples that contained the higher recovery of isotopes are joined by a line, and these values averaged to estimate U_{max} (%). (Adapted from Zheng et al., 2003.)



22.3.2 ANIMALS

Rats (both sexes) at the time they are used are about 9 to 12 weeks old (280 to 320 g). They are kept in a temperature-controlled, 12-hour light/dark cycle facility and fed ad libitum with a Teklad rat chow. Following a 3- to 5-day quarantine period, rats can be randomly placed into several groups according to the experiment. The rats can be pretreated with testing materials for the duration of the study course.

22.3.3 PERFUSION PROCEDURE

At the day of experimentation, rats are anesthetized with 50 mg sodium pentobarbital/kg by ip injection. The skin on the neck is cut open to expose the blood vessels. Both sides of the common carotid arteries are dissected; two surgical threads are imbedded under the carotid arteries. Immediately before cannulation, a node on the carotid arteries toward the heart is made by tightening one string. A cut on the artery is made above the node, followed by insertion of a PE-10 polyethylene tubing (Clay Adams) toward the brain. A second node is then made to secure the tubing inside the blood vessel. Immediately before the start of perfusion, both internal and external jugular veins and the left ventricle of the heart are severed to enable drainage of the perfusate circulating from the brain and to prevent recirculation of the rat blood. By this method, the Ringer solution, both hot and cold, will not mix with rat blood.

Each cannulated artery is initially perfused with a cold (nonradioactive) Ringer solution at 3 to 3.5 mL/min using two independent peristaltic minipumps (Variable-Flow Mini Pump, VWR). The Ringer solution contains (g/L): NaCl, 7.31; KCl, 0.356; NaHCO₃, 2.1; KH₂PO₄, 0.166; MgSO₄·3H₂O, 0.213; glucose, 1.50, sodium pyruvate, 1 mmol/L, and CaCl₂, 2.5 mmol/L, at pH 7.4. The perfusate is circulated through a temperature-controlled water bath system such that the temperature at the tip of the tubing entering the brain is maintained at 37°C. Prior to perfusion, the perfusate must be filtered and presaturated with oxygen. This will enable the extension in perfusion time without anoxia, as reported by Preston et al. (1995). The solution should be continuously oxygenated with 5% CO₂ and 95% O₂ during perfusion. The brain is perfused with nonradioactive Ringer solution for 10 minutes prior to infusion with the hot perfusate (Deane et al., 2004).

The hot perfusate is the Ringer solution containing radioisotopes. The hot perfusate is placed in two syringes and introduced to either side of catheters through two separate three-way tap systems using a slow syringe-driven pump (Harvard Compact Infusion Pump, Model 975). At the time of infusion of the hot perfusate, the peristaltic pumps are turned off, the three-way tap system switched to the syringe pump, and the hot perfusate infused at a flow rate of 0.4 mL/min for up to 30 minutes. Usually [³H]mannitol is used as a reference marker; its distribution volume (V_d < 5 µL/g) in the vascular washed brain is subtracted from the total uptake.

About one minute before the end of the timed perfusion period, a cisterna magna CSF sample can be collected by using a 25 gauge butterfly needle (Becton Dickinson, Sandy, UT) inserted between the protuberance and the spine of the atlas. CSF samples (about 100 to 150 µL) free of blood can be used for the biochemical analyses.





At the end of the timed perfusion periods, the syringe pump is turned off, the three-way tap system switched back to peristaltic pumps, and the brain then washed with cold Ringer solution for 30 seconds.

The brain is then removed from the skull, washed in ice-cold saline, and placed on filter paper saturated with saline, which rests on an ice-chilled glass plate. After removal of the meninges, the choroid plexus can be collected. The rest of brain can be further dissected to collect different regions and processed for capillary depletion assay.

22.3.4 CALCULATIONS

The uptake is expressed as a distributing volume, V_d , calculated as

$$V_d = \frac{C_{\text{brain or CSF}}}{C_{\text{perfusate}}} = (\text{mL/g}) \quad (22.4)$$

where C_{brain} is dpm/g of tissues, and C_{CSF} , $C_{\text{perfusate}}$, dpm/mL of perfusates. The uptake is corrected for residual radioactivity by deducting V_d for [^3H]mannitol from the total radioactivity of the studying compound (e.g., ^{59}Fe) distributing volume.

The unidirectional transport rate constant, K_{in} ($\text{mL/g/min} \times 10^3$), corresponding to the slope of the uptake curve, is determined using the linear regression analysis of V_d against the perfusion time, T (min), from Eq. 22.3

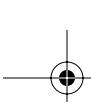
$$V_d = K_{in} T + V_i \quad (22.5)$$

where V_i is the ordinate intercept of the regression line (Zlokovic et al., 1986; Deane and Bradbury, 1990).

22.4 SUMMARY

In vivo experimentation to study the function of the blood–CSF barrier is generally difficult, as the small size of the choroid plexus limits the capability for surgical operations. In situ choroid plexus perfusion in large animals and brain perfusion in rodents are by far the most frequently used techniques in assessing the choroid plexus functions.

The advantages of these in situ perfusion techniques are obvious: first, the techniques better resemble the in vivo condition than in vitro methods such as choroid plexus incubation study or primary culture of plexus cells. Second, the perfused choroid plexus model minimizes the interference by passage of drug molecules via the blood–brain barrier, making it possible to characterize the unique kinetic behaviors of drug molecules at the blood–CSF barrier. Finally, the in situ brain perfusion model eliminates systemic metabolic interference, enabling comparison of transport kinetics between the blood–brain barrier and blood–CSF barrier. The major disadvantages of these in situ techniques pertain to the technical difficulty in choosing large animals for perfusion of choroid plexus, in surgical procedures, and in using large quantities of radiolabeled materials.



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TF1719_C22.fm Page 552 Friday, January 21, 2005 1:38 PM

