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Review

# Separation methods that are capable of revealing blood–brain barrier permeability

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**Abstract**

The objective of this review is to emphasize the application of separation science in evaluating the blood–brain barrier (BBB) permeability to drugs and bioactive agents. Several techniques have been utilized to quantitate the BBB permeability. These methods can be classified into two major categories: in vitro or in vivo. The in vivo methods used include brain homogenization, cerebrospinal fluid (CSF) sampling, voltametry, autoradiography, nuclear magnetic resonance (NMR) spectroscopy, positron emission tomography (PET), intracerebral microdialysis, and brain uptake index (BUI) determination. The in vitro methods include tissue culture and immobilized artificial membrane (IAM) technology. Separation methods have always played an important role as adjunct methods to the methods outlined above for the quantitation of BBB permeability and have been utilized the most with brain homogenization, in situ brain perfusion, CSF sampling, intracerebral microdialysis, in vitro tissue culture and IAM chromatography. However, the literature published to date indicates that the separation method has been used the most in conjunction with intracerebral microdialysis and CSF sampling methods. The major advantages of microdialysis sampling in BBB permeability studies is the possibility of online separation and quantitation as well as the need for only a small sample volume for such an analysis. Separation methods are preferred over non-separation methods in BBB permeability evaluation for two main reasons. First, when the selectivity of a determination method is insufficient, interfering substances must be separated from the analyte of interest prior to determination. Secondly, when large number of analytes is to be detected and quantitated by a single analytical procedure, the mixture must be separated to each individual component prior to determination. Chiral separation in particular can be essential to evaluate the stereo-selective permeation and distribution of agents into the brain. In conclusion, the usefulness of separation methods during BBB permeability evaluation is immense and more application of these methods is foreseen in the future.


*Keywords:* Reviews; Blood–brain barrier

**Contents**



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## 1. Introduction

The concept of the blood–brain barrier (BBB) was initiated in the early 19th century when Paul Elrich showed that aniline dyes, when injected into the bloodstream, did not stain the brain but stained the other organs [1]. In 1913, Goldmann further confirmed that a similar dye injected into cerebrospinal fluid (CSF) of dogs and rabbits could stain the brain but not the bloodstream and other organs [2]. Therefore, it was hypothesized that a barrier between the blood and brain exists which is termed the blood–brain barrier and serves as a barrier for the free entry of molecules into the brain from the blood circulation. The anatomical evidence of this barrier was confirmed using scanning electron microscopy in the late 1960s [3]. The BBB is formed by the brain capillary endothelial cells and is present in all vertebrate animals. The BBB serves two important functions: (i) to protect the brain from foreign substances; and (ii) to maintain an ideal environment for the brain.

The extracellular compartment of the brain can be grouped into two major categories, the cerebrospinal fluid and the brain interstitial fluid (ISF). These two compartments are separated from the blood by the choroid plexus or the blood–CSF barrier, and the brain capillary or BBB, respectively. As shown by the broken lines in Fig. 1, there is no anatomical barrier between CSF and ISF. However, there is a functional barrier between CSF and ISF which arises due to the flow of CSF from the formation site (choroid plexus) to the absorption site (arachnoid villi) [4]. In the case of a human brain, the complete absorption of CSF into systemic circulation (turnover) occurs within 4–5 h [5].

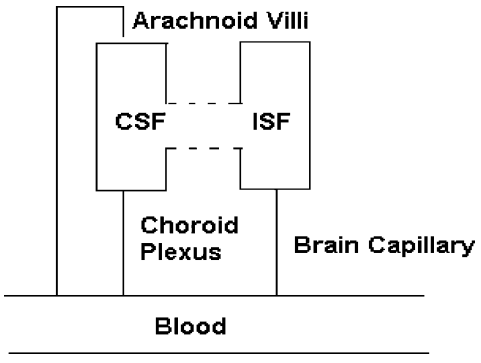


Fig. 1. The two major extracellular compartments of brain.

The brain endothelial cells that make the tubular capillaries are cemented together by intercellular tight junctions. The presence of these tight junctions is responsible for the high electrical resistance across intraparenchymal endothelial cells that may be as high as 8000 $\Omega\,cm^2$ [6]. The presence of these junctions also restricts the transport of solute via the BBB; it eliminates the paracellular pathway of solute movement and also the transcellular bulk flow of circulating solutes. Therefore, solute is generally transported via BBB by two major pathways: lipid-mediated transport and catalyzed transport. The solutes that are lipid soluble and have a molecular weight of less than $6.6 \times 10^{-22}$ to $9.9 \times 10^{-22}$ g (400–600 Dalton) can cross BBB by the former process [7]. Most drugs are transported into the brain by passive diffusion either through the cells or through tight junctions between cells. Various factors which can affect such diffusion include: (i) lipophilicity of the drug; (ii) ionization (pH and $pK_a$); (iii) molecular size; and (iv) plasma protein binding [8–10]. The catalyzed transport includes either carrier-mediated or receptor-mediated processes [11]. Carrier-mediated transport can be facilitated diffusion and active transport mediated. The former process is non-energy dependant and transported down a concentration gradient. The active transport process on the other hand is energy dependent. At least 10 different transport systems have been identified [12]. A number of receptors are expressed both on the luminal and abluminal surfaces of the endothelial cells such as insulin and transferrin receptors. The receptor may act as a transport vector and may affect BBB permeability. A schematic representation of the various pathways involved in the BBB permeability is shown in Fig. 2 [13].

The BBB is composed of two membranes connected in a series: the luminal and abluminal membranes of the brain capillary endothelial cell, separated by a 300 nm thickness of endothelial cytoplasm [14]. The BBB can be broken down by different physiological and pathological conditions, including hypertension, hyperosmolality of the blood, exposure to microwave or radiation, infection, trauma, ischemia and inflammation [15].

Importance of BBB permeability evaluation is an essential task for developing effective drugs for the treatment of central nervous system. Both for drugs already in the market or under development, it is essential to know to what extent a drug enters the BBB. Various in vitro and in vivo methods are now available for determining the BBB permeability characteristics of drugs and nutrients. The objective

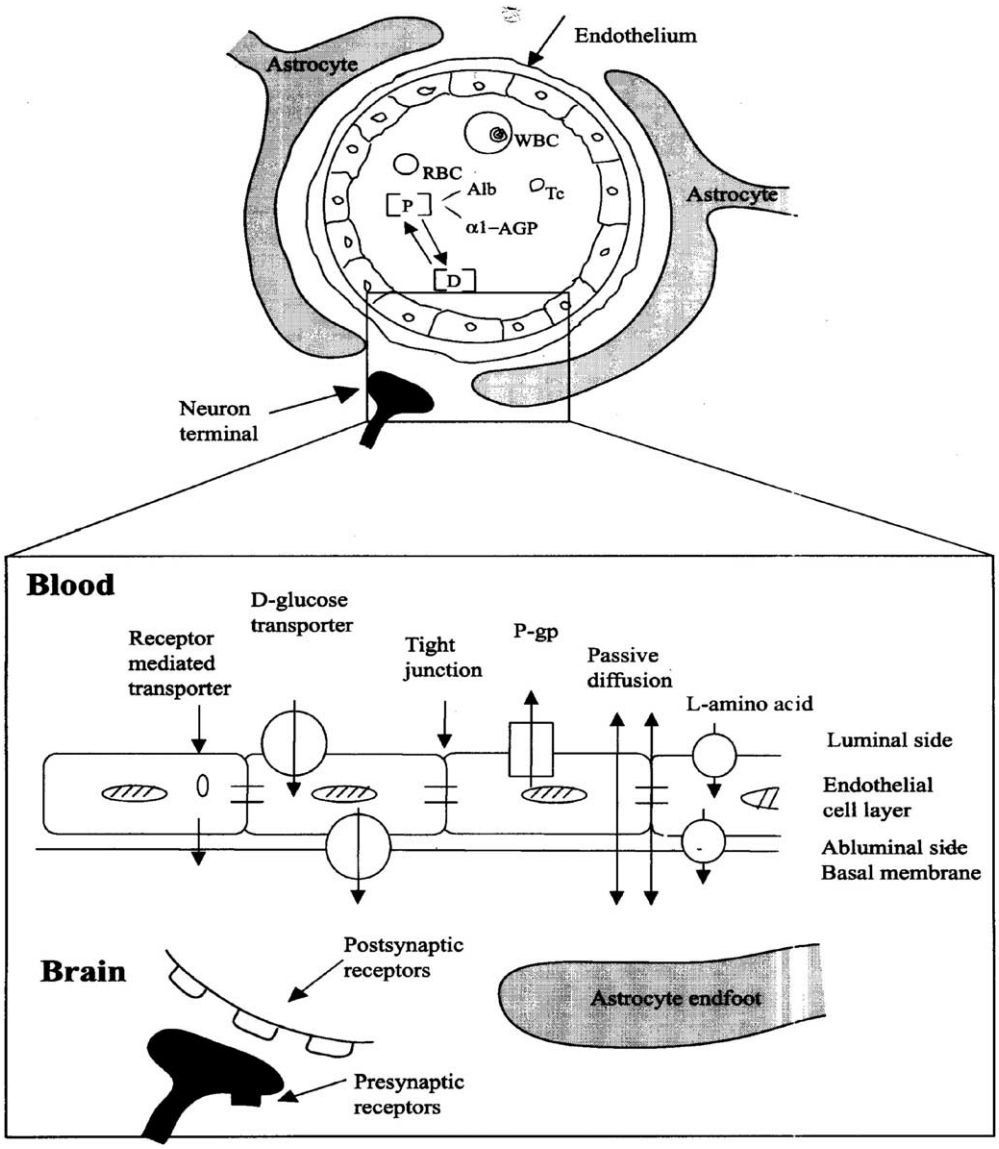


Fig. 2. Blood–brain barrier and transport mechanisms associated with the endothelial cell layer. [P]: protein, [D]: drug, Alb: albumin, Tc: thrombocyte, WBC: white blood cell, RBC: red blood corpuscles. Reproduced from Y. Han [13] with permission.

of this review is to emphasize the application of separation methods in the evaluation of BBB permeability.

Since the main theme of this review is the use of separation methods to reveal BBB permeability, it is necessary to differentiate between analytical methods, i.e., the use of separation versus non-separation techniques. Separation methods are preferred over non-separation methods for two reasons. First, if the selectivity of a determination method is insufficient, interfering substances should be separated from the analyte of interest prior to determination. Secondly, if large number of analytes are to be detected and quantitated by a single analytical procedure, the mixture must be separated into each individual component prior to determination.

In general, separation methods can be classified into two major categories: (i) separation based on substance transformation; and (ii) separation without substance transformation. Separation based on substance transformation includes procedures such as precipitation, volatilization, and electrolysis. Separations without substance transformation may include phenomenon such as partitioning (adsorption, liquid–liquid extraction, solid–liquid extraction and ion exchange phenomenon), differences in particle masses, sizes and shapes (sedimentation, centrifugation, gel filtration), particle charges (ion separation in mass spectrometry (MS), electrophoresis), and differences in vapor pressure (distillation, condensation, crystallization and sublimation). All of these separation methods are based on thermodynamic as well as kinetic principles. In the determination of BBB permeability studies, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the separation methods that have been used extensively. Various separation mechanisms such as adsorption, reversed-phase

matrix, ion-pair, ion-exchange, size-exclusion and gel permeation can be utilized in the HPLC mode. However, separation methods used to quantitate BBB permeability require either a sample preparation step or microdialysis technique with online analysis. The recent trend in this area involves more microdialysis methods, which utilizes the diffusion of an analyte through a membrane with a definite molecular weight cut off.

## 2. In vivo versus in vitro methods used in BBB permeability studies

Drug delivery to the central nervous system presents many logistical problems but BBB permeability poses an important problem for the researcher. Many in vivo methods have been developed for evaluating the BBB permeability. In most cases, radiolabeled test compounds are essential for the in vivo measurements of BBB. If a radiolabeled test compound is not available (especially for newer compounds), a specific and sensitive separation method is necessary to determine the BBB permeability of non-radiolabeled compounds. Due to the vast advances in combinatorial chemistry, a large number of compounds have been developed and need testing. For ethical reasons, convenience and cost, alternative methods to in vivo testing have been developed. Therefore, in vitro models that closely resemble in vivo systems, at least with respect to barrier properties, have been developed and tested in recent years.

### 2.1. In vivo methods

#### 2.1.1. Histochemistry in the measurement of BBB permeability

The use of histochemistry in the evaluation of BBB permeability was credited to Graham and Karnovasy [16]. The development of electron microscopy and the availability of horseradish peroxidase (HRP) as a marker were used to study the vascular permeability of protein from blood into brain or vice versa at a steady state under normal as well as pathological conditions. Trace studies using HRP also have been replaced by immunohistochemical techniques. However, none of these techniques use a so-called separation method (liquid chromatography, LC, or capillary electrophoresis) per se. However, migration of a marker and its identification and quantitation by a microscopic method falls under the definition of a separation method in general.

#### 2.1.2. Pharmacokinetic studies to evaluate the BBB permeability

The delivery of a molecule from systemic circulation into the brain can be evaluated by using pharmacokinetic methods [17]. The amount of solute delivered to the brain can be expressed by percentage of injected dose (ID) delivered per gram of the brain. This amount is dependent on two parameters as shown in Eq. (1): the BBB permeability-surface product (PS) and plasma area under the curve (AUC) at a given time after injection.

$$\frac{\%\mathrm{ID}}{g}\Big|_0^t = \mathrm{PS} \times \mathrm{AUC}\Big|_0^t \quad (1)$$

This PS product is generally determined by three different techniques: (i) single carotid injection technique (for solutes with PS product value more than 10 μl/(min g)); (ii) internal carotid artery perfusion technique (if this value is in excess of 0.5 μl/(min g)); and (iii) the intravenous (i.v.) injection method (can be used virtually for all PS product values).

The carotid artery single injection technique involves two methods, depending on the sampling technique used. Brain uptake index (BUI) studies involve tissue sampling [18] and multiple indicator dilution (MID) method involves venous sampling [19]. The former method uses a diffusible indicator reference while the later technique uses a non-diffusible indicator. The BUI technique uses $^{3}$H-labeled test compound and a $^{14}$C-reference compound or vice versa. Both these compounds are injected as a bolus dose into the carotid artery. The animal is decapitated 5–15 s after injection. The brain is solubilized/homogenized and the radioactivity in the brain as well as in injected solution are counted. BUI is calculated using Eq. (2).

$$\mathrm{BUI} = \frac{([^{3}\mathrm{H}]\mathrm{dpm}/[^{14}\mathrm{C}]\mathrm{dpm})(\mathrm{brain})}{([^{3}\mathrm{H}]\mathrm{dpm}/[^{14}\mathrm{C}]\mathrm{dpm})(\mathrm{injected\ solution})} \times 100$$

The BUI is generally expressed as a ratio of the unidirectional extraction of the test ($E_t$) and reference compound ($E_r$).

$$\mathrm{BUI} = \frac{E_t}{E_r} \times 100 \quad (2)$$

Knowing the cerebral blood flow ($F$), the unidirectional extraction of the test compound ($E_t$) values can be converted to BBB PS products using Renkin–Crone equation (Eq. (3)) [20].

$$E_t = 1 - e^{(-\mathrm{PS}/F)} \quad (3)$$

Most of the earlier BUI methods used tracer techniques to evaluate the BBB PS product. However, some of the recent studies have utilized separation methods to determine the BUI and PS product. Anders et al., have studied the brain uptake of remacemide hydrochloride both by intracarotid tracer technique, and by HPLC determination of the brain extracts in rats [21]. Both studies indicated that this novel anticonvulsant enters the brain intact by passive diffusion process. Sofia et al., have studied the BUI of felbamate in mice, rats and rabbits by the BUI method along with silica-gel thin-layer chromatography and reversed phase LC using a C-18 column and UV detection at 254 nm [22]. The permeability surface (PS) area products of this drug in rats, rabbits and mice were 0.09, 0.16 and 0.30 ml/(min g), respectively. Also, autoradiography has been used in this study

on the frozen brain sections and has revealed a uniform distribution of the drug tracer in the brain. The major disadvantage of this technique is that it is difficult to measure BBB PS products less than 10 μl/(min g).

Internal carotid artery infusion is an extension of the BUI method and is used in larger animals. In this case, the time of the injection is more than one second as used in the case of BUI method. Therefore, this method is more sensitive than the BUI method because of the extended experimental time (15–60 s). The method involves the internal carotid artery perfusion of brain followed by radioactivity measurements of the tissue samples [23,24]. The animal is decapitated and the radiolabeled test compound is measured. The BBB PS product is determined using Eq. (4).

$$\mathrm{PS} = \frac{V_\mathrm{d} - V_\mathrm{o}}{t} \tag{4}$$

where $t$ is the perfusion time in minute.

$$V_\mathrm{d} = \frac{(\mathrm{dpm/g(brain)})}{(\mathrm{dpm/ml(perfusate)})}$$

$$V_\mathrm{o} = \frac{(\mathrm{dpm/g(brain)})}{(\mathrm{dpm/ml(perfusate)})}$$

where $V_\mathrm{d}$ is the volume of distribution for the test compound and $V_\mathrm{o}$ the volume of distribution for plasma volume compounds (example sucrose).

Even though most of the internal carotid artery perfusion techniques have utilized radiolabeled tracers, some of the recent reports have also utilized separation methods like HPLC to determine BBB PS product. Internal carotid artery perfusion technique along with HPLC has been used to measure aminoacyl-transfer RNA activity and cerebral protein synthesis [25]. It has been shown that an inverse relationship existed between the cerebral protein synthesis and plasma phenylalanine concentration in the 200–500 μM range [26]. Gel filtration fast protein liquid chromatography has also been utilized for purification of OX26 mouse monoclonal antibody and used in the targeted delivery of biotin to brain with a covalent conjugate of avidin and a monoclonal antibody to the transferrin receptors in the brain [27].

The intravenous injection method has some advantages over the other two methods described earlier; for example, there is no need to locate the carotid artery, and sensitivity is higher than in the other two methods. The radiolabeled tracer is injected through a canula into the femoral vein. After certain time intervals, groups of animals are sacrificed and arterial blood is collected. The plasma radioactivity is then measured and a percentage of the injected dose/ml of plasma and pharmacokinetic parameters are calculated from a biexponential equation (Eq. (5)).

$$A(t) = A_1 \mathrm{e}^{(-K_1 t)} - A_2 \mathrm{e}^{(-K_2 t)} \tag{5}$$

where $A(t)$ is the percentage of ID/ml at a given time, $K_1$, $K_2$, $A_1$ and $A_2$ are the slopes and intercepts of the two exponents defining the plasma-concentration curve, respectively.

In order to determine the BBB PS product for this study, the animal is sacrificed (0.5–1 h) and brain radioactivity is measured. The PS product is generally calculated from Eq. (6).

$$\mathrm{PS} = \frac{[V_\mathrm{d} - V_\mathrm{o}]C_\mathrm{p}(\mathrm{T})}{\mathrm{AUC}|_0^t} \tag{6}$$

where $V_\mathrm{d}$ is the brain volume of distribution of the test compound, $V_\mathrm{o}$ the plasma volume of distribution of the marker, and $C_\mathrm{p}(\mathrm{T})$ the terminal plasma concentration. One of the major disadvantages of this method is the interference of the peripheral tissue metabolites of the test compound [28]. Chromatographic methods have been utilized to overcome this difficulty but with little effect. It is very difficult to differentiate by chromatography the radiolabeled metabolites formed in the brain from those metabolites formed in the periphery but transported into the brain. Therefore, while using this intravenous technique to determine BBB permeability one should ensure that the experimental agent used will not undergo extensive metabolism during the experiment.

A simple alternative to this intravenous injection technique is also available for the determination of BBB PS product without evaluating the pharmacokinetic parameter [29]. This method uses the cannulation of the femoral artery and the collection of arterial blood throughout the experiment. A single injection of the plasma volume marker, [$^3$H] test compound and [$^{14}$C] sucrose, is performed and arterial blood is collected. At the end of the experiment (0.25–10 min), the concentration of the radioactivity of the test compound in the femoral arterial sample is determined. The BBB PS product is calculated using Eqs. (7)–(9).

$$\mathrm{PS} = \frac{V_\mathrm{d} - V_\mathrm{o}}{t} \tag{7}$$

$$V_\mathrm{d} = \frac{([^3\mathrm{H}]\mathrm{dpm(brain)/g})}{([^3\mathrm{H}]\mathrm{dpm(plasma)/ml})} \tag{8}$$

$$V_\mathrm{o} = \frac{([^{14}\mathrm{C}]\mathrm{dpm(brain)/g})}{([^{14}\mathrm{C}]\mathrm{dpm(plasma)/ml})} \tag{9}$$

### *2.1.3. Imaging techniques used to study BBB permeability*

Radioisotopes have been used also in the development of radio-imaging techniques such as quantitative autoradiography (QAR) and positron imaging tomography (PET). Magnetic resonance imaging, which does not use a radioisotope, has also been used to study the BBB permeability. QAR involves i.v. administration of radiotracer into an experimental animal. Blood samples are collected periodically and radioactivity measured. The animal is sacrificed and the brain is frozen immediately. The frozen brain is sectioned into 20 mm thick sections, may or may not be stained for histology, and placed inside a X-ray cassettes with a X-ray film, and autoradiographed. The film is developed and the distribution and quantitation of the radioactivity is measured generally by an image analysis method [30–32]. PET is a non-invasive tracer technique used to quantitate the BBB PS

product in the human. The two tracers generally used for this technique are $^{82}$Rb with a of half-life of 1.25 min and $^{68}$Ga-ethlenediaminetetra-acetate (EDTA) with a half-life of 68 min [33–36]. BBB permeability has been determined by positron emission tomography (PET) a non-invasive tracer techniques in humans [36]. The other noninvasive methods used in humans include magnetic resonance imaging (MRI) and computed tomography (CT). However, these latter methods are more qualitative than quantitative [37]. BBB permeability with PET uses Ga-68 EDTA as the tracer and requires PET scanning to monitor the tracer kinetics in tissues. A plasma tracer curve is generated from periodic blood sampling. Pharmacokinetic model-based curve fittings are generally utilized to evaluate the BBB permeability. MRI, one of the most useful methods to detect diseased condition in the brain, is utilized. This method reflects the amount of tissue water predominately in the extracellular spaces. With the development of novel contrasting agents (gadolinium-DTPA), it is possible to detect BBB breakdown during different disease states [38,39]. Generally, standard compartmental analysis is used to measure BBB permeability using the MRI technique. None of the three imaging techniques outlined here have utilized separation methods. Major reasons why the separation methods have not been utilized in these techniques is to preserve sensitivity and to minimize invasiveness.

#### 2.1.4. Indicator diffusion method used to quantitate BBB permeability

In this method, an isotopically labeled test compound along with a reference standard which is impermeable to BBB is injected as a bolus via the carotid artery. A series of blood samples are collected from the internal jugular vein immediately. The unidirectional extraction is calculated using Eq. (10) and the BBB PS product is determined using Eq. (11).

$$E = \frac{C_{\text{ref}} - C_{\text{test}}}{C_{\text{ref}}} \tag{10}$$

$$\text{PS} = -\varphi \times \text{CBF} \times \ln(1 - E) \tag{11}$$

where cerebral blood flow (CBF) is the cerebral blood flow and $\varphi$ the apparent distribution volume for the substance in the whole blood.

This method is rapid, allows for a comparison to many other methods, and does not need a radioactive tracer. However, it does not provide the information about the regional distribution of the BBB permeability that can be achieved by PET and MRI techniques. Moreover, this method is limited in its sensitivity [40].

#### 2.1.5. In situ brain perfusion

In situ brain perfusion offers several advantages over other methods. (i) This method provides extended exposure of solute; therefore, it is more applicable for solute which has a poor BBB permeability. (ii) Proper selection of perfusate composition allows kinetic, mechanistic evaluation as well as effect of inhibitors on transport; (iii) Both passive and carrier-mediated transport can be evaluated. (iv) Transport in the brain can be measured in undisturbed BBB. In this method, radiolabeled tracer is added to a suitable perfusate solution. The radiolabeled tracer is perfused through a cannulated carotid artery at a rate of 3.5–4 ml/min. After decapitation, the radiolabeled test as well as the reference compounds are measured and the BBB PS product is quantitated using Eq. (6).

The radiolabeled tracer is quantitated in the brain tissue either by scintillation counting or autoradiography. The only problem encountered in such quantitation is that detection of radiolabeled materials in the brain does not separate the possible metabolites from their parent compound [41]. Therefore, to avoid this difficulty, separation methods like HPLC along with mass spectroscopy have been utilized in confirming the integrity of tracer [42]. This technique is more sensitive than the BUI method because of the long experimental time period used in the former study.

#### 2.1.6. Intracerebral microdialysis

This is one of the few methods which utilizes a separation method extensively for the quantitation of the BBB permeability in an intact animal [43,44]. This method has gained popularity in the evaluation of BBB permeability of compounds because it allows determination of the brain extracellular concentrations of a drug over time. The basic principle involved in intracerebral microdialysis is the stereotatic implantation of a microdialysis probe in a selected area of the brain. The probe consists of a semipermeable membrane and is continuously perfused with a physiological solution at a definite rate. Depending on the molecular weight cut off of the membrane, the molecules can be transported into or out of the perfusate from a higher to a lower concentration. The concentration of the molecules of interest is then monitored in the perfusate using a sensitive separation method. The free concentration of the drug in the brain extracellular fluid is reflected by the concentration measured in the dialysate. An extensive review on the use of microdialysis to study drug transport in the central nervous system (CNS) has also been reported elsewhere [45]. The major advantages of this sample collection technique used in BBB permeability studies are: (i) free concentration of the drug can be measured directly in the brain; and (ii) no sample preparation steps are needed prior to analysis. However, the major limitation of this procedure lies on the sensitivity of the analytical methods used. Since the usual flow rate of the perfusate used is 0.5–2.0 μl/min, a small sample volume generally is collected. If the BBB permeability of the drug is very low, the amount of drug in the dialysate may be considerably low. Therefore, a highly sensitive method of analysis is essential. High sensitivity and selectivity of HPLC with electrochemical or fluorometric detection has been used as the method of detection for the brain microdialysis [46]. The interaction of the drug with the microdialysis probe and with the tubes

may also add additional problems to this method. The three experimental variables that can affect the results of intracerebral microdialysis are the type and nature of the probe used, the postimplantation interval, and the type of animal used (anaesthetized versus freely moving). Direct analysis of sensors has been utilized as non-separation methods during microdialysis sampling. However, most applications use the separation method to isolate the analyte of interest from endogenous sources followed by their quantitation. The two most commonly used separation methods utilized in conjunction with microdialysis sampling are liquid chromatography and capillary electrophoresis. The small sample volume requirement as well as the aqueous sample compatibility are the two main advantages of this system. A review on the analytical considerations for microdialysis sampling is provided elsewhere [47].

Microdialysate samples obtained from brain microdialysis are usually present in aqueous environments with high ionic strength and without proteins. Because of the nature of this type of sample, it is more acceptable to use either HPLC or CE for separation and quantitation of the various analytes. The most compatible and used LC methods for microdialysis samples are reversed-phase and ion-exchange modes. These modes can be applicable either for online or offline microdialysate sample analysis.

*2.1.6.1. Use of reversed-phase chromatography.* Selection of a separation mode such as reversed-phase chromatographic analysis in conjunction with microdialysis sampling techniques depends on the physicochemical properties of different analytes, the nature of the column used, and the flow rate of the microdialysis perfusate. In such studies, one of the major requirements of the detector is its selectivity and sensitivity. In HPLC analysis, the detectors used include immunoassay, ultraviolet absorbance, electrochemical, fluorescence and mass spectrometric modes. In a typical LC assay 5–10 μl of sample is injected. For a perfusion flow of 1 μl/min, the temporal resolution is 5–10 min. The decrease in flow rate of the perfusate also increases the recovery of the probe but further decreases the temporal resolution. No resolution advantages can be gained by the use of narrow bore columns as compared to a regular column. However, use of microbore columns during microdialysis sampling can enhance the sensitivity and rapid analysis of the analytes of interest therefore reducing the mobile phase consumption and the cost. Another important consideration when using the microbore columns is the band broadening by extra-column contribution. This effect can be minimized by reducing the dead volume of the injector, the column, and the connector to an absolute minimum [48–50]. Detection limits in the mid-femtomole mass range of biological amines have been achieved by use of liquid chromatography with electrochemical detection [51]. The low flow rate used in microbore chromatography allows more time for the analytes to interact with the electrode and thereby increases coulometric yields in an EC detector. Online analysis of dopamine, 5-HT, serotonin and its metabolites at a very low concentration has been detected and quantitated by HPLC with an EC detector using a microbore column as shown in Fig. 3 [52]. In order to accommodate various signal outputs due to a varying level in the neurochemicals in the brain, an amperometric detector equipped with a sensitivity programmable controlling software for automatically switching sensitivity inattentively and repeatedly for each sample collection cycle was used in this investigation. Determination of amino acid neurotransmitters during brain microdialysis using HPLC has utilized pre-column derivatization with a fluorescence adduct either *ortho*-phthalaldehyde (OPA) or naphthalenedialdehyde (NDA) [53,54]. Derivatized products are detected by either EC or fluorescence with very high sensitivity. Capillary electrophoretic and laser-induced fluorescence detection has been utilized to measure the glutamate, aspartate, GABA and glutamine concentration in 100 nl microdialysis samples collected every 5–6 s as shown in Fig. 4.

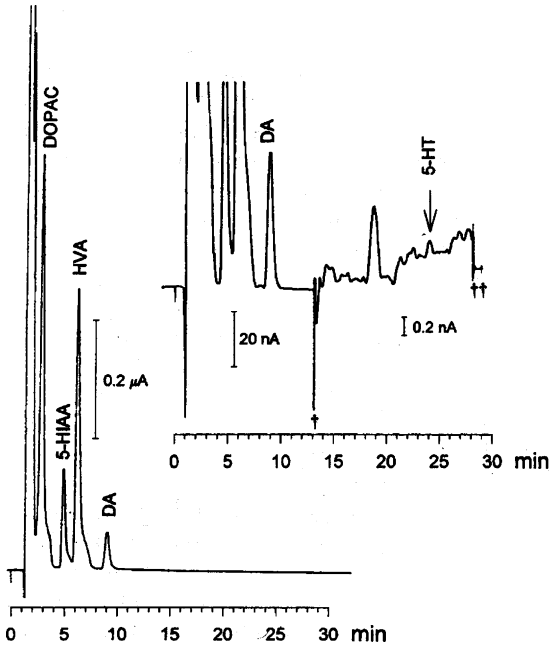


Fig. 3. Chromatogram of dopamine and 5HT collected from a 15 μl rat striatal microdialysate on a 30 min run using a mobile phase containing 0.1 mM EDTA, 1.0 mM sodium octyl sulfate, 17 mM sodium chloride, 50 mM sodium phosphate monobasic (pH 4) and 7 vol.% acetonitrile. The scheduled times for changing sensitivity are indicated by '†' and '††' at time 13.5 and 28 min, respectively. Sensitivity was changed from 0.2 μA, 0.03 Hz to 50 nA, 0.02 Hz, and returned to the initial setting after 5-HT was eluted. The percent HT peak reflects its typical basal level at amplified sensitivity range. Reproduced from Chaurasia et al. [52] with permission.

Separation methods using capillary column LC have been used for the analysis of microdialysis samples. The diameter of the capillary columns with microdialysis sampling is usually 25–100 μm in diameter. The major advantage of this capillary LC column is that relatively large volumes of hydrophobic analytes can be injected onto this column which then can be eluted with a small volume of

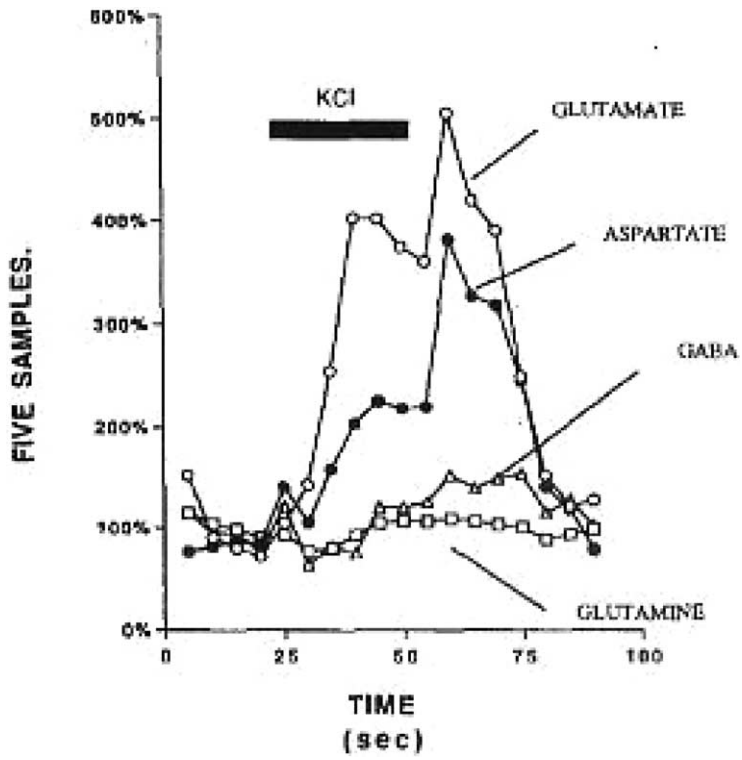


Fig. 4. The increase of glutamate and aspartate levels after perfusing the microdialysis probe with artificial cerebrospinal fluid with KCl 60 mM, while other amino acids as glutamine and γ amino butyric acid did not change significantly. Reproduced from Tucci et al. [54] with permission.

mobile phase [55]. Pre-column complexation with copper II, followed by separation with a capillary column and EC detection, has been used for the quantitation of neuropeptides in microdialysate samples. The detection limit for this assay is in the range of 5–59 pM [56]. Microbore and capillary column chromatography along with mass spectroscopy has also been used to monitor neuropeptide and metabolites of substance P in microdialysis samples in the brain microdialysate [57,58]. Although the microbore or capillary columns have added advantage for separation of microdialysate samples in BBB permeability studies, columns with a conventional diameter are still used for microdialysis samples. Online analysis of microdialysis has enhanced temporal resolution and has avoided problems associated with small volume sample size. The nature of the microdialysate samples which are protein free makes this online separation and analysis possible. One major advantage of such analysis is that the monitoring of the complete physiological condition on the BBB permeability by microdialysis sampling is possible. However, one of the major challenges of online analysis of microdialysate is sample carry-over due to incomplete flushing of the injection line from sample to sample. In order to avoid this problem continuous monitoring of the microdialysate using two sampling valves has been useful. Heppert and Davies studied the concentration of caffeine and its metabolites in the muscle, brain and in jugular vein after IP administration of caffeine in awake rats using the microdialysis sampling technique [59]. A 10-port valve was used online for monitoring the analyte concentration in the brain and the jugular vein. An off-line LC-UV analysis of the microdialysate sample was used to monitor the drug and its metabolites in pectoral muscle.

*2.1.6.2. Use of capillary electrophoresis.* Capillary electrophoresis has become a popular method of separation and quantitation for microdialysis sampling in the BBB permeability studies [60–62]. CE is characterized by its ability to resolve complex aqueous samples with a very high resolution by the application of a high applied direct current (DC) voltage. It can analyze samples of less than 10 nl with high analytical precision [63]. The small sample volume needed for this method is also an added plus for microdialysis sampling which usually generates a very small sample volume. Fig. 5 depicts the use of capillary electrophoresis with EC detection to determine extracellular levels of aspartate, glutamate and alanine in samples from the frontoparietal cortex of the rat with in vivo microdialysis sampling technique [64]. The advantage of using EC detection is that voltametric characterization can be used to identify unknown analytes in the microdialysate. During CE, voltametric characterization can be achieved on a very low volume (1 μl) of microdialysate sample, which allows multiple analysis on a single sample. Determination of neuroactive amino acids by CE in microdialysate sample requires a derivatization procedure prior to detection. Various deriva-

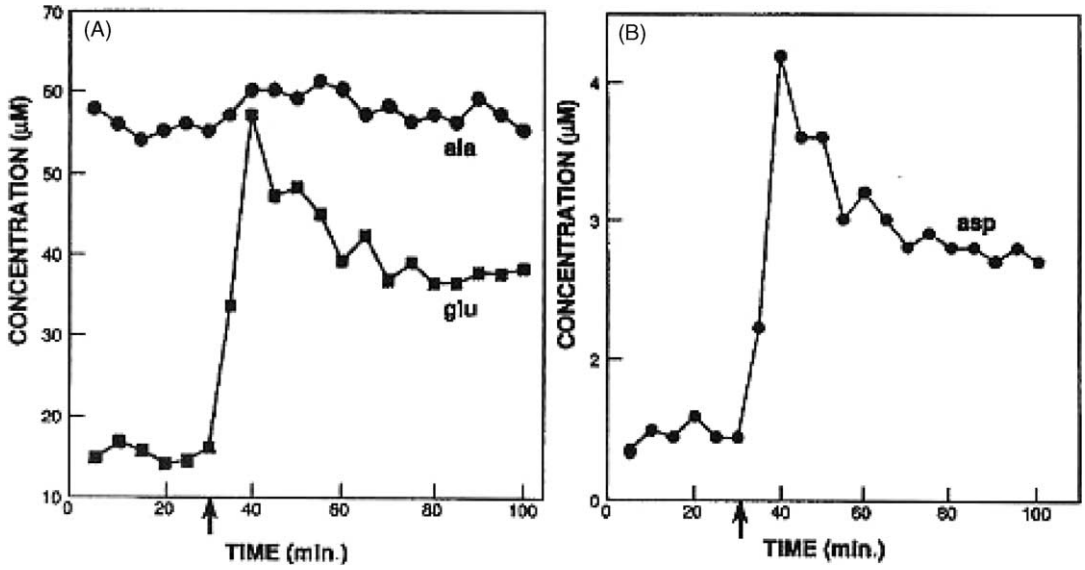


Fig. 5. Concentration–time curves of (A) alanine and glutamate and (B) aspartate in rat brain. Arrow indicates application of potassium stimulation. Reproduced from O'Shea et al. [64] with permission.

tizing agents that have been used include OPA, NDA, imidazoleNDA, and fluorescein isothiocyanate. CE has been used to measure the aspartate and glutamate concentration in the brain [53,54]. Laser-induced fluorescence (LIF), and mass spectrometry (CE-MS) also has been used as detection modes for CE [65–67]. Matrix-assisted laser desorption ionization-MS (MALDI-MS) was used to identify over 80 peptide fragments in a microdialysate sample [67].

Capillary electrophoresis is commonly used to describe the science of electrophoresis performed in a capillary format. The term high-performance capillary electrophoresis (HPCE) is obsolete and not commonly used today. Capillary zone electrophoresis (CZE) on the other hand uses the separation of charged species in aqueous samples on the basis of their speed and direction of movement with the application of a DC current [63]. Capillary gel electrophoresis (CGE) has further advantages in terms of speed, quantitation, and ease of automation [68]. The other modes of detection which have been utilized for CE analysis are isoelectric focusing (IEF) and isotachophoresis (ITP) [69,70]. The micellar electrokinetic capillary chromatography (MEKC or MECC) is another unique separation mode used in capillary electrophoresis. In this mode of separation, a neutral molecule can be separated based on its differential partitioning into charged micelles formed from surfactants incorporated into the background electrolyte in excess of their critical micellar concentration (CMC) [70]. MEKC-LIF has been utilized to monitor γ amino butyric acid (GABA) in human brain microdialysis samples [71]. This study has revealed a significant decrease in extracellular GABA during high-frequency electrical stimulation.

When analyzing biomatrix samples either using CE or MEKC, unwanted shifts in the time axis have been noticed between samples and even between standards and samples. The cause of these shifts can be due to variation in stacking conditions or variation in electroosmotic flow or both. This problem has recently been resolved by the pherogram normalization technique [72].

*2.1.6.3. Microdialysis probe recovery.* In the case of microdialysis, diffusion of low molecular weight compounds into (recovery) or out of (delivery) the probe occurs in response to a concentration gradient between the perfusion fluid and the surrounding medium. The extraction efficiency (EE) or relative recovery of the analyte by the probe is very important in microdialysis experiments because it can influence analyte concentration in the samples. The various parameters which can affect the in vitro as well as in vivo recovery have been extensively reviewed [47,73]. The various methods available to determine relative probe recovery during microdialysis and their advantages and disadvantages are presented elsewhere [73]. In vitro probe recovery determination, using an HPLC method, has been utilized to determine the BBB permeability characteristics of ceftriaxone in a rabbit meningitis model [74]. The ceftriaxone CSF concentration versus time profiles are shown in Fig. 6. This study revealed no significant difference in CSF penetration of ceftriaxone between the ceftriaxone-treated and ceftriaxone-dexamethasone-treated groups. Cerebrospinal fluid penetration of levofloxacin has been reported in a rabbit meningitis model using in vivo microdialysis probe recovery by HPLC in rabbits [75].

*2.1.7. Stero-selective drugs and their BBB permeability*

Many drugs used today are racemic mixtures (mixture of equal parts of the (*S*)- and (*R*)-enantiomers). Some, but not all, chiral drugs are stereo-selectively distributed to the brain. This stereo-selectivity in distribution can be

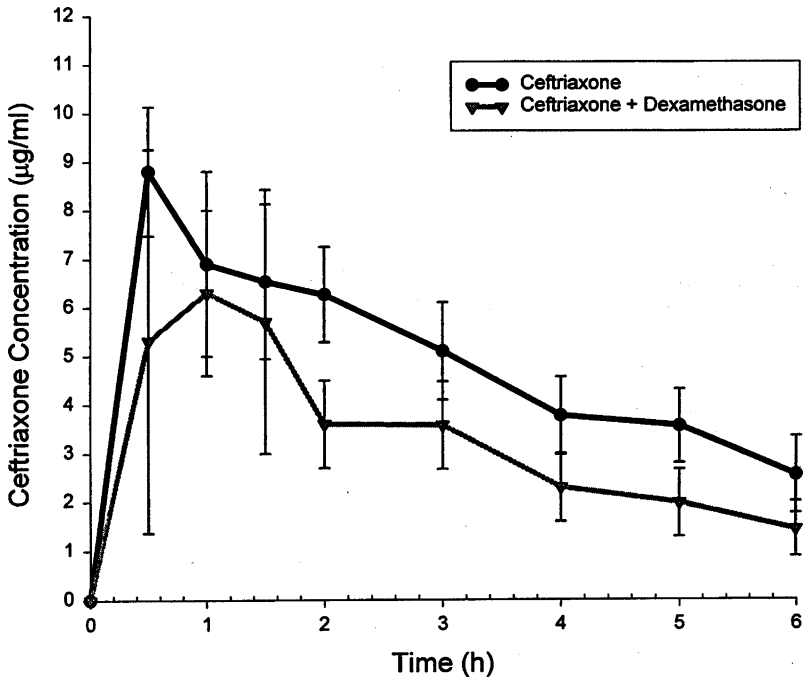


Fig. 6. Ceftriaxone cerebrospinal fluid concentrations vs. time profiles in an experimental animal model (rabbit) of *S. pneumoniae* meningitis. Reproduced from Owens et al. [74] with permission.

accounted for in either stereo-selective plasma protein binding or stereo-selective transport across the BBB [76]. Separation methods like HPLC coupled with chiral columns have been used to assay the pure enantiomers of the racemate in the plasma and brain tissue [13,77]. The two enantiomers of pindolol and propranolol, which have the same $\log P$ values, have been shown to stereo-selectively distribute into the brain [76]. Pretreatment with a P-glycoprotein blocker (verapamil) affected the ratio of brain to plasma concentrations of the enantiomers to a different degree, indicating the possible involvement of stereo-selective transport systems in this case.

### *2.1.8. Immobilized artificial membranes—HPLC to predict BBB permeability*

The drug permeation through BBB has long been considered to depend on several physicochemical properties of drug such as hydrophobicity, molecular weight and hydrogen-bonding potential [7]. The logarithm of octanol–water partition coefficient ($\log P$) has been shown to be directly related to BBB permeability [78]. Levin has also shown a good correlation between BBB permeability with $\log P$ divided by the square root of molecular weight ($\log P/M^{0.5}$) [7]. Peptide lipophilicity has been shown to be a good predictor of BBB permeability [79]. The other methods, which have been used as a good predictor of BBB permeability include hydrogen-bonding capability [80,81], chromatographic capacity parameter [80,82,83], partitioning into liposomes [84], solvatochromic parameters [85], surface activity measurements [86], and computational approaches [87,88]. However, none of these methods described here can be used universally to predict the BBB permeability of a drug, often because of active transport at the BBB. The immobilized artificial membrane (IAM) chromatographic columns, first developed in 1986 for separation of analytes, have been used effectively in predicting the lipophilicity of molecules [89,90]. These columns contain a monolayer of phosphatidylcholine immobilized on a silica support and mimic very closely a membrane bilayer [91]. Reichel and Begley determined the IAM and octadecyl silane (ODS)-HPLC capacity factors ($\log k_{IAM}$ and $\log k_w$) for two sets of compounds such as highly lipophilic (steroids) to highly polar (biological amines) compounds [92]. This study clearly demonstrated that for highly lipophilic compounds, capacity factors, partition coefficients, as well as distribution coefficients, predict equally well the brain uptake of these compounds. However, for polar and ionizable compounds, capacity factor determined by IAM-HPLC is superior over octanol–water partition coefficient, distribution coefficient, and capacity factor determined by reversed phase-HPLC [92,93]. Taillardat-Bertschinger et al. have reported that partitioning of molecules into IAM depends on size, hydrophobicity, and charge of the molecule of interest [94]. When hydrophobic interaction is the dominant factor, the IAM capacity factor correlates well with liposomal partitioning. For hydrophilic solutes, these two systems do not yield the same information and are not interchangeable. Concise reviews on the use of IAM chromatography for drug transport applications [95] and for binding studies has been published elsewhere [96].

### 2.2. In vitro methods

In vitro methods using tissue culture have been utilized for the BBB permeability of drugs and nutrients. It is always useful and advisable to employ in vitro methods in combination with in vivo methods. Two different systems which have been utilized as in vitro models include isolated brain capillaries and cultured bovine brain capillary endothelial cells (BCECs). Isolated brain capillaries are prepared from cerebral gray matter that has been homogenized, centrifuged, then filtered [97]. This method can provide mechanistic and kinetic information on BBB permeability. However, directional transport is difficult to determine as such. Primary cultured BCECs grown on collagen-coated plates or microporous membranes have been used for BBB permeability evaluations [98,99]. Even though polarity of the transport can be evaluated by this method, it may not be suitable for many low permeability compounds, since tight junctions are not well developed in this system. Co-cultures of the above system with astrocytes or astrocyte-conditioned medium have produced monolayers with better developed tight junctions that are close to the in vivo BBB [100–102]. The cell surface is attached to a collagen-coated polymeric filter which resembles the attachment to the basal membrane of capillary. The transport of molecules from apical to basolateral surface or vice versa can be evaluated using this system. Separation methods have been used in conjunction with the in vitro cell culture methods to evaluate the BBB permeability of many new drugs and compounds. Rapid gradient liquid chromatography with a tandem mass spectrometry has been utilized in an in vitro cell culture study to evaluate the BBB permeability characteristics of various new drug entities in a drug discovery process [103]. Bioavailability of ziconotide in brain, its stability and diffusion, has been studied using a cultured bovine brain microvessel endothelial cells (BBMEC) that utilizes HPLC as a separation and quantitation method [104]. The chromatographic profiles from plasma and brain after i.v. administration of radioiodi-

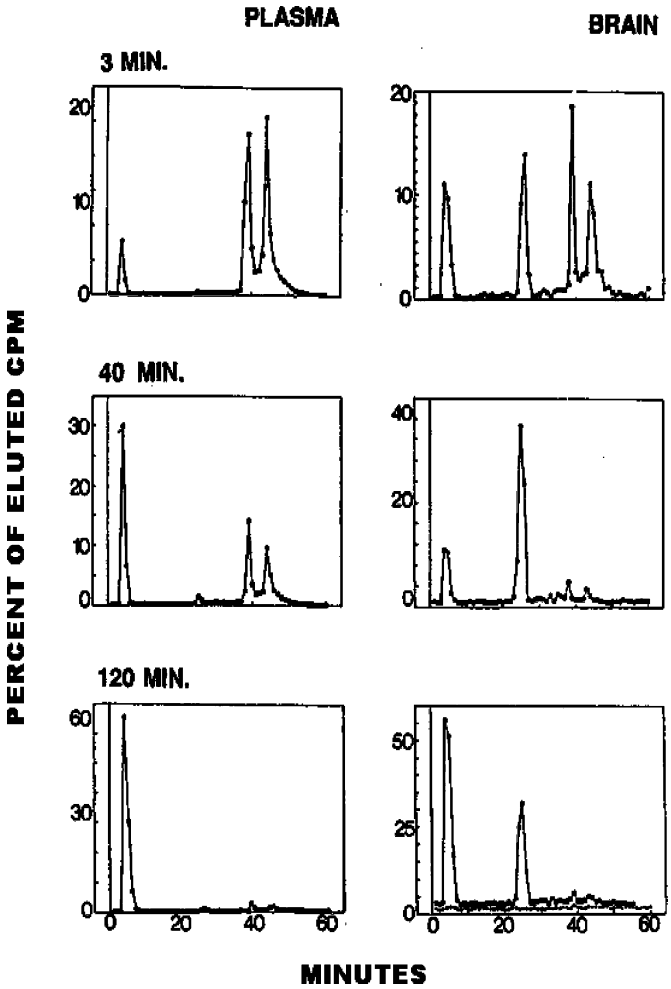


Fig. 7. Chromatographic profiles from plasma (left) and brains (right) at various times after i.v. injection of radioiodinated ziconotide. The peak at 44–45 min represents the intact iodinated peptide, whereas, that 38–39 min represents an in vitro (tissue independent) product (presumably the methionine oxidized from the peptide). The peak eluting at 22–26 min is iodotyrosine. For the 120 min brain sample, results of a prior injection of a similar amount of control extract are also illustrated. Internal standards of ziconotide, as well as external standards of radioiodinated ziconotide were used for peak identification. Reproduced from Newcomb et al. [104] with permission.

nated ziconotide is shown in Fig. 7. A statistically significant amount of radioactivity was found to cross BBB in this study.

## 3. Conclusions

Delivery of drugs to the brain requires that these compounds undergo significant transport through the brain capillary endothelial wall which constitutes the blood–brain barrier. With the advancement of combinatorial chemistry, a large number of compounds of pharmaceutical importance are being developed for CNS diseases. It is very important to know to what extent these molecules enter the CNS. Various in vitro and in vivo methodologies are available for assessing the BBB permeability. None of these methods alone can answer the two main challenges; thus, selectivity of the method can be limited. Secondly, if more than one analyte is to be identified and quantitated in the same sample, none of these methods will be able to accomplish this challenge. Finally, stereo-selective plasma protein binding and stereo-selective transport across BBB can also stereo-selectively distribute a racemic drug into brain. Separation methods are therefore used in conjunction with other methods to over come these difficulties. The microdialysis sampling technique has also added other advantages such as online analysis and the determination of real time data under true physiological conditions. The two separation methods which have been extensively used to evaluate the BBB permeability are reversed-phase HPLC and capillary electrophoresis. The interface of mass spectroscopy to LC has further enhanced the selectivity and sensitivity of the determination. Chiral separation with HPLC has been utilized in conjunction with other methods and is essential to evaluate the stereo-selective distribution of racemic drugs. We believe that more and more new separation methods with high specificity and sensitivity will be developed and used along with other methods available to study the BBB permeability in the future.

## 4. Nomenclature

| | |
|---|---|
| $\varphi$ | apparent distribution volume for the substance in the whole blood |
| AUC | area under the curve |
| BBB | blood–brain barrier |
| BBMEC | bovine brain microvessel endothelial cells |
| BCEC | brain capillary endothelial cells |
| BUI | brain uptake index |
| CBF | cerebral blood flow |
| CE | capillary electrophoresis |
| CGE | capillary gel electrophoresis |
| CMC | critical micellar concentration |
| CNS | central nervous system |
| $C_p(T)$ | terminal plasma concentration |
| CSF | cerebrospinal fluid |
| CT | computed tomography |
| CZE | capillary zone electrophoresis |
| DC | direct current |
| EC | electrochemical |
| EDTA | ethlenediaminetetra-acetate |
| EE | extraction efficiency |
| $E_r$ | unidirectional extraction of reference compound |
| $E_t$ | unidirectional extraction of test compound |
| $F$ | cerebral blood flow |
| GABA | γ amino butyric acid |
| HPCE | high-performance capillary electrophoresis |
| HPLC | high-performance liquid chromatography |
| HRP | horseradish peroxidase |
| i.v. | intravenous |
| IAM | immobilized artificial membrane |
| ID | injected dose |
| IEF | isoelectric focusing |
| ISF | interstitial fluid |
| ITP | isotachophoresis |
| LC | liquid chromatography |
| MALDI | matrix-assisted laser desorption ionization |
| MECC | micellar electrokinetic capillary chromatography |
| MEKC | micellar electrokinetic chromatography |
| MID | multiple indicator dilution |
| MRI | magnetic resonance imaging |
| MS | mass spectrometry |
| NDA | naphthalenedialdehyde |
| NMR | nuclear magnetic resonance |
| ODS | octadecyl silane |
| OPA | *ortho*-phthalaldehyde |
| PET | positron emission tomography |
| PS | permeability surface area product |
| QAR | quantitative autoradiography |
| $T$ | time |
| $V_d$ | volume of distribution of test compound |
| $V_o$ | volume of distribution of plasma volume compound (marker) |

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