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Review

Assaying protein unbound drugs using microdialysis techniques

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Abstract

Compared with traditional sampling methods, microdialysis is a technique for protein unbound drug sampling without withdrawal of biological fluids and involving minimal disturbance of physiological function. Conventional total drug sample consists of unbound drugs and protein bound drugs, which are loosely bound to plasma proteins such as albumin and alpha-1 acid glycoprotein, forming an equilibrium ratio between bound and unbound drugs. However, only the unbound fraction of drug is available for absorption, distribution, metabolism and elimination, and delivery to the target sites for pharmacodynamic actions. Although several techniques have been used to determine protein unbound drugs from biological fluids, including ultrafiltration, equilibrium dialysis and microdialysis, only microdialysis allows simultaneous sampling of protein unbound chemicals from plasma, tissues and body fluids such as the bile juice and cerebral spinal fluid for pharmacokinetic and pharmacodynamic studies. This review article describes the technique of microdialysis and its application in pharmacokinetic studies. Furthermore, the advantages and limitations of microdialysis are discussed, including the detailed surgical techniques in animal experiments from rat blood, brain, liver, bile duct and in vitro cell culture for unbound drug analysis.

Keywords: Reviews; Microdialysis techniques; Proteins; Unbound drugs

Contents

Abbreviations: AUC, area under the concentration versus time curve; BBB, blood–brain barrier; BCF, blood–cerebrospinal fluid; CNS, central nervous system; DHBA, dihydroxyl benzoic acid; LC-ED, liquid chromatography electrochemical detection

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1. Introduction

The first paper involving microdialysis, where it was applied to the study of dopamine neurotransmission, was published in 1974 [1]. Now, a search of Medline at the time of writing revealed 8288 articles for “microdialysis”, 217 articles for “microdialysis and pharmacokinetics” and 394 review articles for “microdialysis”. Microdialysis has rapidly become a widely accepted technique for in vivo sampling in pharmacokinetic studies, allowing measurement of endogenous and exogenous substances in the extracellular fluid surrounding the microdialysis probe [2–12].

Essentially, a microdialysis probe contains a dialysis membrane covering the tip of a probe. As the probe is implanted into blood vessel, tissue or organ, and perfused with a suitable perfusate at a constant flow rate, small molecules diffuse along a concentration gradient toward the probe lumen or away from the probe (Fig. 1). Thus, at least three functions of microdialysis have been demonstrated in in vivo studies. First, for drug administration, as a perfusate-containing drug reaches the membrane, the drug molecules diffuse away from the probe. Second, for drug sampling, when perfusing artificial biological fluids into the microdialysis probe, the endogenous substances or given drug molecules would diffuse into the probe allowing the correction and analysis of the dialysate. As a result, a drug concentration versus time plot can be constructed. Third, for in situ derivatization and analysis, as in our monitoring of hydroxyl radical levels, salicylic acid is used as a trapping reagent contained in the perfusate [13,14]. This salicylic acid would diffuse away from the perfusate into the surrounding tissues or vessels. Consequently, the hydroxyl radical will attach to the benzo-ring of the salicylic acid, forming 2,3-dihydroxyl benzoic acid (2,3-DHBA) and 2,5-DHBA. These two metabolites are measured using an online microdialysis device and by liquid chromatography coupled to electrochemical detection (Fig. 2).

Because the microdialysis technique involves no biological fluid loss from the body, higher temporal resolution of the sampling interval and continuous sampling over long periods of time can be achieved. In addition, microdialysis can be used to increase the data points from a relatively small number of animals. Moreover, multiple sites sampling provide detailed pharmacokinetic information. In contrast to conventional blood sampling method, blood volume loss is not involved. This is important in murine studies, since an adult rat has a blood volume ratio of approximately 70 ml/kg, so total blood volume is approximately 21 ml for a 300 g adult rat. Blood removal volume exceeding 20–25% of the total body volume at one time usually produces signs of hypovolaemia [15]. In order to have minimal disturbance of physiological functions, limited blood sample volume and number of samples have to be considered, resulting in poor temporal resolution using conventional blood sampling. Tissue sample is also usually collected one animal per time point, and following tissue homogenization, complicated sample clean-up is usually required before analysis.

Several methods have been used to measure the analyte from biological sample by protein separation such as conventional protein precipitation, solid-phased extraction and liquid–liquid extraction [16]. However, these methods may not be able to distinguish the protein unbound fraction from the total fraction of drug. Thus, ultrafiltration, equilibrium dialysis and microdialysis have been commonly applied to determine the sampling of unbound fraction of drug from biological samples.

The advantages of microdialysis contribute greatly to the study of pharmacokinetics and drug metabolism. The unbound drug analysis allows access to the information on

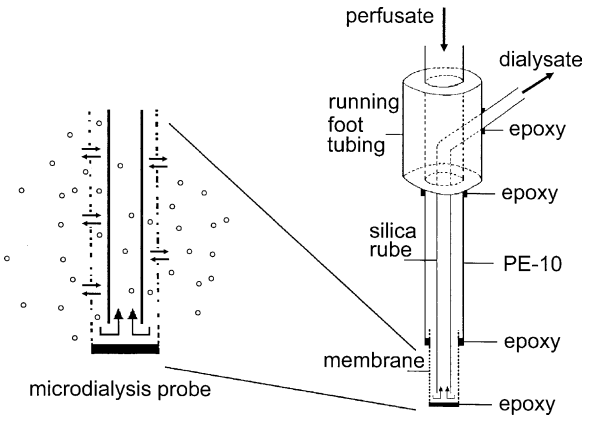

Fig. 1. Structure of a microdialysis probe.

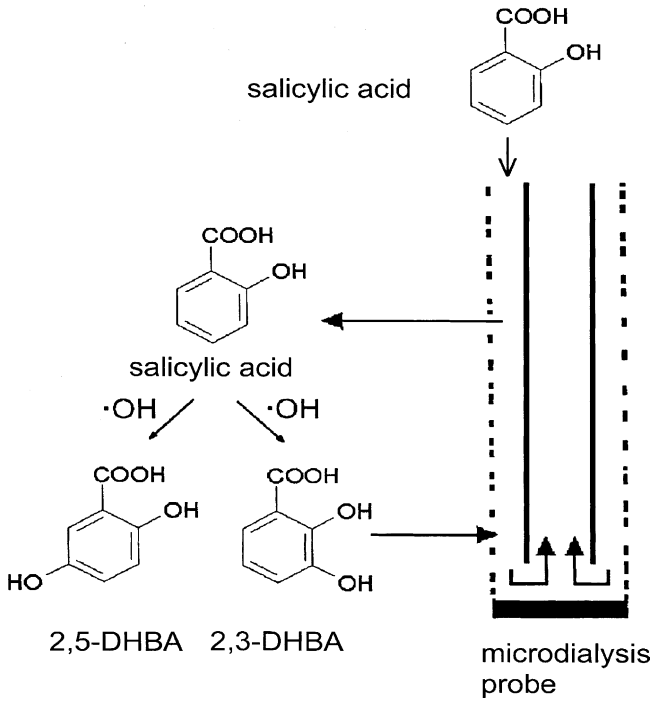

Fig. 2. Enlargement of the tip of a microdialysis probe on the reaction of hydroxyl radicals with salicylic acid through the membrane of the microdialysis probe to 2,3- and 2,5-dihydroxybenzoic acids.

drug transport equilibration across membranes, such as the blood-to-brain and blood-to-bile distributions. This article discusses the practical aspects and surgical procedures of blood, brain, liver, bile, enterohepatic circulation and cell culture microdialysis sampling.

2. Principle of microdialysis

Microdialysis is a technique for in vivo sampling of drugs with concentration gradients in biological fluids, tissues and organs of the body [17]. One basic difference between conventional drug concentration measurements and microdialysis is that the protein unbound form, is measured by microdialysis, whereas drug levels detected from ordinary blood sampling represent the total form. A fundamental difference between microdialysis sampling versus collecting blood samples is that the data obtained are continuous, unlike those usually obtained from blood sampling at specific time points. Microdialysis data contain the mean, or integral, of drug concentrations during each sampling interval. For this reason, they are best represented on a time axis at the midpoint of the sampling interval.

The original design of microdialysis sampling imitated the function of a capillary blood vessel by passive diffusion. The permeation rate of a drug through microdialysis membrane depends on the following factors: concentration of drug, oil/water partition coefficient of drug, and surface area of the dialysis membrane.

A microdialysis probe is usually constructed as a concentric tube where the perfusion fluid enters through an inner tube, flows to its distal end, and then exits the tube to enter the space between the inner tube and the outer dialysis membrane. Following drug administration, the drug molecules in the plasma or tissue extracellular space diffuse into the perfusate and may be recovered to determine their concentrations. Hence, the level of drug concentration can be detected, and the pharmacokinetics may be further studied. The drug concentration data from sampling of extracellular fluid were comparable with the data from ordinary blood sampling. This microdialysis pharmacokinetic characteristic has been evaluated by a multicompartmental model [18,19].

3. Protein binding and equilibrium dialysis

Protein binding drugs are loosely bound to plasma proteins such as albumin and alpha-1 acid glycoprotein, forming an equilibrium ratio between bound and unbound drugs. Most acidic drugs are bound to plasma albumin, whereas basic drugs are bound to alpha-1 acid glycoprotein. Binding to the remaining plasma protein generally occurs to a much smaller extent. The protein binding is usually reversible, so covalent binding of reactive drugs such as acrylonitrile can alkylate highly reactive cysteine of protein [20].

Binding of a drug to plasma proteins limits its concentration in tissues and its pharmacological action, since only the unbound drug is in equilibrium across membranes. Accordingly, after distribution equilibrium is achieved, the intracellular concentration of active, unbound drug is the same as that in plasma, except when carrier-mediated transport is involved. Drug transport, metabolism and excretion are also limited by plasma binding.

In vitro and in vivo protein binding of methotrexate has been assessed by microdialysis, and the results indicated that the relative recovery was independent of methotrexate concentrations. However, in that study, recovery was a factor that has been assessed in the presence of proteins surrounding the dialysis membrane, which was investigated by the physiological buffer, human serum albumin and human plasma. That study revealed that the protein content can directly affect microdialysis probe recovery [21]. Comparing microdialysis sampling with blood sample withdrawal for an in vivo pharmacokinetics of flurbiprofen revealed that simultaneous sampling of blood and intravenous microdialysis has become possible to study the pharmacokinetics of flurbiprofen in an individual rat [22].

In one early study, in order to investigate the variation in microdialysis and ultrafiltration unbound concentrations, both microdialysis sampling via the rabbit femoral vein and collection of whole blood via the rabbit ear vein after valproate injection were used. As a result, concentrations of free valproate in plasma were determined by ultrafiltration method in contrast to microdialysis method. The results indicated that there is no difference in the elimination half-life of valproate determined by microdialysis and ultrafiltration. With correction of dialysate recovery, the area under the concentration versus time curve (AUC) of valproate in the dialysate was also in good agreement with the value obtained from plasma ultrafiltration [23].

Conventional equilibrium dialysis method suffers some limitations for unbound drug sampling, including excessive dialysis time, possibly causing drug and protein degradation and allowing bacterial growth. Alternatively, microdialysis is based on the same basic principle and uses a semi-permeable membrane for collecting unbound drug. Some in vitro experiments have already shown good agreements in the evaluation of unbound drug by equilibrium dialysis and ultrafiltration [24,25]. Additional studies have demonstrated that there was no apparent bias in the correlation of the microdialysis and the equilibrium dialysis data, supporting the use of microdialysis in pharmacokinetic studies [25–27].

4. Ultrafiltration

Removal of proteins and other endogenous large macromolecules from biological samples without extraction or protein precipitation can be achieved using ultrafiltration, which uses cone-shaped membranes that fit on the top of centrifuge tubes or vials. Biological samples are placed into

the cones and centrifuged gently (ca. 200 g). The membrane molecular mass cut-off 25,000–50,000 Da allows only molecules smaller than the exclusion limit to pass through [16]. Ultrafiltration is a rapid and simple method to measure the unbound fraction from biological matrix, whereas equilibrium dialysis is more time-consuming, taking about 3 h before the sample is equilibrated. Due to this major disadvantage of equilibrium dialysis [28], the technique of ultrafiltration is commonly used in clinical applications.

Ultrafiltration has been used in the determination of unbound ropivacaine and bupivacaine in human plasma. The plasma sample (1 ml) was transferred to an ultrafiltration device and centrifuged at 37 °C and 2000 rpm (500 × *g*) for 15 min to give 200–250 μl of filtrate. The ultrafiltrate was injected into column liquid chromatography without further clean-up pretreatment [29].

Koivisto et al. [28] compared the free fraction rate and the precision of ultrafiltration and microdialysis, with results indicating no significant difference with respect to the free fraction between the two methods. Although both methods gave a free fraction of 6% ropivacaine in human plasma, the precision of the ultrafiltration was better. However, microdialysis can effectively be connected to an automatic online analytical system, which is not possible with ultrafiltration [28].

5. Microdialysis experiments

5.1. Blood sampling

Stahle et al. [30] demonstrated that recovery of extracellular lactate in vivo through the microdialysis probe was not directly influenced by changes in the cerebral blood flow. Van Wylen et al. [31] used a cardiac microdialysis probe implanted in the left ventricular myocardium of chloralose-urethane-anesthetized dogs and perfused with Krebs-Henseleit buffer to investigate adenosine concentration and coronary blood flow. The results suggested that cardiac microdialysis is a reliable technique for the sampling of regional intramyocardial interstitial fluid. To investigate long-term sampling, a flexible intravenous microdialysis probe implanted in freely moving rats, for 36 h and for 7 days after surgery showed stable amounts of epinephrine and glucose [32].

For implantation of microdialysis probe into rat blood vessel, a flexible intravenous microdialysis probe is implanted in the jugular vein via the superior vena cava towards the heart (right atrium) and perfused with the anticoagulant citrate dextrose (ACD solution: 3.5 mM citric acid; 7.5 mM sodium citrate; 13.6 mM dextrose), to avoid blood clotting around the dialysis fiber. This probe tip extends about 3 cm to reach the right superior vena cava/atrial junction, and the probe has an active dialysis membrane 1 cm long for drug sampling. The major reason for the location of the probe implanted into the right atrial junction is obtaining a larger blood pool surrounding the probe (Fig. 3) [33]. Although blood flow is impeded when a microdialysis probe is inserted into the jugular vein, the affluent blood from the inferior vena cava provides adequate blood supply for effective blood microdialysis. On the other hand, if the microdialysis probe is implanted into other veins, such as the femoral vein, the blood vessel would be occluded, resulting in poor blood circulation and pool dialysis efficiency. These considerations make the jugular vein the most suitable place for blood sampling for microdialysis. In vivo blood microdialysis sampling has widely been employed to study the pharmacokinetics and metabolism of xenobiotics [34–39].

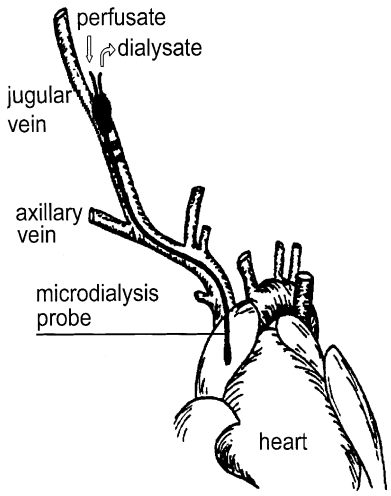

Fig. 3. Position of a microdialysis probe inserted into the jugular vein toward the heart.

One example of this is the study of metformin, where isocratic separation of metformin on a LUNA CN column (250 mm × 4.6 mm, i.d. = 5 μm, Phenomenex, Torrance, CA, USA) was achieved within 10 min. The mobile phase was comprised of acetonitrile–10 mM monopotassium phosphate (pH 5.1) (40:60, v/v) and the flow rate of the mobile phase was 1 ml/min. The mobile phase was filtered through a Millipore 0.45 μm filter and degassed prior to use. The optimal UV detection for metformin was set at a wavelength of 234 nm (Fig. 4).

5.2. Brain sampling

The most important advantage of microdialysis for sampling and studying drug delivery into the brain is its ability to measure the unbound drug concentration in a single experimental animal for both blood and brain over a period of time. This characteristic of unbound drug sampling circumvents the confounding element of drug protein binding in the blood and brain. The central nervous system (CNS) is protected from the peripheral circulation system by the blood–brain barrier (BBB) and the blood–cerebrospinal fluid (BCF) barrier. A drug molecule must penetrates the barriers into the CNS by passive diffusion via endothelial cells or the tight junctions. It is also possible to penetrate the BBB

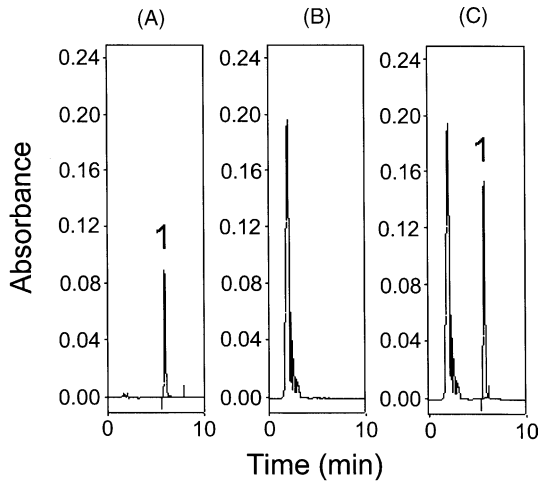

Fig. 4. Typical chromatograms of (A) standard metformin (10 μg/ml), (B) blank blood dialysate from the microdialysis probe before drug administration, and (C) blood dialysate sample containing metformin (21.63 μg/ml) collected 30 min after metformin administration (30 mg/kg, i.v.). (1) Metformin.

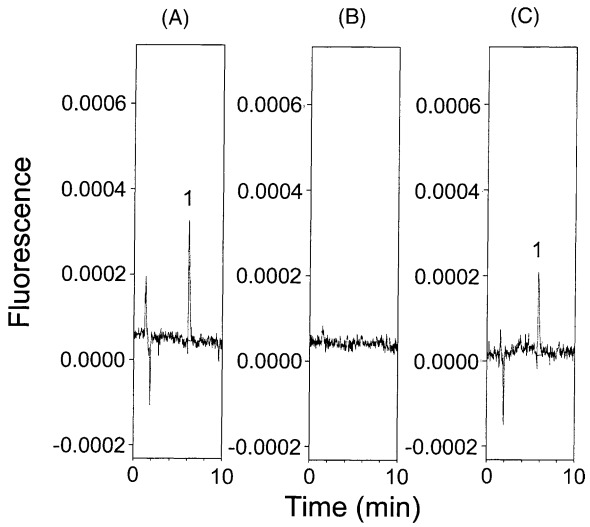

Fig. 5. Typical chromatograms of (A) standard pefloxacin (0.05 μg/ml). (B) Blank brain dialysate from the microdialysis probe before drug administration. (C) Brain dialysate sample containing pefloxacin (0.036 μg/ml) collected from the rat brain microdialysate 20 min after pefloxacin administration (10 mg/kg, i.v.). (1) Pefloxacin.

by active transport. Several specific transporter mechanisms have been identified as efflux pumps on the luminal side of the BBB such as P-glycoprotein [40,41].

Brain penetration is defined as the blood-to-brain distribution, which is calculated by dividing the analyte AUC in brain by its AUC in blood ($k = \mathrm{AUC_{brain}}/\mathrm{AUC_{blood}}$) [42]. Pefloxacin has been found to penetrate the BBB, and this study reveals that the pharmacokinetic profiles of pefloxacin in rat blood and brain are not altered by the treatment of P-glycoprotein modulator [43]. In this study, isocratic separation of pefloxacin from brain dialysate using a reversed-phase C_{18} column (150 mm × 4.6 mm; i.d. = 5 μm; Cosmosil, Kyoto, Japan) has been achieved within 10 min. The mobile phase consisted of citrate buffer (pH 5.0), acetonitrile and triethylamine (83:17:0.1, v/v/v). For this, one liter of citrate buffer (25 mM), was prepared using 25 mM of sodium acetate and 25 mM of citric acid dissolved in triple distilled HPLC grade water and buffered to pH 5.0 using orthorphosphoric acid. This mobile phase was filtered with a 0.45 μm Millipore membrane prior to being used for elution. The chromatographic pump flow rate was set at 1 ml/min. The excitation and emission wavelengths for optimal fluorescence response for pefloxacin were determined to be 330 and 440 nm, respectively [43] (Fig. 5).

5.3. Liver sampling

Since 1991, microdialysis probes have been utilized to monitor the brain and liver extracellular fluid after intravenous aluminum lactate or aluminum citrate injection compared with multiple blood withdrawals. The results indicate that metals can be repetitively sampled in the extracellular space of the liver using microdialysis for toxicokinetic studies [44,45]. Scott and Lunte [46] described a flow-through microdialysis probe for sampling bile while preserving normal bile flow to investigate hepatic metabolism of phenol. After intravenous infusion of phenol, phenol and its major hepatic metabolite were analyzed by liquid chromatography. The results indicate that the bile concentrations of phenol and its metabolite are higher than those in the liver, indicating that the metabolites are actively excreted into the bile. Subsequently, numerous reports have demonstrated that concurrent multiple-site microdialysis sampling is a useful tool for pharmacokinetic and drug metabolism studies in the liver [46–56].

Two types of microdialysis probes have been regularly used in liver sampling. One, the flexible concentric probe is identical to the probe used for sampling in the blood vessel. The other is a simple linear probe, constructed with fused silica as both the inlet and outlet tubing, and containing four to five dialysis membranes between the silica tubing. However, long-term implantation of the probe in the liver tissue runs the danger of some necrosis appearing at the implantation site after 12 h [49].

For liver probe implantation, the liver is exposed by making an incision to the midline at the xiphoid and extending approximately 2–4 cm posterior of the anaesthetized rat. The dialysis probe is inserted into the median lobe of liver toward the head and parallel to the midline and then fixed to the abdominal muscle with thread. After probe insertion, a wash-out period of 1 h was used to clear the extracellular fluid space of substances released from cellular damage caused by the implantation procedure. It is concluded that the microdialysis recovery of a substance from the liver is not generally affected by liver metabolism [57].

In the study of pharmacokinetics of geniposide, isocratic separation of geniposide from liver dialysate on a Nova-Pak reversed-phase column (RP-C_{18}, 150 mm × 3.9 mm,

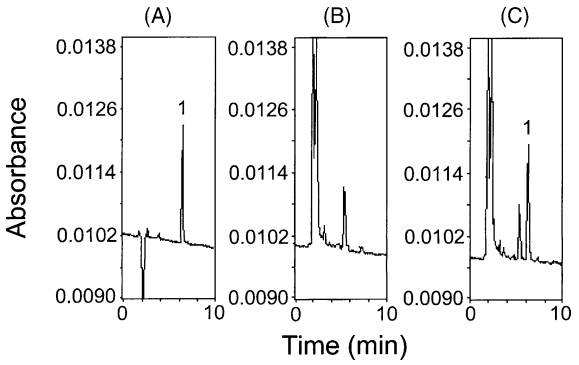

Fig. 6. Typical chromatograms of (A) standard geniposide (1 μg/ml), (B) blank liver dialysate from the microdialysis probe before drug administration, and (C) liver dialysate sample containing geniposide (0.99 μg/ml) collected 10 min after geniposide administration (10 mg/kg, i.v.). (1) Geniposide.

i.d. = 5 μm; Waters, Milford, MA, USA) was achieved within 10 min. The mobile phase was comprised of acetonitrile–methanol–5 mM monosodium phosphate (pH 4.6) (5:15:80, v/v/v), and the flow rate of the mobile phase was 1 ml/min. The UV detection for geniposide was set at a wavelength of 240 nm (Fig. 6).

5.4. Bile sampling

The bile duct in the rat is about 1 mm wide and runs from the hilum of the liver through the pancreatic tissue to the duodenum. The pancreatic ducts merge into the posterior part of the bile duct. There is no gall bladder in the rat, and bile juice together with pancreatic secretions enters the duodenum. Therefore, catheterization of the duct near the hilum of the liver will allow the collection of pure bile (Fig. 7) [46,58–64].

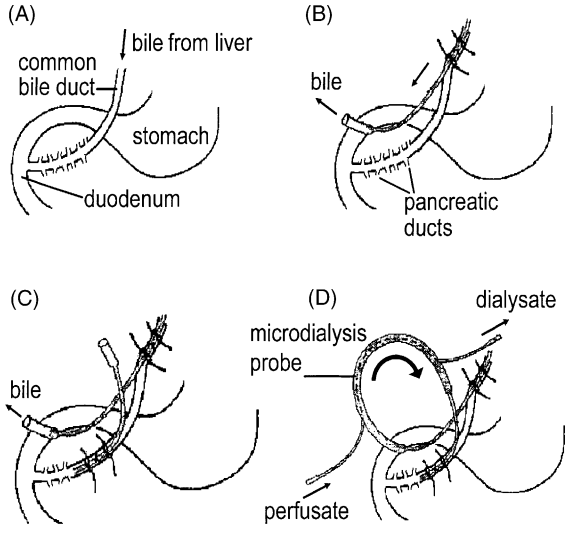

Fig. 7. Schematic procedures for the implantation of a microdialysis probe into the bile duct.

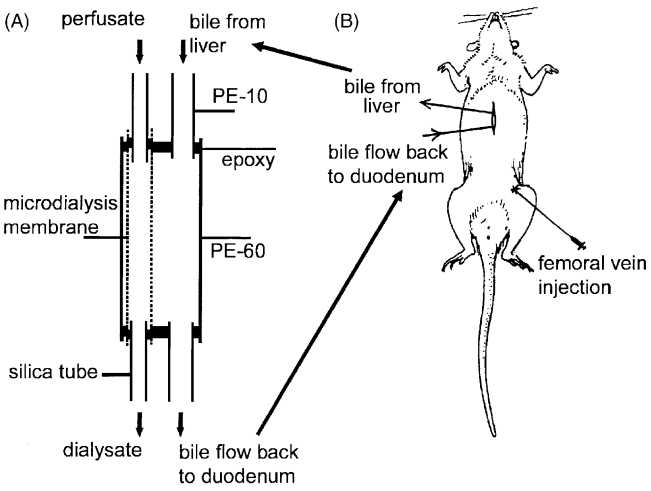

Fig. 8. Animal model for bile sampling, (A) detailed description of a custom-made bile microdialysis probe, and (B) flow-through microdialysis probe used for rat bile sampling.

For bile duct cannulation, a midline abdominal incision of about 2 cm is made in an anaesthetized rat. The duodenum and a small part of the intestine are pulled out to the right and kept moist by covering with a gauze pad soaked with physiological saline. The bile duct can be seen around the region of duodenum, especially if it is traced back from the hilum of the liver (Fig. 7A). Because about 0.5–1 cm of the bile duct near the hilum of the liver is free of pancreatic tissue, this region is used for catheterization. A polyethylene (PE-10) tube is inserted into the anterior region of the bile duct (Fig. 7B). The other PE-10 tubing is inserted into the posterior region of the bile duct (Fig. 7C). Shunt microdialysis probe implantation is connected to the anterior region of PE-10 tubing to conduct bile flow. After bile juice flows through the chamber of the dialysis probe, the small molecules contained in the bile may penetrate the dialysis membrane into the dialysate according to the concentration gradient. The other end of the shunt probe is inserted into the posterior region of PE-10 towards the duodenum, allowing bile to flow into the small intestine (Fig. 7D). The dialysates are collected at 10 min intervals into a refrigerated fraction collector for later analysis.

A diagram of the shunt microdialysis probe is shown in Fig. 8. This is a valuable tool for profiling analytes in the rat bile duct while preserving enterohepatic circulation without disruption of the bile flow. This technique also provides continuous sampling with no net loss in fluid volume and has high temporal resolution during the experimental period.

A simultaneous measurement of unbound camptothecin dialysates in rat blood and bile study indicates that the amount of camptothecin, as estimated from the AUC, in bile versus concentration gradient significantly exceeds that in blood, suggesting that camptothecin might be actively excreted into the bile. Treatment with P-glycoprotein modulator results in a decrease in the concentration versus time

curve of camptothecin in the bile. These results imply that the P-glycoprotein might regulate the hepatobiliary excretion of camptothecin [65].

Here, isocratic separation of ondansetron from bile dialysate using a microbore reversed-phase C_{18} column (150 mm × 4.6 mm; i.d. = 5 μm; Cosmosil, Kyoto, Japan) at ambient temperature was achieved within 10 min. The mobile phase consisted of 25 mM acetate buffer (pH 5.2) and acetonitrile (72:28, v/v), and contained 0.2% octanesulfonic acid sodium salt. The 25 mM acetate buffer was prepared with 25 mM sodium acetate and the pH was adjusted to 4.8 by glacial acetic acid. The mobile phase was filtered with a 0.45 μm Millipore membrane filter. The flow rate was 1 ml/min. The UV wavelength was set at 305 nm (Fig. 9).

5.5. Enterohepatic circulation

The enterohepatic circulation of chloramphenicol may comprise the sequential processes of hepatic uptake from blood, excretion from the liver into the bile, transport of the bile to the duodenum, reabsorption from the intestine, and returning to the liver via portal circulation. If the compound is conjugated in the liver, there are the added processes of liver conjugation and the hydrolysis of the conjugates by intestinal bacteria. These processes form the enterohepatic circulation.

For animal experiments, using a hepato-duodenal shunt connecting a drug-treated donor to another untreated recipient rat and simultaneous blood sampling through microdialysis permit multiple sampling without undue stress and biological fluid consumption. The bile duct of the donor is cannulated proximal to the liver with a 20 cm section of PE-10 tubing, the other end of which is inserted through the bile duct into the duodenum of the recipient rat. To balance

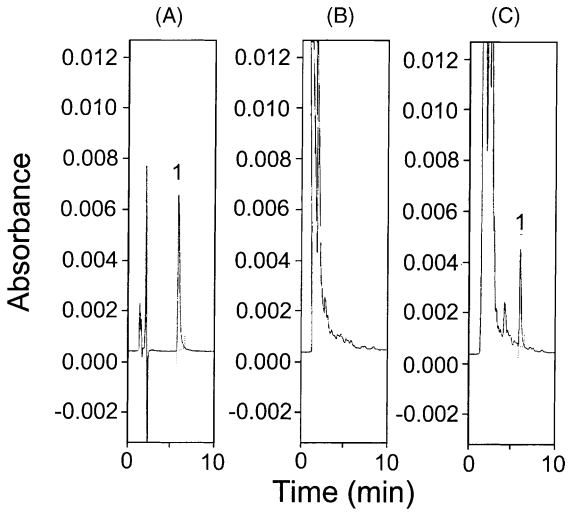

Fig. 9. Typical chromatogram of (A) a standard ondansetron (2 μg/ml), (B) a blank bile dialysate, and (C) a bile dialysate sample containing ondansetron (1.21 μg/ml) collected 24 min after ondansetron (20 mg/kg, i.v.) administration. (1) Ondansetron.

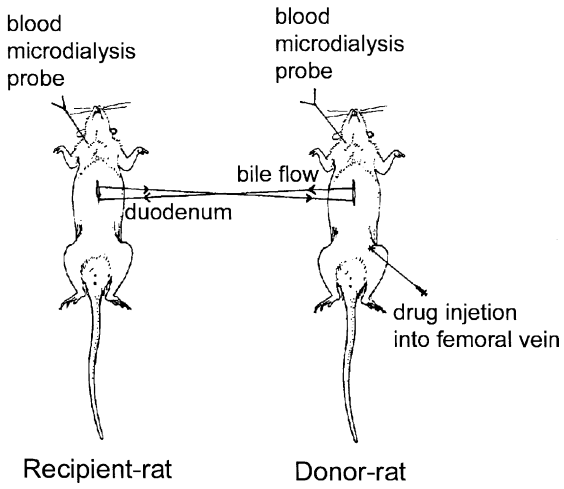

Fig. 10. Hepato-duodenal shunt model for the investigation of enterohepatic circulation. To balance the fluid losses and gains in the donor (drug treatment) and recipient (no drug treatment) rats, the bile duct of the recipient rat was also cannulated to channel the bile back to the donor rat.

the fluid losses and gains in the donor and recipient rats, the bile duct of the recipient rat is also cannulated to channel the bile back to the donor rat (Fig. 10).

For this technique, after about 2 h of surgical stabilization, drug is administered to the donor rat through a femoral cannula. Dialysates from the blood of the donor and recipient rats are collected for later analyses. To estimate quantitatively the degree of enterohepatic circulation taking place in the paired-rats, the AUC in the recipient rats is compared with the AUC in the donor rats ($AUC_{recipient}/AUC_{donor}$) [66]. After chloramphenicol administration (100 mg/kg, i.v.) into the recipient rat, pharmacokinetic parameters calculated from the AUCs of unbound chloramphenicol and chloramphenicol glucuronide show that the extent of recycling ($AUC_{recipient}/AUC_{donor}$) is approximately 1.8 and 4.9% for chloramphenicol and chloramphenicol glucuronide, respectively [66]. Following naringenin administration, the coefficient ratio of enterohepatic circulation for recipient and donor rats is 0.024, which is calculated as the ratio of AUCs between the recipient and the donor rats ($k = AUC_{recipient}/AUC_{donor}$) [67]. The in vivo paired-rat animal model was demonstrated in the study to be potentially useful for studying the pharmacokinetics and enterohepatic circulation of analytes in rats.

5.6. Cell culture

Microdialysis has also been utilized for the measurement of intracellular catecholamines in PC-12 cells [68]. For this, the catecholamines concentrations in the medium are analyzed by microbore liquid chromatography with electrochemical detection (LC-ED). Conventional medium sampling requires time-consuming pretreatments or complicated extraction prior to analysis by conventional LC-ED assays, which may result in sample losses and increases in

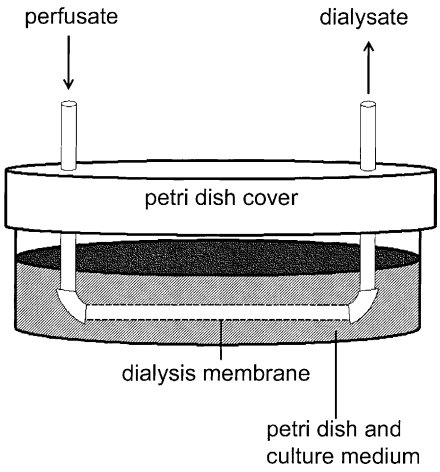

Fig. 11. Schematic diagram of a Petri dish used for microdialysis.

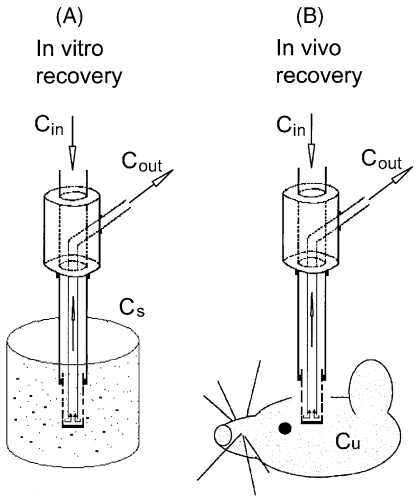

Fig. 12. Microdialysis recovery experiments for (A) in vitro and (B) in vivo.

the amount of time needed to complete the experiments. Alternatively, the microdialysis technique has the advantages of requiring no sample pretreatment, and sample can be directly injected for analysis. This online method speeds up the experimental procedure, provides high sensitivity, minimizes the required sample volume, enhances the detection limits, and decreases degradation of analyzed compounds. We have also developed an online microdialysis device for the direct measurement of pyruvate and lactate in primary liver cell culture medium. This novel sampling device is constructed within a hypoxia chamber. Using these results, dynamic changes in pyruvate and lactate levels in primary liver cell culture medium under hypoxia and reperfusion have been discussed [69].

In this technique, a sampling device is composed of a Petri dish, two transmission tubes, and a dialysis membrane as illustrated in Fig. 11. The Petri dish has a receiving space with an open top. The cover has two holes separated by a predetermined distance. This microdialysis system is similar to those reported by Maas et al. [70] and Miyamoto and Schams [71].

Cell culture coupled to microdialysis technique is relatively efficient, cost-effective, and less vulnerable to human error compared with conventional studies, in which a number of Petri dishes are used. Our previous study has minimized pretreatment procedures for sample preparation, decreased possible contamination from sampling of culture medium, and enhanced the detection sensitivity of catecholamines in PC-12 cell culture medium. Furthermore, this novel microdialysis device can also be applied to the measurement of chemical substances in other culture systems [68].

6. Quantification and validation

However, the concentration data obtained from microdialysis cannot be directly applied to pharmacokinetics or pharmacodynamics. Actually, at least four distinct regions have to be considered during the process of microdialysis, including environments of surrounding tissues, materials of the dialysis membrane, probe geometry and constituents of perfusate. Basically, the perfusate is constantly being pumped through the probe, so the boundary condition is not maintained at equilibrium status, and the drug concentration in the dialysate is some fraction of that in the surrounding matrix. Obviously, estimation of the true drug concentration in tissues sampled by the dialysis probe is dependent upon the in vivo recovery. The transport properties of the microdialysis probe are described by the extraction efficiency or the term of recovery. The diagram of the experiment for recovery is shown in Fig. 12. The recovery is defined as

$$\text{recovery} = \frac{(C_{\text{in}} - C_{\text{out}})}{(C_{\text{in}} - C_{\text{s}})}$$

where C_{in} is the concentration of the perfusate, C_{out} the concentration of the dialysate, and C_{s} the concentration of the sample.

(1) For in vitro recovery by dialysis (R_{dial}) or recovery by gain:

The microdialysis probe is inserted into a sample solution whose temperature is maintained constant by a thermostat (37 °C) and is continuously stirred. A predetermined drug concentration (C_{s}) is added to the sample solution surrounding the probe. The probe is perfused with drug-free physiological buffer ($C_{\text{in}} = 0$) at a constant flow rate, and dialysate concentration of drug (C_{in}) is analyzed by the analytical system. The in vitro recovery by dialysis is estimated as follows:

$$R_{\text{dial}} = \frac{(C_{\text{in}} - C_{\text{out}})}{(C_{\text{in}} - C_{\text{s}})} = \frac{C_{\text{in}}}{C_{\text{out}}}$$

(2) For in vitro recovery by retrodialysis (R_{retro}) or recovery by loss:

The drug is introduced into the perfusate (C_{in}) and its relative loss of drug (L_{retro}) to the medium through the membrane during the microdialysis is determined. The tip of the microdialysis probe is inserted into a drug-free solution ($C_s = 0$). The in vitro recovery by retrodialysis is calculated as follows:

$$L_{retro} = R_{retro} = \frac{(C_{in} - C_{out})}{(C_{in} - C_s)} = \frac{(C_{in} - C_{out})}{C_{in}}$$

(3) For in vivo recovery by retrodialysis:

An experimental rat is used in the in vivo recovery study. Following a series of regular surgical procedures, the microdialysis is placed in the rat blood or tissue, and the probe perfused with biological-buffer containing drug (C_{in}) at a constant flow rate. Before perfusion, the rat is not treated with any drug ($C_s = 0$). Following analysis of dialysate (C_{out}), the in vivo recovery by retrodialysis is calculated as follows:

$$R_{retro} = \frac{(C_{in} - C_{out})}{(C_{in} - C_s)} = \frac{(C_{in} - C_{out})}{C_{in}}$$

The unbound drug concentration (C_u) in the living body is converted by the equation of

$$C_u = \frac{C_{out}}{R_{retro}}$$

Ungerstedt [72] and Scheller and Kolb [73] have demonstrated that the dialysis (recovery by gain) and retrodialysis (recovery by loss) are independent of concentration. Clement et al. [74] showed that neither the dialysis of bupivacaine nor the retrodialysis of ropivacaine in the concentration ranges of 10–200 μg/ml was affected. Evrard et al. [75] demonstrated that there is no statistical significance between dialysis and retrodialysis of flurbiprofen in vitro, nor between retrodialysis of flurbiprofen in vitro and in vivo. To calibrate the microdialysis recovery precisely, Van Belle et al. [76] developed an internal reference technique for in vitro and in vivo microdialysis calibration of carbamazepine and its metabolites in rat brain tissue by adding an internal standard to the perfusate. The results indicate that the internal reference technique is a useful method for estimating the true concentration of exogenous compounds in the extracellular space of tissues.

Telting-Diaz et al. [77] determined in vitro recovery rate using Ringer's solution, plasma and whole blood. Three different theophylline concentrations (1, 10 and 20 μg/ml) were tested for each of the solutions. There was no significant difference between the tested solutions and the different concentrations and microdialysis probes.

Rojas et al. [78] demonstrated the recovery of triamcinolone acetonide in recovered samples by gain, retrodialysis and no-net-flux methods performed under several conditions. The recovery at 37 °C was higher than that at room temperature, but that under stirred or unstirred conditions was the same in either direction of dialysis. Increasing viscosity of the reservoir medium acted to decrease the recovery rate (55% in Ringer's solution compared to 14% in 20% methylcellulose gel). The recovery determined by the no-net-flux method was similar to the retrodialysis result. The results indicate that the conditions of stirring, temperature, viscosity and protein binding in the reservoir medium all affect the in vitro recovery of triamcinolone acetonide.

In the study of the efficiency of microdialysis probe, an in vitro recovery of microdialysis probe was investigated by different perfusion flow rates, showing that the recovery decreases when the flow rate increases. Comparison of the in vitro recovery method, by gain, loss and the zero-net-flux showed that the recovery of gallamine was within the ranges of 38.4–38.9%. In addition, the in vitro recovery before and after in vivo experiment was examined, indicating that the performance of microdialysis probe was stable after being implanted in the muscle tissue, as confirmed by the in vitro experiment performed directly after completion of the in vivo studies [79]. These results suggest that using microdialysis for the study of pharmacokinetics and pharmacodynamics is strongly recommended. For economy, the commercially available microdialysis probes such as CMA-20 can be reused depending on the user, type of membrane and site of implantation [80].

Several reports indicate that in vitro recovery cannot estimate in vivo recovery [81,82]. Furthermore, the efficiency of microdialysis probe may be affected by in vivo conditions, such as the possible interaction of plasma or tissue components with the membrane materials [83].

Yang et al. [84] demonstrated that there is no statistical difference between the recovery by gain and by loss for either fluconazole or UK-54,373. However, the recovery by loss of fluconazole and UK-54,373 in brain tissue is significantly less than the recovery by loss of the same compounds when the probe is placed in artificial CSF at the same perfusion rate. These results also suggest that in vitro recovery is not suitable for estimating the probe recovery in vivo in the brain tissue.

7. Separation method and microdialysis hyphenation

Microdialysis is a continuous process and the sampling interval defines the temporal resolution. The temporal resolution is dependent on the perfusion rate and volume of sample needed, so using a low perfusion rate can increase probe recovery. However, in order to increase the dialysate volume, the perfusion rate should be increased, although the recovery is compromised. Consequently, the concentration of the analytes in the dialysate has been diluted. For this reason, the sensitivity of the analytical method in measuring the desired analyte at the concentrations present in the dialysate is crucial [4,85]. To this end, several analytical methods have been developed, such as liquid chromatography with detections of ultraviolet [86–88], fluorescence [89,90], electrochemistry [91,92], LC-tandem mass spectrometry [93–95], radioimmunoassay [96,97], capillary elec-

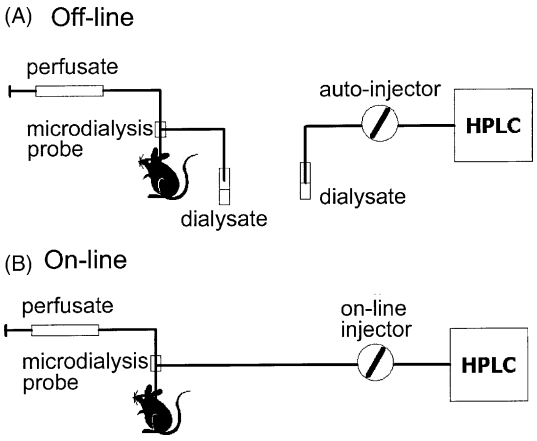

Fig. 13. Microdialysis hyphenation for (A) off-line and (B) on-line systems.

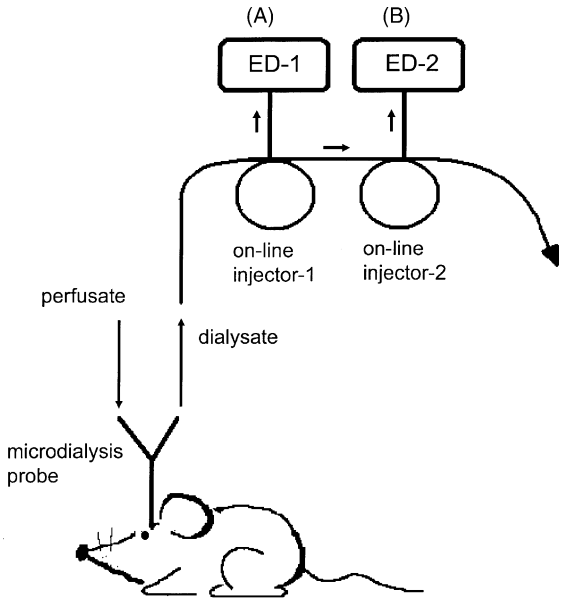

Fig. 14. Schematic diagram for the set-up of two serial online microdialysis injectors and two parallel liquid chromatography systems with electrochemical detectors in freely moving rats.

trophoresis [98,99], gas-chromatography mass spectrometry [100,101], or atomic absorption spectrometry [102] for the analysis of a variety of dialysates [103].

To obtain the samples for off-line analyses (Fig. 13), a series of dialysates are collected for subsequent analysis of the analyte. However, microdialysis off-line sample manipulation is often time-consuming and increases the chance of sample contamination and sample degradation. To circumvent these problems, using online hyphenation the dialysate is automatically injected into the chromatographic system. However, because of the serial connection to the chromatographic system, the temporal resolution will be limited by the analysis time. To balance the perfusion rate, sample volume and detection limit of analyte, a suitable hyphenation between microdialysis device and chromatographic system should be developed. For example, caffeic acid is unstable, being subject to degradation by the non-enzymatic auto-oxidative phenolic browning reaction, which is temperature- and pH-dependent. To minimize the degradation of caffeic acid at physiological pH, an automatic sampling system and a stable analytical system are hyphenated. Thus, an in vivo online microdialysis sampling method coupled to a liquid chromatographic system is used to measure unbound caffeic acid in rat blood [33].

Conventional online hyphenation utilizes a loop to collect dialysate which is subsequently injected for analysis, so the dialysate is used for only a single injection. This design is limited to similar characteristics of the analytes. In order to measure automatically and simultaneously the neurotransmitters of cholinergic and biological amines, two serial online microdialysis injectors and two parallel sets of microbore liquid chromatography with electrochemical detections have been established to assess acetylcholine and biological amines in freely moving rats (Fig. 14) [104].

To investigate the fast releasing events of neurotransmitter release mechanisms, highly temporal resolution has been successfully improved in brain microdialysis for the measurement of neurochemicals. Improving temporal resolution for microdialysis sampling must overcome challenges such as lower sample volume, sensitive detection and fast collection. In 1997, Lada et al. [105] developed a microdialysis online with capillary electrophoresis for the measurement of glutamate and aspartate within 12 s temporal resolution. Shortly thereafter, Tucci et al. [106] measured glutamate by 6 s resolution brain microdialysis with capillary electrophoretic and laser-induced fluorescence detection application. In 2003, Rossell et al. [107] presented a 1 s time resolution brain microdialysis system to determine glutamate in fully awake rats. This study designed an appropriate protocol to overcome the difficulty of the hyphenation of an online derivatizing reactor that restrained the volume to within 30 nl and measurement with capillary electrophoresis laser-induced fluorescence detection [107].

8. Conclusion

Microdialysis provides several advantages for pharmacokinetic and pharmacodynamic studies by in vivo sampling of extracellular fluid in many kinds of tissues and fluids. In contrast to other methods of sampling biological fluids and tissues, microdialysis offers a very clean dialysate, which requires no further clean-up procedure. Microdialysis also allows continuous monitoring of drug absorption, distribution, metabolism and elimination at various tissue sites and various fluids. That there is no biological fluid loss makes microdialysis sampling available for highly temporal and special resolutions. Since no biological fluid is removed from or introduced into the body during the process of microdialysis, minimal perturbation can be achieved. For pharmacokinetic and pharmacodynamic investigation, the dialysate is obtained before, during and after the drug treat-

Table 1
Assaying unbound drugs by microdialysis

Sample	Drug/analyte	Stationary phase	Mobile phase	Detection	References
Blood	Tropisetron	RP-C_{18} 150 mm × 4.6 mm	MeCN–100 mM NaH_2PO_4 (pH 5.0) (26:74, v/v)	UV (284 nm)	[124]
Blood	Caffeic acid	RP-C_{18} 250 mm × 4 mm	MeOH–100 mM NaH_2PO_4 (35:65, v/v, pH 2.5)	UV (320 nm)	[33]
Blood, brain, bile	Omeprazole	Microbore RP-C_{18} 150 mm × 1 mm	MeCN–20 mM NaH_2PO_4 (35:65, v/v) and 0.1 mM 1-octanesulfonic acid	UV (300 nm)	[125]
Blood, brain, bile	Pefloxacin	RP-C_{18} 150 mm × 4.6 mm	Citrate (pH 5.0)–MeCN–triethylamine (83:17:0.1, v/v/v)	Fluorescence (excitation: 330 nm; emission: 440 nm)	[43]
Blood, liver, bile	Berberine	Phenyl 150 mm × 4.6 mm	MeCN–MeOH–0.1 M NaH_2PO_4 (pH 3.0) (35:20:45, v/v/v)	UV (346 nm)	[55]
Blood, brain, liver, bile	Naringenin	Microbore RP-C_{18}	MeCN–0.1 M ammonium acetate (pH 7.1) (28:72, v/v)	UV (283 nm)	[56]
Blood, brain, bile	Baicalein	Microbore RP-C_{18}	MeCN–MeOH–0.1 M NaH_2PO_4 (pH 4.5) (40:8:52, v/v/v)	ED (Ag/AgCl, AP = +0.5 V)	[126]
Liver cell culture	Pyruvate, lactate	Polypore column 100 mm × 4.6 mm	4 mM sulfonic acid (112 μl in 1000 ml double-distilled water)	UV-Vis (546 nm)	[69]
Liver	Glutathione	RP-C_{18} 150 mm × 4.6 mm	0.1 mM monochloroacetic acid, 2 mM sodium hepanesulfonate, 0.046 M NaOH in 2% MeCN	ED (Au–Hg electrodes, working potential 0.15 V vs. Ag/AgCl)	[127]
Skeletal muscle	Norfloxacin	RP-C_{18} 300 mm × 3.9 mm	0.0025 M H_3PO_4, MeCN, MeOH (78:19:3, v/v/v, pH 3 by triethylamine)	Fluorescence (excitation: 276 nm; emission: 445 nm)	[128]

ment, so each animal serves as its own control. This permits a cross-over experiment performed in a single animal.

Using several microdialysis probes, simultaneous sampling from various sites in a single animal avoids the problems associated with intra-animal variability for pharmacokinetic studies. The AUC data from blood could be directly compared with the AUC from brain for brain distribution and blood–brain barrier penetration studies [41,42]. Multiple sites sampling is also utilized in the investigation of hepatobiliary excretion and enterohepatic circulation. The blood-to-brain and blood-to-bile is calculated as AUC_{brain}/AUC_{blood} and AUC_{bile}/AUC_{blood}, respectively. Furthermore, these designs have been used to investigate the mechanism of P-glycoprotein [108–118].

The unbound drug in the dialysate is no longer enzymatically degradable once the drug or its metabolites diffuse into the dialysate and are separated from the protein and other macromolecules [103,119,120]. When the molecular weight cut-off for the membrane of microdialysis is below 20 kDa, ex vivo analysis to examine drug protein binding becomes possible [121]. Because the dialysate is protein-free, it can be directly injected into analytical instruments such as liquid chromatography, capillary electrophoresis and mass spectrometry.

Although microdialysis possesses many advantages for pharmacokinetic and pharmacodynamic studies, it is not without its limitations. The main limitation is its recovery, which can be greatly affected by efficiency of the probe. For sampling of hydrophobic drugs, the compounds are often highly protein-bound and may stick to the membrane of the microdialysis probe. To overcome these problems, bovine serum albumin, glycerol [122] or β-cyclodextrin [123] have been added in the perfusate to increase the recovery of hydrophobic drugs. In sum, the combination of small volume of dialysate and protein binding of the drug, together with low concentration of the analytes is a challenging factor for pharmaceutical analysis. Undoubtedly, a sensitive analytical system is required to further advance microdialysis applications in pharmacokinetic studies. The analytical methods for the measurement of unbound drugs by microdialysis are summarized in Table 1.

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