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Determination of baicalin in rat cerebrospinal fluid and blood using microdialysis coupled with ultra-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

An *in vivo* microdialysis sampling method coupled with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was employed for continuous simultaneous monitoring of unbound baicalin in rat blood and brain. Microdialysis probes were inserted into the jugular vein and brain cerebrospinal fluid (CSF) of Sprague-Dawley rats then, following administration of baicalin at doses of 24 mg/kg via the candal vein, samples were collected every 20 min and injected directly into the UPLC-MS/MS system. *In vitro* recoveries of the probes were 19.26% and 18.38%, while *in vivo* recoveries of the probes were 15.0% and 17.52% for blood and brain, respectively. This improved method offers a rapid quantitative procedure for the determination of baicalin with a retention time of only 1.6 min. The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) based on a signal-to-noise ratio of 5 were 2.37 and 0.1 ng/ml for anticoagulant citrate dextrose (ACD) solution, and 1.185 and 0.3 ng/ml for artificial cerebrospinal fluid (aCSF), respectively. The pharmacokinetics results indicated that baicalin could pass through the blood–brain barrier (BBB) and was detectable in brain dialysate. These *in vivo* microdialysis-based measurements provide a technique for simple sampling and rapid sensitive analysis of unbound baicalin in rat blood and CSF and for further application in pharmacokinetic studies.

1. Introduction

Baicalin (7-glucuronic acid-5,6-dihydroxy-flavone, structure shown in Fig. 1), the predominant flavone glucuronide of the commonly used traditional Chinese medicine (TCM) *Scutellariae radix*, exhibits a variety of pharmacological effects such as anti-viral [1], anti-inflammation [2], anti-oxidation [3,4] and anticancer activities [5–7]. Studies have also revealed that baicalin can act on the dopamine system [8], influence cerebral function and also can relieve fever by affecting the central nervous system (CNS) [9,10].

To quantify baicalin in biological samples, in addition to analytical methods based on HPLC with UV [11] and ECD [12], several HPLC-MS/MS methods have also been developed [13,14]. However, these measurements are hindered by low sensitivity, a long retention time and low throughput. Now, a new sampling technique, *in vivo* microdialysis, combined with an advanced analytical method based on UPLC-MS/MS for the simultaneous determination of unbound baicalin in biological fluids with a higher sensitivity and better continuity has presently been developed. In addition, with regard to the BBB permeability of baicalin, Tsai and Tsai reported it might not be able to cross [15], while Zhang et al. have disagreed with this [16]. In the present study, the new method was used to study the penetration of baicalin from blood into the cerebrospinal fluid (CSF).

Microdialysis, an *in vivo* sampling technique that allows determination of unbound drug concentrations in blood and most tissues, is based on the passive diffusion of compounds down a concentration gradient across a semipermeable membrane with a molecular mass cut-off ranging from 5000 to 50,000 Da. In contrast to other biological samples, because the dialysis membrane is only permeable to small molecules and the analyte can be separated from enzymes by the dialysis membrane, microdialysis samples need no further clean-up and will not undergo further metabolism after collection. Moreover, this sampling method simplifies pharmacokinetic studies by reducing the effect of biological volume changes compared with conventional biological fluid collection assay. Therefore, continuous sampling and simultaneous sampling from multiple sites is made possible by implanting more than one probe in the same animal without affecting the pharmacokinetic profiles while allowing determination of concentration–time profiles involving a high number of samples over short time periods. Till now this technique has been used successfully in the measure-

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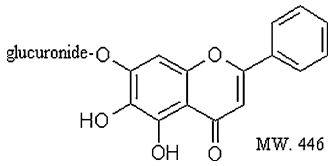

Fig. 1. Chemical structure of baicalin.

ment of neurotransmitters [17,18], and in drug pharmacokinetic and pharmacodynamic studies in animals [19] and humans avoiding exposure of the brain tissue to the perfusion medium and, therefore, minimizing tissue damage. The union of *in vivo* sampling with analytical chemistry has rapidly expanded the field of microdialysis as well as leading to the formation of several microdialysis companies including CMA microdialysis. In addition, when coupled to analytical systems such as mass spectrometry, microdialysis can be used to obtain direct measurements of unbound drug concentrations in the brain following a systemic injection *in vivo* [20].

Generally, a better understanding of the pharmacokinetics of a drug in blood or other target sites will promote the development of improved therapy schedules, drug formulations or improve the molecular structure of drugs meant to act in such areas, e.g. the CNS. Based on pharmacokinetics, only drugs that are not protein bound in biological fluids are effective for therapeutic applications. Now, an *in vitro* study has indicated that the protein binding of baicalin is about 90% [21]. In our study, microdialysis probes were simultaneously inserted into each anesthetized rat to allow biological fluid sampling from the jugular vein and CSF after administration of a single i.v. dose of baicalin. The analyte in the microdialysis samples was determined using an UPLC-MS/MS system using electrospray ionization (ESI) to study the disposition of unbound baicalin in the rat blood and brain. The retention time of baicalin is 1.6 min, representing a significant improvement over previous methods. This new method offers a simultaneous and useful sampling technique using microdialysis followed by a more rapid and sensitive detection method and is suitable for evaluating the BBB penetration of compounds in order to assist in brain-targeting research.

2. Experimental

2.1. Chemicals

Baicalin (98.5%) was purchased from Si Chuan Huatai Pharmaceutical Co. Ltd. (Si Chuan, China). Chromatographic solvents and reagents were obtained from Dikma (Richmond Hill, NY, USA). The dialysis perfusate used was modified aCSF (2.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 145 mM NaCl) for brain and modified ACD solution for blood (3.5 mM citric acid, 7.5 mM sodium citrate and 13.6 mM dextrose) [22].

Triple deionized water was obtained from a Barnstead EASYpure® IIRF/UV ultrapure water system (Dubuque, Iowa, USA) and was passed through a 0.22 μm filter prior to use in all the studies.

2.2. Animals

Adult male Sprague-Dawley rats (about 280 g) were obtained from the Laboratory Animal Center at Shenyang Pharmaceutical University. These animals were specifically pathogen-free and were allowed to acclimatize to their environmentally controlled quarters for at least 5 days with free access to food and water before the start of experimentation. The rats were initially anesthetized with urethane (1.5 g/kg, i.p.), and remained anesthetized throughout the experimental period. The caudal vein was exposed for drug administration, and the body temperature of the rats was maintained at 37 °C with an infrared lamp.

All animal experiments were performed in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee of Shenyang Pharmaceutical University.

2.3. Microdialysis experiment

Blood and brain microdialysis systems consisted of a microdialysis syringe pump (S200, KD Scientific Company, USA), microdialysis probes and a stereotaxic frame. The dialysis probes for blood (10 mm in length) and brain (3 mm in length) were U-shaped and made of hollow cellulose fiber (DM-22, 200 μm inner diameter and 220 μm outer diameter, EICOM Corp., Japan); these were used for both the *in vitro* and *in vivo* studies and had a molecular weight cut-off of 5000 Da. The perfusate was passed through a 0.22 μm nylon filter before use.

2.3.1. Stereotaxic surgery and probe implantation

At the beginning of experiment, heads of the male Sprague-Dawley rats were shaved before placing them in a stereotaxic apparatus (RWD Life Science Co. Ltd., Shenzhen, China). A midline incision of approximately 2 cm was made parallel to the sagittal suture. The bregma was located and used as the reference point for positioning the microdialysis probe. A microdialysis probe was stereotaxically inserted through a cranial burr hole made by a dental drill to a depth of 3.1 mm, using the following coordinates, in relation to the bregma: 1.5 mm lateral, 0.9 mm posterior, and the probe was attached to the skull with dental cement [23]. The blood microdialysis probe was positioned within the jugular vein toward the right atrium and, at the same time, two probes were perfused with ACD solution and aCSF separately, at a flow rate of 3 μl/min, using the S200 microdialysis syringe pump for both delivery of the perfusion solution and sample collection.

2.3.2. Drug administration and collection of biological samples

Drug administration in each group was performed following the successful implantation of the microdialysis probe and stabilization for 2 h with aCSF and ACD solution being perfused. Six animals were used in the experiment and baicalin (24 mg/kg) was given i.v. via the caudal vein. CSF and blood dialysate samples were collected at intervals of 20 min for 7 h, transferred to small polypropylene tubes and analyzed by UPLC-MS/MS within 48 h.

2.4. Microdialysis probe calibration

The concentrations of the drug in the dialysate reflect the concentrations in the (extracellular) fluid around the semipermeable part of the probe. However, as the dialysis procedure is not performed under equilibrium conditions, the concentration in the dialysate will be different from that in the periprobe fluid. The term "recovery" is used to describe this relationship. Microdialysis probe recovery was calibrated by triplicate measurements of *in vitro* recovery ($R_{in\ vitro}$), *in vitro* delivery ($D_{in\ vitro}$) and *in vivo* recovery ($R_{in\ vivo}$), *in vivo* delivery ($D_{in\ vivo}$). *In vivo* recovery was calculated according to $(R/D)_{in\ vitro} = (R/D)_{in\ vivo}$ [24].

2.4.1. In vitro recovery and delivery

The recovery for the microdialysis probe was determined *in vitro* by the no net flux method (NNF) [25,26]. In this experiment, the microdialysis probe was immersed in aCSF at 37 °C containing baicalin (53 ng/ml) as the dialysis medium and perfused at 3 μl/min with aCSF containing different concentrations of baicalin (1.06, 10.6, 31.8, 106, 212 and 318 ng/ml, C_p). Microdialysis samples (C_d, 60 μl) were collected for each concentration

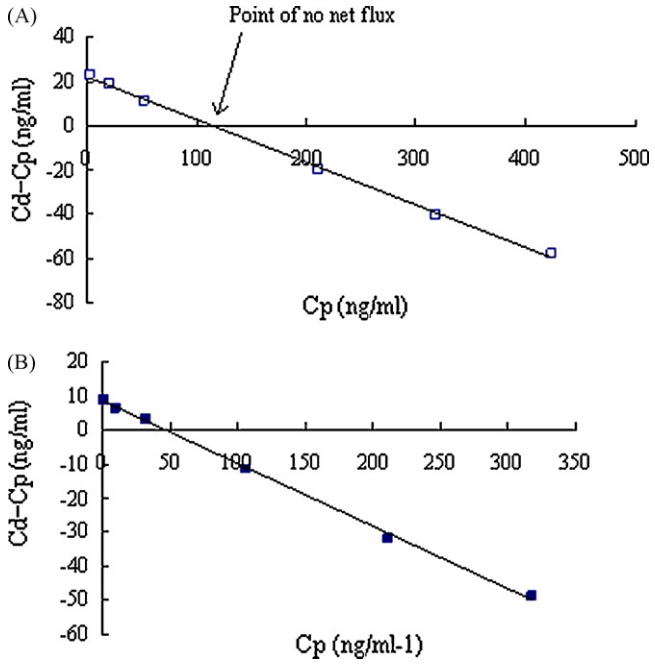

Fig. 2. *In vitro* no net flux determination of the recovery of baicalin from ACD (A) solution and aCSF (B). The slope of the regression line is an estimate of recovery.

($n = 5$) of perfusion solution. The concentration difference between the microdialysate samples and the perfusion solution ($C_d - C_p$) was plotted against the concentration in the perfusion solution (C_p). The recovery was determined from the slope of the linear regression ($R_{in\ vitro} = -\text{slope} \times 100$), while the abscissa intercept represented the concentration in the medium outside the probe. The point of NNF (C_{NNF}) represents a steady-state at which there is no net concentration change across the dialysis membrane, which means that the analyte concentration in the extracellular space of the surrounding tissue is supposed to be equal to the concentration in the perfused dialysate fluid. Therefore, the C_{NNF} value is determined as the C_p value where $C_p - C_d = 0$ and estimates the mean medium concentration of the compound, as shown in Fig. 2.

The *in vitro* recovery of blood dialysis was obtained in a similar manner to the determination of the *in vitro* recovery of brain dialysis. The concentration of baicalin in the vial of dialysis medium was 106 ng/ml, and the six perfusates contained different concentrations of baicalin (2.65, 21.2, 53, 212, 318 and 424 ng/ml).

2.4.2. In vivo recovery

The *in vitro* simulated experiment cannot actually represent the *in vivo* recovery, and *in vitro* microdialysis is simply to confirm that the diffusion of baicalin from one side of the membrane to the other side is equal. In order to calculate the exact baicalin concentration in the dialysate, a retrodialysis method was performed to determine the *in vivo* recovery of baicalin. Three blank rats were prepared and microdialysis probes were implanted as described above and blank aCSF was perfused through the probes at 3 μl/min and stabilized for 1 h. After that, the perfusate (C_p) was changed to three aCSF solutions containing baicalin 4.24, 84.8 and 424 ng/ml in turn and dialysates (C_d) were collected every 20 min on five occasions for each concentration. The *in vivo* recovery was calculated from the formula $R = (1 - C_d/C_p) \times 100\%$.

As in the determination of *in vivo* brain dialysis recovery, the blood microdialysis probe was inserted into the rat jugular vein under anesthesia with three baicalin concentration levels in the perfusate (i.e. 6.36, 127.2 and 636 ng/ml) for the calculation of the *in vivo* recovery of blood dialysis.

2.5. Stability of baicalin in the perfusate

The degradation of labile compounds in biological fluids can yield misleading results from *in vitro* studies. In order to obtain validated methods to stabilize baicalin in biological fluids, studies on baicalin stability and its stabilization were carried out *in vitro* in this study. The stability of baicalin in microdialysis samples collected from CSF and blood was assessed by determining the baicalin concentration after 4, 8, 24 and 48 h storage at 4 °C in a refrigerator and also at ambient temperature.

2.6. Ultra-performance liquid chromatography-tandem mass spectrometry

A Waters ACQUITY™ ultra-performance liquid chromatographic system (Waters Corp., Milford, MA, USA), equipped with a binary pump system coupled to an ACQUITY™ TQD triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) with an electrospray ionization source was used. Chromatographic separation was performed at 35 °C using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm; Waters Corp., Milford, USA). The following gradient was used: solvent A, acetonitrile; solvent B, 0.1% formic acid. The gradient elution was: 0 min, 10% A; 0–0.9 min, linear from 10% to 40% A; 0.9–1.4 min, linear from 40% to 60% A; 1.4–2.5 min, holding at 60% A for 1.1 min and then an immediate reduction to 10% A at 2.5 min; 2.5–3.2 min, initial conditions (i.e. 10% A) for equilibration of the column. The flow rate was kept constant at 0.2 ml/min during the analysis and the sample volume injected was 5 μl.

The mass spectrometer was optimized prior to the analysis by post-column infusion of 500 ng/ml of analytes with the flow rate set at 10 μl/min. Multiple reaction monitoring conditions for each compound were then developed. The multiple reaction monitoring (MRM) analyses were performed by passing molecular ions through the first quadrupole and collision dissociating the molecular ions in the second quadrupole (collision cell). A selected product ion, based on intensity and structural characteristics, was isolated by the third quadrupole and detected with the photomultiplier. The MRM transitions of m/z 447.1/271.0 for baicalin were monitored. The sensitivity was optimized for charged compounds by varying the cone voltage and collision energy. The parameters were: capillary voltage, 3 kV; cone voltage, 40 V; extractor, 3 V; RF lens voltage, 0.1 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow rate, 50 l/h; desolvation gas flow rate, 400 l/h; collision energy, 25 eV. The resolutions of the first and second quadrupoles were 14.5/14.5 and 14.0/14.0 (HM/LM), respectively. Dry nitrogen (≥99.5%) was used as the desolvation and nebulization gas, and argon (>99.999%) was used as the collision gas.

All data were acquired and processed using MassLynx v.4.1 software (Waters Corp., Milford, MA, USA). This approach provided a sensitive and selective analysis that is unique for individual analytes.

2.7. Quantification and method validation

Baicalin standard was dissolved in methanol at a concentration of 212 μg/ml as a stock solution. This stock solution was prepared weekly and the working solutions were diluted with aCSF or ACD to appropriate concentrations daily. The calibration graph was generated from the MRM of increasing amounts of baicalin standard solution. Calibration curves were constructed by the external standard method and were established based on the peak area (baicalin)

versus concentration by using weighted ($w = 1/c^2$) linear regression analysis. For quantification, the concentrations in test samples were obtained from this calibration line.

The accuracy and precision of the method were assessed by determining quality control (QC) samples using six replicate preparations of microdialysis samples at three concentration levels on three separate days. The specificity for baicalin was also evaluated in blank samples collected by microdialysis from CSF and blood in rats that did not receive baicalin.

In addition, the microdialysis samples in the present study were analyzed without prior sample purification except for high-speed refrigerated centrifugation at 20,000 rpm to avoid blocking because of the extremely fine channel of the UPLC.

2.8. Pharmacokinetics and data analysis

Baicalin microdialysate concentrations (C_d) were converted to unbound concentrations (C_u) as follows: $C_u = C_d/R_{in\ vivo}$. Pharmacokinetic calculations were performed on each individual set of animal data using the pharmacokinetic calculation software DAS (drug and statistics) version 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China) by the statistical moment method. The results are represented as the mean ± standard deviation of the mean and the main pharmacokinetic parameters of the baicalin are listed in Table 4.

3. Results and discussion

3.1. Microdialysis

During the *in vitro* experiments, the concentration difference of baicalin between microdialysate samples and perfusion solution ($C_d - C_p$) was plotted against the concentration in the perfusion solution (C_p), shown in Fig. 2. The linear regression functions were: $C_d - C_p = -0.1926C_p + 21.884$, $r = 0.9992$ and $C_d - C_p = -0.1838C_p + 8.5676$, $r = 0.9990$, therefore the *in vitro* recovery of baicalin was 19.26% in ACD solution and 18.38% in aCSF, determined from the slope of the linear regression, while the abscissa intercept concentration (baicalin in the medium outside the probe) was 113.6 and 46.61 ng/ml for ACD solution and aCSF, respectively, following *in vitro* microdialysis This was in agreement with the true concentrations of baicalin in the dialysis medium (106 ng/ml in ACD solution and 53 ng/ml in aCSF). This result indicated that the baicalin probe recovery (from the surrounding medium to perfusate) was similar to the probe delivery (from the perfusate to the surrounding medium), i.e. $(R/D)_{in\ vitro} = 1$, and consequently the determination of the *in vivo* recovery by retrodialysis was reasonable. In a separate study, the average *in vivo* recoveries of baicalin were 15.0 ± 1.26% and 17.52 ± 1.25% for blood and brain, respectively (Table 1). Hence, the actual concentration of baicalin in rat blood and CSF could be corrected by the average recovery of 15.0% and 17.52%, respectively. No difference in recovery values for the same region was observed after the addition of baicalin with different concentrations to the perfusate, indicating that the recoveries from the microdialysis probes in rat blood and brain are independent of the concentration for these experiments, and that retrodialysis can provide *in vivo* recovery values if the surrounding medium properties, perfusion flow rate and temperature are well controlled.

Because of the inverse relationship between the sampling frequency and the sample volume, microdialysis experiments usually produce a multitude of samples with very low volumes. Increasing the microdialysis flow rate will produce larger volumes, but the samples will be more dilute, and the experiments give an average concentration over the sampling interval, so that the higher the sampling frequency, the greater the temporal resolution of the experiment. After thorough consideration of the recovery and sample volume collected, we chose 3 μl/min as the perfusion rate, and 20 min as the sampling interval in the *in vivo* microdialysis experiment after drug administration.

Table 1
In vivo microdialysate recoveries of baicalin from rat blood and brain ($n = 5$)

Concentration (ng/ml)	Recovery (%)
In rat blood	
6.36	16.34 ± 2.64
127.2	13.85 ± 0.71
636.0	14.80 ± 0.49
Average	15.00 ± 1.26
In rat brain	
4.24	18.96 ± 1.93
84.80	16.73 ± 1.73
424.0	16.87 ± 3.07
Average	17.52 ± 1.25

3.2. Mass spectrometry and liquid chromatography

In this assay, an analyte can be identified by both its retention time and molecular weight. Also, triple quadrupole MS allows specific product ions to be monitored. The MRM scan function provides an additional dimension of structurally specific filtering for individual analytes. As a result, the signal-to-noise ratio of an total ion chromatographic (TIC) peak using the MRM scan mode is significantly higher than that obtained using the SIM (selected ion monitoring) scan mode. Overall, the liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques provide a direct, structurally specific measurement of individual components with high sensitivity. In addition, LC-MS/MS systems have minimal baseline drift and can be equilibrated very rapidly.

The positive ion scans of standard solutions of baicalin formed the protonated molecular ions $(M+H)^+$ at m/z 447.1. Low-energy collision tandem mass spectrometric analysis using a collision energies of 25 eV resulted in the formation of a high abundance of product ions for baicalin at m/z 271.0 which was formed by the loss of the glucuronic acid moiety from $[M+H]^+$ ion. Under the CID-MS/MS analysis used, the transition of 447.1 → 271.0 was selected for optimal monitoring of baicalin. The ion dissociation pathways and MS/MS spectra are presented in Fig. 3. A cone voltage of 40 V provided a sufficient response under the selected chromatographic conditions, and no further increase in the response was found when the cone voltage was increased further.

Because of the competition of electrospray ionization between the analytes of interest, LC separation before MS detection proved to be indispensable to prevent sensitivity loss. The mobile phase pH had a significant effect on the retention time of baicalin, with longer retention times being obtained at lower pH values due to the addition of 0.1% formic acid. However, the acid mobile phase is helpful for analysis in ESI positive mode of the compounds of interest. Indeed, the acid pH promotes the protonation of the analyte molecules. In this study, our mobile phase yielded retention times of only about 1.6 min which would allow a high sample throughput. Representative chromatograms of baicalin in dialysis are shown in Fig. 4. Isocratic and linear organic gradient elution were both tested, however, a step gradient of the organic content was found to produce the sharpest peaks with the greatest intensity. The use

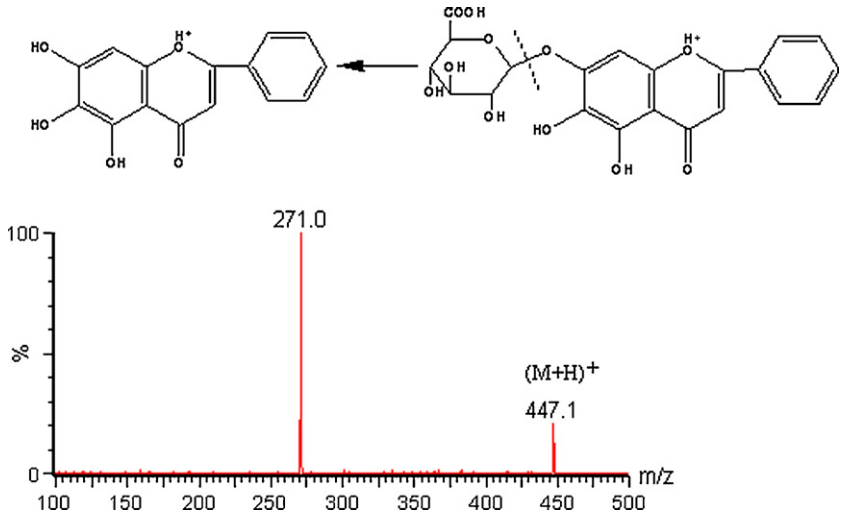

Fig. 3. MS/MS spectra and transitions for baicalin.

of small particles of stationary phase allowed UPLC to improve both peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities) without compromising resolution. Nevertheless, the column with sub-2 μm particles was more easily blocked and, therefore, gradient elution was also used to increase the column life [27].

3.3. Method validation

The lower limit of quantification (LLOQ) was 2.37 and 1.185 ng/ml, and the lower limit of detection (LLOD), based on a baicalin signal-to-noise ratio of 5, was 0.1 and 0.3 ng/ml in ACD solution and aCSF, respectively. Typical equations of the calibration curves were as follows—ACD solution: $y = 139.4x + 8.743$, $r = 0.9986$; aCSF: $y = 68.83x + 21.25$, $r = 0.9969$. Where y represents the baicalin peak area and x represents the concentration of baicalin. The feasibility of quantification of the low concentrations of baicalin in rat CSF and blood during this 7 h collection period after i.v. administration is due to the high sensitivity of the assay. Representative chromatograms of blank dialysate and rat dialysis samples are presented in Fig. 4.

As shown in Table 2, the overall mean precision, as defined by the R.S.D., ranged from 1.8% to 8.1%. Analytical accuracy, expressed as the RE, varied from −6.13% to 8.73%. Thus, the intra- and inter-assay

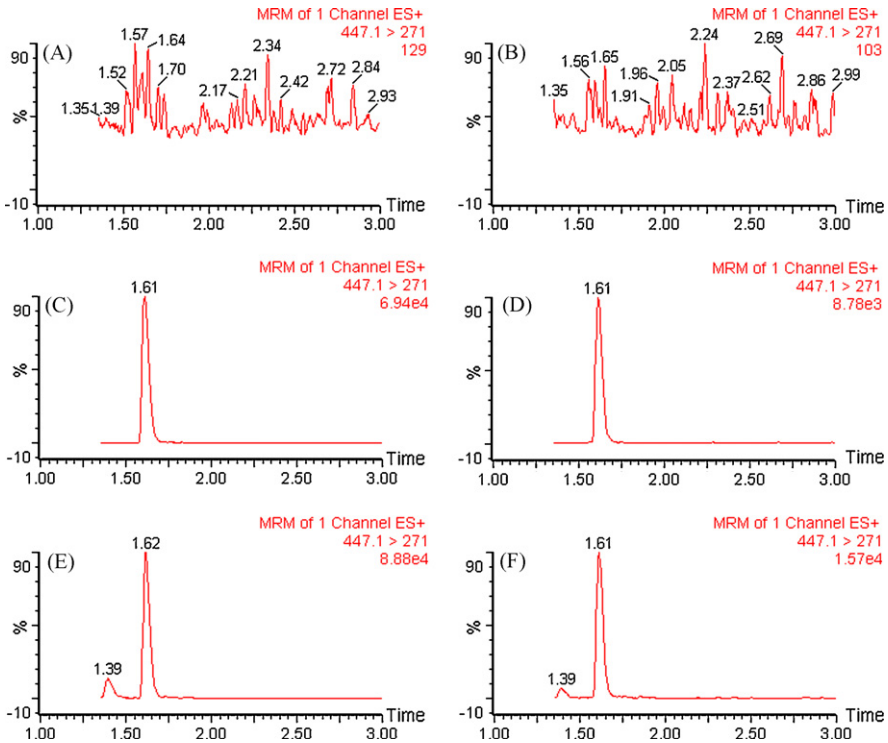

Fig. 4. Typical chromatograms of (A) blank blood dialysate from the microdialysis probe before drug administration; (B) blank brain dialysate from the microdialysis probe before drug administration; (C) ACD solution spiked with baicalin (21.2 ng/ml); (D) aCSF solution spiked with baicalin (6.36 ng/ml); (E) blood dialysate sample collected from rat blood 100 min post-baicalin administration (24 mg/ml, i.v.); (F) brain dialysate sample collected from rat CSF 100 min post-baicalin administration (24 mg/ml, i.v.).

Table 2
Precision and accuracy of the determination of baicalin in dialysis samples

	ACD solution			aCSF		
	1	2	3	1	2	3
Inter-day						
Added *C* (ng/ml)	4.24	50.88	424	4.24	21.2	318
Found *C* (ng/ml)	4.61	48.37	442	3.98	20.7	327
R.S.D. (%)	3.8	7.5	5.5	8.1	2.9	3.6
Accuracy RE (%)	8.73	−4.93	4.23	−6.13	−2.36	2.83
Intra-day						
Added *C* (ng/ml)	4.24	50.88	424	4.24	21.2	318
Found *C* (ng/ml)	4.49	49.23	415	4.56	23.0	340
R.S.D. (%)	4.4	6.8	3.1	2.8	3.0	1.8
Accuracy RE (%)	5.90	−3.24	−2.12	7.55	8.49	6.92

accuracy and precision were found to be acceptable for the analysis of dialysis samples in support of pharmacokinetic studies.

3.4. Matrix effect

A drawback of the use of ESI coupled with microdialysis is its sensitivity to matrix effects when the microdialysis samples are analyzed directly by UPLC-MS/MS without prior sample purification since the microdialysate contained a reasonable amount of non-volatile salts, which generate high background noise and suppress the ionization of analytes resulting in considerable reductions in sensitivity. Recently, some papers have described the use of water or water/ethanol as a perfusion medium instead of aCSF to reduce the salt content in the ion source and to avoid the need for sample clean-up [28]. Also, different groups have applied the column switching technique, which is an elegant solution for desalting dialysis samples [29].

In our study, to exclude interferences from the inorganic salts in the samples, firstly, a chromatographic separation was accomplished by using gradient elution and with a mobile phase containing less organic solvent during the initial part of the chromatographic run, i.e. 10% A (acetonitrile). This resulted in early elution of the inorganic salts and prolongation of the retention time of baicalin to about 1.6 min for desalting, although this retention time is already much shorter than that obtained by previously developed HPLC-UV and HPLC-MS/MS methods. Secondly, due to the 1.6 min retention time, the use of a divert valve that guides the UPLC eluent to waste during the first 1.35 min of a chromatographic run prevented salts from entering the ion source. This approach resulted in minimal ion suppression which is typically observed during electrospray ionization, and reduced potential ion source contamination. As good routine practice, our UPLC-MS/MS system was cleaned up weekly by pumping the H_2O/ACN solution with 0.1% formic acid for 30 min and this system clean-up procedure effectively reduced the chemical noise level in MS detection.

3.5. Baicalin stability in the dialysate

It was found that the degradation of baicalin was pH- and temperature-dependent and the oxidation–reduction reaction involving phenoxyl radicals is the major degradation pathway for baicalin in biological samples [30]. As shown in Table 3, either at 4 °C or at ambient temperature, the concentration of baicalin decreased dramatically over 8 h, especially in aCSF, probably because of the catalytic effect of high concentrations of metal ions in the perfusate. To avoid baicalin degradation, EDTA-Na_2 (0.04%, w/v) and L-cysteine (0.02%, w/v) were added to both the aCSF and ACD solutions. Then no significant degradation of analytes in ACD solution or aCSF was observed after 48 h at 4 °C in a refrigerator. Thus, modified aCSF and ACD solution were used as perfusates during the experiments, and the determination of baicalin in microdialysis samples could be accomplished in 48 h.

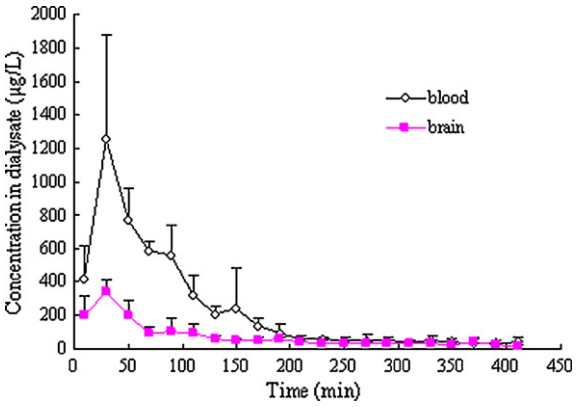

Fig. 5. Mean unbound concentration–time profiles of baicalin in rat blood and CSF after baicalin i.v. administration (24 mg/kg, i.v.).

3.6. Comparison between UPLC-MS/MS and HPLC-MS/MS

Ultra-performance liquid chromatography (UPLC) is a newly developed instrument offering a high power of separation and short analysis time. It involves available reverse phase chromatographic media with a 1.7 μm particle size along with a liquid system that can operate such columns at much higher pressures. In this work, the other previous assay study was improved and, in particular, a higher sensitivity and better chromatographic efficiency were obtained with the UPLC-MS/MS methods compared with the HPLC-MS/MS analysis. As a whole, the major advantages of such UPLC-based method include improved resolution within a shorter retention time, higher analytical sensitivity and more rapid equilibration. Consequently, this method was successfully applied to the baicalin quantification. Considering the need to extend the limits of quantification to lower and lower levels in shorter and shorter times, it is critical to conduct optimization investigations, and the performance of both chromatographic and mass spectral components of the LC–MS systems becomes more and more critical. The goal of increasing overall performance of these techniques can be further enhanced by reducing constraints imposed by the chromatographic separation [31]. It was found from the chromatograms that the hybrid particles used in UPLC columns often showed unique selectivity when compared with conventional HPLC packings [32].

Table 3
Stability of baicalin in dialysate at 50 ng/ml under different conditions

Conditions	Baicalin concentration (%) in ACD					Baicalin concentration (%) in aCSF				
	0 h	4 h	8 h	24 h	48 h	0 h	4 h	8 h	24 h	48 h
25 °C	100	95.25	93.34	87.75	85.27	100	67.26	30.37	18.44	6.32
4 °C in refrigerator	100	95.89	95.31	90.17	88.09	100	80.22	57.16	40.53	20.60
25 °C with 0.04% EDTA-Na_2 and 0.02% L-cysteine (w/v) added	100	98.02	102.35	97.26	98.98	100	101.38	99.35	96.36	96.12

Table 4
Pharmacokinetic parameters of free baicalin in rat blood and CSF after 24 mg/kg, i.v. administration ($n = 6$)

Parameters	In blood	In CSF
T_{max} (min)	30	30
C_{max} (μg/l)	1250.835 ± 624.001	344.235 ± 71.406
$t_{1/2}$ (min)	57.269 ± 30.631	39.032 ± 10.378
AUC_{0-t} (μg min/l)	138468.088 ± 51110.57	44959.629 ± 7960.363
$AUC_{0-\infty}$ (μg min/l)	140470.326 ± 51078.119	45933.796 ± 7986.546
$AUMC_{0-t}$	9446719.99 ± 1879092.213	3869561.119 ± 965933.327
$AUMC_{0-\infty}$	10421913.81 ± 2058557.344	4324597.93 ± 954498.403
MRT_{0-t} (min)	72.321 ± 16.546	85.456 ± 8.854
$MRT_{0-\infty}$ (min)	78.782 ± 18.769	93.735 ± 7.137
CL (l/(min kg))	0.188 ± 0.063	0.534 ± 0.103
V (l/kg)	16.981 ± 12.499	30.396 ± 10.685

3.7. Method application

The time–concentration curves of baicalin at dosages of 24 mg/kg in rat blood and brain are illustrated in Fig. 5 and the parameters obtained are shown in Table 4. The lag-time before peak time (T_{max}) might be due to the dead volume between the sampling site and the point of dialysate collection. Based on these data, it was concluded that baicalin can cross the BBB and distribute into the CSF quickly and reach its peak concentration of 344 μg/l about 30 min after the i.v. administration of 24 mg/kg. Such a conclusion supports the previous results of baicalin affecting cerebral function and also rouses interest in the further study of baicalin from *S. radix* with regard to its effects on learning and memory functions.

4. Conclusion

A serial sampling technique, microdialysis, and a rapid and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the determination of baicalin in rat brain and blood was developed. This method has the advantages of producing less tissue damage, a lower animal requirement, no biological fluid loss and no endogenous interference, combined with sufficient sensitivity for the measurement of unbound baicalin in the microdialysates. This model will be useful for performing pharmaceutical studies of baicalin at multiple sites in one animal. In addition, the UPLC-MS/MS was also used to identify the BBB penetration of baicalin and to help understand the factors that determine its distribution into brain after a single i.v. administration. The results obtained show that baicalin can cross the BBB and be detected in rat CSF.

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