Journal of Medicinal Plants Research Vol. 6(15), pp. 2977-2984, 23 April, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1366
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Rhynchophylline in rat blood by high-performance liquid chromatography-coupled microdialysis

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Accepted 22 March, 2012

Rhynchophylline (RHY) is a pharmacologically active substance isolated from *Uncaria rhynchophylla* which has been used to treat cardiovascular and central nervous system diseases. Microdialysis (MD) technique is a continuous, real-time sampling technique that is very suitable for the evaluation of drug disposition. The present study coupled an *in vivo* MD sampling method with reliable high performance liquid chromatography (HPLC) to determine free RHY in blood. MD probes were inserted into the jugular vein of rats, and blood dialysates were collected at 15-min time intervals for 8 h after intravenous administration of RHY (15 or 25 mg/kg). The method has been validated with good linearity, specificity, accuracy (RE 2.6 to 5.6%) and precision (98.96 to 100.57%) of MD probe recovery. RHY was chemically stable during storage and assay procedures. The pharmacokinetic parameters of unbound RHY were described as: The area under curve (119.96 ±19.29 min/μg/ml, 222.13 ± 27.60 min/μg/ml), T1/2 (46.17 ± 9.18 min, 47.97 ± 9.33 min), mean residence time (65.45 ± 9.15, 69.48 ± 9.45 min) and C_{max} (1.55 ± 0.36, 2.62 ± 0.29 μg/ml). All PK parameters showed positive dose-dependent correlation. In addition, RHY showed a high degree of drug–protein binding. MD sampling may be valuable for pharmacokinetic studies of medicinal plants.

Key words: Microdialysis sampling, rhynchophylline, pharmacokinetics, liquid chromatography, drug–protein binding rate.

INTRODUCTION

Rhynchophylline (RHY; Figure 1) is a major tetracyclic oxindole alkaloid isolated from the stems of *Uncaria* species such as *Uncaria rhynchophylla (MIQ)* Jackson and *Uncaria sinensis* (Oliv.) Havil. RHY is deemed to be a pharmacological active component of these botanicals (Qu et al., 2012). Rhynchophylla has long been used in traditional oriental medicine for its anti-convulsant, anti-pyretic, anti-hypertensive, anti-arrhythmic and anti-thrombotic properties (Zhou and Zhou, 2010; Graeme, 2010). Recent reports reveal that RHY mainly acts on cardiovascular and central nervous system diseases including hypertension, bradycardia, arrhythmia, sedation, vascular dementia, epileptic seizures, drug addiction, and cerebral ischemia (Shi et al., 2003; Zhang et al., 2004). The known pharmacodynamic mechanisms of effect are related to modulation of calcium and potassium ion channels, protection of neural and neuroglial cells, and regulation of central neurotransmitter transport and metabolism (Hsieh et al., 2009; Yuan et al., 2009). RHY is a potential drug candidate with broad therapeutic potential. Therefore, further studies are needed to understand its pharmacokinetic and pharmacodynamic properties.

Pharmacokinetic studies are vital, early phase in drug development. Currently, the U.S. Food and Drug Administration and the European Union's Committee support using clinical microdialysis to obtain pharmacokinetic information (Martin and Hartmut, 2006).

Some studies have examined the pharmacokinetics of

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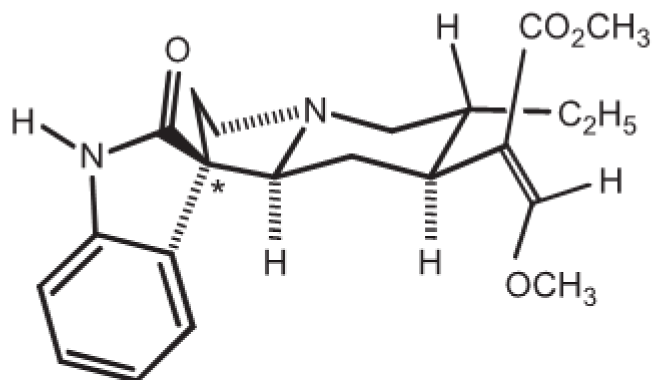

Figure 1. Stereochemical structure of rhynchophylline. The asterisk indicates the spiro C-7 position of a tetracyclic oxindole (Tai-Hyun et al., 2004).

Rhynchophylla alkaloids. It was previously reported (Wang et al., 2010b) that RHY can be detected in the brain 3 h after oral administration of 37.5 mg/kg and with maximum plasma concentration at 3 h (observed values). In addition, eight metabolites of RHY were observed in plasma. However, to our knowledge, no reports have utilized microdialysis approaches to reveal the pharmacokinetics of RHY.

Microdialysis (MD) is a technique that enables continuous monitoring of the free extracellular concentration of solutes in tissues (Elizabeth and William, 2009; Martin and Markus, 2002). Small-caliber probes with a semipermeable membrane are connected to inflow and outflow tubes and are perfused at a low flow rate with a liquid having osmotic properties similar to extracellular fluid. Small molecules are passively exchanged across the membrane between the perfusate and probe surroundings along the concentration gradient. Dialysate is collected from the outflow tube and the concentration of the solute in question is then determined (Olaf et al., 2010; Xiaoping and Julie, 2003). Microdialysis sampling has many merits compared to conventional pharmacokinetic blood sampling approaches (Chandra et al., 2007; Yang et al., 1997). Microdialysis limits blood loss that could change pharmacokinetics and pharmacodynamics. Also, microdialysis samples yield the free form of the drug from blood and tissue which allows proper determination of drug efficacy. Microdialysis samples are pure enough to inject directly into the detection equipment so that errors and losses from sample pretreatment are considerably reduced. In addition, the technique may be performed *in vivo*, is minimally invasive and reflects real-time values (Nele and Charlotte., 2005; Jan-Peer and Stephanie, 2005). These benefits make microdialysis an attractive tool for pharmacological research.

Using the MD method, the present study measured free RHY in rat blood after intravenous administration and determined drug-plasma protein binding. Our results were compared to those reported using a conventional PK method (Wang et al., 2010a). We also investigated MD probe recoveries *in vitro* and *in vivo*. Results obtained here support a sensitive, low-volume, rapid and reliable RHY detection method using microdialysis.

EXPERIMENT

Chemicals and reagents

RHY (purity ≥ 98%) was obtained from the Shanghai Winherb Medical S & T Development Co. Ltd. (Shanghai, China). Carbamazepine used as the internal standard (IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was obtained from Fisher Co. Ltd. (Emerson, IA, USA). Triethanolamine and glacial acetic acid were purchased from the Beijing Chemical Reagent Company (Beijing, China). Ringer's solution (147 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, and 1.3 mM $CaCl_2$) was filtered through a 0.22 μm nylon filter (Millipore, USA) prior to use. All carbamazepine and RHY solutions were prepared in Ringer's solution. To help dissolve RHY, 1% acetic acid was added to RHY and then the volume was diluted with saline to acetic acid < 0.1% Milli-Q (Milford, MA, USA) filtered water was used throughout the study for solution and buffer preparation.

HPLC system

The HPLC system consisted of a Waters 2695 pump/injector, a Waters 996 photodiode array detection detector, a reverse-phase C-18 column (250 × 4.6 mm, 5 μm; Merck KGaA, Darmstadt, Germany), and a guard column (Phenomenex, Torrance, CA). The mobile phase consisted of solvent A and solvent B (65:35, v/v), and set at a flow rate of 1 ml/min; solvent A was acetonitrile, while solvent B consisted of 0.15% triethylamine (pH 7). The column temperature was maintained at 25°C, and the sample temperature was maintained at 15°C. The same volume (30 μl) of 0.2 μg/ml carbamazepine solution was added to all the samples to yield

adequate volumes for analysis. RHY was determined by HPLC at 243 nm. A 10 μl aliquot of each sample was injected into the HPLC apparatus for analysis. All solvents for HPLC were filtered through 0.45 μm filter membranes before injection.

Microdialysis system

The blood microdialysis system consisted of a microinjection pump (CMA/400), a refrigerated fraction collector (CMA/470), a microdialysis probe (AB, Stockholm, Sweden), and a heating pad (CMA/150). A 10-mm length of microdialysis probe with a nominal molecular weight cut-off of 15 kDa was used for blood sampling. The flow rate of the microinjection pump was set at 2 μl/min throughout the micro dialysis experiment; this flow rate was investigated using *in vitro* methodology and was confirmed to be appropriate for the microdialysis experiments. Drug-free samples were collected into a refrigerated fraction collector after RHY administration.

Animal preparations

Animal studies were performed in accordance with the guidelines of the Animal Care and Use Committee of the Chinese Academy of Medical Sciences. Twelve male Sprague–Dawley rats (Vitalriver Experimental Animal Ltd., Beijing, China) were kept in a specific pathogen-free animal house for at least 7 days prior to the experiment until they weighed as 240 to 300 g. The rats were maintained at controlled temperature (22□C ± 2) and humidity (55% ± 5) under 12:12 h light–dark cycles and with free access to food and water. The rats were anesthetized with 1.25 mg/kg urethane (*i.p.*) before surgery and maintained under deep anesthesia throughout the study. The body temperature of the rats was maintained at 37°C throughout the experiment with a heating pad.

Method validation

The method validation assays were carried out according to the currently accepted U.S. Food and Drug Administration bioanalytical method validation protocols (Bioanalytical Method Validation, 2001). A stock solution of RHY (1 mg/ml in Ringer's stored at 4°C) was used to prepare working standard solutions (0.05, 0.1, 0.5, 1, 5, 10, and 50 μg /ml). Working standard solutions were prepared immediately before use. An internal standard (IS) method was used to establish the calibration curve of peak area ratio of RHY:IS against nominal RHY concentrations using linear regression analysis. The calibration curves were required to have a correlation value of at least 0.995 (Tsai et al., 2000). The lowest limit of detection (LOD) was estimated as the amount that could be detected with a signal-to-noise ratio of 3, whereas the lowest limit of quantitation (LOQ) was determined based on a signal-to-noise ratio of 10.

Sample stability

The stability of RHY in Ringer's solution was evaluated as affected by storage duration (1, 3 and 7 days) and storage temperature conditions (-20°C and room temperature) and freeze-thaw effects (three freeze–thaw cycles). All stability studies were conducted at three concentrations of RHY (0.5, 5 and 50 μg/ml) and measured in triplicate. Accuracy was calculated from the nominal concentration (C_0) and the mean value of the observed concentrations (C_S). Precision was determined according to the relative standard deviation (RSD) and was calculated from the mean value of the observed concentrations (C_S) and standard deviation (SD) (Huang et al., 2009). The formulas used are as follows:

$$\text{Accuracy (\%)} = [(C_S - C_0) / C_0] \times 100\% \quad (1)$$

$$\text{Precision (\%)} = (SD / C_S) \times 100\% \quad (2)$$

Recovery of microdialysis probe

The recovery of the microdialysis probe *in vitro* was determined using the incremental method in six replicates. The probe was immersed in Ringer's solution containing RHY (0.5, 5, and 50 μg/ml) (C_0) and then perfused with drug-free Ringer's solution (C_{in}) at a constant flow rate (2 μl/min). The dialysate was collected for analysis (C_{out}), and the *in vitro* relative recovery (RR) was estimated as follows:

$$\text{RR (\%)} = (C_{out} / C_0) \times 100\% \quad (3)$$

Microdialysate concentration (C_{out}) was converted to drug-free concentrations (C_u) as follows (Wu YT et al., 2010):

$$C_u = C_{out} / RR \quad (4)$$

The recovery of the microdialysis probe *in vivo* was determined using the retrodialysis method that estimates the loss of RHY via the probe (Lars et al., 1991). The probe was perfused with Ringer's solution containing RHY (2.5 μg/ml) (C_0), and the dialysate was collected (C_{out}) for analysis. The *in vivo* recovery of RHY was calculated using the following equation:

$$RR_{retro}\text{(\%)} = (C_0 - C_{out}) / C_0 \times 100\% \quad (5)$$

Determination of plasma RHY concentration

After each rat was anesthetized, its jugular vein was isolated by making an incision over the right shoulder of the rat and carefully pulling away the fat and the tissue. The microdialysis probe was inserted into the vein using a cannula. The cannula was then removed and the probe was maintained in the vein by suturing the surrounding muscle. After retrodialysis, the probe was perfused with drug-free Ringer's solution to equilibrate for 2 h. RHY (15 and 25 mg/kg) was then intravenously administered via the femoral vein in a normal saline solution. Blood dialysates (C_{out}) were collected by a fraction collector every 15 min (30 μl) for quantitative analysis, and the drug-free concentration *in vivo* was calculated using Equation 4.

Determination of protein-binding rate *in vitro*

Blood was collected from the anaesthetized rats in a heparinized tube and centrifuged at 3000 rpm for 10 min; the supernatant was used for analysis. The blood microdialysis probe was immersed in the plasma containing different concentrations of javascript:showjdsw('jd_t','j_')RHY (1, 10, or 100 μg/ml). The plasma was positioned at 37ºC and 200 rpm in a gas bath thermostatic oscillator, and the system was kept at 37ºC for one hour before sampling to determine the free concentration of RHY in plasma (Max and Petra, 2011). The protein-binding degree of the RHY in plasma was defined as follows:

$$\text{Binding degree (\%)} = (C_0 - C_u)/C_0 \times 100\% \quad (6)$$

C_0 is the nominal concentration of RHY in plasma, and C_u is the free RHY concentration calculated from the observed concentration (C_{out}) and RR using Equation 4.

Pharmacokinetics and statistics

Statistical analysis was performed using Phoenix WinNonlin 6.1

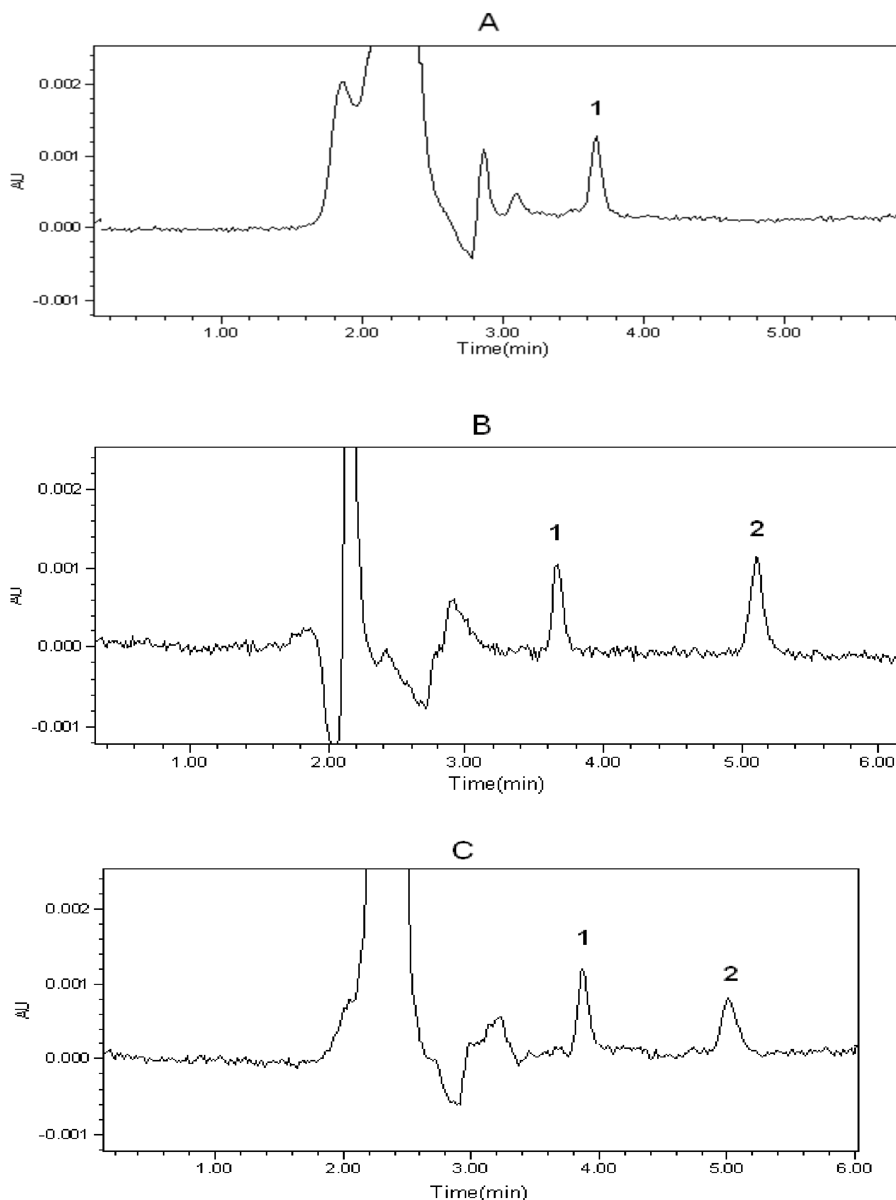

Figure 2. Chromatograms: (A) Representative chromatogram of the blank blood dialysate sample; (B) a standard RHY solution (0.5 μg/ml) spiked into blank blood dialysate; and (C) a blood dialysate sample containing RHY (0.29 μg/ml) collected 0 to 15 min after RHY administration (15 mg/kg i.v.). Peak 1: IS. Peak 2: RHY.

software. Pharmacokinetic parameters were determined by non-compartmental analysis using Kinetica version 4.4.1 (Thermo Fisher Scientific Inc., PA. USA). Statistical calculations were performed using SPSS software version 16.0 (SPSS GmBH, IL, USA).

RESULTS

Determination of RHY in microdialysates by HPLC

A typical chromatogram of RHY in rat blood dialysates is shown in Figure 2. Figure 2A shows the chromatogram of a blank blood dialysate, Figure 2B shows a standard RHY solution (0.5 μg/ml) spiked into blood dialysate, and Figure 2C shows a chromatogram of a blood dialysate sample containing RHY (0.29 μg/ml) collected 0 to 15 min after RHY administration (15 mg/kg i.v.). The retention time was approximately 5.1 min for RHY and 3.7 min for the IS. The RHY calibration curve was constructed over a concentration range of 0.05 to 50 μg/ml prior to analysis of microdialysis samples by HPLC-DAD. The calibration curve was determined as y = 3.1577x + 0.0573 and

Table 1. Stability of RHY at different time and temperature ($n = 3$).

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	RSD (%)	Accuracy (%)
1 day at room temperature			
0.5	0.54 ± 0.03	5.5	104.8
5	5.01 ± 0.07	1.39	100.56
50	50.81 ± 0.43	0.84	100.84
3 days at room temperature			
0.5	0.50 ± 0.03	6.49	101.08
5	4.92 ± 0.13	2.70	100.06
50	49.75 ± 0.43	0.87	100.06
7 days at room temperature			
0.5	0.50 ± 0.02	3.37	101.05
5	4.66 ± 0.23	4.89	96.71
50	50.35 ± 0.69	1.36	100.76
Three freeze–thaw cycles			
0.5	0.54 ± 0.02	3.30	102.07
5	5.14 ± 0.13	2.45	100.79
50	50.94 ± 0.38	0.72	100.53
-20 °C for 7 days			
0.5	0.55 ± 0.04	7.82	105.12
5	5.04 ± 0.09	1.74	99.91
50	50.58 ± 0.17	0.33	100.41

Table 2. Accuracy and precision (intra- and inter-day) for the recovery of RHY in Ringer's solution *in vitro* ($n = 6$).

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	RSD (%)	Accuracy (%)
Intra-day			
0.5	0.51 ± 0.02	4.1	99.44
5	5.05 ± 0.16	3.2	99.82
50	49.31 ± 1.30	2.6	100.25
Inter-day			
0.5	0.49 ± 0.03	5.6	98.96
5	5.05 ± 0.20	3.9	100.57
50	48.05 ± 2.09	4.3	99.35

revealed good linear correlation ($r^2 = 0.9997$). The LOQ for RHY was determined to be 50 ng/ml, and its LOD was calculated to be 10 ng/ml. Stability data are shown in Table 1. RHY dissolved in the blank blood dialysate (0.5, 5, and 50 μg/ml) was determined to be stable under different storage time, handling conditions, and storage conditions. The accuracy ranged from 96 to 104%, and the precision were all less than 10%. These findings demonstrated that RHY had good stability under the experimental conditions.

Recovery of microdialysis probe of RHY *in vitro* and *vivo*

The intra- and inter-day accuracy and precision of probe recovery are listed in Table 2. Determination of probe recovery *in vitro* was simulated by microdialysis *in vivo* via the incremental method, and the probe was immersed in Ringer's solution containing RHY at three concentrations (0.5, 5, and 50 μg/ml). Intra-day precision ranged from 2.6 to 4.1%, and accuracy varied from 99.44

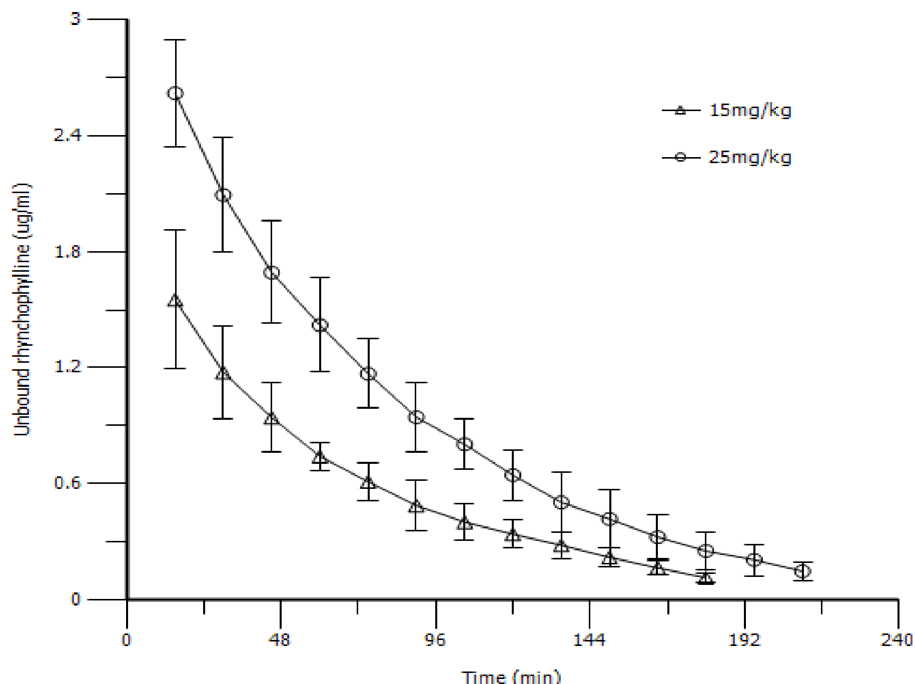

Figure 3. Mean plasma concentration–time profiles of six male rats after administration of RHY (15 or 25 mg/kg i.v.). Each point and bar represents mean ± SD (*n* = 6).

Table 3. Pharmacokinetic parameters of RHY in rats after administrations of 15 and 25 mg/kg i.v.

Parameter	Administration dose	
	15 mg/kg	25 mg/kg
C_{max} (µg/ml)	1.55 ± 0.36	2.62 ± 0.29
$t_{1/2}$ λz (min)	46.17 ± 9.18	47.97 ± 9.33
AUC (min µg/ml)	119.96 ± 19.29	222.13 ± 27.60
CL (ml/min/kg)	199.32 ± 27.36	108.92 ± 14.47
V_d (ml/kg)	13509.13 ± 3982.84	7480.58 ± 1499.96
MRT (min)	65.45 ± 9.15	69.48 ± 9.45

Data are presented as mean ± SD (n=6).

to 100.25%. The results of inter-day precision ranged from 3.9 to 5.6%, while those for accuracy ranged from 98.96 to 100.57%. All data were found to be acceptable for PK analysis. Our research on microdialysis recovery *in vitro* found differences among the probe recovery, so it is very important to test probe recovery *in vivo* before every rat experiment. Results showed that recovery was 22.43% (± 6.45).

Pharmacokinetic analysis of RHY in rat blood microdialysates

Our results showed the pharmacokinetics of free RHY could be determined using microdialysis technology in rat blood. The pharmacokinetic curve of unbound drug versus the time after RHY administration (15 and 25 mg/kg i.v.) is shown in Figure 3, and the pharmacokinetic parameters are listed in Table 3.

The plasma areas under the curve (AUC) of free RHY after 15 and 25 mg/kg i.v. administration were 119.96 (± 19.29) and 222.13 (± 27.60) min/µg/ml. The half-lives ($t_{1/2}$) were 46.17 (± 9.18) and 47.97 (± 9.33) min. The maximum concentrations (C_{max}) were 1.55 (± 0.36) and 2.62 (± 0.29) µg/ml, and the mean residence time（MRT）were 65.45 (± 9.15) and 69.48 (± 9.45) min, respectively. The results reveal good dose-dependent correlation. The clearance (CL) and apparent volume of

Table 4. Comparison the pharmacokinetic parameters of RHY using different sampling methods after administrations of 15 mg/kg i.v.

Parameter	Administration dose (15 mg/kg, i.v.)	
	MD sampling	Blood sampling
$t_{1/2}$ λz (min)	46.17 ± 9.18	81.33 ± 1.8
AUC (min μg/ml)	119.96 ± 19.29	17440 ± 270
CL (ml/min/kg)	199.32 ± 27.36	0.8597 ± 0.013
V_d (ml/kg)	13509.13 ± 3982.84	100.9 ± 0.69

Data are presented as mean ± SD.

distribution (V_d) decreased with increasing RHY concentration. Unbound RHY after administration of the lower dose of RHY (15 mg/kg) was eliminated markedly faster than the higher dose of RHY (25 mg/kg).

Protein-binding rate of RHY *in vitro*

The microdialysis sampling technique described here is a good approach to study drug–plasma protein binding. Using dialysis membranes, small molecules in the sample such as drugs, are transported to collection vials for analysis, and large molecules like plasma proteins and protein-bound drugs can be excluded. The plasma protein binding of RHY at 10 and 100 μg/ml was determined to be 88.62% (± 0.40) and 89.63% (± 0.50), and the 1 μg/ml RHY dose was not detected. These results reveal that plasma RHY is largely protein-bound (Tsai, 2003).

DISCUSSION

Microdialysis is a dynamic sampling technique that is used for continuous measurement of free analyte concentrations in the extracellular fluids of tissues and blood (Pradyot and Susan, 2009; David et al., 2000). Analytes may include endogenous molecules (e.g., neurotransmitters, hormones, glucose, etc.) or exogenous compounds (e.g. pharmaceuticals) to determine their distribution or pharmacokinetics *in vivo*. Microdialysis can be used to collect material from tissues, which is significant for revealing the target organs of a given drug. The implantation of microdialysis probes in rat blood vessels can cause partial obstruction. However, this physiological disturbance is limited compared to traditional PK sampling methods that cause a relatively large loss of blood in rats during an extended PK study. In addition, microdialysis makes it possible to measure unbound drug concentrations in blood and tissue, thus providing true values for drug efficacy. Therefore, the microdialysis technique presents an important link between pharmacokinetic and pharmacodynamic studies.

Experimental conditions, mainly concerning probe recovery, appear to have important effects on the outcomes of *in vivo* microdialysis experiments. Presently, the recovery of free RHY between obverse and reverse dialyses was consistent *in vivo*; this is important evidence to support the validity of the PK calculations *in vivo* (Chandra et al., 2007). Probe recovery was stable for over 8 h. A difference was measured by retrodialysis of the recovery of the probe implanted into rat jugular veins; this difference may be attributed to the nature of the probe and the internal environment. Therefore, the probe recovery for drug concentration calculations must be determined prior to each test.

This experiment was the first time to study RHY pharmacokinetics using microdialysis technology. A previous pharmacokinetic study of RHY in rats used a standard PK blood sampling method (Wang et al., 2010a), but blood loss from this method could affect RHY pharmacokinetics and pharmacodynamics *in vivo*. The microdialysis sampling method aims to overcome this shortcoming. Comparison of the two methods produced noticeable differences (Table 4). For example, after intravenous administration of the same dose of RHY (15 mg/kg), the $t_{1/2}$ values calculated using the microdialysis and traditional methods were 46.17 and 81.33 min, and the CL values were 199.32 and 85.97 ml/kg/min, respectively. This difference was mainly caused by plasma protein binding because only unbound drug is determined by the microdialysis method, whereas the standard PK method determines both free and bound drug concentrations. Free drug concentration is perhaps more important since it correlates better with pharmacological and toxicological effects.

Knowledge of active principle–protein interactions contributes to insights into a drug's pharmacokinetic and pharmacodynamic properties. Only the free-form drug can diffuse through cell membrane and exert biological activity (Max and Petra, 2011). Conventional PK sampling obtains both unbound and protein-bound drugs that are loosely bound to plasma proteins and form an equilibrium between bound and unbound drugs. When the free drug is absorbed by cells or removed from circulation, some bound drug can be separated from binding sites to compensate for the loss of free drug. Plasma proteins that combine with drugs mainly include albumin, α1-acid

glycoproteins, and lipoproteins. Of these, plasma albumin is prominent and always combines with organic acidic drugs, while α1-acid glycoprotein always combines with basic drugs. Presently, RHY, a basic alkali substance, was injected as a hydrochloride salt, and binding to α1-acid glycoprotein may be the main major drug-protein complex. Using a microdialysis technique, the plasma protein binding ratio of RHY was found to be 88.62% (± 0.40) and 89.63% (± 0.50) from the concentrations of 10 and 100 µg/ml, respectively, indicating a high level of plasma protein binding that was unaffected by RHY concentration.

In conclusion, the pharmacokinetics of RHY in rat blood was successfully studied using microdialysis technology. This method was verified to be stable and reliable and could be used for further pharmacokinetic–pharmacodynamic studies of natural medicinal products and phytochemicals.

ACKNOWLEDGEMENTS

Funding for this study was provided in part by research grants from the Key Projects in the National Science and Technology Pillar Program (2008BAI51B02), Major Scientific and Technological Special Project for "Significant New Drugs Formulation" (2009ZX09301-003, 2009ZX09502-015), Doctoral Program of the Higher Education Research Fund (200800231106), and the Fundamental and Public Service Special of Central Research Institutes, Institute of Medicinal Plant Development of Chinese Academy of Medical Sciences (yz-08-01), China.

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