Effect of glycyrrhizic acid on rhein renal penetration: a microdialysis study in rats

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Abstract
1. Rhein (RH), a primary active component isolated from rhubarb, is effective in protecting against the progression of diabetic nephropathy (DN) progression. Glycyrrhizic acid (GA), an active constituent of liquorice, is also considered to be a protective agent against DN. Here, we evaluated the effect of GA on the renal penetration of RH in rats.
2. Plasma and renal pharmacokinetics were profiled to estimate kidney penetration. After rats were anesthetized, the carotid artery was used for blood collection and a microdialysis probe was inserted into the kidney cortex to collect dialysate samples.
3. When co-administered with GA, the \( V_{ss} \) and CL values of RH in plasma increased by 25% and 34%, respectively. The \( C_{\text{max}} \) in kidney dialysates significantly increased 1.3-fold (\( p < 0.05 \)). There was no change in AUC0–\( \infty \) in kidney dialysates, but a significant decrease (2-3 fold) in the plasma was observed. The AUC0–\( \infty \) kidney/AUC0–\( \infty \) plasma ratio of RH, representing kidney penetration, increased by 1.4-fold in the group pre-treated with GA compared to the RH alone group.
4. These results demonstrate that GA increases the renal penetration of RH efficiently and may exert a synergistic effect, although the molecular mechanism of interaction requires further investigation.

Introduction
Diabetic nephropathy (DN) is a significant cause of chronic kidney disease and end-stage renal failure in many countries (Lim, 2014). Commonly used pharmacological interventions include ACE inhibitors, renin inhibitors, allopurinol and statins; however, the active components of Chinese herbs have also recently garnered attention in the development of safe and efficient drugs (Wu et al., 2014). Rhein (RH, 1,8-dihydroxy-3-carboxy-anthraquinone), is a biologically active component of rhubarb (Rheum officinale Baill.), a medicinal herb used in traditional Chinese medicine (TCM). RH has been reported to possess broad pharmacological activity including anti-tumor (Lin et al., 2009), anti-inflammatory (Gao et al., 2014) and anti-bacterial (Azelmatis et al., 2015) properties. Noticeably, RH is very effective in relieving renal lesions in DN (Gao et al., 2010) due to its anti-inflammatory properties. Considering its ability to affect multiple molecular targets (Zeng et al., 2014), RH is seen as a promising new drug for protecting against DN progression.

Combination therapies that provide synergistic and attenuation effects have recently been shown to be more effective than mono-therapies in the treatment of DN (Hofni et al., 2014). Moreover, through the use of compound prescriptions, TCM utilizes secondary “enhancing” or “messenger” drugs that are considered to increase the penetrative effect of the primary drug to its target site, which increases therapeutic efficacy.

Glycyrrhizic acid (GA) is a glycoconjugated triterpene saponin derived from liquorice (Glycyrrhiza uralensis Fisch. [Chinese liquorice]). In TCM, liquorice is one of the most frequently used “messenger” drugs used to direct the primary agent to its targeted organ. It has numerous pharmacological properties, such as anti-inflammatory, anti-viral and hepatoprotective activities (Asl & Hosseinzadeh, 2008). Recently, an increasing number of studies have demonstrated that GA is protective effect against kidney injury and is a potential protective agent against DN (Hou et al., 2014b; Lau et al., 2014; Wang et al., 2014). Therefore, concomitant administration of GA and RH may serve to enhance the renal penetration of RH by contributing to the messenger function of GA, and exert synergistic effects of the two drugs.
This, in turn, may result in an efficient strategy for the treatment of DN.

For pharmacokinetic studies measuring unbound drug concentrations at the pharmacological target site is desirable (Bundgaard et al., 2007). Microdialysis (MD), a semi-invasive sampling technique based on the principles of dialysis (Elmquist & Sawchuk, 1997), is an important technique widely used to measure the tissue levels of unbound drugs. The technique enables direct measurement of unbound drug at the biophase, continuous sampling without net fluid loss and monitoring of local drugs and metabolite concentrations at specific sites (de LT Vieira et al., 2010). In addition, MD can be used to sample multiple sites within the same animal; reducing the number of animals required and improving the quality of the data by minimizing inter-animal variation. Several studies have proven that MD is a powerful tool for assessing drug renal penetration (Araujo et al., 2008; Azeredo et al., 2012; de Araujo et al., 2009).

The aims of the present study were to evaluate unbound levels of RH in kidney and to investigate the effect of GA on RH renal penetration employing an innovative in vivo microdialysis technique.

**Materials and methods**

**Materials**

RH (1,8-dihydroxy-3-carboxy-anthraquinone) (purity >99%) was obtained from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). The authentic standard RH and 1,8-dihydroxyanthraquinone (internal standard [IS]) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). GA was purchased from Tianjin HEOWNS Biotech Co., Ltd. (Tianjin, China). Ultrapure water was obtained from a Milli-Q system (EMD Millipore, Bedford, MA). All other chemicals were of analytical grade.

**Experimental animals**

Adult male Sprague-Dawley (SD) rats, weighing 220 ± 20 g, were obtained from Shanghai Super-B&K Laboratory Animal Corp. Ltd. (Shanghai, China) and were fed a standard diet and provided free access to water. Animals were housed in a room with a 12 h light–dark cycle for at least one week before the study. All rat experiments were performed according to the guidelines for the care and use of animals as established by Zhejiang Chinese Medical University.

**Microdialysis system**

The microdialysis system consisted of a CMA 400 syringe pump (CMA, Stockholm, Sweden), a CMA 470 refrigerated fraction collector and 1 mL gas-tight syringes, which were used to deliver the perfusion fluid through the microdialysis probes. CMA 30 microdialysis probes (membrane length 10 mm, cut-off 6 kDa; CMA/Microdialysis AB, Stockholm, Sweden) were used.

**Experimental design**

Ten rats were randomly distributed into two groups of five. One group received 3 mg/kg body weight of RH intravenously (IV) (RH group). The other group received 3 mg/kg of RH immediately followed by GA (10 mg/kg) by the same route (RH-GA group). The RH and GA solution for IV administration were prepared in water using glycerol as a co-solvent (80:20, v/v), respectively.

**Pharmacokinetic study**

On the day of the experiment, animals were anesthetized with urethane (1.25 g/kg intraperitoneal). Blood was collected through the carotid artery via an indwelling catheter irrigated with heparinized saline (25 IU/mL). The animals were placed in the lateral decubitus position prior surgical removal of the skin and muscle. The kidney was then exposed and a microdialysis probe was inserted into the cortex with the aid of a needle, which was removed after insertion. The probe was perfused with 20% ethanol-Ringer’s solution at a flow rate of 1.0 μL/min and allowed to equilibrate inside the kidney for 1 h before drug administration. Dialysate samples were collected every 30 min for up to 6 h after dosing. Blood samples (~200 μL) were collected at 0.08, 0.15, 0.25, 0.5, 0.75, 1, 2, 3, 4 and 6 h after drug administration in tubes containing heparin, which were immediately centrifuged to separate the plasma (6800 g for 15 min). Plasma and dialysate samples were stored in polypropylene tubes at −80°C until analysis.

**Probe recovery in vivo**

The in vitro recoveries of probes were determined prior to the in vivo calibration by dialysis and retrodialysis method, respectively. Details of validation procedure are given in Supplementary data. After completing the pharmacokinetic experiment on each rat, the in vivo recovery of RH was assessed for each microdialysis probe following a 2-h washout period (Hou et al., 2014a; Hurtado et al., 2014). Three different RH concentrations in the range of 0.525 × 10⁻³–1.05 × 10⁻¹ μg/mL in the perfusion solution were investigated at a flow rate of 1.0 μL/min. Four dialysate samples for each concentration of RH were collected at 30 min intervals in order to determine relative drug loss. The in vivo recovery is described by the equation (Plock & Kloft, 2005):

\[
\text{Recovery (\%)} = \frac{C_{\text{perf}} - C_{\text{dial}}}{C_{\text{perf}}} \times 100,
\]

where \(C_{\text{perf}}\) is the drug concentration in the perfusate solution and \(C_{\text{dial}}\) is the drug concentration in the dialysate sample.

RH average renal recovery in vivo was used to recalculate the true unbound renal levels of the drug in all experiments. RH binding to the outlet tubing was considered negligible; therefore no correction was needed.

**Protein binding**

In order to determine the unbound fraction of RH in plasma samples, an ultrafiltration (UF) method (Howard et al., 2010) was used to separate the unbound fraction from the total plasma concentration. Different RH concentrations (0.5, 5, 15, 20 and 40 μg/mL) were investigated based on the total plasma concentrations observed in the animals after drug administration. Plasma samples spiked with RH were
incubated at 37 ± 1°C for 1 h and aliquots were separated for the determination of total plasma concentration. Aliquots of 1000 μL were placed into the upper part of the Centrifree® Ultrafiltration Devices (49.9 mm, 10 kDa cut-off) (EMD Millipore) and centrifuged at 6600 g for 30 min at 25 ± 1°C. Ultrafiltrates were collected and RH concentration was determined in each sample by HPLC as described in the next section. Duplicates were analyzed for each concentration. The unbound fraction of RH was determined as the ratio between ultrafiltrate and total plasma concentrations. RH binding to the UF device was determined to be in the same range of concentrations using drug solutions.

**Determination of RH in plasma and microdialysate samples**

Prior to analysis, frozen plasma samples were thawed and then transferred to an eppendorf (EP) tube containing 10 μL of IS solution (100 μg/mL) and vortexed for 30 s. Then, 20 μL of 2 mol/L hydrochloric acid was added to the EP tube containing the sample, followed by 0.5 mL of ethyl acetate. The samples were vortexed for an additional 3 min then centrifuged at 12 000 g for 5 min. The supernatant was transferred to a fresh EP tube and dried under vacuum at 50°C. The residue was reconstituted in 100 μL of methanol. Following a second centrifugation (12 000 g for 5 min), 20 μL aliquots of the reconstituted supernatant were injected onto the Waters 2695 Alliance HPLC system (Waters, Milford, MA). Analytes were separated on an Agilent Eclipse XDB-C18 column (150 mm × 4.6 mm internal diameter) protected by a guard column packed with the same material. The mobile phase (methanol–0.1% phosphoric acid mixture [73:27, v/v]) flow rate was 1.0 mL/min. UV absorbance was measured at 254 nm for RH and IS, and column temperature was 30°C. The method was sensitive and accurate within a concentration range of 0.1–50 μg/mL.

The microdialysate samples were analyzed for the concentration of RH by HPLC–MS/MS. The sample was injected directly onto the system without processing and no IS was used. The equipment consisted of a Paradigm MS4B Series HPLC (Michrom Bioresources, Auburn, CA), HTC PAL auto-sampler (CTC Analytics, Zwingen, Switzerland), and an AB SCIEX 4000 QTRAP® equipped with an electrospray ionization (ESI) source (AB SCIEX, Framingham, MA). Chromatographic separation was completed using a CAPCELL PAK C18 MG II column (100 mm × 2.0 mm, 3 μm) (Shiseido, Tokyo, Japan) at 25°C.

A gradient of 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B) was used as follows: 40–60% A from 0 to 10 min, 60–100% A from 10 to 11 min, 100% A from 11 to 15 min, 100–40% A from 15 to 15.1 min and 40% A from 15.1 to 20 min. A 10-μL aliquot of each sample was injected onto the column with a flow rate of 0.2 mL/min. The mass spectrometer parameters were optimized and set as follows: negative ESI with a TurbolonSpray voltage of 4500 V, temperature of 500°C, curtain gas of 55 psi, nebulizer gas of 50 psi, declustering potential of 73 V, collision cell exit potential of 12 V and collision energy of 39 V. Quantification of RH was performed using multiple reaction monitoring with precursor → product fragment transitions of 283.1 → 238.9 m/z. The method was validated according to the Food and Drug Administration guidelines for bioanalytical method validation (Food and Drug Administration, 2013).

In the present study, post extraction spike method (Matuszewski et al., 2003) was applied to determine the matrix effects, which were expressed as the ratio of the mean peak area of analytes spiked in blank microdialysates to that of neat standards at different concentrations (2.1, 21 and 105 ng/mL, respectively, n = 5). Average matrix effect values obtained were 103.02 ± 3.24%, 101.05 ± 1.34% and 98.03 ± 4.32% for RH (2.1, 21 and 105 ng/mL, respectively). Therefore, ion suppression or enhancement from matrix was negligible for the present method. A linear calibration curve using drug peak area was obtained in the range of 0.525–105 ng/mL. Concentrations of 2.1, 21 and 105 ng/mL of dialysate samples represented low, medium and high quality control samples, respectively. The intra- and inter-assay precision values (RSD) for the low, medium and high quality control dialysate samples (2.1, 21 and 105 ng/mL, respectively) were 4.18, 3.91, 4.28, and 5.45, 2.87, 1.41, respectively. The lower limit of quantification for dialysates was 0.525 ng/mL.

**Pharmacokinetic evaluation and statistical analysis**

Pharmacokinetic parameters were determined by Phoenix WinNonlin 6.3 software (Pharsight Inc., Apex, NC) with non-compartmental analysis. The plasma and tissue concentrations versus time profiles were constructed for each individual animal. The area under the plasma (AUC0–∞plasma) and unbound kidney (AUC0–∞kidney) concentration versus time curves were determined by the trapezoidal method. The ratio AUC0–∞kidney/AUC0–∞plasma was calculated as a measure of drug penetration ratio into the kidney. The maximum observed concentration (Cmax) and times to peak concentrations (tmax) were obtained directly from the plot of concentration versus time data.

A student’s t-test analysis was performed to compare results between the two groups in terms of RH and RH-GA using Prism software (Prism 5; GraphPad, La Jolla, CA). A p-value less than 0.05 was the cut-off value for determining statistical significance between data means. All data are expressed as the mean ± standard deviation (SD).

**Results**

The average in vivo recovery was estimated to be 32.0 ± 3.59% as determined by retrodialysis. This was found to be consistent throughout the experiments and between different probes. Therefore, this approach reduces the variability effects between different animals and microdialysis probes.

After taking into consideration RH binding to the UF device (7.64 ± 2.13%), RH protein binding was determined to be 90.03 ± 1.25% over the plasma concentration range observed in the animals (Table 1). This value was constant and concentration-independent, and agrees with previously published data reporting a protein binding of 93.46% in rats (Liu et al., 2013). Subsequently, unbound plasma levels were predicted based on this average value and determination of total concentrations.
Table 1. Protein binding of RH with rats plasma (n = 3).

| Total concentrations (µg/mL) | Protein binding (%) | Mean ± SD (%) |
|-----------------------------|---------------------|---------------|
| 0.5                         | 91.75 ± 1.97        | 90.03 ± 1.25  |
| 5                           | 89.78 ± 0.43        |               |
| 15                          | 90.59 ± 0.08        |               |
| 20                          | 88.35 ± 0.57        |               |
| 40                          | 89.68 ± 1.11        |               |

Figure 1. Mean RH total plasma concentration versus time profiles in rats after single administration of RH (3 mg/kg) and co-administration of RH and GA (10 mg/kg) (n = 5). Data are expressed as mean ± SD.

Figure 2. Mean RH free kidney concentration versus time profiles in rats after single administration of RH (3 mg/kg) and co-administration of RH and GA (10 mg/kg) (n = 5). Data are expressed as mean ± SD.

RH total plasma and unbound kidney concentrations versus time profiles after administration of RH and RH-GA are shown in Figures 1 and 2. The pharmacokinetic parameters are presented in Table 2.

After 10 mg/kg of GA was co-administered, there were no differences in terms of MRT and $T_{1/2}$ both in plasma and kidney dialysates. The $V_{ss}$ and CL values of RH in plasma increased by 25% and 34%, respectively, with no change of those parameters in kidney. However, the $C_{\text{max}}$ in kidney dialysates significantly increased 1.3-fold, from 0.13 to 0.17 µg/mL when RH was co-administered with GA.

There were no statistically significant differences between RH and RH-GA groups in terms of $t_{\text{max}}$ in the kidney. As can be seen from the concentration versus time profiles, the time to peak concentration ($t_{\text{max}}$) values were 1 h in kidney for both the RH group and RH-GA group, demonstrating that rapid penetration of RH into the rat kidney.

There was a statistically significant decrease in the mean AUC$_{0–360}$ and AUC$_{0–\infty}$ in plasma (decreased from 97.58 to 63.21 µg/mL min, from 102.16 to 65.11 µg/mL min, respectively) following co-administration of GA. In contrast, there were no differences in AUC$_{0–360}$ and AUC$_{0–\infty}$ in kidney dialysates between the RH and RH-GA group. The AUC$_{0–\infty,kidney}$/AUC$_{0–\infty,\text{plasma,unbound}}$ ratio gives an indication of kidney penetration ratio and helps explain any observed differences in renal dialysate concentrations. There was a statistically significant increase observed between the AUC$_{0–\infty,kidney}$/AUC$_{0–\infty,\text{plasma,unbound}}$ ratios of the RH group compared to the RH-GA group, which increased from 0.176 to 0.245 (a 1.4-fold increase) ($p < 0.05$, Figure 3).

Discussion

In the present study, the influence of GA on RH renal penetration was investigated to help determine underlying mechanisms regarding the increase in efficacy observed following co-administration (Feng et al., 2015; Sheng et al., 1994). Because accumulation of agents in the interstitial fluid is responsible for their pharmacological responses, a microdialysis technique was used to evaluate the renal penetration of RH. Microdialysis is a very useful technique for determining tissue concentrations of drugs or endogenous compounds in vivo. Recently, Li et al. (2014) applied the microdialysis technique to evaluate the liver distribution of unbound curdione and curcumol in rats. Other studies utilizing microdialysis for pharmacokinetic studies include an investigation of the extracellular concentrations of fosfomycin in lung tissue of septic patients using microdialysis technique (Matzi et al., 2010), the penetration of gatifloxacin into skeletal muscle and lung in rats by microdialysis (Tasso et al., 2008), and the effect of borneol on the brain transportation of kaempferol in rats (Zhang et al., 2015). Similarly, microdialysis has been used to evaluate the penetration of moxifloxacin into rat prostate tissue (Hurtado et al., 2014) and the penetration of levofloxacin through the blood pancreas barrier (Liu et al., 2014). To date there have been few renal penetration studies examining antibacterial agents (de Araújo et al., 2013), anti-fungals (Araujo et al., 2008; Azeredo et al., 2012; de Araujo et al., 2009) or the anti-fibrotic drug, pirfenidone (Togami et al., 2015). Furthermore, to our knowledge, this is the first study to investigate the unbound renal level of an anti-inflammatory agent for the treatment of kidney disease using microdialysis technique. Specifically, microdialysis methods were utilized to examine the renal penetration of RH, a potential protective agent against DN, and the effect of GA on its renal penetration.

The ability of drugs to penetrate tissues is dependent on various factors, including: protein binding, lipophilicity and the physiological mechanisms of drug distribution. High plasma protein binding with test compounds makes it difficult to accurately measure RH renal penetration. In addition,
properties of high lipophilicity and poor water solubility can increase the loss of the compound within the microdialysis probe itself. Several proposed strategies for improving recovery of lipophilic compounds include the addition of binding agents like albumin (Kirbs & Kloft, 2014) or hydroxypropyl-β-cyclodextrin (May et al., 2013) to the perfusate. Additional strategies include the use of isotonic lipoemulsions (Ward et al., 2003) and optimization of the perfusate pH (Tre et al., 2012). In the present study, a 20% ethanol-Ringer’s was used as the perfusate solution, thus improving the microdialysis RH recovery (data not shown). The dialysate sample could be injected directly onto the HPLC–MS, eliminating the need for an additional cleaning step and further improving recovery.

After co-administration of GA with RH, the CL of rat plasma increased by 34% and the AUC decreased by 35%. The results of these pharmacokinetic studies indicate that GA can accelerates the metabolic elimination of RH, which may be attribute to the induction of hepatic CYP3A activity by GA (Tu et al., 2010). The level of RH within the kidney reached a plateau within 60 min whether administered alone or with GA and then displayed a steady decline over the next 300 min. Moreover, the drug concentration in renal tissue is lower than that of plasma. Considering the concentration-independent protein binding property of RH, these results suggest that unbound RH concentrations in renal tissue and plasma do not correlate; therefore, unbound plasma levels of RH are not predictive of drug concentrations in the kidney.

The results of these experiments demonstrate a rapid distribution of RH into the extracellular fluid of rat kidney. This suggests that RH has a relatively high affinity for the kidney, facilitating its efficacy. The AUC_{tissue/plasma} concentration ratio, a recognized standard for evaluating drug penetration ratio, characterizes drug distribution into the tissue interstitium. The AUC_{kidney/plasma,unbound} concentration ratio was found to be 0.176 and 0.245 for the RH and RH-GA groups, respectively. The significant increase after co-administration of GA demonstrates that GA increases RH renal penetration ratio. Co-administration of GA with RH could therefore provide enhanced therapeutic effects of RH. Further studies should be conducted to determine the molecular mechanism of action.

Conclusions
In conclusion, determination of the simultaneous plasma and renal pharmacokinetic parameters facilitated estimation of RH kidney penetration. Considering the lower concentrations and small sample volume (20–30 μL) of renal dialysates, a highly sensitive and selective HPLC–MS/MS method was used to quantify RH in those samples. Our pilot study indicates that RH in kidney can be evaluated by microdialysis, despite its high lipophilicity. The RH renal penetration ratio increased after the drug was co-administered with GA, suggesting that GA is capable of enhancing RH access to the target site by synergistically increasing therapeutic efficacy. The approach described herein might be useful for investigating renal penetration by other drugs used to treat DN disease. Studies are underway to better understand the molecular mechanisms of the GA associated increase in RH renal penetration and the synergistic effect of RH and GA co-administration.

Declaration of interest
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Supplementary material available online

Supplementary data, Table S1, Figure S1