Introduction

Back pain is an important issue in humans, and 70 - 85% of people have back pain at some point in their lives.1 Although multiple factors are involved, the degeneration of the intervertebral disc is a key factor that contributes to lower back pain.2 The structures of the normal intervertebral disc include the central gelatinous nucleus pulposus enclosed by the outer annulus fibrosus and upper and lower cartilaginous end plate.3 The intervertebral disc is the largest avascular structure in the body. The blood supply only reaches the outer 1 to 2 mm of the disc, and the bone-cartilage junction and center of the nucleus in an adult lumbar disc may be 7 to 8 mm from the nearest blood supply.4,5 The nutritional environment of the intervertebral disc cells varies throughout the disc, with a lower oxygen concentration and low pH in the disc center compared to the disc periphery.6 Because of the relative hypoxic characteristics of the intervertebral disc, hypoxia inducible factors play an important role in regulating the survival of intervertebral disc cells and are involved in intervertebral disc degeneration.7,8

Traditional Chinese medicine has been widely used in the Chinese population, and its use is increasing worldwide.9 Many Chinese herbs have been used as therapeutic agents with anti-inflammatory, anti-angiogenic, and anti-MMP expression properties. Furthermore, many herbs have been used to treat musculoskeletal conditions and osteoarthritis.10,11 Honokiol is a major component of Magnoliaceae and its pharmacokinetics are well known.12,13 In previous studies, honokiol demonstrated potential anti-inflammatory, anti-thrombolytic, anti-angiogenic and anti-cancer effects.14–17 It is also a potent inhibitor of the hypoxia-inducible factor (HIF) pathway in a number of cancer and retinal pigment epithelial cell lines,18 which suggests that it may be a potential agent for the treatment of intervertebral disc degeneration. However, the distribution of honokiol into the intervertebral disc has not yet been investigated.

Recently, interest in developing alternative methods of treatment for repairing or regenerating intervertebral disc tissue has increased, particularly regarding biological methods.19 Factors that may influence the solute concentration level include molecular size, charge, matrix properties of the intervertebral disc tissue, convective movement via the diurnal loading cycle of the disc, special tube-like structures and the anisotropic nature of solute diffusivity in the intervertebral disc.20–25 To our knowledge, there have been no reports to date have demonstrated that honokiol penetrates into the intervertebral disc. In this study, we hypothesized that honokiol, which has a relatively
We used the internal standard method to plot the calibration curves with concentrations ranging from 0.5 to 50 μg/mL. The extraction recovery of honokiol was determined using the biological samples of plasma and homogenized intervertebral disc by calculating the ratio of the mean peak area ratio of an analyte spiked before extraction to the mean peak area ratio of an analyte spiked post-extraction as follows: recovery (%) = [(mean peak area ratio of an analyte spiked before extraction)/(mean peak area ratio of an analyte spiked post-extraction)] × 100%. Full validation of the analytical method for lamivudine in rat plasma was performed according to the US Food and Drug Administration bioanalytical method validation guidance (Guidance for Industry: Bioanalytical Method Validation, 2001).

Method validation

The internal standard method was used to plot the calibration curves for the plasma samples and homogenized intervertebral disc samples. Honokiol was dissolved in disodium salt. The calibration curves with concentrations ranging from 0.5 to 50 μg/mL were constructed using the same procedure for the plasma samples and disc samples. All calibration curves required a correlation value of at least 0.995. To confirm the precision and the accuracy of the analytical method, the intra- and inter-day variabilities were measured with three replicates at three different concentrations and were performed on the same day and three successive days, respectively. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentrations (C_{obs}), as follows: accuracy (%) = [(C_{obs} - C_{nom})/C_{nom}] × 100. The precision coefficient of variation (CV) was calculated from the standard deviation and the observed concentrations as follows: precision (%) = standard deviation (SD)/C_{obs} × 100. The extraction recovery of honokiol was determined using the biological samples of plasma and homogenized intervertebral disc by calculating the ratio of the mean peak area ratio of an analyte before extraction to the mean peak area ratio of an analyte spiked post-extraction as follows: recovery (%) = [(mean peak area ratio of an analyte spiked before extraction)/(mean peak area ratio of an analyte spiked post-extraction)] × 100%. Full validation of the analytical method for lamivudine in rat plasma was performed according to the US Food and Drug Administration bioanalytical method validation guidance (Guidance for Industry: Bioanalytical Method Validation, 2001).

In vitro models for disc penetration

The rat tail was used as the in vitro experimental model for the experiment with the intervertebral discs.26 After anesthetization, the rat tail was cut and the intervertebral discs were separated (Fig. 2). The discs were immersed into honokiol solution (50 μg/mL) in a beaker and then the disc was collected at 15, 30, 60, 120 and 240 min after immersion to measure the concentration of honokiol in the disc. Normal saline was used to remove any residual solution contaminating the surface of the disc, and the disc samples were then mixed with 3 volumes of methanol for homogenization. The homogenized samples were placed in an ice bath following centrifugation at 13000g for 10 min at 4°C (Centrifuge 5415R, Eppendorf, Hamburg, Germany). The supernatants were stored at −20°C until analysis.

In vivo model for disc distribution

The experimental animal protocol was approved by the Institutional Animal Care and Use Committee of National Yang-
Ming University (IACUC No. 1030704) and was consistent with the guidelines of the National Research Council, USA. Male Sprague-Dawley rats weighing 240 to 300 g from the Laboratory Animals Center of National Yang-Ming University were used in these studies. During the experiment, the animals were housed in standard cages in a temperature-controlled room with a regular light/dark cycle. Free access to food (laboratory rodent diet 500 g, PMI Feeds, Richmond, IN) and water was available at all times.

The experimental rats were anesthetized with a mixture of urethane (1 g/mL) and α-chloralose (0.1 g/mL) at a dose of 1 mL/kg via intra-peritoneal administration. A heating pad was used to maintain the body temperature of the rat. The surgical sites were shaved and cleaned with 70% ethanol solution. The jugular vein was cannulated for the administration of 30 mg/kg honokiol dissolved in ethanol and PEG 400 (1:4). Blood samples were collected via cardiac puncture with heparinized syringes at 15, 30, 60, 120 and 240 min after dosing. Following centrifugation at 13000 g for 10 min at 4 °C, the plasma was stored at –20 °C until analysis. Rat tails were also collected at the same time points as the blood samples, and the disc samples were separated from the rat tail. The disc samples were washed with normal saline, and then 3 volumes of methanol were added for protein precipitation and homogenization in an ice bath. After centrifugation at 13000g for 10 min at 4°C, the supernatants were stored at –20°C until analysis.

Results and Discussion

To obtain the best analytical conditions, the pH value of the buffer system was optimized. The phosphate buffer system consisting of 10 mM NaH2PO4, pH 2.8 was selected to produce a sharp and narrow peak. The internal standard is a chemical substance and used to calibrate the ratio of analyte signal and internal standard signal in the chromatogram. Survey with literature reports and testing of several potential candidates of the internal standard such as evodiamine, osthole, quercetin, rutin, etc. found that paeonol provides several advantages such as a sharp and narrow peak, suitable UV spectrum with analyte, the peak near the retention time of analyte, no interference with blank chromatogram, easy accurately repeatable, reproducible, etc.

Under the conditions described above, the retention times of paeonol and honokiol were 4.68 and 8.29 min in the plasma and disc samples, respectively. Figures 3 and 4 show the chromatograms and the UV spectrum of analytes after honokiol administration (30 mg/kg, i.v.). The precision and accuracy of the method is defined by examining both the intra-day and inter-day variabilities. Inter- and intra-day accuracy and precision for plasma were determined from the analysis of the calibration curve (concentration range of 5 - 50 μg/mL). For honokiol in rat plasma, the inter-day precisions (RSD%) ranged from 1.21 to 4.10%, while accuracy (bias%) was between –2.02 and 0.08%. Intra-day precision (RSD%) ranged from 1.90 to 6.28% and accuracy (bias%) was between –2.82 and 8.24% (Table 1). For honokiol in rat disc, the inter-day precisions (RSD%) ranged from 1.04 to 3.50%, while accuracy (bias%) was between –1.76 and 4.81%. Intra-day precision (RSD%) ranged from 0.63 to 4.71% and accuracy (bias%) was between –15.3 and 7.23% (Table 2).

Recovery studies were conducted by dividing the peak area ratio of the blank plasma spiked with honokiol at low (5 μg/mL), medium (10 μg/mL), and high (50 μg/mL) concentrations to the
peak area ratio of the concentrations prepared from the standard solutions. The average recoveries of the plasma and intervertebral disc were 94.72 ± 2.21 and 94.97 ± 2.84%, respectively (Table 3).

In our previous report,13 the pharmacokinetics of honokiol showed a biphasic process consisting of a rapid distribution phase followed by a slower elimination phase, and the dose-related response was linear. We further studied the disc distribution of honokiol in this study.

The concentration-time profiles in the in vitro experiments are shown in Fig. 5. In this in vitro study, honokiol gradually penetrates into the disc. The concentration-time curve of honokiol in the in vitro model showed a linear relationship and partition coefficient. The results revealed that honokiol penetrates into the disc by passive diffusion and this phenomenon is similar to oxygen32 and glucose.33 Pottman’s report indicated that oxygen was supplied from blood to the tissues by passive diffusion.
diffusion. Another experiment, the colon cancer cell lines, HT-29 (human) and DHD/K12/TRb (rat) study suggested that the $^{14}$C-glucose was passively diffused across the cell membrane. Actually, several mechanisms may be involved in the cell membrane penetration. The substance diffusion coefficient through the cell membrane may be limited by large molecular weight, hydrophilicity and negative charge. Franze et al. proposed that the low molecular weight heparins exhibit different skin permeability properties. However, the previous study showed that ceftriaxone is undetectable in the in vitro model. We suggested that ceftriaxone was delivered from blood to the tissues by passive diffusion.

The in vivo experiment evaluated the distribution of honokiol into the disc after honokiol administration (30 mg/kg, i.v.) (Fig. 6). The concentration of honokiol in the plasma and intervertebral disc and the concentration ratio of the disc-to-plasma ($C_{\text{disc}}/C_{\text{plasma}}$) at various time points are shown in Table 4. The concentration of honokiol in the plasma and disc were $4.91 \pm 0.89$ and $1.79 \pm 0.92 \mu g/mL$ (6.73 μM) at 15 min after honokiol administration (30 mg/kg, i.v.), respectively. The honokiol concentration ratio of the disc-to-plasma ($C_{\text{disc}}/C_{\text{plasma}}$) is approximately 36.4% at 15 min after honokiol administration, reaching 49.6% at 30 min. Then, the concentration ratio of the disc-to-plasma was approximately 27% from 60 to 240 min.

In a previous study, honokiol inhibited the expression of a hypoxia inducible factor isoform in the concentration range of 5 – 20 μM in a concentration-dependent manner. Compared to our data, the active concentration is obtained for the therapeutic level at 15 and 30 min after honokiol administration (30 mg/kg, i.v.). During the entire time period, the disc-to-plasma concentration ratio of honokiol was between 26.6 and 49.6%.

Both the in vitro disc penetration and in vivo distribution experiments showed that the passive diffusion property of honokiol is governed by the blood supply of the intervertebral disc that reaches the end plate and outer annulus of the intervertebral disc. With blood circulation, the physical characteristics of honokiol remain unchanged and can enter the deep region of the endplate and outer annulus to achieve a more rapid penetration.

**Table 4** The concentration of honokiol in the plasma and intervertebral disc at various times after honokiol administration (30 mg/kg, i.v.)

<table>
<thead>
<tr>
<th>Time/ min</th>
<th>Plasma concentration/ μg mL$^{-1}$</th>
<th>Disc concentration/ μg mL$^{-1}$</th>
<th>Concentration ratio disc-to-plasma, % ($C_{\text{disc}}/C_{\text{plasma}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>$4.91 \pm 0.89$</td>
<td>$1.79 \pm 0.92$</td>
<td>36.4</td>
</tr>
<tr>
<td>30</td>
<td>$3.04 \pm 0.91$</td>
<td>$1.51 \pm 1.27$</td>
<td>49.6</td>
</tr>
<tr>
<td>60</td>
<td>$2.40 \pm 0.51$</td>
<td>$0.64 \pm 0.21$</td>
<td>26.7</td>
</tr>
<tr>
<td>120</td>
<td>$1.80 \pm 0.70$</td>
<td>$0.50 \pm 0.26$</td>
<td>27.8</td>
</tr>
<tr>
<td>240</td>
<td>$2.09 \pm 1.08$</td>
<td>$0.55 \pm 0.45$</td>
<td>26.3</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard deviation ($n = 6$).

**Conclusions**

In summary, a validated reversed-phase HPLC method was developed to determine the in vitro penetration and in vivo distribution of honokiol into the intervertebral disc. The results suggest that the penetration of honokiol into the intervertebral disc is sufficient to achieve a therapeutic concentration after honokiol administration (30 mg/kg, i.v.). The disc-to-plasma concentration ratio of honokiol is between 26.6 and 49.6%. The present study revealed the potential use of traditional Chinese medicine for the treatment of intervertebral disc degeneration. It is suggested that the distribution of potential agents for the treatment of intervertebral disc disease should be studied routinely because of the special physical environment of the intervertebral disc. Moreover, detailed clinical trials are required to confirm the therapeutic efficacy.

**Acknowledgements**

Funding for this study was provided in part by research grants from the National Science Council (NSC102-2113-M-010-001-MY3) Taiwan and TCH10401-62-004 and TCH104-02 from Taipei City Hospital, Taipei, Taiwan.
References