Pharmacokinetic Study on Hyperoside in Beagle's Dogs

AI Guo[1, 2], HUANG Zheng-ming[2*], LIU Chang-xiao[3]

1. Institute of Radiation Medicine, Academy of Military Medical Sciences, Beijing 100850, China
2. Department of Pharmacy, 302 Hospital of PLA, Beijing 100039, China
3. Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China

Abstract: **Objective** To develop and validate a simple, rapid, sensitive, and reproducible HPLC method for determination of hyperoside in plasma of dogs and for the subsequent pharmacokinetic (PK) study. **Methods** An accurate and reproducible HPLC-UV method was developed and validated for the determination of hyperoside in plasma of dogs, using kaempferol as internal standard. The plasma samples of dogs following ig administration of hyperoside were analyzed for the detection of quercetin after enzymatic hydrolysis treatment with combined β-glucuronidase and sulphatase. The analytes were separated on a Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol-buffer solution (0.1 mol/L NH_4Ac + 0.3 mmol/L EDTA-Na_2)-acetic acid (60:40:1) and was delivered at a flow rate of 1 mL/min. The UV detector was set at 370 nm and the column temperature was maintained at 35 ℃. The sample injection volume was 20 μL. Data were collected and analyzed using the ANASTAR software. PK parameters were calculated with DAS software (2.0). **Results** Linear relationships were validated over the range of 0.01－1 μg/mL for hyperoside (r = 0.9997). The intra- and inter-day precision values for all samples were within 10.0%, and the accuracies of intra- and inter-day assays were within the range of 92.4%－102.4%. The validated method was successfully used to determine the hyperoside concentration in plasma of dogs for up to 12 h, after a single ig administration (25 mg/kg). The mean PK parameters for male and female dogs were as follows: C_{max} (0.18 ± 0.05) and (0.16 ± 0.05) μg/mL, $AUC_{0-\infty}$ (0.79 ± 0.34) and (0.86 ± 0.27) μg/(mL·h), $t_{1/2(ka)}$ (0.89 ± 0.41) and (0.88 ± 0.28) h, respectively. Statistical analysis on the PK of hyperoside in male and female groups showed that sex had no significant impact on the PK of hyperoside (P > 0.05). **Conclusion** The method is able and sufficient to be used in drug PK studies of hyperoside.

Key words: Beagle's dogs; HPLC; hyperoside; kaempferol; pharmacokinetics
DOI: 10.3969/j.issn.1674-6384.2012.03.007

Introduction

As an important bioactive compound, hyperoside (Fig. 1A, quercetin-3-*O*-β-*D*-galactoside), has been documented to possess antiviral, antinociceptive, anti-inflammatory, cardioprotective, hepatoprotective, and gastricmucosal-protective effects (Huang, Yang, and Huang, 2009). In our previous studies (Wu *et al*, 2007), we assessed the anti-hepatitis B virus (HBV) effect of hyperoside extracted from *Abelmoschus manihot* (L.) Medic through the human hepatoma Hep G2.2.15 cell culture system and duck hepatitis B virus (DHBV) infection model. Whether the experiments were done *in vivo* or *in vitro*, our results showed that hyperoside possessed anti-HBV activity. In other studies (Geng *et al*, 2009), we found that covalently closed circular DNA (cccDNA) of DHBV could be eliminated efficiently by hyperoside, which may be one of the important mechanisms of the anti-HBV activity of hyperoside.

Up to now only limited data were available on the pharmacokinetics (PK) of hyperoside after ig administration (Chang *et al*, 2005). Hyperoside, with a galactose sugar moiety, was not detected in its intact

* Corresponding author: Huang ZM Address: Department of Pharmacy, 302 Hospital of PLA, Beijing 100039, China
Tel/Fax: +86-10-6693 3233 E-mail: huang_zhengming@sohu.com
Received: November 1, 2011; Revised: January 18, 2012; Accepted: February 5, 2012
Fund: National Nature Science Foundation of China (30572350) and New Drug Foundation of State Administration of Traditional Chinese Medicine (DIX005A)
Online time: May 9, 2012 Online website: http://www.cnki.net/kcms/detail/12.11410.R.20120509.1617.002.html

form by either HPLC-UV or LC-MS-MS methods (Arts *et al*, 2004; Chang *et al*, 2005). In order to investigate the PK profiles of hyperoside during preclinical studies, it was essential to carry out experiments in animals. In this paper, the plasma samples were analyzed by HPLC for the detection of quercetin (Fig. 1B) after enzymatic hydrolysis treatment with combined β-glucuronidase and sulphatase, using kaempferol (Fig. 1C) as internal standard (IS). Subsequently, we applied this method to the PK study on hyperoside in Beagle's dogs after a single ig administration (25 mg/kg).

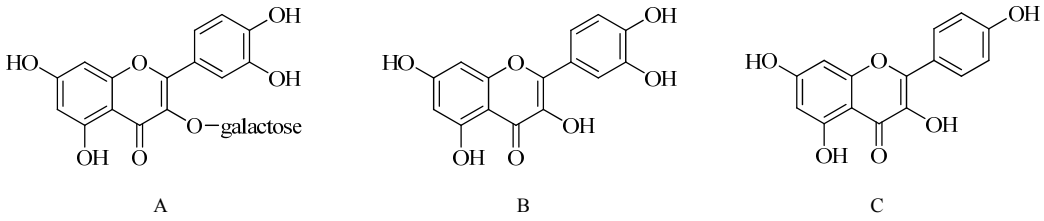

Fig. 1 Chemical structures of hyperoside (A), quercetin (B), and kaempferol (C)

Materials and methods

Chemicals, reagents, and animals

Hyperoside (96.0%) was available from 302 Hospital of PLA (Beijing, China) and the structure of hyperoside was determined by HPLC in comparison with authentic hyperoside (National Institute for Food and Drug Control, Beijing, China). Quercetin (97.3%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Kaempferol (98.3%) used as IS for HPLC assay was obtained from Tianjin Institute of Pharmaceutical Research (Tianjin, China). β-Glucuronidase (including β-glucuronidase 98 000 U/mL and sulfatase 2400 U/mL) was obtained from Sigma (MO, USA). All other chemicals and solvents used were of analytical grade and were obtained from standard commercial sources. Distilled and deionized water was used throughout the study.

Blank plasma sample was obtained from healthy Beagle's dogs without drug therapy. The Beagle's dogs, weighing 7.5－8.6 kg, were purchased from Xingang Animal Experimentation Breeding Center (Shanghai, China) [SCXK (Shanghai) 2002-0014]. The studies were approved by the Animal Ethics Committee of Tianjin Institute of Pharmaceutical Research.

Instruments and operating conditions

The HPLC system consisted of an HI-TECH P4000 Pump, a Shimadzu-10A , and a Lab Alliance AS1000 Auto Sampler. Separation was performed on a Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol-buffer solution (0.1 mol/L NH_4Ac + 0.3 mmol/L EDTA-Na_2)-acetic acid (60:40:1) and was delivered at a flow rate of 1 mL/min. The UV detector was set at 370 nm and the column temperature was maintained at 35 ℃. The sample injection volume was 20 μL. Data were collected and analyzed using the Anastar software.

Pretreatment of plasma samples

The obtained plasma samples (0.5 mL) were transferred into a plastic tube containing 50 μL ascorbic acid (0.5%), 50 μL acetic acid (0.58 mol/L), and 10 μL β-glucuronidase, and vortex-mixed for 1 min. After being incubated at 37 ℃ for 1 h, the samples were added to 5 mL ethyl acetate and 25 μL kaempferol (40 μg/mL) and vortex-mixed for 1 min, then centrifuged (2500 r/min for 10 min) and the upper organic layer was transferred to another set of clean tubes. The supernatants were evaporated to dryness under a stream of nitrogen at 40 ℃. The dry residues were reconstituted in 150 μL methanol, vortex-mixed for 20 s, and then centrifuged for 5 min at 12 000 r/min. The supernatants of 20 μL were injected into the HPLC system for analysis.

Preparation of standard solution

Stock solutions of hyperoside and IS were prepared separately in duplicate by dissolving 0.1 mg of each compound in 1 mL of analytic grade methanol at a concentration of 100 μg/mL, and stored at 4 ℃ for later use. Both the calibration standard samples (0.01－1.0 μg/mL) and the quality control (QC) samples

(0.025, 0.2, and 0.8 μg/mL), which were used in the pre-study validation and during the PK study, were prepared by spiking appropriate amount of the working solution in blank plasma of Beagle's dogs. The IS was diluted with methanol to a final kaempferol concentration of 40 μg/mL.

Method validation

The validation of the method was based on the bioanalytical method validation guidance in *Chinese Pharmacopeia*. The following parameters were considered.

The specificity of this method was investigated by comparing six individual blank plasma samples of dogs. Each blank sample was tested for interference using the present analysis method and compared with the spiked samples. Samples of blank plasma and spiked plasma were processed and analyzed under the same conditions and the retention time of quercetin and kaempferol were detected at the same wavelength of 370 nm.

Calibration standards were prepared and analyzed in triplicate to establish the calibration curve ranging from 0.01 to 1.0 μg/mL. The peak area ratio of quercetin to kaempferol was plotted against concentration. The curves were fitted by least squares linear regression using a weight factor of $1/\chi^2$. Concentration of hyperoside in samples was calculated with the calibration curves. The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve with no more than ± 20% accuracy and 20% precision values.

The concentration of QC samples was 0.025, 0.2, and 0.8 μg/mL which represented of low, medium, and high QC levels, respectively. Intra-day precision and accuracy were evaluated by analyzing each QC sample for five times on the same day, while inter-day precision was evaluated by analyzing each QC sample for consecutive 3 d. The precision of the method was described as relative standard deviation (RSD) among each assay. The accuracy was described as a percentage error of measured concentration versus nominal concentration of hyperoside in QC samples.

Extraction recovery was evaluated at QC samples. It was calculated by comparing the mean peak area (n = 6 at each concentration) of samples spiked before extraction to those spiked after extraction. The extraction recovery of IS was determined in the same way.

Stability of hyperoside in plasma samples of dogs was tested after three freeze-thaw cycles and long-term storage (−20 ℃ for four weeks). For post-preparative stability, the processed QC samples were stored at 25 ℃ for 24 h and analyzed. Six replicates at different concentration (0.025, 0.2, and 0.8 μg/mL) were tested and compared with freshly processed samples at the same concentration.

PK study

The developed method was used to determine the plasma concentration-time profile of hyperoside after ig administration of 25 mg/kg dose. Six Beagle's dogs (half per sex) were included in the study. Plasma samples were collected in heparinized tubes before dosing and at the time points of 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 12 h after ig administration. Plasma was immediately separated by centrifugation at 3000 r/min for 10 min and stored at −20 ℃ until being analyzed. Calculation of the PK parameters was performed by one-compartmental assessment of data using the PK software package (DAS 2.0, Mathematical Pharmacology Professional Committee of Chinese Pharmacological Society).

Statistical analyses were performed using SPSS software version 11.5 (SPSS, Inc., USA). Results were expressed as $\bar{X} \pm s$. All of the experiments were two-sided with the 5% level of significance.

Results and discussion

Method validation

It is believed that the conjugated hyperoside and conjugated quercetin could be changed into quercetin by enzymatic hydrolysis with combined β-glucuronidase and sulphatase. So we analyzed the plasma samples by HPLC for the detection of quercetin after enzymatic hydrolysis, using kaempferol as IS. IS was required to keep accuracy when liquid-liquid extraction method was used. Kaempferol was chosen as IS due to its structure similarity to the target compounds, retention action, and extraction efficiency. During samples preparation, ascorbic acid was used as an anti-oxidant to prevent drug degradation.

Typical chromatograms were shown in Fig. 2. The retention time of quercetin and IS was 7.6 and 12.4 min,

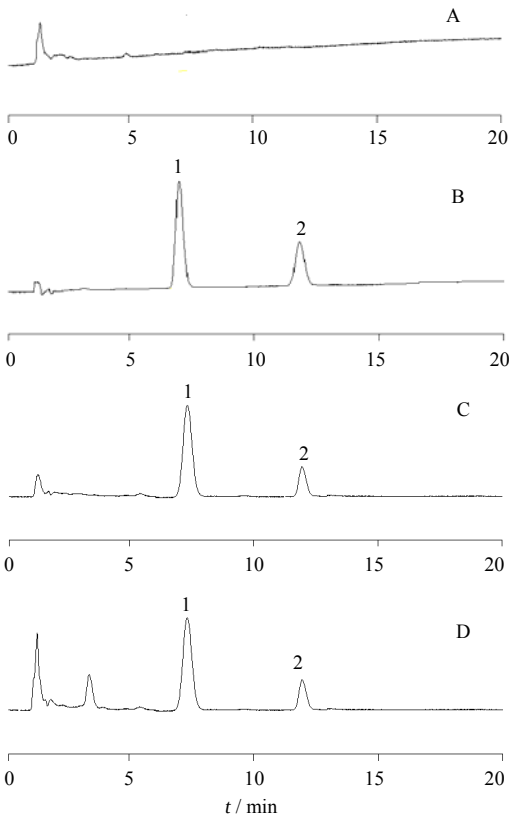

Fig. 2 Representative chromatograms of blank plasma of dogs(A), blank plasma of dogs spiked with quercetin (0.1 μg·mL^{-1}) and IS (B), blank plasma of dogs spiked with hyperoside (0.1 μg·mL^{-1}) after pretreatment as described in paper (C), and plasma sample obtained 1 h after ig administration of hyperoside (D)

1: quercetin 2: IS

respectively. The peaks were sharp and symmetrical with good baseline separation and minimal tailing. Significant interference from endogenous substances was not observed at the retention time of quercetin and IS, indicating the sample preparation method was effective.

Good linearity was obtained for hyperoside in the range from 0.01 to 1 μg/mL. The regression equation of the calibration curve was as follows: Y = 0.355 46X + 0.004 61 (r = 0.9997), where Y and X represented the peak area ratio and hyperoside plasma concentration, respectively. The LLOQ of the method for hyperoside was 0.01 μg/mL.

For intra-assay, the RSD values of QC samples at concentration of 0.025, 0.2, and 0.8 μg/mL were between 3.80% and 9.01%, and the accuracy of QC samples was between 92.4% and 102.4%. For inter-assay, the RSD values of QC samples were less than 9.05% and the accuracy of QC samples was between 98.4% and 101.3%.

The extraction recoveries of hyperoside from plasma of dogs were (101.9 ± 1.1)%, (99.9 ± 3.2)%, and (97.5 ± 2.7)% at the concentration of 0.025, 0.2, and 0.8 μg/mL, respectively. Recovery of IS was (95.4 ± 6.3)% in this method.

Incubation of hyperoside in plasma of dogs at −20 ℃ for four weeks resulted in no discernable loss of material as compared to freshly prepared samples (corresponding RSD values were 5.9%, 6.4%, and 4.9% at the concentration of 0.025, 0.2, and 0.8 μg/mL). Stability of hyperoside in processed samples at room temperature (25 ℃) for 24 h was evaluated. The corresponding RSD values were 3.2%, 4.8%, and 5.6% at low, middle, and high concentration. Results showed that hyperoside was stable for 24 h at 25 ℃ in plasma of dogs. After three freeze-thaw cycles, the corresponding RSD values for the three samples were between 2.7% and 8.2%, which indicated that hyperoside could undergo at least three freeze-thaw cycles.

Application of the method in PK study

The validated method was successfully used to determine the hyperoside concentration in plasma of Beagle's dogs for up to 12 h, after a single ig administration (25 mg/kg). The plasma concentration-time profile within the period of 0—12 h was shown in Fig. 3, and the main PK parameters by sex groups were summarized in Table 1. The mean PK parameters for male and female dogs were as follows: C_{max} (0.18 ± 0.05) and (0.16 ± 0.05) μg/mL, $AUC_{0-\infty}$ (0.79 ± 0.34) and (0.86 ± 0.27) μg/(mL·h), $t_{1/2(ka)}$ (0.89 ± 0.41) and

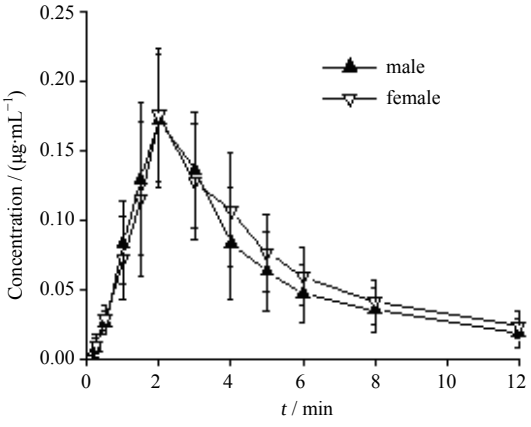

Fig. 3 Concentration-time profiles of hyperoside in plasma of Beagle's dogs after ig administration ($\bar{X} \pm s$, n = 3)

Table 1 Main PK parameters of hyperoside in Beagle's dogs after ig administration ($\bar{X} \pm s$, $n = 3$)

Parameters	Units	Male	Female
A	$\mu g \cdot mL^{-1}$	0.30 ± 0.23	0.28 ± 0.22
k_e	h^{-1}	0.24 ± 0.08	0.18 ± 0.08
k_a	h^{-1}	0.90 ± 0.37	0.84 ± 0.22
$t_{1/2(ka)}$	h	0.89 ± 0.41	0.88 ± 0.28
$t_{1/2(ke)}$	h	3.14 ± 0.88	3.56 ± 1.53
t_{max}	h	2	2
C_{max}	$\mu g \cdot mL^{-1}$	0.18 ± 0.05	0.16 ± 0.05
$AUC_{0\text{-}12h}$	$\mu g \cdot mL^{-1} mL \cdot h$	0.73 ± 0.20	0.80 ± 0.30
$AUC_{0\text{-}\infty}$	$\mu g \cdot mL^{-1} \cdot h$	0.79 ± 0.34	0.86 ± 0.27
CL/F	$L \cdot h^{-1} \cdot kg$	38.41 ± 9.63	35.20 ± 19.79
V/F	$L \cdot kg^{-1}$	173.80 ± 69.22	168.27 ± 75.33

(0.88 ± 0.28) h, respectively. Statistical analysis on the PK of hyperoside in male and female groups showed that sex had no significant impact on the PK of hyperoside ($P > 0.05$).

Conclusion

In previous reports, some methods had been developed for the quantification of hyperoside in various herbal medicines and biological fluids. However, most of them were mainly used for the determination of several active components, such as hyperoside, and for the PK study of hyperoside in rats. For instance, Wang *et al* (2010) reported an HPLC method to simultaneously determine five active compounds which were vitexin-4″-*O*-glucoside, vitexin-2″-*O*-rhamnoside, vitexin, rutin, and hyperoside in plasma of rats after iv administrating the hawthorn leaves extract; Ying *et al* (2011) developed an HPLC method to simultaneously determine three active compounds which were vitexin-4″-*O*-glucoside, vitexin-2″-*O*-rhamnoside, and hyperoside in plasma of rats following ig administrating the hawthorn leaves extract.

In order to investigate the PK profiles during preclinical studies, an accurate and reproducible HPLC method with UV detection was developed and validated for the determination of hyperoside in plasma of dogs after ig administration. In this method, the plasma samples were analyzed for the detection of quercetin after enzymatic hydrolysis treatment with combined β-glucuronidase and sulphatase, using kaempferol as IS. The method was found to be convenient, accurate, and proved suitable for PK study. The PK data could be useful for further studies of hyperoside.

References

Arts IC, Sesink AL, Faassen-Peters M, Hollman PC, 2004. The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides. *Br J Nutr* 91(6): 841-847.

Chang Q, Zuo Z, Chow MS, Ho WK, 2005. Difference in absorption of the two structurally similar flavonoid glycosides, hyperoside and isoquercitrin, in rats. *Eur J Pharm Biopharm* 59(3): 549-555.

Geng M, Wang JH, Chen HY, Yang XB, Huang ZM, 2009. Effects of hyperin on the cccDNA of duck hepatitis B virus and its immunological regulation. *Acta Pharm Sin* 44(12): 1440-1444.

Huang K, Yang XB, Huang ZM, 2009. Research progress in pharmacological activities of hyperoside. *Herald Med* 28(8): 1046-1048.

Liu X, Wang D, Wang SY, Meng XS, Zhang WJ, Ying XX, Kang TG, 2010. LC determination and pharmacokinetic study of hyperoside in rat plasma after intravenous administration. *Yakugaku Zasshi* 130(6): 873-879.

Wang SY, Chai JY, Zhang WJ, Liu X, DU Y, Cheng ZZ, Ying XX, Kang TG, 2010. HPLC determination of five polyphenols in rat plasma after intravenous administration of hawthorn leaves extract and its application to pharmacokinetic study. *Yakugaku Zasshi* 130(11): 1603-1613.

Wu LL, Yang XB, Huang ZM, Liu HZ, Wu GX, 2007. *In vivo* and *in vitro* antiviral activity of hyperoside extracted from *Abelmoschus manihot* (L.) Medic. *Acta Pharm Sin* 28(3): 404-409.

Ying X, Meng X, Wang S, Wang D, Li H, Wang B, Du Y, Liu X, Zhang W, Kang T, 2011. Simultaneous determination of three polyphenols in rat plasma after orally administering hawthorn leaves extract by the HPLC method. *Nat Prod Res* 26(6): 585-591.