Metabolic Pharmacokinetics of Isoflavones in the Roots of *Pueraria lobata* in Rats

Hsiu-Mei Chiang[1,6], Yi-Rou Yeh[2], Pei-Dawn Lee Chao[3], Su-Lan Hsiu[3], Yu-Chi Hou[4], Ying-Chang Chi[1], Kuo-Ching Wen[5]

[1]Institute of Pharmaceutical Chemistry, [2]Institute of Chinese Pharmaceutical Science, [3]School of Pharmacy, [4]School of Chinese Medicine, [5]School of Cosmeceutics, China Medical University, Taichung; [6]Department of Nursing, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan, R.O.C.

Purpose. Puerariae radix, the root of *Pueraria lobata*, is widely used in clinical Chinese medicine. It is abundant in isoflavones including puerarin, daidzin, and daidzein. This study investigated the metabolic pharmacokinetics of isoflavones after orally administrating Puerariae radix to Spraque-Dawley rats.

Methods. The concentrations of puerarin, daidzin and daidzein in commercial extract of Puerariae radix were simultaneously quantified by a gradient high performance liquid chromatographic method (HPLC). Seven rats were orally given the commercial extract of Puerariae radix and blood samples were withdrawn at specific time points. Serum samples were assayed by HPLC prior to and after enzymatic hydrolysis with β-glucuronidase and sulfatase. Pharmacokinetic parameters were calculated using noncompartment model of WINNONLIN.

Results. The contents of puerarin, daidzin and daidzein in the commercial extract of Puerariae radix were 33.9, 1.3 and 0.9 mg/g, respectively. After oral administration of Puerariae radix, the parent forms of puerarin, daidzin and daidzein were not detected in serum; however, the sulfates and glucuronides of daidzein were found in the blood, predominantly as daidzein sulfates.

Conclusions. Daidzein sulfates and glucuronides may play important roles in the biological activities of isoflavones in Puerariae radix and are worthy of further investigation. **(Mid Taiwan J Med 2005;10:57-64)**

Key words

daidzein, daidzin, isoflavone, pharmacokinetics, *Pueraria lobata*, puerarin

INTRODUCTION

Flavonoids possess numerous beneficial biological properties including antiallergic [1-3], antiviral [4], anti-inflammatory [1,5], antioxidation [6,7], and antitumor [8] activities. Isoflavones, a family of flavonoids possessing estrogenic properties, have been attracting much attention because hormone replacement therapy has been reported to cause adverse effects. Isoflavones including genistin, genistein, daidzin and daidzein have been extensively studied. They are known to have antithrombotic [9], antiallergic [9] and estrogenic [10,11] activities and have been shown to decrease blood lipid levels, increase bone density [12,13], lower the risk of cancer [14-17], protect against cardiovascular deseases [18,19], reduce oxidative stress [20-22] and modulate immune responses [23].

Received : 20 January 2005. Revised : 5 March 2005.
Accepted : 1 April 2005.
Address reprint requests to : Kuo-Ching Wen, School of Cosmeceutics, China Medical University, 91 Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C.

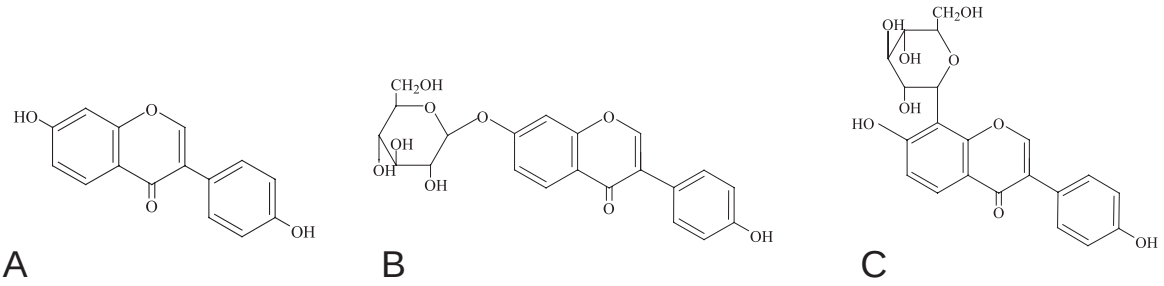

Fig. 1. Chemical structures of isoflavones. A: Daidzein. B: Daidzin. C: Puerarin.

Puerariae radix, the root of *Pueraria lobata* (Leguminosae), is an important Chinese herb for treating angina pectoris, hypertension, influenza, neck stiffness, diarrhea and deafness. It possesses various pharmacological activities including anti-hypertensive, hypolipidemic, anti-diabetic, cardioprotective and estrogen-like effects and has been shown to aid in recovery from alcohol abuse and alcoholism [24-26]. Puerariae radix contains isoflavones including puerarin, daidzin and daidzein (Fig. 1).

Despite many pharmacodynamic studies on Puerariae radix, the metabolic pharmacokinetics of isoflavones in Puerariae radix still remain unclear. Commercial herbal extract is the most common dosage form in Taiwan. Therefore, this study investigated the metabolic pharmacokinetics of isoflavones after orally administrating a commercial extract of Puerariae radix to rats.

MATERIALS AND METHODS

Chemicals

Puerarin (purity 88%), daidzin (purity 98%), β-glucuronidase (Type B-1, from bovine liver), sulfatase (Type H-1, from *Helix pomotia*) and ethyl paraben were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Daidzein (purity 97%) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Acetonitrile, methanol and ethyl acetate were LC grade and purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). L (+)-Ascorbic acid was obtained from Riedel-deHaen (Seeize, Germany). Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study.

Quantitation of isoflavones in Puerariae radix commercial extract

A commercial extract of Puerariae radix (50 mg powder, containing 65% starch), purchased from a Chinese drug store in Taichung, was extracted twice with 10 mL of 70% aqueous methanol by ultrasonication for 30 min and then filtered. Sufficient methanol was added to the combined filtrates to make 20.0 mL for later analysis.

Ethyl paraben solution (20 μL, 100.0 μg/mL in methanol as internal standard) was added to 180 μL of sample, and 20 μL was subjected to HPLC analysis. Gradient elution with a mixture of acetonitrile and 0.05% phosphoric acid as the mobile phase was conducted as follows: 11/89 (0 min); 18/82 (10 min) and 50/50 (20 min). The detection wavelength was set at 240 nm and the flow rate was 1.0 mL/min. The calibrators ranged from 1.6 to 200.0 μg/mL for puerarin, 0.6 to 40.0 μg/mL for daidzin and 0.3 to 10.0 μg/mL for daidzein.

Drug administration and blood collection

Seven male Sprague-Dawley rats weighing 390 to 490 g were fasted for 12 h before administration of Puerariae radix commercial extract. Food was withheld for an additional 3 h after drug administration. Rats were orally given water suspension of the commercial extract of Puerariae radix at 4.0 g/kg (0.5 g/mL). Water was supplied ad libitum. Blood samples (0.5 mL)

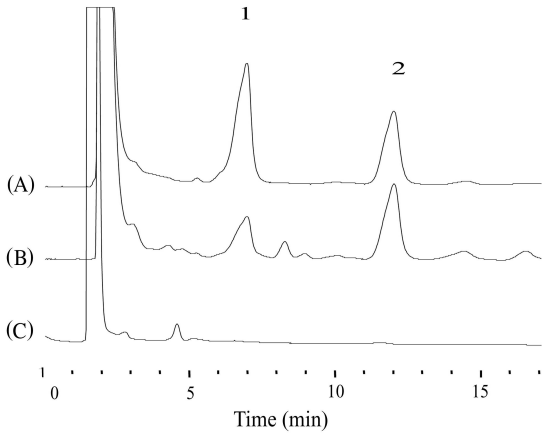

Fig. 2. HPLC chromatograms of serum. A: Daidzein (2.0 μg/mL, 1) and ethyl paraben (1.0 μg /mL, 2) spiked in blank serum. B: Hydrolyzed serum collected at 60 min after oral administration of commercial extract of Puerariae radix (4 g/kg) to one rat. C: Blank serum.

were withdrawn via cardiopuncture at 0, 5, 30, 60, 120, 240, 360, 480, 720, 1440 and 2880 min after dosing. This animal study adhered to *The Guidebook for the Care and Use of Laboratory Animals* (2002) (Published by The Chinese Society for Laboratory Animal Science, Taiwan, R.O.C.).

Quantitation of isoflavones and their conjugated metabolites in serum

Serum was deproteinized by the addition of a 4-fold volume of methanol. The supernatant was concentrated under nitrogen and subjected to HPLC analysis to determine the parent forms of puerarin, daidzin and daidzein. The conjugated metabolites of isoflavones in serum were determined by hydrolysis with sulfatase or glucuronidase. Our enzymolysis process essentially followed a previous study but with slight modification [27]. Serum (100 μL) was incubated at 37˚C with either 50 μL of β-glucuronidase (1666 units/mL in pH 5 acetate buffer) for 14 h or sulfatase (1000 units/mL in pH 5 acetate buffer) for 2 h in the presence of 25 μL of ascorbic acid (200 mg/mL). After hydrolysis, serum was partitioned with 175 μL ethyl acetate containing 5.0 μg/mL of ethyl paraben as internal standard and then centrifuged at 9860 g for 15 min. The ethyl acetate layer was evaporated to dryness under nitrogen and reconstituted with the mobile phase for HPLC analysis.

For calibrator preparation, sera were spiked with standard solutions of daidzein to afford serum standards in the concentration range of 0.1 to 6.4 μg/mL. The pretreatments and HPLC conditions were identical to those described above except for enzyme treatment. The calibration curve for serum was plotted after linear regression of the peak area ratios (daidzein to the internal standard) against concentrations of daidzein.

Validation of serum assay method

The precision and accuracy of the analytical method were evaluated by intra-day and inter-day analysis of triplicate serum standards within one day and over a period of three days. Recovery studies were conducted by spiking daidzein into blank serum and water in triplicates to afford concentrations of 0.5, 2.0, 4.0 μg/mL. The accuracy of this method was assessed by comparing the concentrations of daidzein obtained from serum with the corresponding ones from water [28]. Lower limit of quantitation (LLOQ) represents the lowest concentration of analyte that can be determined with acceptable precision and accuracy, whereas limit of detection (LOD) represents the lowest concentration of analyte that can be detected with S/N > 3.

Data analysis

The peak serum concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained from experimental observations. The pharmacokinetic parameters were calculated by a noncompartment model run on WINNOLIN (version 1.1 SCI software, Statistical Consulting Inc., Apex, NC, USA). The area under the serum concentration-time curve (AUC_{0-t}) was calculated by the trapezoidal rule to the last point.

RESULTS

The HPLC chromatogram showed that

Table. Pharmacokinetic parameters of daidzein glucuronides and sulfates in 7 rats after oral administration of Puerariae radix commercial extract (4 g/kg)

Parameters	Daidzein glucuronides (A)	Daidzein sulfates (B)	Difference % (B-A)/A
T_{max} (min)	390.0 ± 190.9	651.4 ± 204.3	67.0 ± 78.8
C_{max} (nmol/mL)	1.5 ± 0.2	9.6 ± 2.0	540.0 ± 151.2*
AUC_{0-t} (nmol · min · mL^{-1})	1887.2 ± 178.0	8898.2 ± 1080.8	371.5 ± 73.5*
MRT (min)	1270.1 ± 38.4	1041.2 ± 100.1	–18.0 ± 8.5

Data expressed as mean ± SE. T_{max} = time to peak serum level; C_{max} = concentration of peak serum level; AUC_{0-t} = area under blood concentration–time curve to the last point; MRT = mean residence time. *$p < 0.05$.

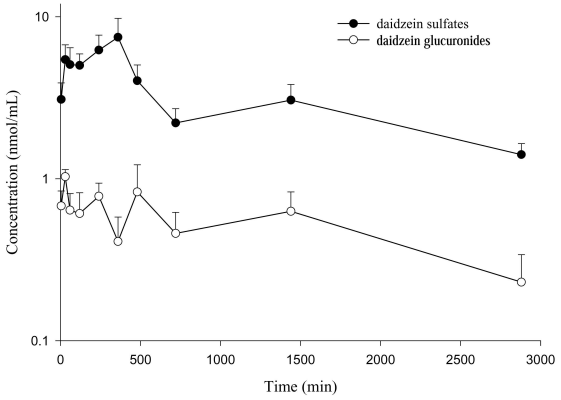

Fig. 3. Mean (± SE) serum concentration-time profiles of diadzein sulfates (●) and glucuronides (○) after orally administrating Puerariae radix commercial extract (4 g/kg) to 7 rats.

puerarin, daidzin and daidzein in the commercial extract of Puerariae radix as well as the internal standard, ethyl paraben, were well resolved by gradient elution within 25 min. Good linearity was shown in the range of 1.6 to 200.0 μg/mL for puerarin, 0.6 to 40.0 μg/mL for daidzin and 0.3 to 10.0 μg/mL for daidzein. Validation of this assay method indicated that all coefficients of variation for intra-day and inter-day were less than 10%, and the relative errors were below 20%. The LLOQ was 3.1 μg/mL for puerarin, 0.3 μg/mL for daidzin and 0.6 μg/mL for daidzein whereas the LOD was 0.02, 0.02 and 0.01 μg/mL, respectively. The commercial extract contained 33.9 mg/g puerarin, 1.3 mg/g daidzin and 0.9 mg/g daidzein.

The parent forms of puerarin, daidzin and daidzein were not detected in serum samples after being deproteinized with methanol. The concentrations of daidzein glucuronides and daidzein sulfates in serum were determined by HPLC after enzymolysis. The chromatogram is shown in Fig. 2. A time study reveled that the optimal duration needed for hydrolysis was 14 h for glucuronides and 2 h for sulfates. Good linearity of daidzein in serum ranged from 0.1 to 6.4 μg/mL. Validation of this assay method indicated that all coefficients of variation for intra-day and inter-day analysis were below 5.9% and the relative errors were below 8.7%. The recoveries of daidzein from serum were 89.8% to 99.7%. LLOQ and LOD were 0.1 μg/mL and 0.01 μg/mL, respectively.

The mean serum profiles showed that the levels of daidzein sulfates were much higher than those of daidzein glucuronides (Fig. 3). The pharmacokinetic parameters of daidzein conjugates revealed that C_{max} and AUC_{0-t} of sulfates were 540% and 371% higher than the C_{max} and AUC_{0-t} of glucuronides (Table).

DISCUSSION

Previous studies which reported the biological fates of puerarin in rats [29,30] have stated that puerarin was detected in urine and serum after oral administration of puerarin. In the present study, we did not detect any puerarin in the serum. This discrepancy may be explained by the fact that the detection sensitivity of the UV detector used in this study is lower than that of the tandem mass spectrometer used in the previous study [30]. On the other hand, in comparison with pure puerarin, the coexisting

complex constituents in the Puerariae radix extract might inhibit the absorption of puerarin. Moreover, the incubation of puerarin with rat feces indicated that no daidzein was liberated from puerarin (data not shown), indicating that the hydrolysis of this C-glycoside by enterobacteria was not feasible.

The optimal hydrolysis time for daidazein sulfates and glucuronides in serum was determined in our preliminary study to be 2 h and 14 h, respectively, instead of 3 h reported by a previous study for hydrolyzing both conjugates [31]. Several studies have reported the biological fate of daidzin [27,31,32]. They found that only conjugates of daidzein were present in urine and bile, whereas neither daidzin nor daidzein could be detected after oral administration of daidzin. Our result was largely in good agreement with the results reported in those studies. The permeation of daidzin through intestine is limited because of its poor lipophilicity. In recent years, two β-glucosidases, lactase phlorizin hydrolase (LPH) and cytosolic β-glucosidase (CBG) isolated from human small intestine mucosa were shown to hydrolyze flavonoid glycosides into more lipophilic aglycones [33-35]. Furthermore, in an unpublished fermentation study using feces of rats and humans, we demonstrated that daidzin can be rapidly hydrolyzed by colon microflora into daidzein. Therefore, daidzin can serve as a prodrug of daidzein, which is more lipophilic and absorbable by enterocytes. Furthermore, daidzein was further metabolized into sulfates and glucuronides by gut and liver. Our results showed that serum levels and $AUC_{0\text{-}t}$ of daidzein sulfates were significantly higher than those of glucuronides in rats, which was consistent with a previous report [36]; however, our result contradicts another study which stated that glucuronides were the major metabolites [31]. Daidzein sulfates showed a rather long residence time as demonstrated by the detectable concentration in serum up to 48 h post dosing of Puerariae radix. There were several peaks of daidzein sulfates and daidzein glucuronides in the serum profiles, suggesting enterohepatic circulation of these metabolites.

In conclusion, the isoflavones in Puerariae radix were predominantly present as daidzein sulfates and glucuronides in the bloodstream, although sulfates were the dominate form after oral administration to rats. These conjugated metabolites may play important roles in the biological activity of Puerariae radix and are worthy of further investigation.

ACKNOWLEDGMENTS

This work was supported in part by the National Science Council, R.O.C. (NSC 92-2320-B-039-043) and the China Medical University, Taichung, Taiwan, R.O.C. (CMU92-C-02, CMU93-P-09).

REFERENCES

1. Middleton E, Kandaswami C. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In: Harborne JB, ed. The Flavonoids: Advances in Research Since 1986. London, UK: Chapman and Hall, 1993;619-52.
2. Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radic Biol Med* 1994; 16:845-50.
3. Hope WC, Welton AF, Fielder-Nagy C, et al. In vitro inhibition of the biosynthesis of slow reacting substances of anaphylaxis (SRS-A) and lipoxygenase activity of quercetin. *Biochem Pharmacol* 1983;32: 367-71.
4. Li BQ, Fu T, Yan YD, et al. Inhibition of HIV infection by baicalin--a flavonoid compound purified from Chinese herbal medicine. *Cell Mol Biol Res* 1993;39: 119-24.
5. Galati EM, Monforte MT, Kirjavainen S, et al. Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. *Farmaco* 1994;40:709-12.
6. Bors W, Heller W, Michel C, et al. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol* 1990;186:343-55.
7. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple

peels. *J Agric Food Chem* 2003;51:609-14.
8. Wang HK, Xia Y, Yang ZY, et al. Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. [Review] *Adv Exp Med Biol* 1998;439:191-225.
9. Choo MK, Park EK, Yoon HK, et al. Antithrombotic and antiallergic activities of daidzein, a metabolite of puerarin and daidzin produced by human intestinal microflora. *Biol Pharm Bull* 2002;25:1328-32.
10. Doerge DR, Sheehan DM. Goitrogenic and estrogenic activity of soy isoflavones. [Review] *Environ Health Perspect* 2002;110(Suppl 3):349-53.
11. Xu X, Duncan AM, Merz BE, et al. Effect of soy isoflavone on estrogen and phytoestrogen metabolism in postmenopausal woman. *Cancer Epidemiol Biomarkers Prev* 1998;7:1101-8.
12. Potter SM, Baum JA, Teng H, et al. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am J Clin Nutr* 1998;68(6 Suppl):1375-9.
13. Uesugi T, Fukui Y, Yamori Y. Beneficial effects of soybean isoflavone supplementation on bone metabolism and serum lipids in postmenopausal Japanese women: a four-week study. *J Am Coll Nutr* 2002;21:97-102.
14. Han R. Highlight on the studies of anticancer drugs derived from plants in China. *Stem Cells* 1994;12:53-63.
15. Messina MJ, Persky V, Setchell KD, et al. Soy intake and cancer risk: a review of the in vitro and in vivo data. [Review] *Nutr Cancer* 1994;21:113-31.
16. Chan HY, Leung LK. A potential protective mechanism of soya isoflavones against 7,12-dimethylbenz[a]anthracene tumour initiation. *Br J Nutr* 2003;90:457-65.
17. Farhan H, Wahala K, Cross HS. Genistein inhibits vitamin D hydroxylases CYP24 and CYP27B1 expression in prostate cells. *J Steroid Biochem Mol Biol* 2003;84:423-9.
18. Anderson JW, Major AW. Pulses and lipaemia, short- and long-term effect: potential in the prevention of cardiovascular disease. *Br J Nutr* 2002;88(Suppl 3): 263-71.
19. van der Schouw YT, de Kleijn MJ, Peeters PH, et al. Phyto-oestrogens and cardiovascular disease risk. [Review] *Nutr Metab Cardiovasc Dis* 2000;10:154-67.
20. Arora A, Nair MG, Strasburg GM. Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch Biochem Biophys* 1998;356:133-41.
21. Guo Q, Rimbach G, Moini H, et al. ESR and cell culture studies on free radical-scavenging and antioxidant activities of isoflavonoids. *Toxicology* 2002;179:171-80.
22. Ruiz-Larrea MB, Mohan AR, Paganga G, et al. Antioxidant activity of phytoestrogenic isoflavones. *Free Radic Res* 1997;26:63-70.
23. Zhang R, Li Y, Wang W. Enhancement of immune function in mice fed high doses of soy daidzein. *Nutr Cancer* 1997;29:24-8.
24. Yue HW, Hu XQ. Pharmacologic value of radix Puerariae and puerarin on cardiovascular system. [Review] *Zhongguo Zhong Xi Yi Jie He Za Zhi* 1996; 16:382-4. (In Chinese; English abstract)
25. Lee JS, Mamo J, Ho N, et al. The effect of Puerariae radix on lipoprotein metabolism in liver and intestinal cells. *BMC Complement Altern Med* 2002;2:12.
26. Wang X, Wu J, Chiba H, et al. Puerariae radix prevents bone loss in ovariectomized mice. *J Bone Miner Metab* 2003;21:268-75.
27. Kim DH, Yu KU, Bae EA, et al. Metabolism of puerarin and daidzin by human intestinal bacteria and their relation to in vitro cytotoxicity. *Bio Pharm Bull* 1998;21:628-30.
28. Yang CY, Tsai SY, Chao PDL, et al. Determination of hesperetin and its conjugate metabolites in serum and urine. *J Food Drug Anal* 2002;10:143-8.
29. Yasuda T, Kano Y, Saito K, et al. Urinary and biliary metabolites of puerarin in rats. *Biol Pharm Bull* 1995; 18:300-3.
30. Prasain JK, Jones K, Brissie N, et al. Identification of puerarin and its metabolites in rats by liquid chromatography-tandem mass spectrometry. *J Agric Food Chem* 2004;52:3708-12.
31. Yasuda T, Kano Y, Saito K, et al. Urinary and biliary metabolites of daidzin and daidzein in rats. *Biol Pharm Bull* 1994;17:1369-74.
32. Setchell KD, Brown NM, Zimmer-Nechemias L, et al. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr* 2002;76:447-53.
33. Mackey AD, Henderson GN, Gregory JF 3rd. Enzymatic hydrolysis of pyridoxine-5'-beta-D-glucoside is catalyzed by intestinal lactase-phlorizin hydrolase. *J Biol Chem* 2002;277:26858-64.

34. Day AJ, Gee JM, DuPont MS, et al. Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochem Pharmacol* 2003;65:1199-206.
35. Wilkinson AP, Gee JM, Dupont MS, et al. Hydrolysis by lactase phlorizin hydrolase is the first step in the uptake of daidzein glucosides by rat small intestine in vitro. *Xenobiotica* 2003;33:255-64.
36. Piskula MK. Factors affecting flavonoids absorption. *Biofactors* 2000;12:175-80.

葛根異黃酮於鼠體內之代謝動力學

江秀梅[1,6] 葉怡柔[2] 李珮端[3] 徐素蘭[3] 侯鈺琪[4] 姬盈璋[1] 溫國慶[5]

中國醫藥大學 藥物化學研究所[1] 中國藥學研究所[2] 藥學系[3] 中醫系[4] 藥用化妝品學系[5]

仁德醫護管理專科學校 護理科[6]

目的 葛根爲臨床上廣泛使用之中藥，含豐富之異黃酮成分包括葛根素、大豆苷及大豆苷元，本研究探討大鼠口服葛根後，異黃酮之代謝動力學。

方法 利用HPLC梯度沖提法同時定量葛根濃縮散劑中葛根素、大豆苷及大豆苷元之含量。七隻大鼠口服葛根濃縮散劑後於特定時間點採血，血清檢品分別以β-葡萄糖醛酸酶及硫酸酶水解。水解前後之血清檢品分別以HPLC定量，並利用WINNONLIN之非室體模式計算動力學參數。

結果 葛根濃縮散劑中之葛根素、大豆苷及大豆苷元之含量分別爲33.9、1.3及0.9 毫克/克。口服葛根後血清未檢出原型之葛根素、大豆苷及大豆苷元，而以大豆苷元之葡萄糖醛酸及硫酸結合態代謝物存在於血流，且主要爲硫酸結合態代謝物。

結論 此等結合態代謝物於葛根異黃酮之活性上可能扮演重要的角色，值得進一步研究。（中台灣醫誌 2005;10:57-64）

關鍵詞

大豆苷元，大豆苷，異黃酮，藥物動力學，葛根，葛根素

聯絡作者：溫國慶

地　　址：404台中市北區學士路91號

中國醫藥大學 藥用化妝品學系

收文日期：2005年1月20日　　修改日期：2005年3月25日

接受日期：2005年4月1日