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Comparison of urinary kinetics between traditional decoction and concentrated powders of Puerariae Radix in healthy men

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ABSTRACT

Puerariae Radix (PR), the roots of *Pueraria lobata* (Leguminosae), is widely used in clinical Chinese medicine and also as a food in oriental countries. PR is a rich source of isoflavones including puerarin, daidzin and daidzein. This study was aimed to compare the urinary kinetics of isoflavones between traditional decoction (TD) and concentrated powders (CP) of PR in healthy man. Ten male volunteers were given two dosage forms of PR in a crossover design and their urine was collected at specific intervals until 36 h. Urine samples were assayed by an HPLC method before and after hydrolysis with β-glucuronidase and sulfatase. The results indicated that the parent forms of puerarin, daidzin and daidzein were not detected in urine, whereas daidzein sulfates/glucuronides were predominant, mainly sulfates. The half-lives of daidzein sulfates/glucuronides were 5-7 h. In conclusion, the isoflavones were exclusively metabolized to sulfates/glucuronides of daidzein following administration of TD and CP of PR. Through the comparison of urine kinetics between two dosage forms, we suggest that the standardization of the contents of daidzin and daidzein, which are bioavailable, is more important than puerarin content for the efficacy of PR.

Key words: isoflavone, *Pueraria lobata*, puerarin, daidzin, daidzein, pharmacokinetics, metabolites

INTRODUCTION

In recent years, due to the adverse effects arisen from hormone replacement therapy (HRT), natural isoflavones attract increasing attention because of their phytoestrogen properties. In literatures, isoflavones in soybeans including genistin, genistein, daidzin and daidzein have been extensively studied. Their potential usefulness include antiallergic[1] and estrogenic activities[2,3] as well as beneficial effects on blood lipid, bone density[4,5], cancer risk[6-9], cardiovascular protection[10,11], oxidative stress[12-14] and immune responses[15].

Puerariae Radix (PR), the root of *Pueraria lobata* (Leguminosae), is an isoflavone-rich herb widely used in clinical Chinese medicine to treat influenza, stiff neck and cardiovascular accidents. PR is also a food in oriental countries. In Taiwan, traditional decoction (TD) and concentrated powders (CP) of PR are the two dosage forms used clinically, whereas only CP was paid by National Health Insurance. The major isoflavones in PR are puerarin, daidzin and daidzein (chemical structures shown in Figure 1). To understand the biological fate of isoflavones is an important tool to explore the rationale of the clinical implications of PR. Although the plasma and urinary kinetics of isoflavones in soy flour has been reported[16], limited information is available in regard to the metabolism and pharmacokinetics of isoflavones in PR. This study was aimed to investigate the urinary kinetics of PR and

to compare the relative bioavailability of isoflavones between TD and CP of PR in healthy men.

MATERIALS AND METHODS

I. *Chemicals*

Puerarin (purity 80%), daidzin (purity 97%), daidzein (purity 98%), β-glucuronidase (type B-1, from bovine liver), sulfatase (type H-1, from *Helix pomotia*, containing 14,000 units/g of sulfatase and 498,800 units/g of β-glucuronidase) and ethyl paraben were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol and ethyl acetate were of LC grade and purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). L(+)-Ascorbic acid was obtained from Riedel-de Haën (Seelze, Germany). Other reagents were of HPLC grade or reagent grade. Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study.

II. *Preparation of TD and Quantitation of Isoflavones*

The crude drug of PR was purchased from a Chinese medicine store in Taichung, Taiwan. The origin of the crude drug was verified by microscopic examination. Four L water was added to 200 g crude drug and heated on a gas stove. After boiling, gentle heating was continued until the volume was reduced to less than 2 L. The mixture was filtered while hot and sufficient hot water was added to make 2 L which was immediately divided into aliquots (150 mL each) and then frozen at -30_℃ for later use.

PR was quantified as described previously [17]. Briefly, TD (3 mL) was added with 7 mL methanol and the supernatant (180 µL) was added with 20 µL ethyl paraben solution (100 µg/mL in methanol as the internal standard), and 20 µL was subject to HPLC analysis. Gradient elution using 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 - 25 min). The detection wavelength was set at 240 nm and the flow rate was 1.0 mL/min. The calibrators of puerarin, daidzin and daidzein were at concentration ranges of 1.6 - 200.0, 0.6 - 40.0 and 0.3 - 10.0 µg/mL, respectively.

III. *Source of CP of PR and Quantitation of Isoflavones*

Eight commercial products of CP of PR were purchased from Chinese Medicine stores at several cities in Taiwan. The powder (50.0 mg) of each product was accurately weighed and extracted twice with 10 mL 70% methanol. The combined extract was diluted properly and subject to HPLC analysis as described above for the TD.

IV. *Healthy Volunteers and Drug Administration*

Ten healthy male volunteers, aged 20 - 21 y, 54 - 84 kg, with their informed consents provided were included in this study. Routine biochemical tests indicated that their hepatic and renal functions were in good condition. They did not smoke or drink and had not taken medication for at least 2 weeks. In addition, they were asked

to take soy-free diet for 2 weeks before PR administration and throughout the study. The TD and CP of PR were given to the subjects in a crossover design and the washout period between two treatments was 2 weeks. The TD (150 mL) was warmed in a microwave oven and given to each subject followed by 100 mL of warm water. The CP (2.65 g) was administered with 250 mL of warm water. Fasting was continued for another 4 h. This human study protocol had been approved by the Institutional Review Board, China Medical University Hospital, Taichung, Taiwan (DMR93-IRB-33).

V. *Urine Collection and Quantitation of Isoflavones and the Conjugated Metabolites*

Urines of volunteers were collected before and at 0 - 2, 2 - 4, 4 - 6, 6 - 8, 8 - 10, 10 - 12, 12 - 24 and 24 - 36 h after drug administration. The isoflavones and conjugated metabolites in urine were determined before and after hydrolysis with sulfatase and β-glucuronidase, respectively. Urine (400 μL) was added with 100 μL β-glucuronidase (1666 units/mL) and sulfatase (containing 1000 units/mL of sulfatase and 35,600 units/mL of β-glucuronidase) in acetate buffer (pH 5.0), 100 μL of ascorbic acid (200 mg/mL) and incubated at 37 ℃ for 14 h and 2 h, respectively. After hydrolysis, urine was partitioned with 600 μL ethyl acetate (containing 5 μg/mL ethyl paraben as the internal standard) and then centrifuged at 10,000*g* for 15 min. The ethyl acetate layers were evaporated under nitrogen to dryness and reconstituted with

50 µL mobile phase before HPLC analysis.

For calibrator preparation, blank urine was spiked with various standard solutions of daidzein to afford urine standards in the concentration range of 0.2 - 64.0 µg/mL. The procedures were identical with those described above for urine samples except for the addition of enzyme-free buffer. The calibration curve for urine was plotted after linear regression of the peak area ratios (daidzein to the internal standard) with concentrations of daidzein.

VI. *Validation of the Assay Method for Urine*

The precision and accuracy of the analytical method was evaluated by intraday and interday analysis of triplicate urine standards within one day and over a period of three days. Recovery studies were conducted to further assess the accuracy of this method. LLOQ (Lower Limit of Quantitation) represents the lowest concentration of analyte that can be determined with acceptable precision and accuracy, whereas LOD (Limit of Detection) represents the lowest concentration of analyte that can be detected with S/N＞3.

VII. *Data Analysis*

The concentrations of conjugated metabolites of daidzein in urine were multiplied by the respective urine volume, which was collected in each time interval, to obtain the total amount excreted in the sampling time. The renal excretion rates

were calculated by dividing the urinary recovery with the specific collection time interval. The apparent elimination rate constants were estimated by Sigma-Minus method in which the slope of the regression line obtained when the natural logarithm of the metabolite amount not yet excreted was plotted versus time and then transformed into half-lives.

RESULTS

The contents of isoflavones in eight commercial products of PR are shown in Table 1. The product 5 contains the highest concentration of puerarin and was selected for this study. A dose of CP (2.65 g) contained 215.7, 8.5 and 9.0 μmol of puerarin, daidzin and daidzein, respectively, and a dose of TD (150 mL) of PR contained 190.5, 20.6 and 21.9 μmol of puerarin, daidzin and daidzein, respectively. After giving both dosage forms to volunteers, none of the parent forms of puerarin, daidzin and daidzein was detected in urine (data not shown). The concentrations of glucuronides and sulfates/glucuronides of daidzein were determined by HPLC method indirectly through hydrolysis with glucuronidase and sulfatase, respectively. The optimal hydrolysis condition was determined in a preliminary study, which indicated that the optimal reaction times needed for glucuronidase and sulfatase were 14 h and 2 h, respectively in the presence of ascorbic acid under anaerobic condition.

Good linearity of daidzein in blank urine was found in the concentration range of 0.2 - 64.0 μg/mL. Validation of this method indicated that all coefficients of variation for intraday and interday analysis were less than 6.9%, and the relative errors were below 10.0%. The recoveries of daidzein from urine were 94.8-111.8% at concentrations of 5.0, 2.5 and 1.25 μg/mL. LLOQ and LOD were 0.2 μg/mL and 0.03 μg/mL, respectively.

Owing to considerable amount of β-glucuronidase in the sulfatase (type H-1) used in this study, treatment with this enzyme resulted in the hydrolysis of both sulfates and glucuronides. Figure 2 shows the mean renal excretion rate of daidzein sulfates/glucuronides and daidzein glucuronides of ten volunteers during each urine collection interval following administration of TD and CP of PR. The cumulated urinary recoveries of daidzein glucuronides and daidzein sulfates/glucuronides over 36 h are shown in Figure 3. The total amounts of daidzein glucuronides and daidzein sulfates/glucuronides excreted in urine were 10.6 and 55.1 μmol after administration of TD, 3.9 and 27.1 μmol after administration of CP, respectively.

Through comparison of the released amounts of daidzein between treatments with sulfatase and glucuronidase, the concentrations of daidzein sulfates in urine were estimated. The urinary recovery of daidzein sulfates was found about 4-5 folds of daidzein glucuronides after administration of both dosage forms. Through calculation by Sigma-Minus method, the half-lives of daidzein sulfates/glucuronides were about 5-7 h.

DISCUSSION

Quantitation of the isoflavones in CP of PR showed great variability of the contents of puerarin, daidzin and daidzein for about 30-fold, 10-fold and 10-fold among eight products, respectively. The standardization of PR products by the manufacturer is essential to maintain consistent quality. The contents of three isoflavones in all products were consistently ranked in the order of puerarin > daidzin > daidzein, indicating that glycosides were more abundant than aglycones.

The analytical method of urine with satisfactory precision, accuracy and recovery was developed and validated in this study. The major isoflavones found in human urine were sulfates/glucuronides of daidzein, whereas the parent forms of puerarin, daidzin and daidzein were not detected. The absence of puerarin and daidzin, isoflavone glycosides, in human urine was in good agreement with many previous studies reporting that flavonoids glycosides were not absorbed *per se*[18-21]. In addition, this fact was also consistent with our previous finding that puerarin and daidzin were not present in the serum of rats administered with CP of PR[17].

Being an O-glycoside, daidzin was not permeable through the membrane of enterocytes. In recent years, two enzymes with activity toward flavonoid glycosides were isolated from human small intestine mucosa: lactase phlorizin hydrolase (LPH) and cytosolic β-glucosidase (CBG), which could hydrolyze daidzin into more

lipophilic daidzein that was absorbable[22-24]. Besides, daidzin can be rapidly hydrolyzed to daidzein by colonic microflora as demonstrated in fermentation with ~~of~~ rats and humans feces (data not shown). Therefore, daidzin serves like a precursor of daidzein. However, daidzein was not detected in urine, instead, its sulfates and glucuronides of daidzein were found. It can be proposed that daidzin was hydrolyzed to daidzein in gut lumen and then extensively transformed into sulfates and glucuronides by gut and/or liver during the first pass.

In regard to the determination of daidzein conjugates, the optimum reaction times for glucuronidase and sulfatase have been determined to be 14 h and 2 h, respectively, in this study. However, two previous studies reported that the hydrolysis took 3 h or 15-18 h for both conjugates [25,26]. The discrepancy of reaction time might be due to different amounts of enzyme used among studies. Owing to the presence of considerable amount of glucuronidase in the sulfatase used in this study, only 2 h was needed for the optimal hydrolysis of sulfates/glucuronides. In all urine specimens, comparison of daidzein liberated between treatments with sulfatase/glucuronidase and glucuronidase showed that daidzein sulfates were the major metabolites. This result was in good agreement with our previous finding in the serum of rat administered with CP[17], but contradictory to another study reporting daidzein glucuronides as the major metabolites in rat urine after administration of daidzin[25]. This differential

conjugation pathway between sulfation and glucuronidation might be due to the difference of matrix[27].

With regard to the bioactivities of daidzein conjugates, they were found to be weakly estrogenic and capable of activating human natural killer cells[28,29]. In addition, two recent studies have reported that the serum metabolites of PR induced the apoptosis of breast cancer cells and promoted peripheral nerve regeneration[30,31]. Therefore, the sulfates/glucuronides of daidzein warrant more bioactivity studies to better understand the clinical implication of PR.

About half of daidzein sulfates/glucuronides in urine were excreted within 0 - 8 h after administration of both dosage forms of PR and they were detectable up to 36 h. The excretion of daidzein glucuronides and daidzein sulfates/glucuronides after ingestion of TD were significantly higher than those after ingestion of CP through the urine collection period. In this study, the doses of two dosage forms were designed to contain equal amount of total isoflavones including puerarin, daidzin and daidzein. The content of daidzein/daidzin in TD (42.5 μmol) was 2.4-fold of CP (17.5 μmol); whereas the content of the major isoflavone puerarin (190.5 μmol) in TD was lower than CP (215.7 μmol) by 8.5%. When the total urinary recoveries of daidzein sulfates/glucuronides during 0-36 h between two dosage forms were compared, the amount (55.1 μmol) excreted after administration of TD was 2.1-fold of that of CP

(27.1 μmol), which was comparable with the relative abundance of daidzein/daidzin between two dosage forms. Accordingly, we can infer that daidzin and daidzein were absorbable, whereas the C-glycoside puerarin was not bioavailable, although puerarin was the major isoflavone in PR. Unexpectedly, the total urinary recoveries of daidzein sulfates/glucuronides after administration of TD (55.1 μmol) was greater than the total intake dose of daidzin/daidzein (42.5 μmol). Likewise, the total urinary recovery of daidzein sulfates/glucuronides (27.1 μmol) after administration of CP was greater than the total intake dose of daidzin/daidzein (17.5 μmol). These facts implied that other O-glycosides of daidzein besides daidzin may also be present in PR. Due to the unknown contents of the total O-glycosides of daidzein in TD and CP, the relative bioavailability between two dosage forms can not be calculated. The half-lives of daidzein sulfates/glucuronides were about 5-7 h. Considerable variation of half-lives of the conjugated metabolites was observed among individuals, which may be explained by the inter-subject differences with regard to the variability in enterobacteria, metabolizing enzymes or drug transporters.

A previous study has detected puerarin in urine and serum after oral administration of puerarin in rats [21]. However, the present study did not detect any puerarin in human urine. Consistently, our previous study has not detected puerarin in the serum of rats administered with CP of PR [17]. Being a C-glycoside of isoflavone,

puerarin was found not transformed to the aglycone daidzein through incubation with the ~~of~~ rats and human feces in our laboratory (data not shown), indicating that puerarin was resistant to the hydrolysis by enterobacterial enzymes. Therefore, unlike daidzin, puerarin could not be metabolized to absorbable daidzein in gut lumen, which can be accounted for by the chemical nature of C-glycoside. The present study added human evidence illustrating that puerarin was neither absorbable nor hydrolyzable to absorbable aglycone.

In conclusion, the isoflavones were exclusively metabolized to sulfates and glucuronides of daidzein following administration of TD and CP of PR. Through the comparison between two dosage forms, we suggest that the quantitation of daidzin and daidzein, representing bioavailable isoflavones, is more important than puerarin in PR.

ACKNOWLEDGMENTS

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Legends for figures:

Figure 1. Chemical structures of daidzein (a) daidzin (b) and puerarin (c)

Figure 2. Urinary excretion rate (μmol/h) of daidzein sulfates/glucuronides; daidzein glucuronides during each collection interval in 10 volunteers after intake of traditional decoction (TD) and concentrated powders (CP) of Puerariae Radix.

Figure 3. Cumulated urinary recoveries (μmol) of daidzein sulfates/glucuronides; daidzein glucuronides in 10 volunteers after intake of traditional decoction (TD) and concentrated powders (CP) of Puerariae Radix.

Title for table:

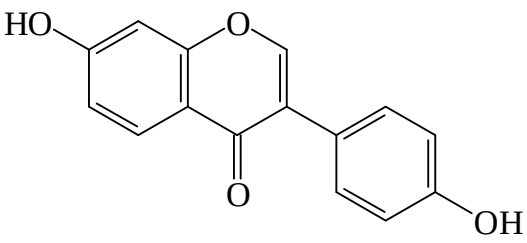
Table 1. Contents (mg/g) of puerarin, daidzin and daidzein in eight commercial products of the concentrated powders of Puerariae Radix.

Table 1

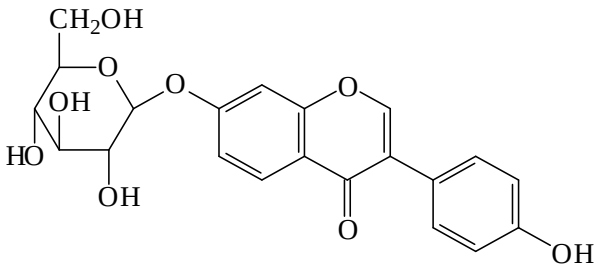
Constituents / Samples	puerarin	daidzin	daidzein
1	3.6 ± 0.0	0.80 ± 0.0	0.3 ± 0.0
2	2.9 ± 0.1	0.5 ± 0.0	0.1 ± 0.0
3	3.6 ± 0.3	1.0 ± 0.1	0.3 ± 0.0
4	3.6 ± 0.2	1.2 ± 0.1	0.2 ± 0.0
5	34.0 ± 2.7	1.3 ± 0.0	0.9 ± 0.0
6	20.0 ± 0.4	5.6 ± 0.2	0.7 ± 0.0
7	6.5 ± 1.1	0.6 ± 0.1	0.3 ± 0.2
8	4.4 ± 0.6	0.5 ± 0.1	0.3 ± 0.1
Mean	9.8 ± 0.7	1.4 ± 0.1	0.4 ± 0.0

Data are expressed as Mean ± SD (n = 3)

Figure 1.



(a)



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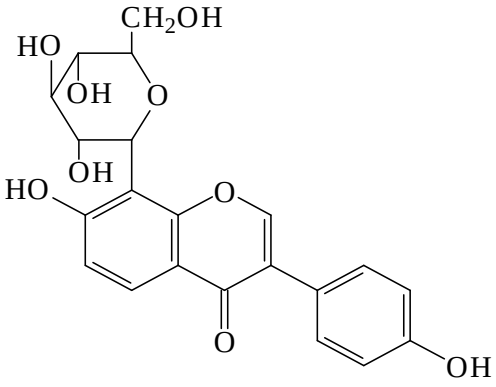


(c)

Figure 2.

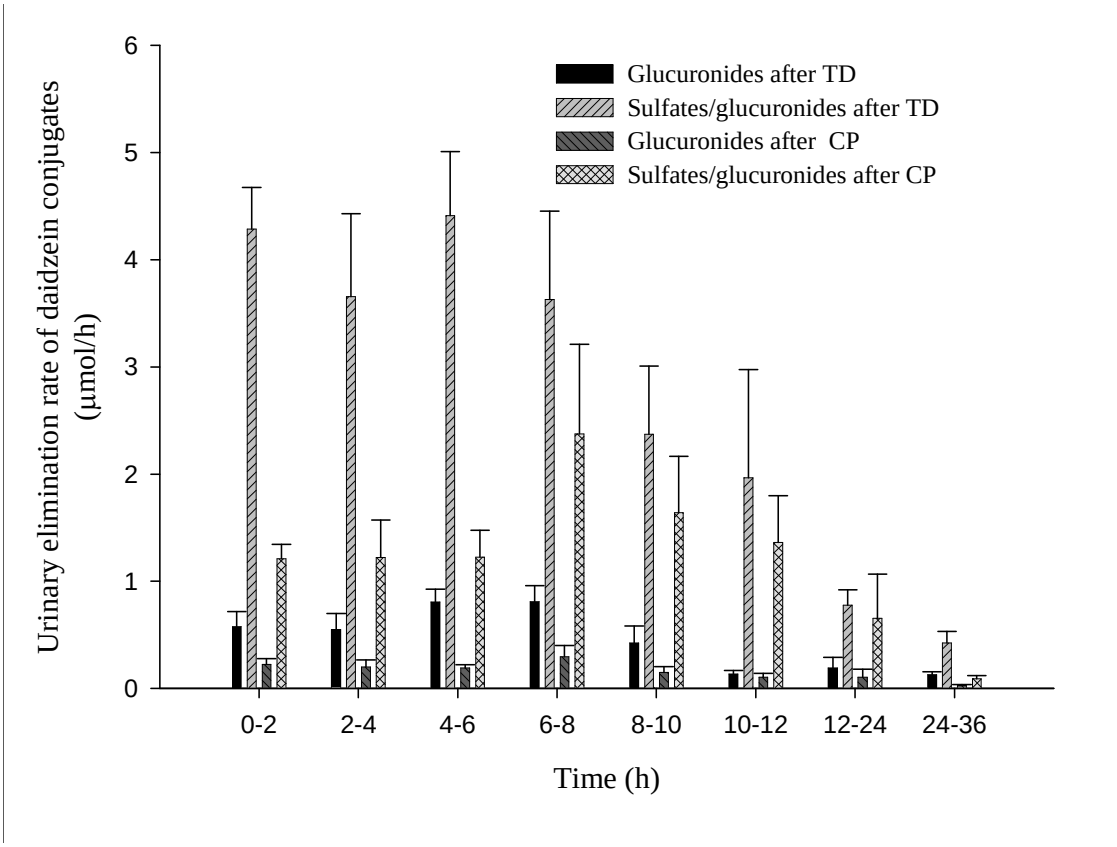
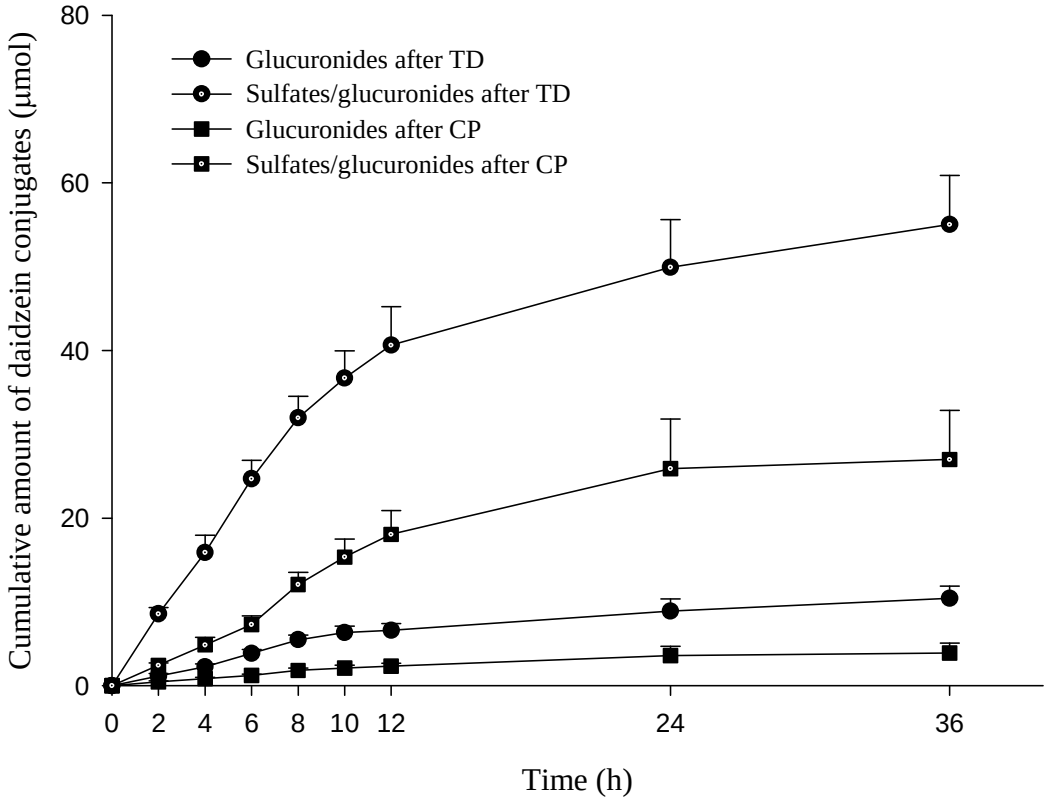

Urinary elimination rate of daidzein conjugates (µmol/h)
Glucuronides after TD
Sulfates/glucuronides after TD
Glucuronides after CP
Sulfates/glucuronides after CP
0
1
2
3
4
5
6
0-2
2-4
4-6
6-8
8-10
10-12
12-24
24-36
Time (h)

Figure 3.


Glucuronides after TD
Sulfates/glucuronides after TD
Glucuronides after CP
Sulfates/glucuronides after CP
Cumulative amount of daidzein conjugates (µmol)
0
20
40
60
80
0
2
4
6
8
10
12
24
36
Time (h)

葛根水煎劑與濃縮散劑於健康人體尿藥動力學之比較

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摘要

葛根為常用中藥，富含異黃酮成分，如 puerarin、daidzin、daidzein 等。本研究比較葛根水煎劑與濃縮散劑於健康~~中國~~人體的尿藥動力學。十位男性志願受試者口服葛根水煎劑與濃縮散劑，以交叉設計給藥，收集尿液 36 h。尿液樣品於水解之前與之後利用 HPLC 分析。研究結果顯示，尿中異黃酮主要以 daidzein 之 sulfates、glucuronides 等結合態代謝物的形式存在。Daidzein sulfates/glucuronides 的半生期約 5-7 h。綜言之，口服葛根水煎劑與濃縮散劑後，異黃酮幾全代謝成 daidzein sulfates/glucuronides，主要為 sulfates。藉由葛根兩種劑型間尿藥動力學之比較，結果顯示 daidzin、daidzein 是可為生物體利用的異黃酮，因此對葛根之療效而言，其含量比 puerarin 重要。

關鍵詞：異黃酮，葛根，puerarin， daidzin，daidzein，尿藥動力學，代謝物