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Determination and Disposition Kinetics of Phellamurin Metabolites in Rat Brain

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ABSTRACT

Phellamurin is a flavanone glycoside that is abundant in the leaves of *Phellodendron wilsonii* Hayata et Kanehira (Rutaceae). The disposition kinetics of phellamurin metabolites in rat brain was investigated in this study. Doses of 100 mg/kg and 200 mg/kg were orally administered to rats. At 0.33, 2, 4 and 6 hr after dosing, rats were dissected and the brain concentrations of the aglycone neophellamuretin were determined by an HPLC method prior to and after hydrolysis with β-glucuronidase. The mobile phase was acetonitrile: H_2O (containing 1% acetic acid, 38:62, v/v). Our results indicated that neophellamuretin glucuronide might be able to cross the blood-brain barrier and its concentration in the brain was dose-dependent at the two doses investigated, whereas the aglycone neophellamuretin was present in a trace amount in the brain.

Key words: phellamurin, neophellamuretin, neophellamuretin glucuronides, brain distribution

INTRDUCTION

Flavonoids have attracted much attention in recent years because of their beneficial pharmacological activities including antioxidation[(1)], free radical scavenging[(2)], anticancer[(3)] as well as antiviral activity[(4)], and their additional ability to modulate drug efflux systems[(5-8)]. Phellamurin, 3,4',5,7-tetrahydroxy-8-isoprenylflavanone-7-glucoside (Figure 1), is a flavanone glycoside that is abundant in the leaves of *Phellodendron wilsonii* Hayata et Kanehira (Rutaceae) which is endemic in Taiwan and commonly used in clinical Chinese medicine[(9)]. In our previous study, the metabolic pharmacokinetics of phellamurin was reported[(10)]. It was found that the aglycone neophellamuretin accounted for 20% of the exposure of circulating metabolite of phellamurin, whereas neophellamuretin glucuronides accounted for the other 80%. The present study attempted to investigate the disposition kinetics of phellamurin metabolites in rat brain at doses of 100 mg/kg and 200 mg/kg.

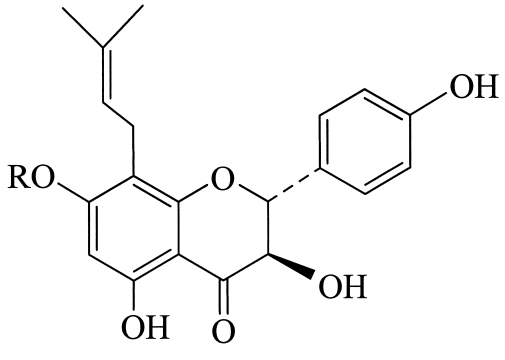
MATERIALS AND METHODS

I. *Chemicals*

β-Glucosidase (from almonds) and β-glucuronidase (from *Helix pomatia*, containing 121,900 units/mL of β-glucuronidase and 4,100 units/mL of sulfatase) were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Acetonitrile (LC grade) and ethyl acetate (LC grade) were obtained from Alps (Taipei, Taiwan) and Mallinckrodt Baker, Inc. (U.S.A). Hesperetin (95%) was purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Dimethylacetamide was obtained from Wako (Osaka, Japan). PEG 400 and hydrochloric acid were purchased from Merck (Germany). Milli-Q plus water (Millipore, Bedford, MA, U. S. A.) was used for all preparations.

II. *Instrumentation and HPLC Conditions*

The HPLC apparatus included two pumps (LC-10AT, Shimadzu, Japan), a communication bus module (CBM-10A, Shimadzu, Japan), a diode array detector (SPD-M10A,



Phellamurin : R = Glucose
Neophellamuretin : R = H

Figure 1. Structures of phellamurin and neophellamuretin.

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Shimadzu, Japan) and CLASS-LC 10/M10A software (Shimadzu, Japan). The RP-18e column (LiChrospher 100, 5 μm, 250 × 4 mm) was equipped with a guard column (LiChrospher 100, 5 μm). The mobile phase was acetonitrile-1% acetic acid (38:62) and the flow rate was 1 mL/min with the detection wavelength set at 287 nm.

III. *Preparation of Neophellamuretin*

Neophellamuretin standard was obtained from hydrolysis of phellamurin by β-glucosidase. The detailed procedures had been described in a previous report[10].

IV. *Preparation of Calibration Curve*

Various concentrations of 50 μL neophellamuretin solution (in MeOH) were spiked into 450 μL brain homogenate to afford a series of brain standards consisting of 0.1, 0.2, 0.3, 0.6, 1.2, 2.5 and 5.0 μg/mL. To each 500 μL brain homogenate, 200 μL acetate buffer (pH 5) and 5 μL of ascorbic acid (200 mg/mL) were added, followed by the addition of 750 μL of EtOAc (containing 1.0 μg/mL hesperetin). The mixture was vortexed for 20 sec and then centrifuged at 9860 × g for 15 min, the EtOAc layer was removed and evaporated to dryness under N-EVAP, then the residue was reconstituted with 160 μL CH_3CN, of which 20 μL was subjected to HPLC analysis.

The peak-area ratios (neophellamuretin to hesperetin) of brain standards were determined. A calibration curve was calculated by linear regression of the peak-area ratios against neophellamuretin concentrations.

V. *Validation of Assay Method*

The precision and accuracy of the method were assessed by intra-day and inter-day assays of brain standards. Recoveries were determined in quartet and calculated based on the detected concentrations of neophellamuretin in brain compared to those in Ringer solution.

VI. *Animals and Drug Administration*

Twenty four male Sprague-Dawley rats weighing between 250 and 350 g were fasted for 12 hr before dosing. The oral solution of phellamurin (40.0 mg/mL) was prepared by dissolving in a solvent containing water: PEG 400 : dimethylacetamide (5:4:1). Rats were administered with phellamurin at the doses of 100 mg/kg and 200 mg/kg via gastric gavage. At 0.33, 2, 4 and 6 hr after dosing, triplicate rats were dissected and the brains were taken, carefully washed with ice cold Ringer solution and then frozen at -30˚C for later analysis.

VII. *Assay of Brain Samples*

After thawing, 0.5 g of brain was homogenized with 1.0 mL Ringer solution using TRI-R STIR-R (model S63C, TRI-R instruments, Rockville center, N.Y.). For the assay of free form neophellamuretin, the process described above for brain standards was followed. For the assay of neophellamuretin with its glucuronides/sulfates, brain homogenate samples (500 μL) were mixed with 50 μL ascorbic acid and 200 μL β-glucuronidase (β-glucuronidase 89.4 units/mL, sulfatase 3.3 units/mL in acetate buffer, pH 5), and then incubated anaerobically at 37˚C for 1 hr for hydrolysis as had been previously determined. The subsequent procedures were identical as for brain standards.

VIII. *Pharmacokinetic Analysis*

The elimination constants were calculated by using WINNONLIN (version 1.1; SCI software, Statistical Consulting Inc., Apex, NC). The maximum brain concentration (C_{max}) and the corresponding time (T_{max}) were recorded as observed.

RESULTS AND DISCUSSION

In this study, an HPLC method was established for the assay of neophellamuretin in brain. Neophellamuretin was well separated with hesperetin as the internal standard and both could be eluted within 20 min. The retention times of neophellamuretin and internal standard were 15.8 min and 9.2 min, respectively (Figure 2). The regression line was y = 0.321X – 0.005 (r = 0.9999). The good linearity were found within the ranges of 0.2~5.0 μg/mL. The precision and accuracy for intra-day and inter-day assays were satisfactory, as shown in Table 1. The LOQ (limit of quantitation) was 0.2 μg/mL and the LOD (limit of detection) was 6.2 ng/mL. The recoveries of neophellamuretin from brain at concentrations of 5.0, 1.2 and 0.3 μg/mL were 87.6 ± 2.1%, 86.6 ± 0.8% and 82.9 ± 1.2%, respectively, as shown in Table 2.

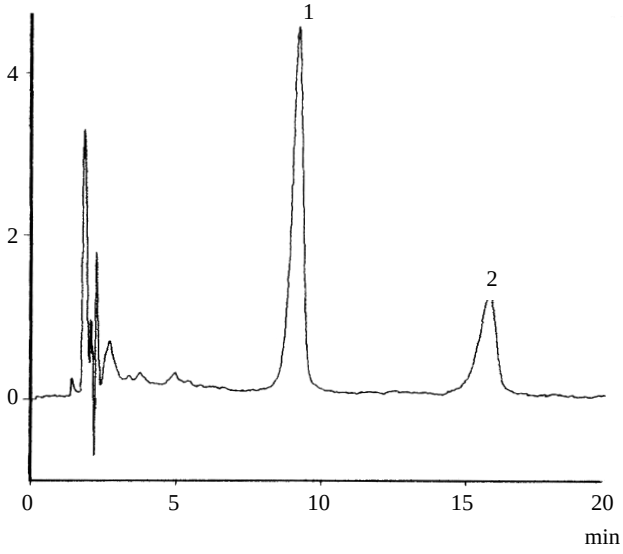

Figure 2. HPLC chromatogram of hesperetin as internal standard (1) and neophellamuretin (1.4 μg/mL) (2) in a brain sample at 20 min after oral dosing of 200 mg/kg phellamurin to a rat.

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Table 1. Intraday and interday analytical precision and accuracy of neophellamuretin in brain

Conc.	Precision				Accuracy	
	Intra-day		Inter-day		Intra-day	Inter-day
μg/mL	Mean ± S.D	(C.V.%)	Mean ± S.D.	(C.V.%)	Relative error (%)	Relative error (%)
5.0	$5.0 \pm 0.0_5$	1.0	$5.0 \pm 0.0_5$	1.0	0.2	–0.2
2.5	$2.5 \pm 0.0_8$	3.2	$2.5 \pm 0.0_9$	3.7	0.9	0.4
1.2	$1.3 \pm 0.0_7$	5.7	$1.3 \pm 0.0_4$	3.1	1.7	1.2
0.6	$0.6 \pm 0.0_1$	1.6	$0.6 \pm 0.0_3$	4.6	–3.0	–0.3
0.3	$0.3 \pm 0.0_2$	7.0	$0.3 \pm 0.0_1$	2.1	–8.7	–9.1
0.2	$0.2 \pm 0.0_1$	9.2	$0.2 \pm 0.0_1$	7.5	4.3	3.8

Table 2. Recovery (%) of neophellamuretin from rat brain

	5.0 μg/mL	1.2 μg/mL	0.3 μg/mL
1	90.5	85.1	85.2
2	88.8	86.6	81.1
3	83.5	88.0	82.4
Mean ± S.E.	87.6 ± 2.1	86.6 ± 0.8	82.9 ± 1.2

Mean brain concentration-time profiles of neophellamuretin glucuronides after oral administration of phellamurin are shown in Figure 3 for doses of 100 mg/kg and 200 mg/kg. The T_{max} occurred at 20 min after dosing, indicating fast brain distribution of neophellamuretin glucuronides. The brain concentrations of neophellamuretin glucuronides after the dose of 200 mg/kg were much higher than those after 100 mg/kg. The C_{max} of neophellamuretin glucuronides was 1.7 ± 0.4 μg/g of brain at the dose of 200 mg/kg, whereas that was only 0.4 ± 0.1 μg/g at the dose of 100 mg/kg, although our previous study found that there was no significant difference in serum levels between the administrations of the two doses[10].

Our previous study indicated that when phellamurin was administered orally, the parent compound was not detected in serum, as hydrolysis to neophellamuretin occurred before absorption. The rate and extent of neophellamuretin absorption were quite good. About 80% of the absorbed neophellamuretin was circulating in plasma as its glucuronides, the other 20% was in the form of aglycone neophellamuretin[10]. Our present results found that neophellamuretin glucuronides

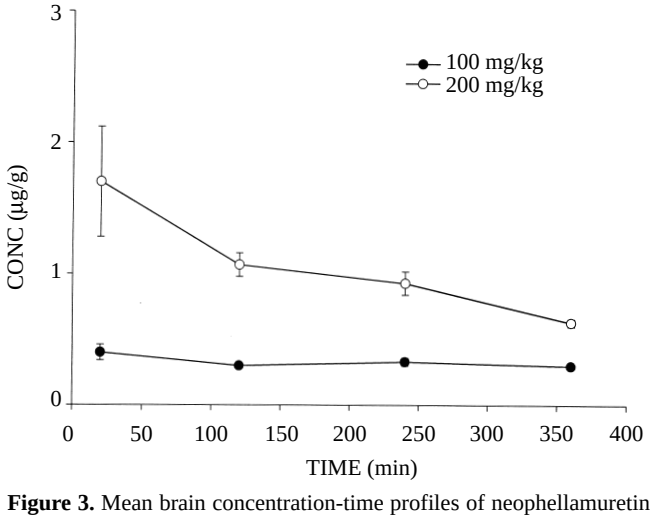

Figure 3. Mean brain concentration-time profiles of neophellamuretin glucuronides after oral dosing of 100 mg/kg and 200 mg/kg phellamurin to rats (n=3 for each time point).

accounted for the majority of the metabolites in brain and only a trace of neophellamuretin aglycone was detected, but lower than LOQ. Glucuronides in general are considered as highly polar metabolites unable to cross the blood-brain barrier. Although it has been discussed that morphine glucuronidation occurred in human brain tissue, the capacity is very low compared to that of the liver, indicating that morphine glucuronides can penetrate into the brain despite their high polarity[12]. This study also indicated that neophellamuretin glucuronides might be able to cross the blood-brain barrier. Glucuronides represent metabolites that are not generally inactive and may contribute to drug action either directly or indirectly[13].

Our previous drug interaction study found that concurrent oral administration of phellamurin significantly decreased the total exposure of cyclosporin in plasma by 55.7% in rats[14]. The pharmacokinetic and statistical analysis indicated that phellamurin markedly decreased the absorption of cyclosporin. Cyclosporin is a substrate of both CYP 3A4 and Pgp (P-glycoprotein)[15,16], the product of *mdr* (multidrug - resistance) genes. Therefore, the decreased absorption of cyclosporin could be accounted for by the induction of CYP3A4 or/and Pgp by phellamurin or its metabolites. Because phellamurin has been demonstrated to inhibit the efflux of a Pgp substrate rhodamine 123 in an everted sac study[14], the major causative agents for the interaction could be in turn explainable by its major metabolites, neophellamuretin glucuronides which formed very rapidly with a T_{max} at 30 min after oral dosing of phellamurin[10]. Pgp is expressed in various normal human tissues such as small intestine, kidney, liver and capillary endothelial cells of brain and testis[17-20], and is a defense mechanism limiting oral bioavailability and CNS accumulation of drugs[21]. Therefore, it is suspected that the presence of neophellamuretin glucuronides in brain might induce brain Pgp and result in limitation of CNS accumulation of other drugs. The effects of phellamurin metabolites on the metabolizing enzyme CYP 3A4 and the drug efflux pump Pgp are worthy of further investigation.

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黃柏素-7-葡萄糖苷之代謝物在大白鼠腦中之分析及動力學

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摘　要

黃柏素-7-葡萄糖苷是台灣黃蘗的樹葉中含量頗豐的一種雙氫黃酮類配醣體。本研究探討黃柏素-7-葡萄糖苷之代謝物在大鼠腦中之動力學。大鼠口服100 mg/kg和200 mg/kg兩種劑量，於給藥後0.33，2，4及6小時將大鼠犧牲，分析腦中黃柏素-7-葡萄糖苷之代謝物。定量方法係採用高效液相層析法，檢測腦組織經β-葡萄糖醛酸酶水解之前及之後所含新黃柏素之濃度。移動相為乙腈：水（含1%醋酸，38：62，v/v）。結果顯示新黃柏素葡萄糖醛酸可能穿越血腦障蔽，其腦中之濃度呈現劑量相關性，而新黃柏素在腦中的含量則極微。

關鍵詞：黃柏素-7-葡萄糖苷，新黃柏素，新黃柏素葡萄糖醛酸，腦中分析