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Measurement of daphnoretin in plasma of freely moving rat by liquid chromatography

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Abstract

Daphnoretin (7-hydroxyl-6-methoxy-3,7′-dicoumaryl ether), isolated from *Wikstronemia indica* C.A. Mey. (*Thymelaceae*), has been reported to induce rabbit platelet aggregation through protein kinase C activation and anticancer activity. In this study, we developed an automated blood sampling system coupled to a simple and sensitive HPLC system to determine plasma concentration of daphnoretin in rats. This method was applied to investigate the pharmacokinetics of daphnoretin in a freely moving rat. Separation of daphnoretin in the rat plasma was achieved using a reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm) with a mobile phase of methanol–10 mM NaH_2PO_4 (adjusted to pH 3.0 with H_3PO_4) (55:45, v/v), and the flow rate of 1.0 ml/min. The UV detector was set at 345 nm. The automated blood sampling system (DR-II has been applied for blood sampling in a conscious and freely moving rat. The blood samples were centrifuged at 3000 × *g* for 10 min and the plasma samples were then deproteinized by acetonitrile containing an internal standard (khellin 1 μg/ml). After centrifugation (8000 × *g* for 10 min), the aliquot of supernatant was injected into the HPLC system for analysis. The concentration–response relationship from the present method indicated linearity over a concentration range of 0.05–1.00 and 1.00–100 μg/ml. Intra- and inter-assay precision and accuracy of daphnoretin fell well within the predefined limits of acceptability (≤15%). After daphnoretin (500 mg/kg) was given orally, the maximum concentration was 0.17 μg/ml at the time of 5 min. The oral bioavailability was about 0.15%.
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Keywords: Daphnoretin; Liquid chromatography; *Wikstroemia indica*

1. Introduction

The poisonous plant *Wikstroemia indica* C.A. Mey (*Thymelaceae*) has been used in Chinese folk medicine as herbal remedy for the treatment of syphilis, arthritis, whooping cough [1] and cancer [2]. The herb has also been used for medication in the Far East for the relief of fever, pain and wounds. The herbal ingredient daphnoretin (7-hydroxy-3,7-dicoumaryl ether; Fig. 1) has been reported for in vitro antitumor use [3,4] and the activation of protein kinase C [5–7]. However, intoxicating syndrome of the herb includes vomiting and diarrhea.

To date, no any assay method has been reported for the determination of daphnoretin in biological fluids. Importantly, to ensure the effectiveness in vivo and prevent harmful toxicological consequences, a validated assay method is required to monitor the concentration of daphnoretin in biological fluids. The aim of this study was to develop an automated blood sampling system coupled with a liquid chromatographic method to detect daphnoretin in a freely moving rat. Application of the assay to the pharmacokinetics of daphnoretin is demonstrated. In addition, the LC–tandem mass spectrometry (LC–MS–MS) was used to determine the molecular weight of purified daphnoretin.

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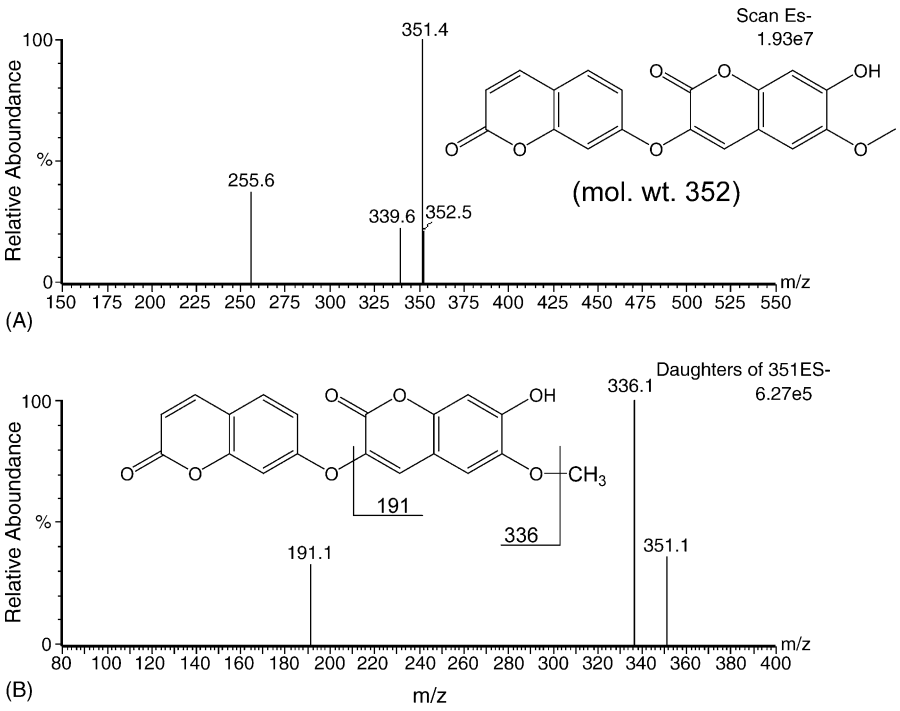

Fig. 1. Chemical structure of daphnoretin (molecular weight 352). (A) Full scan mass spectrum of daphnoretin, and (B) its daughter ions in LC–tandem mass spectrometry with electrospray positive-ion mode.

2. Experimental

2.1. Isolation and purification of daphnoretin

Daphnoretin was isolated from *W. indica*. The dried and crushed roots of *W. indica* (3.6 kg) were extracted three times with 95% EtOH (30 l, 50 °C) for 16 h (for each extraction). The ethanolic extract (210 g) was then partitioned successively between H_2O and EtOAc, followed by *n*-BuOH (each 1 l 3×). The EtOAc extract (72 g) was subjected to silica gel column chromatography with a gradient of EtOAc in *n*-hexane, and four fractions were collected. Fraction 2 (12.6 g) was rechromatographed over silica gel (30% EtOAc in *n*-hexane) to give compound daphnoretin (2.7 g). Daphnoretin: recrystallized from $CHCl_3$–MeOH to give light yellow needles, m.p. 239–240 °C; ^{1}H NMR ([$^2H_6$] dimethyl sulfoxide, (DMSO-d_6) δ 3.81 (3H, s, C$\underline{H}_3$-6), 6.37 (1H, d, J = 9.5 Hz, H-3′), 6.85 (1H, s, H-8), 7.11 (1H, d, J = 8.5 Hz, H-6′), 7.18 (1H, s, H-8′), 7.20 (1H, s, H-5), 7.70 (1H, d, J = 8.5 Hz, H-5′), 7.87 (1H, s, H-4), 8.04 (1H, d, J = 9.5 Hz, H-4′); ^{13}C NMR (DMSO-d_6) δ 56.0 (6-O$\underline{C}H_3$), 102.7 (C-8), 104.0 (C-8′), 109.4 (C-5), 110.1 (C-4a), 113.4 (C-6′), 113.9 (C-3′), 114.4 (C-4a′), 130.0 (C-5′), 130.9 (C-4), 135.7 (C-3), 144.0 (C-4′), 145.6 (C-6), 147.4 (C-8a), 150.3 (C-7), 155.0 (C-8a′), 156.9 (C-2), 159.6 (C-7v), 159.9 (C-2′) [8]; elecrospray ionisation (ESI) MS *m*/*z* 351 $[M-H]^-$, 336 $[M-CH_3–H]^-$, 191 [6-methoxy, 7-hydroxycoumarin ion]$^-$.

Standard daphnoretin was dissolved in methanol at a concentration of 1 mg/ml using a brown glass vial and stored at a 4 °C refrigerator. Solvents and reagents of liquid chromatographic grade were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Liquid chromatography

HPLC was performed with a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), a Rheodyne Model 7125 injector equipped with a 20 μl sampling loop and an ultraviolet detector (Linear Model 340, San Jose, CA, USA). Separation was achieved by a LiChrosorb RP-18 column (Merck, 250 mm × 4 mm i.d.; particle size 5 μm). The mobile phases consisted of methanol–10 mM NaH_2PO_4 (adjusted to pH 3.0 with H_3PO_4) (55:45, v/v) with a flow-rate 1 ml/min. The detection wavelength was 345 nm. Output data from the detector were integrated using an EZChrom chromatographic data system (Scientific Software, San Roman, CA, USA).

2.3. Method validation

All calibration curves were required to have a correlation value of at least 0.995. The intra- and inter-assay variabilities were determined by quantitating six replicates at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml using the HPLC method described above on the same day and six consecutive days, respectively. The lowest concentration of the linear regression defined the limit of quantitation (LOQ). The accuracy (% bias) was calculated from the mean value of observed concentration (C_{obs}) and the nominal concentration (C_{nom}) as follows: accuracy:

(% bias) = $[(C_{obs} - C_{nom})/C_{nom}] \times 100$. The relative standard deviation (R.S.D.) was calculated from the observed concentrations as follows: precision (% R.S.D.) = [standard deviation (S.D.)/C_{obs}] × 100.

2.4. LC–tandem mass spectrometry

LC–MS–MS analysis was performed using a Waters 2690 with a 996 photodiode array detection (DAD) system together with an automatic liquid chromatographic sampler and an autoinjection system hyphenated to a Micromass Quattro Ultima tandem quadrupole mass spectrometry (Micromass, Manchester, UK) equipped with an ESI source. The separation was achieved using a reversed-phase C_{18} column (150 mm × 4.6 mm i.d.) (Agilent, USA). The solvent delivery system was kept constant at 200 μl/min and the mobile phase consisted of 50% methanol. The volume of injection was 10 μl. For operation in MS–MS mode, a mass spectrometer with an orthogonal Z-spray ESI interface was used. The infusion experiment was performed using a Mode 22 multiple syringe pump (Harvard, Holliston, MA, USA). During the analyses, the ESI parameters were set as follows: capillary voltage, 2.0 kV for negative mode; source temperature, 80 °C; desolvation temperature, 200 °C; cone gas flow, 60 l/h; and desolvation gas flow, 450 l/h. The cone voltage of *m*/*z* 315 was 60 V and the collision voltages were 20 eV. All LC–MS–MS data were processed by the MassLynx version 4.0 NT Quattro data acquisition software.

2.5. Animals

All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of the National Research Institute of Chinese Medicine. Male specific pathogen-free Sprague–Dawley rats were obtained from the Laboratory Animal Center of the National Yang Ming University, Taipei. The animals had free access to food (Laboratory rodent diet 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to being used in experiments, at which time only food was removed. Six Sprague–Dawley rats (280–320 g) were initially anesthetized with pentobarbital (50 mg/kg, i.p.), and remained anesthetized throughout the experimental period. During the period of surgery, the body temperature of rats was maintained at 37 °C with a heating pad. After surgery, the rats were installed in the experimental cage and allowed to recover for 1 day.

2.6. Blood sampling and sample preparation

Daphnoretin (500 mg/kg) was given orally and the femoral vein was exposed for daphnoretin injection (10 mg/kg). The automated blood sampling system DR-II (Eicom Kyoto, Japan) has been applied for blood sampling in conscious and freely moving rats. A 150 μl blood sample was withdrawn from the jugular vein into a heparin rinsed vial with fraction collector according to a programmed schedule at 5, 10, 20, 30, 40, 50 min, 1, 1.5, 2, 2.5 and 3 h after dosing. Each blood sample was centrifuged at 3000 × *g* for 10 min. The resulting plasma sample (50 μl) was vortex-mixed with 100 μl of internal standard (khellin, 1 μg/ml) solution. The denatured protein precipitate was separated by centrifugation at 8000 × *g* for 10 min. An aliquot (20 μl) of the supernatant was directly injected onto the HPLC for analysis. Data from these samples were used to construct pharmacokinetic curves of daphnoretin concentration in blood versus time. The same sample handling process was used for the determination of precision.

Prior to using the blood sampling module with experimental animals, we performed in vitro experiments to evaluate fluid delivery precision and carry-over level via the pumping tubes. Two sets of whole blood samples, which contained 10 and 25 μg/ml daphnoretin respectively, were siphoned through the sampling module tubes. Each set of samples had three replicates.

2.7. Pharmacokinetic application

Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Pharsight, Mountain View, CA, USA) by noncompartmental method. The area under the concentration–time curve (AUC) and the area under the first moment curve (AUMC) were calculated according to the log linear trapezoidal method. The clearance (Cl) was calculated as follows: Cl = dose/AUC.

3. Results and discussion

3.1. Mass spectrometry

Daphnoretin was purified from *W. indica* C.A. Mey. (*Thymelaceae*) and its molecular weight was identified by

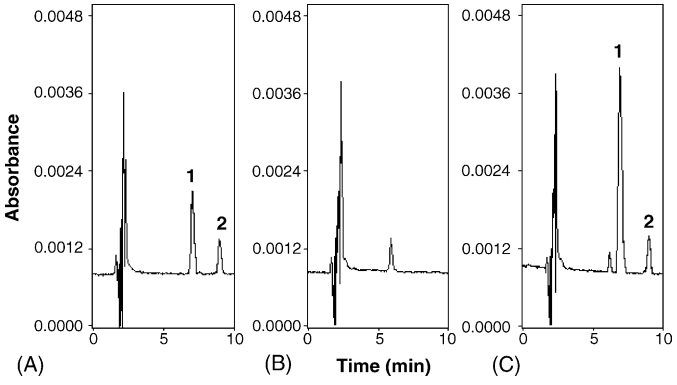

Fig. 2. Chromatograms of daphnoretin. Part (A) shows a chromatogram of a standard of daphnoretin (1 μg/ml). Part (B) shows a chromatogram of a drug-free plasma extract. Part (C) shows the chromatogram of a plasma sample containing daphnoretin (2.84 μg/ml) collected from a rat plasma 10 min after daphnoretin administration (10 mg/kg, i.v.). (1) Daphnoretin; (2): internal standard (khellin).

Table 1
Standard curves of HPLC assay of daphnoretin in rat plasma

	Slope	Intercept	r
Day 1	0.439	−0.0258	0.999
Day 2	0.437	−0.0272	0.999
Day 3	0.469	−0.0774	0.996
Day 4	0.451	−0.0617	0.998
Day 5	0.452	−0.0463	0.997
Day 6	0.441	−0.0237	0.999
Mean	0.448	−0.0437	0.998
S.D.	0.012	0.0222	0.001

LC–tandem mass spectrometry. The spectrum revealed a base peak at *m*/*z* 351 corresponding to the quasi-molecular ion $[M-H]^-$ (Fig. 1A). When collided with reagent gas, the selected parent ion *m*/*z* 351 produced the daughter ion spectrum shown in Fig. 1B. In this figure, we attribute the base peak at *m*/*z* 336 to the loss of one methyl group at the C-6 position. The other peak *m*/*z* 191 corresponds to 6-methoxy-7-hydroxy coumarin.

3.2. Chromatography

Daphnoretin in rat plasma was quantitated using LC coupled with ultraviolet detection. Peak scanning by the photodiode array (Waters 2695 module coupled to 2696 detector; Waters Millipore, Milford, MA, USA) detection revealed the maxima wavelength of daphnoretin to be 345 nm. Fig. 2A shows a chromatogram of a standard of daphnoretin (1 μg/ml). Fig. 2B shows a chromatogram of a drug-free plasma extract, illustrating a clean, stable baseline with no interfering endogenous peaks. Run-time was set at 10 min, and no carry-over peaks were detected in subsequent chromatograms of plasma samples. Fig. 2C shows the chromatogram of a plasma sample containing daphnoretin (2.84 μg/ml) collected from rat plasma 10 min after daphnoretin administration (10 mg/kg, i.v.). The analytes were well separated using the present chromatographic conditions. The retention times were 7.1 and 9.1 min for daphnoretin and internal standard, respectively, and no peak distortions were visible.

Table 3
Pharmacokinetic data after daphnoretin administration (10 mg/kg, i.v. and 500 mg/kg, p.o.) in rats

Parameters	10 (mg/kg, i.v.)	500 (mg/kg, p.o.)
AUC (min μg/ml)	117 ± 5	17 ± 2
$t_{1/2}$ (min)	51 ± 4	93 ± 26
Cl (ml/(kg min))	0.086 ± 0.004	0.031 ± 0.002

Data are expressed as mean ± S.E.M. from six individual experiments for each group.

3.3. Linearity

Linear least-square regression analysis of the calibration graph on six different days demonstrated linearity between the response and the nominal concentration of daphnoretin over the range of 0.05–100 μg/ml. Table 1 shows the equations of the standard curves of daphnoretin on six different days. The results of linear regression analysis show that the correlation coefficients of all standards curves were better than 0.995. The data show the excellent reproducibility of the sample analysis.

3.4. Limit of detection

The limit of detection (LOD) of daphnoretin in rat plasma was determined to be 0.01 μg/ml at a signal-to-noise ratio of 3. The lower limit of quantitation was 0.05 μg/ml.

Table 2
Method validation for the intra- and inter-assay precision (% R.S.D.) and accuracy (% bias) of the HPLC method for the determination of daphnoretin

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	Precision (R.S.D., %)	Accuracy (bias, %)
Intra-assay			
0.05	0.053 ± 0.005	9.4	6.7
0.10	0.099 ± 0.011	11.1	−1.2
0.50	0.503 ± 0.032	6.4	0.6
1.0	0.981 ± 0.038	3.9	−2.0
5.0	5.099 ± 0.190	3.7	−1.9
10.0	10.074 ± 0.296	2.9	0.7
50.0	50.118 ± 1.699	3.4	0.2
100.0	101.802 ± 1.962	1.9	1.8
Inter-assay			
0.05	0.054 ± 0.006	11.1	8.3
0.10	0.093 ± 0.003	3.2	−6.8
0.5	0.506 ± 0.008	1.6	1.1
1.0	1.032 ± 0.049	4.7	3.2
5.0	4.956 ± 0.133	2.7	−1.1
10.0	9.968 ± 0.400	4.0	−0.3
50.0	50.144 ± 0.879	1.8	0.2
100.0	101.908 ± 1.968	1.9	1.9

Observed concentration data are expressed as mean ± S.D. ($n = 6$).

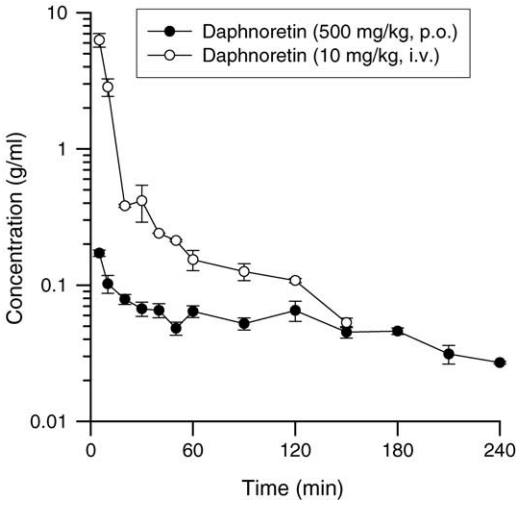

Fig. 3. Concentration vs. time curves of daphnoretin after drug administration (500 mg/kg, p.o. and 10 mg/kg, i.v.) in rats.

3.5. Precision, accuracy and recovery

The intra- and inter-assay precision and accuracy values are presented in Table 2. The overall mean precision, defined by the R.S.D., ranged from 11.1 to 1.6%. Analytical accuracy, expressed as the percent difference of the mean observed values compared with known concentration varied from 8.3 to −6.8%. The extraction recovery of daphnoretin in rat plasma was 99.70% at the concentration of 1 μg/ml.

3.6. Carry-over examination

The collected blood samples were determined to maintain high recovery levels, 98.3% of 10 μg/ml and 99.3% of 25 μg/ml. During the interval of two spiked sample collections, one blank whole blood sample was flushed through the tubes. The results of the flushed blank determination indicate that no measurable carry-over was found in the automated blood sampling system.

3.7. Pharmacokinetic application

This method was developed for the application in pharmacokinetic study of daphnoretin in a freely moving rat. Fig. 3 illustrates the concentration versus time profiles of daphnoretin with a single intravenous and oral dose administration to six individual rats for each group. The area under the concentration versus time curves were 117 ± 5 and 17 ± 2 min μg/ml for intravenous (10 mg/kg) and oral (500 mg/kg) doses, respectively. The oral bioavailability $(AUC_{p.o.}/dose)/(AUC_{i.v.}/dose)$ of daphnoretin in freely moving rat was 0.15% (Table 3).

It has been reported that either anesthesia or conscious restraint traditional methods may cause stress to the animal and affect the pharmacokinetics [9]. This automated blood sampling system provides a major advantage by allowing free movement, which can minimize the stress caused by the restraint or anesthesia. Recently, pharmacokinetic studies associated with automated blood systems have been reported [10–12]. Lee and Sarna [13] indicate that stress may delay gastric emptying and slow the absorption of drugs in the gastrointestinal tract.

In conclusion, an automated blood sampling connected to a liquid chromatographic assay using ultraviolet detection was developed to monitor daphnoretin in rat plasma. This method is potentially useful evaluating biological fluids and additional pharmacokinetic studies.

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