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Short communication

High-performance liquid chromatography assay of gnetol in rat serum and application to pre-clinical pharmacokinetic studies

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ABSTRACT

A method of analysis of gnetol (2,3′,5′,6-tetrahydroxy-*trans*-stilbene) in biological fluids is necessary for the study of its kinetics and disposition in plants. A simple high-performance liquid chromatography (HPLC) method was developed for the determination of gnetol in rat serum using a reverse-phase, isocratic system. Separation was achieved using a Phenomenex® Luna C_{18} (2) column with ultraviolet detection at 305 nm. The standard curves were linear ranging from 0.5 to 100 μg/ml. The mean extraction efficiency was >90.5%. Precision of the assay was <14% (R.S.D.), and was within 14% at the limit of quantitation (0.5 μg/ml). Bias of the assay was lower than 15%, and was within 15% at the limit of quantitation. The assay was applied successfully to the study of serum disposition of gnetol in rats.

1. Introduction

The naturally occurring stilbenoid, gnetol (2,3′,5′,6-tetrahydroxy-*trans*-stilbene), is a structural analog of resveratrol, a well known polyphenolic compound with anti-inflammatory, anti-cancer, and cardioprotective properties [1–3]. Gnetol has been isolated extensively in the genus *Gnetum* specifically in the species *Gnetum gnemon* [4], *Gnetum montanum* [5], *Gnetum hainanense* [6], and *Gnetum klossii* [7]. Various species of *Gnetum* are used in traditional medicine throughout Asia. Specifically, the seeds and fruits of *G. gnemon* commonly called Melinjo are used as traditional foods throughout Southeast Asia [8]. Extracts of *G. montanum* have been used in traditional Chinese medicine for the treatment of arthritis and bronchitis [5]. Because of the history of traditional use, extracts of this genus are now being sold as health food supplements.

Gnetol's close structural similarity to resveratrol may suggest similar pharmacological activities. However, only one publication has documented any of the activities of gnetol. Ohguchi et al. have shown that gnetol possesses strong inhibitory action on tyrosinase, the rate limiting enzyme in melanin synthesis, and gnetol can inhibit melanogenesis in murine B16 melanoma cells [9]. Other research reports on gnetol are limited in the literature with no HPLC detection methods or pharmacokinetic studies for gnetol being published. It is the purpose of the present study to develop a novel, isocratic, reversed-phase HPLC method using ultraviolet (UV) detection for the determination of gnetol and its application to evaluate the *in vivo* pharmacokinetics of gnetol in a rat model.

2. Experimental

2.1. Chemicals and reagents

Gnetol was supplied by the Sabinsa Corporation (NJ, USA). Daidzein, dimethyl sulfoxide (DMSO), and β-glucuronidase from *Escherichia coli* type IX-A were purchased from Sigma Chemicals (MO, USA). HPLC grade acetonitrile and water were purchased from J.T. Baker (NJ, USA). Silastic® laboratory tubing was purchased from Dow Corning Corporation (MI, USA). Intramedic® polyethylene tubing was purchased from Becton Dickinson Primary Care Diagnostics, Becton Dickinson and Company (MD, USA). Monoject® 23 gauge (0.6 mm × 25 mm) polypropylene hub hypodermic needles were purchased from Sherwood Medical (MO, USA). Synthetic absorbable surgical sutures were purchased from Wilburn Medical US (NC, USA). Rats were obtained from Simonsen Laboratories (CA, USA). Ethics approval for animal experiments was obtained from Washington State University.

Abbreviations: HPLC, high-performance liquid chromatography; UV, ultraviolet; PAR, peak area ratio; R.S.D., relative standard deviation; S.E.M., standard error of the mean; DMSO, dimethyl sulfoxide; LOQ, limit of quantitation; V_d, volume of distribution.

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2.2. HPLC system and conditions

The reversed-phase HPLC system used was a Shimadzu LC-2010A (Kyoto, Japan). Data analysis was accomplished using Shimadzu EZStart 7.4 software (Kyoto, Japan). A Phenomenex® Luna C_{18} (2) analytical column (250 mm × 4.6 mm; 5 μm) was used with UV detection at 305 nm (CA, USA). The mobile phase consisted of acetonitrile, water and formic acid (35:65:0.04, v/v/v) that was filtered and degassed prior to use with isocratic separation carried out at a flow rate of 0.5 ml/min.

2.3. Stock and working standard solutions

Methanolic stock solutions of gnetol and the internal standard (daidzein) were prepared at a final concentrations of 100 μg/ml. These solutions were protected from light and stored at −20 °C between uses for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solution of gnetol by sequential dilution with blank rat serum, yielding a series of concentrations namely, 0.5, 1, 5, 10, 50, and 100 μg/ml.

2.4. Sample preparation

To the working standards or samples (0.1 ml), 25 μl of daidzein was added as well as 1 ml of cold acetonitrile to precipitate proteins. Samples were then vortexed for 1 min (VWR Scientific, PA, USA) and centrifuged at 10,000 rpm for 5 min (Beckman Coulter, Inc., CA, USA). The supernatant was collected and evaporated to dryness under a stream of compressed nitrogen gas. The residue was reconstituted with 200 μl of mobile phase, vortexed for 30 s and centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to HPLC vials and 100 μl of it was injected into the HPLC system. All serum samples including calibration curve and pharmacokinetic samples were prepared in the same manner.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using six different concentrations of gnetol (0.5–100 μg/ml). The between-run precision and accuracy of the assays were estimated from the results of calibration curve samples on six different days within one week. The precision was evaluated by the relative standard deviation (R.S.D.). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [10].

2.6. Recovery

Recovery of gnetol from biological fluids was assessed ($n = 3$) at 1, 10, and 100 μg/ml and the recovery of the internal standard was evaluated at the concentration used in sample analysis (100 μg/ml). One milliliter of cold acetonitrile was added to precipitate the proteins in serum, which was followed by centrifugation at 10,000 rpm for 5 min. The supernatant was transferred to a new vial and injected for HPLC analysis. The extraction efficiency was determined by comparing the peak area ratio (PAR) of gnetol and daidzein to the PAR of corresponding concentration injected directly in the HPLC system without extraction.

2.7. Freeze–thaw stability of gnetol samples

The freeze–thaw stability of gnetol was evaluated at six concentrations over a range of 0.5–100 μg/ml. These samples were analyzed without being frozen at first, and then stored at −20 °C and thawed at room temperature (25 ± 1 °C) for three cycles.

2.8. Pharmacokinetic disposition of gnetol in rats

Male Sprague–Dawley rats ($n = 3$, average weight of 180 g) were anaesthesized using isoflurane and a silastic catheter was cannulated into the right jugular vein. The animals were allowed to recover and fasted overnight. On the day of experiment, the rats were dosed intravenously with 10 mg/kg gnetol in 2% DMSO dissolved in polyethylene glycol 600. A series of blood samples (0.30 ml) was collected at 0, 1, 15, 30 min, and then at 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h. Following centrifugation of the blood samples in regular centrifuge tubes, serum was collected and stored at −20 °C until analysis. Serum samples (0.1 ml) were run in duplicate with or without the addition of 40 μl of 500 U/ml β-glucuronidase from *E. coli* type IX-A and incubated in a shaking water bath at 37 °C for 2 h to liberate any glucuronide conjugates [11].

2.9. Data analysis

Quantification was based on calibration curves constructed using PAR of gnetol to internal standard, against gnetol concentrations using unweighted least squares linear regression. Pharmacokinetic analysis was performed using data from individual rats for which the mean and standard error of the mean (S.E.M.) were calculated for each group. Pharmacokinetic modeling was completed using WinNonlin® software (Ver. 1.0).

3. Results and discussion

3.1. Chromatography

Separation of gnetol and the internal standard in biological fluids was achieved successfully (Fig. 1). There were no interfering peaks co-eluted with the compounds of interest (Fig. 1). The retention times of gnetol and the internal standard, daidzein, were 10.6 and 13.9 min, respectively. The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in biological fluid, linearity, limit of quantitation (LOQ), freeze–thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. A range of mobile phases and flow rates were attempted in the development of this assay. This includes a range of ratios of acetonitrile and water and methanol and water with varying flow rates between 0.2 and 1.0 ml/min. The optimal separation was achieved when the combination of acetonitrile, water and formic acid was 35:65:0.04 (v/v/v) and the flow rate of 0.5 ml/min.

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 0.9999$) were demonstrated between PAR of gnetol to the internal standard over a range of 0.5–100 μg/ml. The mean regression lines from the validation runs were described by gnetol (μg/ml) = $0.0417x - 0.0195$. The LOQ of this assay was 0.5 μg/ml in biological fluids with the corresponding between day R.S.D. of 13.7% and bias of 13.0%. The back-calculated concentrations of samples were within the acceptance criteria (Table 1).

3.3. Precision, accuracy and recovery

The within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of gnetol in rat serum was <14% over a wide range of concentrations (Table 1). The intra- and

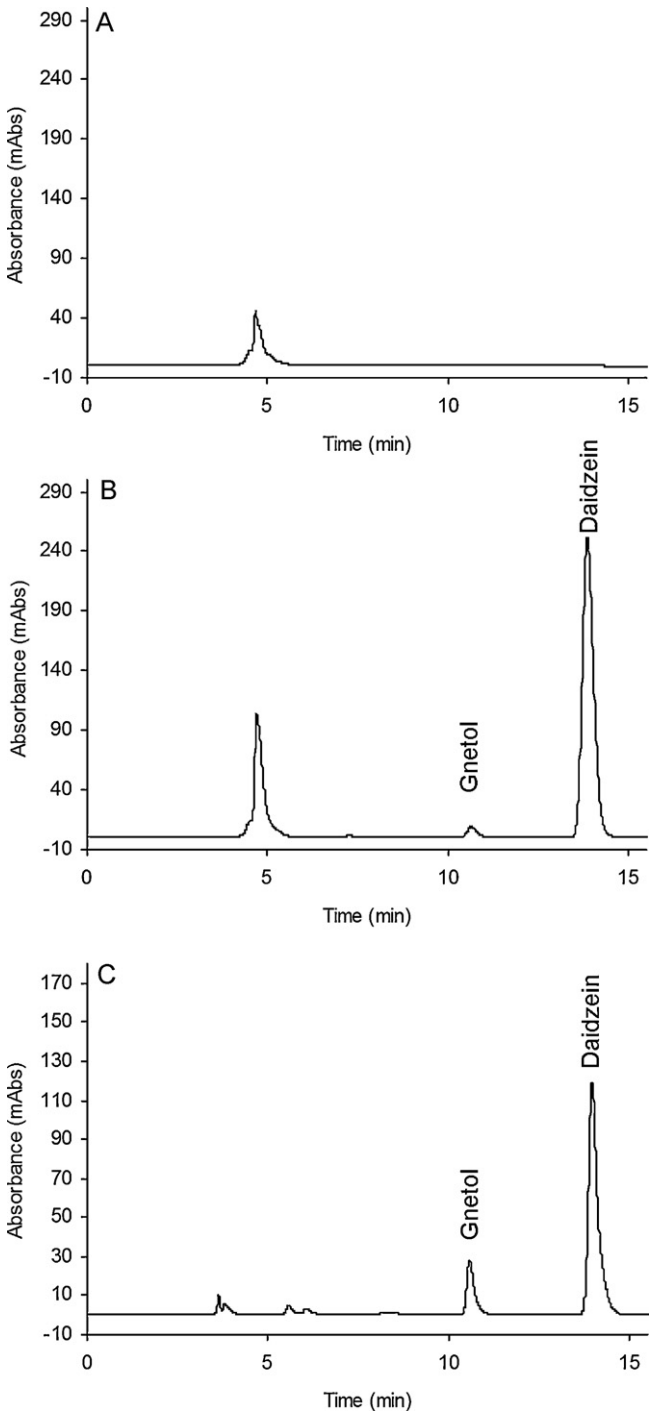

Fig. 1. Representative chromatograms of (A) drug-free serum demonstrating no interfering peaks co-eluted with the compounds of interest, (B) serum containing gnetol at the LOQ of 0.5 μg/ml with internal standard, daidzein, and (C) a pharmacokinetic sample of free gnetol 1 min after IV administration of gnetol at 10 mg/kg.

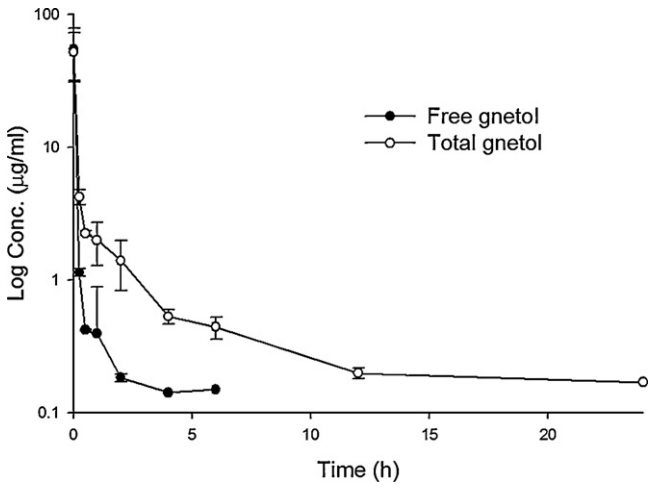

Fig. 2. Disposition in serum of free gnetol and the total gnetol (both free and glucuronidated forms) following IV administration of gnetol (10 mg/kg) to rats ($n = 3$, mean ± S.E.M.).

inter-run bias assessed during the replicate assays for gnetol varied between −8.86 and 14.4% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for gnetol from biological fluids varied from 90.5 to 102.7% with the use of acetonitrile extraction. Other extraction procedures were attempted including methanol and ethyl acetate extractions, but these procedures ultimately had lower recovery. High recovery from biological fluids with the use of acetonitrile extraction suggests there is negligible loss of gnetol during the protein precipitation process.

3.4. Stability of gnetol samples

No significant degradation was detected after the samples of gnetol in biological fluids following three freeze–thaw cycles. The recoveries of gnetol were respectively from 96.7 to 100.7% following three freeze–thaw cycles.

3.5. Pharmacokinetic study of gnetol in rats

The HPLC method was applied to the determination of gnetol in pharmacokinetic studies in rats ($n = 3$). To our knowledge, this is the first report of gnetol pharmacokinetics in any species. Following administration of gnetol IV, the serum disposition was examined (Fig. 2). Gnetol was detected in serum both in its aglycone form and as a glucuronide. This rapid glucuronidation of gnetol parallels other stilbene compounds including pterostilbene [12] and resveratrol [13]. Pharmacokinetic parameters include a relatively short half life of 1.19 h and a large volume of distribution (V_d) indicating distribution of gnetol into tissues (Table 2).

Table 1
Within- and between-day precision and accuracy of the assay for gnetol in rat serum ($n = 6$, mean, R.S.D., and bias).

Gnetol concentration (μg/ml)						
Added	Observed		R.S.D (%)		Bias (%)	
	Within-day	Between-day	Within-day	Between-day	Within-day	Between-day
0.5	0.572	0.565	4.01	13.7	14.4	13.0
1.0	0.987	0.933	2.46	11.9	−1.31	−6.73
5.0	5.43	4.98	1.46	6.91	8.59	−0.434
10	9.44	9.11	1.06	6.54	−5.61	−8.86
50	51.6	50.9	2.98	0.712	3.18	1.78
100	100.0	99.8	0.0866	0.257	0.0108	−0.191

Table 2
Pharmacokinetic parameters of gnetol after IV administration at 10 mg/kg ($n = 3$).

PK parameter	Mean ± S.E.M.
V_d (L/kg)	5.60 ± 3.37
$t_{1/2}$ (h)	1.19 ± 0.49

4. Conclusions

In summary, the developed HPLC method for gnetol is sensitive, reproducible, and accurate. It has been applied in the study of the pharmacokinetics of gnetol in rats for the first time. The HPLC method presented here has also been used in our laboratory in the determination of serum and urine disposition of gnetol (unpublished data). Further studies are ongoing in our laboratory to characterize the pharmacological activities of gnetol as well as other polyphenolic compounds.

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