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High-performance liquid chromatographic analysis of pterostilbene in biological fluids using fluorescence detection

Connie M. Remsberg, Jaime A. Yáñez, Kathryn A. Roupe, Neal M. Davies *

College of Pharmacy, Department of Pharmaceutical Sciences and Pharmacology and Toxicology Graduate Program, Washington State University, Pullman, WA 99164-6534, United states

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Abstract

A method of analysis of pterostilbene [trans-3,5-dimethoxy-4′-hydroxystilbene] is necessary to study the kinetics of in vitro and in vivo metabolism and determine its concentration in foodstuffs. A novel and simple high-performance liquid chromatographic method was developed for determination of pterostilbene in rat serum. Serum proteins (0.1 mL) are precipitated with cold acetonitrile after addition of the internal standard, pinosylvin. Separation was achieved on a Phenomenex C18 column (250 mm × 4.60 mm) with fluorescence excitation at 330 nm and emission at 374 nm. The calibration curves were linear ranging from 0.5 to 100 μg/mL. The mean extraction efficiency was >99%. Precision of the assay was <15% (CV), and was within 14% at the limit of quantitation (0.5 μg/mL). Bias of the assay was lower than 14%, and was within 9% at the limit of quantitation. The assay was applied successfully to the study of pterostilbene pharmacokinetics in rats.

Keywords: Reversed-phase HPLC; Fluorescence detection; Pterostilbene; Stilbene

1. Introduction

Pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene) $C_{16}H_{16}O_3$, MW 256.296 g/mol (Fig. 1a), is a naturally occurring stilbene found in deerberry and rabbiteye blueberries, several varieties of grapes, and in the tree species *Pterocarpus marsupium* and *Guibourtia tessmanii* [1–6]. Much of the current interest in pterostilbene may be that it is a dimethylated analogue of resveratrol, a stilbene shown to have anti-cancer activity across many cell lines. In fact, pterostilbene has demonstrated anti-cancer activity in vitro in B-16 melanoma cells, HL60 promyelocytic leukemia cells, leukemia cells that express the anti-apoptotic oncogene Bcr-Abl and cells that express the multidrug resistant (MDR) phenotype [7–9]. In addition, pterostilbene has also been demonstrated to have anti-diabetic and anti-oxidant properties [10–13]. The pharmaceutical application of several stilbenes including pterostilbene has recently been reviewed [14].

In order to recognize the metabolism kinetics of pterostilbene, it is important to understand its metabolic pathways in biological fluids. Currently, no study has been published on the pharmacokinetics of pterostilbene. Before evaluating the biotransformation of pterostilbene, a selective and sensitive assay for its quantification must be developed. Pterostilbene has been previously quantified via high-performance liquid chromatography (HPLC) using gradient elution, gas chromatography after methylation and mass spectrometry [1,2,4,15]. To our knowledge, there are no validated analytical methods in the literature to quantify pterostilbene in biological matrices. The present study describes a novel, isocratic, reversed-phase HPLC method for the determination of pterostilbene and its application to in vivo kinetic studies.

2. Experimental

2.1. Chemicals and reagents

Pterostilbene and pinosylvin was purchased from Sequoia Research Products Ltd., Oxford, United Kingdom. HPLC grade

* Corresponding author. Tel.: +1 509 335 4754; fax: +1 509 335 5902.
E-mail address: ndavies@wsu.edu (N.M. Davies).

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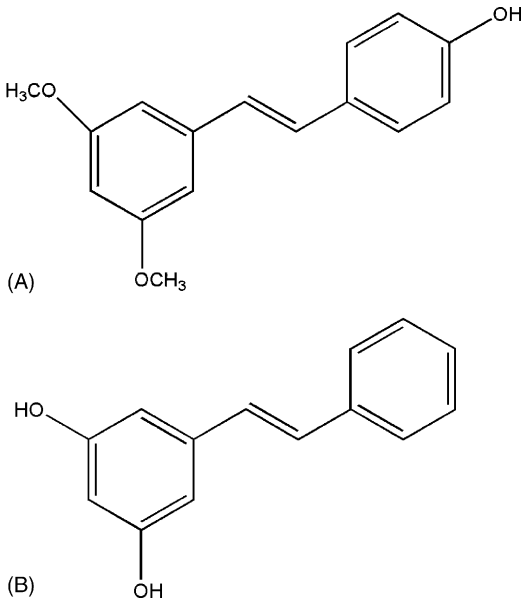

Fig. 1. Structures of: (A) pterostilbene and (B) internal standard, pinosylvin.

methanol, acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Rats were obtained from Charles River Laboratories. Ethics approval for animal experiments was obtained from Washington State University.

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AD pump, a SIL-10A auto-injector, a RF-535 fluorescence detector and a SCL-10A system controller. Data collection and integration were achieved using a Shimadzu CR501 chromatopac integrator. The analytical column used was a Phenomenex C18 (250 mm × 4.60 mm). The mobile phase consisted of acetonitrile and HPLC water (50:50, v/v) that was filtered and degassed under reduced pressure prior to use. Separation was carried out isocratically at ambient temperature and a flow rate of 1 mL/min, with fluorescence detection excitation at 330 nm and emission at 374 nm.

2.3. Stock and working standard solutions

Methanolic stock solutions of pterostilbene (1 mg/mL) and the internal standard, pinosylvin (1 mg/mL), were prepared. The pinosylvin solution was diluted with methanol to make a working internal standard (IS) solution of 10 μg/mL. These solutions were protected from light and stored at 4 °C between uses for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solutions of pterostilbene by sequential dilution with blank rat serum, yielding a series of concentrations namely, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL.

Quality control (QC) samples were prepared from stock solution of pterostilbene by dilution with blank rat serum to yield target concentrations of 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL. The QC samples were divided into 0.1 mL aliquots in micro centrifuge tubes and stored at −20 °C before use.

2.4. Sample preparation

To the working standards or samples (0.1 mL), 25 μL of internal standard solution (10 μg/mL) was added. The mixture was precipitated with 1 mL cold acetonitrile, vortexed (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 5000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was transferred to new Eppendorf tubes and evaporated to dryness by a stream of nitrogen gas. The residue was reconstituted with 200 μL of mobile phase, vortexed for 30 s and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to HPLC vials and 50 μL was injected into the HPLC system.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested using six different concentrations of pterostilbene, namely 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL. The between-run precision and accuracy of the assays were estimated from the results of eight replicate assays on 8 different days during a 2-week period. 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 [16].

2.6. Recovery

Recovery of pterostilbene from rat serum was assessed ($n = 3$) at 0.5, 5.0 and 50.0 μg/mL. A known amount of pterostilbene was spiked into 0.1 mL rat serum to give the above concentrations. One milliliter of cold acetonitrile was added to precipitate the proteins in the serum, which was followed by centrifugation at 5000 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 pterostilbene and IS to the PAR of corresponding concentration injected directly in the HPLC system without extraction.

2.7. Stability of pterostilbene samples

The freeze-thaw stability of pterostilbene was evaluated at six concentrations 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL. These samples were analyzed in triplicate without being frozen at first and then stored at −20 °C and thawed at room temperature (25 ± 1 °C) for three cycles.

The stability of pterostilbene in reconstituted extracts during run-time in the HPLC auto-injector was investigated using pooled extracts from QC samples of six concentration levels 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL. Samples were kept in the sample rack of the auto-injector and injected into HPLC sys-

tem every 4 h, from 0 to 24 h at the temperature of auto-injector ($26 \pm 1\,^{\circ}C$).

2.8. Pharmacokinetic disposition of pterostilbene in rats

Male Sprague–Dawley rats ($n = 3$, 250 g) were anaesthetized using halothane and a silastic catheter was cannulated into the right jugular vein. The animals were placed in metabolic cages where they recovered overnight and were fasted for 12 h before dosing. On the day of the experiment, the animal was dosed intravenously with pterostilbene (20 mg/kg) in polyethylene glycol 600. A series of blood samples (0.5 mL) were collected at 0, 1, 10 min, 0.5, 1, 2, 4, 6 and 24 h. The cannula was flushed with 0.25 mL saline after each sample collection. Following centrifugation of the blood samples, serum was removed and stored at $-20\,^{\circ}C$ until analyzed. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Washington State University.

2.9. Data analysis

Quantification was based on calibration curves constructed using peak area ratio (PAR) of pterostilbene to internal standard, against pterostilbene concentrations using unweighted least squares linear regression. The apparent decomposition rate constants (k_{app}) were estimated from the slope of the log-linear phase of declining concentration versus time plots. The half-lives ($t_{1/2}$) were calculated using the following equation: $t_{1/2} = 0.693/k_{app}$. Data were expressed as the mean ± standard deviation (S.D.) of replicate determinations. Pharmacokinetic parameters were estimated using WinNonlin (Version 1.0).

3. Results and discussion

3.1. Chromatography

Separation of pterostilbene and the internal standard (IS) in rat serum was successfully achieved. There were no interfering peaks co-eluted with the compounds of interest (Fig. 2A). The retention times of pterostilbene and IS were approximately 7 and 15 min, respectively (Fig. 2B–D).

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. Various compositions of HPLC were tested to achieve the best resolution of pterostilbene. The optimal separation was achieved when the combination of acetonitrile and water was 50:50 (v/v) and the flow rate of 1 mL/min.

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 0.999$) were demonstrated between PAR of pterostilbene to the internal standard and the corresponding serum concentrations of pterostilbene over a range of 0.5–100 μg/mL. The mean regression lines from the validation runs were described by pterostilbene (μg/mL) = $0.0767 \pm 0.0059x + 0.0666 \pm 0.0121$.

The LOQ of this assay was 0.5 μg/mL in biological fluids with the corresponding between day relative standard deviation of 14.83% and bias of 9.22% for pterostilbene. The back-calculated concentration of QC samples was within the acceptance criteria.

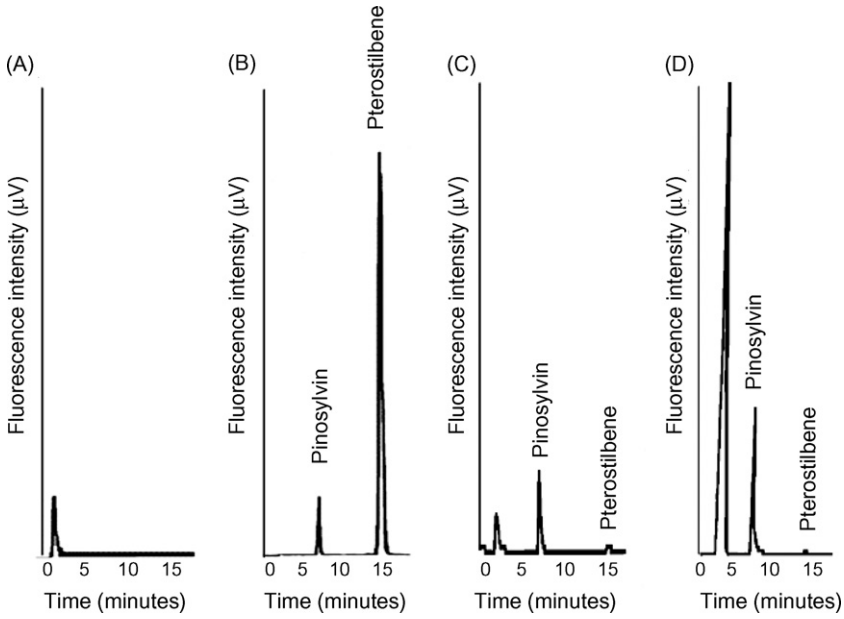

Fig. 2. Representative chromatogram of: (A) blank serum demonstrating no interfering peaks co-eluted with the compounds of interest; (B) rat serum containing IS and pterostilbene with concentration of 50 μg/mL; (C) rat serum containing IS and pterostilbene with concentration of 0.5 μg/mL (LOQ); (D) chromatogram of a real pharmacokinetic sample containing a concentration of pterostilbene close to LOQ. Separation was achieved isocratically using a reverse-phase HPLC method. A Phenomenex C18 column was used with fluorescence excitation at 330 nm and emission at 374 nm. The mobile phase consisted of acetonitrile and HPLC water (50:50, v/v) with a flow rate of 1.0 mL/min.

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

Pterostilbene concentration (μg/mL)						
Added	Observed		R.S.D. (%)		Bias (%)	
	Within-day	Between-day	Within-day	Between-day	Within-day	Between-day
0.5	0.57	0.55	14 05	14.83	13.89	9.22
1	1.11	1.09	12.20	14.77	10.95	8.97
5	4.47	5.27	0.88	13.59	−10.69	5.39
10	9.50	9.08	7.07	5.07	−4.99	−9.15
50	49.44	49.97	1.68	2.96	−1.11	−0.06
100	100.38	101.30	0.45	3.19	0.38	1.30

3.3. Precision, accuracy and recovery

The within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of pterostilbene in rat serum was <15% over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for pterostilbene varied between −10.69% and 13.89% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for pterostilbene from biological fluids varied from 107.9% to 117.2% (Table 2). In addition, the recovery of pinosylvin was 98.8% at the concentrations used in the assay. High recovery from biological fluids suggested that there was negligible loss of pterostilbene during the protein precipitation process. Additionally, the efficiencies of extraction of pterostilbene and pinosylvin were comparable.

3.4. Stability of pterostilbene samples

No significant degradation was detected after the samples of pterostilbene in biological fluids following three freeze-thaw circles. The recoveries of pterostilbene were from 82.01% to 112.87% following three freeze-thaw cycles for pterostilbene quality control (QC) samples of pterostilbene or pinosylvin. There was no significant decomposition observed after the reconstituted extracts of pterostilbene were stored in the auto-injector at room temperature for 24 h. The measurements were >97.4% of the initial values for extracts of pterostilbene in biological fluids of 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL, respectively, during the storage in the auto-injector at room temperature for 24 h.

3.5. Pharmacokinetics of pterostilbene in rats

The HPLC method has been applied to the determination of pterostilbene in pharmacokinetic studies in rats ($n = 3$). To our knowledge, there are no previously published studies or information of the pharmacokinetics of pterostilbene in any species. Following administration of pterostilbene intravenously, the serum disposition was examined (Fig. 3). The total serum clearance of pterostilbene was determined to be 3.10 ± 1.66 L/h/kg. The volume of distribution of pterostilbene is 5.56 ± 2.53 L/kg, which is greater than total body water, suggesting pterostilbene is highly distributed in tissue. The serum concentrations of pterostilbene appeared to decline rapidly with a mean elimination half-life of 2.38 ± 0.84 h. The mean area under the curve (AUC), representing the total amount of drug exposure in the serum over time, was 15.37 ± 5.02 μg h/mL.

Table 2
Recovery of pterostilbene from rat serum

Concentration (μg/mL)	Recovery (%) (mean ± S.D.)
0.5	107.9 ± 2.9
5	117.2 ± 3.1
50	115.8 ± 2.1

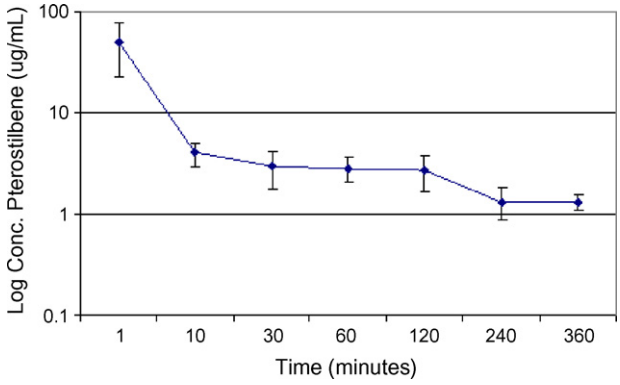

Fig. 3. Pterostilbene disposition in serum following administration (20 mg/kg) to rats ($N = 3$, mean ± S.E.M.).

4. Conclusions

In summary, the developed HPLC method for pterostilbene is sensitive, reproducible and accurate. It has been applied successfully in the study of the pharmacokinetics of pterostilbene in rats for the first time. Using this method, it is possible to analyze a large number of biological samples in a relatively short time period. The HPLC method presented here has also been used in our laboratory in the determination of urinary excretion of pterostilbene, and in examining the concentrations of pterostilbene in organic and conventional blueberry products. Further studies are ongoing in our laboratory to further characterize the pharmacological and toxicological activities of pterostilbene as well as other stilbene and flavonoid compounds.

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References

[1] A.M. Rimando, K. Wilhelmina, J.B. Magee, J. Dewey, J.R. Ballington, J. Agric. Food Chem. 52 (2004) 4713–4719.
[2] M. Adrian, P. Jeandet, A.C. Douillet-Breuil, L. Tesson, R. Bessis, J. Agric. Food Chem. 48 (2000) 6103–6105.
[3] R. Pezet, V. Pont, Plant Physiol. Biochem. 26 (1988) 603–607.
[4] A.C. Douillet-Breuil, P. Jeandet, M. Adrian, R. Bessis, J. Agric. Food Chem. 47 (1999) 4456–4461.
[5] M. Manickam, M. Ramanathan, M.A. Farboodniay Jahromi, J.P.N. Chansouria, A.B. Ray, J. Nat. Prod. 60 (1997) 609–610.
[6] V. Fuendjiep, J. Wandji, F. Tillequin, D.A. Mulholland, H. Budzikiewicz, Z.T. Fomum, A.M. Nyemba, M. Kock, Phytochemistry 60 (2002) 803–806.
[7] P. Ferrer, M. Asensi, R. Segarra, A. Ortega, M. Beniloch, E. Obrador, M.T. Varea, G. Asensio, L. Jorda, J.M. Estrela, Neoplasia 1 (2005) 37–47.
[8] M. Roberti, D. Pizzirani, D. Simoni, R. Rondanin, R. Baruchello, C. Bonora, F. Buscemi, S. Grimaudo, M. Tolomeo, J. Med. Chem. 46 (2003) 3546–3554.
[9] M. Tolomeo, S. Grimaudo, A.D. Cristina, M. Roberti, D. Pizzirani, M. Meli, L. Dusonchet, N. Gebbia, V. Abbadessa, L. Crosta, R. Barucchello, G. Grisolia, F. Invidiata, D. Simoni, Int. J. Biochem. Cell Biol. 37 (2005) 1709–1726.
[10] M. Manickam, M. Ramanathan, M.A. Farboodniay Jahromi, J.P.N. Chansouria, A.B. Ray, J. Nat. Prod. 60 (1997) 609–610.
[11] L.A. Stivala, M. Savio, F. Carafoli, P. Perucca, L. Bianchi, G. Maga, L. Forti, U.M. Pagnoni, A. Albini, E. Prosperi, V. Vannini, J. Biol. Chem. 276 (2001) 22586–22594.
[12] A.M. Rimando, M. Cuendet, C. Desmarchelier, R.G. Mehta, J.M. Pezzuto, S.O. Duke, J. Agric. Food Chem. 50 (2002) 3453–3457.
[13] R. Amorati, M. Lucarini, V. Mugnaini, G.F. Pedulli, J. Org. Chem. 69 (2004) 7101–7107.
[14] K. Roupe, C. Remsberg, J. Yáñez, N. Davies, Curr. Clin. Pharmacol. 1 (2006) 81–101.
[15] B. Paul, I. Masih, J. Deopujari, C. Charpentier, J. Ethnopharmacol. 68 (1999) 71–76.
[16] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249–255.