Preparative Enzymatic Synthesis and HPLC Analysis of Rhapontigenin: Applications to Metabolism, Pharmacokinetics and Anti-Cancer Studies

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ABSTRACT

Purpose: A facile method was established to enzymatically synthesize rhapontigenin from the glycosylated parent compound rhaponticin. A novel and simple high-performance liquid chromatographic method was developed for the determination of rhapontigenin. The assay was successfully applied to both the *in vitro* and *in vivo* metabolic kinetic study of rhapontigenin. **Methods:** Serum, or microsomes (0.1 mL) was precipitated with acetonitrile after addition of the internal standard, daidzein. Separation was achieved on an amylose tris 3,5 dimethylphenylcarbamate column (150 x 4.6mm, ID, 5μm) with UV detection at 324nm. Hep G2 hepatoma cells were treated with rhapontigenin or rhaponticin (0-250 μg/mL) and cell viability was measured. **Results:** The calibration curves were linear ranging from 0.5 to 100 μg/mL. The mean extraction efficiency was > 99%. Precision of the assay (coefficient of variation) was <5%, including the limit of quantitation (0.5 μg/mL). Bias of the assay was lower than 5%. The limit of detection was 100 ng/mL for a 0.1 mL sample. One glucuronidated metabolite of rhapontigenin has been identified. Preliminary pharmacokinetic data revealed the presence of a glucuronidated metabolite in the serum and a terminal elimination $t_{1/2}$ of ~6h. Rhapontigenin demonstrated concentration-dependent anti-cancer activity with an IC_{50} 115 μg/mL in HEP G2 cells while rhaponticin showed no activity across the concentrations tested *in vitro*. **Conclusions:** The preparative enzymatic synthesis method has demonstrated utility to provide sufficient rhapontigenin for pharmaceutical studies. Rhapontigenin is an active anti-cancer compound. The developed HPLC assay is sensitive, reproducible and accurate and can be applied to pharmacokinetic and metabolism studies.

INTRODUCTION

Rhapontigenin, (3, 3’, 5 –trihydroxy-4’-methoxy-stilbene) $C_{15}H_{16}O_4$, MW: 258 [Table 1], is a stilbene found in Korean rhubarb rhizomes, and is most abundant in the *Rhei undulatum* species [1]. Rhaponticin, the glycosylated parent compound of rhapontigenin has long been employed in Korea, Japan, and China as an oral haemostatic agent in treating Oketsu, a disease characterized by poor circulation, pain, and chronic inflammation [2] [Table 1]. Rhaponticin has also been recommended by health professionals in Asian countries to treat and prevent allergies [3]. Rhapontigenin, the aglycone of rhaponticin, has been suggested to be the active molecule [3, 4, 5]. Recent research has shown rhapontigenin to be a potent anti-allergic, anti-coagulant, and anti-inflammatory compound [6-8].

Rhapontigenin is structurally similar to the anti-cancer stilbene resveratrol, which is present in red wine [9]. Considerable scientific studies have demonstrated potent anti-cancer activity of resveratrol across many cancer cell lines [9]. Given the similarity in structure of resveratrol [Table 1], it is possible that rhapontigenin also possesses potent anti-cancer activity. Recent investigation has found rhapontigenin to be a potent inhibitor of the human cytochrome P450 1A1 enzyme, which is implicated in the biotransformation of a number of carcinogenic and immunotoxic compounds [10]. Additionally, rhapontigenin has been shown to be an inhibitor of CYP 1B1, an enzyme that is expressed and detected in a number of cancers such as prostate and breast cancers [11].

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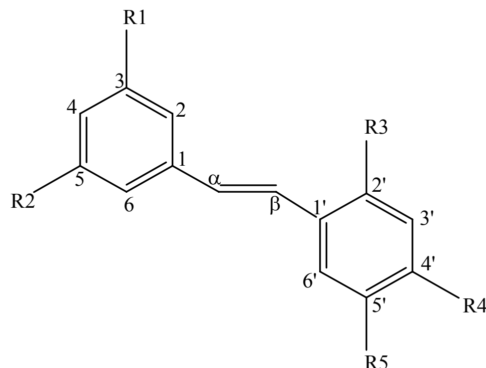
Table 1: Structures of Stilbenes



Compound	R1	R2	R3	R4	R5
Rhapontigenin	OH	OH	H	OCH_3	OH
Resveratrol	OH	OH	H	OH	H
Rhaponticin	*O*-Glucose	OH	H	OCH_3	OH
Piceatannol	OH	OH	H	OH	OH
Pinosylvin	OH	OH	H	H	H

Although used in traditional Asian medicine, rhapontigenin has not been thoroughly investigated pharmaceutically. This is likely due to the fact that it is not yet commercially available for purchase from chemical companies. In order to elucidate the metabolism kinetics of rhapontigenin, knowledge of its metabolic pathways in biological fluids is of considerable importance. To our knowledge, no study has been published characterizing the *in vitro* metabolism of rhapontigenin, and there is no pharmacokinetic information or validated assays to measure rhapontigenin described in the literature. Before performing studies of biotransformation, a facile method of producing pure rhapontigenin is necessary and development of a selective and sensitive assay for rhapontigenin is needed. The present study describes a simple method of enzymatic synthesis of rhapontigenin from commercially available rhaponticin. Furthermore, a selective, isocratic reversed-phase HPLC method for the determination of rhapontigenin and its metabolites in rat serum and its application to *in vitro* and *in vivo* kinetic studies is detailed.

EXPERIMENTAL

Chemicals and reagents

Daidizen, halothane, rhaponticin, β-glucosidase from almonds, total protein reagent, protein standard solution, monosodium glucose-6-phosphate, tetraethylammonium acetate tetrahydrate, β-nicotinamide adenine dinucleotide phosphate (β-NADP) sodium salt hydrate, and glucose-6-phosphate dehydrogenase, Trypsin-EDTA, Trypan blue, phosphate-buffered saline (PBS), resazurin, sodium bicarbonate, penicillin-streptomycin, and insulin were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol, reagent alcohol, acetonitrile, and water were purchased from J. T. Baker (Phillipsburg, NJ, USA). Solid phase extraction (SPE) C-18 columns were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Dulbecco's Modified Eagle Medium (D-MEM) and RMPI 1640 medium were purchased from Gibco Industries Inc. (Langley, OK, USA). Fetal bovine serum (FBS) was purchased form Equitech-Bio Inc. (Kerrville, TX, USA).

Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT pump, a SIL-10AF auto injector, a photodiode-array SPD-10A *VP* UV/VIS spectrophotometric detector and an SCL-10A system controller. Injection volume was 150 μL. Data collection and integration were accomplished using Shimadzu EZ start 7.1.1 program software.

The analytical column used was an amylose tris 3, 5 dimethylphenylcarbamate (150 × 4.6 mm, ID, 5 μm) (Chiral Technologies Inc. Exton, PA, USA). The mobile phase consisted of acetonitrile

and 0.1% phosphoric acid (30:70, v/v), filtered and degassed under reduced pressure prior to use. Separation was carried out isocratically at ambient temperature, and a flow rate of 1.0 mL/min, with UV detection at 324 nm.

Mass Spectrometry Conditions

Samples were applied to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems Sciex, ON, Canada) using negative ion electrospray under similar chromatographic conditions to those mentioned above with the exceptions that an Agilent 1100 series HPLC system (Palo Alto, CA, USA) was employed, consisting of: autosampler, binary pump, degasser, and UV detector and phosphoric acid was omitted as a modifier while 1 mM $(NH_4)HCO_3$ was used to maintain neutral pH. The mass spectrometer was operated under conditions optimized for rhapontigenin at the chromatographic flow conditions (0.5 mL min^{-1}) as follows: The Ionspray needle was maintained at -4500 kV, with nitrogen as drying gas 1 (setting 40), drying gas 2 (setting 25), curtain gas (setting 10), and collision gas (setting 4). The turbospray interface was maintained at 400 °C. The declustering potential (DP), collision energy (CE), and exit potential (EP) were optimum at 30 V, 45 eV and 10 V, respectively. Both the Q1 and Q3 quadrupoles were maintained at unit resolution (0.7 Da width at half height). The characteristic fragmentation reactions include *m*/*z* 257 for rhapontigenin and *m*/*z* 419 for rhapontigenin glucuronide.

Nuclear Magnetic Resonance (NMR) Conditions

^{1}H NMR (499.85 MHz), ^{13}C NMR (125.67 MHz) spectroscopic analyses were carried out on a Varian Inova 500 MHz spectrometer. Proton spectra of rhaponticin (6 mg in 700 µl DMSO-d_6) were collected at 22 ^{o}C and referenced to the residual proton signal at 2.49 ppm, whereas the ^{13}C spectra were referenced to the solvent signal at 39.5 ppm. Proton spectra of rhapontigenin were obtained in benzene-d_6 at 22 ^{o}C and were referenced to the residual proton signal at 7.15 ppm. The phenolic OH protons were in fast exchange with the bulk water in the benzene-d6 solution and hence appear at the bulk water chemical shift which is a 1.35 ppm.
A gradient enhanced phase-sensitive ^{1}H-^{13}C HSQC spectrum was obtained for rhaponticin using the standard Varian pulse sequence and was collected with sweep widths (acquisition times) of 4,614 Hz (222 ms) in t2 (^{1}H) and 16967 Hz (15.1 ms, 256 x 2 hypercomplex increments) in t1 (^{13}C). The data were then processed in F2 by applying a Gaussian function with a 0.101 s time constant prior to Fourier transformation. The F1 processed data utilized a linear prediction of the original 256 real points to 512 points, apodizing with a Gaussian function using a 0.026 s time constant, zero filling to 2K complex points and followed by Fourier transformation. A gradient selected ^{1}H-^{13}C HMBC was also acquired for rhapontigenin using the standard Varian pulse sequence and was collected with sweep widths (acquisition times) of 4,614 Hz (222 ms) in t2 (^{1}H) and 16214 Hz (24.7 ms, 400 increments) in t1 (^{13}C). The data were processed in F2 and F1 by applying a sine-bell function prior to Fourier transformation. The data in F1 was extended to 800 real points by linear prediction, zero filled to 4096 points and Fourier transformed.

Enzymatic Synthesis of Rhapontigenin

A 0.01M tetraethylammonium acetate buffer was made by adding 261 mg tetraethylammonium acetate to 100 mL HPLC water in a volumetric flask. The pH was adjusted to 5.0 using 1M HCL. 4 mL buffer was filtered and added to a clean glass test tube. 20 mg rhaponticin was weighed carefully and added to the prepared buffer. The rhaponticin solution was sonicated and vortexed until dissolved. The rhaponticin solution was then placed in a 37 ^{o}C shaking water bath. Next, an enzyme solution was prepared by adding 1 mL buffer to 6 mg β-glucosidase. The enzyme solution was shaken gently and directly added to the rhaponticin solution. The resulting solution was incubated for 72 hours. 200 µL aliquots of the incubate were taken every 24 hours and the reaction progression was monitored via HPLC [Figure 1].

The final incubate was transferred to a solid phase extraction C-18 column and the eluted solution was collected in a test tube. The SPE column was washed with HPLC water and the aqueous fraction was collected in a separate test tube. Finally, the SPE column was eluted with 2 mL methanol and the methanol fraction was collected in a glass test tube and dried under a gentle stream of nitrogen gas. The resulting powder was weighed, transferred to a screw top glass vial and stored at 4°C. The successful enzymatic synthesis of rhapontigenin was verified by

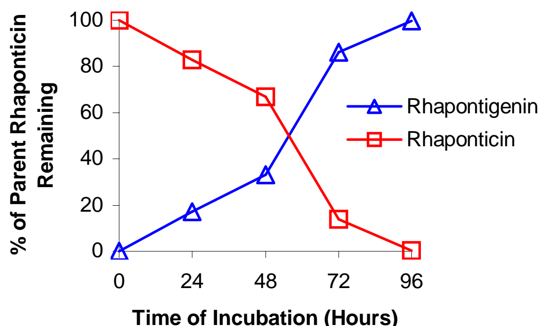


Figure 1:. HPLC-monitored enzymatic reaction of rhaponticin into rhapontigenin via β-glucosidase.

NMR and mass spectrometry with an *m/z* ratio of 256.6 and a fragmentation pattern consistent with rhapontigenin [Table 2-3 Figures 2-4]. The melting point of the newly synthesized rhapontigenin was determined employing a Thomas Hoover Capillary Melting Point Apparatus® (Arthur H. Thomas Company, Philadelphia, PA, USA). The heat setting was set at 3.9 and the temperature was ramped at 1.0 °C/ minute. Visual determination of the melting point showed the uncorrected onset temperature to be 182.0 °C and the end point to be 184.0 °C.

Stock and Working Standard Solutions

Methanolic stock solutions of rhapontigenin (1 mg/mL) and daidzein (1 mg/mL) were prepared. The daidzein solution was subsequently diluted with methanol to make a working internal standard (IS) solution of 10 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 rhapontigenin by sequential dilution with blank rat serum, yielding a series of concentrations namely 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 µg/mL), in four replicates.

Table 2: H'NMR values for Rhapontigenin: (Benzene-d_6)

OH	Exchanging with Benzene-d_6
H2	6.18
H4	5.40
H6	6.18
Hα	6.77
Hβ	6.90
H2'	6.85
H3'	6.39
OCH_3'	3.10
H6'	5.91

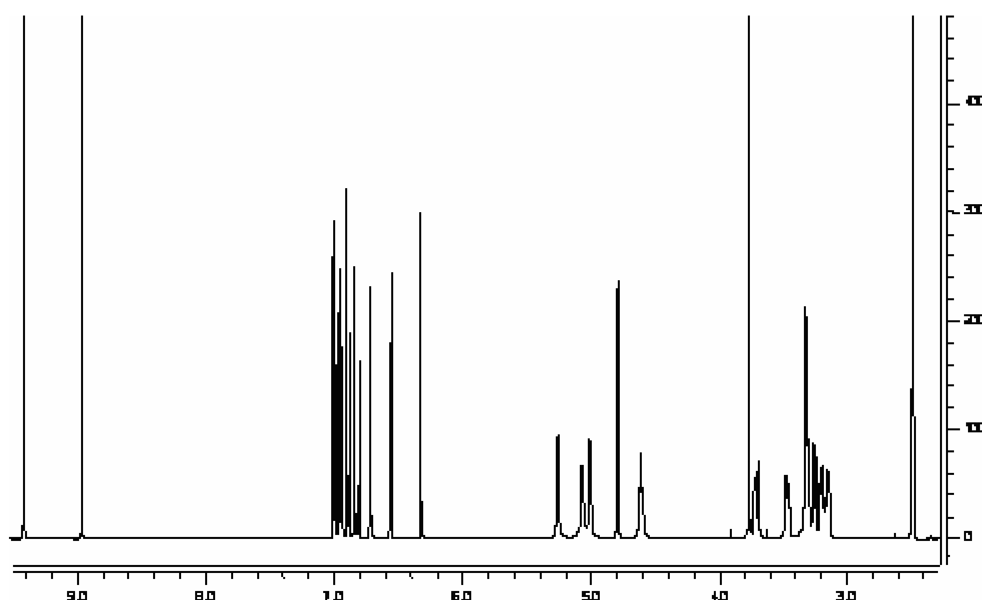


Figure 2:. H' NMR spectra of rhaponticin

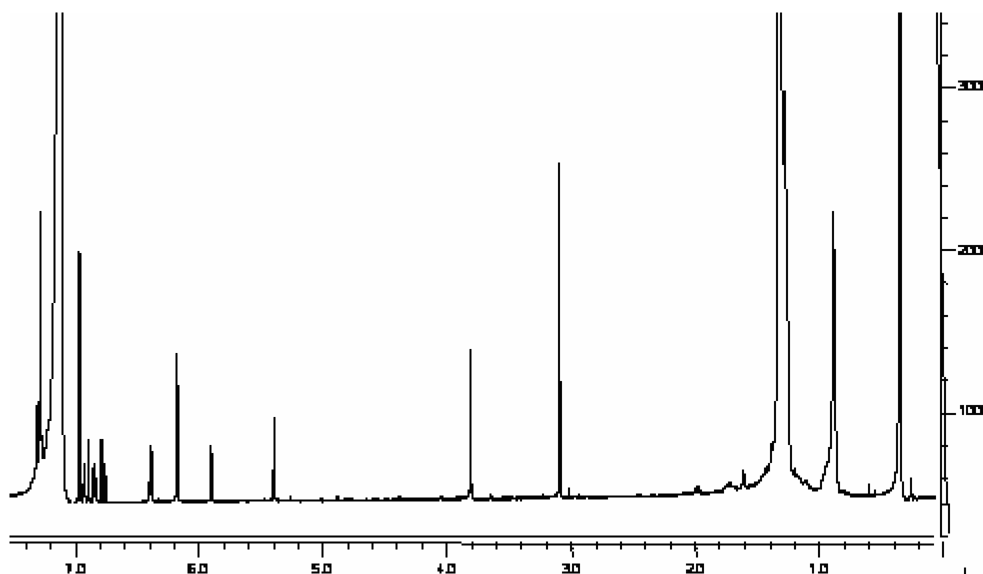


Figure 3: H' NMR spectra of rhapontigenin.

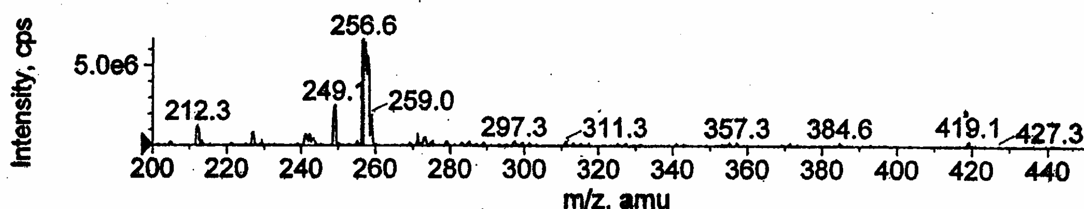


Figure 4: Mass spectrum of Rhapontigenin

Quality control (QC) samples were prepared from the stock solution of rhapontigenin 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 0.5 mL aliquots in screw-capped test tubes and stored at –20 °C before use.

Sample preparation

0.1 mL of internal standard solution (10 µg/mL) was added to working standards or samples (0.1 mL). The mixture was precipitated with 1.0 mL ice-cold acetonitrile and was centrifuged at 8,000 x g for 5 min using Beckman microfuge. Following transfer of the supernatant to new vials, the residue was placed in sample vials 150 µL of the supernatant was injected onto the column.

Precision and accuracy

The within-run precision and accuracy of the replicate assays (*n*=6) were tested by using six different concentrations, namely 0.5, 1, 5, 10, 50 and 100 µg/mL. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples of six different days within one week. The precision was evaluated by calculating the coefficient of variation (CV) using ANOVA. The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration. The values of CV and bias were within 15%, at all concentrations tested [12].

Recovery

Recovery of rhapontigenin from rat serum was assessed (n=6) at 0.5, 1.0, 5, 10, 50, 100 µg/mL. A known amount of rhapontigenin was spiked into 0.1 mL rat serum to give the above concentrations. The proteins present in the serum were precipitated with 1mL ice-cold acetonitrile and the supernatant was analysed by HPLC following centrifugation at 8,000 x g for 5 min. The extraction efficiency was determin ed by comparing the peak area ratio (PAR)

of rhapontigenin to IS in the serum matrix to that in water.

Stability of Rhapontigenin Samples

The stability of rhapontigenin samples were assessed under five different conditions. The stability of rhapontigenin in rat serum at room temperature (22 ± 1 ºC) and at –20 ºC was investigated using QC samples of five concentration levels, 1, 5, 10 µg/mL in four replicates.

The freeze-thaw stability of rhapontigenin was evaluated at three concentrations 1, 5, and 10 µg/mL, using QC samples. These samples were analyzed in triplicate without freezing, and then stored at –20 °C and thawed at room temperature (22 ± 1 ºC) for three cycles.

The stability of rhapontigenin in reconstituted extracts during run-time in the HPLC auto-injector was investigated, using pooled extracts from QC samples of three concentration levels, 1, 5, 10 µg/mL. Samples were kept in the sample rack of the auto-injector and injected into HPLC system every 6h, from 0 - 24 h, at the temperature of auto-injector (26 ± 1 °C).

The stability of reconstituted extracts was also tested at –20 °C for one week. The reconstituted extracts of six concentrations, 0.5, 1, 5, 10, 50 and 100 µg/mL were allocated in injection vials, stored at –20 °C and injected onto the column on day 0 and day 1.

The stability of reconstituted extracts was also tested at –20 °C for one day. The reconstituted extracts of six concentrations, 0.5, 1, 5, 10, 50 and 100 µg/mL were allocated in injection vials, stored at –20 °C and injected onto the column on day 0 and day 1.

The light stability of rhapontigenin in stock solution was also tested at room temperature for one day. Samples were exposed to laboratory (fluorescent overhead) illumination for up to 24 hours and injected onto the column from time 0 to 24 h post illumination.

Table 3: ^{1}H'NMR and ^{13}C'NMR for Rhaponticin: (DMSO-d_6)

C1	138.99		
C2	100.52	H2	4.79
C3	158.67		
C4	102.77	H4	6.33
C5	158.14	OH5	9.44
C6	107.12	H6	6.57
Cα	125.93	Hα	6.83
Cβ	128.29	Hβ	6.98
C1'	129.83		
C2'	112.83	H2'	7.01
C3'	146.39	3'-OH	8.97
C4'	147.54		
4'-OCH_3	55.59	4'-OCH_3	3.77
C5'	111.95	H5'	6.89
C6'	118.43	H6'	6.95
Glucose Ring Assignments:			
C1''	100.50	H1''	4.79
C2''	73.21	H2''	3.19 2''-OH 5.26
C3''	76.62	H3''	3.25 3''-OH 5.07
C4''	69.69	H4''	3.14 4''-OH 5.01
C5''	77.04	H5''	3.30
C6''	60.65	H6''a	3.46 H6''b 3.70 6''-OH 4.61

Stability of Rhapontigenin in Rat Serum

Rhapontigenin was incubated in rat serum at 37.0 ± 0.1 °C in a thermostatically controlled shaking water bath. Prior to the kinetic study, the incubation media were equilibrated to the temperature of the study. Kinetic studies were initiated by the addition of a stock solution of rhapontigenin to incubation media, yielding an initial concentration of 10 µg/mL. At pre-determined time intervals, samples (0.5 mL) were removed and the reaction was stopped by adding equal volume of ice-cold acetonitrile and mixing immediately. Samples were analysed by HPLC following centrifugation at 8,000x g for 5 min using Beckman microfuge.

Pharmacokinetics

Male Sprague Dawley rats (n=3, 300-325 g, Harlan, Indianapolis, IN, USA) were anaesthetized using halothane and a silastic catheter was cannulated into

the right jugular vein. The animals were placed in metabolic cages, allowed to recover overnight and fasted for 12 h before dosing. On the day of experiment, the animals were dosed intravenously with an IV dosing solution of rhapontigenin (10 mg/kg) in 5% dimethyl sulfoxide (DMSO) and polyethylene glycol 400 (PEG-400). Serial blood samples (0.25 mL) were collected at 0, 1 min, 10 min, 0.25, 0.5, 1, 2, 4, 6, 12 and 24 h. After each sample collection, the cannulas were flushed with 0.25 mL of saline. Following centrifugation of the blood samples, serum was collected and stored at – 70 °C until analysed. The experimental animal protocols were approved by the Institutional Animal Care and Use Committee of Washington State University.

Rat Liver Microsomes Preparation

Male rat liver microsomes were prepared from adult male Sprague-Dawley rats using previously published procedures [13,14]. The fresh rat livers were cut from euthanized rats and put into ice-cold saline, weighed, and minced. Samples were homogenized using a motorized homogenizer (four strokes) in ice-cold homogenization buffer (50 mM pH 7.4 potassium phosphate buffer, 250 mM sucrose, 1 mM EDTA) and centrifuged at 7700 x *g* for 15 min at 4 °C. The supernatant collected was then centrifuged again at 18,500 x *g* for 15 min at 4 °C. After the pellet was discarded, the supernatant was centrifuged again at 85,600 x *g* for 1.0 h at 4 °C to yield microsome pellets. The microsomes were resuspended in microsome washing buffer (10 mM pH 7.4 potassium phosphate buffer, 0.1 mM EDTA, and 150 mM KCl) and centrifuged again at 85,600 x *g* for 1.0 h at 4 °C to yield microsomes. The microsome pellet was then resuspended in 250 mM sucrose, aliquoted into vials (0.5 mL/vial), and stored at -80 °C until use.

Microsome Protein Concentration

Protein concentration of microsomal protein was determined using a protein assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as standard.

Phase I Metabolism

Studies of metabolic kinetics of rhapontigenin were conducted in the presence of cofactors which included 10 mM $MgCl_2$ and an NADPH-generating system (7.5 mM glucose 6-phosphate, 0.3 mM β-NADP and 0.42 unit/mL glucose-6-phosphate dehydrogenase), in 100 mM phosphate buffer containing 1 mM EDTA (pH 7.4) under carbogen gas at 37.0 ± 0.1 °C in a shaking (75 rpm) water bath. The parent drug was added as a methanolic stock solution of 1.0 mg/mL (at a volume of 0.5% in the final incubation mixtures) and was pre-incubated in the incubation buffer for 5 min at 37 ± 0.1 °C. The reaction was initiated by adding the cofactors. At pre-determined time intervals, samples (0.5 mL each) were withdrawn and the reaction was terminated immediately by adding 50 µL of 94% acetonitrile/ 6% glacial acetic acid. Samples were then extracted and analysed by HPLC.

Phase II Metabolism

The incubation procedures for measuring uridine diphosphate-glucuronosyltransferase (UGT) activities using microsomes were as follows: 1) Microsome (final concentration ~0.05 mg protein/ml) was mixed with each of the following: $MgCl_2$ (0.88 mM), saccharolactone (4.4 mM), and alamethicin (0.022 mg/ml). 42 µM of rhapontigenin in a 50 mM potassium phosphate buffer (pH 7.4) was added as the substrate and finally uridine diphosphoglucuronic acid (3.5 mM) was added to activate the reaction. 2) The mixture was incubated at 37 °C for 10, 20, 30, or 60 min; and 3). The reaction was stopped by the addition of 50 µL of 94% acetonitrile/6% glacial acetic acid.

Cell Culture

Hep-G2 (human hepatoma) cell line was obtained from the American Type Culture Association (ATCC, Rockville, MD.) and maintained in Dulbecco's Modified Eagle Medium (D-MEM). The Cell line was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin (10mg/1L) insulin (4mg/mL) and was incubated at 37°C in a 5% CO_2 atmosphere.

Cell Number

The optimal cell seeding numbers for each cell line was determined by preliminary cell seeding number experiments. Cells were seeded in numbers 1 x 10^4, 2 x 10^4, 3 x 10^4 and so on until the final cell seeding number 1 x 10^5 per well in a 96 well plate (Costar

3595). Cell plates were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. Following incubation, medium was aspirated and alamar blue (resazurin) fluorescent dye solution was diluted in fresh medium to make a 10% resazurin solution. The 10% solution was added directly to cells. The cell plates were incubated at 37°C in a 5% CO_2 atmosphere for 3 hours. The cell plates were subsequently removed from the incubator and placed at room temperature in a darkened drawer to protect from light for 30 minutes. Next, the cell plates were placed into the Cytoflour®4000 fluorescence multi-well plate reader (Applied Biosystems, USA). Fluorescence was read at an excitation of 485 nm and an emission of 530 nm. Standard curves of cell seeding number against fluorescence were generated. Hep G2 cells were seeded at a density of 5000 cells/well.

Alamar Blue Assay

Alamar Blue (resazurin) fluorescent dye is a facile and accurate assay that has recently gained scientific popularity in determining the cytotoxicity of many cell lines [15]. The resazurin non-fluorescent compound is metabolised into the fluorescent compound resorufin by intact and viable cells. This emission of fluorescence can be quantified using a cell plate reader and the number of viable cells following treatment can be determined. Cells were counted and seeded on 96 well plates. The seeded cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Rhaponticin and rhapontigenin were dissolved in methanol the day of the experiment and were diluted in medium to yield concentrations of 0.1, 1, 10, 50, and 100 µg/mL. Following aspiration of the medium, cells were treated with the stilbene solutions. Additional cells were treated with either methanol diluted in medium or medium only. Treated and control cells were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. After cell plates were removed from the incubator, medium was aspirated and replaced with 10% alamar blue (resazurin) fluorescent dye diluted in fresh medium. Cell plates were incubated at 37°C in a 5% CO_2 atmosphere for an additional 3 hours. Following incubation, cell plates were placed in a darkened environment for 30 minutes at room temperature. Next, the cell plates were placed into the Cytoflour®4000 fluorescence multi-well plate reader (Applied Biosystems, USA). Fluorescence was read at an excitation of 485 nm and an emission of 530 nm. The viable cell number (as a percent of control) in each cell line exposed to varying concentrations of stilbene was measured.

Data analysis

Rhapontigenin was identified with and without β-glucuronidase by its retention time relative to the internal standard (IS) on HPLC chromatograms. Quantification was based on calibration curves constructed using the peak area ratio (PAR) of rhapontigenin to (IS), against rhapontigenin concentrations using unweighted least squares linear regression. The percentage of metabolism products was estimated as the ratio of PAR of metabolite to PAR of patent drug at time zero. The apparent decomposition rate constants (k_{app}) were estimated from the slope of log-linear phase of declining concentration versus time plots. The half-lives ($t_{½}$) were calculated using the following equation: $t_{½} = 0.693/ k_{app}$. Data were expressed as the mean ± standard deviation (SD) of replicate determinations. Pharmacokinetic parameters were estimated using WinNonlin® (version 1.0).

RESULTS

Chromatography

There were no interfering peaks co-eluted with the compounds of interest (Figure 5). Separation of rhapontigenin and the internal standard in rat serum were achieved successfully. The retention times of rhapontigenin and IS were approximately 22 min and 16 minutes, respectively (Figure 6).

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in rat serum, linearity, limit of quantitation (LOQ), limit of detection (LOD), freeze-thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Various conditions of HPLC were tested to achieve the best resolution of rhapontigenin. The retention times of analytes were found to be very sensitive to the percentage of acetonitrile in the mobile phase. The optimal separation was achieved when the combination of acetonitrile and phosphoric acid was 30:70 (v/v) and the flow rate was 1.0 ml/min.

Based on spectrophotometer analysis of rhapontigenin reconstituted in mobile phase prior to HPLC analysis, UV detection was set at 324 nm.

Linearity, LOQ and LOD

An excellent linear relationship (r^2 = 0.998) was demonstrated between peak area ratio (PAR) of rhapontigenin to IS and the corresponding serum concentrations of rhapontigenin over a range of 0.5 to 100 µg/mL.

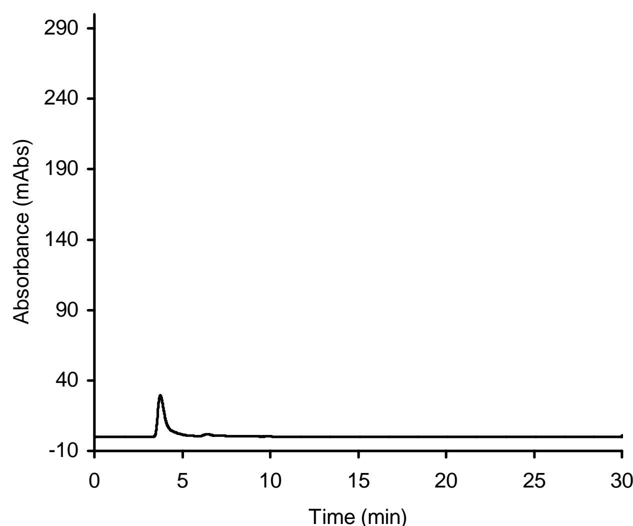


Figure 5: Blank rat serum demonstrating no interfering peaks co-eluted with the compounds of interest.

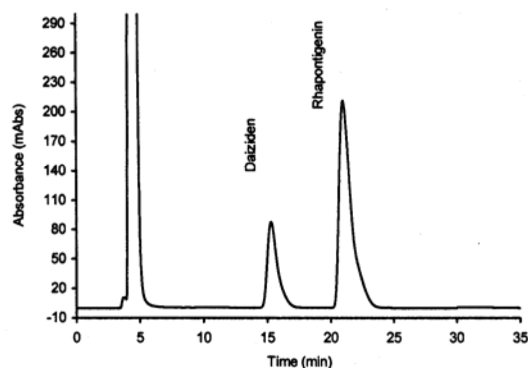


Figure 6: Rat serum containing daidzein (internal standard) and rhapontigenin with at a concentration of 10 µg/mL.

The mean regression line from the validation runs was described by rhapontigenin µg/mL= Peak area ratio x 1.4366+2.2237. The LOQ of this assay was 0.5 µg/mL in rat serum with the corresponding relative standard deviation and bias of 0.83 and 8.8%, respectively. This calibration curve was cross-validated with QC samples of rhapontigenin in microsomes. The back-calculated concentration of QC samples in these matrices was within the acceptance criteria. The LOD of rhapontigenin was estimated to be 0.1 µg/mL in rat.

Precision, Accuracy and Recovery

The within- and between-run CV calculated during replicate assays (*n*=6) of rhapontigenin in rat serum were <5% over a wide range of rhapontigenin concentrations. The intra- and inter-run bias assessed during replicate assays varied between –4.7 and 14.6%. Precision and accuracy studies indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for rhapontigenin from rat serum varied from 99 to 100.2%. High recovery of rhapontigenin from rat serum suggested that there was negligible loss during the protein precipitation process, and the efficiencies of extraction of rhapontigenin and IS were comparable.

Stability of Rhapontigenin Samples

No significant degradation was detected after the samples of rhapontigenin in rat serum were stored at room temperature for 3 h, or in a freezer at or below –20 °C for 4 weeks, or after undergoing one freeze-thaw cycle. Under ambient conditions for 3 h, there was > 99% of rhapontigenin recovered across concentrations. When stored in a freezer at –20 °C, recoveries of rhapontigenin were >99% after 1 and 4 weeks. The recoveries were >95% following three freeze-thaw cycles in all concentrations tested (1 µg/mL, 5 µg/mL, and 10 µg/mL). There was no significant decomposition observed after the reconstituted extracts of rhapontigenin were stored in the auto-injector at room temperature for 24 h or in freezer at –20 °C for 1 week. The measurements were >99% of the initial values for all concentrations during the storage in the auto injector at room temperature for 24 h. When stored in a freezer at –20 °C, the recovery was >99% within one week at all concentrations investigated.

Metabolism of Rhapontigenin in Rat Liver Microsomes under a NADPH Generating System

The HPLC method has been applied to the determination of rhapontigenin and its metabolic products in the Phase I metabolic kinetic study of rhapontigenin in rat liver microsomes. Rhapontigenin was added individually to microsomes in a concentration of 10 μg/mL. Following the incubation of rhapontigenin as parent drug at 37 °C in rat liver microsomes with an NADPH generating system, no observable peaks were detected and no decrease in parent rhapontigenin was evident, suggesting no appreciative oxidative metabolism is apparent.

Pharmacokinetics of Rhapontigenin in Rats

The HPLC method has been applied to the determination of rhapontigenin in pharmacokinetic studies in rats. There are no previously published studies or information of the pharmacokinetics of rhapontigenin in any species. Following administration of rhapontigenin there was an apparent terminal elimination half-life of ~6h for the parent compound [Figure 7].

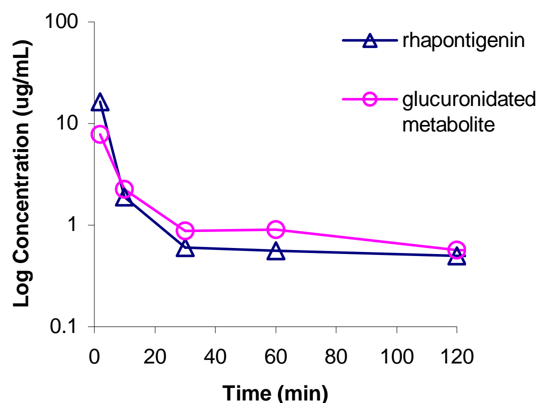


Figure 7: Mean intravenous pharmacokinetics of rhapontigenin in male Sprague Dawley rats (n=3).

One previously unidentified metabolite was detected with a retention time of 4 minutes in the solvent front. The metabolite was measured indirectly by treating samples with β-glucuronidase and measuring the increase in parent compound. The metabolite was isolated and the *m/z* ratio and fragmentation pattern was determined using mass spectrometry. The metabolite was shown to have an *m/z* ratio of 419 and a fragmentation pattern that is consistent with glucuronidation [Figure 8]. The pharmacokinetics of rhapontigenin appears to be qualitatively very similar to previous reports of resveratrol in the rat where a glucuronide metabolite is also present in plasma [19].

Metabolism of Rhapontigenin in Rat Liver Microsomes under a UGT Generating System

The HPLC method has been applied to the determination of rhapontigenin and its metabolic products in the phase II metabolic kinetic study of rhapontigenin in rat liver microsomes. Rhapontigenin was added individually to microsomes in a concentration of 10 μg/mL. Following the incubation of rhapontigenin as parent drug at 37 °C in rat liver microsomes with the UGT enzyme, a rapid and significant decrease in rhapontigenin was detected. A metabolic peak was observed, eluting at 4 minutes, which could not be resolved from the solvent front. The amount of this metabolite detected increased over time coinciding with the reduction of parent rhapontigenin [Figure 9].

This suggests that extensive glucuronidative metabolism is apparent, which was confirmed by mass spectrometry analysis. The glucuronidated metabolite had an *m/z* ratio of 419 and a fragmentation pattern consistent with glucuronidation. Due to the fact that the metabolite eluted with the solvent front, the quantification of metabolite was determined indirectly using β-glucuronidase to hydrolyze the metabolite back to parent compound. This technique has been extensively used in metabolism and pharmacokinetic research to determine metabolite concentration over time [16, 17, 18]. β-glucuronidase was added to a set of microsomal samples instead of acetic acid/acetonitrile stop solution. These samples were analyzed via HPLC along side of original microsomal samples exposed to the stop solution. HPLC analysis confirmed the absence of the glucuronidated metabolite. This same peak at the same retention time was also apparent in the rat serum

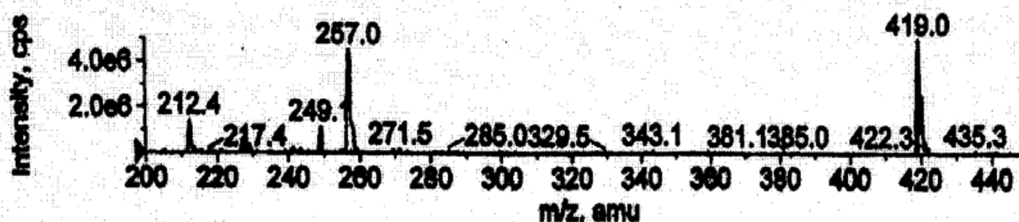


Figure 8: Mass spectrum of the glucuronidated metabolite of Rhapontigenin

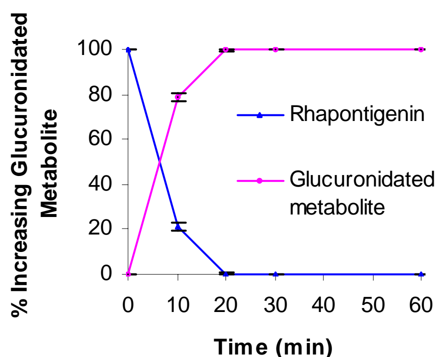


Figure 9: Phase II Microsomal metabolism of Rhapontigenin in rat liver microsome.

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Anti-cancer Activity of Rhaponticin and Rhapontigenin in Hep-G2 Cells.

The anti-cancer activities of rhaponticin and rhapontigenin were tested in the Hep-G2 hepatoma cell line. Analysis of cell viability as a percent of the control following exposure showed greater activity in cells treated with rhapontigenin compared to the parent compound rhaponticin. At the highest concentration tested (250 μg/mL), the viable number of cells detected was 10% of that of the control. Comparatively, rhaponticin showed little pharmacological activity [Figure 10]. The IC_{50} of rhapontigenin in the Hep-G2 cell line was determined by pharmacodynamic modeling using WinNonlin® (version 1.0) and was determined to be 115.0±49.3 μg/mL.

DISCUSSION

Rhapontigenin is a stilbenoid compound that has been shown to be an active anti-allergenic, anti-inflammatory and anti-cancer agent. Recent investigation has found rhapontigenin to be a potent inhibitor of human cytochrome P450 1A1 enzyme. This enzyme is implicated in the biotransformation of a number of carcinogenic and immunotoxic compounds. In addition to potently inhibiting P450 1A1, rhapontigenin is also an inhibitor of CYP 1B1. This enzyme is expressed and detected in a number of cancers such as prostate and breast cancers. These data demonstrating the possible anti-cancer activity of rhapontigenin illustrates the necessity for further characterization, testing, and development of this compound. However, rhapontigenin is not commercially available and a facile method for its synthesis is imperative in order to develop an assay for its quantification as well as to further study its pharmacological properties. This current research describes a novel method for the enzymatic synthesis of rhapontigenin from its glycosylated parent compound rhaponticin. Moreover, the development and validation of this HPLC assay methodology is described and its applicability to the study of pharmacokinetics, metabolism and anti-cancer activity of rhapontigenin has been demonstrated.

Our metabolism data shows that rhapontigenin is extensively glucuronidated. This same pattern of metabolism has been demonstrated for other structurally similar stilbene compounds namely piceatannol, pinosylvin, and resveratrol [Table 1] [16-19] in which the glucuronidated metabolite is extensive and present in both *in vivo* male rat serum samples and in *in vitro* male rat liver

microsomal fractions. In addition, the preliminary pharmacokinetic characterization of rhapontigenin appears to be very similar to those of piceatannol, pinosylvin, and resveratrol.

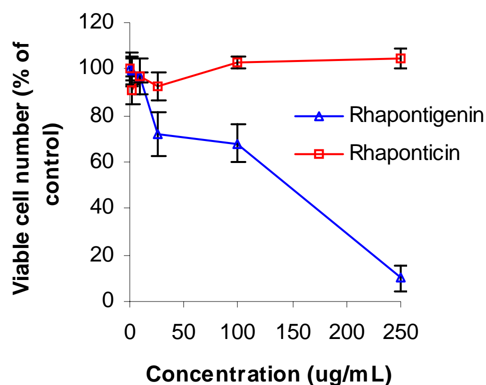

Figure 10: Anti-cancer activity of Rhaponticin and Rhapontigenin in Hep-G2 cells.

Our data from the alamar blue assay, which measured Hep G2 hepatoma cell viability following treatment with rhapontigenin and rhaponticin, demonstrated that rhapontigenin possesses anti-cancer activity with an IC_{50} 115.0±49.3 µg/mL. Comparatively, rhaponticin showed insignificant activity. This data further supports the concept that rhapontigenin is the active molecule compared to its glycosylated parent compound, rhaponticin, and that rhaponticin must be biotransformed into rhapontigenin to elicit anti-cancer activity.

CONCLUSIONS

In summary, the developed enzymatic synthesis method for rhapontigenin is facile and facilitates preparative rhapontigenin production and may have utility for preparation and purification of other stilbene and flavonoid glycosylates to be resolved into their respective aglycones. The developed HPLC assay is sensitive, reproducible, accurate and specific. It has been successfully applied to the preliminary study of pharmacokinetics and metabolism of rhapontigenin in rats and is the first report of these pharmacometric properties in any species. Using this HPLC method, large numbers of biological samples can be analyzed in a relatively short period of time. Further studies are ongoing in our laboratory to further characterize the pharmacokinetics and metabolites of rhapontigenin and other stilbenes as well as their pharmacological and toxicological activities.

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