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Development of reversed-phase high performance liquid chromatography methods for quantification of two isomeric flavones and the application of the methods to pharmacokinetic studies in rats

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ABSTRACT

Isomers 5,7-dihydroxy-3,6,8-trimethoxy-2-phenyl-4H-chromen-4-one (5,7–dihydroxy-3,6,8 trimethoxy flavone) (flavone A) and 3,5-dihydroxy-6,7,8-trimethoxy-2-phenyl-4H-chromen-4-one (3,5-dihydroxy-6,7,8-trimethoxy flavone) (flavone B) have recently demonstrated differential antineoplastic activities against pancreatic cancer *in vitro*. These studies also indicated that these compounds target highly tumorigenic cells while sparing normal cells. The *in vivo* antitumor activities of these flavones have not been determined, and detection protocols for these compounds are needed to conduct pre-clinical assays following intravenous dosing. Here, we report methods developed using acetonitrile to extract two flavone isomers and corresponding internal standards, celecoxib and diclofenac, from rat plasma. Separation was achieved using a Shimadzu liquid chromatography system with a C18 column and mobile phase acetonitrile/water (60:40 and 70:30 for flavones A and B, respectively) containing 0.2% acetic acid and 0.05% triethylamine at a flow rate of 0.4 mL/min and detection at 245 nm. Calibration curves ranging from 250 to 2500 ng/mL and 2500 to 100,000 ng/mL for both flavones were linear ($r^2 \geq 0.99$) with the lower limits of quantification being 250 ng/mL. Recovery of concentrations 250, 1000, 2500, 5000, and 100,000 ng/mL ranged from 87 to 116% and 84 to 103% ($n = 3$) for flavone A and B, respectively. Stability of both flavones after a freezing/thawing cycle yielded a mean peak ratio ≥0.92 when compared to freshly extracted samples. Intravenous administration of a 20 mg/kg dose in rats yielded half-lives of 83.68 ± 56.61 and 107.45 ± 53.31 min with clearance values of 12.99 ± 13.78 and 80.79 ± 35.06 mL/min/kg for flavones A and B, respectively.

1. Introduction

Throughout history people have used plants for medicinal purposes. In the Andean mountains of South America, people often rely on a group of plants commonly known as vira-viras to treat different ailments [1]. Many of the bioactive compounds found in these plants are part of a class of polyphenolic secondary metabolites called flavonoids, a large family of compounds characterized by a C6–C3–C6 phenylbenzopyrone backbone. They are further subclassified as flavones, isoflavones, flavanones, flavonols, anthocyanidins, and chalcones. Over 5000 flavonoids have been discovered, but the potential health benefit of only a few have been studied [2]. Pharmacological properties of flavonoids include antioxidant [3,4], antiviral [5,6], antidiabetic [7], as well as protective properties of the gastrointestinal tract [8], and cardiovascular system [9]. Anticancer studies have shown some flavonoids to prevent or inhibit the development of cancer in rodents [10–14]. The antineoplastic properties of flavonoids are due to their effect on cell growth, inhibition of kinase activity, induction of apoptosis, suppression of the secretion of matrix metalloproteinases, and/or tumor invasive behavior [12,15,16].

Gnaphalium elegans and *Achyrocline bogotensis* are two of the species identified generically as vira-viras that are traditionally used in the treatment of various cancers. A pair of isomeric flavones, 5,7-dihydroxy-3,6,8 trimethoxy flavone (flavone A) and 3,5-dihydroxy-6,7,8–trimethoxy flavone (flavone B) were isolated from bioactive fractions of *G. elegans* and *A. bogotensis* respectively [1,17–19]. *In vitro* studies suggest that flavone A and flavone B have

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differential antineoplastic activity against cancer cell lines, which may depend upon cellular tumorigenic and differentiation status. Indeed, flavones A and B target cancer cell lines derived from colon, pancreas, breast, and prostate that have been categorized as being highly tumorigenic. Among these, flavone A demonstrated preferential activity against the better differentiated cell lines, while flavone B was shown to be active against the poorly differentiated cells. Furthermore, flavones A and B demonstrated significant cytotoxic activity on cancer cell lines of the pancreas and the colon [1].

Only 6% of the 46,420 new cases of pancreatic cancer projected to be diagnosed during 2014 will survive another 5 years. Of the 96,830 new cases of colon cancer predicted to be diagnosed in 2014, only 40% will be detected at an early stage to have a 90% 5 year survival rate. However, once the cancer has spread to distant organs, the 5 year survival rate drops to 13% [20]. Thus, new treatments for pancreatic and colon cancer are urgently needed. *In vivo* testing and mechanistic analysis are warranted to further understand the antineoplastic properties of these compounds.

In this study, we developed methods to quantify the concentration of flavone A or flavone B in rat plasma, using high performance liquid chromatography (HPLC) analysis with celecoxib or diclofenac as internal standards, respectively. These methods were used to determine the pharmacokinetic parameters of flavone A and flavone B in rats administered a 20 mg/kg dose *via* intravenous injection.

2. Experimental

2.1. Chemicals and standards

5,7-Dihydroxy-3,6,8 trimethoxy flavone (flavone A) and 3,5-dihydroxy-6,7,8-trimethoxy flavone (flavone B) were extracted as described previously [1]. NMR analysis was done to verify that compounds were ≥99% pure in a Bruker 400 MHz spectrometer (Billerica MA). The results were compared to spectra previously obtained [17,19]. Celecoxib was purchased from Toronto Research Chemicals (Toronto, ON, CA). Diclofenac sodium was purchased from MP Biomedicals, LLC (Solon, OH). HPLC grade acetonitrile, acetic acid, triethylamine and water were purchased from Fisher Scientific (Pittsburgh, PA). Polyethylene glycol 400 was purchased from Electron Microscopy Sciences (Hatfield, PA).

2.2. Stock solution and standards

Stock solutions of flavone A (100 μg/mL) and celecoxib (25 μg/mL) were prepared with acetonitrile/water/acetic acid/triethylamine (60:40:0.2:0.05). Stock solutions of flavone B (100 μg/mL) and diclofenac (25 μg/mL) were prepared with acetonitrile/water/acetic acid/trimethylamine (70:30:0.2:0.05). All stock solutions were stored protected from light at 4 °C.

2.3. Sample preparation

Calibration curves for flavone A was prepared by spiking 100 μL of blank plasma with 100 μL flavone A, 100 μL celecoxib (25 μg/mL), and 200 μL of organic solvent (acetonitrile). The samples were vortex mixed for 5 s before centrifugation for 15 min at 4400 rpm. The supernatant was removed to a clean tube and evaporated using a Labconco vacuum concentrator (Kansas City, MO). 200 μL of mobile phase was used to reconstitute the residue and 100 μL of sample was injected into the HPLC column. Calibration curves for flavone B were prepared in similar manner with diclofenac as the internal standard. Analysis was conducted in triplicate.

2.4. HPLC conditions and quantitation

Assays were performed with a Shimadzu liquid chromatography system (Shimadzu Scientific Instruments Inc., Columbia, Maryland, USA) consisting of a DGU-20A Prominence degasser, LC020AB solvent delivery system, SIL-20A$_{HT}$ auto sampler, CBM-20A communication bus module, SPD-M20A diode array detector, and CTO-20A column oven with a C18 (100 × 4.6 mm, 2.6 μm; ACE, Aberdeen, Scotland) column. Mobile phases used were acetonitrile/water/acetic acid/triethylamine 60:40:0.2:0.05 (for flavone A HPLC assay) and 70:30:0.2:0.05 (for flavone B HPLC assay). Detection was at 245 nm with a temperature of 30°C. Flow rate was 0.4 mL/min with run times of 11 and 10 min respectively. LC solutions program was used to collect and analyze the data. Plotting the peak area ratios of flavone against internal standard in Excel were used to make the calibration curves.

2.5. Accuracy and precision

Calibration curves were used to determine the accuracy and precision of the methods. The coefficient of variation (CV) of three replicates performed on the same day was used to determine the precision for intra-day analysis and the CV of replicates performed on three consecutive days was used for the precision of inter-day analysis. Accuracy was determined by how close the calculated concentration was to the known concentration. Data is presented as mean ± standard deviation.

2.6. Recovery

Five concentrations (250, 1000, 2500, 5000 and 100,000 ng/mL) were used to determine the percent of flavone recovered by the extraction method. Recovery was determined by comparing the peak area ratio of extracted samples to the peak area ratio of non-extracted standard of the same concentration. Experiment was performed in triplicate.

2.7. Stability

Three concentrations (1000, 5000, and 100,000 ng/mL) were used to determine the stability of the flavones after freezing/thawing cycle. 100 μL of blank plasma was spiked with 100 μL flavone and put in −20 °C for 24 h. After thawing, 100 μL of internal standard and 200 μL of acetonitrile were added to the sample. Extraction was completed as describe above. The peak area ratio of frozen samples were compared to the peak area ratio of freshly prepared samples. Experiments were performed in triplicate.

2.8. Animals and drug administration

Male Sprague-Dawley rats weighting 269–305 g and fitted with jugular vein cannulas were purchased from Charles River (Raleigh, NC). Animals were housed in a 12 h light-dark cycle and experiments were carried out within the Animal Care Committee of East Tennessee State University guidelines. During the experiment, rats were housed in metabolic cages. Flavones were mixed in polyethylene glycol 400 and administered by intravenous injection to deliver a 20 mg/kg dose of flavone A ($n = 6$) or flavone B ($n = 6$). Serial blood samples (250 μL) were collected at 0, 5, 15, 30, 60, 90, 120, 240, and 360 min post dosing. After 3 min of centrifugation at 10,000 rpm, plasma was collected and stored at −20 °C.

2.9. Pharmacokinetic analysis

Outliers were determined using SPSS Statistics software version 21. The non-compartmental component of Phoenix WinNonlin