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Short Communication

Determination of visnagin in rat plasma by liquid chromatography with tandem mass spectrometry and its application to *in vivo* pharmacokinetic studies

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ABSTRACT

A rapid and sensitive method for the determination of visnagin in rat plasma was developed using liquid chromatography tandem mass spectrometry (LC–MS/MS). The plasma samples were processed by protein precipitation with methanol:acetonitrile (25:75) and warfarin was used as the internal standard. After vortex mixing and centrifugation, the supernatants were diluted with water (1:5) and then directly injected onto a Phenomenex Synergi Max RP column (75 mm × 2.0 mm ID, 4 μm) with isocratic elution at a flow rate of 0.2 mL/min. The mobile phase consisted of water and methanol (15:85, v/v) containing 0.1% formic acid and 5 mM ammonium acetate. The total run time (injection to injection) was less than 4 min. Detection of the analytes was achieved using positive ion electrospray in the selected reaction monitoring (SRM) mode. The linear standard curve ranged from 1.0 to 5000 ng/mL and the precision and accuracy (inter- and intra-run) were within 4.5% and 4.3%, respectively. The method, which is rapid, simple, and precise, was successfully used to support a visnagin pharmacokinetics study.

1. Introduction

Visnagin is a furanocoumarin derivative and one of the major constituents in *Ammi visnaga* L. (Khella, apiaceae). Khella was used by ancient Egyptians as a treatment for kidney stone disease. Visnagin has cardiovascular effects due to calcium channel blocking actions [1,2]. It can inhibit vascular smooth muscle contraction and seems to dilate peripheral and coronary vessels and increase coronary circulation [1–5]. Visnagin also has negative chronotropic and inotropic effects and reduces peripheral vascular resistance [2,6]. Khella extracts appear to have some antimicrobial activity; this might be attributable to both the khellin and visnagin constituents, which both seem to have antifungal, antibacterial, and antiviral activity [7].

We showed previously that Khella extract prevents urolithiasis (kidney stone formation) by decreasing calcium oxalate crystal growth in the stone forming rat model [8,9]. In addition, khellin and visnagin, the main compounds in Khella extract, decreased calcium oxalate crystal growth in rats as well. Although, visnagin has been studied for therapeutic use for over 10 years, its pharmacokinetics have not been investigated.

Various analytical methods have been used to identify and quantify visnagin, including thin-layer chromatography (TLC) [10,11], high performance liquid chromatography (HPLC) with solid-phase extraction (SPE) [12,10], capillary electrophoresis [13] and HPLC [14–19]. All of these methods involved multi-step extractions, large volumes of organic solvent and had large sample volume requirements. In order to fully characterize the pharmacokinetics of visnagin, we wanted to collect blood samples in each animal at multiple time points, which limits the per sample blood volume to not more than 10% of total blood volume. Therefore, a simple, sensitive, and robust method with small sample volume requirements was needed.

The purpose of this study was to develop a sensitive and highly selective method based on liquid chromatography–tandem mass spectrometry (LC–MS) to determine visnagin in rat plasma.

2. Experimental

2.1. Chemical and reagents

The analytes, visnagin (>97%) and warfarin (98%), shown in Fig. 1, and polyethylene glycol 200 (PEG 200) were purchased from Sigma (St. Louis, MO, USA). All chemicals used in the study were analytical grade. Ammonium acetate, formic acid (88%), and acetonitrile were obtained from VWR (West Chester, PA, USA). Methanol was purchased from ThermoFisher Scientific (Pittsburgh, PA, USA). HPLC grade deionized water was prepared using a Barnstead Nanopure Diamond UV ultra pure Water System (Dubuque, IA, USA).

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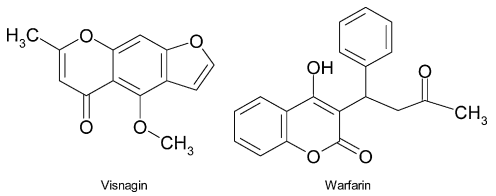

Fig. 1. Chemical structures of visnagin and warfarin (internal standard).

2.2. Preparation of visnagin standards and quality control samples

The stock solution of visnagin was prepared in methanol at a concentration of 0.5 mg/mL and used to spike standards and the quality control (QC) samples. Standards were prepared by spiking blank rat plasma at seven concentrations ranging from 1.0 to 5000 ng/mL. For validation, QC samples were prepared by spiking rat plasma at three concentration levels (20, 850 and 4000 ng/mL). The standards and QC were stored at −20 °C until analysis. The internal standard solution was prepared by dissolving warfarin in methanol:acetonitrile (25:75, v/v) to produce the final concentration of 125 ng/mL and stored at 4 °C.

2.3. LC–MS/MS condition

The LC–MS/MS system consisted of a Surveyor HPLC autosampler, Surveyor MS quaternary pump and a TSQ Quantum Discovery triple quadrupole mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). The TSQ Quantum was equipped with an electrospray (ESI) ionization source and operated in the positive ion mode. The ESI source spray was set orthogonal to the ion transfer tube. The MS/MS conditions were optimized by infusing visnagin in methanol while the ESI source parameters were tuned for maximum abundance of $[M+H]^+$ ions of visnagin at the LC flow rate of 0.2 mL/min. For quantification, the TSQ Quantum was operated in high-resolution selected reaction monitoring mode (H-SRM). The instrument parameters included an ion transfer tube temperature of 325 °C, spray voltage of 5.0 kV and source CID set to 5 V. Nitrogen was used as the sheath and auxiliary gas and set to 35 and 10 (arbitrary units), respectively. The collision energy was 27 eV for visnagin and warfarin and the collision gas (argon) pressure was set to 1.5 mTorr. The product ion spectrum for visnagin showed the loss of *m/z* 15 (methyl radical) from the protonated molecular ion ($[M+H]^+ = m/z$ 231), producing a molecular fragment with mass 216. Thus, the H-SRM scheme followed transitions of the $[M+H]^+$ precursor to selected product ions with the following values: *m/z* 231 → 216 for visnagin and *m/z* 309 → 163 for warfarin. The instrument was operated in enhanced (high) resolution mode with the peak width (full width at half-maximum, FWHM) set to 0.2 *m/z* at Q1 and to 0.70 *m/z* at Q3.

2.4. Chromatographic conditions

Chromatography was performed on a Phenomenex Synergi Max RP, 75 mm × 2.0 mm ID, 4 μm analytical column (Torrance, CA, USA) at ambient temperature. The mobile phase used for analysis was 0.1% formic acid, 5 mM ammonium acetate in deionized water and methanol (15:85, v/v) delivered at a flow rate of 0.2 mL/min. The mobile phase was degassed and filtered through 0.45 μm Nylon 66 membrane before use. The autosampler rinse solution was comprised of isopropanol, acetonitrile, and water (35:35:30, v/v/v) containing 0.1% formic acid and the sample tray was maintained at 10 °C. Data were acquired and processed using ThermoFinnigan XCalibur® software version 1.4, service release 1 (ThermoFisher, San Jose, CA, USA).

2.5. Sample preparation

The internal standard solution (125 ng/mL, 200 μL) was added to visnagin standards, QCs or samples (50 μL). After shaking for 2 min on a vortex shaker, samples were centrifuged at 10,000 rpm for 15 min. An aliquot of clear supernatant (100 μL) was transferred to 1.5 mL tubes and diluted with DI water (500 μL). The samples were then transferred to autosampler vials and 10 μL of sample was injected on the column.

2.6. Method validation procedure

Calibration curves were constructed by linear regression of the peak area ratio of visnagin to warfarin (*Y*-axis) and the nominal standard visnagin concentration (*X*-axis) with a weighting factor of $1/y^2$. Concentrations of QCs and samples were calculated using the regression equation of the calibration curve. Standards at all concentration were analyzed in duplicate.

The method was validated with respect to selectivity, processed stability, carry-over, linearity, precision, accuracy, extraction recovery and matrix effect [20]. The stability of the analytes post-processing was assessed by analyzing QC samples immediately after processing and then daily for 3 days while stored in the autosampler at 10 °C. Carry-over was evaluated by placing vials of blank solvent at several locations in the analysis set. The accuracy and precision of the assay was determined by the analysis of QC samples at visnagin concentrations of 20, 850 and 4000 ng/mL. Replicate QC samples ($n = 8$) were analyzed in each of the three runs to determine intra-run and inter-run precision and accuracy. Precision is presented as percent relative standard deviation values (%R.S.D.), which were calculated using one-way ANOVA with run as the grouping variable. Accuracy was calculated as the percent error in the calculated mean concentration relative to the nominal visnagin concentration (%R.E.). For the assay to be considered acceptable, the precision and accuracy determined at each QC concentration level was required to be within 15%. The extraction recovery and absolute matrix effect were evaluated for visnagin samples prepared at concentrations of 20 and 4000 ng/mL and for the internal standard warfarin at a concentration of 500 ng/mL. Each set of samples was analyzed in triplicate. Extraction recovery was determined by comparing peak areas of the visnagin from spiked matrix (blank plasma containing visnagin) in the same manner and spiked after extraction with the same standard concentration. Matrix effect on ionization was evaluated by comparing the visnagin peak areas of samples spiked post-extraction with corresponding peak area ratios of visnagin standards prepared in the injection solution.

2.7. Pharmacokinetic study

A male Sprague–Dawley rat (weighing 250 g) purchased from Harlan (Indianapolis, IN, USA) was used in this study. The animal was housed in a plastic cage and allowed to adapt to the environment for one week before being used for experiments. The animal was maintained on 12 h/12 h light/dark cycle and received standard chow and water *ad libitum* during the study. The animal experiment was performed according to the policies and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida, Gainesville, FL, USA (NIH publication #85-23).

For the pharmacokinetics study, visnagin (10 mg/kg in 2% ethanol and 2% PEG 200) was administered orally by gavage. Plasma samples (300 μL per blood sample) were collected from the sublingual vein into Vaccuette® heparinized tubes prior to dosing and at 10, 20, 30 min, 1, 4, and 6 h. Before blood collection, the rat was anaesthetized with halothane and the blood loss was replaced with 1 mL normal saline. The blood sample was centrifuged for 15 min at

4000 rpm at 4 °C. The supernatant was transferred into tubes and stored at −20 °C until analysis.

2.8. Data analysis

Pharmacokinetic parameter estimates were obtained by non-compartmental analysis using WinNonlin® software package version 5.0 (Pharsight Corporation, Mountain View, CA, USA).

3. Results and discussion

3.1. Chromatographic method

Extracted ion chromatograms are shown in Fig. 2. Fig. 2A shows a blank rat plasma sample (no visnagin or warfarin), Fig. 2B is the limit of quantitation (LOQ) standard for visnagin (1 ng/mL) in plasma, Fig. 2C shows a plasma sample obtained 6 h after oral administration (concentration = 95.4 ng/mL) and Fig. 2D depicts the internal standard warfarin (500 ng/mL). The retention times were approximately 1.6 min and 1.9 min for visnagin and warfarin, respectively. The peaks of visnagin and warfarin were well separated without interference from any endogenous substance. During method development, we discovered that it was important for visnagin and warfarin to be chromatographically separated because visnagin affected warfarin ionization such that there was an inverse relationship between the visnagin concentration and warfarin response.

3.2. Method validation

Validation of the assay method was conducted according to the United States Food and Drug Administration (FDA) guidelines with respect to selectivity, stability, carry-over, linearity, precision, and accuracy. The calibration curve was linear over the concentration range of 1.0–5000 ng/mL with a mean ± S.D. correlation coefficient

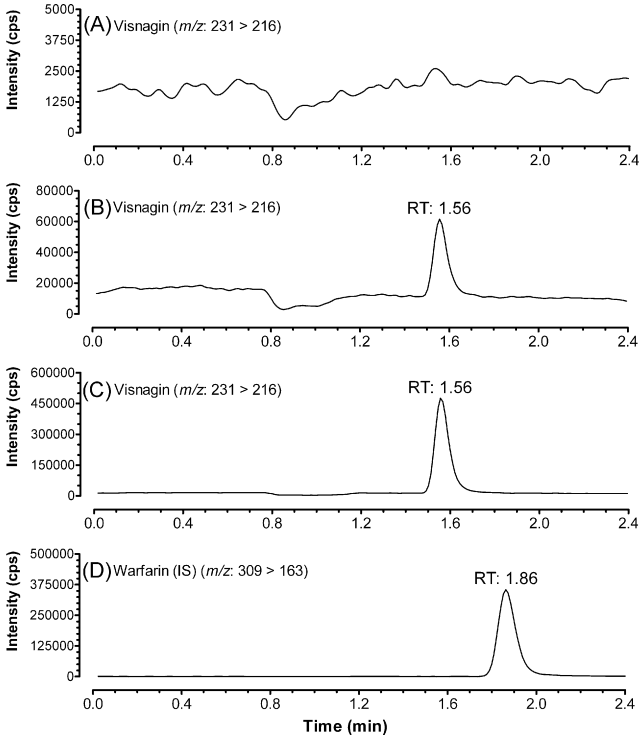

Fig. 2. The extracted LC–MS/MS chromatograms of: (A) blank rat plasma; (B) visnagin lower limit of quantitation (1.0 ng/mL); (C) plasma sample from a rat obtained 6.0 h after oral administration of visnagin (concentration = 95.4 ng/mL); and (D) warfarin (500 ng/mL) as the internal standard.

Table 1
Intra-run ($n = 8$) and inter-run ($n = 24$) precision (estimated by one-way ANOVA) and accuracy for analysis of visnagin in rat plasma.

		Quality control samples (ng/mL)		
		20	850	4000
Accuracy	Mean bias (%R.E.)	2.9	3.5	4.3
Precision	Intra-run (%R.S.D.)	3.6	4.0	3.1
	Inter-run (%R.S.D.)	4.5	4.3	4.4

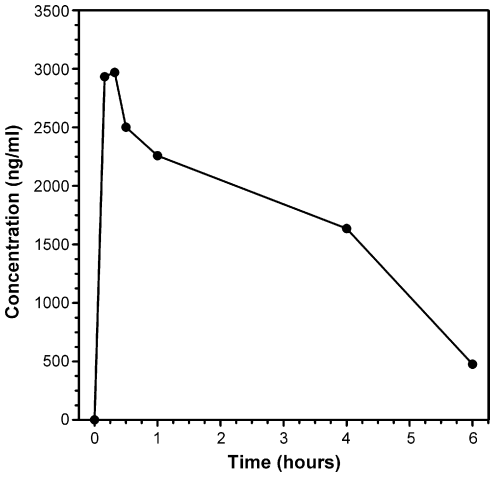

Fig. 3. Concentration–time profile of visnagin in plasma obtained from a single rat after oral administration. The solid line represents the best fit to the data.

(R^2) of 0.9922 ± 0.0008. Precision is represented as the relative standard deviation (%R.S.D.) and accuracy was calculated as the relative error (%R.E.) from the respective nominal concentration. The maximum acceptable limit for precision and accuracy was set at 15%. The intra-run and inter-run precision (%R.S.D.) was ≤4.5% and accuracy (%R.E.) was ≤4.3% (Table 1). Visnagin and warfarin were both stable in processed samples held in the autosampler at 10 °C for at least 72 h with mean calculated values within 10% of the nominal concentration. There was no evidence of sample carry-over. In addition, the matrix effect assessed by spiking samples post-processing showed <10% difference from spiked injection solvent. The mean extraction recoveries ($n = 6$) for visnagin were 100.8% and 100.4% at concentration of 20 and 4000 ng/mL, respectively. The extraction recovery for warfarin was 107.1%.

3.3. Application to study visnagin pharmacokinetics in rats

The visnagin concentration–time profile obtained after oral administration is shown in Fig. 3. The maximum concentration (C_{max}) was 2969 ng/mL, which was observed at 0.33 h (T_{max}). The area under the concentration–time curve (AUC), calculated based on the trapezoidal rule, was 11.9 h × mg/L, the apparent clearance estimated after oral administration (CL/F) was 0.84 L/kg, and the terminal elimination half-life was 2.3 h.

4. Conclusion

The application of LC–MS/MS for analysis of visnagin in rat plasma resulted in a sensitive, simple and robust method. The method was developed and validated with respect to selectivity, carry-over, linearity, precision and accuracy. The method requires only a small amount of sample (50 μL) and can be applied for quantitative determination of visnagin in rat plasma. Therefore, the present method is applicable for pharmacokinetic studies of visnagin in rats.

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