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Short communication

Simultaneous determination of fraxin and its metabolite, fraxetin, in rat plasma by liquid chromatography-tandem mass spectrometry and its application in a pharmacokinetic study

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ABSTRACT

For the first time, a rapid and sensitive high-performance liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of fraxin and its metabolite, fraxetin, in rat plasma, using esculin as the internal standard (IS). The plasma samples were precipitated with methanol before separation on an Nova-Pak C_{18} column (150 mm × 3.9 mm, 3 μm) using a mobile phase consisting of 0.1% formic acid and methanol (55:45) at a flow rate of 0.8 mL/min. The analytes were detected by multiple reaction monitoring in the negative ion mode with the mass transitions at *m/z* 368.9→ *m/z* 191.9 (fraxin), *m/z* 206.9→ *m/z* 191.8 (fraxetin) and *m/z* 339.0→ *m/z* 176.9 (esculin, IS). The results demonstrated that the calibration curves for both analytes have good linearity ($r \geq 0.995$) over a concentration range of 5.00–3000 ng/mL. The assay was validated according to the regulatory bioanalytical guidelines and proved acceptable. The intra- and inter-day precisions (R.S.D.%) were within 10.9% for both analytes, whereas the deviation of assay accuracies (R.E.%) ranged from −5.3 to 1.0%. The method was successfully applied to a pharmacokinetic study after a single oral dose of fraxin at 50 mg/kg to rats.

1. Introduction

Cortex Fraxini, called "Qin pi" in Chinese, has a long history of use as a Chinese herbal folk medicine, belonging to the 'heat-clearing' category according to the classification of traditional Chinese medicine (TCM). It is the dried bark of different *Fraxinus* species, *such as Fraxinus chinensis* Roxb., *Fraxinus chinensis* Roxb. var.*acuminate* Lingelsh., *Fraxinus rhynchophylla* Hance and *Fraxinus stylosa* Lingelsh. Cortex Fraxini is commonly used as an antibacterial, analgesic and anti-inflammatory agent and clinically effective in the treatment of hyperuricemia, arthritis, diarrhea and bacillary dysentery in TCM [1].

Fraxin, a main active component isolated from Cortex Fraxini, has a wide spectrum of bioactivities, including anti-inflammatory, anti-hyperuricemia, diuresis and protection of cells from oxidative stress [2–4]. Fraxin can be extensively metabolized to fraxetin in vivo [5], which is also an effective constituent isolated from Cortex Fraxini and possesses a variety of activities such as antioxidant, anti-inflammatory, antithrombotic, antiviral, anticarcinogenic and neuroprotective effects [6–9].

Although fraxin has been used clinically for many years, its pharmacokinetic characteristics remain unclear. In order to elucidate their pharmacokinetic properties, a sensitive method for simultaneous determination of fraxin and its metabolite fraxetin in bio-samples is urgently needed. Analytical methods for the single or simultaneous quantification of fraxin or fraxetin in crude herbs or in the Cortex Fraxini agent have been reported using high performance thin layer chromatography-ultraviolet and high-performance liquid chromatography (HPLC) coupled with ultraviolet or tandem mass spectrometry (MS/MS) detection [10–13]. However, these methods are not sensitive to meet the quantitative demands of biological samples with low concentrations and complex components given their lowest detection limits of 53.8 and 24.0 ng/mL for fraxin and fraxetin, respectively. Thus, this study aimed to develop a rapid, sensitive and specific LC–MS/MS method for the quantification of fraxin and fraxetin in rat plasma. And the developed method was supposed to be applied to a pharmacokinetic study of fraxin and fraxetin after oral administration of fraxin at 50 mg/kg in rats.

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Table 1
Mass spectrometry parameters.

Analyte	RT (min)	MRM (*m*/*z*)	CE (V)	DP (V)
Fraxin	1.8	368.9→206.9[a]	−30	−90
		368.9→191.6	−40	−66
Fraxetin	2.3	206.9→191.8[a]	−20	−60
		206.9→163.9	−30	−60
Esculin (IS)	1.7	339.0→176.9[a]	−27	−76
		339.0→132.5	−54	−76

[a] Transition used in the quantification.

2. Experimental

2.1. Chemicals and reagents

Fraxin, fraxetin and esculin (internal standard, IS) were purchased from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China). The purity of three standards was higher than 98.9%. Chromatographic grade methanol and acetonitrile were bought from Tedia Company (Fairfield, OH, USA). Formic acid of analytical grade was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water up to a resistivity of 18.2 MΩ prepared by a Milli-Q water purification system (Millipore, Mississauga, Canada) was used throughout the study.

2.2. Liquid chromatography and mass spectrometry

The LC–MS/MS analysis was carried out on an ekspert^{TM} ultra LC 110-XL system (SCIEX, Concord, Ontario, Canada), composed of a quaternary pump, an autosampler, a column oven, and an AB QTRP 4500 mass spectrometer equipped with an electrospray ion source. Analyst Version 1.6.2 software was used for data acquisition and analysis. LC separation was performed on an Nova-Pak C_{18} column (150 mm × 3.9 mm, 3 μm; Waters, Wexford, Ireland) protected by a C_{18} guard column (30 mm × 2.00 mm, Phenomenex, Torrance, CA, USA), and the column oven was set at 35 °C. The mobile phase consisted of 0.1% formic acid and methanol (55:45), delivered at a flow rate of 0.8 mL/min. The run time was 3.0 min, and the injection volume was 10 μL.

The ESI–MS data were performed in negative ionization mode by multiple reaction monitoring (MRM). For each compound, two mass fragments were monitored, one fragment used for quantification and the other for the additional confirmation of identity. The MRM transitions can be found in Table 1. The compound dependent parameters like the collision energy (CE) and de-clustering potential (DP) were adjusted to provide the highest sensitivity. The independent parameters remained constant. The ion temperature (TEM) and ion spray voltage were maintained at 500 °C and −4500 V. Curtain gas (CUR), source gas1 (GS1) and gas 2 (GS2) were 50, 40 and 40 psi, respectively. Entrance potential (EP) and collision cell exit potential (CXP) were set at 10 V and 7 V, respectively. Dwell time was set at 150 ms for each transition.

2.3. Preparation of calibration standards and quality control (QC) samples

The stock solution of fraxin, fraxetin and esculin (IS) were separately prepared in methanol at a concentration of 400 μg/mL. Mixed standard solution of fraxin and fraxetin were serially prepared by diluting the stock solutions with methanol:water (50:50, v/v) to yield final concentrations of 5.00, 10.0, 30.0, 100, 300, 1000 and 3000 ng/mL. Mixed QC samples were prepared independently in blank plasma at concentrations of 8.00, 500 and 2500 ng/mL. A 100 ng/mL of IS solution was prepared by diluting the stock solution with methanol:water (50:50, v/v). The standard and IS solutions were stored at 4°C and the QC samples were stored at −20 °C.

2.4. Sample preparation

A simple protein precipitate method was employed for the extraction of fraxin, fraxetin and IS from rat plasma. A 30.0 μL aliquot of the IS solution and 30.0 μL of methanol:water (50:50, v/v) was added to 30.0 μL plasma sample. The sample mixture was deproteinized with 125 μL of methanol. The protein precipitate was removed via centrifugation at 11,300*g* for 5 min, and 10.0 μL of the supernatant was then injected into the LC–MS/MS system.

2.5. Assay validation [14]

The selectivity was assessed by comparing the chromatograms of from six different rats blank plasma with their corresponding spiked plasma. Each sample was tested using the proposed extraction procedure and LC–MS/MS conditions.

Linearity was assessed by assaying calibration curves in rat plasma with seven levels covering a range of 5.00–3000 ng/mL for fraxin and fraxetin in duplicate in three consecutive runs. And the curves were fitted using a linear weighted ($1/x^2$) least-squares regression method by measuring the peak area ratio of the analytes to IS. The LLOQ was defined as the lowest concentration at which the both the precision and accuracy were less than 20% by analyzing the six replicates of samples spiked with each analyte.

The intra- and inter-day accuracy (as relative error, R.E.) and intra- and inter-day precision (as relative standard deviation, R.S.D.) were based on assay of six replicate QC samples (8.00, 500 and 2500 ng/mL) on three consecutive runs. The criterion of acceptance was ±15% deviation from the nominal value for the precision and accuracy.

The recovery of fraxin and fraxetin using protein precipitation was evaluated by comparing the mean peak areas of the regularly prepared samples (n = 6) with the values of spike-after-extraction samples, which represented the 100% recovery, at 8.00, 500 and 2500 ng/mL. To prepare the spike-after-extraction samples, blank human plasma was processed according to the sample preparation procedure mentioned above. All supernatants were mixed with the appropriate standard solutions of fraxin and fraxetin. The recovery of IS was evaluated by comparing the mean peak areas of six regularly prepared samples with those of spike-after-extraction samples. The matrix effect of the assay was evaluated by comparing the peak areas of analytes from the spiked-after protein precipitation samples with those of standard solution at 8.00 and 2500 ng/mL.

The stability of analytes in rat plasma was evaluated by analyzing replicates (n = 3) of the plasma samples at 8.00 and 2500 ng/mL placed on storage for 35 days at −20 °C, for 4 h at room temperature (23 °C) and after three freeze/thaw cycles from −20 °C to room temperature. The auto-sampler stability was studied by reanalyzing the extracted samples kept in the auto-sampler at room temperature for 12 h. Samples were considered stable if assay values were within ±15% of the nominal values.

2.6. Application of the method

This developed method was employed to study the pharmacokinetic profiles of fraxin and fraxetin after oral dose of fraxin at 50 mg/kg. The animal welfare and experimental protocols were approved by the animal care committee of Xuzhou medical college. Six Male Sprague-Dawley rats (SD rat) (190–240 g) were provided by Xuzhou Medical College Experimental Animal Center (Xuzhou, China). The rats were fasted overnight with free access to water prior to drug administration. Blood samples (about 0.4 mL)