Journal of Chromatography B 1067 (2017) 71–88

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Determination of the phytochemical composition of *Jingning fang* and the *in vivo* pharmacokinetics of its metabolites in rat plasma by UPLC–MS/MS

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ARTICLE INFO

Keywords:
Thermo Q Exactive™
Plus Orbitrap™
Mass spectrometer
Chemical constituents
HPLC-QTOF/MS/MS
Pharmacokinetics

ABSTRACT

Jingning fang (JNF) is an effective Traditional Chinese Medicine (TCM) which is used for the treatment of Attention Deficit Hyperactivity Disorder (ADHD). To clarify the bioactive constituents of JNF, a Thermo Q Exactive™ Plus Orbitrap™ mass spectrometer was used in this study. More than 127 chemical compounds were isolated and identified tentatively in the JNF extract, while 42 prototype constituents with 4 potential metabolites were identified tentatively in rat plasma. A method for simultaneous determination of polygalaxanthone III (PAIII), sibiricose A_5 (A_5), sibiricose A_6 (A_6), 3, 6′-disinapoyl sucrose (3,6′-DISS), tenuifoliside C (TEC), tenuifolin B (TNB), verbascoside (VCE), heterophyllin B (HEB) and schisandrin (SCH) in rat was developed and validated using polydatin (PLN) and psoralen (PSN) as internal standards. All calibration curves proved favorable linearity ($R^2 \geq 0.9923$) in linear ranges. The lower limit of quantification (LLOQ) was 2.5 ng/mL for PAIII, A_5, 3, 6′-DISS, TNB, VCE, HEB and SCH, 1.0 ng/mL for A_6 and TEC, respectively. Intra-day and inter-day precisions didn't exceed 14.0% for all the analytes. Extraction recoveries and matrix effects of analytes and IS were acceptable. The validated method has been successfully applied to the pharmacokinetics (PK) studies of the nine compounds in JNF. These findings are useful for predicting the bioactive components of JNF, and will aid in optimizing dose regimens of the drug.

1. Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the most common neuro-behavioral disorder of childhood, and it affects up to 1 in 20 children worldwide. Individuals with ADHD often present with inattentiveness, hyperactivity, impulsiveness and affective disorders [1,2]. The ADHD symptoms in approximately 80% of children persist into adolescence, and may even continue into adulthood. The effects of ADHD significantly impact on the affected individual throughout childhood and well into adult life, especially if not managed optimally. People with ADHD tend to have lower occupational status, poor social relationships, depression, marital discord, and are more likely to commit vehicle traffic offences [3].

Jingning Fang (JNF) is a TCM formula used for the treatment of ADHD in China. It is composed of seven medicinal herbs including Pseudostellariae Radix, Rehmanniae Radix Praeparata, Schisandrae Chinensis Fructus, Lycii Fructus, Polygalae Radix, Acori Tatarinowii Rhizoma and Poria with the weight ratio of 2:2:1:1:2:1:1. JNF is a clinical prescription created by Doctor Junhong Wang at the Dongzhimen Hospital of Beijing University of Chinese Medicine for the treatment of ADHD in the clinic. Liu [4] has reported that JNF shows better clinical efficacy and safety than orthodox drugs in the treatment of children with ADHD of Qi Yin deficiency. In addition, it has very minimal side effects. Thus, JNF has promising potential for the development of a new anti-ADHD drug. It is important to identify the bioactive constituents of JNF in the elucidation of its clinical effectiveness. The bioactive constituents which are not absorbed into the blood should not be considered "real bioactive constituents" even though strong bioactivity is observed *in vitro* assays [5]. Moreover, therapeutic effects of TCM are also determined by the plasma concentrations of bioactive constituents. Therefore, in order to explore the effective constituents in JNF, it is necessary to identify the major compounds absorbed into the blood, as well as their pharmacokinetic profiles.

In this study, the constituents of JNF and the absorbed prototype constituents and their metabolites in rat plasma after i.g administration

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http://dx.doi.org/10.1016/j.jchromb.2017.09.019
Received 14 July 2017; Received in revised form 25 August 2017; Accepted 12 September 2017
Available online 21 September 2017
1570-0232/

of JNF were identified using a Thermo Q Exactive™ Plus Orbitrap™ mass spectrometer [6], based on a database of chemical information derived from the literature on the individual herbs contained in JNF. A rapid validated high-performance liquid chromatography method was developed for evaluating the pharmacokinetics of several JNF-derived metabolites in rat plasma by using the ultra-performance liquid chromatography coupled to triple-quadrupole mass spectrometry [7].

2. Materials and methods

2.1. Chemicals and materials

The herbs that make up JNF, Pseudostellariae Radix (the dried root or rhizome of *Pseudostellaria heterophylla* (Miq.) Pax ex Pax et Hoffm.), Rehmanniae Radix Praeparata (the dried root of *Rehmannia glutinosa* Libosch.), Schisandrae Chinensis Fructus (the dried fruit of *Schisandra Chinensis* (Turcz.) Baill.), Lycii Fructus (the fruit of *Lycium barbarum* L.), Polygalae Radix (the dried root of *Polygala tenuifolia* Willd *or Polygala sibirica* L.), Acori Tatarinowii Rhizoma (the dried root bark of *Acorus tatarinowii* Schott) and Poria (the dried sclerotia of *Poria cocs* (Schw.) Wolf), were purchased from Beijing Tongrentang Pharmaceutical Co. Ltd. (Beijing, China). PAIII, 3, 6′-DISS, VCE, SCH, PLN and PSN, RTN (all 98%; pure) were purchased from the National Institute for Food and Drug Control (Beijing, China). A_5, A_6, BTE, TFA, TFB, TFN, SDB, DSR, SNB, TEC, TNB and HEB (all 98%; pure) were bought from Welch Materials (Shanghai, China). The standards mentioned above are listed in Table 1. HPLC-grade methanol and acetonitrile were bought from Fisher (USA). HPLC-quality water was obtained using a Cascada™ IX-water Purification System (Pall Co., USA). All other chemicals were of analytical grade.

2.2. Instrumentation

2.2.1. Liquid chromatography

The UPLC separation was performed on an ACQUITY UPLC™ BEH C18 column (100 × 2.1 mm, 1.7 mm). For the qualitative analysis of the constituents in JNF, the mobile phase consisted of 0.1% (v/v) FA in water (A) and methanol (B), at a flow rate of 0.3 mLmin^{-1}. The following gradient elution scheme was used: 0–5 min, 5–5% B; 5–50 min, 5–99% B; 50–55 min, 99–99% B; 55–55.1 min, 99–5% B; and 55.1–60 min, 5–5% B. The injection volume was 10 μL. For the pharmacokinetic studies, the mobile phase consisted of 0.2% (v/v) FA in water (A) and 0.2% (v/v) FA in methanol: acetonitrile (1;1, v:v, B) at a flow rate of 0.2 mLmin^{-1}. The following gradient elution for the negative mode was used: 0–1 min, 20–20% B; 1–3 min, 20–75% B; 3–5 min, 75–100% B; 5–7 min, 100-100% B; 7–7.10 min, 100–20% B; and 7.10-10.00 min, 20-20% B. In the positive mode, the gradient elution used was: 0–1 min, 20-20% B; 1–3 min, 20–75% B; 3–5 min, 75–100% B; 5–6 min, 100–100% B; 6–6.10 min, 100–20% B; and 6.10–10.00 min, 20–20% B. The injection volume was 10 μL.

2.2.2. Mass spectrometric conditions

For the qualitative analysis of the constituents in JNF, Thermo Q Exactive™ Plus Orbitrap™ mass spectrometer was used. Mass spectrometric parameters were: Sheath gas flow rate of 35; Aux gas flow rate of 10; Sweep gas flow rate of 0; S-lens RF level of 50. Spray voltage was set to 3.5 kV for the positive ion mode and −4.5 kV for the negative ion mode. Capillary temperature was set to 320 °C. Aux gas heater temperature was set to 350 °C. The *m/z* ranged from 100 to 1200 and 150–2000 in the positive and negative ion modes. For the pharmacokinetic studies, the detection of PAIII, A_5, A_6, 3,6′-DISS, TEC, TNB in Polygalae Radix, VCE in Rehmanniae Radix Praeparata, HEB in Pseudostellariae Radix and SCH in Schisandrae Chinensis Fructus was done on an AB SCIEX Triple Quad™ 4500 (Applied Bio-systems, Foster City, CA, USA) with an electrospray ionization source (Turbo Ionspray) maintained at a temperature of 500 °C.Other mass spectrometric parameters were: curtain gas flow pressure of 10 psi; medium CAD gas setting; ion spray voltage of 5500 V or −4500 V; and ion gas 1 and 2 at 50 psi. Other parameters with high sensitivity are shown in Table 2. The data were processed with AB SCIEX Analyst 1.6 Software (Applied Biosystems).

2.3. Preparation of JNF

72 g of Schisandra chinensis was crushed into powder and then extracted by reflux for thrice, each time for 60 min, each with 10-fold volume of 60% ethanol. Radix pseudostellariae, Radix rehmanniae preparata, Barbary wolfberry fruit, Polygala tenuifolia and Wolfiporia extensa were decocted 12 times with water, and extracted thrice, each time for 90 min. The volatile oil of Rhizoma acori tatarinowii (72 g) was extracted 8 times by steam distillation each time for 8 h. The volatile oil was used as β- cyclodextrin complex. The decoction was mixed with the water extract obtained earlier, concentrated to approximately 1.5 g/mL (at 60 °C, 1.5 g plant material per 1 mL water), and precipitated with 60% ethanol for 24 h. All the filtered supernatants were combined and evaporated under reduced pressure at 70 °C in a rotary evaporator. Subsequently, a certain amount of concentrated extract was dissolved in water to obtain an oral solution of JNF with a yield of

Table 1
The classification, acronyms and function of the 18 compounds used as standards in the study

	compound	acronyms	action
Xanthones	Polygalaxanthone III	PAIII	quantification standards
Sucrose esters	3, 6′-disinapoyl sucrose	3,6′-DISS	quantification standards
	sibiricose A_5	A_5	quantification standards
	sibiricose A_6	A_6	quantification standards
	tenuifoliside A	TFA	qualitative standards
	tenuifoliside B	TFB	qualitative standards
	tenuifoliside C	TEC	quantification standards
Phenylpropanoid glycosides	Verbascoside	VCE	quantification standards
Lignans	Schisandrin	SCH	quantification standards
	schisandrol B	SDB	qualitative standards
	deoxyschisandrin	DSR	qualitative standards
	schizandrin B	SNB	qualitative standards
Stilbenes	Polydatin	PLN	internal standard
Coumarins	Psoralen	PSN	internal standard
Flavonoids	rutin	RTN	qualitative standards
Alkaloids	betaine	BTE	qualitative standards
Polygala saponins	tenuifolin	TFN	qualitative standards
	Tenuifolin B	TNB	quantification standards
cyclicpeptide	heterophyllin B	HEB	quantification standards