Journal of Chromatography B 1092 (2018) 244–251

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Simultaneous quantification of 33 active components in *Notopterygii Rhizoma et Radix* using ultra high performance liquid chromatography with tandem mass spectrometry

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ARTICLE INFO

Keywords:
Notopterygii Rhizoma et Radix
Coumarins
Phenolic acid esters
Quality control
Ultra high performance liquid chromatography with tandem mass spectrometry

ABSTRACT

A method of ultra high performance liquid chromatography with tandem mass spectrometry was developed for the simultaneous quantification of 33 active components including 26 coumarins and 7 phenolic acid esters in *Notopterygii Rhizoma et Radix* (NRR). Chromatographic separation was achieved on a Kinetex® C_{18} column (100 mm × 2.1 mm i.d.; 2.6 μm) with a gradient elution in 18 min. Electrospray ionization tandem mass spectrometric detection was conducted in the positive and negative ionization modes with multiple reaction monitoring. All of 33 analytes showed good linearity ($R^2 \geq 0.9919$) within the test range. The relative standard deviations of the precision, repeatability and stability were not exceeding 4.97%, and the recoveries were in the range of 85.37%–115.00%. The matrix effect on the response of target analyte was not obvious. The validated method was successfully applied to quantify the 33 components in ten batches of NRR samples. Quantitative results showed the coumarins and phenolic acid esters with large difference in level of content in the herb samples. Furthermore, hierarchical cluster analysis and principal component analysis were applied to classify and discriminate these samples. The variations of isoimperatorin, notopterol, bergamottin, nodakenin, phenethylferulate were suggested as important indicators of NRR quality. This work may serve for quality identification and comprehensive evaluation of NRR.

1. Introduction

Notopterygii Rhizoma et Radix (NRR, Qianghuo in Chinese), the dried roots and rhizomes of *Notopterygium incisum* Ting ex H. T. Chang (*N. incisum*) and *Notopterygium franchetii* H. de Boiss. (*N. franchetii*), belongs to Apiaceae and is mainly grown in Sichuan province of China. NRR is an important constituent of traditional Chinese medicine (TCM) [1] and it is documented in every version of Chinese pharmacopoeia. Modern pharmacological research has demonstrated the diaphoretic, analgesic, anti-oxidant, anti-inflammatory, anti-viral, anti-arrhythmic and immunosuppressive effects of NRR [2]. Coumarins are a group of bioactive components naturally existing in NRR that have been recognized as major contributors for the pharmacological effects of this herb [3–10]. Through further analysis of chemical composition, dozens of phenolic acid esters, such as isopropylferulate, benzylsalicylate, *p*-hydroxyphenethyl anisate, (−)-bornylferulate, phenethylferulate, 4-methoxyphenethylferulate, and 4-methyl-3-*trans*-hexenylferulate, have been isolated from NRR and also demonstrated to be its bioactive components [11–13].

In view of the current situation, a variety of methods, including high-performance thin layer chromatography (HPTLC) [14], gas chromatography-mass spectrometer (GC–MS) [15], and high performance liquid chromatography (HPLC) coupled with a range of techniques, such as fluorescence detection (FLD) [16], diode array detection (DAD) [14, 15] and MS [17], have been employed for the determination of NRR. However, all these methods had a number of different shortcomings, such as, low sensitivity and selectivity for HPTLC and HPLC–DAD, narrow application scope for GC–MS and HPLC–FLD, and long assay time for HPLC–FLD, HPLC–DAD and HPLC–MS. Additionally, these methods only determinated a few analytes and were mainly focused on the quantification analysis of coumarins, including notopterol, isoimperatorin, nodakenin, bergaptol, bergapten, and bergamottin [18, 19]. In the aspect of quantitative determination of phenolic acid esters, HPLC was employed to analyse the content of *p*-hydroxyphenethyl anisate in *N. incisum* and *N. franchetii* and HPLC–MS was employed to analyse the content of *p*-hydroxyphenethyl anisate in

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https://doi.org/10.1016/j.jchromb.2018.06.006
Received 4 September 2017; Received in revised form 18 April 2018; Accepted 3 June 2018
Available online 08 June 2018
1570-0232/ © 2018 Published by Elsevier B.V.

different commercial parts of *N. incisum* with a low limit of detection (LLOD) value of 1.5 μg/L [19, 25]. It is well known that the multiple constituents contribute to the therapeutic effects of TCM. The variations of therapeutic effects of NRR are not limited to the contributions of a few coumarins, and it should also include the pharmacological effects of many other active coumarins, phenolic acid esters and so on. Moreover, the contents of chemical components tend to have a great effect on the efficacy. Therefore, there is a need to develop a selective, rapid and sensitive analytical method to quantify as many bioactive components of NRR as possible.

Ultra high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC–MS/MS), a powerful technique for quantification of chemicals in complex matrices, has been widely used to identify the minor components in TCM [20–24]. Compared with conventional HPLC, UHPLC is proven to be a more efficient chromatographic separation tool, which not only obtains higher analytical sensitivity and better peak shapes, but also gives a greater resolution within a shorter retention time. Accordingly, UHPLC–MS/MS was employed for the quantitative analysis of NRR.

In this study, a selective, rapid and sensitive UHPLC–MS/MS method was established for the simultaneous quantification of 33 active components including 26 coumarins and 7 phenolic acid esters in NRR for the first time. After it was validated, the method was successfully employed to quantify the active components in ten batches of NRR samples collected from different regions. Finally, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied to classify and discriminate the NRR samples. This work would provide a valuable reference for improving the quality control (QC) of NRR.

2. Materials and methods

2.1. Reagents and materials

Methanol (MeOH), acetonitrile (ACN) and formic acid were of LC–MS grade from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were available products of at least analytical grade. Deionized water was purified by Milli–Q system (Bedford, MA, USA). Reference standards of 33 compounds including capillarin (**1**), 7-isopentenyloxy-6-methoxy-coumarin (**2**), osthenol (**3**), scopoletin (**4**), isoimperatorin (**5**), psoralen (**6**), bergapten (**7**), isopimpinellin (**8**), bergaptol (**9**), cnidilin (**10**), pabulenol (**11**), oxypeucedanin hydrate (**12**), demethylfuropinarine (**13**), notopterol (**14**), 5-dehydronotopterol (**15**), notoptol (**16**), bergamottin (**17**), anhydronotopoloxide (**18**), nodakenetin (**19**), angenomalin (**20**), columbianetin (**21**), bergaptol-5-*O*-β-D-glucopyranoside (**22**), nodakenin (**23**), decuroside V (**24**), decuroside I (**25**), forbesoside (**26**), isopropylferulate (**27**), benzylsalicylate (**28**), *p*-hydroxyphenethyl anisate (**29**), (−)-bornylferulate (**30**), phenethylferulate (**31**), 4-methoxyphenethylferulate (**32**), 4-methyl-3-*trans*-hexenylferulate (**33**) (as shown in Fig. 1) were isolated and identified in our previous reports [13, 25–28]. The individual purity of each reference standard was confirmed over 98% according to LC–MS analysis.

The NRR samples were collected from different regions of Sichuan province of China with the exception that batch QH-1 was purchased from Hehuachi medicinal materials market in Chengdu city of China, and they were identified by Dr. Shun-Yuan Jiang of Sichuan Academy of Chinese Medicine Sciences (Chengdu, China). Voucher specimens were deposited at the State Key Laboratory of Natural and Biomimetic Drugs (Peking University, Beijing, China). Detail information of ten batches of NRR samples is shown in Table 1.

2.2. Preparation of standard solution

Stock solutions of 33 compounds (each 0.2 mg/mL) were individually prepared by dissolving accurately weighed reference standards in MeOH. A mixed standard solution containing all 33 reference standards was prepared and serially diluted with MeOH to appropriate concentrations to produce working solutions for quantitative analysis. The solutions were stored at 4 °C.

2.3. Sample extraction optimization and sample preparation

To obtain an efficient and appropriate extraction process, variables such as extraction method, extraction solvent, material ratio and extraction time were optimized by using sample QH-1. As a result, both refluxing and ultrasonic methods could achieve adequate extraction of chemical components. Considering the convenience of operation, the ultrasonic method was selected as the final extraction method. Subsequently, MeOH, chloroform ($CHCl_3$), acetone were chosen as candidate solvents, and the test results suggested that MeOH was the best choice for extraction of these components, especially for coumarin glycosides. Next, different material ratios (1/500, 1/250, 1/125) were analysed, and the material ratio of 1/250 was demonstrated to be more suitable. Then, the extraction time (30 and 60 min) was investigated, and the results showed that the target components could be sufficiently extracted within 30 min. According to above results, the optimal sample preparation method was found to be the extraction of 0.2 g sample with 50 mL of MeOH in an ultrasonic water bath for 30 min.

NRR samples were pulverized and passed through a 40 mesh sieve. An accurately weighed 0.2 g grinded powder was added to a flask and extracted in an ultrasonic bath (40 kHz, 200 W) with 50 mL of MeOH for 30 min. The extraction temperature was not exceeding 35 °C. Additional MeOH was added to make up the lost weight. The extracted solution was then filtered through a 0.22 μm Millipore filter prior to injecting into the UHPLC–MS/MS system.

2.4. Apparatus and analytical conditions

UHPLC–MS/MS 8050 system (Shimadzu Corp., Kyoto, Japan) was consisted of a Shimadzu 30 AD liquid chromatography system (LC–30AD binary pump, an SIL–30AC autosampler, an SPD-M30A PDA detector, and a CTO–20AC column oven) and a 8050 triple quadrupole mass spectrometer equipped with a heated electrospray ionization source. Comprehensively considering the resolution, baseline and analysis time, the UHPLC conditions were obtained as described below, which achieved a good separation effect. The chromatographic separation was achieved on a Kinetex® C_{18} column (100 mm × 2.1 mm i.d.; 2.6 μm; Phenomenex, Inc., Torrance, CA, USA) with a guard column, maintained at 30 °C. The mobile phases A and B were 0.1% v/v aqueous formic acid and ACN, respectively, and the flow rate was 0.4 mL/min. The gradient conditions were as follows: 0–5 min, 20–35% B; 5–7 min, 35–46% B; 7–12 min, 46–49% B; 12–14 min, 49–85% B; 14–15 min, 85–95% B; 15–15.1 min, 95–20% B; 15.1–18 min, 20% B. The injection volume was 1 μL.

The MS parameters were set as follows: drying gas (N_2) flow rate, 10.0 L/min; nebulizing gas flow rate, 3.0 L/min; heating gas flow rate, 10.0 L/min; interface voltage, 3 kV; detector voltage, 1.8 kV; interface temperature, 300 °C; desolvation temperature, 250 °C and heat block temperature, 400 °C. Data acquisition was performed using the LabSolutions LCMS Ver. 5.6 software (Shimadzu Corp., Kyoto, Japan).

The MS conditions of 33 analytes were optimized in both positive and negative ion modes. For optimization of multiple reaction monitoring (MRM) conditions, each analyte was scanned under the MS scan mode to obtain the precursor ion and further scanned under the product ion mode to obtain the product ions using automated software. The spectra were acquired over a mass range from *m/z* 100 to 1000. Furthermore, the parameters of quadrupole 1 pre-rod bias (Q1), collision energy (CE), quadrupole 3 pre-rod bias (Q3) and dwell time (DT) were also optimized to get the richest relative abundance.

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