

Full Length Research Paper

High-performance liquid chromatography with Photodiode Array (HPLC-PAD) quality control of menoprogen, a traditional Chinese Medicine (TCM) formula used for the management of menopause

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Accepted 10 December, 2012

The development and optimization of quality control and assurance methods for plant based medicines to assure batch-to-batch consistency and standardization is an essential part of the production process. This is even more important for Traditional Chinese Medicine (TCM) formulas that may contain several different medicinal plant drugs. The goal of the present work was to optimize high-performance liquid chromatography-photodiode array detection (HPLC-PDA) fingerprinting method for a TCM herbal formula (menoprogen) used for the management of menopause. Ten batches of the combination herbal product were obtained and extracted in methanol-water. The extracted samples were analyzed by HPLC-PDA and separated on a Kromasil ODS C18 column (4.6 × 250 mm, 5 μm) with a mobile phase of 0.1% phosphoric acid (0.1%) -acetonitrile in a gradient elution over 135 min using a PDA (205 nm) for analysis and compared with reference standards. The developed HPLC-PDA method clearly identified the main 21 common peaks from a methanol extract of 10 batches of menoprogen with excellent precision and reproducibility. The results demonstrate that the HPLC-PDA chemical fingerprinting methods described in this work are feasible for the comprehensive quality evaluation of the medicinal plant formulation, and thus can be used for the chemical standardization and batch-to-batch consistency of this product.

Key words: Botanicals, fingerprinting, herbals, photodiode array, medicinal plants, menopause; menoprogen, traditional Chinese medicine.

INTRODUCTION

Methods and procedures employed for the quality control and assurance of medicinal plant based drugs involves taxonomic, chemical, spectroscopic and microbial protocols. Botanical taxonomic identification must be performed to assure correct species identification (Mahady et al., 2001; van Breemen et al., 2007).

Macroscopic and microscopic examinations assure the identity and purity of medicinal plants, while microbial evaluations monitor the plant materials for the presence and levels of microbial contaminants. All of these protocols are part of the good manufacturing practice (GMP) guidelines in the manufacturing process of products. Procedures for the quality control and assurance analysis of active and/or marker chemical compounds in herbal products are accomplished by colorimetric, spectroscopic and/or chromatographic methods (Mahady et al., 2001; van Breemen et al., 2007).

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Colorimetric and spectroscopic methods are older analytical procedures that may be non-specific and dependence on such practices alone has declined in recent years. Chromatographic procedures are currently the method of choice for the analysis of botanical chemical constituents (Drasar and Moravcova, 2004; Lin et al., 1996; Mahady et al., 2001). High performance liquid chromatography (HPLC) is capable of resolving complex mixtures of polar and non-polar compounds, and is currently the method of choice for both qualitative and quantitative analysis of herbal extracts and products. HPLC can provide rapid, inexpensive (as compared with mass spectrometry), and characteristic chemical profile patterns for the purpose of standardization and quality control.

The goal of standardization is to produce finished herbal products that contain a consistent level of active ingredients (chemical standardization) and thus produce predictable pharmacological and biological effects (biological standardization). For chemical standardization purposes, the quality of herbal medicines is determined by the amount of active and/or marker components. Many herbal medicines contain more than one active/marker compounds, and may contain different classes of chemical constituents that work together for a synergistic therapeutic effect. Thus, for chemical identification of botanical or herbal drug it is necessary to obtain a characteristic HPLC fingerprint of specific medicinal plant (herbal) formula that shows the presence (or absence) of the major chemical components and can be used for quality control of raw materials and finished herbal products. In this way, the chemical chromatographic fingerprint can be used in a more meaningful way for controlling the quality of medicinal plant samples or finished herbal products (Jiang et al., 2005; Zschocke et al., 1998). Both the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have concluded that appropriate fingerprint chromatograms should be used to assess the consistency of botanical products (EMA, 2001; USFDA, 2004). In 2000, the State Food and Drug Administration (SFDA) of China officially stated that herbal medicines should be standardized by chromatographic fingerprints (State-FDA 2000). Chemical fingerprinting has also been accepted by the World Health Organization (WHO) as a strategy for identification and quality evaluation of herbal medicinal products (WHO, 2011).

Although multicomponent herbal mixtures have not been part of the mainstream of botanical supplements in the USA, there has been a significant increase in their use over the past year (Elaine, 2011). A good example of this is traditional Chinese medicine (TCM) and multicomponent herbal formulas from India, Mexico and other countries. One such TCM formula is marketed as "Menoprogen" in Europe and the USA for the management of menopausal symptoms (Liu et al., 2009).

Menoprogen is based on an ancient TCM formula that

contains semi-purified extracts of five well-known medicinal plants, which have been used as dietetic and medical materials in China for thousands year (Liu et al., 2009; Ma et al., 2010; Wei et al., 2004). Menoprogen was developed in China to treat menopausal symptoms, and is comprised of semi-purified extracts the medicinal plants: Fructus Lycii (*Lycium chinensis* fruits, Chinese goji berries, Solanaceae); Semen Cuscutae (*Cuscuta chinensis* Lam., Convolvulaceae), Radix Rehmanniae (*Rehmannia glutinosa* Libosch., roots, Scrophulariaceae), Fructus Mori (*Morus alba* fruits, Moraceae) and Flos Carthami (*Carthamus tinctorius* L., safflower, Asteraceae), which are commonly used in TCM and are all described in either the Chinese Pharmacopoeia and/or the WHO Monographs (Ma et al., 2010; Mahady et al., 1999, 2007).

For the final herbal product, the major medicinal plants are prepared by extraction in 70% ethanol and then subjected to specific semi-purification using column chromatography that maintains a highly concentration of the active principles from the raw plant materials, namely the polysaccharides and flavonoids. Although this herbal product has been pharmacologically and clinically used in the management of menopausal symptom for over ten years in China (Liu et al., 2009; Ma et al., 2010; Lu et al., 2008), the formula has been recently modified, and new optimized chemical quality control measures are needed for the revised product. Thus, using modified protocols for quality control of Menoprogen previously published by our research group (Wang et al., 2008), we report an optimized HPLC-photodiode array (PDA) method in this work to select the optimal wavelength for the chemical constituents in the new Menoprogen formula. The aim of this study was to optimize the sensitive and efficient HPLC method coupled with photodiode array detection (HPLC-PDA) method for use as a fingerprinting tool for rapid authentication of quality control for batch-to-batch consistency of this TCM formulation for use in animal and human studies, and to determine the peak of compound as much as possible.

MATERIALS AND METHODS

Instrumentation and reagents

High-performance liquid chromatography (HPLC) was performed using an Agilent/HP 1100 series HPLC system consisting of a vacuum degasser, thermo stated column compartment and photodiode array detector (PDA; Agilent, Palo Alto, CA, USA). A sonicator was used for extraction of the capsules. Reverse osmosis water (18M, simplicity 185, Millipore, France) was used for all the solutions and dilutions. The HPLC-grade methanol and acetonitrile was obtained from SK Chemicals (South Korea); the phosphoric acid and sodium hydroxide were obtained from Nanjing Chemical Reagent Corporation

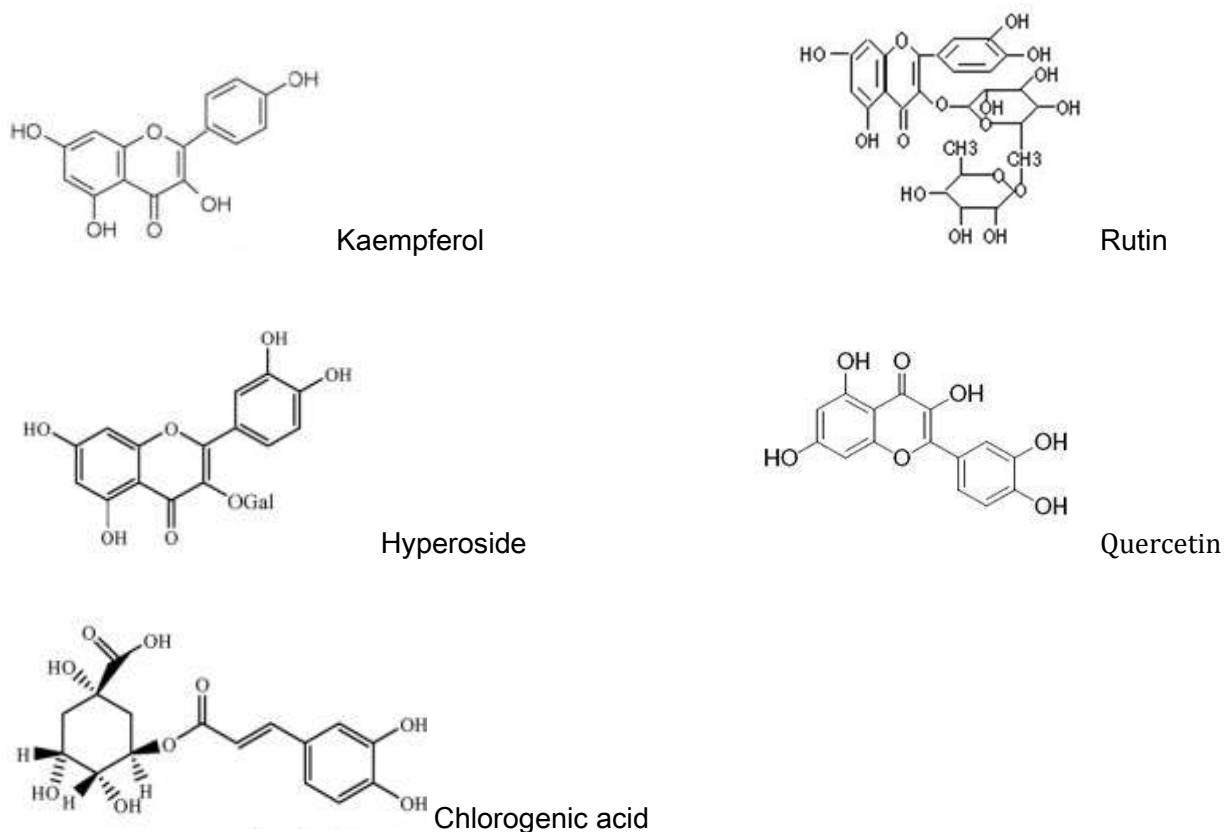


Figure 1. Chemical structures of kaempferol, hyperoside and chlorogenic acid, used as reference standards for the development of an HPLC chemical fingerprint for Menoprogen capsules.

(Nanjing, China). Deionized water was prepared using a Millipore water purification system (Billerica, MA, USA).

Reference standards of chlorogenic acid, rutin, hyperoside, quercetin and kaempferol (Figure 1) were purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). All have been shown to have biological activities. Hyperoside is one of the key chemical constituents of *Semen Cuscutae*. Rutin is one of the key chemical constituents of *Flos Carthami* and *Fructus Mori*. Quercetin is one of the key chemical constituents of *Semen Cuscutae* and *Flos Carthami*. Chlorogenic acid is one of the key chemical constituents of *Fructus Lycii*, and kaempferol is one of the common chemical constituents of *Flos Carthami* (Mahady et al., 2007).

Sample and standard solutions preparation

Ten batches of Menoprogen capsules were obtained from Nanjing Moresoft Manufacturing Company (Nanjing, China) and were manufactured under GMP. The batch numbers used were as follows: 20020615, 20021218, 20030512, 20040209, 20051202, 20051202, 20060206, 20061201, 20071203, and 20070521. The individual

analytical standard solutions of the contents from the combination product capsules were prepared by accurately weighing for 1 g of the contents, and dissolving the corresponding contents into 10 ml of methanol and sonication for 60 min at room temperature. The extracted solution was filtered through a 0.45 μm membrane filter before the injection of the samples into the HPLC system for analysis.

The reference standards of chlorogenic acid, rutin, hyperoside, quercetin and kaempferol were prepared using the same methods as described for the samples earlier mentioned.

High-performance liquid chromatography (HPLC) methodology

The HPLC separations were performed using a Kromasil C18 reverse phase column, 5 μm , 250 \times 4.6 mm i.d. with a column temperature at 25°C and a mobile phase consisted of varying concentrations of acetonitrile and 0.1% phosphoric acid (adjusted with 1M sodium hydroxide to pH 3.0 \pm 0.2) using a gradient program (Table 1) with a flow rate at 1.0 ml/min (Wang et al.,

Table 1. HPLC gradient elution program.

Time (min)	0	50	86	135
Acetonitrile (%)	10	20	48	100
0.1 (%) phosphoric acid	90	80	52	0

2008). PDA detector was set at 205 nm for obtaining chromatograms. UV spectra were acquired from 200 to 400 nm. The loading volume was 10 μ l.

Method validation

Injection precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. The injection precision was determined by replicate injections of the same sample solution for five HPLC runs and by determining the relative standard deviation (RSD) of relative retention time and relative peak areas.

Reproducibility

The reproducibility of the method was checked by determining precision on the same instrument, and by determining the relative standard deviation of the relative retention times. The reproducibility was assessed by analyzing five independently prepared samples of the capsule extracts.

Sample stability testing

The stability of the drug in solution during analysis was determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of the dry solution for 48 h under laboratory bench conditions ($25 \pm 1^\circ\text{C}$). An accurately weighed quantity of the sample was prepared and the solution was subjected to HPLC analysis at time points of 0, 4, 8, 12, 24 and 48 h.

Similarity calculation for the HPLC fingerprinting chromatograms

Similarity calculations of the HPLC fingerprinting chromatograms were performed using the Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A, State Pharmacopoeia Commission), which was recommended by SFDA of China to be used for evaluating similarities of

different chromatograms by calculating the correlative coefficient and/or cosine value of vectorial angle (Feng et al., 2009; Zhao et al., 2007).

RESULTS AND DISCUSSION

Method validation

Precision

The injection precision showed that the relative standard deviation (RSD) of relative retention time and relative peak area did not exceed 0.16 and 1.42%, respectively.

Reproducibility

The reproducibility showed that the relative standard deviation of relative retention time and relative peak area was below 0.15 and 1.68%, respectively.

Sample stability testing

The precision of sample stability tests showed that the RSD of relative retention time and relative peak area were found less than 0.18 and 1.87%, respectively. All outcomes by this test as well as the repeatability and the injection precision showed that the extracted sample solution remained stable for 48 h at room temperature.

Optimization of extraction conditions

Sample extraction and pretreatment conditions are the most critical procedures to define for HPLC fingerprinting analysis of the chemical constituents present in traditional Chinese medicine products. These fingerprints are often complicated due to the chemical complexities of the active and marker chemical constituents present in these herbal formulations. Thus, the development of optimal extraction solvents and extraction times are important for proper consistency and the identification of characteristic compounds present in a chemical fingerprint that can be used for quality control purposes and batch to batch standardization.

The results depicted in Figure 2 show that different extraction solvents (water, 100% methanol and 50% (v/v) methanol-water mixtures) significantly impacted the peak areas for the chemical components of the Menoprogen capsules. The results indicated that methanol was the best solvent and the extraction rate was up to 80%. So methanol was chosen as the optimal extraction solvent, as it produced the most defining HPLC chromatogram.

The time factors that is, 30, 60, and 120 min, on the extraction efficiency of the components in the sample

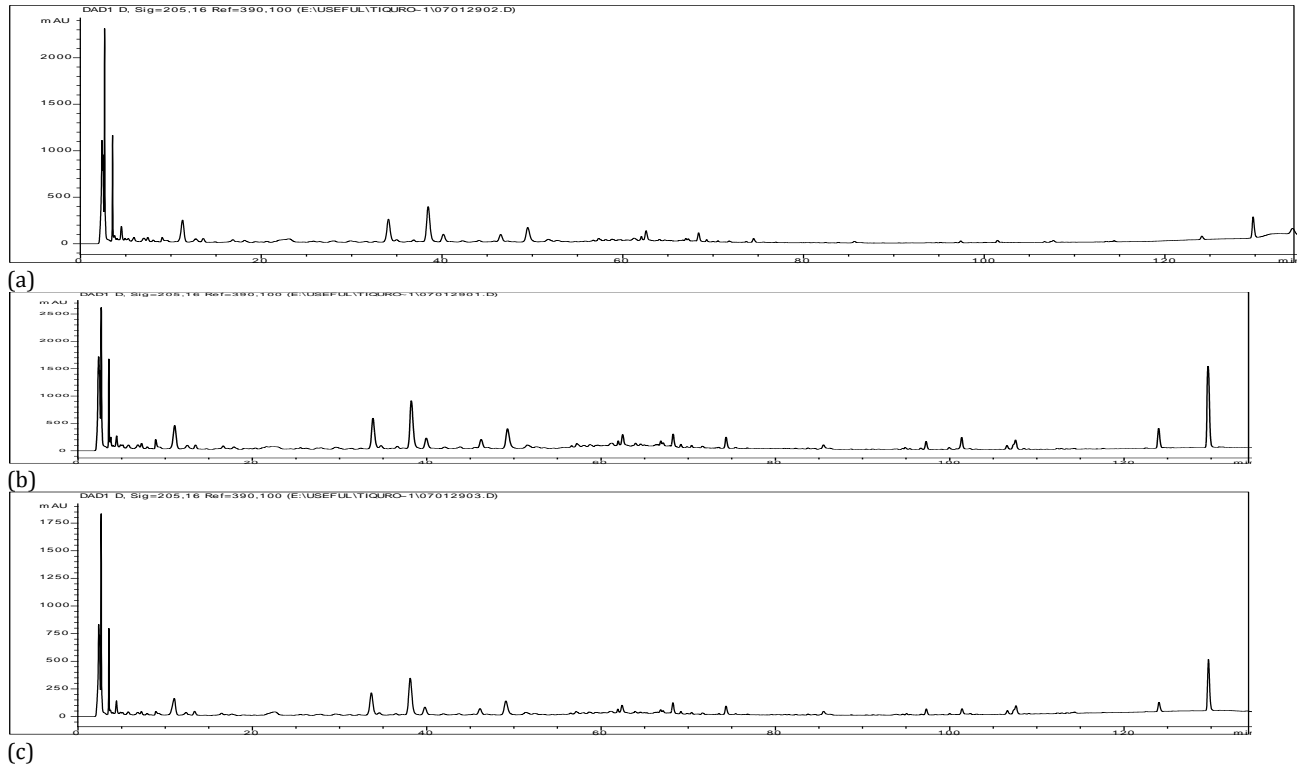


Figure 2. Comparison of the extraction solvents for extraction efficiencies of components in Menoprogen capsules. a) aqueous extraction; b) 100% methanol; c) 50% methanol.

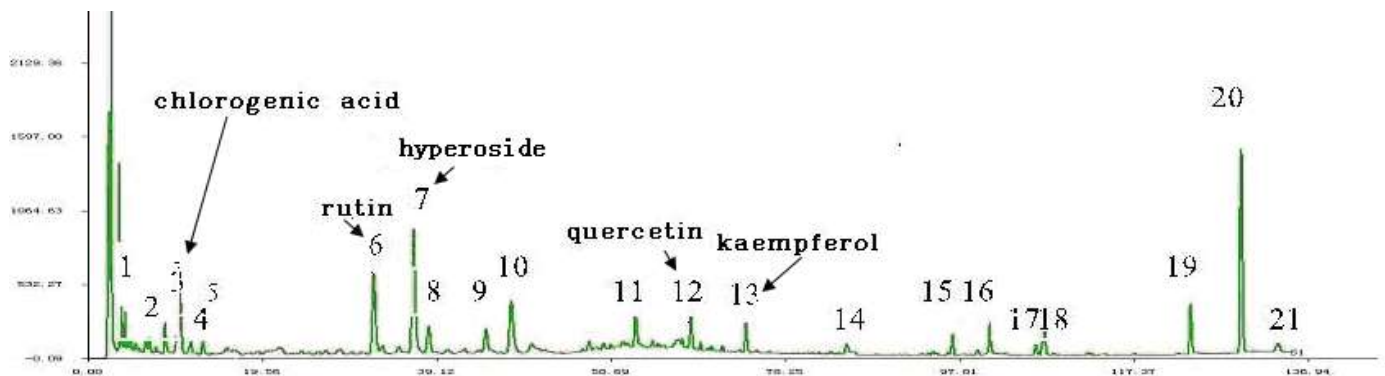


Figure 3. The fingerprint HPLC chromatography profile of the extracted solution of Menoprogen capsules. Twenty-one characteristic peaks are observed in the chromatogram of the sample solutions from Menoprogen capsules. Peak 3 = chlorogenic acid; Peak 6 = rutin; Peak 7 = hyperoside; Peak 12 = quercetin; Peak 13 = kaempferol.

solutions of Menoprogen capsules were explored, suggesting that the 60 min extraction was the most optimal extraction time (data not shown).

Validation of HPLC fingerprinting for batch-to-batch standardization

The method validation of fingerprint analysis was

performed using the relative retention time (the ratio of peak retention time of sample constituents to the reference standard) and the relative peak area (the ratio of peak area of sample constituents to the reference standard). Among the twenty-one characteristic peaks observed in the chromatogram of the sample solutions from menoprogen capsules (Figure 3). Peak 7 (hyperoside) was selected as a reference standard due to its high and stable content in the chromatogram. The

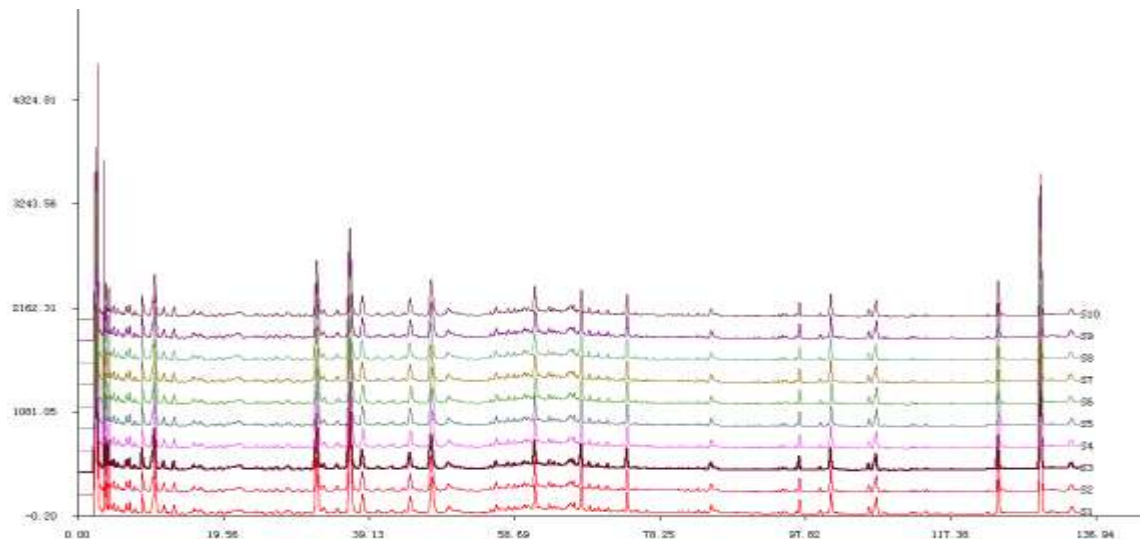


Figure 4. Stacked HPLC fingerprint chromatograms of ten batches of Menoprogen capsule extracts showing all 21 peaks in each batch. S1= Batch # 20020615, S2= Batch #20021218, S3= Batch #20030512, S4= Batch #20040209, S5= Batch #20051202, S6= Batch #20051202, S7= Batch #20060206, S8= Batch #20061201, S9= Batch #20071203, S10= Batch #20070521.

Table 2. Values of similarity of ten batches of Menoprogen capsules.

Batch	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Value of similarity	0.9996	0.9994	0.9995	0.9995	0.9994	0.9993	0.9997	0.9997	0.9990	0.9996

relative peak areas (RPA) and relative retention times (RRT) of other characteristic peaks were obtained on the basis of this substance.

The reproducibility and repeatability of the HPLC fingerprinting method were evaluated by analysis of the precision and reproducibility of the chromatograms. Precisions of all 21 peaks were in the ranges of 0.06 ~ 0.16% and 0.31 ~ 1.42% for RSDs of RRA and RPT ($n = 5$), respectively. The reproducibility test was performed with five sample solutions extracted from one batch of menoprogen capsules. The RSD of the relative retention times and the relative peak areas were 0.08 ~ 0.15% and 0.29 ~ 1.68% ($n = 5$), respectively. The stability test was performed with sample solutions over 24 h. RSD of both, the relative retention times and the relative peak areas, were 0.13 ~ 0.18% and 0.43 ~ 1.87% ($n = 5$), respectively. These results indicated that the conditions used in the qualitative chromatographic fingerprint analysis are reliable, valid and satisfactory.

Analysis of batch-to-batch consistency by HPLC fingerprint

Ten different batches of menoprogen capsules were examined immediately after production using the

developed HPLC-PDA fingerprinting analysis, and their stacked chromatograms are shown in Figure 4. Peaks that existed in all 10 batches of samples were assigned as “common peaks” for menoprogen capsules. The batch-to-batch consistency of the fingerprint chromatograms of 10 batches of menoprogen capsules are shown in Figure 4 and Table 2. Taken together the chromatograms demonstrate that this HPLC-PDA fingerprinting method for identifying and using absolute quantitation of a number of specific chemical marker compounds (standards) is useful for characterization of the complete mixtures of herbs in menoprogen capsules and can be further used to determine batch-to-batch reproducibility for the production of Menoprogen capsules for animal and human studies.

The difficulties involved in assessing methods for the quality control and assurance of botanical drugs and complex formula mixtures seen in TCM are due to the inherent chemical complexities of natural products. This process is made even more difficult considering that medicinal plants are dynamic living organisms and are therefore impacted by growing conditions, environment and processing methods. Thus, the batch-to-batch consistency of the chemical constituents (chemical standardization) of herbal products is extremely important in terms of quality control and standardization of these

products particularly if they are used for preclinical and clinical investigations. Chemical standardization is also critical to achieve in order to progress to biological standardization of extracts and final products for sale to the public. In this work, we have optimized an HPLC–PDA analytical method for the TCM product (Menoprogen) that has been marketed in China and Europe for the management of menopausal symptoms. This optimized method was based on a preliminary report that we had published in 2008 (Wang et al., 2008). Since then, the TCM formula had been modified, and in this report we demonstrate that the HPLC fingerprinting method developed was reliable, with validated repeatability and precision for the newly modified formula. Thus, using this method, the average fingerprints of 10 batches of samples from Menoprogen capsules were obtained using a standardized procedure and showed consistent chemical quality results. The fingerprint of the herbal product showing 21 “common peaks” represents the characteristic chemical constituents of this novel herbal formula.

Conclusion

The optimized extraction and HPLC-PDA methods described herein are feasible for the quality control of menoprogen, and thus can be used for the chemical standardization and batch-to-batch consistency. Pharmacological methodology for the biological standardization of the product using receptor binding assays, cell culture assays and real-time PCR are also currently being developed.

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