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Multiple Chromatographic and Chemometric Methods for Quality Standardization of Chinese Herbal Medicines*

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Abstract: Quality standardization of complementary medicine is fundamental for industry and practice as it underpins the quality, safety and efficacy of Chinese herbal medicines. Current herbal standardizations are often based on the quantitative analysis of a single compound, which may not reflect the total characteristic, bioactive and toxic nature of the herbs or products. Therefore, there is a need to establish an internationally recognized methodology for quality standardization of Chinese herbal medicines.The analytical methods reviewed in this article are pharmacognosy, TLC, HPLC, LCMS, CE and chemo-metrics. This article also covers the developments and applications of these methods in quality standardization. Recent advances show that a combination of these methods creates an overall chemical profile of each herb. This is supported by results reviewed in this article and obtained in our laboratory tests on medicinal herbs including Hypericum perforatum, Morinda officinalis and Centella asiatica. Significant variations in active components have been observed between herbal samples and products. It is proposed that the identification of active components, pharmacological activities and eventual clinical applications are required for a comprehensive quality standardization system. Our findings indicate that the combination of various chromatographic and chemometric methods will advance the methodology of quality standardization and enhance the overall confidence in herbal medicine for the health practitioner and the public.
Keywords: Capillary electrophoresis; Chemo-metrics; Herbal medicines; High performance liquid chromatography; Liquid chromatography mass spectrometry; Pharmacognosy; Quality standardization; Thin layer chromatography.

收稿日期:2009-11-04

* 国家科学技术部等 14 部委和广东省人民政府共同主办的"2009'传统医药国际科技大会暨博览会"第七分会——"传统医药的疗效、安全性评价与质量控制"论文,该论文为大会学术委员会评选的优秀论文。

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Abbreviations

CE: Capillary electrophoresis; DAD: Diode array detector; GAP: Good agricultural practices; GMP: Good manufacturing practice; HPLC: High performance liquid chromatography; LC-MS: Liquid chromatography-mass spectrometry; TCM: Traditional Chinese medicines; TLC: Thin layer chromatography

Introduction

The prevalent use of herbal medicines has raised concerns over their quality, efficacy and safety due to their ready availability. Many herbal products are sold without prescription and consist of a decoction of several herbal materials defined in a formula. Thus, the clinical application of a particular herbal medicine is the synergistic effect of multiple chemical components. In this case, the pharmaceutical approach of analyzing a single component cannot be applied in discerning the quality of an herbal preparation. Thus, quality control methods which reflect the holistic approach of complementary medicines have to be developed in order to determine the chemical basis of herbal medicines [1].

Quality control is crucial to ensure the safety and correct handling of herbal medicines. There have been numerous reports on the toxicity and misidentification and substitution of plant species [2]. Herbal medicines have been reported to contain heavy metals and synthetic prescription or non-prescription drugs. They may originate from mineral components, contamination or adulteration[3].

In the past, researchers and manufacturers relied solely on traditional usage and documented monographs which might not have fully characterized the herbal species. Also, many of the procedures and analytical conditions outlined in the documents used limited technology. The current edition of the Pharmacopoeia of the Peoples' Republic of China 2005 (Volume 1)[4] includes 1,146 items of raw herbal materials and processed herbs, lipids, extracts, formulary and single herb products. Thus, it is vital that herbal documentation such as Pharmacopeias stay abreast with current research and updated procedures which allow both qualitative and quantitative assessment of herbal preparations.

Quality standardization is a basic requirement for all medicines to ensure reliability of the product, bioequivalence and reproducibility of clinical effects[5]. Quality standardization of herbal medicines, as defined by Heinrich et al, (2004)[6], is the establishment of reproducible pharmaceutical quality by comparing a product with established reference substances and by defining minimum amounts of one or several compounds or groups of compounds[6].

In this work, an alternative definition was proposed: Quality standardization of herbal medicines is the study of quality standards which reflect the intrinsic quality, efficacy and safety of medicines by employing a combination of pharmacognostic, analytical and biological methods.

Thus, multiple chromatographic methods were proposed as a comprehensive platform (Figure 1) for the quality evaluation of herbal medicines. The methods include pharmacognostic and DNA fingerprinting, quantitative TLC, HPLC, CE and carbohydrate determination. The analysis would generate information on the various types of chemical components contained in the herbs and provide chemical, bioactive and toxic markers. To compare the large amount of data derived from samples or products, statistical analysis via chemometrics was used to generate patterns, or groups. If the analysis is correlated with biological activity or clinical studies, then a new standard can be set.

During the last few years, the fingerprint method has been developed for quality control of Chinese herbal medicines to monitor batch-to-batch consistency[8]. Fingerprinting in herbal evaluation represents the characteristic pattern of the constituents using one or many identification techniques such as pharmacognosy, chromatography and chemical electrophoresis.

Pharmacognosy Approaches

Pharmacognosy is the first step of quality control assessment as GAP guidelines define that the quality and authenticity of the final botanical product are directly related to the proper identification and authenticity of the source material [7].

The World Health Organization [9] recommends that medicinal plant materials are categorized according to their sensory, macroscopic, microscopic and molecular charac-

teristics. Jones et al. (2006) [10] review the advances in pharmacognosy. Various molecular markers such as Random Amplified Polymorphic DNAs (RAPDs), Restriction Fragment Length Polymorphisms (RFLPs), Microsatellites and various Polymerase Chain Reaction (PCR)-based DNA markers such as Sequence Characterized Amplified Regions(SCARs), Sequence tagged sites (STS) and Inter-simple Sequence Repeat Amplification (ISA), Amplified Fragment Length Polymorphic DNAs (AFLPs) and Amplicon Length Polymorphisms (ALPs) are currently used for plant drug analysis[11]. From Joshi's work(2004), it appears that DNA markers may have several benefits as the uniqueness for each species is not governed by age, physiological conditions or environmental factors as with botanical constituents[12]. DNA fingerprinting identifies existence of the correct genotype but does not disclose the phytochemical or active principle component. Consequently, DNA fingerprinting should be used as a complement tool with other pharmacognostic techniques[13].

Thin Layer Chromatography

Planar chromatography (such as TLC/HPTLC) provides a powerful, flexible and inexpensive separation technique for the analysis of multiple herbal samples at any given time [14]. Because of the advent of the CAMAG video store system, qualitative and semi-quantitative analysis of the analytes can be achieved and visualized.

Validation of TLC methods for herbal analysis is continually developed[15] and the technique is expanding. In a paper by Zarzycki(2008)[16], thermo-stated micro-thin-layer chromatography was capable of separating more than 10 spots in one direction or up to 180 spots for two dimensional runs. TLC was enhanced to obtain instantaneous mass spectra from substance zones[17]. Planar electro-chromatography (PEC) was improved by applying high pressure and was commonly referred to as pressurized planar electro-chromatography(PPEC) [18]. Electro-osmotic TLC (EOTLC) was improved by using low volatility mobile phases to eliminate evaporation of the mobile phase[19]. For quantitative evaluation of TLC, a recent paper [20] improved image processing based on a charge-coupled device (CCD)camera. Thus, TLC remains as the forerunner in obtaining the first characteristic fingerprint profile of an herbal sample.

High Performance Liquid Chromatography

Due to its superior precision, high resolution and capacity to analyze thermally labile and non-volatile compounds, high performance liquid chromatography (HPLC) is extensively applied in the quality control of (Chinese) herbal medicine[21]. HPLC is comprehensive in its quantitative and qualitative assessment for quality consistency[22]. For Huang-qi (Radix astragali), HPLC was combined with ELSD and DAD Yu (2007) to determine 12 flavonoids and groups of saponins[23]. A reversed-phase high-performance liquid chromatography method with pulsed amperometric detection was examined for the determination of glycosides[24].

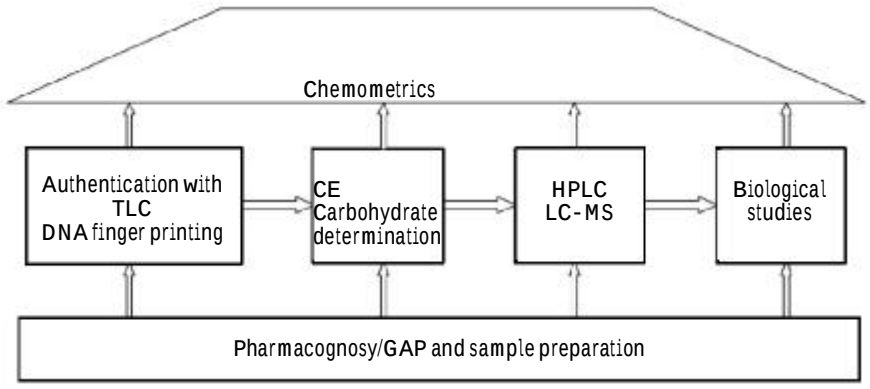

Figure 1 Multiple chromatographic methods for quality standardization of herbal medicines [7]

A HPLC-DAD method was developed to separate marker compounds of Dahuang Huanglian Xiexin Decoction (DHXD), which is composed of Rheum officinalis, Coptis sinensis and Scutellaria baicalensis. The HPLC method was applied to simultaneously determine baicalin, palmatine, berberine, baicalein, aloe-emodin, rhein, emodin, chrysophanol and physcion in the decoction. The results showed that inclusion of S. baicalensis in the DHXD formula changed the concen-

trations of anthraquinones, as well as other marker compounds (Figure 2). The chromatographic peaks of the individual herb and the DHXD formula were identified by comparing the relative retention time and the UV absorption spectrum to reference compounds. Together with LC/MS/MS, 20 chromatographic peaks were observed and are shown in Figure 3. The determination of the marker chemicals, the identification of chemical components and determination of their source herbs in the formula provide new analytical evidence on the chemical basis and combinational principles, and, therefore, the quality control of DHXD and other decoctions[25].

Liquid Chromatography-mass Spectrometry

As an adjunct to HPLC, liquid chromatography mass spectrometry (LC-MS and MS/MS) has played an increasingly important role in chemical profiling and quantitation of the complex matrices of herbal medicines[26]. The technique has also played a vital role in toxic screening of herbal medicines[27]. Ding and colleagues (2007) have recently quantitatively determined alkaloids in Corydalis yanhusuo using LC-MS/MS and LC-DAD[28]. Several studies have reported the utilization of LC-MS methods in profiling flavonoid glycosides[29~30], terpenes and saponins[31~33].

High throughput has been achieved with ultra-performance liquid chromatography with time-of-flight mass spectrometry[34]. Novel atmospheric pressure photo ionization (APPI) technique provides high sensitivity LC-MS analyses for low polar herbal constituents such as phytosterols[35~36]. With these improvements, the above studies clearly demonstrate that MS/MS is a powerful technique for the quantitative analysis and characterization of fingerprint pattern in herbal medicines.

Capillary Electrophoresis

In recent years, capillary electrophoresis (CE) is becoming more popular in the separation and quantitation of mixed natural compounds with high peak capacity, low sample consumption, low reagent consumption, high speed analysis, high efficiency, excellent mass sensitivity and cost-effectiveness[37~38].

The most widely used CE techniques for herbal medicines include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), non-aqueous CE (NACE), chiral CE, CE-mass spectrometry (MS), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary isotachphoresis (CITP), affinity capillary electrophoresis (ACE), microchip CE and multiplexed CE (MCE)[39].

CE analyses of alkaloids, flavonoids, coumarins, terpenes, organic acids, anthraquinones and a few miscellaneous analytes including lectins, lignans and sugars have been published[40]. A group of flavonoids, including rutin, kaempferol, quercetin, myricetin and apigenin from Centella asiatica[41], was separated by CZE, Suntornsuk and Anurukvorakun. With the application of MEKC, the separa-

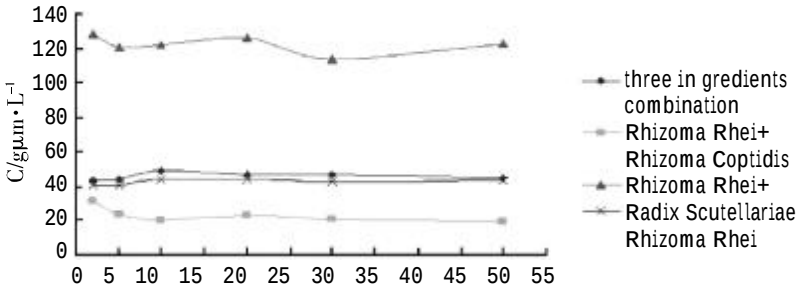

Figure 2 The influence on the concentration of anthraquinones by formula combination[25].

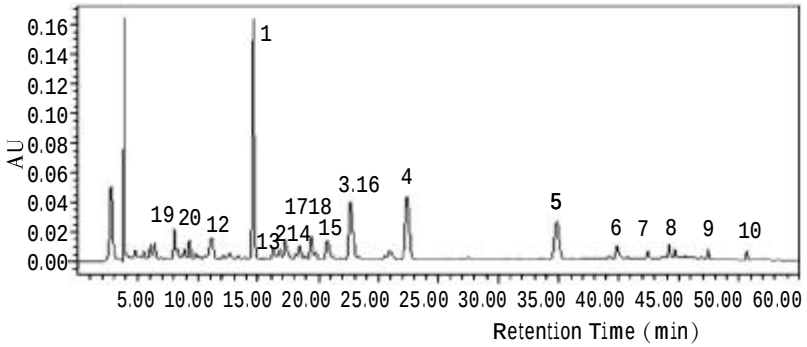

Figure 3 Major chemical components in DHXD identified by HPLC-DAD
1) bacailin, 3) palmatine, 4) berberine, 5) baicalein, 6) aloe-emodin, 7) rhein, 8) wogonin, 9) emodin, 10) chrysophonol, 13) columbamine, 15) coptisine, 16) wogonoside[25].

tion, identification and quantification of protocatechuic aldehyde, salvianolic acid B and gallic acid from "Shuangdan" granule were accomplished. In 2007, Dinelli et al. separated pinoresinol, lariciresinol and hinokinin from Triticum aestivum L using CE-MS [42]. Separation and quantitative analysis of alkaloids in Corydalis species by NACE-ESI-MS has been achieved[43].

Improvements in separation capabilities, detection sensitivity, reliable quantitation and commercial instrumentation are bringing widespread acceptance of CE[44]. Thus, CE, especially when coupled with other powerful analytical methods, will significantly contribute to a better understanding of the solution behavior of herbal medicines.

Chemometrics

The complexity of herbal preparations has made quality standardization a difficult task[1]. In most of cases, therapeutic activities (and sometimes toxicity effects) of (Chinese) herbal medicines are derived from synergistic action (s) of multiple secondary metabolites in the single herb or between the herb(s) within a formula[45]. The fore mentioned analytical (and/or hyphenated) techniques can readily produce the (phyto) chemical profiles of the target system[46]. In herbal medicine, application of fingerprinting, combined with chemo-metric models (such as pattern recognition model), induces the speed and reliability of translating process data into information that can be assessed in an objective manner[47]. Here, common peaks or regions of the herbal profile are compared using similarity index and linear correlation coefficient[48]. Computer-based software such as CASE and SPSS allows peak identification and matching, background and retention time correction and can evaluate many different samples at once[49]. There are numerous attempts demonstrating the potential application of fingerprinting evaluation using chemometric method for quality control of (Chinese) herbal medicines [50~51]. Qualitative and quantitative analysis of the HPLC fingerprints of Ginkgo biloba extract was examined by the involution similarity method[52]. HPLC fingerprinting was combined with chemometric models (principal component analysis (PCA) and hierarchical cluster analysis (HCA))in evaluating various samples of Cortex cinnamomi [49]. From 30 sample fingerprints that originated from different locations and different species, 17 common peaks were identified. PCA and cluster analysis were further applied and successfully discriminated according to different species and regions. Ni et al., 2008 combined both LC-DAD and LC-MS to produce a characteristic fingerprint of 46 Eucommia bark samples originating from different locations and identified unknown peaks (resinols, geiposidic acid and chlorogenic acid) in the samples[51]. Pattern recognitions (PCA and HCA) were further applied and successfully discriminated differences in the samples according to their phyto-chemical profiles. The study demonstrated potential application of fingerprinting evaluation (when produced by multi-analytical techniques) combined with chemometrics in the quality control of (Chinese) herbal medicines.

Our Work

The research undertaken in our labs has focused on combining analytical methods in order to assess the quality of herbal extracts and preparations. St John's work is a popular herbal extract used to treat mild-to-moderate depression[53]. Naphthodianthrones (hypericin) phloroglucinols (hyperforin) and flavonoids are the main secondary constituents. Poor quality and characterization of herbal medicines used in clinical trials may contribute to the variation of clinical outcome[8]. We were focused on the HPLC of commercial preparations combined with chemometrics to analyze the synergism of the extract. It was found that significant variability of the phyto-chemical contents, including hyperforin and hypericin, was observed between the different commercial products. Thus, current standardization of hypericin content should be reviewed.

Morinda officinalis is a common Chinese herbal medicine indicated for pain and impotence. Its active components are iridoids, anthraquinones, oligosaccharides and polysaccharides[54]. Currently, there is no quality standardization for Morinda. Thus, the various components will require several analysis modes and we are currently developing qualitative and quantitative separation (TLC, CE, HPLC) and carbohydrate methods to address this. It was shown that a validated CE model gave good separation and detection of the iridoids, with excellent linearity.

Centella asiatica is a popular Ayurvedic medicine possessing anti-inflammatory and healing effects[55]. The main constituents are triterpenoid glycosides. Most separation methods gave poor resolution and validation. We are currently developing TLC, CZE and HPLC techniques which will enable comparison of the quality of the herb grown in Australia with samples obtained from other countries. A combination of the above techniques has provided fingerprints that are suitable for the identification, comparison and standardization of Centella asiatica.

Concluding Remarks

Herbal medicines are characterized by many varieties, numerous components, multiple pharmacological actions and variable clinical applications. The current herbal standards are predominantly based on the analysis of one marker compound. Consequently, the marker compounds may not reflect the synergistic nature of herbal medicines. In the last few decades, the quality standardization, fingerprinting and bioequivalence concepts had been proposed as new methods for the quality control of herbal medicines.

The multiple chromatographic platform proposed here includes various pharmacognostic and chromatographic techniques to define the quality characteristics of a specific herbal extract containing the combination of multiple components. The development of improved analytical technology has made concurrent assessment of herbal samples possible. The multiple chromatographic approaches described in this article can be extensively applied for species authentication, quality standardization and, ultimately, for regulated quality control of all herbal medicines.

Acknowledgements

This project is supported by the International Science Linkages Australia China Special Found 2007, CH0701667 established under the Australian Government's innovation statement, Backing Australia's Ability and the University of Sydney International Program Development Fund (IDPF, 2008). We also acknowledge the China Australia Special Fund from the China National Science Fund Commission (Quality Standardisation, anti-inflammation and anti-depressant activities of Hypericum species and Morinda Officinalis, Yang Dp, 2007).

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