Comprehensive Principle of HPLC Fingerprint in the Construction and Evaluation of Chinese Herbal Medicine

Jian Gu, PhD, Rui Tan, PhD, Shengmao Li, MD

Abstract

The establishment of a Chinese herbal medicines quality control system is at the core of the modernization development of Chinese medicine. High Performance Liquid Chromatography (HPLC) fingerprint technology is applied to quality control of Chinese herbal medicines from the perspective of systems biology and depends on the classical theory of Chinese medicine system together with information processing technology. In the view of the characteristics of Chinese medicine and the development and application of the top-notch technology in recent years, we tried to integrate information and establish a standard evaluation system of Chinese medicine by using standard chromatographic fingerprints, with a view to improve Chinese medicine quality control and modernization of Chinese medicine development.

[N A J Med Sci. 2010;3(2):107-112.]

Key Words: *Chinese medicine, HPLC fingerprint*

Introduction

Chinese medicine (CM) has been widely used in many oriental countries (e.g., China, Japan and Korea) for thousands of years. It has developed into a unique holistic health care system for the prevention, diagnosis and treatment of diseases. As is regarded as an organic whole—a notion called holism - which is the soul of CM, the human body is dynamically regulated with self-healing capabilities to balance a diseased state, which is also known as homeostasis. This approach is a fine-tuned method at individual levels. Instead of addressing a disease with a single chemical compound or aiming at relieving a single symptom, CM prefers to present as single herbs or composite formulae containing many compounds during the decoction. Those may be the main reasons why quality control of CM is more difficult than that of Western drugs.

During the past decade, researchers have made people realize the prevalence of chronic diseases,[1] limitations of Western medicine, and considerable efficacy of CM. That's why the CM usage has expanded globally and gained ever-increasing interest and acceptance in many different countries.[2] It was estimated that about 1.5 billion people use CM worldwide.[3] However, the knowledge about the chemical, pharmacology, clinical, toxicology of CM has been limited. The efficacy and safety of CM is mostly based on long-term clinical experience instead of modern scientific evaluation. The data on CM about safety and efficacy as well as quality control (QC) is far from sufficient to meet the criteria supporting its use worldwide.[4]

Currently, the QC of CM tends to employ a characteristically Western method:

a) Choose one or two markers (pharmacologically active components in herbs or formulas) and test them with a qualitative identification method.

b) Evaluate the quality and authenticity based on the data above. This method could reflect and control the quality in some degree, but could not give a complete picture of a TCM. The reasons are the fact that CM is a complex, multi-constituents systems. CM has the integrated effects as well as dynamic adjustments effects on multiple target sites.[5] Each constituent in CM is usually responsible for its therapeutic effects but no single active constituent for the overall efficacy.[6,7]

c) Moreover, there are many factors affecting the chemical constituents in CM, such as plant origins, habitat, harvest season, origin processing, etc.

In order to ensure the reliability and repeatability of pharmacological and clinical research, it is necessary to determine the majority constituents rather than only one or two kinds. This means that the all constituents could be regarded as the active 'compound' in the QC process. Therefore, chromatographic fingerprint following by the development of several chromatographic techniques, such as HPTLC (High Performance Thin Layer Chromatography), HPLC, GC (Gas Chromatography) or CE (Capillary

Received 30 January 2010; Revised 28 March 2010; Accepted 8 April 2010

Jian Gu, PhD
Southwest University for Nationalities, Chengdu, China 610051
Rui Tan, PhD * (Corresponding author)
School of Life Science and Engineering,
Southwest Jiaotong University
No.111, Erhuanlu Beiyiduan
Chengdu, People's Republic of China, 610031
Tel: +86-28-87600993 Fax: (86)-28-87601450
Email: ruirui_tan@yahoo.com.cn
Shengmao Li, MD
Southwest Jiaotong University, Chengdu, China 610031

Electrophoresis) coupled with UV (Ultraviolet Rays), DAD (Diode-Array Detector), ELSD (Evaporative Light-scattering Detector), MS (Mass Spectrometer) or MS2 (MS-MS), is necessary to apply for QC. The fingerprint pattern is based on considering the whole chromatographic profile as a chemical feature covering the relative compositions. It appears to offer a more logical tool for evaluating the quality of CM.

At present, HPLC fingerprint technology is regarded as a primary means in the QC study of CM. It is sensitive, accurate, simple and fast, widely suitable for almost all the compounds in CM. Our paper focused on HPLC fingerprint technology research in recent years and aims to construct comprehensive information systems and evaluation systems of HPLC fingerprint for the purpose of improving CM QC systems research.

Construction Program of Comprehensive Information in HPLC Fingerprint

1. General steps of the establishment

1.1. Source control and standards [8-11]

The original herbs, manufacturing procedures, pieces, extracts, intermediate goods of CM production and preparations, all should be taken into consideration in QC. The representative source of herbs is the primary factor that is related to the effectiveness of QC in follow-up products. That is why it is necessary to do a normative research on the test samples to achieve a comprehensive understanding.

We suggest classifying the background information of Chinese herbal medicines including pieces, extract, and intermediate goods. In terms of their medicinal material name, the origin, medicinal site, origin processing, and source identification, the creation of database managements in computer programming and electronic ID (e-id) makes experimenter convenient. We also suggest that the background information of Chinese herbal medicines, pieces, extract, intermediate goods, whose chromatographic fingerprint standard have been formed initially, should be arranged and consolidated.
Refer to specific contents as follows:

a) Name:

The Chinese name, the Chinese phonetic name and the English name of Chinese medicinal materials; piece; extract; intermediate goods; for plant herbals, their family name of the original plant, the plant name, and the Latin name; for the medicines originating in or produced by animals, their family name of the original animal, the animal name, and the Latin name.

b) Source:

Habitat points to the traditional authentic origin, rich resources areas, GAP bases or other origins. Plant herbals include root, stem, leaf, plant epidermal, floral, fruit department, seeds department, or the whole plant, without the limitation of the Pharmacopoeia. Harvest seasons of test samples should be noted so that the evidence would be available to investigate the relationships between internal growth development and the external environmental factors and lay the foundation for systems biology. The record of the harvest season is also beneficial to investigate dynamic changes of the effective components associated with their internal growth, development and external environmental factors. In order to guarantee the qualities, easy-to-package-and-transport medical materials need to be processed locally to ensure physical integrity, appropriated moisture content, and little destruction of effective ingredients. The normal methods include sorting, washing, slicing, peeling, boiling, steaming and drying, etc. There are some discrepancies of chemical components between natural plants and medical herbs, which are processed via domestic standardization through the local process. At the same time, the specific circumstances and the special needs should also be described in a note to back up data in further research. All of the data are the prerequisite preparation of data tracking and investigations.

To ensure medicine quality and convenience of packaging, storage, transport, the medicinal herbs need origin-processing to achieve physical integrity, moderate moisture, less component damage and so on. Commonly used methods include sorting, washing, slicing, peeling, boiling, steaming and drying, etc. The medicinal herbs after processing are often different from original plant in chemical constituents. Therefore, it is necessary to consider how processing influences the chemical composition if there is a need to understand the chemical composition of original plant.

c) Sample retention and "electronic identity card":

Sample retention quantity should remain generally not less than three times of the actual test amount for subsequent research needs and experimental data traceability. Storage environment, containers, methods, and so should be marked, such as the use of high-temperature, chemical reagents for pest control.

1.2. The establishment of CM standard HPLC fingerprint

a) Standardization of pre-treatment method [11,12]

Usually making a pre-treatment on test samples to meet the requirements of HPLC separation and maintain the purity by removing the substance damage for the column. Meanwhile the solvent with the same or similar polarity compared with the mobile phase is a suitable solvent. Considering the physical and chemical properties in Chinese herbal medicines pieces as well as preparations, we should adopt appropriate methods of extraction. For example, Chinese herbal medicines and solid pieces can be extracted by the traditional extraction methods, such as ultrasound extraction, and solvent extraction. Solid-phase extraction can effectively remove interference substances and the substances that may damage the column. It also can effectively concentrate and enrich the composition of the test sample. In terms of the systems biology in quality research of CM, there are two principles need to be noticed: firstly, method should be simple. It also can fully reflect the composition of test sample, and reduce accidental error from operator; secondly, it costs

less, and is easy to be applied. Also the standards of the method should be easier to be formed for the quality control.

The procedures of the pre-treatment include the preparation of the test sample solution and the preparation of the reference solution. In the reference preparation, it is very important to determine the type, the quantity, the concentration and the preparation method of reference. Methods depend on the active ingredients of pieces and preparations. If the active ingredients have been fully understood, it is better to use the active ingredients as reference; if the active ingredients have not been comprehensibly understood, the conclusion is supposed to be based on a large amount of experimental data, and combined with the extraction and separation of chemical composition analysis. At this stage the main point is the distinction, but the less contacting with pharmacological effect limits the choices of reference. If the chemical substances study could be based on the interaction between the components group and the impact of the body, combing with the clinical research, we can more systematically and scientifically select the reference, and determine its preparation methods.

b) Chromatographic analysis conditions standardized [12,13]

Identification of chromatographic methods: according to the physicochemical property of test sample identify the analysis method. The samples, the neutral, the weak acid, and the weak alkaline compounds all can be soluble in water or organic solvents then RP-HPLC (Reverse Phase HPLC), this situation is the best. If the test sample is ion compounds, the ion HPLC is suitable. If we could not get a good separation by RC-HPLC (Radiogaschromatographic HPLC), the NPC (Normal Phase Chromatography) is also suitable for the separation and analysis for the neutral, the weak acid or weak alkaline compounds. For example, if the test sample is strongly hydrophilic or hydrophobic, NPC is a good choice. RP-HPLC is the most widely used method; our paper is mainly based on the parameters optimization in the RP-HPLC as an example to expatiate the establishment.

Optimizing chromatographic conditions: We should consider equipment conditions and the nature of the test sample to optimize the chromatographic conditions.

(1) Effective and stabilized column:

Choose the suitable column to guarantee the quality of analysis, identify and document the specifications of column. It includes stationary phase, filler particle size, length, diameter, the plate number N based on the designated k, column pressure, selective α, peak asymmetry factor and so on. For the late experimental retrospect, it is better to give the reasons of the selection and applicability. The components of the samples are complex, they often contain such types of compounds as acid, alkaline or neutral whose polar intensity changed in a large range as well as different molecular weight. C_{18} (Octadecylsilyl, ODS) column is the first choice to ensure good separation, the stability, and durability in the actual analysis.

(2) The choice for type and concentration of mobile phase:

The degree of polarity and the concentration of mobile phase directly related to the retention value and the quality of separation of the test sample. It is supposed to use the mobile phase without UV absorption and the low viscosity, such as ACN (Acetonitrile) and MeOH (Methyl Alcohol). THF is rarely used because the significant absorption is under 250 nm. Because we cannot know the exact chemical composition before analysis, we generally use strong reservations solvent elution, such as 80% CAN, to clear almost all components. A large number of experimental studies proved that retention value would increase by about three times if organic phase concentration decreased 10%. This principle can be used to identify the organic concentration related to retention value.

Because of the complexity of components in CM, the gradient elution is better for separation. We can carry out gradient elution all of the linear: the organic phase concentration B% changes from 0% to 100% the organic in 60 minutes; to observe the scope of retention time; according to time of initial peak and the termination, to estimate the polarity of components and judge the concentration of organic phase B%. Thereby it is possible to shorten the time of analysis and change gradient rate (% / min). If there are serious peaks overlap in some regional or too much distance between the peaks, it is necessary to adopt piecewise linear gradient method. If separation is not very good, it should be considered to change the solvent type in the same gradient conditions to improve the resolution. If separation results are still not ideal, this following sequence can be followed: changing column (C_{18} column, cyano-column, phenyl-column, etc.), and/or changing the temperature to improve isolation. It is necessary to keep trying this process to explore the conditions and summarizing experience. Especially in gradient elution, re-balance is required. Because it is time-consuming and costly, the analysis would be simplified if it is possible to use computer to simulate elution conditions. After get a better separation, it is necessary to record the original data of several major changes in the mobile phase and the concentration.

c) Considering other conditions:

General velocity is controlled in the range from 1.0 to 2.0ml / min and sample volume is controlled in general ≤ 25μl, to have certain sensitivity as well as to prevent the column overload. For special purposes, it can be adjusted slightly. Column temperature is directly related to column stability, solvent viscosity, solvent vapor pressure. Increasing column temperature can reduce the retention value and change the distance between peaks simply. Choosing a more appropriate wavelength to determine the further optimization process, the detection wavelength can be identified through the entire ultraviolet absorption peak. If a detection wavelength cannot meet QC, one or more the embodiment of active components of herbs, which have acknowledged characteristics of detection wavelength, are then selected and indirectly

regarded as the detection wavelength substitute by which its certain wavelength can be identified as the case.

d) Operation methods standardization

(1) Procedures Standardization:

Each step of the procedure requires a more stable method, the procedure sequence should be identified according to the physical and chemical characteristics of the sample, forming a lot of analysis data and accumulating a record, then gradually developing into standards.

(2) Experimental operation standardization:

Because chromatographic fingerprint is related to quantitative analysis, the operation of the normative and accuracy is very important. For example, reducing accidental error caused by the choice principle of crush size, the degree of drying, the accuracy of weighing, the membrane pore size of ultra filtration should be stringently required. Experimental operation standardization lays a good foundation for qualitative analysis and quantitative calculation, as well as making a preparation for further profile-effect research.

(3) Standardization of data records:

In chromatographic fingerprint studies, we often require chromatographic conditions optimization many times, and the observation of sample preparation methods many times. The standardization of data recording format and content is convenient for the next experiment, as well as for the fingerprint evidence-based comparative analysis. A reasonable and clear data record is the basis of study.

(4) Standardization of Maintenance:

The same test sample, in the case of different HPLC instruments or experimenters under the same chromatographic conditions, often shows a different result, which brings difficulties for the latter profile-effect research. So it is proposed that experimenters should pay full attention to equipment factors of instability. We should fully understand the differences and characteristics of various types of chromatography (such as limit of detection, limit of quantization, the signal to noise ratio S / N ', etc.) and ensure maintenance and overhaul regularly to provide a hardware foundation for effective, accurate, and comprehensive study.

e) The determination of methodology verification process

In order to ensure the validity and reliability, there is a need to determine the limit allowed when operating conditions change, including specificity, accuracy, precision, range and robustness.

(1) Specificity:

Combining CM theory, these should determine the specificity of pre-treatment methods, chromatographic conditions, and the analysis methods. If necessary, they should be in conjunction with other analytical techniques, such as MS, CE, online testing methods in order to increase credibility.

(2) Accuracy:

Instrument structural quality, residues of test samples in the equipment, the uneven nature of a test sample and the number of test samples that do not have statistical significance, will lead to inaccurate data. In order to ensure the accuracy, controlling resolution, and the overall separation efficiency is feasible.

(3) Precision:

It inspects discrete level under certain conditions from the perspective of the statistical, including repeatability, intermediate precision and reproducibility. Intermediate precision includes differential influence of discrete data in the same laboratory, different dates, personnel, equipment and so on.

(4) Robustness:

Which impacts the results the most among the factors, and how does the stability of the results withstand changing of the factors? Robustness solves this problem. The size of the changing degree indicates the tolerance ability of the method. The greater stability of the methods, the more reliable of the method will be.

(5) Universal application:

Universal application is the prerequisite for combing with systems biology research. The verification of the universal is one of the very important factors for the widely used and in-depth study of fingerprint. We can evaluate the universal of the method through accuracy, precision, stability of the study, actual experimental conditions as well as the cost.

f) The establishment of Fingerprints standards

(1) Establish principles:

Excluding interference in the solvent peaks and impurities, those should be as much as possible as follows: the shared characteristic peaks in the same sample, or characteristic peaks region; and the discriminative characteristic peaks in different samples, or characteristic peaks region, even less content. The objective is to reflect the comprehensive chemical information of the sample as much as possible - typical, representative and reference.

(2) Establishing Method:

There are two methods for establishing the standards fingerprint: typical fingerprint selection and shared pattern generated method. The former one is suitable for a small number of samples with a small difference in fingerprints. However, because of the complexity of CM, directly choosing from a large number of the samples with differences is not scientific. It often contains great arbitrariness. Therefore the latter method that generates the fingerprint by computer simulation is more widely used in analysis. The latter method is carried out based on two algorithms, average vector method and the median vector method. If the sample results are uniform, the average vector method can be used, but if there are great differences in the individual samples, the median vector method would be better. Compared with the average vector method, the median vector method is more robust.

1.3. The analysis and evaluation status quo of standard HPLC fingerprint [11-13]

Chromatographic fingerprint is a description of the overall chemical properties of CM. All information is a collection of the vectors with size and direction, so chromatographic fingerprint analysis and evaluation are actually vector comparison in the degree of similarity. Then apply chemistry measurement theory. The correlation coefficient and the consistency coefficient are often used to describe the relationship among different vectors.

a) Qualitative Evaluation Principle

(1) Based on the different perspective of the evaluation

Use the graphics function of fingerprints: the collected ASC Ⅱ data will be in gauss simulation. Then generate a standard fingerprint for comparison.

(2) Based on the evaluation in the different depth

Locality: Just collecting the data of peak area or peak the height of representative for obtaining the information. It is preliminary but not comprehensive.

Integrity: Choosing all peaks that lay a foundation for systems biology research.

b) Quantitative analysis

(1) Data preprocessing:

The peak area is calculated by using the automatic integral function. The laboratory personnel only need to adjust the signal to noise ratio and the minimum peak area. However, with poor resolution, especially peak inversion and serious baseline drift, it is not satisfactory and the data do not have true credibility. Therefore, a good resolution is the basis for the effective data. The retention time drifts are caused by the complexity of the CM and the fluctuation of experimental conditions, so it is normal and inevitable. Xie Peishan 11 used the spectrum information to make the relevant curve between spectroscopy and chromatography, calibrating the retention time drift, to achieve the correct judgment for the complex components. The uneven curve of chromatogram is the result of equipment noise. Reducing noise could improve SNR (signal to noise ratio) and optimize chromatography curve. The current common smoothing methods include window moving average method, window moving polynomial least squares smoothing method, Fourier transform, Wavelet transform, etc. Comparing the window moving polynomial least squares smoothing method with window moving average method, the former has not only removed the noise for the purpose of improving the SNR, but also reflected the real situation of the chromatography curve.

(2) Data Compression:

The information expressed by chromatographic fingerprinting and multiple data points features will produce an enormous amount of data. Keeping data characteristics is extremely necessary. The application of multi-resolution wavelet transform and Fourier transform as well as other signal processing tools have made some progress in effective data compression and de-noising.

(3) Similarity Computing:

Two kinds of commonly methods: Correlation coefficient method, which is the description of changes trends in the degree of similarity and consistency coefficient (cosine angle) method, which is the description of fitting extent in the degree of similarity. How to choose appropriate methods of evaluation is in need of attention, a standardized similarity evaluation system is the important basis. Targeting a variety of similarity calculation method at present, we recommend the purpose of the study; research methodology, the study object, and the follow-up study are all guidelines for appropriate evaluation methods choosing.

2. The building program of standard chromatographic fingerprint integrated data in modernization of CM

Consolidating and summarizing the source control, standard chromatographic fingerprint system, the efficacy information under CM theory and the pharmacokinetic research results at this stage to form a standard system in modernization of CM, which provides comprehensive data for the standardization of QC and traditional theory exploration, and promotes innovation in pharmaceutical industry modernization. Liu Xunhong[14] used Visual FoxPro (VFP) 6.0 programming language of the process, collected the fingerprinting in various types of traditional Chinese medicine and the related information. They developed a DTCMF (The Database of TCM Fingerprint) database, including chromatography, spectroscopy, electrophoresis and DNA fingerprinting, and x-ray diffraction and thermal analysis spectrum. Database can be run independently in all the current common operating system, with common, practical and ease-of-use characteristics. Qu Jinghui[15] thought that the fingerprint database of CM has been lagging behind the fingerprint technology. According to the wide range of CM resources, we should use administrative means and technical measures. It will avoid duplication of low-level research and the wastage of resources and manpower. Lu Peizhang[16] claimed that because of the overall synergies characteristics, liquid chromatography is used as the main means of chromatography, combing with mass spectrometry or the various spectral analysis. Forming HPLC intelligent and unified a common database to obtain comprehensive information of components is necessary. Wang Qin[17] establishes a pluralistic multi-mode multi-layered database of information mining as a platform for practicing of fingerprint to achieve information, standardization, and standardized in CM.

Nowadays, the development of a fingerprint database has extended gradually from the nonlinear integrated macro system to a comprehensive database. In order to improve the modernization of CM, this research will provide the multi-disciplinary participation and a holographic, pluralistic, standards fingerprint database.

References

1. Macau Chinese philosophy society, philosophical thinking of Chinese medicine basic theories, Beijing, Chinese ancient books publishing house. 2005.
2. Patwardhan B, Vaidya ADB, Chorghade M. Ayurveda and natural product drug discovery. Curr Sci. 2004;86(6):789-799.
3. Dobos GJ, Tan L, Cohen MH, Mclntyre M, Bauer R, Li X. Are national quality standards for traditional Chinese herbal medicine sufficient? Current governmental regulations for traditional Chinese herbal medicine in certain Western countries and China as the Eastern origin country. Complementary Therapies in Medicine. 2005;13(3): 183-190.
4. WHO, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines. 2000:1.
5. Xue TH, Roy R. Studying Traditional Chinese Medicine. Science. 2003; 300 (5620):740-741.
6. Qiu J. Traditional medicine: A culture in the balance. Nature. 2007;448(7150):126-128.
7. Bell IR, Koithan M. Models for the Study of Whole Systems. Integr Cancer Ther. 2005;5(4):293-307.
8. Li J-S. TCM Identification. Shanghai Science and Technology Publishing House. 1996.
9. States Pharmacopoeia Commission, the Chinese Pharmacopoeia. 2005.
10. Zhou Y. Chinese medicine fingerprint technology, Beijing, Chemical Industry Press. 2002.
11. Xie P. Chinese medicine chromatographic fingerprint, Beijing, People's Medical Publishing House. 2004.
12. Sen Deer (snydeh L.R.), Zhang Y, Wang J, Zhang B. The establishment of the high-performance liquid chromatography, Beijing, Chinese Press. 2001.
13. Li F. Chinese medicine high-performance liquid chromatography, Beijing, People's Medical Publishing House. 1999.
14. Liu X, Yu S, Cai B, Qian JD. Comprehensive database of TCM fingerprint. Chinese Traditional Patent Medicine 2005;27(10):1117-1119.
15. Qu J-H, Liao Q-M, Zhang X. The establishment technology of Fingerprint database, Medical Information 2006;19(2):190-191.
16. Lu P, Liang X, Xiao H. Chinese components Intelligent unified fingerprint database, Chemical Progress. 1999;19(2):199-201.
17. Wang Q, Cao J, Ye Z, Che Z. Establishment and Application of Primary TCM Compound Formulae Fingerprint Database, Chinese Archives of Traditional Chinese Medicine. 2006;24(4):659-662.