# Study of High-Performance Liquid Chromatogramphy Fingerprint for Traditional Chinese Medicine Yigongningxue Oral Liquid

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**Summary**: A simple, reliable and accurate fingerprinting method has been developed for quality control of YiGongNingXue oral liquid. The separation was done by reverse phase chromatography using Zorbax eclipse XDB $C_{18}$ column (250mm×4.6mm i.d. with 5.0μm particle size) and detection at 246nm. Methanol (5-95 % in 70 min)-buffer solution (water-phosphoric acid, pH 3.0, 95-0 % in 70 min) as mobile phase for linear gradient elution. The flow rate was 1.0 $mL \cdot min^{-1}$ and the column temperature was at 25℃. The similarity of 20 batches of YGNX oral liquid was more than 90 %. Also 15 common peaks of chromatogram have been detected, ten of them were identified by comparing fingerprint chromatogram with reference substances. The HPLC fingerprint can be used to control the quality of YiGongNingXue oral liquid.

## Introduction

It is well known that the traditional Chinese medicine (TCM) has a long therapeutic history over thousands of years in ancient China and Asian countries, and currently it is attracting ever-increasing attention worldwide [1-3]. YiGongNingXue (YGNX) oral solution (produced by Hubei Shientang Pharmaceutical Co., Ltd. of China, national drugs surveillance administrative bureau standard WS-5092 (B-0092)-2002, country medicine accurate character B20020100) consisted of twelve commonly used chinese drugs including Radix Fructus Psoraleae, Coastal Glehnia Root, Radix Polygoni Multiflori, Fructus Ligustri Lucidi, Fructus Schisandrae, Radix Rubiae, Herba Leonuri and Radix Glycytthizae, etc. YGNX oral solution is used for the treatment of invigorating vital energy, nourishing YIN, enhancing kidney-function and hemostasis. Moreover, it is also effective for the cure of menometrorrhagia, prolonged and/or irregular menstruation. On the basis of spectroscopic analysis and comparison with the data of known compounds, the components are elucidated as glycyrrhizin (GLY), ammonium glycyrrhizinate (AG), psoralen (PS), isopsoralen (IS), schisandrin (SC), imperatorin (IM), isoimperatorin (ISO), emodin (EM), oleanolic acid (OA), mollugin(MO), respectively. These components have bioactivities, and their chemical structures are shown in Fig. 1.

The European Medicines Agency has stipulated that an appropriate fingerprint chromatogram should be used to assess the consistency of botanical drugs. The Ministry of Science and Technology of China also requires standardization of herbal medicine by use of chromatographic fingerprints. Normally, only few of markers or pharmacologically active constituents were employed to assess the quality and authenticity of the complex Chinese prescription. However, the therapeutic effects of TCM are based on the complex interaction of numerous ingredients in combination, which are totally different from that of chemical drugs. There are many kinds of chemical fingerprint analysis methods to control the quality of TCM, such as thin-layer chromatography, gas chromatography, high-performance liquid chromatography, and so on [4-7]. Among these methods, high-performance liquid chromatographic fingerprint has played a more and more important role. Fingerprinting has also been introduced and accepted by World Health Organization (WHO) as a strategy for the identification and quality control of TCM. Till now, there is no chromatographic fingerprint for the quality control of YGNX oral liquid have been reported. So we focused on developing a novel, accurate and valid chromatographic fingerprint

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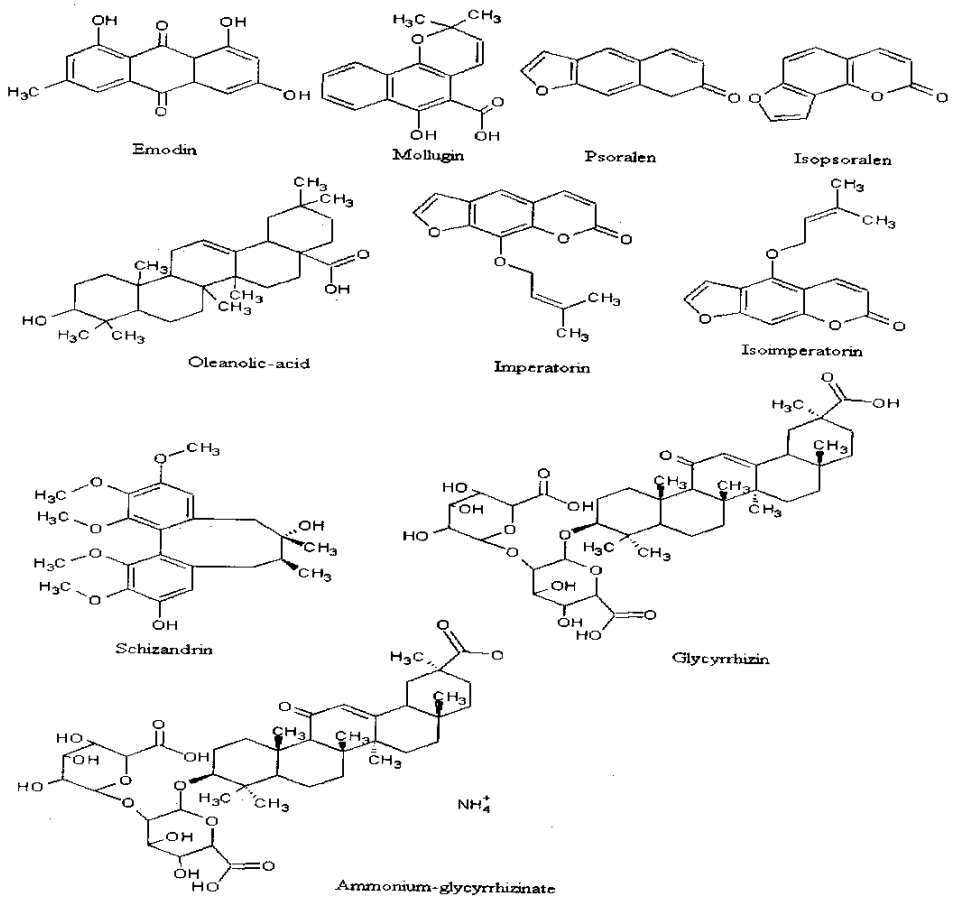


Fig. 1: Molecular structures of the investigated components.

method to control the quality of YGNX oral liquid by HPLC in this research.

## Results and Discussion

### *Optimization of Extraction Conditions*

An orthogonal experiment was employed in order to optimize the extraction conditions. It involved four factors: (A) solvent volume; (B) proportion between methanol and water; (C) extraction time; (D) sonication time. The experimental factors and corresponding levels were shown in Table-1, and orthogonal designs $L_9$ ($3^4$) were presented in Table-2. The optimal condition for extraction of YGNX oral solution could be obtained by intuitionistic analysis of the experimental results

Table-1: Factors and levels for the optimization of extraction conditions.

| Factors | Levels | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| A: solvent volume (mL) | 10 | 20 | 30 |
| B: methanol concentration (%) | 40 | 60 | 80 |
| C: extraction times | 1 | 2 | 3 |
| D: sonication time (min) | 5 | 10 | 15 |

of the orthogonal design. In order to fully show the quality of a medicine, the more relative intensities of all peaks the better in a fingerprint. So the relative sum area of the five biggest peaks, which was more than 70 % of the area of all peaks, was used as a criterion for the selection of the optimal sonication conditions. $K_1$, $K_2$ and $K_3$ was the sum of $A_{5p}$[a] while one of the four factors was fixed and other three factors were changed, respectively. For example: $K_1^A$

Table-2: The results and analysis of orthogonal design.

| Run no. | A: solvent volume (mL) | B: methanol concentration (%) | C: extraction times | D: sonication time (min) | $A_{5p}$[a] |
|---|---|---|---|---|---|
| 1 | 10 | 40 | 1 | 5 | 316 |
| 2 | 10 | 60 | 2 | 10 | 626 |
| 3 | 10 | 80 | 3 | 15 | 625 |
| 4 | 20 | 40 | 2 | 15 | 502 |
| 5 | 20 | 60 | 3 | 5 | 543 |
| 6 | 20 | 80 | 1 | 10 | 594 |
| 7 | 30 | 40 | 3 | 10 | 620 |
| 8 | 30 | 60 | 1 | 15 | 618 |
| 9 | 30 | 80 | 2 | 5 | 644 |
| $K_1$ | 1567 | 1438 | 1528 | 1503 | |
| $K_2$ | 1637 | 1787 | 1772 | 1840 | |
| $K_3$ | 1882 | 1863 | 1788 | 1745 | |
| $k_1$ | 522 | 479 | 509 | 501 | |
| $k_2$ | 546 | 596 | 591 | 613 | |
| $k_3$ | 627 | 621 | 596 | 582 | |
| Range | 105 | 142 | 87 | 112 | |
| Optimized scheme | A3 | B3 | C3 | D2 | |
| Primary and secondary order | 3 | 1 | 4 | 2 | |

[a] $A_{5p}$ represents the area sum of 5 peaks.

is the sum of $A_{5p}{}^{a}{}_1$, $A_{5p}{}^{a}{}_2$ and $A_{5p}{}^{a}{}_3$. $K_2{}^{A}$ is the sum of $A_{5p}{}^{a}{}_4$, $A_{5p}{}^{a}{}_5$ and $A_{5p}{}^{a}{}_6$. $K_3{}^{A}$ is the sum of $A_{5p}{}^{a}{}_7$, $A_{5p}{}^{a}{}_8$ and $A_{5p}{}^{a}{}_9$. $k_1$, $k_2$ and $k_3$ is the average value of $K_1$, $K_2$ and $K_3$, respectively. According to statistic analysis theory, the biggest range of the four factors was 142 of factor B; the smallest was 88 of factor C. It means that the substance B was the most important factor in the extract conditions of YGNX oral solution, which changed a little, the sum area would change greatly. Optimized factors' ordering was obtained according to range analysis (Table-1). The optimal condition was presented in detail in sample preparation.

*HPLC Conditions*

To develop a fingerprint for YGNX oral liquid, an optimized strategy for HPLC conditions was performed. In order to obtain a good resolution, the tried mobile phase systems were shown in Table-3. Both systems with acetonitrile had longer duration of analysis than those with methanol. The methanol-water system had the same analytical time as the methanol-buffer solution system, but the former had a poor resolution. So the methanol (5-95 % in 70 min)-buffer solution (water-phosphoric acid, pH 3.0, 95-0 % in 70 min) system was chosen for its well baseline resolution and suitable duration for analysis. The linear gradient was applied in order to ensure the good repeatability without reducing their resolutions. In order to obtain a sufficiently large number of detectable peaks on the HPLC chromatogram, the spectra of all main peaks were investigated and 246nm was selected as detection wavelength.

Table-3: The tried mobile phases in optimization of HPLC condition.

| Systems | Gradients |
|---|---|
| Methanol (M) and water (W) | (1) 5-80% M and 95-20% W in 50 min<br>(2) 5-80% M and 95-20% W in 70 min<br>(3) 5-100% M and 95-0% W in 70 min |
| Methanol (M) and buffer solution (B, water-phosphoric acid, pH 3.0) | (1) 5-80% M and 95-20% B in 50 min<br>(2) 5-80% M and 95-20% B in 70 min<br>(3) 5-100% M and 95-0% B in 70 min |
| Acetonitrile (A) and water (W) | (1) 5-80% A and 95-20% W in 60 min<br>(2) 5-80% A and 95-20% W in 80 min<br>(3) 5-100% A and 95-0% W in 70 min |
| Acetonitrile (A) and buffer solution (B, water-phosphoric acid, pH 3.0) | (1) 5-80% A and 95-20% B in 60 min<br>(2) 5-80% A and 95-20% B in 80 min<br>(3) 5-100% A and 95-0% B in 70 min |

*Validation of Methodology*

The analytical methods of binary fingerprinting have been validated based on the retention time and the peak area. The results, which clearly demonstrated the reproducibility of the sample preparation, were listed in Table-4. The injection precision of the fingerprint was in the range of 0.07-0.27 % (n = 6) for retention times and 0.63-1.67 % (n = 6) for peak areas. The intra-day precisions (R.S.D.) of the fingerprint were 0.08-0.56 % ($n$ = 5) for retention times and 0.68-3.82 % ($n$ = 5) for peak areas, while the inter-day precisions (R.S.D.) were within the range of 0.12-0.50 % ($n$ = 15) for retention times and 1.49-4.43 % ($n$ = 15) for peak areas, respectively.

Table-4: Analytical method validation results.

| Peak no. | R.S.D. of retention time (%) | | | R.S.D. of peak area (%) | | |
|---|---|---|---|---|---|---|
| | Injection ($n$ = 6) | Intra-day ($n$ = 5) | Inter-day ($n$ = 15) | Injection ($n$ = 6) | Intra-day ($n$ = 5) | Inter-day ($n$ = 15) |
| 1 | 0.18 | 0.21 | 0.22 | 0.86 | 2.38 | 2.05 |
| 2 | 0.17 | 0.12 | 0.12 | 1.44 | 2.17 | 1.60 |
| 3 | 0.15 | 0.22 | 0.22 | 1.07 | 2.09 | 3.17 |
| 4 | 0.27 | 0.56 | 0.50 | 0.75 | 3.14 | 2.49 |
| 5 | 0.08 | 0.08 | 0.14 | 1.45 | 1.31 | 1.93 |
| 6 | 0.18 | 0.24 | 0.24 | 1.14 | 3.82 | 3.61 |
| 7 | 0.18 | 0.26 | 0.25 | 0.63 | 1.11 | 2.31 |
| 8 | 0.20 | 0.20 | 0.28 | 0.95 | 3.30 | 2.85 |
| 9 | 0.20 | 0.13 | 0.13 | 1.67 | 2.21 | 1.49 |
| 10 | 0.21 | 0.29 | 0.28 | 0.82 | 3.15 | 4.43 |
| 11 | 0.16 | 0.28 | 0.25 | 0.49 | 1.30 | 1.55 |
| 12 | 0.07 | 0.30 | 0.28 | 0.96 | 1.68 | 2.89 |
| 13 | 0.16 | 0.37 | 0.33 | 1.61 | 3.37 | 3.73 |
| 14 | 0.14 | 0.22 | 0.22 | 1.35 | 0.68 | 2.85 |
| 15 | 0.09 | 0.29 | 0.24 | 1.15 | 3.20 | 2.26 |

*Standardization of HPLC Fingerprint of YGNX Oral Liquid*

It is well known that the standard HPLC fingerprint must be representative of oral liquid preparation. For this purpose, 20 different batches were analyzed by SES to generate the representative standard fingerprint (Fig. 2). The chromatograms of 20 samples were somewhat different on the number of some peaks, which illustrated the difference on the quality of YGNX oral liquid from batch to batch. No. 200706 was selected as the typical fingerprint, for it had the biggest similarity in 20 batches (Table-5). About 15 peaks were shown in the standard chromatogram of batch No. 200706 and among them, 10 of characteristic peaks were unambiguously identified in the same (Fig. 2). Based on their UV spectra, migration, standard addition and LC-MS (data not shown), peak 1- peak 10 were identified as GLY, PS, IS, SC, IM, AG, ISO, OA, EM, MO, respectively.

## Experimental

*Instrumentation*

The HPLC system 1100 series (Agilent Technologies, Palo Alto, CA, USA) equipped with the ChemStation software (Agilent Technologies) and comprised of a quaternary solvent delivery pump, an online vacuum degasser, a thermostated compartment and a diode array detector, were used for the chromatographic analysis. The peak identification was based on the retention time and the DAD spectrum against the standard presented in the chromatogram.

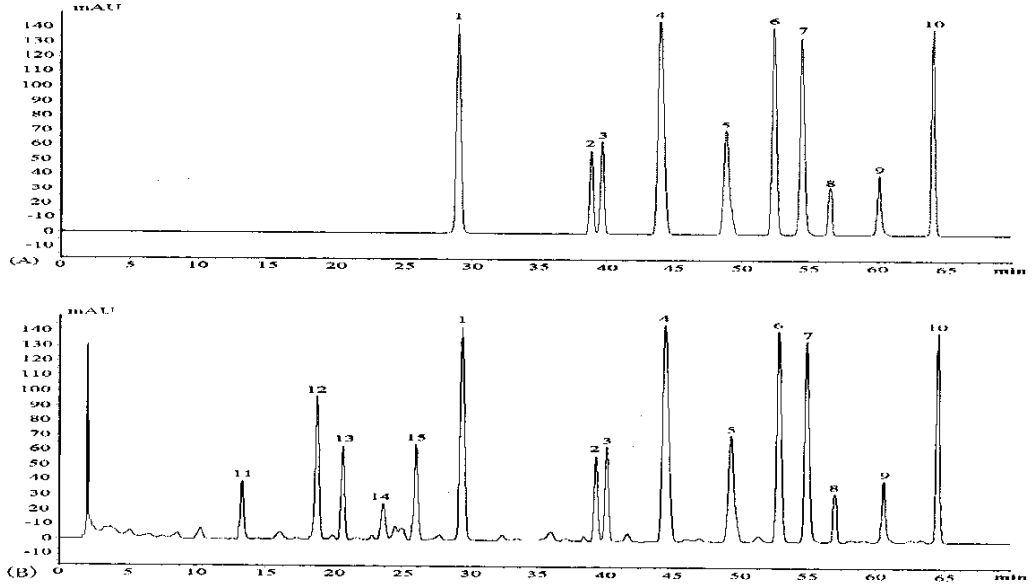


Fig. 2: The typical fingerprint HPLC chromatograms of the standard solution (A) and the real sample solution (B) at 246 nm. The peaks marked were 1 = GLY, 2 = PS, 3 = IS, 4 = SC, 5= IM, 6 = AG, 7 = ISO. 8 = OA. 9 = EM, 10 = MO, respectively.

Table-5: The similarities of 20 chromatograms.

| No. of batches | Similarities [a] | No. of batches | Similarities [a] | No. of batches | Similarities [a] | No. of batches | Similarities [a] |
|---|---|---|---|---|---|---|---|
| 200401 | 0.936 | 200411 | 0.953 | 200509 | 0.957 | 200607 | 0.947 |
| 200403 | 0.911 | 200501 | 0.946 | 200511 | 0.956 | 200609 | 0.975 |
| 200405 | 0.969 | 200503 | 0.940 | 200601 | 0.964 | 200611 | 0.972 |
| 200407 | 0.965 | 200505 | 0.927 | 200603 | 0.923 | 200703 | 0.979 |
| 200409 | 0.958 | 200507 | 0.939 | 200605 | 0.932 | 200706 | 0.985 |

*Materials and Reagents*

Twenty batches (Table-5) of YGNX oral liquid were supplied by Hubei Shientang Pharmaceutical Co., Ltd. (Hubei, China). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Phosphoric acid (analytical grade) was purchased from Gaojing chemical industry company (Hangzhou, China). Other reagents were all of analytical grade. HPLC-grade water was purified by use of a Milli-Q system (Milford, MA, USA).

*Reference Compounds*

Standard substances: MO (lot code: 110884-200604), EM (lot code: 110756-200110), GLY (lot code: 111610-200604), AG (lot code: 110731-200513), PS (lot code: 110739- 200511), IS (lot code: 110738-200511), SC (lot code: 110857-200507), IM (lot code: 110826-200511), ISO (lot code: 110827-200407), OA (lot code: 110709-200304). All of them were provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), whose chemical structures were shown in Fig. 1. These reference compounds were dissolved in methanol and then injected into HPLC after filtration with a 0.45μm filter.

*Sample Preparation*

A representative example of extraction is given below: YGNX oral liquid (10mL) was dried under vacuum to get the residue, then the residue was extracted with 20 mL methanol-water (6:4, v/v) solution in an ultrasonic water bath for 10min. The extraction was repeated three times. After cooling, the extracted solution was mixed and filtrated through analytical filter papers. The filtered solution was evaporated at 45°C to dryness by vacuum. The dry extract was dissolved in 10.0 mL methanol-water (50:50, v/v) and suspended particles were then filtered through a syringe filter (0.45μm) and injected directly into the HPLC system for analysis. Percentage of method, number of extraction and sonication time are mentioned in Table-2 for other examples.

*HPLC Procedures*

The best mobile phase consisted of methanol (5-95% in 70 min)- buffer solution (water-phosphoric acid, pH 3.0, 95-0 % in 70 min). The flow-rate was 1.0 mL·min$^{-1}$ and DAD detector was set at 246 nm for acquiring chromatograms. All separations were carried out on a Zorbax eclipse XDB $C_{18}$ column (250 mm×4.6 mm I.D. with 5.0 μm particle size, Agilent, American) at column temperature 25°C. Other systems tried are given in Table-3.

*Method validation*

The validation of the analytical method was carried out with sample solutions. The instrument/injection precision (repeatability) was obtained by analyzing the variations of retention time and peak area of six injections. The intra-day and inter-day precisions of the method were evaluated using multiple preparations of the same sample. Five replicate samples were prepared and analyzed in a single day and on three different days.

*Data Analysis*

Data analysis was performed by a professional software named Similarity Evaluation System (SES) for Chromatographic Fingerprint of the complex Chinese prescripition (Version 2004A), which was recommended by SFDA (State Food and Drug Administration), used for evaluating similarities of different chromatograms by calculating the correlative coefficient or cosine value of vectorial angel [8-11]. In this article, all of the results were calculated by the correlative coefficient.

**Conclusion**

Chromatographic fingerprint analysis in plant extracts or herbal medicines have been reported in many papers recently [3, 9, 12-14]. Unfortunately, all of the analysis methods for fingerprints in those

papers were so tedious that they were impossible to be accepted in general quality standards of herbal medicines. In the present work, an impersonal, valid and rapid fingerprint analysis method was developed and applied for the YGNX oral liquid. The average fingerprint of 20 batches of samples from different time was obtained, 15 common peaks represents the major constituents of this complex Chinese prescription. The similarity of 20 batches of YGNX oral liquid was more than 0.911, which shows the preparations from different time were consistent.

Comparing the quantification of a few markers or pharmacologically active constituents, the chromatographic fingerprint has more predominance in showing the authenticity of a medicine. For example, only ten in fifteen peaks were identified in the fingerprint of YGNX oral liquid. Another advantage of using chromatographic profiles for quality assessment of herbal products is that it is often unnecessary to know the individual components that make up the fingerprint [15]. The results demonstrate that the method is feasible for comprehensive quality evaluation of YGNX oral liquid.

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