Comparison on HPLC Fingerprints between *Fraxini Cortex* and Its Eye Drop

XIE Rui-fang[1†], ZHAO Qiu-hua[2†], LI Zhi-cheng[3], ZHOU Xin[1*]

1. Department of Pharmacy, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China
2. Department of National Medical, Youjiang Medical University for Nationalities, Baise 533000, China
3. Department of Surgery, Nanhui Hospital, Shanghai 201300, China

Abstract: **Objective** To compare the HPLC fingerprints of *Fraxini Cortex* and its eye drop. **Methods** Using esculin and esculetin as reference substances, HPLC method was established for fingerprint chromatography of *Fraxini Cortex* and its eye drop. The similarity was analyzed by similarity evaluation and system cluster analysis. **Results** For *Fraxini Cortex*, eight peaks were separated, among which four common peaks were obtained; for eye drop, eleven peaks were separated including six common peaks. Similarities of both fingerprints were good. Cluster analysis further showed that different sources resulted in the variance of ingredients in crude drug. Both materials and eye drop had some common peaks such as esculin and esculetin, on the other hand, they had some different peaks which might be caused by extracting process. **Conclusion** HPLC fingerprint chromatogram could be applied for the quality control of *Fraxini Cortex* and its preparations; the pharmaceutical process may be responsible for the variance of ingredients.

Key words: esculetin; esculin; eye drop with *Fraxini Cortex*; *Fraxini Cortex*; HPLC fingerprint; similarity evaluation; system cluster analysis

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Introduction

Fraxini Cortex is one of the most used traditional Chinese medicinal materials in China and is listed in *Pharmacopoeia of People's Republic of China 2010*. It is mainly harvested in Shanxi, Hebei, Sichuan, Henan, Liaoning, and Jilin provinces, etc. According to the theory of traditional Chinese medicine (TCM), this herb could clinically clear away heat, remove dampness, brighten eye, and so on. Its main ingredients are coumarin including esculin, esculetin, fraxetin, etc.

Fraxini Cortex has been often prepared into eye drop combined with *Borneolum Syntheticum*. The eye drop has been adopted for more than 40 years in clinic with good therapeutic effect for keratitis, conjunctivitis, and especially electric ophthalmia. It could have the eye uncomfortable symptoms relieved such as red, itch, dry, fatigue, and so on. Compared to other antibiotic eye drop, its drug tolerance is lower since it is made from natural herb (Lu *et al*, 2002).

However, *Fraxini Cortex* is often obtained from different origins, and the cultural manner, harvest time, and pretreatment process are not always the same, the manufacturing processes of eye drop could not keep complete uniform, and all these reasons may result in the significant variance of eye drop. As a result, the quality control of *Fraxini Cortex* and its eye drop is critical to ensure the quality and safety, especially for hospital preparation.

The HPLC fingerprint has been considered to be a useful method in identification and quality evaluation of herbs (Su *et al*, 2012), the previous articles established the HPLC analysis of *Fraxini Cortex* and its eye drop (Yang *et al*, 2011; Zhou and Gu, 2004), but HPLC were not able to comprehensively reflect the information of

* Corresponding author: Zhou X E-mail: 2479707904@qq.com
First author: Xie RF E-mail: 510706346@qq.com
† Parallel first author: Zhao QH
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chemical structures. In this article, the chromatographic fingerprints (Hao, Wang, and Zhang, 2012) of *Fraxini Cortex* and its eye drop were attempted to establish.

Materials and methods

Instruments

An Agilent 1100 series HPLC-DAD system comprising binary pump, auto-sampler, thermostat column compartment, and DAD (Agilent, USA) were employed for acquiring chromatograms, UV spectra, and 3D plots.

Chemicals, solvents, and materials

Esculetin and esculin were purchased from National Institute for Food and Drug Control (Beijing, China). Methanol of analytical grade (Merck) was utilized for the preparation of sample and reference solutions. Acetonitrile of HPLC grade (Merck), deionized water generated from Milli-Q water system (Millipore, USA), and phosphate acid (Shanghai Wujin Chemical Regent Factory) were used for the preparation of mobile phase. Plant material samples of *Fraxini Cortex* were collected as the previous paper (Zhao *et al*, 2011). Eighteen batches of *Fraxini Cortex* and *Borneolum Syntheticum* eye drop were prepared, and the batches number were set according to production date.

Sample preparation and analysis

Sample preparation The sample powder (0.5 g) of different batches of *Fraxini Cortex* was respectively accurately weighed, and introduced into a 10 mL amber vial with methanol. The mixture was sealed, ultrasonically extracted for 20 min and centrifuged at 10 000 r/min for 10 min. The eighteen batches of eye drop were directly centrifuged at 10 000 r/min for 10 min. The blank eye drop without *Fraxini Cortex* was prepared using the same method of normal eye drop. All the supernatants of crude drug and eye drop were filtered through 0.22 μm membrane filters. An aliquot of 5 μL solution was injected for HPLC analysis. The chromatographic fingerprints of *Fraxini Cortex* and its eye drop were documented as the condition of our previous paper (Yang *et al*, 2011).

Chromatographic conditions Analyses were performed on an Agilent reversed-phase XDB-C_{18} column (250 mm × 4.6 mm, 5 mm) at 40 ℃. The mobile phase consisted of 0.1% phosphoric acid in deionized water (A) and acetonitrile (B), and the volume ratio was 95:5. The elution was performed at a solvent flow rate of 1.0 mL/min, the diode-array detector (DAD) was set at 340 nm to record the chromatograms and the injection volume of each sample, and the standard solution was 5 μL.

Calibration and detection limits

The calibration reference solution (5 μL) was injected into HPLC instrument. The calibration curves were constructed by plotting the chromatographic peak area (*A*) versus the compound amount injected and there was a good linear relationship when the correlation coefficient was above 0.9. The limits of detection (LOD) and quantification (LOQ) for marker compounds under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

Precision, repeatability, and accuracy

To assess the intra-day variations, the same solution was determined in triplicate within a day. The inter-day precision was with the same solution over three consecutive days by three injections per day.

To estimate the repeatability, five different working solutions were prepared from the same decoction and analyzed.

To evaluate the accuracy of this method, known amounts of reference stubstances were respectively spiked to the same sample at the low-, mid-, and high-dose and analyzed as described above. The average recoveries were calibrated by the formula: recovery (%) = (amount found − original amount)/amount spiked, and relative standard deviation or RSD (%) = SD/mean.

Data analyses

Similarity tests among samples were performed using the professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2004A). The matching chromatogram among the fingerprints of samples was performed by a multipoint calibration mode based on the retention time (t_R) and UV spectra. In this test, all samples were examined to generate a reference chromatogram as the representative reference fingerprint and the similarity of each chromatogram against this standard chromatogram was then calculated using the nearest neighbor and cosine method (equations 1 and 2).

$$C_{xy}=\frac{(x,y)}{\|x\|\|y\|} \quad (1)$$

$$\cos\theta=\frac{\sum_{i-1}^{n} x_i y_i}{\sqrt{\sum_{i-1}^{n} x_i^2}\sqrt{\sum_{i-1}^{n} y_i^2}} \quad (2)$$

The hierarchical clustering analysis (HCA) was performed to analyze the data from HPLC chromatograms of *Fraxini Cortex*. All above were implemented by using SPSS software (SPSS for Windows 11.0, SPSS Corporation, USA).

Results and discussions

Validation of HPLC method

Calibration, detection limits, and precision Under the developed method, esculin and esculetin were separated according to time sequence, and the calibration equations were Y = 5676.9 X + 121.14, r = 0.9998 and Y = 13 701 X − 9.2001, r = 0.9999, respectively. The RSD values of the intra-day and inter-day precision were found not exceeding 2%, suggesting that the instrument have a good precision. Meanwhile, the LOD values for esculin and esculetin were and 1.2 and 2.0 μg/mL, and the LOQ values were 2.4 and 4.0 μg/mL.

Reproducibility and accuracy As shown in Table 1, the recovery test for six compounds showed mean recovery rates were between 90% and 110%, indicating that the accuracy of the method was acceptable. The RSD values of repeatability test were below 30% (Table 2), also demonstrating that the extraction method of samples was stable.

Table 1 Recovery for assay of esculin and esculetin ($\bar{x} \pm s$, n = 3)

Ingredients	Control content / (μg·mL^{-1})	Measured value / (μg·mL^{-1})	RSD / %	Recovery / %[b]
esculin	744.40	673.03 ± 27.35	4.06	90.41
	864.64	803.75 ± 15.28	1.90	92.96
	973.22	944.33 ± 16.24	1.72	97.03
esculetin	35.88	37.71 ± 1.27	3.37	105.11
	41.16	44.52 ± 1.88	4.23	108.18
	45.63	48.49 ± 3.45	7.11	106.26

a: measured value = total content – sample content; b: recovery = measured value / control content

Table 2 Relative retention time (RRT) and relative peak area (RPA) reproducibility of common peaks in *Fraxini Cortex* (n = 5)

Peak No.	Average RRT	RSD of RRT / %	Average RPA	RSD of RPA / %
1	0.4668 ± 0.0005	0.10	0.0040 ± 0.0007	17.52
2	0.6232 ± 0.0025	0.41	0.0050 ± 0.0014	27.37
3	0.8732 ± 0.0039	0.45	0.0033 ± 0.0011	32.55
4 esculin	1	0	1	0
5 esculetin	1.5839 ± 0.005	0.31	0.1288 ± 0.0035	2.71
6	1.8436 ± 0.0076	0.41	0.0205 ± 0.0025	12.31
7	2.8102 ± 0.0183	0.65	0.4348 ± 0.0071	1.63
8	3.3411 ± 0.0222	0.66	9.6718 ± 0.5351	5.53

HPLC fingerprints of *Fraxini Cortex*

To establish a representative chromatographic fingerprint, twelve authentic batches of crude drug from Shanxi, Liaoning, Sichuan, and Hebei provinces were analyzed using HPLC method. Among acquired chromatograms, eight peaks had satisfied baseline separation (Fig. 1A). The contents of two marker compounds, esculin and esculetin, were within the range of calibration curves for all samples. However, the contents of esculin in most batches from Shanxi were less than the reference material (Table 3).

The representative peaks existing in all batches of samples were assigned as common peaks. Among these peaks, peak 1 (esculin) indicated the most abundance, with good resolution and demonstrated pharmacological activities (Table 3). Therefore, it was chosen as

the reference peak. Altogether there were four common peaks (Fig. 1A), of which the t_R was 17.6 (esculin), 27.3 (esculetin), 50.3, and 57.2 min (fraxetin), respectively, according to the chromatograms of reference compounds. The area percentage of these four peaks in all peak area was 97.32%, above the 90% limit which specified by the *Fingerprint Guideline of Traditional Chinese Medicine Injection in China* (2004 edition).

Most of the similarity values were above 0.9, and there was no obvious difference among most of these materials. HCA further showed that the materials from Shanxi province were basically one sort while other resources belonged to another sort (Fig. 2). It showed that different sources resulted in the variance of ingredients in *Fraxini Cortex*.

HPLC fingerprint of eye drop

The chromatographic fingerprints of eighteen batches of eye drops showed that eleven peaks had good baseline separation under the developed condition (Fig. 1B). Six common peaks were obtained, of which the t_R was 16.6 (esculin), 26.0 (esculetin), 30.0, 45.3, 57.6, and 65.5 min, respectively (Table 4). The area percentage of these common peaks in all peak areas was 97.32%. The similarities of *A* from most batches were above 0.9 except several values. It showed that there was

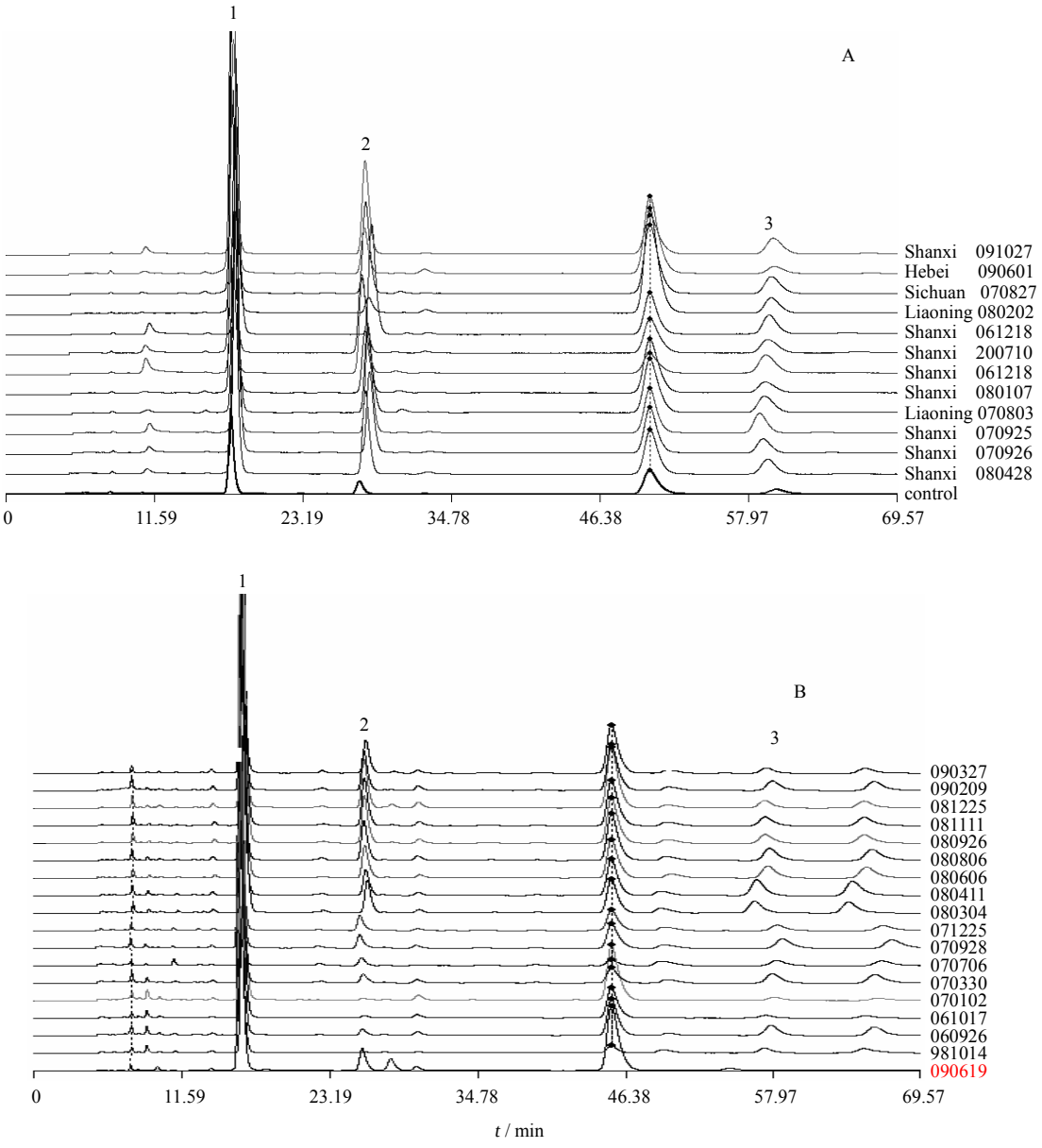

Fig. 1 Chromatographic fingerprints of twelve batches of *Fraxini Cortex* (A) and different batches of eye drop (B)

1: esculin 2: esculetin 3: fraxetin

Table 3 t_R and peak area (*A*) values of common peaks in twelve batches of *Fraxini Cortex*

Origin	Batch No.	Esculin			Esculetin			Fraxetin	
		t_R1 / min	A1	Content / (mg·g^{-1})	t_R2 / min	A2	Content / (mg·g^{-1})	t_R3 / min	A3
Shanxi	20080428	18.1	2386	7.9832	28.4	992	0.1462	60.0	328
Shanxi	20070926	17.4	2627	8.8313	27.5	946	0.1394	57.8	325
Shanxi	20070925	17.4	2726	9.1801	27.9	1137	0.1672	58.1	423
Liaoning	20070803	17.4	2929	9.8950	27.6	819	0.1208	58.7	295
Shanxi	20080107	17.1	2109	7.0094	27.0	1213	0.1785	57.1	469
Shanxi	2006121812	17.8	1312	4.1981	28.0	1299	0.1910	59.5	514
Shanxi	2007102502	17.9	2476	8.2971	28.1	1062	0.1563	60.0	368
Shanxi	2006120107	17.6	2294	7.6554	28.0	1327	0.1950	58.8	484
Liaoning	20080202	17.6	3975	13.5783	28.1	182	0.0278	59.4	391
Sichuan	20070827	17.8	4015	13.7207	28.2	1136	0.1672	60.0	458
Hebei	20090601	18.1	4368	14.9721	28.6	558	0.0828	61.0	179
Shanxi	20091027	18.1	2047	6.7870	28.6	1169	0.1719	60.6	418
/	121415-200702	18.8	3896	11.6051	29.4	767	0.1187	62.7	528

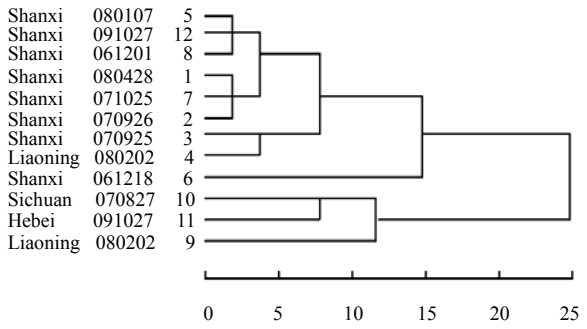

Fig. 2 HCA of twelve batches of *Fraxini Cortex*

no significant difference among these batches of eye drop.

Comparison of *Fraxini Cortex* and its eye drop

Compared with *Fraxini Cortex*, the fingerprints of its eye drop had more peaks than crude drug. Among common peaks, although the t_R had a few differences, both *Fraxini Cortex* and its eye drop had esculin and esculetin, which could be confirmed by recovery test. However, crude drug had the peak of fraxetin (t_R = 59.5) while its eye drop did not. In front of No. 2 peak (esculetin) in eye drop, there was an extra small peak

Table 4 t_R and *A* values of common peaks in eye drop

Batches	Esculin			Esculetin			Fraxetin	
	t_R1 / min	A1	Content / (mg·mL^{-1})	t_R2 / min	A2	Content / (mg·mL^{-1})	t_R3 / min	A3
981014	16.2	3423	0.5816	25.8	113	0.0089	57.0	319
060926	16.4	7800	1.3526	26.0	245	0.0186	58.1	773
061017	16.1	7176	1.2427	25.7	121	0.0095	57.0	189
070102	16.4	11948	2.0834	25.8	976	0.0719	58.3	210
070330	16.3	5523	0.9515	25.8	371	0.0277	57.9	752
070706	16.4	1514	0.2454	26.0	310	0.0233	58.5	390
070928	16.5	7205	1.2479	26.2	526	0.0391	59.4	782
071225	16.6	5715	0.9853	26.3	576	0.0427	59.2	403
080304	15.9	7983	1.3849	25.3	1178	0.0866	55.5	942
080411	15.9	7546	1.3078	25.3	949	0.0700	55.8	1305
080606	16.4	7444	1.2899	25.9	1277	0.0939	57.5	984
080806	16.2	9333	1.6226	25.7	1522	0.1117	57.4	989
080926	16.3	10144	1.7656	25.8	1848	0.1356	57.2	701
081111	16.0	12561	2.1913	25.5	1848	0.1355	56.8	560
081215	16.4	10079	1.7541	26.2	1484	0.1090	58.2	736
090209	16.0	22859	4.0053	25.7	1231	0.0905	57.6	651
090327	16.0	10185	1.7727	25.6	1242	0.0913	56.9	382
090619	16.2	11545	2.0123	25.9	884	0.0652	57.6	651

(Fig. 1B). The chromatogram of *Fraxini Cortex* sample spiked with its eye drop (Fig. 3) showed three peaks in eye drop (Fig. 1B) were extra peaks which the materials did not have (Fig. 1A). The chromatogram of blank eye drop sample without *Fraxini Cortex* had none peaks. This result indicated the extra peaks were not from *Borneolum Syntheticum* and supplementary materials. Where did these peaks come from? According to the prepared process of eye drop, *Fraxini Cortex* was extracted with water (pH < 10). When the hydrolyzed condition was alkaline, lactone rings in coumarin structure could be opened and changed into *cis*-*O*-hydroxylcinnamic acid sodium, which could return again to lactone ring if alkaline condition comes back to acid. But if the heating time is long under alkaline condition, the open lactone ring will switch from *cis* structure to *trans* and not able to close again. So new hydrolysates were generated (Fig. 4). This could be the reason, but need to be further confirmed. The results indicated that the pharmaceutical process could be responsible for the transformation of the chemical structures in eye drop.

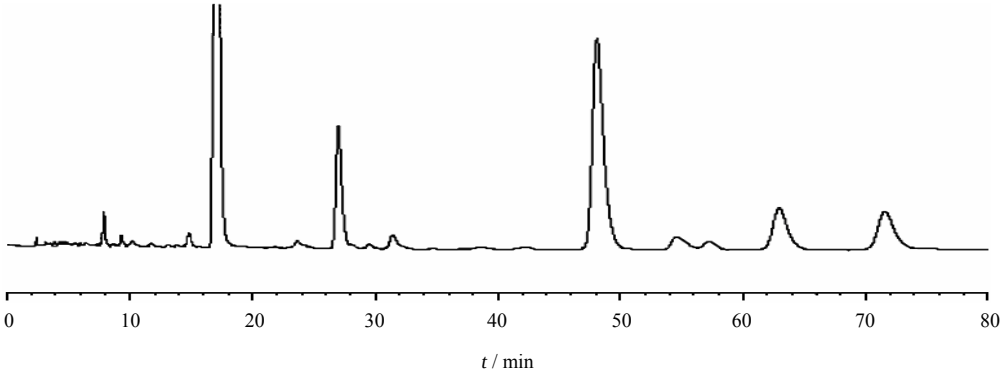

Fig. 3 Chromatographic fingerprint of *Fraxini Cortex* spiked with eye drop

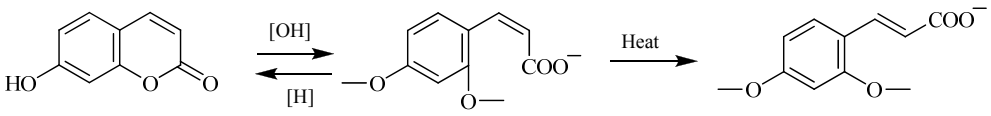

Fig. 4 Hydrolyzing process of coumarin

Conclusion

In this article, the chemical fingerprints and quantitative data of *Fraxini Cortex* and its eye drop are obtained and compared. The HPLC fingerprint chromatogram could be applied for the quality control of *Fraxini Cortex* and its eye drop; different sources and manufactories could result in variance of constituents of crude drug; the pharmaceutical process may make the ingredients change.

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