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Journal of Chromatography A, 1022 (2004) 139–144
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Comparison of high-speed counter-current chromatography and high-performance liquid chromatography on fingerprinting of Chinese traditional medicine

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Received 28 April 2003; received in revised form 10 September 2003; accepted 19 September 2003

Abstract

Active constituents of Chinese traditional medicine (CTM) are influenced by soils, climates, and growth stages. Therefore, fingerprint is important in quality control of CTM. Our studies showed for the first time that high-speed counter-current chromatography (HSCCC) could be applied in the development of fingerprint of CTM. Twelve components were separated from extracts of three crude samples of *Salvia miltiorrhiza Bunge*, which were collected from different growth locations. All 12 components of each sample were isolated within 13 h coming with good correspondence by using HSCCC. Relative standard deviation (R.S.D.) values of the retention time in HSCCC separation was less than 3% satisfied the precision requirements of national standards in the fingerprint development. As a new approach, HSCCC was compared to the conventional approach-HPLC in our experiments. The fingerprint developed by HPLC comprised 11 peaks that collected within 45 min. R.S.D. values of retention times of the corresponding peaks in HPLC analysis were very small (maximum 0.66% and average 0.10%). In conclusion, HSCCC was a feasible method to be used in the development of fingerprint of CTM.

Keywords: Counter-current chromatography; Pharmaceutical analysis; Finger printing; *Salvia miltiorrhiza*

1. Introduction

Secondary metabolites depending on growing circumstances usually are active constituents of CTM. As a result, a CTM may have variable chemical components and contents according to the different soils and climates that growing in as well as growth stages when being harvested [1]. It is absolutely necessary to develop analytic methods of CTM for quality control. Among all the quality control systems, fingerprint has gained more and more attention recently after it is applied widely in quality control of CTM [2]. Fingerprint is a kind of method to show chemical information of medicines with chromatograms, spectrograms and other graphs by chemical analytical techniques. The major feature of fingerprints is commonness of the same kind of medicine from different collections.

Chromatography, ultraviolet spectroscopy (UV), infrared spectrometry (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS) are practically used in development of fingerprints, which means there can be more than one way to express the chemical information of a CTM sample [1]. Each technique indicates part of characters of chemical information based on its principle. Therefore, a fingerprint can be composed of several sets of graphs, for example chromatograms and spectrograms, when necessary. There are limitations of UV, IR, NMR and MS in fingerprinting. Thus, chromatography, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), is recommended by Chinese Pharmacopoeia for fingerprinting [2]. Among them, TLC has been used in the past 10 years. TLC is fast and easy to operate. But the precision of TLC is poor. HPLC is of high precision and popularly used in fingerprinting. However, HPLC strictly requires complicate pretreatments of samples to remove residual solid particles and eliminate irreversible adsorptive loss of samples onto the solid support matrix [3]. Furthermore, some samples with high viscosity

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doi:10.1016/j.chroma.2003.09.038

and samples easy to adsorb on to the solid support matrix are not suitable for HPLC to analysis or separate. GC is of high precision, but with limited application. High-speed counter-current chromatography (HSCCC) is a kind of liquid–liquid partition chromatography without any solid matrix, which eliminates irreversible adsorption of samples on solid support in the conventional chromatographic column [4]. It has been successfully applied to analysis and separation of various natural products [5], especially for polar substances [6]. Cheap instruments and commonly used chemicals make HSCCC more cost-effective than HPLC. Therefore, HSCCC potentially can be more popular in fingerprinting.

So far HSCCC technique has not been applied in development of fingerprint of CTM in previous reports. As a new approach in fingerprinting, HSCCC was compared with HPLC, the conventional technique applied in fingerprinting, in our present studies. Due to the difference between HSCCC that is semi-preparative and HPLC that is mostly analytical and limited experiment conditions, our studies focused on the feasibility of this method by using HSCCC in developing fingerprints of *Salvia miltiorrhiza Bunge*.

Salvia miltiorrhiza Bunge, a popular CTM, has been extensively used in treatments for many kinds of disorders such as coronary artery disease and angina pectoris [7]. The major active constituents of *Salvia miltiorrhiza Bunge* are tanshinones [8]. Separation and purification of *Salvia miltiorrhiza Bunge* by different types of counter-current chromatography has been reported. Tian prepared four tanshinones by using HSCCC [9] and eight tanshinones by using multidimensional counter-current chromatography [10]. Li separated eight tanshinones [8] and 1 water-soluble phenolic compound by using HSCCC [11].

In this study, HSCCC was applied to separate more components from *Salvia miltiorrhiza Bunge* and to develop the fingerprint of this medicinal plant. Twelve components were identically separated from each of three different collections of *Salvia miltiorrhiza Bunge* in 13 h, whereas HPLC identified 11 peaks in 45 min. The feasibility and precision of HSCCC used as a method of fingerprinting were discussed in our present report.

2. Materials and methods

2.1. Instruments and materials

HSCCC (TBE-300) is from Shenzhen Tauto Biotech, China, with three preparative coils connected in series (diameter of tube = 2.6 mm, total volume = 300 ml) and a 20 ml sample loop. The HPLC system is from Shimadzu, Japan with a 20 μl sample loop. The column used was Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan). Ethanol and *n*-hexane were analytical-grade chemicals from Atoz Fine Chemicals, Tianjin, China. Reverse osmosis water (18 MΩ, Milli-Q, Milipore, USA) was used for all solutions and dilutions. Acetonitrile and aqueous trifluoroacetic acid (TFA) were chromatographic-grade chemicals from Fisher Scientific, UK.

2.2. Solvent system for HSCCC

Solvent system A: *n*-hexane–ethanol–water (10:5.5:4.5, v/v). Solvent system B: *n*-hexane–ethanol–water (10:7:3, v/v) [5]. Each mixture was equilibrated thoroughly in a funnel at room temperature. The upper phase and lower phase were separated before use.

2.3. Sample preparation

The powdered dried roots (20 g) of *S. miltiorrhiza Bunge* from Hebei, Shandong and Jiangsu province were added to 50 ml of *n*-hexane–ethanol (1:1, v/v). The mixture was shaken for 45 min. It was then centrifuged at 10,000 × *g* for 10 min, and the supernatant was saved. The same extraction procedure was repeated once and the supernatants were combined. The extract was diluted with water at a ratio of 1:2 and equilibrated for 2 h. The organic phase was separated and washed with 30% aqueous ethanol until the water phase was almost colorless. The organic extracts were dried by rotary vaporization at 40 °C to yield the final crude samples [5]. 100 mg crude sample was dissolved in the lower phase of solvent system A before HSCCC separation.

2.4. HSCCC separation

Preparative separation was performed using a stepwise elution with solvent systems A and B in sequence. First, the coiled column was filled with the upper phase of solvent system A. Then, the apparatus was rotated at 900 rpm, and at the same time, the lower phase of solvent system A was pumped through the column at a flow-rate of 2.0 ml/min. After the mobile phase emerged in the effluent and hydrodynamic equilibrium was established in the column, 6 ml of the sample solution containing 100 mg of the crude tanshinones was injected through the valve. The effluent was monitored with a UV-Vis detector at 280 nm and the peak fractions collected separately. After 470 min of elution, the mobile phase was changed to the lower phase of system B until the end of the separation.

2.5. HPLC analysis

The column used was a Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan). The mobile phase was solvent A (0.1% aqueous TFA) and solvent B (0.1% TFA + acetonitrile) in the gradient mode as

follows: 0–5 min, 0% B; 5–25 min, 0–70% B; 25–40 min, 70% B; 40–41 min, 70–0% B. The flow-rate was 1.0 ml/min. The effluent was monitored at 280 nm.

2.6. UV-Vis spectrophotometer scanning

Each peak fraction and standard sample was scanned by a UV-Vis spectrophotometer at 900–200 nm.

3. Results and discussion

3.1. Fingerprint development of S. miltiorrhiza Bunge by HSCCC

The main components in the crude sample of *S. miltiorrhiza Bunge* were tanshinones [8]. The medicinal plants from three different provinces were separated by HSCCC as described in Section 2.4. Step-wise elution showed better performance than one-step elution in previous studies [9]. An optimized step-wise elution strategy was performed in our studies: 0–470 min, in solvent system A; then in solvent system B. HSCCC system was performed at a speed of 900 rpm and at a flow-rate of 2 ml/min [8]. Retention of the stationary phase was 78.8%, which assured the resolution of separation. In our experiments, 12 distinct peak fractions were eluted, respectively, from the three crude samples within 13 h (Fig. 1). More peak fractions were eluted in our studies than previous reports [8,9] after an optimized elution strategy was applied in separation of tanshinones from *Salvia miltiorrhiza Bunge* by HSCCC.

The content of each peak fraction varied greatly in different samples as shown in Fig. 1 and Table 1, which confirmed that location and climate had great impact on the quality of CTM. A feasible quality control system is therefore necessary for CTM.

Table 1
Relative contents of peaks in HSCCC separation

Peak no.	Hebei (%)	Shandong (%)	Jiangsu (%)
1	2.0	2.8	1.4
2	0.3	0.1	0.1
3	0.5	0.1	0.1
4	2.0	2.5	2.3
5	2.4	1.0	1.9
6	0.5	0.9	0.3
7	19.5	12.9	15.3
8	5.3	20.2	9.1
9	9.0	1.6	4.2
10	10.6	8.0	13.6
11	40.0	48.7	48.5
12	8.2	1.3	3.3

Conditions: (column) multilayer coil of 2.6 mm i.d. tube with a total capacity of 300 ml; rotary speed: 900 rpm; stationary phase: the upper phase of solvent system A; mobile phase: 0–470 min, the lower phase of solvent system A and after 470 min, the lower phase of solvent system B; flow-rate: 2 ml min^{-1}; detection at 280 nm; sample size: 100 mg; retention of stationary phase: 78.8%.

Table 2
Retention times of peaks of HSCCC separation in HPLC analysis

Peak no.	Retention time (min)			
	Heibei	Shandong	Jiangsu	Average
1	27.67	27.69	27.65	27.67
2	27.60	27.64	27.63	27.62
3	29.00	29.00	29.01	29.00
4	28.29	28.28	28.27	28.28
5	29.13	29.14	29.13	29.13
6	30.91	30.94	30.90	30.92
7	30.79	30.77	30.79	30.78
8	29.46	29.42	29.43	29.44
9	31.07	31.21	31.21	31.16
10	32.29	32.29	32.31	31.30
11	34.69	34.64	34.66	34.66
12	35.54	35.53	35.52	35.53

Conditions: (column) reversed-phase Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm); mobile phase: solvent A (0.1% aqueous TFA) and solvent B (0.1% TFA + acetonitrile) in the gradient mode; flow-rate: 1.0 ml min^{-1}; detection at 280 nm.

The 12 peak fractions from each crude sample were collected, respectively. Their retention times in HPLC analysis and absorption spectrum in UV-Vis scanning were identified individually (Tables 2 and 3). Although retention times and absorption spectrums of peak fraction 1 were similar to those of peak fraction 2, there was not enough evidence to argue that the two peak fractions were the same constituent. Therefore, we consider peak fractions 1 and 2 were different. Retention time and absorption spectrum of peak fractions 1–12 from three crude samples were in good correspondence so they were consistent in all samples [12]. The good correspondence of the peaks of all crude samples made it an applicable fingerprint for *S. miltiorrhiza Bunge*. Thus, the peak profile of the 12 components made up the fingerprint of *S. miltiorrhiza Bunge* with universal features.

All 12 components can be defined as common peaks in the fingerprint, which means non-common peak area is 0, less than the national standard (10%). Retention time was an important parameter in the fingerprint [1], so precision of HSCCC could be defined by relative standard deviation (R.S.D.) of retention time. R.S.D. values of the 12 peaks were less than 3% (maximum 2.9% and average 2.7%) as shown in Table 4, which met the demands of the national standard.

If semi-preparative HSCCC with 300 ml capacity was replaced by analytical HSCCC with 50 ml capacity, precision could be increased greatly and run time could be decreased evidently [5]. Temperature was not the most important parameter in HSCCC separation [13]. So it was not discussed in this study.

HSCCC was proven to be feasible as a new method to develop the fingerprint of CTM in our studies. When applying

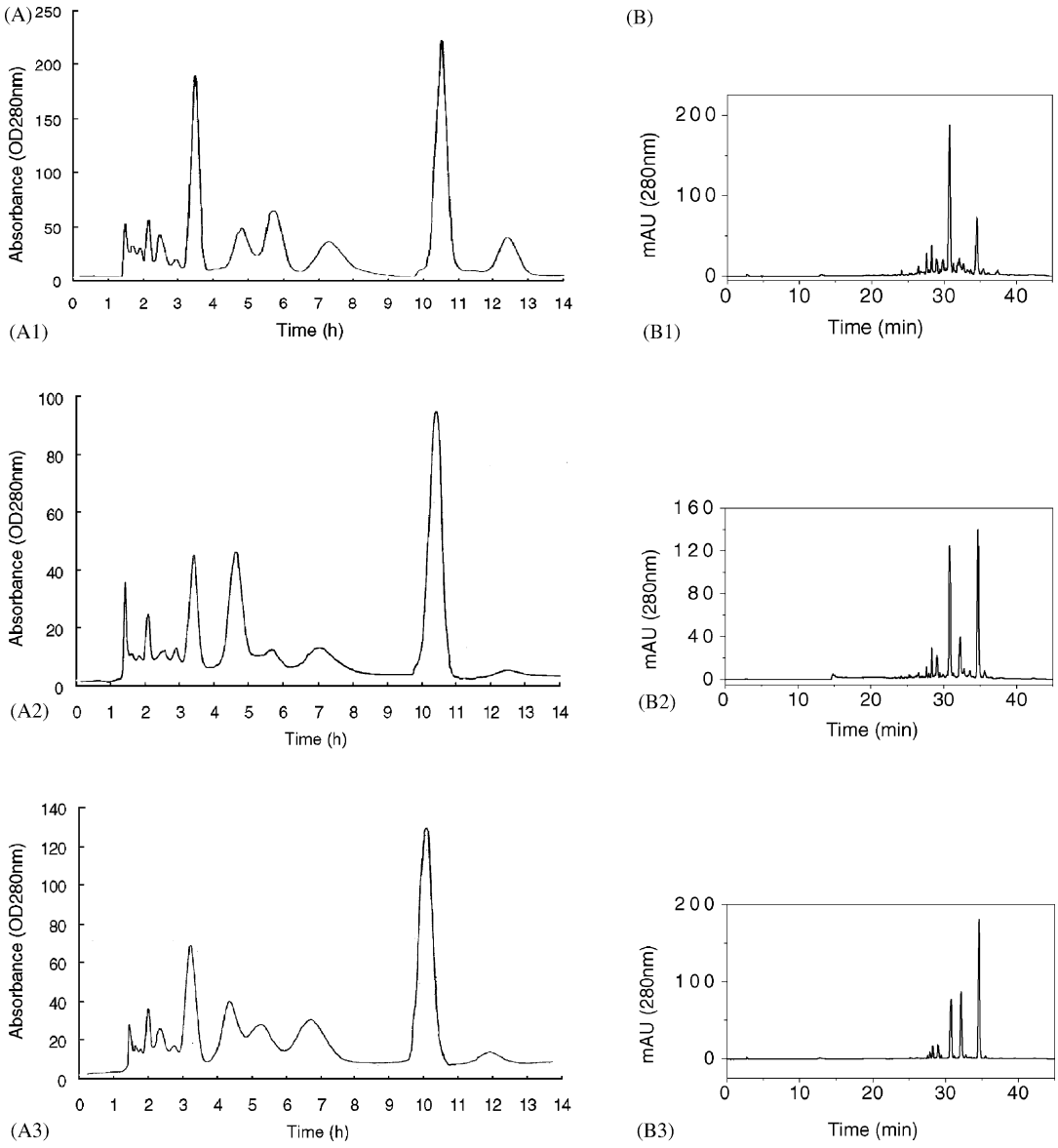

Fig. 1. (A) Chromatograms of crude samples of *Salvia miltiorrhiza Bunge* from three different provinces by HSCCC separation; (A1) sample from Hebei; (A2) sample from Shandong; (A3) sample from Jiangsu. Conditions: (column) multilayer coil of 2.6 mm i.d. tube with a total capacity of 300 ml; rotary speed: 900 rpm; stationary phase: the upper phase of solvent system A; mobile phase: 0–470 min, the lower phase of solvent system A and after 470 min, the lower phase of solvent system B; flow-rate: 2 ml min^{-1}; detection at 280 nm; sample size: 100 mg; retention of stationary phase:78.8%. (B) Chromatograms of crude samples of *Salvia miltiorrhiza Bunge* from three different provinces by HPLC analysis; (B1) sample from Hebei; (B2) sample from Shandong; (B3) sample from Jiangsu. Conditions: (column) reversed-phase Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm); mobile phase: solvent A (0.1% aqueous TFA) and solvent B (0.1% TFA + acetonitrile) in the gradient mode; flow-rate: 1.0 ml min^{-1}; detection at 280 nm.

HSCCC in practice to develop a fingerprint, some factors need to be considered carefully, including the sample stability (10 collections of samples should be included at least), the precision and repeatability of apparatus.

3.2. Fingerprint development of S. miltiorrhiza Bunge by HPLC

HPLC, being conventionally applied in analysis and separation of natural products, is recommended by Chinese Pharmacopoeia in development of the fingerprint. As a comparison to HSCCC, HPLC was used to develop fingerprint of *S. miltiorrhiza Bunge* in our studies as described in Section 2.5 [1].

There were 11 common peak fractions shown, respectively, in the three samples by HPLC (Fig. 1). It is known that the correspondence of peaks in a group of chromatograms can be preliminarily determined by the retention time in HPLC analysis [1]. There is professional software for further analysis of similarity of a group of chromatograms. In this study, we deterred preliminary correspondence based on the retention time.

Table 3
Absorption wavelengths of peaks of HSCCC separation in UV-Vis spectroscopy analysis

Peak no.	Absorption peak no.	Absorption wavelength (nm)		
		Hebei	Shandong	Jiangsu
1	1	276	277	278
2	1	276	276	277
3	1	233	233	234
	2	270	270	269
4	1	241	240	241
	2	291	292	293
	3	333	335	336
	4	418	417	417
5	1	224	225	224
	2	280	277	279
6	1	222	220	221
	2	254	253	254
	3	270	271	270
7	1	220	219	219
	2	265	264	264
	3	363	363	362
	4	452	457	454
8	1	247	246	246
	2	425	425	424
9	1	247	247	247
	2	326	325	326
	3	326	325	326
	4	366	365	367
10	1	227	227	227
	2	294	292	293
	3	512	512	512
11	1	224	224	224
	2	253	253	253
	3	271	270	270
	4	356	357	357
	5	468	468	469
12	1	275	277	274

Conditions: scanned by a UV-Vis spectrophotometer at 900–200 nm.

The fingerprint developed by HPLC was composed of the 11 common peaks eluted in 45 min. R.S.D. values of retention times of the corresponding peaks in HPLC analysis were very small (maximum 0.66% and average 0.10%) as shown in Table 5. HPLC showed great advantages in precision and running time over semi-preparative HSCCC. Total peak area of non-common peak was less than 10%, which met the standards.

There were 12 peaks in HSCCC separation and 11 peaks in HPLC analysis due to some differences between two systems. There is solid matrix in HPLC column, which possibly lead to adsorption of some constituents. Therefore, it was hard to find correlations between peak areas in HSCCC and in HPLC. Fingerprints of *Salvia miltiorrhiza Bunge* could be developed by either HSCCC or HPLC. HSCCC and HPLC can express different parts of chemical information of *S. miltiorrhiza Bunge*.

Table 4
Retention times of peaks in HSCCC separation

Peak no.	Retention time (min)			R.S.D. of retention time (%)
	Hebei	Shandong	Jiangsu	
1	78	80	76	2.6
2	89	93	94	2.9
3	102	105	108	1.2
4	116	121	121	2.4
5	137	145	141	2.8
6	159	168	165	2.8
7	190	199	192	2.4
8	263	273	260	2.6
9	312	326	315	2.3
10	400	417	398	2.6
11	578	610	596	2.7
12	688	723	701	2.5

Conditions: (column) multilayer coil of 2.6 mm i.d. tube with a total capacity of 300 ml; rotary speed: 900 rpm; stationary phase: the upper phase of solvent system A; mobile phase: 0–470 min, the lower phase of solvent system A and after 470 min, the lower phase of solvent system B; flow-rate: 2 ml min^{-1}; detection at 280 nm; sample size: 100 mg; retention of stationary phase: 78.8%.

3.3. Comparison of HSCCC and HPLC on fingerprinting of Chinese traditional medicine

Commonness and difference of the two methods were shown in Table 6. Generally speaking, HPLC had some advantages in precision and running time, and HSCCC had some advances in scales of crude samples and non-common peak area.

Although the precision of semi-preparative HSCCC is lower than analytical HPLC, R.S.D. values of retention times of HSCCC are less than 3%, which satisfying the national standards. Isolation and fingerprint development can be completed simultaneously by semi-preparative HSCCC. Moreover, large amounts of yields will benefit the possible studies of structure/bioactivity following HSCCC separation. Of course, analytical HSCCC with 50 ml capacity

Table 5
Retention times of peaks in HPLC analysis

Peak no.	Retention time (min)				R.S.D. of retention time (%)
	Hebei	Shandong	Jiangsu	Average	
1	27.59	27.56	27.58	27.58	0.06
2	27.91	27.87	27.91	27.90	0.08
3	28.30	28.28	28.30	28.29	0.04
4	29.00	28.97	29.02	29.00	0.09
5	29.42	29.43	29.42	29.42	0.02
6	30.77	30.78	30.82	30.79	0.05
7	30.92	31.31	31.23	31.15	0.66
8	32.18	32.11	32.19	32.16	0.14
9	32.73	32.67	32.78	32.73	0.17
10	34.64	34.59	34.65	34.63	0.09
11	35.55	35.51	35.56	35.54	0.07

Conditions: (column) reversed-phase Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm); mobile phase: solvent A (0.1% aqueous TFA) and solvent B (0.1% TFA + acetonitrile) in the gradient mode; flow-rate: 1.0 ml min^{-1}; detection at 280 nm.

Table 6
Comparison of HSCCC and HPLC in the study of fingerprinting

	HSCCC	HPLC
Scale of apparatus	Semi-preparative	Analytical
Separation column	300 ml	150 mm × 4.6 mm i.d.
Scale of crude sample	mg–g	μg
Temperature	Not controlled	40 °C
Run time	13 h	45 min
R.S.D. of t_R (%)	1.2–2.9	0.02–0.66
Non-common peak area	0	<10%

Conditions of HSCCC: (column) multilayer coil of 2.6 mm i.d. tube with a total capacity of 300 ml; rotary speed: 900 rpm; stationary phase: the upper phase of solvent system A; mobile phase: 0–470 min, the lower phase of solvent system A and after 470 min, the lower phase of solvent system B; flow-rate: 2 ml min^{-1}; detection at 280 nm; sample size: 100 mg; retention of stationary phase: 78.8%. Conditions of HPLC: (column) reversed-phase Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm); mobile phase: solvent A (0.1% aqueous TFA) and solvent B (acetonitrile) in the gradient mode; flow-rate: 1.0 ml min^{-1}; detection at 280 nm.

would be much better to be applied in fingerprinting and be compared with analytical HPLC.

Acknowledgements

We thank for Drs. Huajie Wen and Lisa Madden for critically reviewing the manuscript.

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