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A method for the production and characterization of fractionated libraries from Chinese herbal formulas

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Abstract

High-throughput screening (HTS) for pharmaceutical leads requires sufficient number of samples with vast chemical diversity. In this paper, we proposed Chinese herbal formulas as an attractive source for HTS and introduced a strategy for the production of high-quality fractionated libraries. An offline two-dimension liquid chromatography protocol was developed to separate medium- and low-polar extract (MLPE) of Chinese herbal formulas, which implemented the production of semi-purified mixture libraries. HPLC coupled with diode-array detector (DAD) and mass spectrum (MS) analysis was performed to obtain MS and UV spectrum of library components. The detected components were characterized by retention, molecular weight and UV absorbance assisted by WiseProcessor, a customer-developed software to automatically process analytical data. Based on the current understanding in pathophysiology and pharmacology, multiple cell-based bioassays were performed to screening the library samples. Through validation and dereplication process, bioactive compounds could be identified rapidly. The combination of off-line two-dimension liquid chromatography separation, HPLC-DAD-MS analysis and computer-aided data processing is reliable and efficient for the utilization of Chinese herbal formulas as valuable sources for HTS. As a demonstration, a library sample set was generated from Qi-Xue-Bing-Zhi Formula, an efficient Chinese herbal formula to treat atherosclerosis. Several bioactive compound s were quickly identified from this library through the screening and dereplication process.
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Keywords: Chinese herbal formulas; Fractionated library; HPLC-DAD-MS; High-throughput screening; Medium- and low-polar extract

1. Introduction

The advent of high-throughput screening (HTS) has a profound impact on drug discovery today. This approach can be characterized by several characteristics: high-density screen assays, efficacy with low (nanogram to microgram) quantities of sample, cost- and time-efficiency and capability to screen large number of samples against multiple molecular targets [1,2]. However, significant challenge for the successful random screening is to provide sufficient number of samples with vast chemical diversity. Natural products offer structural diversity that is not rivaled by the combinatorial chemistry [3,4]. Thus, recent effort to meet the demand results in a variety of libraries generated from natural sources [5], such as pure compound libraries [6], pre-fractionated extract libraries [7,8] and semi-synthetic natural-product-based compound libraries [9].

Traditional Chinese Medicine (TCM) may also serve as an attractive source for HTS for pharmaceutical leads, which remains to be explored. Formulas feature largely in TCM remedies, which are prepared from multiple crude drugs before use by patients [10,11]. The therapeutic value and safety of many Chinese formulas have been demonstrated in clinical application for hundreds of years and numerous modern scientific investigations [12–15]. It has been estimated that there were nearly 100,000 multi-drug formulas recorded in Chinese medicine classics [16]. There are more than 12,000 crude drugs, which are mostly commonly used herbs [17]. Therefore, Chinese herbal formulas (CHF) offer huge structural diversity and various biological activities. Moreover, the preparing process of formula remedies enriches the bioactive constituents and reduces the toxic constituents presented in preparations, which may

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enhance the probability of hitting and reduce the risk of clinical failure.

However, so far no systematic method to utilize CHF for HTS of leads has been suggested, since the technical difficulties related to production and analysis of libraries generated from CHF remain to be overcome. The challenges in this area are twofold: first, how to rapidly generate high-quality libraries from these complex mixtures that fit HTS program; second, how to characterize the library components.

In the present work, we reported an offline two-dimension liquid chromatography protocol and related data handling method to generate and characterize semi-purified fractionated libraries from CHF for the screening of pharmaceutical leads. A schematic representation of the method was illustrated in Fig. 1. Now, this method had been used routinely in the authors' laboratory to support drug discovery programs.

We also describe here the process of sample preparation, analysis, data handling and screening, and illustrate it using Qi-Xue-Bing-Zhi Formula, a six-herb formula for treating atherosclerosis. A sample set was produced from the formula and characterized by HPLC coupled with diode-array detector and mass spectrum (HPLC-DAD-MS) analysis. Several bioactive compounds were quickly identified through multiple bioassays and dereplication process.

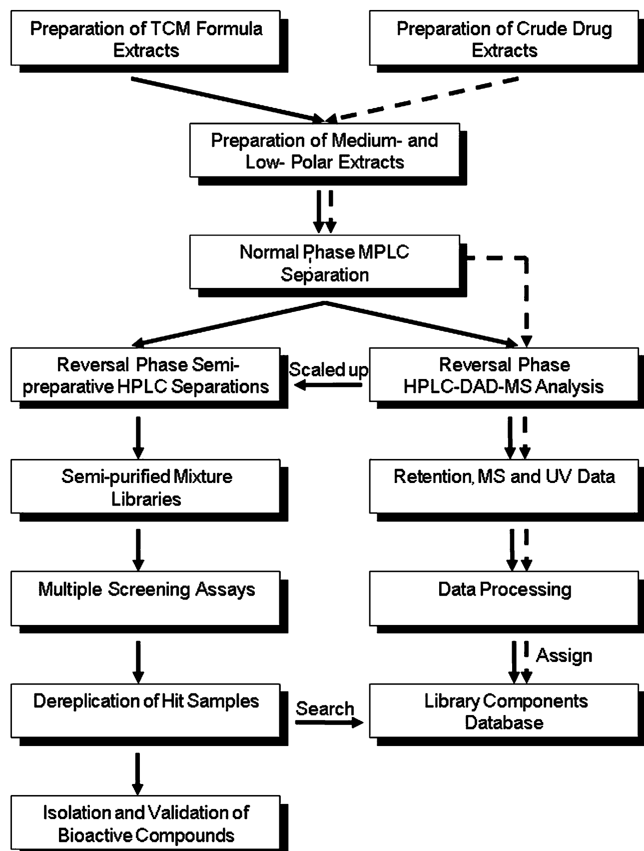

Fig. 1. Schematic representation of the method for the production and characterization of fractionated CHF libraries for HTS.

2. Experimental

2.1. Instrumentation

A medium pressure liquid chromatographic (MPLC) system combinating with a C-615 Pump Manager, double C-605 Pump Modules, C-660 Fraction Collector and a C-630 UV Monitor (Büchi Labortechnik AG, Postfach, Switzerland) and a C-690 glass column (1.5 mm × 200 mm) with 20-g silica gel were used for the normal-phase (the first dimension) separation. An 1100 preparative HPLC (Aglient Technologies, Palo Alto, CA, USA) was used for reversal-phase (the second dimension) separation. And the analysis was performed using an Agilent 1100 series HPLC-DAD-MS system (Aglient Technologies, Palo Alto, CA, USA).

2.2. Preparation of medium- and low-polar extract

In traditional Chinese remedies, crude drugs are proportionally mixed and decocted before oral administration by patients. Some preparations require that the crude drugs be added in a pre-determined order. Decoction of CHF was prepared in the traditional way. After filtration, the decoction was concentrated using a rotary evaporator below 60 °C in vacuum, and then brought onto a glass column (4.0 cm × 80 cm) wet-packed with D101 absorbent resins (water content: 65–70%, granularity: 0.25–0.84 mm, surface area: 500–550 m^2/g, pore diameter: 90–100 Å, density: 0.65–0.75 g/ml, purchased from Tianjing Resin Factory, Tianjing, China). The column was rinsed with three-column volume of water and subsequently 70% ethanol. Finally, three-column volume of 95% ethanol was applied as the final mobile phase to elute the compounds from the column. The neutral and long-chain lipids retained on the resins. The 95% ethanol effluent was collected as medium- and low-polar extract (MLPE) and dried by rotary evaporation below 60 °C in vacuum. The corresponding extracts of individual crude drug (each weight of 100 g) were prepared with the same procedure.

2.3. Medium pressure liquid chromatography (MPLC) separation

The normal-phase chromatography separation was performed on the MPLC system. The MLPE (500 mg) was dissolved in 10 ml methanol and absorbed onto 800 mg silica gel powder. The dried power was brought onto a 20 g silica gel column (1.5 mm × 200 mm). Firstly, the column was rinsed with three-column volume of hexanes to remove neutral lipids. Next, the MPLC separation was performed using a step-by-step gradient of (1) 60% hexanes, 40% EtOAc ($\varepsilon \approx 0.19$), (2) 40% hexanes, 60% EtOAc ($\varepsilon \approx 0.27$), (3) 20% hexanes, 80% EtOAc ($\varepsilon \approx 0.36$), (4) 100% EtOAc ($\varepsilon \approx 0.45$), (5) 50% EtOAc and 50% methanol ($\varepsilon \approx 0.59$), (6) 100% methanol ($\varepsilon \approx 0.72$). The flow rate was 10 ml/min. The collector was set to collect fractions of three-column volume of elute per gradient step (∼150 ml). Consequently, the resulting MPLC fractions were concentrated and dried in vacuum. The weight of MPLC fractions ranged from 35 to 80 mg.

2.4. Semi-preparative HPLC separation

Reversal-phase semi-preparative HPLC separation was performed on a Zorbax 300SB-C_{18} column (9.4 mm × 250 mm, 5 μm, Aglient) with Zorbax PrepHT guard cartridge columns (21.2 mm). Each 20 mg MPLC fraction was injected into the semi-preparative HPLC system. The mobile phase consisted of (A) 0.5% aqueous acetic acid and (B) methanol. The flow rate was 2 ml/min. The gradient started at 40% B and linearly increased to 90% B in 100 min. Every MPLC fraction was time separated into 8–10 sub-fractions automatically. The HPLC sub-fractions were dried in vacuum, and their weight ranged from 0.5 to 8.0 mg.

2.5. HPLC-DAD-MS analysis condition

The analysis was performed using a Zorbax StableBond C_{18} column (4.6 mm × 250 mm, 5 μm, Aglient) with a C_{18} guard column (Hanbon Science & Technology Co., Ltd., Jiangsu, China). To analyze the MPLC fractions of formulas and relative crude drug extracts, a universal analysis method was used. Briefly, samples were dissolved in methanol as 1 mg/ml solutions and centrifuged to remove particles before injection. The mobile phase consisted of (A) 0.5% aqueous acetic acid and (B) methanol using a linear gradient (from 40% B to 90% B in 100 min). The flow rate was 0.5 ml/min. The temperature of column was 30 °C. The UV spectrum was recorded from 190 to 400 nm. Two separate injections for each sample were performed in positive ion mode and negative ion mode, respectively, with electrospray ionization. The full scan mass spectrum was recorded over the range of *m/z* 100–1200. Temperature of drying gas was 350 °C with gas flow rate of 10.0 l/min and a nebulising pressure of 310 kPa. The value of fragmentor voltage was set at 120 V.

2.6. Data processing

To begin, AIA (Analytical Instrument Association standard format) files containing on-line MS signal data, retention time, peaks area, peaks height and so on were exported from Aglient LC/MS ChemStation (Rev.A.09.03) and transferred to a custom-developed program WiseProcessor. This program performed a denoizing and peak-detection algorithm, which was based on Windig's component detection algorithm [18], and then extracted mass spectrum for each peak. In general, one detected peak was considered as a component. Williams' algorithm [19] for automatic interpretation of mass spectra was briefly modified and employed. According to pre-determined criteria based on the numerical relationship among the quasi-molecular ions, adduct ions and dimmers, molecular weights of components were inferred. Subsequently, the results generated from positive ion mode and negative ion mode were combined, providing a summary report containing a systemic ID for each component, associated with retention time, molecular weight and supporting data. According to the retention time listed in the report, UV absorbance of each peak was extracted manually from ChemStation if available.

To assign components of library samples to crude drugs, a pair-wise algorithm was developed to search corresponding item in each summary reports of crude drug extracts. It contained a moving window that was centered over peak time point in the chromatogram of a library sample. Due to fluctuations of retention time, the moving window was allowed to have a width of ±1.5 min. In the window region, if one or more match molecular weight data (allowed to have a dispersion of 0.5) could be found in the peak list of crude drug extracts, this component was assigned.

3. Results

3.1. Library production

Since the low success rate of converting lead compounds into drugs often due to unfavorable absorption, distribution, metabolism and elimination (ADME) properties, there is now a clear trend to move away from huge and diverse 'random' libraries towards smaller and drug-like subsets [20]. Drug-like properties evaluation has often focused in profiling key physicochemical properties, such as molecular weight, charge and solubility. Some counting rule filters have been suggested, for instance Lipinski's "rule of five": limit range for $MW < 500$, computed octanol–water partition coefficient ($C \log P$) ≤ 5, hydrogen bond donors ($OHs + NHs$) ≤ 5 and acceptors ($Ns + Os$) ≤ 10 [21]. Accordingly, highly hydrophilic compounds, large molecular weight tannins, proteins, polysaccharides and long-chain lipids are considered to be non-drug-like molecules. This work aimed not only to discover bioactive components in CHF, but also to provide drug-like chemical entities for HTS.

Adsorption–desorption process on macroporous resin is an efficient, environment-friendly and robust method with moderate purification effect for the recovery and concentration of plant-derived extracts [22]. D101, a type of non-polar polystyrene absorbent resin is often used to prepare polar constituents, such as phenolic compounds [23,24], saponins [25], glycosides [26,27] from crude plant extracts. Generally, water is used as the initial mobile phase to remove large molecular weight tannins, proteins, polysaccharides; and then relatively low concentration (20–70%) of ethanol solution is used to obtain target constituents. We found that most of these polar constituents did not meet "rule of five". However, medium- and low-polar constituents, such as terpenoids, coumarins, lignans, lactones, aglycons of flavonoids and anthraquinones retained on the resins, which are readily eluted by 90–95% ethanol solution. Thus, D101 absorbent resin chromatography was employed to prepare MLPE of herbal formulas, which mainly consisted of small molecules with proper physicochemical proprieties.

On the other hand, cytotoxic effects, overlapping biochemical effects, non-specific binding and biological interaction of multiple components can interfere with bioassays in HTS program. These are often observed when complex mixture samples were screened. Hence, a requirement for the approach is that the number of components in each library sample should be limited. In the practice, multi-herb formula extracts are very complex

mixtures consisting of various phytochemical constituents, such as alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, lactones and so on. Therefore, the libraries production method should be efficient and robust to separate the complex mixtures and yield a large number of library samples consisted of a small number of compounds per sample. We, therefore, developed an offline two-dimensional liquid chromatography protocol for the high-resolution separation and fractionation of MLPE. A normal phase MPLC system was employed for the first dimension chromatographic separation and yielded six fractions. To minimize the compound duplicates in adjacent fractions, a step-by-step gradient elution protocol was adopted.

Subsequently, the second preparative chromatography separation was performed on a semi-preparative reversal-phase HPLC system by which each MPLC fraction was time fractionated yielding 8–10 sub-fractions. Generally, a total of 40–60 sub-fraction set were made from MLPE of a formula, which consisted of 1–6 main components per sample and primarily from 0.5–8.0 mg material. Fig. 3 showed a typical series of chromatograms of sub-fractions obtained from one semi-preparative HPLC run. Taken advantages of automatization and reproducibility of HPLC and MPLC instruments, this approach was efficient and capable to produce fractions in a small number of compound mixtures format and meet the quantity demand of HTS program, which were collectively called "semi-purified fractionated CHF library".

3.2. Library analysis

To determine the components of library samples, profile analysis of MPLC fractions was performed. We employed a reversal-phase HPLC coupled with diode-array detection (DAD) and electrospray ionization mass spectrometry (ESI-MS) detection. This separation used a shallow linear gradient, which possessed the advantage of great resolution of the eluting components. Since the two-dimension chromatographic separation provided higher resolution, the number of overlapping peaks was significantly reduced and clear spectrum of most detectable peaks can be obtained. A clear spectrum provided interpretable data for molecular weight assignment or structure analysis. Generally, 20–40 components were distinguished in a chromatogram. Therefore, there is no need to develop special analytical methods for every library sample and analyze them one by one. Moreover, the universal analytic method permits continuous and nearly unattended operation of instruments equipped with automatic sampler.

3.3. Data processing

A computer-based solution for chromatographic data processing is necessary when profile analysis for a large number of samples was performed. We developed a software application (WiseProcessor), which integrated chromatographic signal preprocessing, peak detection, molecular weight inferring and component assignment. A flowchart overview of data processing for the characterization of library components was illustrated in Fig. 2.

The profiling analysis for complex mixtures using HPLC-MS, particularly using electrospray as an ionization method, always results in chromatograms with a high level of background and noise. In order to reduce noise in HPLC-MS data and facilitate peak detection, Windig's component detection algorithm (CODA) was adopted [18]. In substance, CODA is a variable selection procedure. It calculates a "similarity index" between each original total ion current chromatogram and generates smoothed and mean-subtracted version, thereby providing a chromatogram with low noise and low background.

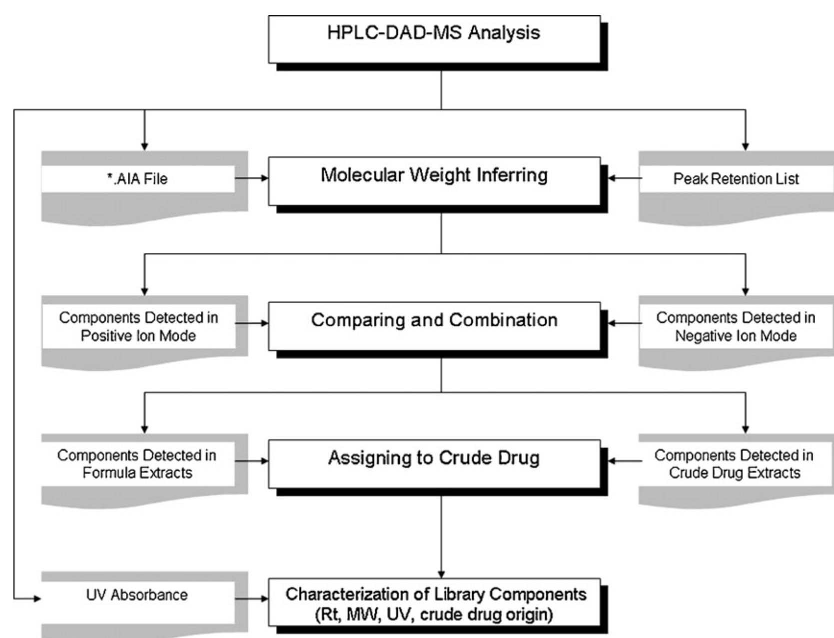

Fig. 2. A flowchart overview of data processing for the characterization of library components based on HPLC-DAD-MS analysis.

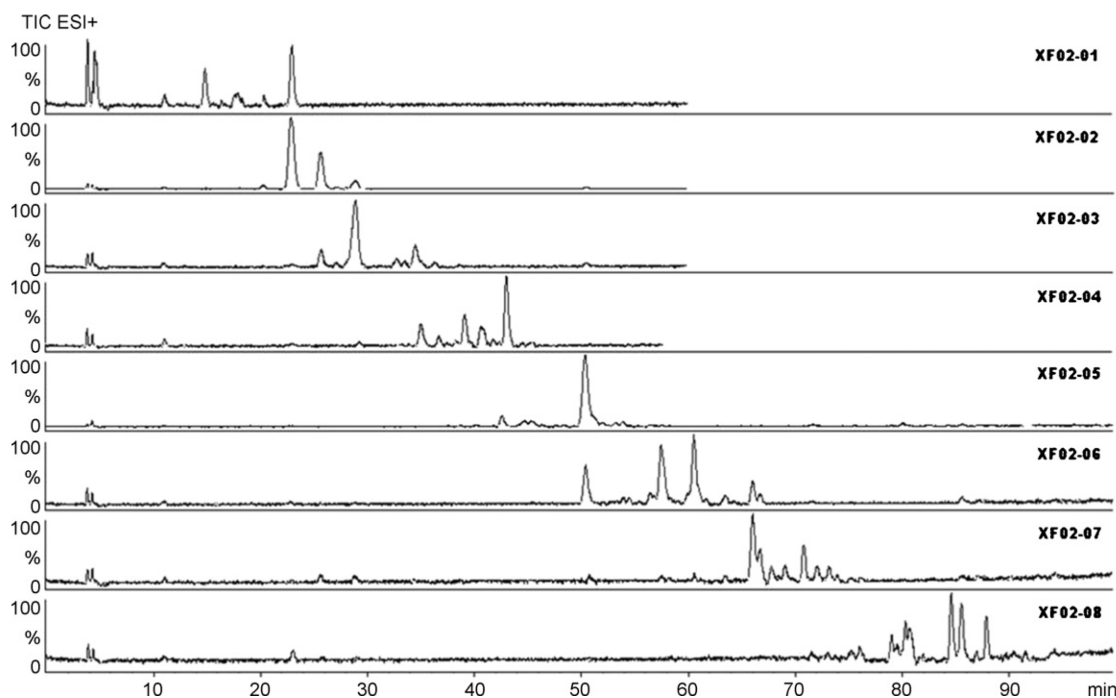

Fig. 3. An example showing that HPLC-MS (TIC in positive ion mode) chromatograms of eight fractionated CHF library samples (XF02-01—XF02-08) generated from an MPLC fraction. MLPE of Qi-Xue-Bing-Zhi Formula was separated using a normal phase MPLC system, yielding six fractions. And then each fraction was fractionated via preparative scale reversal-phase HPLC yielding eight sub-fractions. In most cases, each sub-fraction contained no more than six major components.

HPLC-MS with electrospray ionization is a powerful approach for obtaining molecular weight information for unknown compounds [28]. Using a generic chromatography and ionization condition, to interpret spectrum and infer molecular weight automatically is possible. William has developed a software application that facilitates molecular weight assignment based on a preliminary set of interpretive rules [19]. Since the chromatography and ionization conditions were different with that of William's report, the interpretive rules needed to be modified in the present work. Moreover, to determine various types of compounds and to enhance the reliability of the processing, we also developed two sets of interpretive rules for the data from positive and negative ion mode, respectively. The program compared the results from the two modes, establishing a uniform report containing retention time, molecular weight and supporting data. With the described chromatographic condition, the unambiguous determination of the molecular weight for detectable components was possible in most of the instances (>80%). Accordingly, molecular weight distribution of CHF library components can be illustrated by a histogram (as shown in Fig. 4), which indicated that compounds with molecular weight below 500 Da were in majority (>70%). In this way, certain components could be characterized using retention time and molecular weight. Moreover, UV absorbance data of some components were extracted and provided additional structure information.

It was the first step to assign library components to crude drug for dereplication. A pair-wise searching algorithm was designed, which used a moving window to overcome the retention time shifts. Generally, about 80% library components could be assigned to crude drugs based on retention time and molecular weight. In the course of assignment, some compounds appeared in multiple crude drugs and some were only observed in one. The former were designated as ubiquitous compounds, the latter diagnostic. For the diagnostic compounds, molecular weight and UV absorbance were used as search queries in searches against commercially available databases (such as Dictionary of Natural Products) and in house database, a number of compounds could be tentatively identified.

Therefore, most components of a semi-purified fractionated CHF library could be characterized by retention time, molecular weight, UV absorbance and crude drug origin. In addition, some of them could be tentatively identified.

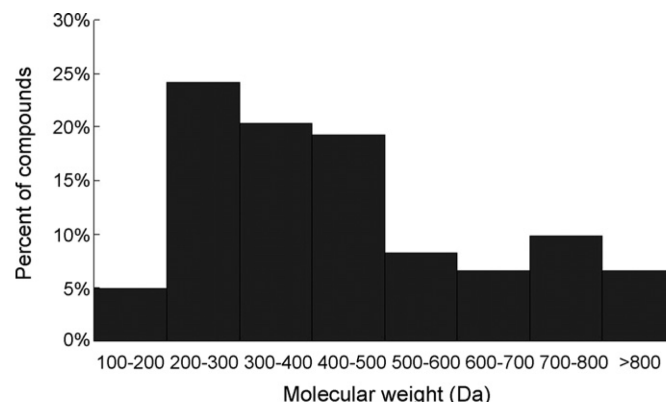

Fig. 4. Distribution of the molecular weights of detected compounds in the sample set generated from Qi-Xue-Bing-Zhi Formula. Molecular weight of 162 detected components in the sample set had been assigned according to the HPLC-MS analytical data. Among them, about 70% had molecular weight below 500 Da.

3.4. Screening and dereplication

Next, semi-purified fractionated CHF library samples underwent HTS using various bioassays, which were relevant to its therapeutic effect. The components of "hit" samples were analyzed using the generic HPLC-DAD-MS method. Since retention time, molecular weight, UV absorbance and crude drug origin of the major components in "hit" samples were known, the isolation of pure compounds from plant materials directly became a convenient process in most cases. Thus, compounds of active samples were isolated from individual crude drug. Occasionally, some pure samples of tentatively identified compounds gathered from commercial channels. Their bioactivities were validated in secondary bioassays.

Since a large number of natural-derived compounds had already been discovered and reported in previous literatures, it is time and cost-saving to perform a "dereplication" process for elucidation of their chemical structures [29]. Generally, tentatively identification could be achieved by the comparison of molecular weight, UV absorbance with literature data. Further, the chemical structures were confirmed by spectroscopic analysis or by comparison with the authentic standards. A schematic overview of the procedure was presented in Fig. 5.

As a case study, a semi-purified fractionated library consisting of 48 samples was generated from Qi-Xue-Bing-Zhi Formula, an efficient herbal formula for treating atherosclerosis. HPLC-DAD-MS analysis was performed to obtain chemical information of the library. More than 170 components in the sample set were detected by mass spectrum detector (MSD), in which 108 were observed significant UV absorbance simultaneously. 76.4% of detectable components were assigned to crude drugs, in which 54.1% were ubiquitous compounds. Based on molecular weight, UV absorbance, 36 diagnostic compounds were the tentatively identified (Table 1).

Based on the considerations of molecular mechanisms that contribute to the pathological process in atherosclerosis, we screened the library samples using cell-based multiple bioassays (as described in supporting information) for components that activated estrogen receptors (ERs), peroxisome proliferator-activated receptors (PPARs) or inhibited nuclear factor κB (NF-κB) activation and so on, which were promising therapeutic targets for the treatment of atherosclerosis. The primary screening was performed in 96-well plate format assisted by a Biomek1000 Laboratory Automation Workstation (Beckman Coulter). As a result, several sub-fractions were found to exhibit significant bioactivities, and therefore, were further analyzed using the described HPLC-DAD-MS method. Through dereplication and validation processes, a number of bioactive compounds had been identified (shown in Table 2).

4. Discussion and conclusion

In general, large-scale random libraries have yielded only a quality lead molecules. For receptor and enzyme targets, on an average one lead compound is identified for 120,000 compounds screened [20]. A typical semi-purified fraction library originated from 176 plant families and 561 genera has been reported whose hit rates are 0.5% or less [8]. In the present study, we introduce CHF as valuable sources for HTS of pharmaceutical leads. We introduced a strategy for the production of high-quality fractionated CHF libraries with the feature of superior hit-rates in the screening program. This approach produces biased library

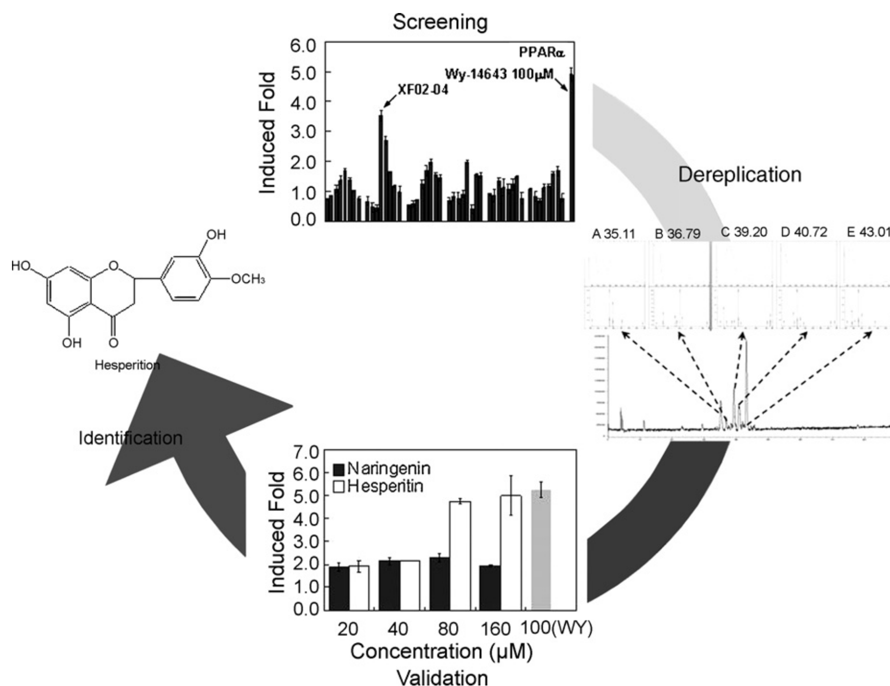

Fig. 5. The program to screen, dereplicate and validate a bioactive compound from semi-purified fractionated CHF libraries. In the 48 samples of Qi-Xue-Bing-Zhi Formula, XF02-04 was observed to activate PPARα in the primary screening, and then was analyzed using HPLC-DAD-MS for dereplication. Accordingly, five major components had been determined and pure compounds were isolated from crude drugs. A flavonoid compound from *Citrus aurantium*, hesperetin possessed potency to activate PPARα in the secondary bioassay. The active compounds were identified by comparison with the authentic standards.

Table 1
Tentatively identified compounds in the library generated from Qi-Xue-Bing-Zhi Formula

ID	Chemical	Compound type	MW	λ_{max} (nm)	Crude drug
XF01-02c	Limone	Terpenoid	470	287	CA
XF01-03c	Auraptenol	Benzopyranoid	260	258,324	CA
XF01-04c	Citrusal	Benzopyranoid	260	256,323	CA
XF01-05c	(*Z*)-6,7-Epoxyligustilide	Benzofuranoid	206	276	LC
XF01-06c	Senkyunolide G	Benzofuranoid	208	284	LC
XF01-08c	Senkyunolide A	Benzofuranoid	192	281	LC
XF01-09c	3-butylphthalide	Benzofuranoid	190	230,276	LC
XF01-15c	Nobiletin	Flavonoid	402	248,270	CA
XF01-16c	*Z*-ligustilide	Benzofuranoid	190	280,328	LC
XF01-17c	Cnidilide	Benzofuranoid	194	234,315	LC
XF01-21c	Tangeritin	Flavonoid	372	272,323	CA
XF01-25c	Diligustilide	Benzofuranoid	380	282	LC
XF01-35c	Auraptene	Benzopyranoid	298	325	CA
XF02-02c	Ferulic acid	Simple aromatic	194	236,323	LC
XF02-04c	Senkyunolide I	Benzofuranoid	224	275	LC
XF02-05c	Senkyunolide H	Benzofuranoid	224	276	LC
XF02-11c	Naringenin	Flavonoid	272	286,328	CA
XF02-12c	Hesperetin	Flavonoid	302	282	CA
XF02-13c	Equisetan	Simple aromatic	222	316	LC
XF02-14c	Kaempferol	Flavonoid	286	265,365	CT
XF02-17c	Marmin	Benzopyranoid	332	325	CA
XF02-21c	2-Methoxy-safynol	Aliphatic	214	258	CT
XF02-22c	Dehydrosafynol	Aliphatic	198	256	CT
XF02-23c	Safynol	Aliphatic	200	254	CT
XF03-01c	Umbelliferone	Benzopyranoid	162	324	CA
XF03-02c	Senkyunolide L	Benzofuranoid	242	272	LC
XF03-08c	Benzoylpaeoniflorin	Terpenoid	584	234	PL
XF04-01c	Oxybenzoylpaeoniflorin	Terpenoid	600	227,260	PL
XF04-02c	Paeoniflorin	Terpenoid	480	231,274	PL
XF04-05c	Galloylpaeoniflorin	Terpenoid	632	232	PL
XF05-01c	Albiflorin	Terpenoid	480	231,274	PL
XF05-03c	Naringin	Flavonoid	580	283,328	CA
XF05-04c	Neohesperindin	Flavonoid	610	284,328	CA
XF05-09c	Poncirin	Flavonoid	594	284,326	CA
XF06-19c	Saikosaponin b_2	Terpenoid	780	252,262	BF
XF06-20c	$2'$-*O*-acetylsaikosaponin b_2	Terpenoid	822	252,262	BF
XF06-21c	Saikosaponin b_1	Terpenoid	780	250,260	BF

The abbreviation for crude drugs: PL vs. *Paeonia lactiflora*; LC vs. *Ligusticum chuanxiong*; CT vs. *Carthamus tinctorius*; CA vs. *Citrus aurantium*; SP vs. *Prunus persicae*; BF vs. *Bupleurum falcatum*.

for screening of bioactive compounds against putative molecular targets relevant to adaptive diseases, and focuses on MLPE, which presents proper physicochemical proprieties. Therefore, it ensures relatively high probability to detect bioactive entities.

Table 2
Screening assays and identified active compounds in the library generated from Qi-Xue-Bing-Zhi Formula

Screening assay	Active compound (minimal effective concentration)
Activate ERα	Naringenin (10 μM) and hesperetin (10 μM)
Activate ERβ	Naringenin (5 μM)
Activate PPARα	Hesperetin (80 μM)
Activate PPARγ	Naringenin (20 μM) and hesperetin (30 μM)
Inhibit NF-κB activation	Z-ligustilide (5 μM) and senkyunolide A (10 μM))
Inhibit superoxide anion production	Umbelliferone (25 μM)
Stimulate endothelium-derived NO	Hesperetin (10 μM)

In addition, the fractionated CHF library offers considerable structural diversity and may give structure-activity information, since bioactive compounds obtained through this program are often belonging to homologue families.

Because of the complexity of CHF extracts containing clusters of structurally similar compounds, separation with acceptable resolution requires combined application of multiple separation techniques. Multi-dimensional chromatography has been developed to improve the peak capacity and applied in planar chromatography [30], GC [31], HPLC [32] and proteomics research [33] successfully. In the present study, by combined use of normal phase MPLC and reversal-phase HPLC, we developed an effective separation method to produce and characterize semi-purified fractionated CHF libraries for HTS. The library samples generated by this process were in a semi-purified mixture format with a low number of duplicate compounds. The first dimension separation also reduces the overlapping of elute components in HPLC-DAD-MS analysis, thus clear MS and UV spectrum can be obtained. The approach presents significant feature

comparing with reported methods to generate natural-derived libraries [5–8] that the library production and analysis employ a generic chromatographic separation method. The advantages of this approach included: analysis of a large number of library components in a few injections, time-saving for method development, and facility to isolate pure compounds from crude drugs.

We described here how to screen bioactive compounds from fractionated CHF library and illustrated it using Qi-Xue-Bing-Zhi Formula, which was used for the treatment of atherosclerosis for a long time. Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries [34]. The relationship between atherosclerosis and the metabolic abnormalities in lipid has been well established. Over the past decade an extensive body of research has revealed that some families of nuclear receptors are promising therapeutic targets for the treatment of atherosclerosis and dyslipidemia [35]. Among them, PPARα, which is highly expressed in liver, kidney, heart and muscle, plays a prominent role in the control of lipid and lipoprotein metabolism. Many investigations have demonstrated that PPARα agonists induce synthesis of apoA-I and apoA-II, lower hepatic apoC-III production, increase lipoprotein lipase-mediated lipolysis, and stimulate uptake of fatty acids and their β-oxidation in liver [36]. PPARγ is predominantly expressed in adipose tissues and controls adipocyte differentiation and glucose metabolism. PPARγ agonists, thiazolidinediones (TZDs), improve insulin sensitivity, lower glucose lever and lower plasma triglyceride and free fatty acid [37]. Furthermore, recent reports also showed the differential inhibition of the macrophage foam-cell formation and cholesterol efflux promotion by PPARα and γ in vivo [38]. ER has long been considered to play an important role in cardiovascular protection, especially for postmenopausal women [39]. Particularly noteworthy facts with this respect include that estrogen is shown to lower LDL, suppress oxidation of lipoprotein and up-regulate endothelial nitric oxide production [40]. A number of plant-derived compounds have been demonstrated to possess protective activity at the risk of atherosclerosis and arterial degeneration through the effects on arterial walls and lipoprotein metabolism, especially among older women [41].

Moreover, a large number of investigations have established a fundamental role for inflammation in mediating all stages of atherosclerosis [42]. One of the key regulators of inflammation is the transcription factor NF-κB, which has been regarded as a proatherogenic factor because of its regulation of many of the proinflammatory genes linked to atherosclerosis. Activated NF-κB has been demonstrated in human atherosclerotic lesions, especially in smooth muscle cells, macrophages, and endothelial cells [43]. The inflammatory process also results in abnormalities in endothelial function [44], characterized by the overproduction of superoxide anion and decrease in production and/or biological activity of endothelium-derived nitric oxide (NO) [45]. Therefore, to inhibit NF-κB activation, suppress superoxide anion generation or improve endothelium-derived NO production is expected attenuation of atherosclerotic lesions and affording therapeutic benefits.

As a case study, we tested bioactivities of library samples generated from Qi-Xue-Bing-Zhi Formula against the mentioned targets. In those cell-based multiple bioassays, some library samples exhibited potency to induced PPAR/ER controlled reporter gene expression, inhibit NF-κB activation, suppress superoxide anion generation from endothelial cells or promote endothelium-derived NO release. Though more targets relevant to the pathological process needed to be considered, the present results adequately demonstrated that the library samples were suitable for cell-based screening assays in high-density format.

In conclusion, we believe that the present method for production and characterization of fractionated CHF libraries will improve the utilization of traditional Chinese medicine for modern drug discovery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2007.12.004.

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