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Review

Strategy for analysis and screening of bioactive compounds in traditional Chinese medicines

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Abstract

Traditional Chinese medicines (TCMs), due to their long time clinic test and reliable therapeutic efficacy, are attracting increased global attention served as excellent pools of bioactive compounds for the discovery of new drugs. However, hundreds or even thousands of components are usually contained in traditional Chinese medicines and only a few compounds are responsible for the pharmaceutical and/or toxic effects. The large numbers of other components in traditional Chinese medicines make the screening and analysis of the bioactive components extremely difficult. By the way, the combination effect of bioactive components on the pharmacological activity makes it very difficult to clear the therapeutic mechanism of TCMs. Therefore, some strategies have to design for screening of bioactive compounds in traditional Chinese medicines, which further leads to disclose the therapeutic mechanism of TCMs in molecular level. The review will summarize the present state of the art of screening strategy for active compounds in traditional Chinese medicines, and the chromatography methods for screening and analysis of bioactive compounds in traditional Chinese medicines will be emphasized.

Keywords: Reviews; Screening; Bioactive compounds

Contents

Abbreviations: AGP, α_1-acid glycoprotein; CE, capillary electrophoresis; ChEI, cholinesterase inhibitor; CMC, cell membrane chromatography; GABA, γ-aminobutyric acid receptors; GC/MS, gas chromatography/mass spectrometry; FAC, frontal affinity chromatography; FIA, flow injection analysis; HBV, Hepatitis B virus; Hce-8693, human cecum undifferentiated adenocarcinoma cell; HCV NS3-NS4A protease, Hepatitis C virus NS3-NS4A protease; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; HSA, human serum albumin; 5-HT, 5-hydroxytryptamine (Serotonin); 5-HT1A, 5-hydroxytryptamine (Serotonin) 1A; 5-HT2, 5-hydroxytryptamine (Serotonin) 2; IBMC, immobilized biomembrane chromatography; ILC, immobilized liposome chromatography; KBV200 cell, human oral epithelioma cell; LC/MS, liquid chromatography/mass spectrometry; MS, mass spectrometry; M-TMCA, methyl-3,4,5-trimethyoxycinamic acid; MTT method, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric method; NMDAR, *N*-methyl-D-aspartate receptor; NMDA, *N*-methy l-D-aspartate; PAA, human pulmonary adenoma; PC_3, human prostatic carcinoma; PEP, prolyl endopeptidase; TCMs, traditional Chinese medicines; TMCA, 3,4,5-trimethyoxycinamic acid; TSN, Tanshinone IIA; YiXinTongMei, a traditional Chinese medical recipe for treating coronary heart disease

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1. Introduction

Traditional Chinese medicines (TCMs) as we know it today has a long history dating back several thousands of years. The origin of TCMs was associated with the legendary testing of many herbs for their medicinal properties by the folk hero, Shen Nong [1]. His experience and work in these areas was eventually recorded in *Shen Nong Ben Cao Jing* (*The Herbal Classic of the Divine Plowman*) in about 2700 BC. Up until the first and second BC, the establishment of the Yin and Yang doctrine under the influence of Confucius helped to reinforce the use of herbal materials for relieving illnesses and the first major medical text *Huang Di Nei Jing* (*Yellow Thearchy's Inner Canon*) occurred in China. With the development of theory and clinical practice, China has accumulated a rich body of empirical knowledge of the use of medicinal plants for the treatment of various diseases. In the Ming Dynasty (1368–1644), a major medical literature *Ben Cao Gang Mu* (The Comprehensive Herbal Foundation) written by Li Shi-Zhen in 1590 discussed 1892 medicinal substances and contained over 1000 illustrations and over 10,000 medicinal formulas, which indicates the contemporary Chinese had grasped comprehensive pharmaceutical knowledge and clinical experience. So far, there have been 12,806 medical resources found in China, including 11,145 medicinal plants, 1581 medicinal animals and 80 medicinal minerals [2]. Furthermore, a total of 2375 products have been compiled in the Pharmacopoeia of People's Republic of China (2000 edition) [3]. Such ample Chinese natural medicinal resources provide valuable materials for the discovery and development of new drugs of natural origin. Moreover, a vast majority of active components have been discovered from these Chinese medical products for anticancer, anti-bacterial, anti-fungal, anti-viral, promoting the immunological function activity, such as camptothecin, taxol, vinblastine, vincristine, podophyllotoxin and colchicine that exhibit antineoplastic activity [4], tripterygium wilfordii multiglycoside, simomenine, total glucosides of astragalus showing anti-inflammatory activity [5], etc. More important, the clinic medicinal experience of more than 2000 years and integrated theory system for diagnosis and treatment open a shortcut for discovering new drugs from these natural products. In the past decades, a large number of effective analysis tools have been used for analyzing the constitutes of TCMs in order to control the quality and discover bioactive compounds, especially the chromatography methods provide impressive separation technique for complex mixture. Furthermore, the coupling of chromatography method and spectroscopy techniques, such as mass spectrometry, nuclear magnetic resonance spectroscopy, infrared spectroscopy, etc. can conveniently provide a number of structural information of the separated components. Thereby, we can simultaneously determine the structure of separated components or other information in one program. These techniques provide strong support for the study of TCMs. However, so far it is still very difficult to obtain specific bioactive compounds, even if from a well-document formulas for specific disease therapy. Because the amount of active compounds with a number of interfering ingredients is usually low, and their action mechanism is not clear yet. Therefore, some strategies have to design for screening of bioactive compounds in TCMs. This review will summarize the present state of the art of screening strategy for active compounds in TCMs, and the chromatography methods for screening and analysis of bioactive compounds in TCMs will be emphasized.

2. Pharmacological screening with animal models

The routine process for screening is to extract single ingredient or single distilled fraction from TCMs, determine its bioactivity by the classic pharmacological means. The whole animal model is the most classic pharmacological screening model, which is very important at the aspect of medicine evaluation because it can apparently responses the efficacy, side effect and toxicity of medicines in whole. Although this method is high cost and low efficient, at present it is still a primary way to drug discovery and evaluation.

2.1. Pharmacological screening in biofluids

In conventional pharmacological screening of TCMs, some components are directly extracted from single medicine or formulas by some separation means, and then pharmacological evaluation is carried out to determine the bioactivity of these components respectively. Although the method is very straightforward and objective, some deficiencies have been found in screening of TCMs: (1) generally, the Chinese medicines are taken orally and some components are changed by gastrointestinal or liver metabolism, therefore, the actual

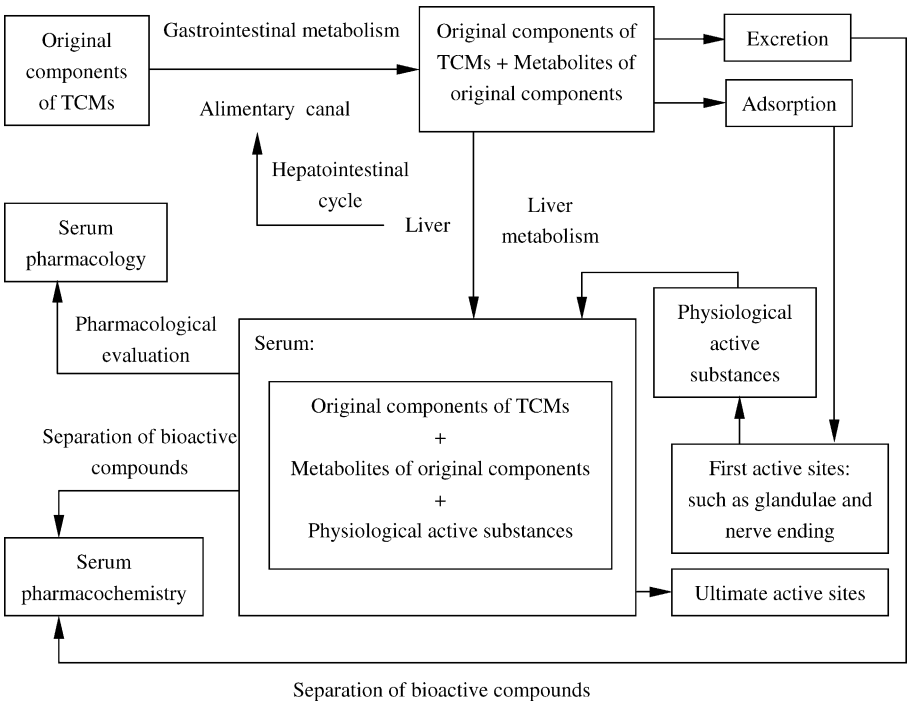

Fig. 1. Schematic procedures for serum pharmacology and serum pharmacochemistry.

active components are not always the components in original Chinese medicines. (2) The components of Chinese medicines is very complicated, many inactive compounds interfere the in vivo experiment results so that the authentic evaluation for efficacy is very difficult. In 1988 and 1992, Homma et al. [6] proposed serum pharmacological screening strategy based on hypothesis that the active compounds should appear in blood and urine with appropriate concentrations and urinary excretion rates after the administration of medicines which can be subdivided into serum pharmacology and serum pharmacochemistry. The schematic of method is illustrated in Fig. 1. The method of serum pharmacochemistry utilizes the physiology of animal to separate the bioactive components of TCMs into fluids of animal, and then these bioactive components in the fluids can be subsequently extracted and identified by common techniques. The method is very straightforward and helpful to know about the real active components in TCMs. Furthermore, it separates the possible bioactive components from numerous compositions in TCMs and makes the determination of bioactive components of TCMs more easily. In their research, a very popular Chinese medicine in Japan consisting of 10 different plant extracts, *Saiboku-To*, used for the treatment of bronchial asthma, was studied. β-D-Glucuronigase-treated urine samples collected before and after the administration of *Saiboku-To* to healthy and asthmatic subjects were analyzed by high performance liquid chromatography (HPLC) with a multichannel ultraviolet absorption detector. Three new peaks were found in the post-administrative urine that were eventually identified as magnolol, a major component in *Magnolia officinalis*, 8,9-dihydroxydihydromagnolol and liquiritigenin, metabolites from *M. officinalis* and *Glycyrrhiza glabra*, respectively, which were suggested as possible candidates for antiasthmatic agents in *Saiboku-To*. Futher studies of TCMs *Daisaiko-to* and *Shosaiku-to* were carried out with this screening approaches [7–9], and number of possible bioactive compounds were discovered.

In China, Wang [10] studied the active components of TCMs *Radix Polygalae*, *Artemisia Capillaris Thunb*., *Vaccinium Vitis-idaea L*. and *Rhizoma Atractylodis Macrocephalae* by serum pharmacological screening, and 3,4,5-trimethyoxycinamic acid (TMCA) and its metabolite of methyl-3,4,5-trimethyoxycinamic acid (M-TMCA) were identified as the actual bioactive components, Tenuifoliside A and C is only the pro-drug. Ding et al. [11] made a preliminary study on the TCM of *Radix Rehmannia Glutinosa* by the serum pharmacochemistry, and the aqueous macromolecular fraction was regarded as the active principle fraction of *Radix Rehmannia Glutinosa*. Yang et al. [12] also studied the *Lisgusticum chuanxiong Hort*. by a similar way, the ferulic acid that a generally considered active component in *Lisgusticum chuanxiong Hort*. was not the real bioactive compound for anti-release of 5-HT (Serotonin) from platelet and inhibiting Ca^{2+} transport toward rabbit blood endotheliocytus.

Han and Ling [13] and Zhang et al. [14] reviewed the screening of TCMs of anti-tumor activity and anti-hepatofibrosis activity by serum pharmacology, respectively. Three results can usually be found in serum pharmacology experiment: (1) the original medicines have the specific efficacy, but the serum collected after oral administration of the original medicines does not have the efficacy [15], which turns out that the conventional pharmacology experiment

responses unauthentic results at some time; (2) the original medicines does not have the specific efficacy, but the serum after oral administration have the efficacy [16], which indicates the real bioactive compounds from the transformation of some components in original medicines by absorption and metabolism in vivo; (3) both original medicines and the serum after oral administration have the efficacy [17], which indicates the bioactive compounds might exist in the original medicines.

2.2. Pharmacological screening with organ and tissue models

The tissues and organs model can response the effect of drug under the physiological condition and even under the pathological condition. It overcomes some deficiency of whole animal model: firstly, the amount of screening sample is cut down. As usual the amount of sample in the whole animal model need more than 1–5 g (according to the dosage and the size of used animal), whereas the amount of sample used in tissues and organs model is low as one tenth as the whole animal model or less. Secondly, the labour intensity is reduced and the scale of screening is enlarged. Multisample screening can be performed at the same time so that the efficiency is improved and the cost is reduced. Thirdly, it decreases the interference of other in vivo factors and is prone to obtain the authentic evaluation of pharmacology. Although the organ and tissue models have been widely used in drug screening, a few of examples can be seen that it is used in screening of TCMs.

Su et al. [18] carried out the preliminary screening of 12 TCMs for inhibitor of blood vessel growth by chicken embryo and it was found that the *Rhizoma Curcumae* and *Radix Curcumae* had the efficacy. Pharmacological screening of TCMs with organs and tissues was proposed by Liu and coworkers [19]. They analyzed the components distributed in rat organs of brain, stomach, liver and kidney etc. and serum and urine by HPLC, LC/MS and GC/MS after feeding the TCMs *Rhizoma Chuanxiong*, *Herba Ephedrae* and *Rhizoma Gastrodiae*. It was found that seven and four components entered the blood and brain in *Rhizoma Chuanxiong*, respectively. Vanillin, Senkyunolide H, Senkyunolide I and its isomer, ferulic acid and two undetermined compounds were detected in serum and Senkyunolide H, Senkyunolide I and its isomer, ferulic acid were detected in brain. In the study of *Rhizoma Gastrodia*, they observed that the distribution of Gastrodin and Gstrodigenin in the organs was liver > stomach > kidney and stomach > kidney > liver, respectively.

As we know, many the original components of TCMs usually put into effect after biologically transforming in vivo, namely, their metabolites result in the efficacy [20]. Recently, a method by in vitro metabolism with liver tissues for screening of TCMs was designed in our lab. In contrast to the method of in vivo metabolism by the whole animal model, it can be obtained the enough amount of sample by in vitro metabolism because the method can be scaled up easily only if we can provide enough liver tissues. Furthermore, we can readily control the process and good reproducibility can be obtained. A TCM of *Rhizoma Chuanxiong* was added into the rat live tissue homogenate for incubation of the period of time and the mixture was handled, the resulting sample was analyzed by HPLC [21]. As shown in Fig. 2, compared with chromatogram before metabolizing, some peaks disappeared and a new peak appeared after metabolizing. By HPLC, we can easily prepare the fraction of new peak so as to further identify its structure and evaluate its efficacy by pharmacological experiment.

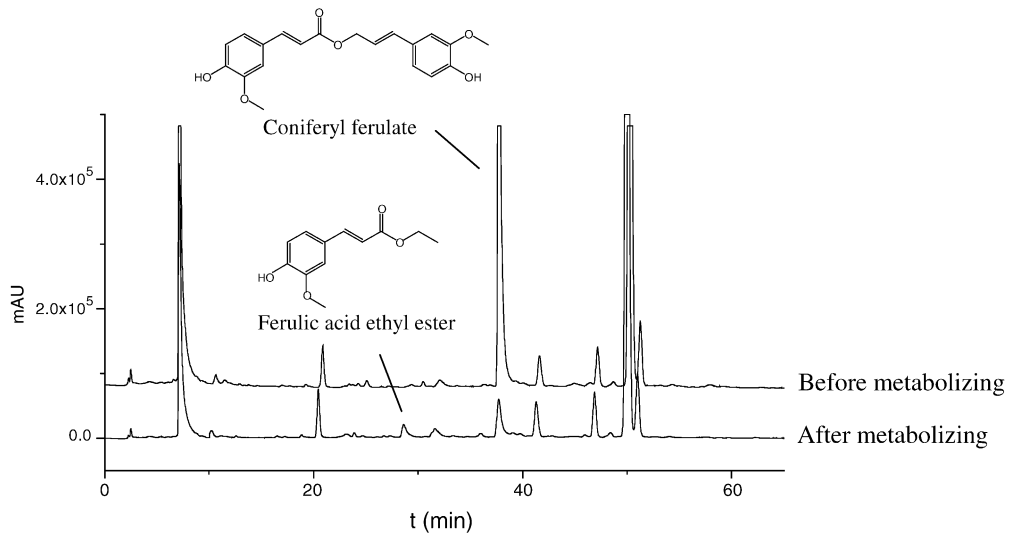

Fig. 2. Comparative chromatograms for ethanol extract of *Rhizoma Chuanxiong* before and after in vitro metabolism with rat liver tissue. Conditions: column, Kromasil C18 column (200 mm × 4.0 mm i.d.); flow rate, 0.8 ml/min; detection wavelength, 280 nm; mobile phase, 0–90 min, from acetonitrile/water (25/75, v/v) to acetonitrile/water (85/15, v/v) with linear gradient elution.

3. Screening methods with cellular models

In recent years the field of cell-based screening is expanding rapidly as innovations in target selection and instrumentation increase the number of targets that can be efficiently screened in cellular formats. Cell-based screens can be configured to provide a broad range of data on drug activity, mechanism of action and drugability. Compared with the whole animal models, the cellular models based on different disease and mechanism are more adaptive to large-scale drug screening because the resource and culture of cells are relatively economical and easy. It has become a very important means in screening and evaluation of TCMs. According to the selection of cell, it can be subdivided into normal cell models and pathological cell models.

Zhang et al. [22] screened the ethanolic extracts of 100 common traditional Chinese drugs, which are widely used in many prescriptions in treatment of cancer in China for multidrug resistance of KBV200 cell line in vitro with MTT method. The result showed 9 extracts having multidrug resistance reversal activity. They were the extracts of *Fructus Lagenariae Sicerariae*, *Radix Glycyrrhizae*, *Poria*, *Herba Andrographitis*, *Radix Sophorae Tonkinensis*, *Caulis Mahoniae*, *Folium Artemisiae Argyi*, *Rhizoma Curcumae*, *Fructus Cnidii*. Other five extracts showed cytotoxic on KBV200 cell line. Wei et al. [23] also screened 50 herbs usually used in Guangxi Province for their anti-tumor activity by MTT method and found that 29 herbs showed the anti-tumor activity to liver cancer cell. Lu et al. [24] studied the selective inhibition of a polysaccharide from *Acanthopanax giraldii Harms var. hispidus Hoo* (AGP) on human gastric cancer cells, human embryonic tendocytes and human lymphocytes, respectively, and it was observed that AGP exerted selective inhibition on the activity and function of human gastric cancer cells. Zhang et al. [25] studied on in vitro anti-tumor activity of total steroidal glycoside from the Root of *Cynanchum Auriculatum* by determining cell growth curve, MTT test, protein content assay and morphological observation. The results showed that it exhibited potent cytotoxic effect on all four solid tumor cell lines, Hce-8693, PC_3, Hela and PAA in a concentration-dependent manner. Except the well-known Taxol, Camptothecin and vincristine, β-elemene from *Rhizoma Curcumae*, matrine *from Radix Sophorae Flavescentis*, colchicines and so on exhibited the excellent anti-tumor or anti-cancer activity in clinic practice [26,27]. Xiong et al. [28] reported the screening of 27 TCMs for inhibiting the HIV by infected H_9 cell line and found 11 TCMs including *Radix Sophorae Flavescentis*, *Flos Lonicerae*, *Spica Prunellae*, etc. showed the activity. Mi et al. [29] carried out the antiviral study of 21 TCMs for screening the antiviral agents by means of 2.2.15 cell model. The extracts of *Phyllanthus urinarin* and *Polygonum cuspidatum* exhibit obvious effects on duck hepatitis B virus and human hepatitis B virus, while the extract of *Eclipta alba* showed limited inhibition on HBV DNA polymerase.

4. Screening based on the activity of receptors and enzymes

In essence, the production of biological activity (efficacy) of drugs is closely associated with the binding with enzymes, receptor, DNA, RNA, protein, etc. in vivo, which provides an important path to screen drugs in the molecular level. So far, about 600 drug targets have been found and identified and with the development of biotechnology, it can also be expected that more drug targets will be gradually disclosed in future. The receptor and enzyme models have become one of the fundament for modern high throughput screening of drugs. Compared with the above models, the receptor and enzyme models are rapid, economical, high sensitive and specific. The models remedy some deficiency in whole animal models and provide the possibility of functional screening. As for Chinese medicines, although their treatment theory is different from the western medicines, but the pharmacological effects of all active components in TCMs should also be based on the molecular mechanism.

Wang et al. [30,31] had firstly used the receptor and enzyme models for screening the TCMs. More than 400 extracts from 150 common TCMs were systematically screened with 11 receptors and enzymes as the binding partners. The bioactive compounds Alantolactone, Medicarpin, Ursolic acid, etc. were found. Gao et al. [32] established and optimized the receptor model based on human muscarinic 1 receptor (M1) for high throughput screening of 400 aqueous extracts of TCMs, and three extracts of TCMs were identified to have potential agonists for M1 receptor. Sun et al. [33] screened 22 TCMs for *N*-methyl-D-aspartate receptor (NMDAR) antagonist activity and abate NMDA-induced neurotoxicity in cultured cortical neurons with the patch-clamp technique. Aqueous extracts of *Scutellaria baicalensis*, *Stephania tetrandra*, and *Salvia miltiorrhiza* blocked currents induced by NMDA but none of these extracts blocked NMDA-induced neuronal death. These TCM drugs may exert therapeutic effects due to their Mg^{2+} content.

Zhu et al. [34] assessed a methanol extract of "Gouteng" (*Uncaria rhynchopylla*) hooks and stems for its ability to inhibit the binding of radioligands to 13 different receptors and the extract inhibited ligand-receptor binding by more than 60% to α_2-adrenoceptors, dopamine 1, 5-HT1A (Serotonin), opiate, $GABA_A$ and $GABA_B$ (γ-aminobutyric acid) receptors. Bioassay-guided fractionation resulted in the isolation of ursolic acid (muscarinic and sulphonylureas activities), husutine (α_2- and β-adrenoceptor, 5-HT1A and 5-HT2, opiate and sulphonylureas activities) and epiallocorynanthine (β-adrenoceptor, 5-HT1A and 5-HT2, and opiate receptor activities). In addition to receptors, some potential drug candidates can be straightly found from the inhibiting action to specific enzyme. Huperzine A, a selagine-type alkaloid was found to have powerful and reversible anticholinesterase activity and its inhibitory effect is three times as potent as that of physostigmine, a well-known cholinesterase inhibitor. Clinical trials indicated that huperzine A was a promising

candidate as a cholinesterase inhibitor (ChEI) in the treatment of Alzheimer's disease [35]. Xiong et al. [36] further synthesized some huperzine derivatives and found among them isovanihuperzine A (IVHA) deserves further study as a novel ChEI due to its potent AChE inhibition and its lower toxicity than huperzine A. Tang et al. [37] studied inhibitory activity of 35 TCMs, including extracts of traditional medicines or clinically useful recipes, herbs and isolated compounds against human immunodeficiency virus reverse transcriptase in vitro. Baicalin was found to be a noncompetitive inhibitor of HIV RTase, with an effective concentration (IC50) of 22 mM. el-Mekkawy et al. [38] isolated a new highly oxygenated triterpene named ganoderic acid alpha from a methanol extract of the fruiting bodies of *Ganoderma lucidum* together with 12 known compounds. Ganoderiol F and ganodermanontriol were found to be active as anti-HIV-1 agents with an inhibitory concentration of 7.8 mg/ml for both, and ganoderic acid B, ganoderiol B, ganoderic acid C1, 3beta-5alpha-dihydroxy-6beta-methoxyergosta-7, 22-diene, ganoderic acid alpha, ganoderic acid H and ganoderiol A were moderately active inhibitors against HIV-1 protease with a 50% inhibitory concentration of 0.17–0.23 mM.

Fan et al. [39] found that methanol extract from the underground part of *Rhodiola sacra S. H. Fu* shows significant inhibitory activity against prolyl endopeptidase (PEP) from Flavobacterium meningosepticum. Examination of the constituents of the extract resulted in the isolation of 19 known compounds. Among these, seven compounds protocatechuic acid, gallic acid, (−)-epigallocatechin 3-*O*-gallate, 3-*O*-galloylepigallocatechin-(4beta → 8)-epigallocatechin 3-*O*-gallate, sacranoside A, arbutin and 4-*O*-(beta-D-glucopyranosyl)-gallic acid showed the PEP inhibition. Luo et al. [40] immobilized polyclone antibodies of compound A synthesized by adding a linker spacer to the benzene ring of RD3-4078 (Fig. 3) that has high inhibitory activity to HCV NS3-NS4A protease (IC508.5 μg/mL) for mimicking HCV NS3-NS4A protease to screen affinity compounds from an extract of the traditional Chinese Herb *Phyllanthus urinaria L.* by frontal affinity chromatography (FAC) coupled with mass spectrometry. The compounds of brevifolin, brevifolin carboxylic acid, corilagin, ellagic acid, and phyllanthusiin U were observed to have high inhibitory activity.

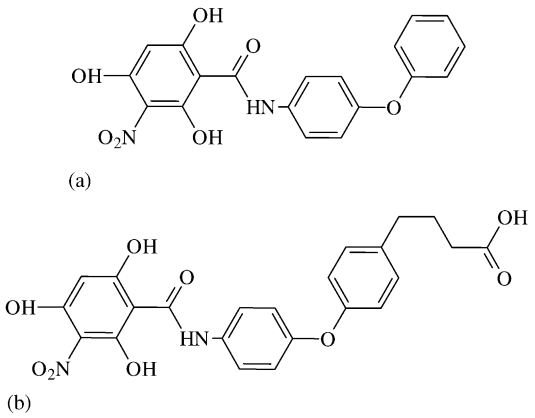

Fig. 3. Structures of RD3-4078 and compound A. (a) RD3-4078; (b) compound A.

5. Study of bioactive compounds in TCMs by chromatographic methods

As we know, the TCMs are complex mixtures containing up to hundreds or even thousands of different constituents, but only a few compounds are responsible for the pharmaceutical and/or toxic effects. The large numbers of other components in the TCMs make the screening and analysis of the bioactive components extremely difficult. Chromatography is one of main techniques applied in the field because of its powerful separation efficiency and sensitive detection. The rapid development in studies of TCMs is at least partly attributed to chromatographic method. Together with the experiment of pharmacology, both make the basic path of the research of bioactive components in TCMs. However, current chromatographic techniques are not still satisfactory because it is very difficult to distinguish the bioactive component peaks from the other numerous component peaks appeared in a chromatogram, which is a main bottleneck in the whole screening process. For the sake, some methods have been proposed in the recent several years.

5.1. Screening and analysis of bioactive compounds with immobilized plasma proteins biochromatography

Separation of TCMs by conventional chromatography such as gas chromatography (GC), reversed-phase high-performance liquid chromatography (RP-HPLC) and normal-phase (NP) HPLC is always based on the physicochemical interactions between the known biologically active compounds and the mobile phase and stationary phase. Therefore, there is no correlation between their retention and bioactivities. Affinity chromatography is based on the biological interactions between biologically active compounds and immobilized proteins, enzymes and antibodies. It has been successfully applied to rapidly probe drug–protein binding and to study anti-cooperative, non-cooperative and cooperative protein–ligand interactions [41–43]. Chaiken redefined the extension of affinity chromatography as molecular biochromatography that was described as "a means to use matrix-mobile interactant systems to study mechanisms of biomolecular interactions and therein to attain an understanding of such interactions which are often not easily achieved by solution method alone" [44]. As we know, most synthetic drugs bind, more or less, to plasma protein [45,46]. Albumin is the most abundant plasma protein and often accounts for the entire drug binding in plasma. Binding to albumin can have physiological significance in the transport, modulation and inactivation of metabolites and drug activities, as well as serve as a protective device in the binding and

inactivation of potential toxic compounds to which the body is exposed. The majority of drugs bind to serum albumin quantitatively. Since drug binding to albumin is readily reversible, the albumin–drug complex serves as a circulating drug reservoir that releases more drugs as the free drug is biotransformed or excreted. Albumin binding thus decreases the maximum intensity but increases the duration of action of many drugs. Therefore, it is valuable to study the possibility for screening and analysis of the biologically active components in TCMs by the molecular biochromatography through their biological interaction with immobilized human serum albumin (HSA).

Wang et al. [47,48] firstly introduced the novel strategy for screening and analysis of the biologically active components in *Angelica sinensis* (Oliv.) Diels with immobilized HSA on silica as the stationary phase. Ten major peaks were obtained from the methanol extract of *Angelica sinensis* with an optimized mobile phase of 50 mM phosphate buffer (pH 7.4)/acetonitrile (95/5, v/v) shown in Fig. 4a. Among them, two principal peaks identified as ferulic acid and ligustilide are the principal biological active components, which agrees very well with the results in the literatures. It was shown that molecular biochromatography is an effective way of analyzing and screening biologically active components in TCMs. Furthermore, a quality control method of TCMs that is very important in the identification and characterization of medicine materials and crude drugs was also developed based on the quantitative determination of ferulic acid and ligustilide in solutions of *Angelica sinensis* (Oliv.) Diels extracted with methanol and water. It was observed that the concentrations of ferulic acid and ligustilide in solution extracted with methanol were two and fifty-three times higher than those in solution extracted with water, respectively. In the study of the methanol extract from *Artemisia capillaris Thunb*. that has been used to cure hepatitis A for a long period of time, Wang et al. [49] obtained five major peaks and several minor peaks shown in Fig. 4b by molecular biochromatography with HSA as stationary phase, and two of them were identified as scoparone (SCO, 6,7-dimethoxycoumarin) and capillarisin (CAP). Moreover, Kong et al. [50] resolved the aqueous extracts from four kinds of TCMs on the HSA column and observed that four major active peaks appeared in chromatograms of *Angelica sinensis* (Oliv.) Diels and *Radix astragal*, respectively, three in *Rhizoma Chuanxiong* and two in *Paeonia lactiflora*. In addition to serum albumin, α_1-acid glycoprotein (AGP) is another important carrier, mainly binding basic drugs [51], and perhaps could be used as a complement to serum albumin for the ligand of the stationary phase in molecular biochromatography to screen and analyze the active components in TCMs. Wang et al. [52] studied the methanol extract from *Radix Salviae Miltiorrhizae* and its remedy of YiXinTongMei, which has been used to treat heart diseases, by molecular biochromatography with α_1-acid glycoprotein as the stationary phase and obtained more than ten peaks (Fig. 5). Tanshinone IIA (TSN) was ultimately identified as one of the principal bioactive components. Moreover,

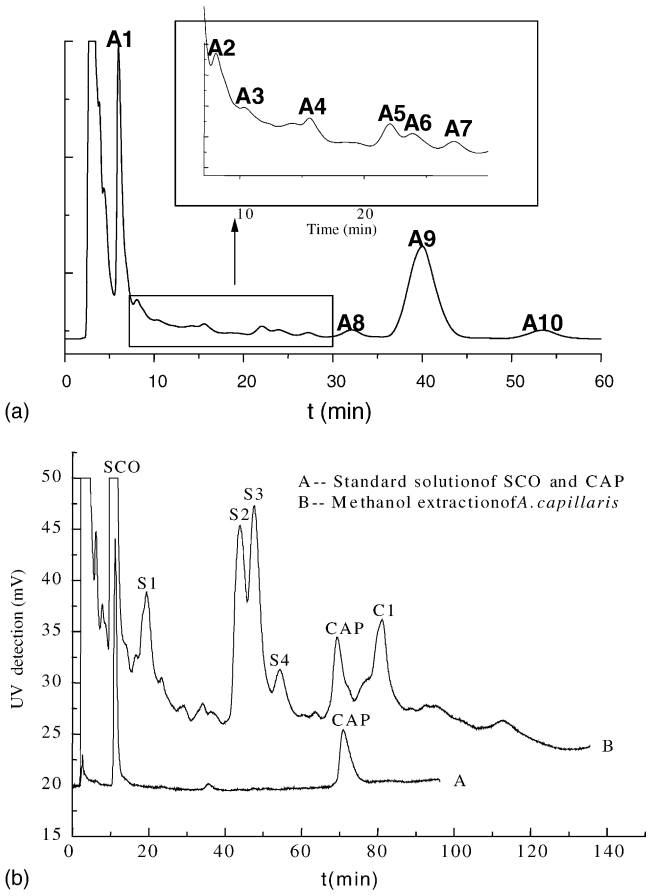

Fig. 4. Chromatograms for (a) methanol extract of *Angelica sinensis* and (b) *Artemisia capillaries Thunb*. on the immobilized human serum albumin stationary phase. Condition: column, 150 mm × 4.6 mm i.d. packed with human serum albumin immobilized on silica (7 μm); column temperature, 35 °C; flow-rate, 0.8 ml/min. Mobile phase: (a) 50 mM phosphate buffer (pH 7.4)/acetonitrile (95/5, v/v); (b) linear gradient elution from 10 mM phosphate buffer (pH 7.4) to 15% acetonitrile in 10 mM phosphate buffer (pH 7.4) in 90 min, and the latter eluent was run for another 60 min; UV detection wavelength, (a) 205 nm and (b) 238 nm. Peaks in chromatogram (a): (A1) ferulic acid; (A9) ligustilide. Peaks in chromatogram (b): (SCO) capillarisin, (CAP) scoparone.

Wang et al. [53] applied the same method to screen and analyze the biologically active components of *Rhizoma Chuanxiong*. Five major peaks and a number of small peaks were resolved based on their affinity for AGP and HSA, respectively, and three of them were identified as ferulic acid, chuanxiongzine and ligustilide regarded as effective components via standard compounds. Furthermore, Wang et al. [52] probed into the possibility for fast differentiation of the TCM sources by the comparison of the fingerprint of chromatograms for six typical TCMs on the AGP stationary phase. It was observed that different TCMs showed different fingerprint characteristics (Fig. 6) under the same operating conditions. Even for the same plant, *Rhizoma cimicifugae* from three different geographical sources, although there were common biologically active components were clearly observed (Fig. 7).

Application of affinity chromatography to studies of TCMs and natural products has significant advantages. First,

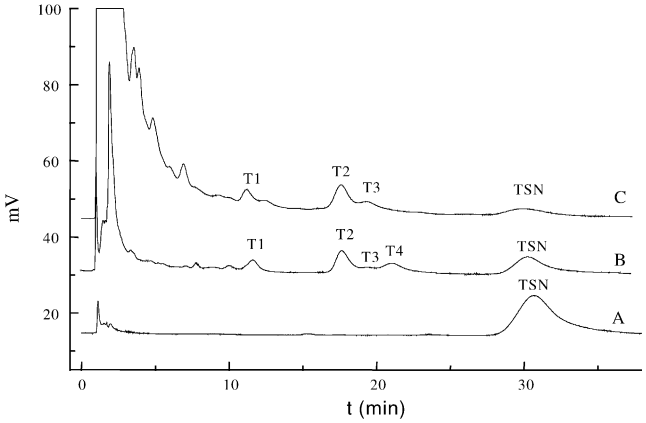

Fig. 5. Chromatogram for methanol extract of *Radix Salviae miltiorrhizae* on the immobilized α_1-acid glycoprotein stationary phase. Condition: column: 150 mm × 4.0 mm i.d. packed with α_1-acid glycoprotein immobilized on silica (5 μm); column temperature, 30 °C; flow-rate, 0.8 ml/min; mobile phase, 15% acetonitrile in 20 mM phosphate buffer (pH 6.0); UV detection wavelength, 268 nm. Samples: (A) standard sample of Tanshinone IIA (TSN); (B) *Radix Salviae miltiorrhizae*; (C) remedy of YiXinTongMai, a traditional Chinese medical recipe for treating coronary heart disease.

the interactions of biologically active compounds with proteins, enzymes and DNA can be probed, even if these compounds have not yet been identified. Second, the biologically active components and their biochemical change during the process of Chinese medicines can be rapidly evaluated and monitored. Third, the interactions occurring between biologically active compounds can be studied by adding some effective components screened from Chinese remedies or endogenous compounds to the mobile phase. Therefore, it might be expected that molecular biochromatography should play important role in disclosing the mystery of TCMs.

5.2. Screening and analysis of permeable compounds with immobilized biomembrane chromatography

The permeability of drugs across cell membrane plays an important role in influencing the bioactivity expression of drugs because a vast majority of drugs just exhibit their bioactivity after they go into the cell. Therefore, it may become a quick and reliable way to identify the bioactivity of drugs by investigating drug–biological membrane interactions. Reversed-phase high performance liquid chromatography (RP-HPLC) can be a tool to model solute–membrane partitioning, but it only primarily model the hydrophobicity interaction between solutes and membrane whereas the interactions between solutes and the polar lipid head groups of membrane are not well included, which is also believed to be important for the partition of drugs to biological membranes. A novel chromatography method, immobilized biomembrane chromatography (IBMC), has also been developed recently as a powerful model to study drug–membrane interactions in vitro [54,55]. It can be subdivided into cell membrane chromatography (CMC) and immobilized liposome chromatography (ILC).

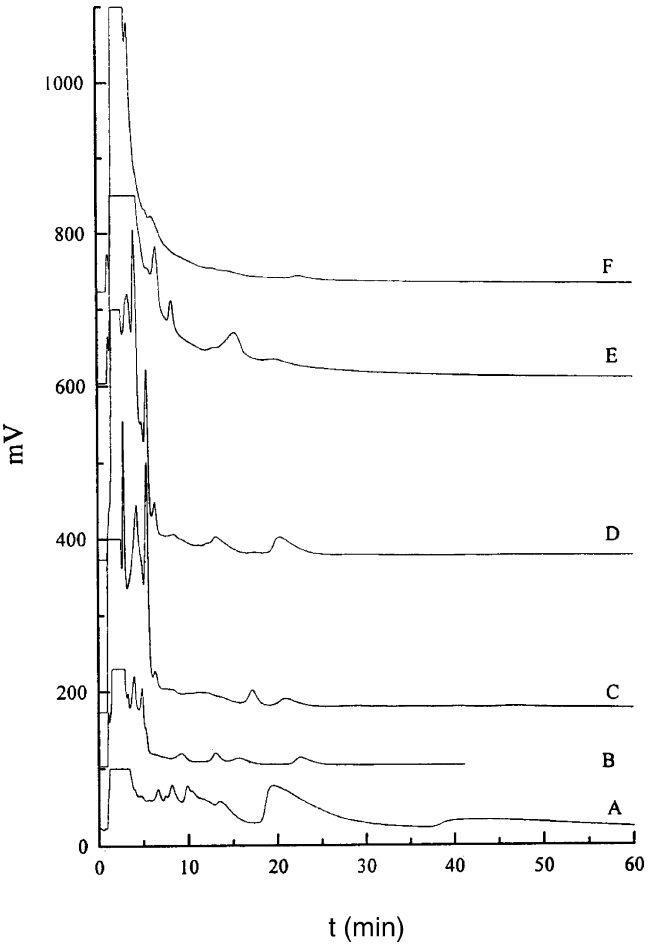

Fig. 6. Chromatograms patterns for methanol extracts of six TCMs on the immobilized α_1-acid glycoprotein stationary phase. Condition: column: 150 mm × 4.0 mm i.d. packed with α_1-acid glycoprotein immobilized on silica (5 μm); column temperature, 30 °C; flow-rate, 0.8 ml/min; mobile phase, 20 mmol/L phosphate buffer (pH 7.0)/acetonitrile (85/15, v/v); UV detection wavelength, 205 nm. Samples: (A) *Radix Polygalae*; (B) *Radix Astragali*; (C) *Radix Angelicae sinensis*; (D) *Rhizoma Chuangxiong*; (E) *Radix et Rhizoma Rhei* and (F) *Radix Paeoniae alba*.

Liposome formed by phosphatidylcholine is the main components found in cell membrane. It can well mimic the lipid bilayer structure and the fluidity of biological membranes. So immobilized liposome chromatography can be used as a mimic cell membrane system for probing the penetration ability of drugs through biological membranes. Mao et al. [56] firstly introduced the technique for screening and analyzing permeable compounds in traditional Chinese medicines (TCMs). More than ten peaks were resolved from the methanol extract from *Radix Angelica sinensis* based on their interactions with coated liposome stationary phase shown in Fig. 8. Two of them, ligustilide and ferulic acid were identified from their MS spectrum and via standard samples, which are well documented as the primary bioactive components in *Radix Angelica sinensis*. It turned out the method can be a powerful tool for screening and analyzing the bioactive components in TCMs. However, soft gel beads such as sepharoses and sephadexes that were still the main

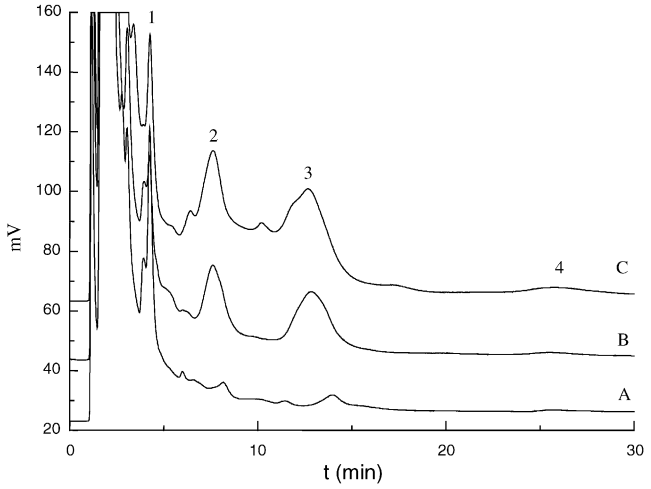

Fig. 7. Comparative chromatograms for methanol extracts of *Rhizoma Cimicifugae* from three different origins on the immobilized α_1-acid glycoprotein stationary phase. Condition: Column: 150 mm × 4.0 mm i.d. packed with α_1-acid glycoprotein immobilized on silica (5 μm); column temperature, 30 °C; flow-rate, 0.8 ml/min; mobile phase, 20 mmol/L phosphate buffer (pH 7.0)/acetonitrile (85/15, v/v); UV detection wavelength, 268 nm. Samples: (A) Shanxi Province in China; (B) Heilongjiang Province in China and (C) USA. Chromatographic peaks 1, 2, 3 and 4 are characteristic compounds for *Rhizoma Cimicifugae*.

supports of liposome immobilization in liquid chromatography research presently impeded further development of the technique because they all suffer from some drawbacks, such as large particle size, wide size distribution and poor mechanical strength that resulted in the low column efficiency and low separation speed. For effectively avoiding these drawbacks above, Mao et al. [57] proposed a covalent coupling method for preparing the covalently immobilized unilamellar liposome stationary phase on porous silica. Experimental results indicated that the stability of covalently coupled liposome column was obviously superior to that of coated

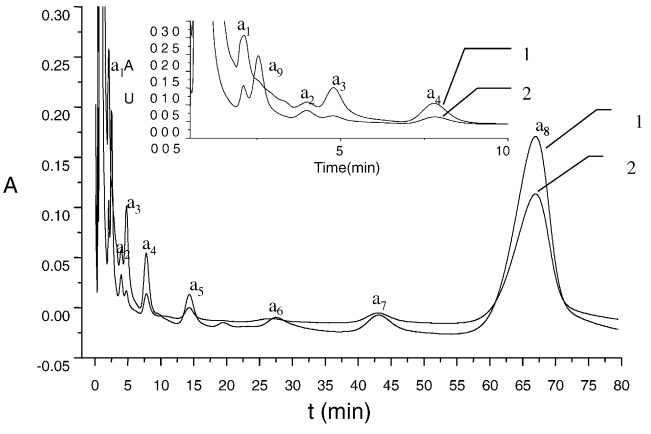

Fig. 8. Chromatograms of *Radix Angelica Sinensis* extract on the immobilized liposome chromatography. Conditions: mobile phase, 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl; flow rate, 1 mL/min; detection wavelength, 210 nm for chromatogram 1 and 280 nm for chromatogram 2. The inset shows an expansion of the first 10 min of the separation. Peak a8 is ligustilide.

liposome column but the selectivity of both columns was basically identical. Both columns were applied in the separation and analysis of a crude extract of traditional Chinese medicine, *Ligusticum Wallichii* and six main peaks can be distinguished in both chromatograms with well comparable retention. Better repeatability was achieved on the covalently coupled column than the coated liposome column after three continuous injections due to the facile loss of coated phosphatidylcholine on the coated liposome column.

The activity, toxicity, distribution, and other processes of drugs in human body always depend on their absorption, while intestine plays the most important role in the process of compound absorption. This is because the intestine epithelial cell membrane is the first and major barrier to drugs given orally, although most compounds must penetrate one or more cell membrane including target cell membrane to exhibit activity [58]. So the permeability of drugs across intestine has been considered as one of most important coefficients to evaluate their bioactivity. Because biomembrane chromatography with the immobilizing liposome as stationary phase could closely mimic the interaction of analytes with biological membranes including intestine epithelial cells, it can be used as a tool for prediction of drugs absorption in intestine [55]. Mao et al. [59] found the weighed retention value of drugs under three pH on the immobilized liposome chromatography existed good correlation with their cell absorption rate constants P_{app}. Therefore, biomembrane chromatography can be applied as a model in vitro to study absorption and distribution of active components from TCMs in human body.

Cell membrane chromatography is another important type of biomembrane chromatography with immobilized biological cell membranes on silica supports, which can apparently reflect the interaction between analytes and cell membrane or membrane receptors. He et al. [60,61] firstly introduced the technique for the study of TCMs. By changing the different cell membranes, the bioactive components of TCMs for specific targets can be screened. This technique has been applied to study the bioactive components of vasodilatation in *Angelica sinensis* [62], *Cladonia alpestris* [63], *Herba eplmedii* [64], *Semen cuscutae* [65] and *Leontice robustum* [66] by immobilized rabbit vascular cell membrane as stationary phase. It was found that the *n*-butanol extract of *Leontice robustum* (HMQ-4) showed the affinity to rabbit vascular cell membrane, whereas the ethyl ether, ethyl acetate and petroleum ether extracts did not show the affinity. Therefore, it can be preliminary confirmed that the *n*-butanol extract contained the bioactive components of vasodilatation. Subsequently, the *n*-butanol extract HMQ-4 was separated into five compositions and they were further tested by the immobilized rabbit vascular cell membrane chromatography. The compositions of HMQ-42 and HMQ-44 exhibited the affinity. Eventually, the HMQ-44 was determined as the dominated effective active component by in vitro pharmacological test although its structure was not still illustrated and the retention value of the component on the immobilized rabbit vascular

cell membrane chromatography was found to have a good correlation with its in vitro pharmacological activity. By the same method, they screened the bioactive components of vasodilatation: DG-21 from the ethyl ether extract of *Angelica sinensis*, YYH-214 and YYH-216 from the ethyl ether extract of the *Herba eplmedii*, T_{21} from the ethyl ether extract of *Semen cuscutae*, and TBH2-6 from the ethyl ether extract of *Cladonia alpestris*. In addition, they screened the bioactive component for inhibiting the cardiac muscle in *Cladonia alpestris* by the immobilized cardiac muscle cell membrane chromatography. In a word, the method proposes a simple and convenient way for studying the interaction of active components in TCMs with membrane in vitro and screening active components for specific targets on cell membrane.

5.3. 2-D HPLC for screening and analysis of bioactive compounds

Despite the impressive increase in the resolving power of conventional chromatographic columns, the number of compounds that can be separated into individual peaks in a single run (peak capacity) is limited by the peak width, which in turn depends on the efficiency of the column. Generally, peak capacity is proportional to the square root of the number of theoretical plates available for the separation. For example, a peak capacity of up to about 60 can be theoretically achieved on a 15-cm HPLC column with a plate count of 15,000. However, the theoretical calculation shows that less than 37% of the peak capacity can be used to generate peak resolution. This decreases the number of separated peaks to only 22, provided the selectivity of the column allows the separation of all of the components. When the selectivity is not sufficient for such a separation, some peaks can overlap and actually represent more than one compound. Overlap must also occur if the overall number of compounds in a sample exceeds the peak capacity of the column. Since the number of components in some complex biochemical or environmental samples may easily exceed the actual peak capacity of the available HPLC system. As mentioned above, the traditional Chinese medicines are very complex samples by themselves and generally contain up to hundreds or even thousands of different compounds. Therefore, the separation and analysis of components in TCMs become extremely difficult by the conventional chromatographic system. Multidimensional separation techniques provide dramatic improvements in peak capacity. In a two-dimensional (2-D) separation, the total capacity is equal to the product of the peak capacities in both dimensions, resulting in much higher resolution. So far, comprehensive two-dimensional liquid chromatography system as a typical form of multidimensional separation system has been widely used to characterize and separate biomolecules [67], polymers [68], and other complex mixtures [69] due to its high peak capacity, powerful separation and resolution ability since it appeared in 1990 [70]. However, the study of TCMs by the 2-D HPLC for screening and analysis of bioactive compounds is still very infrequent.

Our group [71] has built a comprehensive two-dimensional liquid chromatographic separation system based on the combination of a CN column and an ODS column for the separation of a traditional Chinese medicine *Rhizoma Chuanxiong*. Much more components of *Rhizoma Chuanxiong* can be easily separated and identified with the comprehensive two-dimensional liquid chromatography than traditional one-dimensional liquid chromatography. Moreover, the on-line mass spectrometry further reduced the chances of coeluting peaks passing undetected and obtained molecular weight information to accurately identify the chromatographic peaks. More than 52 components were separated in less than 215 min (Fig. 9) and 10 of these compounds were simultaneously determined by this comprehensive two-dimensional system without tedious pretreatment. The comprehensive two-dimensional liquid chromatography system herein shows its high peak capacity, sensitivity and powerful resolving potential in separation and identification of complex traditional Chinese medicines. Zhang et al. [72] conducted comprehensive two-dimensional separation by capillary liquid chromatography coupled with capillary micellar electrokinetic chromatography for analysis of traditional Chinese medicines, Liquorice and an ancient prescription, Cheng-Qi-Tang consisted of three traditional Chinese medicines *Dahuang* (*R. officinale Baill*), *Zhishi* (*Citrus aurantium L.*) and *Houpu* (*Magnolia officinalis Rehd. Et Wils.*), which exhibited impressive separation ability and peak capacity. More than 110 components were fairly well resolved in Liquorice and hundreds of compounds were present in the 2-D chromatogram of Cheng-Qi-Tang. The total peak capacity of the comprehensive 2-D system could be as high as 2000. Such powerful resolving potential of comprehensive 2-D chromatography system will be well expected in future study of traditional Chinese medicines. Moreover, a comprehensive two-dimensional liquid chromatographic separation system based on the combination of an immobilized HSA column and an ODS column has also been applied for the separation of active components in traditional Chinese medicine *Rhizoma Chuanxiong* with effective improvement of resolution, and a typical 2-D chromatogram is shown in Fig. 10. By the way, we can estimate the binding strength of components with the immobilized as well as their hydrophobicity simultaneously.

5.4. Microdialysis-HPLC techniques for evaluation of bioactive compounds with human serum

It is well known that a drug in blood is bound to a greater or lesser extent to plasma proteins such as albumin and α-acid glycoprotein and that the concentrations of bound and free species are in equilibrium. Studies on drug–protein binding are important in pharmacology and pharmacokinetics [45,46,73], because drug–protein interaction affects the pharmacological activities and side effects of the drug as well as the drug distribution and elimination. The unbound drug alone is supposed to diffuse from the blood to the

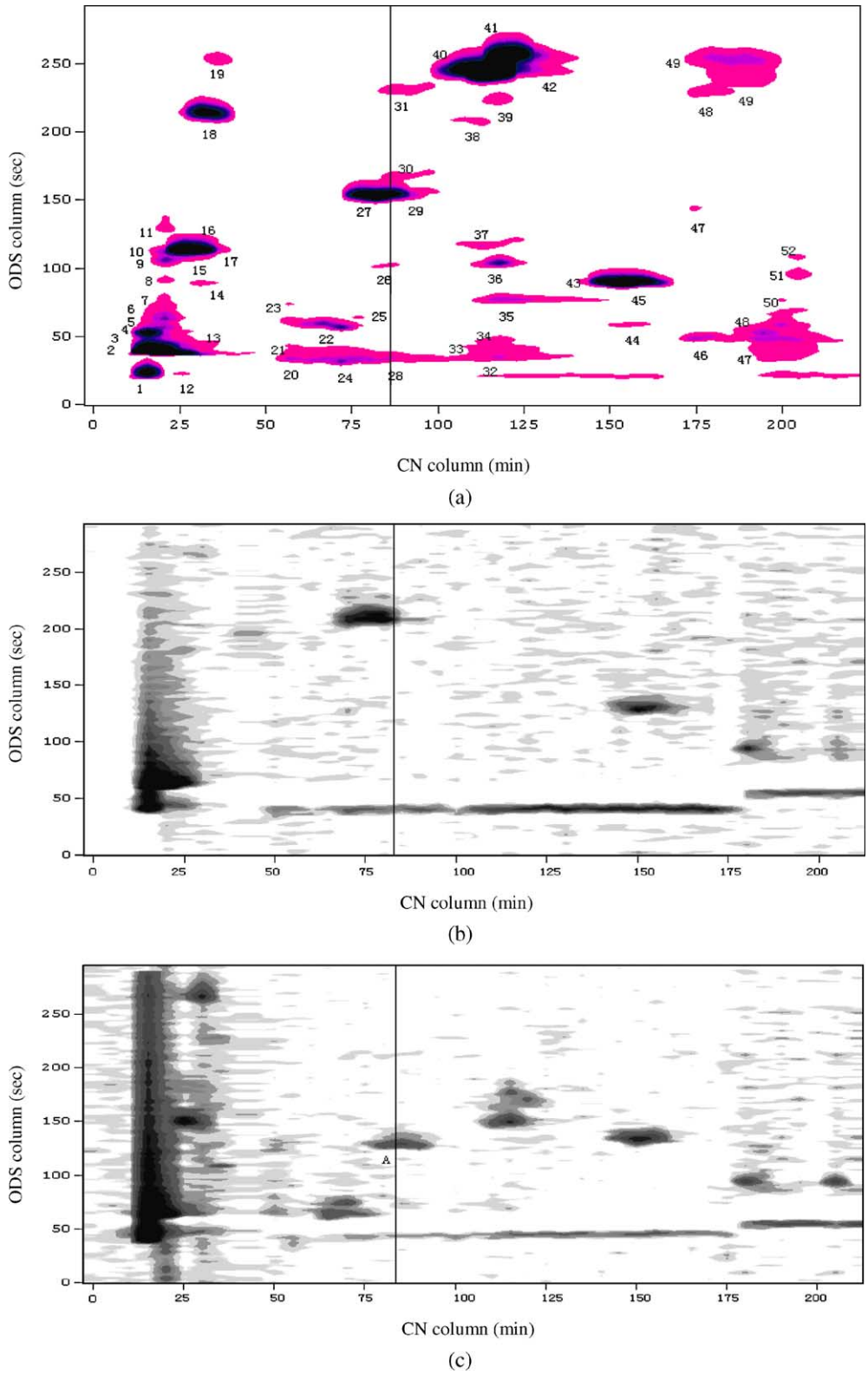

Fig. 9. Comprehensive two-dimensional chromatograms of *Rhizoma Chuanxiong*. Experimental conditions: CN column as the first dimensional sepration; flow rate, 0.04 ml/min; C18 column as the second dimensional separation; flow rate, 0.7 ml/min; detection wavelength, 250 nm. (a) The depths of the two-dimensional plot determined by the relative UV absorbance. (b) The depths of the two-dimensional plot determined by the counts per second using APCI positive ion mode. (c) The depths of the two-dimensional plot determined by the counts per second using APCI negative ion mode. Peaks: (4) protocatechuic acid; (6) caffeic acid; (15) ferulic acid; (16) vanillin; (18) senkyunolide I; (19) senkyunolide H; (27) 4,5-dihydro-3-butylphthalide; (30) 3-butylphthalide; (41) ligustilide; (42) 3-butylidenephthalide.

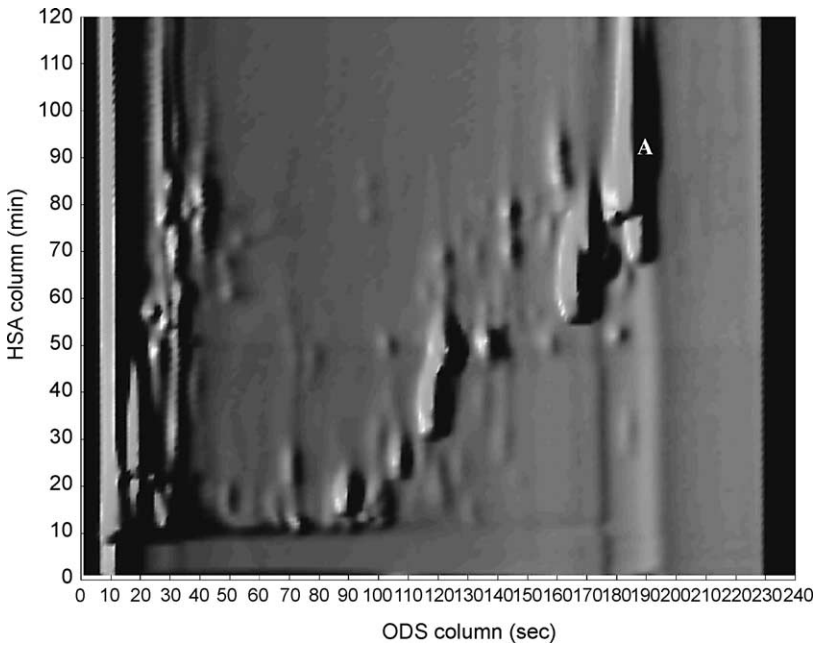

Fig. 10. Comprehensive two-dimensional liquid chromatogram for extract of *Rhizoma Chuanxiong* based on the combination of an immobilized HSA column and an ODS column. Conditions: column 1, immobilized HSA column (150 mm × 4.6 mm i.d.); flow rate, 0.049 ml/min; mobile phase, 0–40 min, 20 mM phosphate buffer (pH 6.0), 40–240 min, from 20 mM phosphate buffer (pH 6.0) to acetonitrile/20 mM phosphate buffer (pH 6.0) (17/83, v/v) with linear gradient elution and the latter eluent was ran for another 240 min. Column 2, Kromasil C18 Column (20 mm × 2 mm i.d.); flow rate, 2.0 ml/min; mobile phase, 0–210 s, from water to methanol/water (70/30, v/v) with linear gradient elution, 210–240 s, water. Detection wavelength, 220 nm; column temperature, 37 °C. Peak A is ligustilide.

extravascular active sites and to exhibit the pharmacological activity and/or the side effect. Various methods have been developed for determining the drug–protein, e.g. the immobilizing plasma protein biochromatography as mentioned above. However, unfortunately it can only embody the interaction of TCMs with one type of protein in plasma. Microdialysis has been extensively applied to monitor continuously the concentration of unbound drug and neurotransmitter in vivo [74–76]. Microdialysis sampling allows the determination of the concentrations of unbound drugs after a dialysis membrane has been placed in the drug–protein mixed solution. The technique is based on the kinetic dialysis principle in which substances diffuse down their concentration gradient. The microdialysis probe is usually a tubular membrane mounted on a double cannula made of fused silica and plastic. A perfusion solution is pumped at a low-rate (1–5 μl/min) through the inlet of the probe and collected at the outlet, yielding a sample ready for analysis. The dimensions of the probe, i.e. the membrane length, diameter, and molecular weight cut-off, can be varied according to the requirements of application. The method is time saving and even simpler than equilibrium dialysis. Microdialysis has also the advantage that the technique is easy to be automated and can be on-line hyphenated with many analytical techniques such as LC, capillary electrophoresis (CE), flow injection analysis (FIA), mass spectrometry (MS), etc. and compared with the above method of biochromatogrphy, it can determine the comprehensive results of TCMs interacting with multiple proteins of plasma in solution, which reflects the more actual status of TCMs in plasma. In our laboratory, the technique of microdialysis combined LC had been used to study the interactions between HSA and some western medicines such as carbamazepine [77], sulfamethoxazole[78] and fenoprofen [79]. The results were

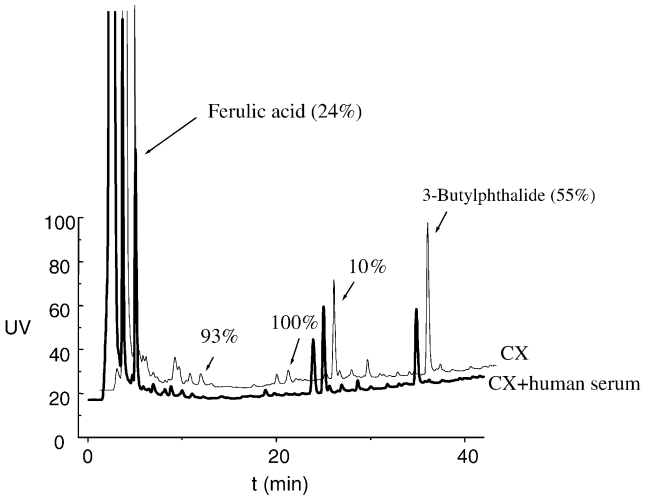

Fig. 11. Comparative chromatograms for microdialysis samples from the extract of *Rhizoma Chuanxiong* and mixed solution of *Rhizoma Chuanxiong* (CX) with plasma. Conditions: column, Kromasil C18 column (200 mm × 4.6 mm i.d.); flow rate, 0.8 ml/min; detection wavelength, 215 nm; mobile phase, 0–5 min, acetonitrile/water (15/85, v/v), 5–35 min, from acetonitrile/water (15/85, v/v) to acetonitrile/water (30/70, v/v) with linear gradient elution.

in agreement with the literatures where the high performance frontal analysis was used. It demonstrated the possibility of the method for further studying binding behavior of the active components in TCMs to proteins in plasma. This work is ongoing in our laboratory and some preliminary results have been achieved. The chromatograms of microdialysis samples from the extracted solution of *Rhizoma Chuanxiong* and the mixed solution of *Rhizoma Chuanxiong* with plasma are shown in Fig. 11. It can be apparently seen that some peaks exhibited the affinity to proteins in plasma and the recovery conveniently indicated their affinity capacity to proteins in plasma. Two primary active components were identified as ferulic acid and 3-butylphthalide, and the 3-butylphthalide showed the higher affinity capacity than ferulic acid. The method provides a simple way to study the binding behaviors of active components in TCMs with proteins in plasma with automatic operation.

6. Perspective

Analysis and screening of bioactive components in traditional Chinese medicines is a very formidable task. However, it is luck for us that the rapid development of modern analysis and separation methods offers a variety of means for discovering the mystery of traditional Chinese medicines. Even so, some strategies have to be designed for the sake. The whole animal model is very important for medicine evaluation because it can apparently responses the efficacy, side effect and toxicity of medicines in whole, but it is very limited in high cost and low efficiency so that could not compatible with the requirement of modern high throughput screening. On the contrary, the cellular models and receptor and enzyme models carry out the screening with the specific targets based on the cellular and molecular levels, respectively. They are rapid, economical, high sensitive and specific. Therefore, compared with the whole animal models, they are more adaptive to high throughput and large-scale drug screenings due to their diversity and low cost. However, in fact, the causes of most of diseases are relative with multi-target action except a few of the diseases. Therefore, despite the screening based on cellular and molecular models can discover the component with specific activity from original TCMs and even response the mechanism information of drug action, they can not response the authentic pharmacological action and can not also embody the characteristic of multi-component and multi-target action of TCMs. So it may be an effective and rational way by combining these models on the different levels to discover the actual bioactive components in TCMs. Chromatographic methods have become very effective and powerful tools for screening the bioactive components in TCMs except as a separation means. Some novel chromatographic methods have been designed for simulating the adsorption, distribution, metabolism and excretion (ADME) process of drug in body based on immobilizing the biomembranes, plasma proteins or other functional biomacromolecules such as hepatoenzymes. They can be used as the screening tools as well as predictive profiling of key pharmacokinetic parameters of an active molecule (adsorption, distribution, metabolism and excretion). The combination of these biochromatography builds a platform of TCMs screening with comprehensive consideration of ADME properties. Therefore, the platform is very effective and promising for its use in screening of bioactive components in TCMs because it is well coincident with the characteristic of multi-component, multi-target and multi-channel action of TCMs.

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