Cardiac Muscle Sarcolemma Chromatographic Stationary Phase and Its Potential Application in Drug Screening

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Abstract

A new bioactive packing material for liquid chromatography, sarcolemma chromatography stationary phase (SCSP), is presented. Its surface characteristics are investigated, and it is found that the acceptors embedded in sarcolemma remained bioactive for more than a week. The retention behavior of antagonists and activators related to cardiac muscle sarcolemma on the SCSP chromatographic column shows the screening function of the SCSP column, and the retention behavior of the active components in the aether extract from the Chinese herb *Ligusticum chuanxiong Hort.* on the SCSP column reveals, to a certain extent, the separation function of the SCSP column. These suggest that SCSP is a potentially useful material in drug screening.

Introduction

Traditional herbal medicine is a rich source for modern, molecular-target specific drug discovery (1). In the last several decades, a tremendous amount of effort has been invested to isolate individual compounds from traditional herbal medicine and to determine their chemical structures (2). High-throughput screening of large compound libraries has become a central component of modern drug discovery programs. Such a broad-based screening of collections of low-molecular weight molecules has indeed resulted in the discovery of compounds that modulate the activity of targets associated with many different therapeutic areas, but this generally requires considerable resources, especially when a large number of compounds need to be acquired and screened (3). The concept of target-based libraries as alternatives to large, maximally diverse compound libraries is one that has generated considerable interest in the field of drug discovery. This relatively new approach, in which knowledge of the ligand specificity of the biological target is used to design or select compounds, offers the possibility of discovering drugs more efficiently (4).

A bioassay that uses living things to test the effect of the chemicals is an indispensable technique for drug screening. The conventional bioassay, however, involves troublesome handling procedures, such as pipetting, and requires many expensive reagents (5). In recent years, rewarding attempts to overcome these shortcomings have emerged. Based on the specific affinity of the immobilized cell (5–8), protein (9–11), and acceptor (12–14), a series of drug screening models have been successfully developed; however, these models cannot be called perfect at present. Among them, the cell models cannot provide information about the very site the individual compound (drug candidate) combines; furthermore, the nature of the cells may be changed in these models. As for protein models, the immobilized protein columns are expensive (11), and the restrictive nature of the bound acceptor diminishes the protein's ability to fold and reduces its activity (15). Acceptor models have been proven effective for studying the interaction between acceptors and drug candidates, but they still have some limitations (11). An ever-present challenge is that preparing acceptors is difficult because they are of trace quantity and not easy to purify (16). More seriously, the purified acceptors may lose their biologic activity, which is indispensable for its proper function when parting from the surroundings.

Efforts to unravel the complex network of biomolecular interactions within the functioning cell have revealed that many proteins and drugs attain optimal activity by associating with sarcolemma (17). In this study, based on the specific recognition of the acceptors embedded in sarcolemma, a new bioactive packing material of liquid chromatography, sarcolemma chromatography stationary phase (SCSP), is presented, which has many advantages such as keeping acceptors in site and in maintaining its integrity and biological activity.

SCSP was prepared by having living rabbit cardiac muscle sarcolemma self-fused on the surface of the silica carrier; thus, the integration and enzymatic bioactivity of sarcolemma was best retained, as well as the surroundings of membrane receptors. In this study, the screening and separation functions of the SCSP column were displayed. The new bioactive packing material can be further extended to explore its capability for the site-specific interaction between drugs and membrane acceptors in vivo.

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Experimental

Instrument

A high-performance liquid chromatograph (HPLC) (Waters, Milford, MA) with a 2996 detector, 1525 pump, and Empower personal chromatography data-processing system was used. An L8-80 ultracentrifuge (Beckman, Fullerton, CA), ISM-5800 scanning microscope (JEOL, Tokyo, Japan), PHI-5400 X-ray photoelectron spectroscopy (Perkin Elmer, Wellesley, MA), CGY-100 pump (Beijing, China), and SCQ250 supersound producer (Shanghai, China) were used.

Reagents and material

Noradrenaline bitartrate injection (010301, Shanghai Harvest Pharmaceutical, Shanghai, China); phentolamine mesylate injection (020502, Shanghai Haipu Pharmaceutical, Shanghai, China); isoprenaline hydrochloridum injection (990301, Shanghai Harvest Pharmaceutical); dobutamine hydrochloride injection (020207, Jiangsu Huanghai Pharmaceutical, Jiangsu, China); promethazine hydrochloride injection (020117, Wuxi Pharmaceutical, Wuxi, China); propylthiouracil (020712, Zhengzhou Aodong Medicine, China); and spironolactone (980201, Shanghai Hengshan Pharmaceutical, Shanghai, China) were used.

The mobile phase of 0.05 mol/L was prepared by dissolving a certain amount of NaH_2PO_4 and Na_2HPO_4 in distilled water and adjusting to pH 7.4 with a solution of 1.0 mol/L HCl. It was filtered through a 0.45-µm membrane (Shanghai, China) with a vacuum pump (Tianjin Do-Chrom Technology, Tianjin, China) prior to use.

The sample solutions of 0.1 mg/mL were prepared in 5-mL flask by dissolving a certain amount of injections with the mobile phase.

The Chinese herb *Ligusticum chuanxiong Hort.* was yielded from the Sichuan Province of China and appraised by Prof. Yi Ren, who works at School of Life Science of Shaanxi Normal University of China. The aether extract of *Ligusticum chuanxiong Hort.* was prepared by steeping 10 g of the herb powder in three batches, with a total 100 mL aether, and concentrating the extract solution to 20 mL. The extract solution was filtered through a 0.45-µm membrane with a vacuum pump prior to use.

All chemicals used in this work were of analytical regent grade or higher. Macroporous ball silica (5 µm, 10 nm) was purchased from the Institute of Chemistry of the Chinese Academy of Science (Beijing, China). Rabbits of either sex were provided by Laboratory Animal Research Center of the Fourth Military Medical University of China (Xi'an, China).

Experiment

Activation of silica carrier

Silica (5.0 g) and HCl (100 mL 1.0 mol/L) were delivered into a flask. The mixture underwent circulation reflux under stirring for 2 h, and then, after having been washed until pH 7 was reached in the supernatant, the silica was heated in the oven at 120°C for at least 7 h.

Preparation of cardiac muscle sarcolemma

Referring to Lee's method (18), five rabbits of 2.0 kg were sacrificed, and the 20 g ventriculus cordis muscle obtained was frittered, homogenized with 20 mL mobile phase solution, and then the crude homogenate was centrifuged at 12000 rpm for 30 min twice to remove the cellular debris (in the form of precipitation). The supernatant produced was centrifuged at 32000 rpm for 30 min, such that the 150 mg cell member precipitation was obtained, which was then suspended in 10 mL mobile phase solution and stored at 4°C for further experiments.

Preparation of SCSP

SCSP was prepared as follows: 4.6 g silica activated and 10 mL suspension solution of the sarcolemma were mixed and joggled until approaching adsorption equilibrium. Then the mixture was centrifuged at 2000 rpm for 10 min. The precipitation produced was washed and stored at 4°C.

The determination of the bioactivity of SCSP versus time

According to the method in the literature (19,20), a mixture of given amounts of Na^+, K^+, Mg^{2+}, a given volume of a buffer, and a certain amount of freshly prepared SCSP was placed in a thermostatic water bath at 37°C for 10 min, and then a given amount of adenosine triphosphate (ATP) was added into the mixture. Ten minutes later, ATP decomposed to adenosine diphosphate (ADP) and inorganic phosphate (Pi) under the action of enzyme on SCSP. The reaction was ended by adding trichloroacetic acid and cooling the mixture. The produced Pi content was determined by spectrophotometry. The described procedure was run once every 30 min; thus, a series of Pi contents and consequent enzyme activity IU (µ mol/g × h) (i.e., the produced Pi contents "µ mol" under the action of 1 g enzyme within 1 h) were obtained for the different times SCSP was used.

Preparation of SCSP chromatography column

The SCSP prepared was transferred into 50-mL mobile phase solution and was packed in the chromatographic column (250 × 4.6-mm i.d.) by the slurry method under high pressure. In the same way, an SCSP chromatography column of 50 × 4.6-mm i.d. was prepared.

Chromatography experiment

The SCSP chromatographic columns were 50 × 4.6-mm i.d. or 250 × 4.6-mm i.d., and the silica columns were 50 × 4.6-mm i.d. The mobile phase was a 0.05 mol/L phosphate buffer solution of pH 7.4. The optimum flow rate of the mobile phase was set at 0.3 mL/min. The detected wavelength was at 278 nm. The injection volume of each sample solution was 20 µL. Under such conditions, various antagonists and activators corresponding to acceptors in SCSP and not in SCSP were analyzed, as well as aether extract from the Chinese herb *Ligusticum chuanxiong Hort.*

Results and Discussion

Special surface characteristics of SCSP

By comparing scanning electron microscope results, Figure 1B (SCSP) with Figure 1A (silica carrier), it was found that the surface of SCSP appeared to be almost completely coated by the cardiac muscle sarcolemma, and this may result from the

irreversible adsorption of silicon hydroxyl groups for biopolymers and self-fusion of the broken pieces of sarcolemma on the surface of the activated silica.

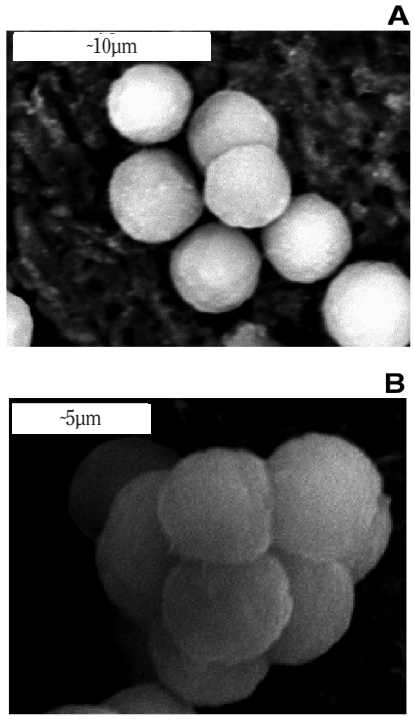

Figure 1. Scanning electron micrograph of silica carrier and SCSP surface: silica carrier (A) and SCSP (B).

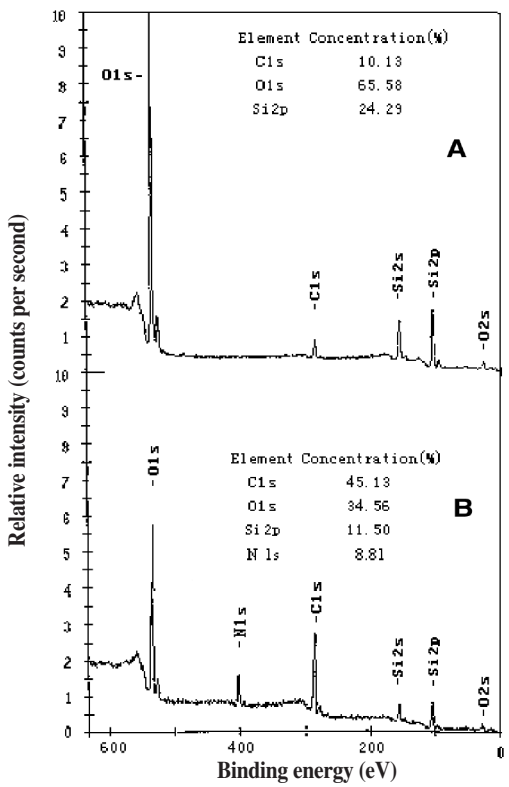

Figure 2. X-ray photoelectron surface energy spectrums of silica carrier and SCSP: silica carrier (A) and SCSP (B).

By comparing X-ray photoelectron spectroscopy results, Figure 2B (SCSP) with Figure 2A (silica carrier), it was shown that, as to SCSP, surface silica content clearly decreased. The reason why the surface silica peak didn't disappeared completely may be because of the fact that the thickness of sarcolemma is about 8 nm and the penetrating thickness of X-ray photoelectron spectroscopy is about 10 nm. Other reasons include the fact that the surface oxygen content decreased slightly, a surface nitrogen peak appeared, and that surface carbon content clearly increased. The described results further confirmed that the surface of SCSP was effectively coated by the sarcolemma. It must be emphasized that the usual chemical modification of the surface of the stationary phase was in no way capable of achieving these effects because of steric and other reasons (21).

Bioactivity of SCSP

In order to investigate whether the acceptors immobilized on the surface of SCSP remained bioactive, a study on the enzyme bioactivity of the SCSP surface versus time was carried out, which virtually reflected the bioactivity of the acceptors immobilized. As is shown in Figure 3 (series one), SCSP remained bioactive over a long period of time, and the relation between enzyme bioactivity (Y, IU) and the time (X, hour) SCSP was used fit an equation:

$$Y = 6.80 \times 10^{-7}X^4 - 2.11 \times 10^{-4}X^3 + 2.43 \times 10^{-2}X^2 - 1.41X + 86.1 \quad \text{Eq. 1}$$

with a regression coefficient of 0.9999. The experimental results suggested that the bioactivity of the receptors decreased along with the increasing time SCSP was used, and it decreased with a certain law. However, the acceptors immobilized remained bioactive for at least one week. Thus, SCSP was expected to be useful in the study on the interaction between drugs and acceptors.

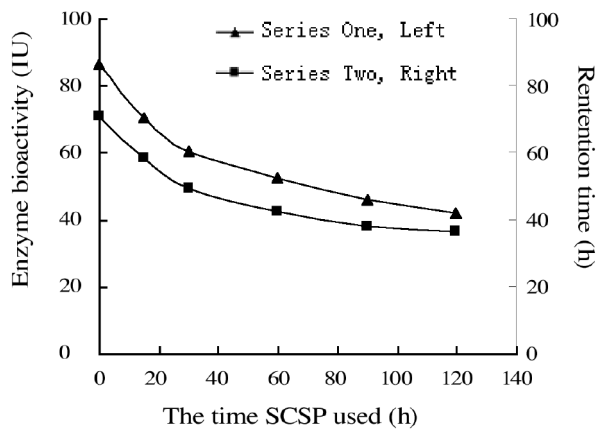

Figure 3. Series one: the law of bioactivity of SCSP varying along with time SCSP prepared. Series two: the law of retention time varying along with the time SCSP prepared. Chromatographic conditions (series two): column, (250 × 4.6-mm i.d.); mobile phase, 0.05 mol/L NaH_2PO_4-Na_2HPO_4 buffer (pH 7.4); flow rate, 0.3 mL/min; detection wavelength, 278 nm; column temperature, 37°C.

Special screening function of SCSP chromatographic column

By comparing the retention characteristics of phentolamine mesilate (α acceptor antagonists) on the SCSP column (Figure 4B) with those on silica column (Figure 4A), the retention times were found to be obviously different. Further experiments indicated the retention time on SCSP varied along with the time the SCSP column was used, but the retention time on the silica column didn't. When a 25-cm long SCSP column was used, the relation between retention time (*Y*, Min) and time (*X*, hour) SCSP was used fit an equation:

$$Y = 4.61 \times 10^{-7}X^4 - 1.43 \times 10^{-4}X^3 + 1.72 \times 10^{-2}X^2 - 1.10X + 707 \quad \text{Eq. 2}$$

with a regression coefficient of 0.9999, as is shown in Figure 3 (series two). It was obvious that there was a close relation between the law of the retention time varying and the law of enzyme bioactivity of SCSP varying. It could be inferred that the acceptors on SCSP well accounted for the retention behavior on SCSP (i.e., bioactive receptors in sarcolemma could selectively recognize some components and combine with them). When the bioactivity of the receptors decreased, the specific affinity decreased with it. Thus, the retention time of a certain component shortened.

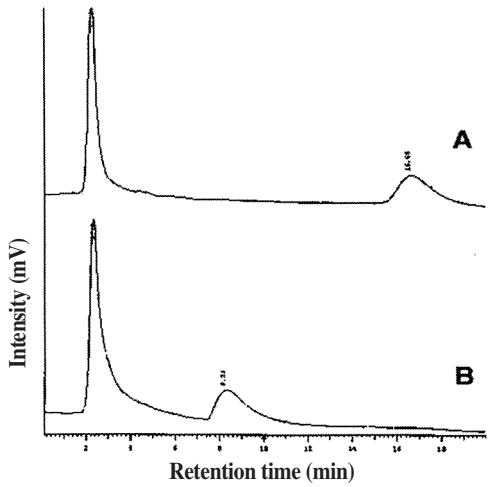

Figure 4. Chromatogram of phentolamine mesilate with silica and SCSP column: silica column (A) and SCSP column (B). Chromatographic conditions are the same as in Figure 3, except for the column (50 × 4.6-mm i.d.).

In order to verify the assumption, many antagonists or activators corresponding to known acceptors in cardiac muscle sarcolemma were tested, including noradrenaline bitartrate (α acceptor activator), phentolamine mesilate (α acceptor antagonists), dobutamine hydrochloride (β_1 acceptor activator), isoprenaline hydrochloridum (β_1 acceptor activator), and promethazine hydrochloride (H_1 acceptor antagonists). All antagonists were proven to adhere on the SCSP chromatographic column. Figure 5 shows the chromatograms of noradrenaline bitartrate (A), phentolamine mesilate (B), and dobutamine hydrochloride (C). Several kinds of antagonists corresponding to acceptors, which don't exist in cardiac muscle sarcolemma, were tested, including propylthiouracil (thyroxine acceptor antagonists) and spironolactone (androgen acceptor antagonists). All were proven not to adhere on the SCSP chromatographic column.

These results indicate that whatever combines with known acceptors in cardiac muscle sarcolemma will be retained on the SCSP chromatographic column, whereas that which doesn't combine will not. The specialty of the SCSP chromatographic column suggests that it would be of interest to drug screening (22).

Separation function of SCSP chromatographic column

The retention behavior on the SCSP chromatographic column of the aether extract from the Chinese herb *Ligusticum chuanxiong Hort*. was also studied. Whether the 50- × 4.6-mm i.d. SCSP column or the 250- × 4.6-mm i.d. SCSP column was used, five peaks appeared in the chromatograms. The chromatogram of the latter is shown in Figure 6. Their capacity factors (k) were 0.40, 1.47, 2.87, 4.44, and 10.15, respectively. Distribution coefficients (K) were 2.68, 9.85, 19.23, 29.75, and 68.01, respectively; separation factors alpha were 3.68, 1.95, 1.55, and 2.29, respectively; the theoretical plate numbers were 34, 100, 230, 56, and 100. These results showed that the SCSP column had a separation function to a certain extent, though it was weak. The sepa-

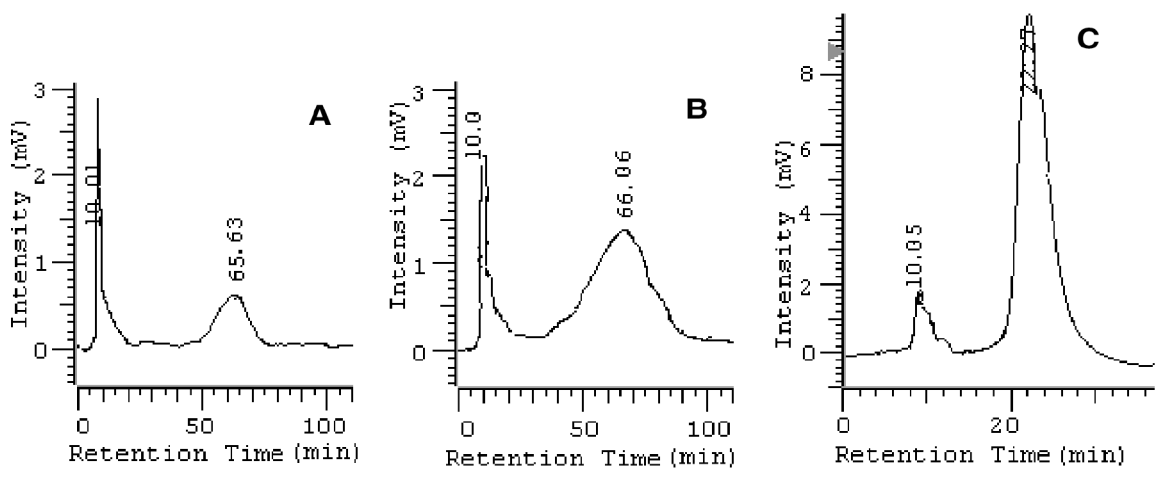

Figure 5. Chromatograms with SCSP column: noradrenaline bitartrate injection (A), phentolamine mesylate injection (B), and isoprenaline hydrochloridum injection (C). Chromatographic conditions were the same as in Figure 3.

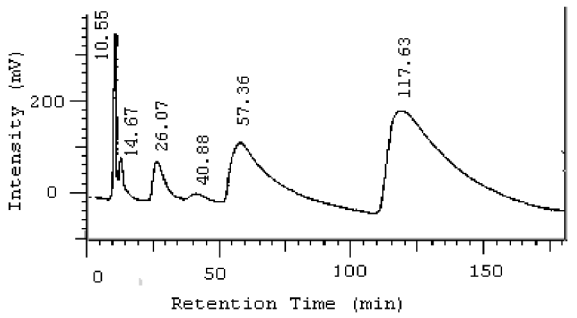

Figure 6. Chromatogram of the aether extract from the Chinese herb *Ligusticum chuanxiong Hort.* with the SCSP column. Chromatographic conditions were the same as in Figure 3.

ration function may result from the fact that there were various different kinds of acceptors in cardiac muscle sarcolemma, and their affinity magnitudes for corresponding drug candidates often differ from each other. Thus, the SCSP chromatographic column may be useful in drug screening and even in the primary separation of some complicated active components, especially in medicinal plants.

Conclusion

Based on the specific recognition of sarcolemma acceptors, SCSP, as a new bioactive packing material of liquid chromatography, may make the SCSP-based chromatographic system very effective in the screening and separation of active components in medicinal plants, though it still requires further study and perfection. The system is expected to be useful for the study of the specific interaction between drug and acceptor in vivo.

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