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Separation of Lipopolvsaccharides and Heparin Sodium by Activated Charcoal Adsorption and Ultrafiltration Process

C.Y. LI[1], Y.F. NAI[1], X. QIAN[1], H.Y. LI[1] and G.P. PENG[1,2,*]

[1]Pharmacy College, Nanjing University of Chinese Medicine, Nanjing, P.R. China
[2]Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Nanjing, P.R. China

*Corresponding author: Tel/Fax: +86 25 86798186; E-mail: guopingpeng@126.com

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In this study, a harmful lipopolvsaccharides (endotoxin) was separated by membrane and activated charcoal from another polysaccharide with 12 kDa molecular weight, heparin sodium, which was widely used as an injectable anticoagulant. The concentration of endotoxin was investigated by dynamic turbidity method. Meanwhile, removal efficiency of endotoxin from heparin was 80.9, 5.8 and 98.8 % with 1.0 % (w/w) activated charcoal, 200 kDa polyether sulfone membrane and 200 kDa hybrid membrane, respectively. The loss of heparin was 15.7, 4.8 and 4.1 % correspondingly. Due to the similar polysaccharide of heparin and endotoxin, activated charcoal absorption may be limited by saturating and competitive adsorption. Compared with the endotoxin rejection and heparin retention, hybrid ultrafiltration membrane showed many advantages and also provided theoretical support for industrial manufacturing of heparin injection for removing endotoxin.

Keywords: Endotoxin, Ultrafiltration, Activated charcoal, Hybrid, Polythersulfone.

INTRODUCTION

Heparin sodium injection is widely used for the treatment of anticoagulant therapy in prophylaxis and treatment of venous thrombosis and its extension[1-3], use by one billion persons in the world. Heparin, a polysaccharide, is made of pork or beef sausage mucous membrane. When highly stringent measures against microbial contamination are not taken, the heparin might be contaminated by endotoxin from the accidental microbial contamination[4]. Refer to industrial removal pyrogen process from injections[5,6], activated charcoal absorption is a traditional process for pyrogen removal during intravenous injection preparations[7].

Pyrogens are fever-inducing substances usually derived from microorganisms. Pyrogenic contaminations of several classes of injectable drugs represent an undeniable major health risk[8]. Endotoxin has a natural surfactant characteristic with a large hydrophilic polysaccharide chain and a hydrophobic fatty acid tail[9-11], which accounts for the varying character of the endotoxin micelle molecular weight (10 kDa to 1000 kDa)[12].

Ultrafiltration is one of the molecular sieve filtration, which has been used increasingly in the separation stage in biological production processes in recent decades[13,14]. Ultrafiltration can be used to remove or to reduce the level of endotoxin in solutions[13,15], however, such removal is accompanied by a high loss of heparin[16], heparin could be satisfactorily retained but the endotoxin removal rate may decline when the membrane molecular weight cut off (MWCO) is increased. This contradiction is a serious constraint to the application of ultrafiltration technology in the pharmaceutical industry. The objectives of this research were to demonstrate the removal process of activated charcoal and ultrafiltration, identify a way to retain heparin sodium while effectively removing endotoxin.

EXPERIMENTAL

Two kinds of material ultrafiltration membrane were selected for this study. 50 and 100 kDa polyethersulfone membrane was bought from Synder Membrane Technology Co., Ltd. Hybrid spiral membrane was provided by Nanjing Tuozhu Medical Technology Co., Ltd. Heparin sodium was purchased from Aladdin Reagent (Shanghai) Co., Ltd. Heparin sodium standards was bought from Sigma-Aldrich Co. LLC. Endotoxin standard substances were obtained from the National Institute for the Control of Pharmaceutical and Biological Products in China. Limulus amebocyte lysate (LAL) was manufactured by Zhanjiang Bokang Ocean Creature Co., Ltd. in China. Activated charcoal manufactured by Hangzhou wood factory activated carbon factory.

Determination of endotoxin concentration: Endotoxin concentration of different heparin samples were analyzed by turbidity method with endotoxin detector (ACC International CO., LTD in American, Mode: PKF). Briefly, a standard substance (140 EU/mL) was diluted to 2.0, 0.5, 0.125 and 0.03125 EU/mL. A 100 µL portion of each dilution was mixed with the same volume of limulus amebocyte lysate according to the manufacturer's instructions. The reaction mixtures were incubated at 37 °C and the optical density (OD) was simultaneously monitored. The time required to reach a designated turbidity (OD: 0.02) of the reaction mixtures was measured by PKF endotoxin detector.

Analyses of the serial samples were performed using least square method. The relationship between endotoxin concentration and reaction time was as follows:

$$\log T = 3.02416 - 0.39616 \log C \quad (1)$$

where T is the reaction time (s) and C is the endotoxin concentration (EU/mL). The related coefficient r = -0.9993. The endotoxin concentration of the stock solution and filtrate was calculated using eqn. 1.

Analysis of heparin sodium: A high-performance liquid chromatography manufactured by Agilent Technologies Co., Ltd. (Mode: 1200) with 2 × 250 mm anion-exchange column equipped with a variable wavelength detector (202 nm wavelength) and an Agilent chromatography workstation were used to determinate the concentration of heparin sodium. The detection conditions were as follows: solvent A, 0.1 % KH_2PO_4 aqueous solution; solvent B, sodium perchlorate - phosphate aqueous solution.

Activated charcoal adsorption measurements: The effects of activated charcoal adsorption on endotoxin and heparin were studied as follows. Endotoxin standard substances were dissolved in 200 mL heparin sodium injection with 1000 EU/mL to analyze the adsorption of 0.125, 0.25, 0.5 and 1.0 % (w/w) activated charcoal, respectively. During the experiment, the mixed solutions with different activated charcoal heat and bring to a boil, stirring constantly. Boil and stir 15 min, let cool and make up the volume to around 200 mL, filtrate through a 0.45 µm membrane. The removal rate was calculated using eqn. 2. Subsequently, several diagrams were designed using the endotoxin concentration and removal rate as the ordinate and activated charcoal concentration similar to the abscissa to analyze the adsorption relationship between endotoxin, heparin and activated charcoal.

$$R = \left(1 - \frac{C_f}{C_s}\right) \times 100\ \% \quad (2)$$

where C_f and C_s are the solute concentrations in filtration solution and stock solution, respectively.

Saturated adsorption of endotoxin and heparin sodium in membranes: In order to exclude the adsorptive effect of endotoxin and heparin sodium on membranes, it was necessary to find the saturated adsorption. All the ultrafiltration membranes were stored in 25 mmol/L sodium hydroxide aqueous solutions to protect the membranes from the contaminant of endotoxin. They were rinsed by purified water until the pH value was neutral before ultrafiltration use.

The adsorption effect of membranes on endotoxin was studied. 1 000 EU/mL endotoxin heparin sodium solutions (1 L) were filtrated by ultrafiltration membranes to analyze the saturated adsorption. During the experiment, the pipeline of feed solution, filtrate and rejected solution were placed in the same tube. The filtration operational pressure was controlled under 0.3 kg/cm^2 and the temperature was set to 22 to 25 °C to maintain the same experiment condition. The filtrate flux was then measured to calculate the circulation volumes. The adsorption was calculated using eqn. 3. Subsequently, several diagrams were designed using the adsorption as the ordinate and circulation volume similar to the abscissa to find the saturated adsorption.

$$\text{Adsorption} = (C_s - C_c) \times V \quad (3)$$

where V is the solution volume and C_s and C_c are the solute concentrations in the stock solution and circulation solution, respectively.

Separation of endotoxin from heparin by ultrafiltration: The rejection characteristics of endotoxin and heparin sodium were evaluated to analyze the performance of the membranes, excluding the effects of saturated adsorption. The mixture of endotoxin (1000 EU/mL) and heparin sodium (50 mg/mL) with 1 Lwas filled in stock tube and filtrated by polyethersulfone and hybrid membranes. The concentration of endotoxin and heparin sodium in the filtrate was determined after the adsorption of membranes had saturated. The removal rate was calculated by eqn. 2.

RESULTS AND DISCUSSION

Remove endotoxin from heparin sodium solution by activated charcoal: Dynamic turbidity method was performed using limulus amebocyte lysate to quantify the endotoxin concentration[17,18]. The endotoxin adsorption capacity with different concentration activated charcoal was investigated. The results are showed in Fig. 1 that the adsorption first increased as activated charcoal concentration adding and then the adsorption capacity remained stable when its concentration reached 0.25 %. But the endotoxin concentration was still above 190 EU/mL as the activated charcoal increased to 1 % in contaminated heparin sodium solution with 1000 EU/mL endotoxin. However, the safety concentration of endotoxin in heparin sodium injection was 15.625 EU/mL by intravenous therapy.

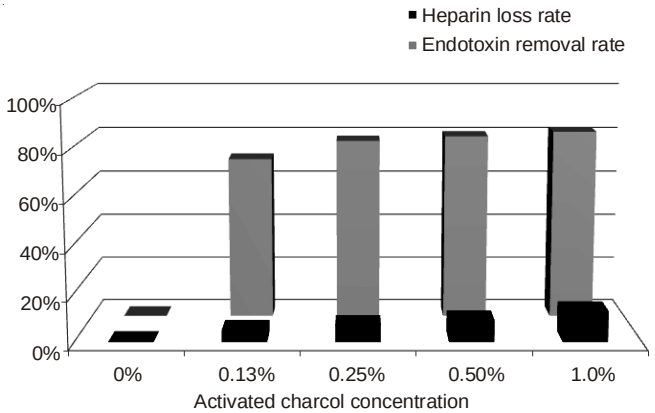

Fig. 1. Heparin sodium loss rate and endotoxin removal rate with different concentrations of activated charcoal

The pharmaceutical preparation technology determines the quality of production, however, the activated charcoal

cannot guarantee the endotoxin limited requirements of heparin sodium injection. In the removal progress of endotoxin, the heparin loss with activated charcoal was shown in Fig. 1, in 50 mg/mL heparin sodium, the loss rate of heparin sodium has close positive relationship with activated charcoal concentration. The adsorptions of activated charcoal were lower than 16 % with the four concentrations when the kinetic adsorption between the heparin sodium and activated charcoal was balanced.

These results indicate that endotoxin was adsorbed onto activated charcoal by kinetic equilibrium adsorption. Due to the similar polysaccharide of heparin and endotoxin, activated charcoal absorption may be limited by saturating and competitive adsorption, therefore, this method could not ensure the safety of heparin sodium injection.

Separate endotoxin from heparin sodium solution by polyethersulfone ultrafiltration membranes: The saturated adsorption of 50 and 100 kDa membranes was investigated to eliminate the influence of ultrafiltration results. As shown in Fig. 2 and 3, the saturation absorptive capacity of membranes were different in heparin sodium solution with 1000 EU/mL endotoxin.

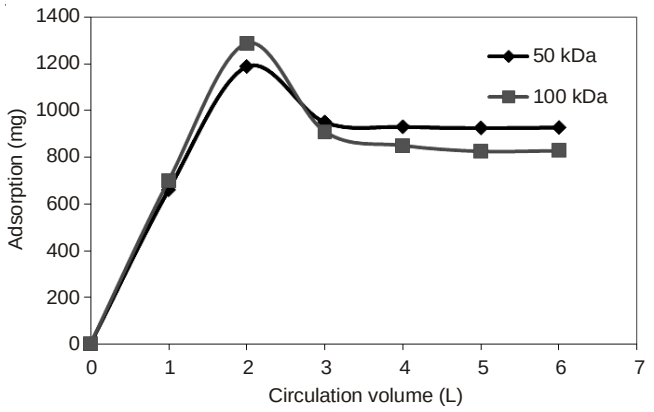

Fig. 2. Heparin sodium adsorption curves of 50 and 100 kDa polyethersulfone ultrafiltration membranes

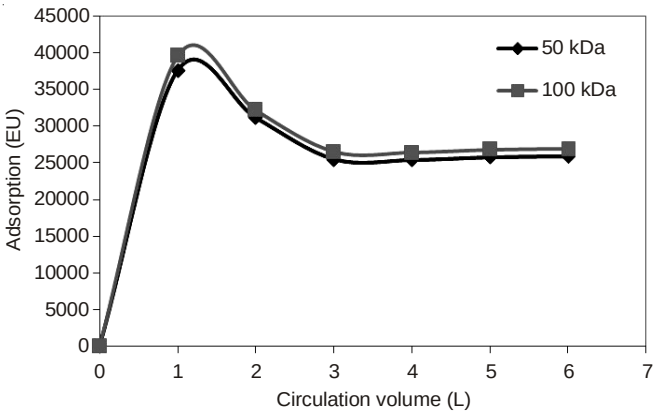

Fig. 3. Endotoxin adsorption curves of 50 and 100 kDa polyethersulfone ultrafiltration membranes

The adsorption of polyethersulfone membranes first increased and then remained stable when the circulation volume was increased. The saturation absorptive characteristics of endotoxin and heparin sodium were different with the same molecular weight cut off, as the circulation equilibrium volume was approximately 4 L. Therefore, the filtrate circulation volume of 5 L was chosen for subsequent filtration experiments and the adsorption constant was affected by the molecule structure and membrane material. The saturated adsorptions lower than 900 mg when the kinetic adsorption between the heparin sodium and the membranes was balanced. However, after saturated adsorptions of the two membranes, the transmissions of heparin sodium were 30.3 and 95.2 % and then endotoxin rejections were 27.6 and 5.8 %, respectively (Fig. 4).

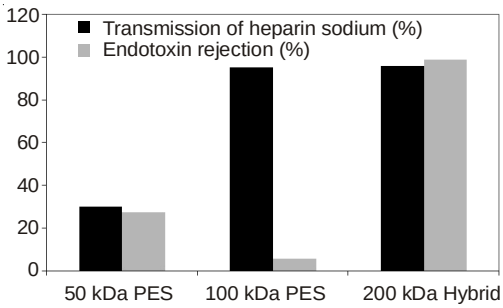

Fig. 4. Heparin sodium transmission and endotoxin rejection with different ultrafiltration membranes

Separate endotoxin from heparin sodium solution by hybrid ultrafiltration membrane. On the basis of above experiment, 200 kDa hybrid ultrafiltration membrane was selected to separate endotoxin from heparin sodium solution. After saturated adsorptions of the two membranes (Fig. 4), the transmission of heparin sodium was 95.9 % and endotoxin rejection was 98.8 %, respectively.

The filter material of hybrid ultrafiltration membrane was PES/PVDF blends, so the hydrophobic of polyethersulfone/PVDF blends was greater than polyethersulfone. Meanwhile, the endotoxin was an amphiphile with a large hydrophilic polysaccharide chain and a hydrophobic fatty acid tail and its monomer could be found in aggregated form. In ultrafiltration progress, endotoxin was dispersed and could pass through the polyethersulfone membrane when its molecular size was smaller than the radius of the membrane pores. But the aggregated endotoxin was protected in the hybrid membrane surface, endotoxin was rejected and separated from heparin sodium solution efficiently by 200 kDa hybrid membrane without any contradiction, such like competitive adsorption.

Conclusion

Compared with activated charcoal, the hybrid membrane has the advantages of separating endotoxin from heparin. The saturated adsorption defect of activated charcoal could not ensure the safety of heparin sodium injection. In the progress of removing pyrogens by hybrid membrane, the endotoxin micelles could stably exist in heparin sodium injection, but the hydrophilic micelle was influenced by hybrid membrane materials. The affinity between the endotoxin micelles and the membrane material also gradually weakened when the membrane material changed from hydrophilic to hydrophobic. The endotoxin micelle was preserved and rejected by hybrid membrane surface when its micelle size was bigger than the radius of the membrane pores. Significantly, the results provide us with a potential way to separate endotoxin from macromolecular

component and to modify various membrane materials. The ultrafiltration technology provides useful information to resolve the contradictions between target composition retention and hazardous molecules rejection.

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