Journal of Chromatography A, xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Preparation and application of a restricted access material with hybrid poly(glycerol mono-methacrylate) and cross-linked bovine serum albumin as hydrophilic out layers for directly on-line high performance liquid chromatography analysis of enrofloxacin and gatifloxacin in milk samples

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ARTICLE INFO

Article history:
Received 2 July 2018
Received in revised form 24 August 2018
Accepted 31 August 2018
Available online xxx

Keywords:
Restricted access material
Cross-linked bovine serum albumin
Antibiotics
Protein exclusion
Direct injection

ABSTRACT

A novel restricted access material (RAM) was prepared through combination of hydrophilic polymer poly(glycerol mono-methacrylate) and cross-linking bovine serum albumin (BSA) for direct biological analysis. In the material preparation, the poly(styrene-co-divinylbenzene) and poly(glycerol mono-methacrylate) were grafted on silica successively by atom transfer radical polymerization. Then the BSA was adsorbed on the material and cross-linked through an in-column process. The BSA recoveries of the resulted RAM were higher than 99.3%. Small molecules such as alkylbenzene and quinolone antibiotics could be retained with a reversed phase mechanism. The column packed with RAM had good long term stability and could last at least five months without significantly changed in its efficiencies. An on-line SPE/HPLC method for the analysis of enrofloxacin and gatifloxacin in milk samples was established by using the resulted RAM as the solid-phase extraction material. The detection and quantitation limits of two antibiotics were 8.22 and 27.4 ng mL^{-1}, respectively. In the method validation, least recovery 88.5% with relative standard deviation of less than 3.9% was obtained. It demonstrated that the method is reliable and the material can be used for the direct analysis of veterinary drug residues in biological samples.

1. Introduction

In complex biological samples analysis, sample preparation is often the key step of the method development [1]. Meanwhile, it can be the great source of error and time consumption of the analysis [2]. Presence of macromolecules in the sample matrix is the major problem because they not only interfere the detection of the low molecular weight compounds but also damage the columns in the HPLC analysis [3]. To solve this problem, various restricted-access materials (RAMs), such as internal-surface reversed phase (ISRP) [4], shield hydrophobic phase (SHP) [5] have been developed [6–11]. Among theses RAMs, protein-coated octadecylsiyl silica gel (ODS) columns that utilized bovine serum albumin (BSA) [12] or cross-linked BSA [13] as restricted access barrier have also been reported for drugs analysis in plasma samples. The results demonstrate that coating of BSA is a convenient way for making RAM columns. With both functions of macromolecules exclusion and small molecules retention [14], RAMs have shown great advantages in the direct HPLC injection in the complex biological sample analysis.

We reported a novel RAM [Sil-p(St/DVB)-g-pGMMA] in our previous paper [15]. In the Sil-p(St/DVB)-g-pGMMA, the poly(styrene-co-divinylbenzene) [p(St/DVB)] bonded silica was used as the inner structure and poly(glycerol monomethacrylate) (pGMMA) was the external hydrophilic surface. To meet the application requirements, the amount of silica gels for synthesis of Sil-p(St/DVB)-g-pGMMA was scaled up from 30.0 g to 545.0 g. It was found that the protein recoveries of Sil-p(St/DVB)-g-pGMMA

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https://doi.org/10.1016/j.chroma.2018.08.067
0021-9673/

from the scale-up process did not meet the expectations which was attributed to imperfect covering of p(St/DVB). To increase the hydrophilic webs on the material surface, further coating by cross-linked BSA was conducted by an in-column process. The process was performed by injection of BSA solutions into Sil-p(St/DVB)-g-pGMMA columns and then cross-linked with glutaraldehyde, which created a hybrid protein-coated and pGMMA surface. The resulting product Sil-p(St/DVB)-g-pGMMA-i-BSA not only has good protein exclusion ability but also the excellent column durability. To examine the applicability of the material, an on-line HPLC analysis of quinolone antibiotics from milk samples was developed.

Abuse of antibiotics causes high level of veterinary drug residues in animal-derived products. Human consumption of contaminated foods can lead to the enrichment of antibiotics in the human body, resulting in the drug resistance that threatens human health [16]. Therefore, antibiotic residue analysis is an important issue in the food safety control areas. Enrofloxacin (ENR) and gatifloxacin (GTFX) are quinolone antibiotics (QNs) that are widely employed in the prevention and treatment of animal diseases [17]. Milk is one of the main foodstuffs for human intake protein. Due to the presence of fats and proteins in the matrix [18], analysis of QNs in milk samples often requires multi-step pretreatment including protein precipitation, concentration [19,20] and/or off-line solid-phase extraction (SPE) [21–23]. Therefore, it is highly desirable to explore simple, efficient and environment-friendly methods for the determination of residual QNs.

In the present research, the newly developed RAM: Sil-p(St/DVB)-g-pGMMA-i-BSA was used for the on-line SPE process to determine ENR and GTFX in milk samples. Compared with the classical deproteinization steps, the on-line SPE method coupling with HPLC reported in this work allows the direct injection of biological samples, preventing coprecipitation of drug in the precipitation process. The results demonstrated that the method is efficient and reliable. It can be employed in the real sample analysis.

2. Experimental

2.1. Materials and reagents

Amorphous silica gel (particle size: 38–48 μm, specific surface area: 362 $m^2 g^{-1}$) was purchased from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). Enrofloxacin (ENR) was obtained from Senbeijia Biotechnology Co., Ltd. (Nanjing, China). Gatifloxacin (GTFX) was purchased from Bide Pharmatech Ltd. (Shanghai, China). Methylbenzene (MeBz) and Ethylbenzene (EtBz) were provided by Guangfu Fine Chemical Research Institute (Tianjin, China). Bovine serum albumin (BSA) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Glutaraldehyde (GA, 25% in water) was from HWRK Chem. Co., LTD. (Beijing, China). Sodium cyanoborohydride (CH_3BNNa) was provided by Woerjiming Technology Development (Beijing, China). Milk was purchased from the local super-market. Unless otherwise noted, all other reagents were of analytical grade and used as received. Phosphate buffer consisting of NaH_2PO_4 and Na_2HPO_4 was prepared according to the Handbook of Biochemistry and Molecular Biology [24].

2.2. Synthesis of Sil-g-p(St/DVB)-g-pGMMA

Sil-g-p(St/DVB)-g-pGMMA particles were synthesized using atom transfer radical polymerization (ATRP) according to our previous publication with some modifications [15]. In the synthesis, the p(St/DVB) and poly(glycidyl methacrylate) (pGMA) layers were grafted on the surface of silica gel successively by ATRP method. After hydrolyzation, Sil-p(St/DVB)-g-pGMMA was obtained. The procedures were described in the Supplementary material.

2.3. Immobilization of BSA on the surface of Sil-g-p(St/DVB)-g-pGMMA

Sil-g-p(St/DVB)-g-pGMMA particles were slurry-packed into a large stainless steel column (250 × 25 mm i.d.). The column was connected to an HPLC system. The mobile phase was Na_2HPO_4-NaH_2PO_4 buffer (0.1 mol L^{-1}, pH 7.0)/ACN/THF/IPA (90/10/1/1, v/v) with a flow rate of 1.0 mL min^{-1}. A 500 μL 5.0 mg mL^{-1} BSA aqueous solution was injected twenty five times successively with a total volume of 12.5 mL. The purpose was to ensure that the BSA was adsorbed as much as possible on the surface of Sil-g-p(St/DVB)-g-pGMMA material. A quantitative recovery of BSA from the column was achieved after the injections. Then a 5% GA aqueous solution was loaded into the column by an HPLC pump with a flow rate of 2.0 mL min^{-1} for 25 min. After the column was incubated for 2 h at room temperature, it was balanced with 0.3 M CH_3BNNa solution for 25 min at a flow rate of 2.0 mL min^{-1}. The in-column reaction was allowed to take place for 2 h at room temperature, before the column was rinsed with water and methanol successively. The resulted Sil-g-p(St/DVB)-g-pGMMA-i-BSA particles were vacuum dried at room temperature. The synthetic schematic of the Sil-g-p(St/DVB)-g-pGMMA-i-BSA were shown in Fig. 1.

Elemental analysis was employed to estimate the amount of BSA adsorbed on surface of the materials. The amount of the coated BSA on unit area of the material was calculated by the following equation: $n_{BSA} = \Delta N\% / (Mr \times Nr \times Ss)$. In the equation, ΔN% is the nitrogen content increment in 1.0 g of particle after immobilizing of BSA. Mr is the mass of the nitrogen atom. Nr is the numbers of nitrogen atom in one BSA molecules. Ss is the specific surface area of the silica gel.

2.4. Chromatographic evaluation

The Sil-g-p(St/DVB)-g-pGMMA-i-BSA particles were dry packed into three different length columns (10.0, 3.0 and 1.0 cm) with same 4.6 mm i.d. for subsequent chromatographic evaluation.

A LC-20A HPLC system (Shimadzu, Japan) equipped with three LC-20AT pumps and a SPD-20A UV detector were employed for the experiments.

2.4.1. Determination of hydrophobicity and binding capacities of RAMs

The ACN/H_2O (40/60, v/v) solution at a flow rate of 1.0 mL min^{-1} was employed as the mobile phase. The alkylbenzene was detected at 254 nm. The column hold-up time (t_0) was determined by acetone. The retention factors and selectivity factors were calculated by $k = (t\text{-}t_0)/t_0$ and $\alpha = k_2/k_1$, respectively. The binding capacities (Q) of the RAM columns were determined by the frontal chromatography. The solution of ACN/H_2O (40/60, v/v) containing alkylbenzene (4.5 mg mL^{-1}) was used as mobile phase and pumped into the column at a flow rate of 1.0 mL min^{-1}. Q was calculated by $Q = c\upsilon t$, in which c is the concentration of alkylbenzene in the solution, υ is the pumping flow rate, and t is the retention time corresponding to the half-height of the platform in the frontal chromatogram.

2.4.2. Evaluation of protein exclusion efficiency

The protein exclusion efficiency of the RAM columns was characterized by the recoveries of BSA, calculated by the ratio of the BSA peak areas from the RAM columns to those obtained without columns. A BSA aqueous solution (5.0 mg mL^{-1}) was employed as the sample and the mobile phase was Na_2HPO_4-NaH_2PO_4 buffer (0.1 mol L^{-1}, pH 7.0)/ACN/THF/IPA (90/10/1/1, v/v). The BSA was detected at 280 nm and the flow rate of the mobile phase was 0.5 mL min^{-1}.

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