Journal of Chromatography B, 1003 (2015) 67–73

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Determination of 11 quinolones in bovine milk using immunoaffinity stir bar sorptive microextraction and liquid chromatography with fluorescence detection

Kai Yao, Wei Zhang, Linyan Yang, Jianfang Gong, Liuan Li, Tianming Jin, Cun Li*

College of Animal Science and Veterinary Medicine, Tianjin Agricultural University, 300384 Tianjin, People's Republic of China

ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form 7 September 2015
Accepted 9 September 2015
Available online 15 September 2015

Keywords:
Immunoaffinity stir bar
Quinolone
Monoclonal antibody
Bovine milk
Liquid chromatography

ABSTRACT

A sensitive, selective and reproducible immunoaffinity stir bar sorptive microextraction (SBSME) coupled with liquid chromatography-fluorescence method for determination of 11 quinolones (QNs) in bovine milk was developed and validated. It is first report of a broad-specificity monoclonal antibody to QNs that has been immobilized to glass bar for preparation of a re-usable immunoaffinity stir bar. Analytes were extracted by placing stir bar in milk and shaking on a rotary shaker for 30 min at 30 rpm, followed by liquid chromatography and fluorescence detection. The newly developed method has limits of detection for each QN from 0.05 to 0.1 ng/g with intra-day and inter-day precision ranging from 3.2 to 11.9% and from 5.2 to 12.5%, respectively. This allowed us to quantitatively analyze drugs in bovine milk with the advantage of significantly simplified sample preparation. The proposed method was successfully applied to the bovine milk samples analyses with QNs, demonstrating its rare application in animal food safety analysis.

1. Introduction

Quinolones (QNs) are synthetic antibacterial agents that have been widely used in human and animals for treatment of intestinal, urinary and respiratory infections [1]. Their antimicrobial mechanism against gram-negative bacteria and gram-positive bacteria can be explained as follows: QNs can affect bacterial reproduction by inhibiting topoisomerase and DNA gyrase enzymes [2]. In order to promote production and maintain animal health, the improper use of antibacterials are becoming more common, which leads to drug residues in edible animal products and poses serious threats to consumers' health, including development of resistant bacterial strains, liver damage, allergic hypersensitivity and gastrointestinal reactions. The European Union (EU) has set maximum residue limits of quinolones in milk and other tissues to minimize the incidence of toxicity and to regulate the use of QNs in animals [3]. MRLs for several QNs such as enrofloxacin, ciprofloxacin and marbofloxacin in bovine milk ranged from 75 to100 ng/g. Thus, there is an urgent need to develop a highly specific, sensitive and efficient method to quantify QNs in milk.

A number of methods has been developed for determination of QNs residues, many of them based on polarization immunoassay [4], enzyme-linked immunosorbent assay [5], capillary electrophoresis with laser-induced fluorescence [6,7], micellar electrokinetic capillary with indirect laser-induced fluorescence [8] and liquid chromatography (LC) with DAD [9], MS/MS [10–12] or fluorescence [13,14]. Bovine milk is considered a complex matrix and contains many types of interfering substances, such as lipid and protein. These substances often interfere with analyte determination and become a major problem when analytes are at trace concentration, so a cleanup step to remove their interference is inevitable part of sample preparation prior to the measurement of analytes in bovine milk [15]. The majority of current cleanup methods are based on solid-phase extraction [16–18] and liquid–liquid extraction [19,20]. These methods of analytes separation and concentration require additional cleanup procedures and large numbers of organic solvents, which are laborious, expensive and time-consuming due to many steps and the lack of selectivity.

Immunoaffinity solid phase microextraction (SPME) was first reported for determining theophylline in serum samples in 2001 [21]. It is a form of immunoaffinity chromatography (IAC) in which highly selective antibodies are bound to a solid support material such as glass bar, fused silica capillary or stainless steel rod. To date, immunoaffinity SPME or immunoaffinity in-tube SPME devices have been mainly used for detecting theophylline

* Corresponding author. Fax: +86 22 23784590.
E-mail address: hhlicun@163.com (C. Li).

http://dx.doi.org/10.1016/j.jchromb.2015.09.008
1570-0232/

[21], 7-aminoflunitrazepam [22], benzodiazepines [23], fluoxetine [24], interferon alpha [25], and penicillin binding protein 2a [26]. Immunoaffinity column is one of the most powerful method for purifying QNs in complicated matrices [27–29]. However, the preparation process for immunoaffinity column requires large amount of monoclonal antibodies (mAbs), and the cleanup procedure is complex. As far as we know, no method about immunoaffinity SPME has been published for determination of a class of veterinary drugs in animal-derived food. Therefore, the developed method in this study is mainly focused on construction of an immunoaffinity stir bar by immobilizing mAbs on a glass bar for purification and extraction of 11 QNs from bovine milk which need little amount of mAbs and organic solvent. The extraction is followed by the quantification of QNs with high performance liquid chromatography (HPLC) with programmable fluorescence detection (FLD). The method was validated according to the cross-reactivity, binding capacity, linearity, recovery, accuracy, precision and the stability of immunoaffinity stir bar, and was expected to be easy, sensitive, reproducible and environmental-friendly.

2. Materials and methods

2.1. Reagents and materials

All chemicals used were of analytical grade. Acetonitrile was obtained from Merck (Darmstadt, Germany). Sulfuric acid (98%), hydrogen peroxide (30%), sodium hydroxide, sodium chloride, potassium chloride, potassium phosphate monobasic and sodium phosphate dibasic were purchased from Tianjin Chemical Reagent No. 3 Plant (Tianjin, China). Formic acid was purchased from Tianjin Fuyu Fine Chemical Co., Ltd. (Tianjin, China). Methanol (MeOH) was obtained from Tianjin Kangchao Biopharmaceutical Tech Co., Ltd. (Tianjin, China). Ethanol absolute was purchased from Tianjin Jinke Fine Chemical Research Institute (Tianjin, China). (3-aminopropyl)-triethoxysilane (APTES), glutaraldehyde grade II (25% aqueous solution) and ethanolamine were obtained from Sigma-Aldrich (St. Louis, MO, USA). All water was obtained from a Milli-Q system (Bedford, MA, USA) and was collected at 18 MΩ or higher. Borosilicate glass bars (5 mm × 10 cm) were obtained from Beijing Glass Instrument Factory (Beijing, China).

Marbofloxacin (MAR), danofloxacin mesylate (DAN), flumequine (FLU), enrofloxacin (ENR) and orbifloxacin (ORB) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The ciprofloxacin (CIP) was purchased from Sigma–Aldrich (St. Louis, MO). Ofloxacin (OFL), norfloxacin (NOR) and levofloxacin (LEV) were purchased from National Institutes for Food and Drug Control (Beijing, China). Lomefloxacin (LOM) and Fleroxacin (FLE) were purchased from National Institute For The Control Of Pharmaceutic (Beijing, China). The purity of all drugs was greater than 99%. The mAb to QNs, named XXQ-6-2-C1 were produced by our laboratory against CIP. The mAb showed cross-reactivities with 11 QNs in the range of 47.7–128% (Table 1).

Individual QNs stock solutions (100 μg/mL) were prepared by dissolving 10 mg of standard in 2 mL 0.03% sodium hydroxide and make a final volume of 100 mL with MeOH. Working solutions were prepared daily by a step-by-step dilution with MeOH to a final concentration of 1 μg/mL. All stock solutions were stored in amber glass bottles at 4 °C in the dark for 3 months. Phosphate buffered saline (PBS) consisted of 1.8 mM phosphate monobasic, 11.4 mM sodium phosphate dibasic, 0.14 M sodium chloride and 2.7 mM potassium chloride and the pH was adjusted to 7.4 with 1 M sodium hydroxide. The PBS containing 0.01% sodium azide was prepared by dissolving 0.1 g sodium azide to a final volume of 1 L with PBS.

Table 1
Retention time, column capacities, and cross-reactivities for the QNs.

Analytes	MW[a]	CC[b] (pmol/cm^2)	CR[c] (%)	T_R[d] (min)
CIP	332	2.15	128.0	8.753
LOM	352	1.25	52.5	10.464
DAN	358	0.57	71.1	10.500
FLE	370	0.48	55.2	7.986
OFL	362	0.35	68.1	7.888
ENR	360	0.54	114.3	12.212
NOR	320	1.69	100.0	7.782
MAR	363	0.46	54.2	6.648
LEV	371	0.43	69.6	7.825
ORB	396	0.38	56.1	13.958
FLU	262	0.43	47.7	29.808

[a] MW, molecular weight.
[b] CC, column capacity.
[c] CR, cross-reactivity.
[d] T_R, retention time.

2.2. Instruments

The vortex mixer was purchased from North-Biotechnology Co., Ltd. (Beijing, China), and the centrifuge was purchased from Hunan Cence Instrument Co., Ltd. (H1650, Hunan, China). The heating magnetic stirrer was from Tianjin Honour Instrument Co., Ltd. (EMS-9$_A$, Tianjin, China). Samples were agitated during extraction and desorption process using a rotary shaker purchased from Junyi Electrophoresis Instrument Co., Ltd. (JY15B, Beijing, China). The 12-sample nitrogen evaporator with a heating bath was purchased from Organomation Associates Inc., (Berlin, MA, USA). The HPLC system was from Agilent Technologies 1260 Infinity (Santa Clara, USA), and was equipped with a quaternary pump and fluorescence detector. The chromatographic separation of the QNs was carried out on an Inert Sustain C_{18} column (150 × 4.6 mm i.d., 6 μm, Shimadzu, Tokyo, Japan) with gradient elution. Mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). The flow rate was 0.8 mL/min and the column temperature was set to 35 °C. The injection volume was 20 μL. The gradient program and wavelengths for excitation and emission are shown as follows: 0–20 min, 15% B; 20–27 min, 15–50% B; 27–35 min, 50% B; 35–45 min, 50–90% B; 45–55 min, 90–15% B. The excitation/emission wavelengths were programmed at 297/515 nm for MAR (0–7.3 min), at 280/450 nm for NOR, LEV, OFL, FLE, CIP, LOM, DAN, ENR and ORB (7.3–20 min), and at 320/365 nm for FLU (20–35 min). The retention time of each QN was shown in Table 1.

2.3. Immunoaffinity stir bar preparation

The mAbs were bound to glass bars using glutaraldehyde (GA) as the coupling reagent according to Yuan et al. [21]. and Lord et al. [22,23]. Briefly, the lower halves of glass bars were cleaned and etched by immersing in a mixture of 70 mL 98% sulfuric acid and 30 mL 30% hydrogen peroxide. After the mixture lowered to room temperature, heating mixture in water bath at 80 °C for 1 h. Bars were then thoroughly rinsed with water, absolute ethanol and water. Particular care was taken in case of contamination. The cleaned parts of bars were silanized with ethanolic APTES solution (5 mL APTES, 5 mL water and 90 mL absolute ethanol) for 24 h at room temperature, followed by rinsing with water and absolute ethanol. The bars were placed in a vacuum oven with nitrogen flushed at 70 °C overnight or 15 h. Bars were activated by placing in 100 mL of PBS containing 2.5 mL GA for 7 h, followed by rinsing with water. After rinsing, the GA activated surface were immersed in 10 mL mAb PBS solution (0.5 mg/mL) to a depth of 3 cm and stirred for 24 h at 4 °C using a magnetic stirrer. It was found that the specific binding of bars and antibody reached saturation when antibody concentration over 0.6 ng/mL[23]. Subsequently, the bars