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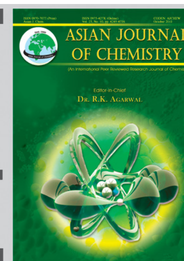

On-Line Solid-Phase Extraction of Fluoroquinolone Residues from Milk with Enrofloxacin-Imprinted Monolithic Column

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On-line solid-phase extraction method was developed by enrofloxacin-imprinted monolithic column (monolithic MIP) coupling with reversed-phase high-performance liquid chromatography for preconcentration and separation of fluoroquinolone residues in milk. The monolithic MIP was prepared by *in situ* polymerization in a stainless-steel chromatographic column. An enrichment factor of 6.70 along with a good sample clean-up effect was obtained. High recoveries for three fluoroquinolones such as enrofloxacin, ciprofloxacin and norfloxacin were achieved, which was in the range of 82.0-94.0 % with precision lower than 5.6 %. The limit of detection and limit of quantitation of the proposed method were in a range of 4.31-7.62 μg/kg and 14.37-25.40 μg/kg, respectively. The proposed method was successfully applied to on-line preconcentration, separation and determination of the fluoroquinolones in the milk sample.

Key Words: Molecularly imprinted monolithic column, On-line, Solid-phase extraction, Enrofloxacin, Milk.

INTRODUCTION

Fluoroquinolones are a class of important antibacterials, which have been developed rapidly in recent years and have wide applications in clinical medicine of human and animals. Enrofloxacin is a high bactericidal activity against major pathogenic bacteria found in diseased animals[1,2]. However, the residues of fluoroquinolones in edible animal tissues and food produced from animal are potentially hazardous to human health. In order to control the amount of fluoroquinolone residues in harmful concentrations, many methods have been proposed for the analysis of the drugs in biological matrices, such as capillary electrophoresis[3,4] and liquid chromatography[5,6]. However, a method development for the selective extraction and determination of these compounds from the complex matrix is necessary.

The molecularly imprinted polymers (MIPs) are a rapidly growing research focus possessing recognition ability and high selectivity for the template molecule, which is used in many analytical fields[7,8], particularly as absorbents for molecularly imprinted solid phase extraction (MISPE)[9-12]. Most of the molecularly imprinted solid phase extraction application was investigated in an off-line mode and the procedures was tedious procedures[13,14]. However, on-line solid-phase extraction procedure is simple, one-step and easy. Recently, the molecularly imprinted monolithic column as stationary phase had been applied in HPLC to separate analytes, such as the sulfamethoxazole[15], theophlline[16], nateglinide[17] and strychnine[18].

In this work, a monolithic enrofloxacin-imprinted polymer (monolithic MIP) had been prepared by *in situ* polymerization in a stainless-steel column. The obtained monolithic molecularly imprinted polymer showed high specific affinity to the template molecular and the monolithic molecularly imprinted polymer was used as the precolumn on-line coupled with C18 column for extraction of enrofloxacin and its analogues from milk samples. The present method was simple, rapid and efficient in sample analysis.

EXPERIMENTAL

Enrofloxacin (ENR), ciprofloxacin (CIP) and norfloxacin (NOR) were obtained from Beijing Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol, acetonitrile (ACN) and hexane (HPLC grade) were obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Trichloroacetic acid and anhydrous sodium sulfate were obtained from Tianjin Reagent Chemicals Co., Ltd. (Tianjin, China). All other chemicals and solvents were of analytical grade. Solutions prepared for HPLC were through a 0.45 μm PVDF syringe filter before use. Doubly deionized water was used throughout. Commercial whole milk was purchased from the market. Samples for HPLC were filtered through a 0.45 μm membrane filter.

Chromatographic separation of the fluoroquinolones was performed using an HPLC (LC-6A, Shimadzu Corporation, Kyoto, Japan) equipped with a LC-6A pump and UV3000 detector (Beijing Tong Heng Innovation Technology Co., Ltd) at a wavelength of 280 nm. The samples were separated on C18 stainless column (Venusil XBP C18, 4.6 × 150 mm, 5 μm) and eluted with a mobile phase consisting of a mixture of acetonitrile (ACN) -0.05 % trifluoroacetic acid (TFA) (20:80; v/v) at pH = 2.54. The flow rate was 0.8 mL/min at ambient temperature, the injection volume 10 μL. The SEM micrographs of the monolithic materials were obtained in a KYKY-2800B scanning electron microscopy (Beijing, China) at 25 kV.

Preparation of monolithic molecularly imprinted polymer column: The polymerization strategy was conducted according to previous work[19]. The monolithic molecularly imprinted polymer was directly prepared by *in situ* polymerization technique in a stainless steel chromatographic column of using 125 mm × 4.6 mm i.d. Template molecule (ENR, 0.5 mmol) was dissolved in acetonitrile (2 mL), to which the functional monomer (MAA, 2 mmol) was subsequently added. After stirring for 0.5 h, the cross-linker (EGDMA, 10 mmol), porogenic solvent (1-dodecanol, 2 mL) and free radical initiator (AIBN, 0.08 mmol) were added stepwise. The mixture was sonicated for 5 min and purged with nitrogen gas for 5 min before filled into a stainless steel column. The column was sealed under nitrogen protection and dipped into a 50 ºC water bath for 24 h. After polymerization, the column was connected with a HPLC pump and washed exhaustively on-line with methanol-acetic acid (4:1, v/v) to remove the template molecule and the porogenic solvent. At last, it was washed with methanol until no residue of template was found and a stable baseline was achieved. A non-imprinted monolithic column (NIP) was prepared in the absence of template and treated in an identical manner.

Morphology analysis: After the chromatographic experiments had been completed, the monolithic molecularly imprinted polymer was washed with methanol-acetic acid (85:15, v/v) for 4 h and methanol for 1 h. The column fitting was removed and the monolithic polymer was pushed out of the column using a flow-rate of 5 mL/min. The polymer was dried under vacuum at 50 ºC for 24 h and cut into pieces with a razor blade. Surface analysis of polymer was carried out in a KYKY-2800B scanning electron microscopy (Beijing, China) at 25 kV.

Breakthrough curves of enrofloxacin on the molecularly imprinted polymer and non-imprinted monolithic polymer columns: The monolithic molecularly imprinted polymer and non-imprinted monolithic polymer were thoroughly flushed with pure acetonitrile until a stable baseline was observed at 280 nm, the monolithic column was shortly removed from the LC system and the tube from the reservoir to the inlet of the monolithic column was filled with a solution of the enrofloxacin in acetonitrile. Subsequently, it was applied as precolumn to on-line connect with HPLC system and 0.10 mg/mL of enrofloxacin solution was loaded at a flow rate of 0.8 mL/min and simultaneously recording the signal at 280 nm, the breakthrough curves were obtained.

Pretreatment of milk samples[20]: The samples were stored at -20 ºC. A sample of milk (2 g) was accurately weighed and were transferred to a 50 mL polypropylene centrifuge tube. After a 10 min equilibration period, 7 mL of 2.5 % trichloroacetic acid (TCA)/acetonitrile (ACN) (25:75, v/v) was added and vortex-mixed for 30 s. Then the mixture was left undisturbed for 10 min following mixture with 4 g of anhydrous sodium sulfate thoroughly and incubated for 5 min. The samples were centrifuged at 3000 rpm for 20 min. The sample residues were extracted once again with 7 mL of 2.5 % TCA/ ACN (25:75, v/v). The solutions were combined to the above supernatant after centrifugation, to which 10 mL of *n*-Hexane was added. After vortex mixed for 15 s and centrifugation for 10 min, the upper layer was discarded and the lower layers were transferred to glass tubes and concentrated under a stream of nitrogen at 45-50 ºC. The concentrated residues were dissolved in 1 mL of acetonitrile and filtered through 0.45 μm filter membrane. 10 μL of the filtrate was used for chromatography analysis.

Procedure for on-line SPE-HPLC determination of enrofloxacin: The schematic diagram of on-line SPE-HPLC system is similar to that previously reported by Han *et al.*[21]. Firstly, the precolumn was conditioned with acetonitrile at a flow rate of 0.5 mL/min. Secondly, the 10 μL extraction solution of milk was applied to the conditioned precolumn and the precolumn was washed with 20 % acetonitrile in water. Thirdly, the compounds retained to the precolumn were eluted by the mobile phase. The analysis was performed on the analytical column at a flow rate of 0.8 mL/min.

RESULTS AND DISCUSSION

Characteristic of the monolithic molecularly imprinted polymer: The morphology analysis of the monothic molecularly imprinted polymer was shown in Fig. 1. The results indicated many macropores and flow-through pores embedded in the network skeleton of the monolithic molecularly imprinted polymer. These macropores and channels allowed mobile phase to flow through the monolithic column with low flow resistance and thus enables fast mass transfer of the solutes.

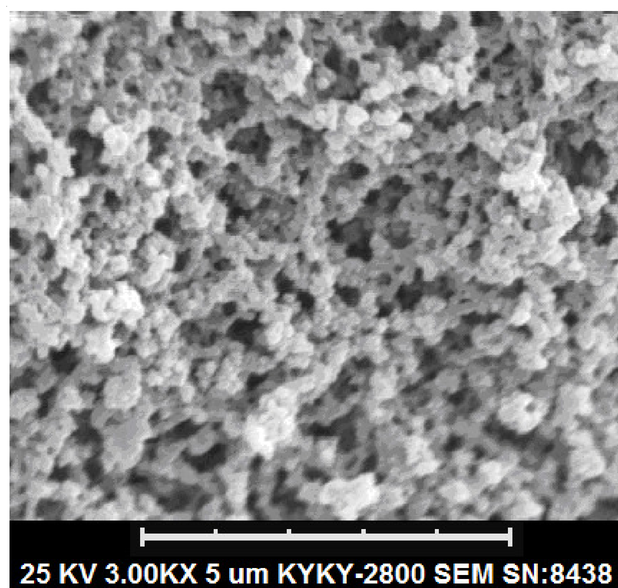

Fig. 1. Scanning electron micrograph of the ENR-imprinted monolithic HPLC column

Breakthrough curve: The study on the adsorption capacity of the monolithic molecularly imprinted polymer for enrofloxacin is helpful to verify the workability of the monolithic column to extract the analogues from the milk. Fig. 2 illustrates the breakthrough curves of enrofloxacin on the monolithic molecularly imprinted polymer and non-imprinted monolithic polymer. It can be seen that the breakthrough time is 18.13 min for the molecularly imprinted polymer and 6.04 min for the non-imprinted monolithic polymer. Considering the void volume of the column, the adsorption capacity of the monolithic molecularly imprinted polymer and non-imprinted monolithic polymer for enrofloxacin were 2.94 and 0.61 mg/mL, respectively, which indicates the specific adsorption capacity of the obtained molecularly imprinted polymer was high enough to use it as the sorbent material for solid-phase extraction.

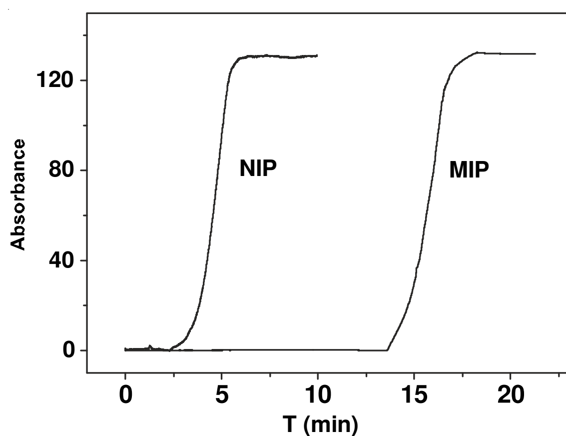

Fig. 2. Breakthrough curves of enrofloxacin on non-imprinted monolithic polymer (NIP) and monolithic molecularly imprinted polymer (MIP). Mobile phase: acetonitrile in addition of 0.10 mg/mL enrofloxacin; the flow rate: 0.8 mL/min

Preconcentration and clean-up of enrofloxacin of the milk samples: To demonstrate the potential of the monolithic molecularly imprinted polymer for preconcentration and clean-up of complex matrices, the spiked milk sample was detected by HPLC directly and on-line SPE-HPLC (Fig. 3b and c). The results show that most of the interfering compounds were removed by the purification of the fluoroquinolones extract on the monolithic molecularly imprinted polymer and the preconcentration for fluoroquinolones by on-line SPE-HPLC was obviously. The enrichment factors (EF) obtained by comparing the slopes of the linear portion of the calibration curves before and after the molecularly imprinted solid phase extraction were 6.70, 7.43 and 9.75 for enrofloxacin, ciprofloxacin and norfloxacin, respectively. It demonstrates that a real contribution of clean-up and enrichment for target analytes is obtained by the molecularly imprinted polymer monolith and it can be applied on the analysis of real sample.

Analysis of milk samples: The milk samples were purchased from markets and analyzed by the on-line SPE-HPLC. No residue of enrofloxacin, ciprofloxacin and norfloxacin was observed in any of the samples (Fig. 3a), which demonstrates that misuse of these kinds of antibiotics is not extensive. The mean recoveries of enrofloxacin, ciprofloxacin and norfloxacin in milk evaluated by three spiking samples with concentrations (0.05, 0.1 and 0.2 mg/kg) were 88.0-94.0, 82.0-89.5 and 84.0-87.8 %, respectively, with relative standard deviations (RSDs) lower than 5.6 % (Table-1). The limits of detection (LOD, S/N = 3) and the limits of quantitation (LOQ, S/N = 10) of the proposed method were 4.31 and 14.37 μg/kg for enrofloxacin, 5.39 and 17.97 μg/kg for ciprofloxacin, 7.62 and 25.40 μg/kg for norfloxacin, respectively.

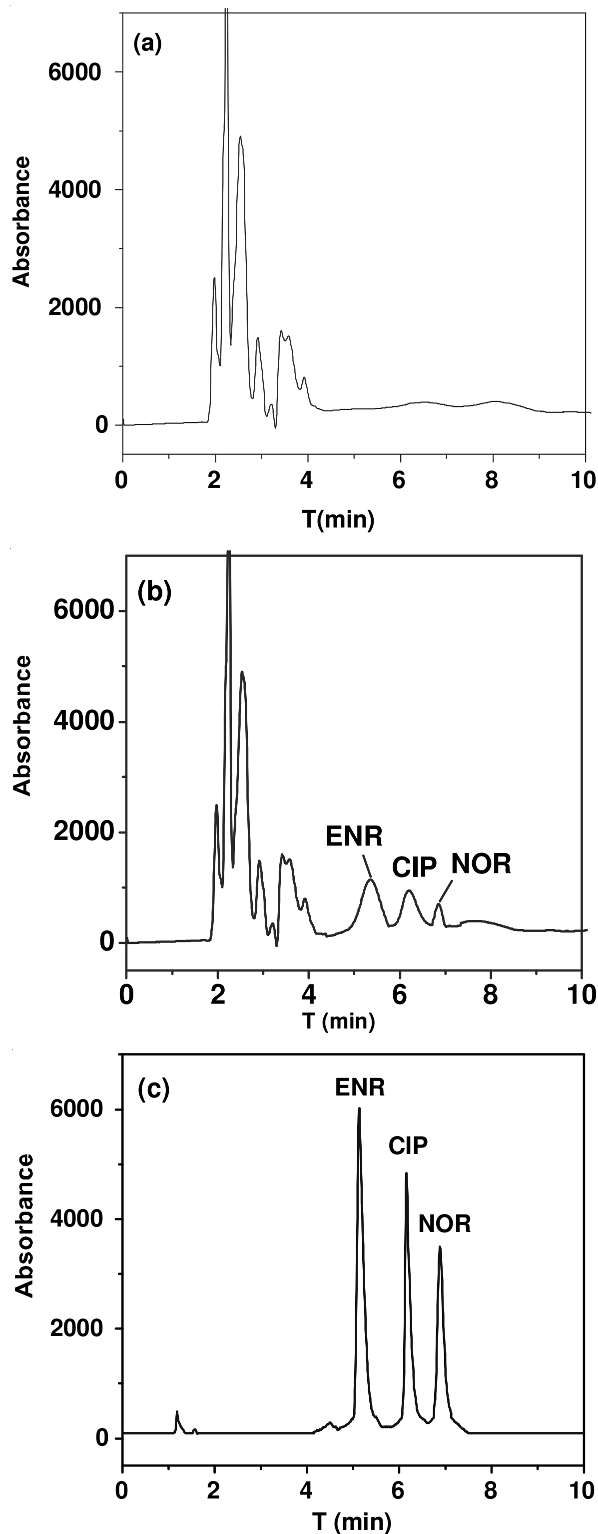

Fig. 3. Chromatograms of the blank milk (a), the spiked milk (b) the spiked milk after on-line SPE procedure (c). The mobile phase: acetonitrile-0.05 % trifluoro acetic acid (20:80, v/v); the flow rate: 0.8 mL/min; the samples spiked concentration: 0.2 mg/kg; injection volume: 10 μL

TABLE-1
AVERAGE RECOVERIES (R), RELATIVE STANDARD DEVIATION (RSDs, n = 3), LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ) OF THREE FLUOROQUINOLONES OBTAINED AFTER ON-LINE SPE OF THE SPIKED MILK SAMPLES

Analyte	Spiked level (mg/kg)	Detected (mg/kg)	R (%)	RSD (%)	LOD[a] (µg/kg)	LOQ[b] (µg/kg)
Enrofloxacin	0.05	0.044	4.7	88.0	4.31	14.37
	0.1	0.091	5.4	91.0		
	0.2	0.188	3.8	94.0		
Ciprofloxacin	0.05	0.041	5.6	82.0	5.39	17.97
	0.1	0.086	4.3	86.0		
	0.2	0.179	3.2	89.5		
Norfloxacin	0.05	0.042	4.9	84.0	7.62	25.40
	0.1	0.086	3.8	86.0		
	0.2	0.175	2.6	87.8		

[a]LOD calculated as three times the signal-to-noise ratio; [b]LOQ calculated as 10 times the signal-to-noise ratio

Conclusion

The enrofloxacin-imprinted monolithic column was prepared by *in situ* polymerization. It shows good flow-through property, high selectivity and affinity. On-line SPE-HPLC offers a promising method for selective separation and determination of the fluoroquinolone from biologic matrices.

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