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Research Article

Rapid Separation and Determination of Metronidazole Benzoate and Other Antiprotozoal Drugs by Pressurized Capillary Electrochromatography

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A novel method for the rapid separation and determination of five polar 5-nitroimidazoles in water from different sources by pressurized capillary electrochromatography has been developed. Compared with the gradient elution mode and the packed nonpolar columns which were usually utilized for the separation of 5-nitroimidazoles, a simple isocratic elution mode and low-cost homemade polar molecularly imprinted polymer monolith were used in the experiment. Electrochromatographic conditions such as pH of buffer, organic modifier, concentration of buffer, and separation voltage were optimized. At 320 nm UV wavelengths, the five 5-nitroimidazoles could be baseline-separated rapidly in less than 11 min with the separation voltage of +20 kV in 10 mmol/L sodium dihydrogen phosphate-disodium hydrogen phosphate buffer solution (pH 4.82) containing 30% acetonitrile. Under the optimum conditions, the linear ranges of the metronidazole, secnidazole, tinidazole, ornidazole, and metronidazole benzoate were 0.50–100.00, 0.50–100.00, 0.80–500.00, 0.80–100.00, and 5.00–500.00 μg/mL, respectively, and the detection limits of these analytes were 0.11–0.73 μg/mL. Column efficiencies of 43 000, 36 000, 34 000, 14 000, and 29 000 plates/m were obtained for metronidazole, secnidazole, tinidazole, ornidazole, and metronidazole benzoate, respectively. The recoveries of different water samples were about 85.0–95.8%. Additionally, the proposed method has been successfully applied to the rapid separation of 5-nitroimidazoles in the locally available pure milk sample by simple pretreatment.

1. Introduction

Structurally related antiprotozoal drugs, namely, nitroimidazole derivatives including metronidazole (MNZ), secnidazole (SNZ), tinidazole (TNZ), ornidazole (ONZ), and metronidazole benzoate (MB), etc., are a class of veterinary drugs and are used widely as antibacterial and antiprotozoan drugs in poultry and swine dysentery [1]. In some countries, they are also incorporated into feed products as feed additives to prevent diseases and promote growth in livestock and aquaculture. However, because of their potential mutagenic and carcinogenic properties, the abuse of them is very harmful to human health [2, 3]. In the EU, nitroimidazole application to food-producing animals and other products has been prohibited [4, 5]. So, a rapid, simple, and reliable analytical method is needed to prevent and control the illegal use of them. Moreover, the rapid analysis method for MB and other polar nitroimidazole derivatives still represents a challenge and holds a significant interest for separation scientists.

In addition to spectrophotometry [6–9], electrochemistry, voltammetry [10], and thin-layer chromatography [11], the methods commonly used for the separation and determination of MB or MB-mixed other compounds in various matrices, such as blood plasma, urine, drug, and aqueous solution samples, were high-performance liquid chromatography (HPLC) [12–17] and ultra performance liquid chromatography (UPLC) [18] using highly or totally aqueous mobile phases combined with nonwetted nonpolar packed columns (such as C_8 and C_{18}) and gradient elution

mode, which can lead to a number of issues such as bubble formation. They are also approach-complicated and time-consuming. Upon the literature survey, it was observed that there was no validated method for separation and determination of MB and other 5-nitroimidazoles in milk or other samples.

Capillary electrochromatography (CEC) fuses the high separation efficiency of CE with the various retention mechanisms of liquid chromatography (LC). As a high efficiency, prominent selectivity, and novel-promising miniaturized chromatography technique, CEC gains much attention in recent years [19, 20]. However, because of too high salt concentration or intrinsic limitation of the packed column, some problems such as "dry out" and bubble formation were frequently encountered in "pure" CEC. Pressurized capillary electrochromatography (pCEC) is a powerful separation system in which a mobile phase is driven by both a pressurized flow and an electroosmotic flow (EOF), which can improve stability and reproducibility of the experimental system by avoiding the above problems [21].

Molecular-imprinted polymers (MIPs) have seen increased use in contaminant or trace analysis as suitable materials for applications where analyte selectivity is essential [22]. MIP monoliths represent a novel method and a promising tool to be used in separation processes; however, an example that uses the MIP monolithic column in CEC for the separation of a group of structurally related compounds was relatively rare in the literature [23, 24]. In recent years, they all confirmed that these MIP polymers with mixed functional monomers yielded better selectivity than that of using single-functional monomers [25–27]. In our laboratory, a homemade MIP monolithic column using (S)-ornidazole as template situ polymerization of 2-hydroxyethyl methacrylate (HEMA), dimethylaminoethyl methacrylate (DMAEMA), and ethylene dimethacrylate (EDMA) was prepared [23], which offers unique pore structure with high permeability and good selectivity, and applied for rapid separation of MB and other 5-nitroimidazole derivatives in isocratic elution high pCEC for the first time. The effects of separation conditions were studied in detail, respectively. The mechanism of separation was simply discussed, and the potential applicant of this method in water and milk sample was also investigated.

2. Materials and Methods

2.1. Materials. MNZ, TNZ, ONZ, and MB were supplied by the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). (S)-ONZ was purchased from Xin An Medicine Co., Ltd (Xi'an, China). SNZ was purchased from the Xi'an Ziguang Institute of Biochemistry (Xi'an, China). Functional monomers, crosslinker, and 3-trimethoxysilyl propyl methacrylate were purchased from Aldrich (Milwaukee, WI, USA). AIBN was purchased from the Forth Chemical Reagent Plant (Shanghai, China). Toluene and dodecanol were purchased from the Tianjin Chemical Reagent Plant (Tianjin, China). Phosphate, disodium hydrogen phosphate, sodium dihydrogen phosphate, HPLC-grade methanol, and ACN were purchased from the Chemical Reagent Corporation (Shanghai, China). Each of the standard stock solutions (1.0 mg mL^{-1}) was made in methanol and stored refrigerated in dark bottles at −20°C. Water was purified with a Millipore Milli-Q purification system (Milford, MA, USA). The fused-silica capillaries with a dimension of 100 μm (ID) and 375 μm (OD) were purchased from the Yongnian Optic Fiber Plant (Hebei, China).

2.2. Preparation of MIP Monoliths. In order to improve the stability of the MIP monoliths, the inner wall of the capillary was treated with γ-MAPs, according to the procedure reported previously [28]. The mole ratio of template ((S)-ONZ) to functional monomers (HEMA and DMAEMA) was 1 : 1.5. The mole ratio of HEMA to DMAEMA was 1 : 1. The mole ratio of functional monomers (HEMA and DMAEMA) to crosslinker (EDMA) was 1 : 4. The concentration of toluene in a binary porogenic solvent mixture (toluene and dodecanol) was 12% (wt.%). According to the abovementioned ratios, (S)-ONZ, functional monomers, crosslinker, and AIBN (1 wt.% with respect to the monomers) were dissolved in porogenic solvents to form homogenous solution, which was sonicated for 15 min and purged with nitrogen for 10 min to remove oxygen. The polymerization mixture was injected into the pretreated capillary. The ends of the capillary were sealed with rubber stoppers and submerged in a 56°C water bath for 24 h. The obtained MIP monolith was washed with methanol/acetic acid (9/1, v/v) using an HPLC pump to remove the template and the residual reagents. The detection window was made by burning off 1-2 mm of the coated polymer outside after the polymer bed.

2.3. Apparatus and Procedures. The Trisep™ 2010GV pCEC system (Unimicro Technologies, Inc., Pleasanton, CA, USA) consisted of a UV/Vis detector (190–800 nm), two microvolume pumps, a high-voltage power supply (+30 and −30 kV), a microfluid manipulation module (including a six-port injector), and a data acquisition module [29]. The samples injected were delivered to the injection valve and introduced in the internal 0.8 μL sample loop and then carried to the four-port split valve by the mobile phase flow. After splitting in the four-port valve, the flow entered the capillary column under constant supplementary pressure controlled by a back-pressure regulator [26]. A positive voltage was applied to the outlet of the column, and the inlet of the column was connected to the split valve and grounded. The isocratic elution system was used in all experiments. Scanning electron micrography (SEM) of the monolith was carried out on an XL30E scanning electron microscope (Philips, The Netherlands). All the buffer solutions were prepared using double-distilled water and filtered through 0.22 μm membrane. Before use, mobile phase solution was degassed in an ultrasonic bath for 20 min. Applied voltage was firstly ramped from 0 to + 10 kV (or + 20 kV) and then operated at +10 kV (or +20 kV). UV detection was performed at 320 nm. Before pCEC experiments, the monolith was conditioned with the

mobile phase for 1 h and equilibrated for about 30 min after every mobile phase changed.

2.4. Sample Preparation. Take 5 mL of the milk sample in a 50 mL centrifuge tube and then add 20 mL of aqueous trichloroacetic acid (2%, v/v). After 1 min of vortex shaking, place in an ultrasonic bath for 20 min, shake for 10 min, and then centrifuge for 10 min at 4000 rpm. Filter the supernatant with a 0.22 μm membrane for pCEC analysis.

3. Results and Discussion

3.1. Characteristics and Selectivity of the MIP Monolith. Based upon our experiments, a low-polar porogen mixture consisting of toluene-dodecanol was found to yield a suitable, rigid porous MIP monolith. The SEM images show aggregates of nanometer-sized globular particles which are surrounded by interconnected large through-pores that permit bulk flow through the capillary (Figure 1). The small size of the globular units offers good diffusion properties, and thus the rapid access of the solute to the imprinted sites in globules gives a shorter separation time. Furthermore, the covalent attachment points of the polymer to the capillary wall can also be observed, which assured definite stability of the stationary phase.

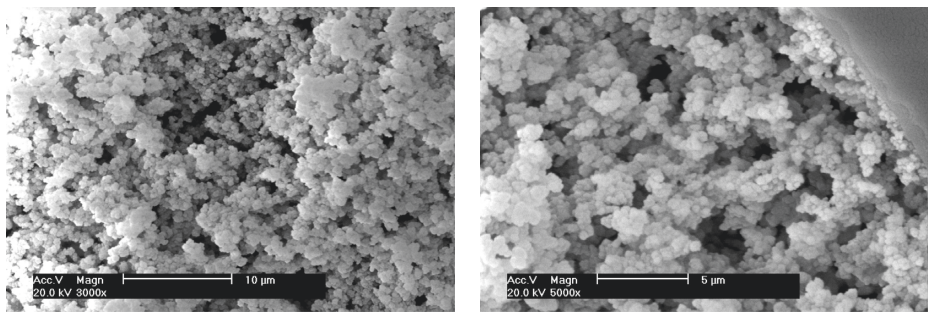
A nonimprinted polymer (NIP) monolith (in the absence of template) was prepared and treated in an identical manner with MIP monolith. In our experiment, the NIP monolith was found to have less flow resistance than the MIP monolith. The selectivity of the MIP monolith was assessed by parallel experiments using MIP and NIP monoliths (Figure 2). Shorter retention time and poorer resolution for the five 5-nitroimidazole drugs in NIP monolith were observed (Figure 2). The experiment results also indicated that the selectivity depended on not only chromatographic retention but also imprinting cavities on the MIP monolith. These results confirmed that the template molecule has a great influence on the morphology and performance of the result columns. Deng et al. have also observed the same phenomena [30].

3.2. Optimization of the Chromatographic Separation

3.2.1. Effect of the pH. The effect of the pH value of buffer solution on the molecular recognition abilities was studied by varying the pH value of sodium dihydrogen phosphate-disodium hydrogen phosphate solutions from 4.50 to 5.80 in aqueous solution before mixing with 30% ACN (Figure 3). The pKa values of the studied 5-nitroimidazole drugs (MNZ pKa 2.52, SNZ pKa 1.16, TNZ pKa 1.82, and ONZ pKa 2.3) were lower than 3.0. The pKa value of DMAEMA is about 8.4, and the HEMA is a neutral compound. When the pH value of buffer solution was in the range from 4.50 to 5.80, the studied 5-nitroimidazole drugs remained unprotonated and had a migration nearly identical to the neutral solute. The DMAEMA in the polymer matrix is a main EOF promoter. With the increase of the pH value of the buffer solution from 4.50 to 5.80, the protonated degree of DMAEMA slightly decreased, thus causing the decrease of the EOF and the longer retention times. The increase of the pH is beneficial to the hydrogen-bonding formation between the analyte and functional groups in the imprinted cavities thus the ability of molecular recognition increased, and then the resolution between MNZ and SNZ increased. So, pH 4.82 was chosen because it provided the appropriate resolution and analysis time.

3.2.2. Effect of ACN Concentration. In pCEC, the organic modifier in the mobile phase is an important parameter. ACN is the most widely used organic modifier in pCEC. The effect of the mobile phase of ACN concentration on separation of the five 5-nitroimidazole drugs was studied, and different concentrations of ACN, in the range of 30–70% (v/v), were added to a 10 mM sodium dihydrogen phosphate-disodium hydrogen phosphate solution with pH 4.82 (Figure 4). It is well known that the hydrogen-bonding interactions in the process of molecular recognition are stronger with the increase of ACN concentration in the hydro-organic mobile phase. However, with the increase of ACN concentration, the decrease of the viscosity of the solution and the increase of zeta potential caused the increase of EOF; so the retention times was shorter, resulting in a poorer recognition. In addition, it could be seen from Figure 4 that the separation degree and retention of 5-nitroimidazole drugs decreased with the increase of ACN concentration. From Figure 4, with the decrease of ACN concentration, it can be seen that MB has longer retention time than ONZ, and the difference of retention time between MB and ONZ also increases. From Figure 2 (MIP monolith and NIP monolith), it can be seen that MB has always longer retention time than ONZ. It can be deduced that the retention of 5-nitroimidazole drugs was due to hydrophobic interaction, hydrogen-bonding and electrostatic interactions with the imprint cavities in the polymer. So, 30% ACN was the optimized organic content.

3.2.3. Effect of Applied Voltage. The effect of applied voltage on the separation performance of the column was investigated by applying voltages +5 kV, +10 kV, +15 kV, and +20 kV, using the mobile phase containing 30% ACN in 10 mM sodium dihydrogen phosphate-disodium hydrogen phosphate solution with pH 4.82 under constant pressure (Figure 5). Methanol was used as the marker of the EOF mobility. The EOF linearly increased with the increase of the voltage from +5 kV to +20 kV with a correlation coefficient of 0.9428, which suggested the Joule heating was negligible in the system under the experimental conditions. When the EOF velocity was faster, the mobility times of analytes would be shorter and rapid separation could be realized on the MIP monolith (Figure 5). However, too high voltage would cause large current and lead to a greater Joule heating. So, +20 kV was the optimized voltage. Good flow characteristics of the MIP monolith and a supplement pressure of 1000 psi avoid air bubble formation. Under the optimum conditions, the column efficiency (plates/m) was 43 000 for MNZ, 36 000 for SNZ, 34 000 for TNZ, 14 000 for ONZ, and 29 000 for MB.



(a) (b)

Figure 1: SEM of the end of the MIP monolithic stationary phase in a fused-silica capillary column.

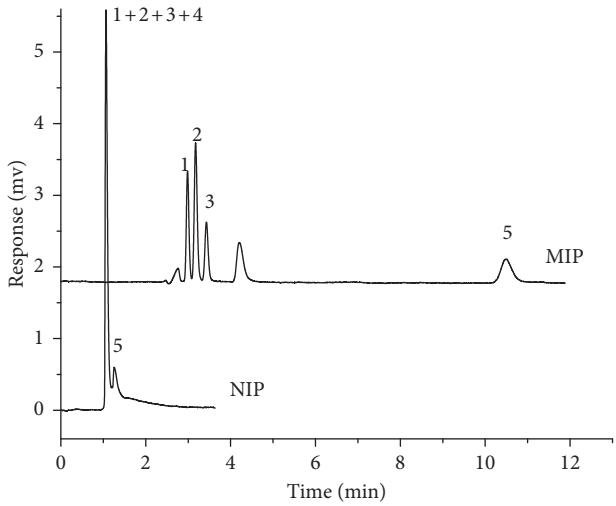


Figure 2: Comparison between MIP monolith and NIP monolith. Capillary column: 51 cm column length; 30 cm effective length. Electrochromatographic conditions: mobile phase, ACN/10 mM sodium dihydrogen phosphate-disodium hydrogen phosphate buffer (30/70, v/v, pH 4.82); voltage: +20 kV; flow rate: 0.1 ml/min; supplement pressure: 1000 psi. 1 MNZ, 2 SNZ, 3 TNZ, 4 ONZ, and 5 MB.

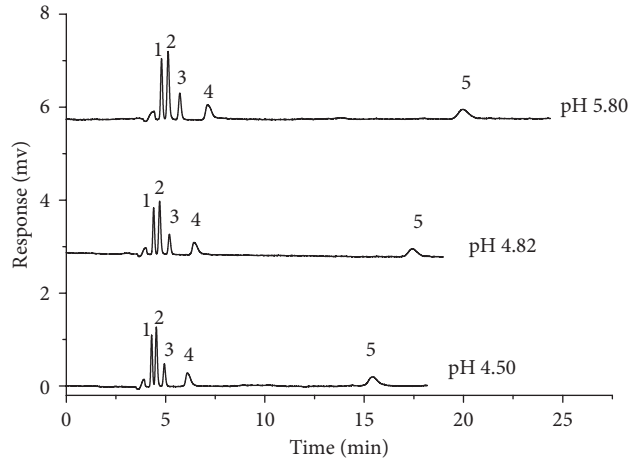


Figure 3: Effect of pH of buffer solution on separation of 5-nitroimidazole compounds. MIP monolith: 51 cm column length; 30 cm effective length. Electrochromatographic conditions: mobile phase, ACN/10 mM sodium dihydrogen phosphate-disodium hydrogen phosphate buffer (30/70, v/v); voltage: +10 kV; flow rate: 0.1 ml/min; supplement pressure: 1000 psi. 1 MNZ, 2 SNZ, 3 TNZ, 4 ONZ, and 5 MB.

3.3. Linear Response Range, Detection Limit, and Precision. Under the optimized experimental conditions, the linear regression analysis of five 5-nitroimidazoles was constructed by plotting the peak area against analyte concentration. The linear ranges, correlation coefficients, relative standard deviations, and the detection limits are summarized in Table 1. The reliability and reproducibility of the chromatographic performance of the MIP monolith was examined using 0.05 mg/mL of five 5-nitroimidazoles for four repeated injections, and the percent of relative standard deviation (RSD) values illustrated that the results were highly reproducible.

3.4. Application for Separation of 5-Nitroimidazoles in Real Water Sample. In order to assess the specificity of the method, a water blank sample was analysed. No interference peaks were observed around the retention time of the 5-nitroimidazoles. These results demonstrate that real sample

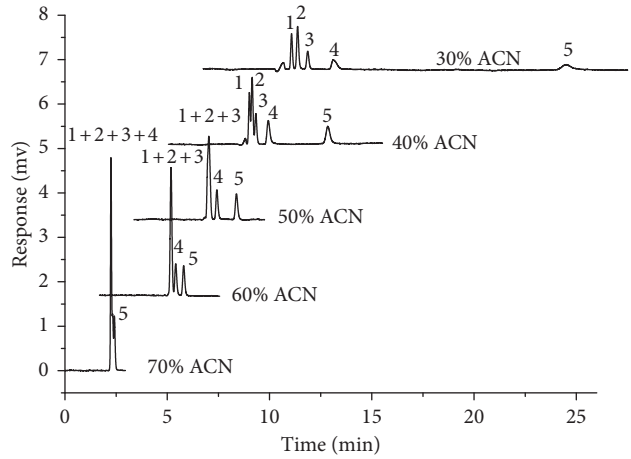


Figure 4: Effect of content of ACN on separation of 5-nitroimidazole compounds. Electrochromatographic conditions: mobile phase: ACN/10 mM sodium dihydrogen phosphate-disodium hydrogen phosphate buffer (pH 4.82). Other conditions and the order of peak can be seen in Figure 3.

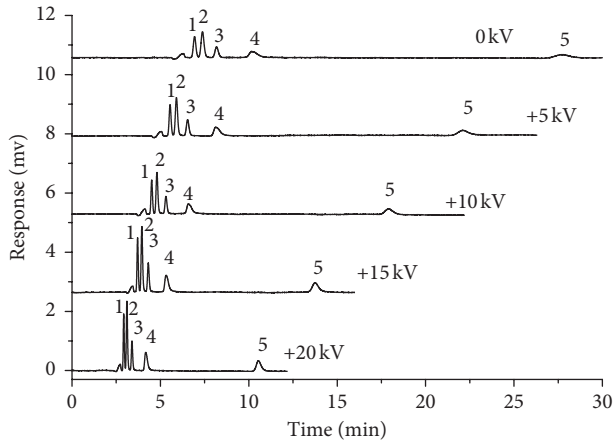


Figure 5: Effect of the applied voltage on separation of 5-nitroimidazole compounds. Electrochromatographic conditions: pH 4.82 and applied voltage 0 kV, +5 kV, +10 kV, +15 kV, and +20 kV. Other conditions and the order of peak can be seen in Figure 3.

Table 1: Linear range, detection limit, and precisions.

Analyte	Linear range (μg/mL)	Correlation coefficient (R^2)	Detection limits[a] (μg/mL)	% RSD time	% RSD area
MNZ	0.50–100	0.9952	0.11	1.0	1.69
SNZ	0.50–100	0.9952	0.15	1.21	2.11
TNZ	0.80–500	0.9990	0.22	1.47	1.48
ONZ	0.80–100	0.9984	0.13	1.72	2.37
MB	5–500	0.9955	0.73	2.57	1.84

[a]Calculated as three times the signal-to-noise ratio.

matrices had no effect on the performance of the proposed method. In order to verify the applicability of the MIP-pCEC method, the developed method was applied to the analysis of 0.05 mg/mL 5-nitroimidazoles mixture in the river (Minjiang River, Fuzhou, China) and pond water samples (Pond near Fuzhou University, Fuzhou, China), respectively. Each water sample spiked with five kinds of 5-nitroimidazoles was filtered through a 0.45 μm membrane and then analysed using the optimized chromatographic conditions. Recoveries were estimated by comparing the concentrations from spiked water samples determined by the calibration equations and the corresponding added concentrations. For the river water sample, the recoveries were found to be 93.2% for MNZ, 95.8% for SNZ, 85.0% for TNZ, 93.6% for ONZ, and 90.6% for MB, respectively. For the pond water sample, the recoveries were found to be 95.0% for MNZ, 95.8% for SNZ, 86.5% for TNZ, 93.7% for ONZ, and 94.6% for MB, respectively. The RSDs for four repeated injections were less than 3.8%.

3.5. Application for Separation of 5-Nitroimidazoles in Milk Sample. In order to enlarge the applications of the obtained MIP monolith, the monolith was applied to the rapid separation of five 5-nitroimidazole drugs in the locally available pure milk sample (AoNiu). The sample was simple pretreated according to the procedure stated in Section 2.4, and the analysis was performed using the optimized chromatographic conditions. The chromatogram is shown in Figure 6. The added standards were successfully separated from the milk sample. The result confirmed that the development method for the fast separation of the real sample is feasible (Figure 6).

4. Conclusion

In the present work, a homemade MIP monolith which contained polar tertiary amine groups and alcohol groups in the imprinted cavities was firstly and successfully applied to the rapid separation and determination of five 5-nitroimidazoles in water samples and milk samples. The effects of electrochromatographic conditions, such as voltage, the composition of the mobile phase on the separation of 5-nitroimidazoles have been systemically investigated and also suggested that the separation mechanism of analytes on the MIP monolith was mainly based on the interplays of hydrophobic, hydrogen-bonding, and electrostatic interactions and imprinting cavities on the MIP monolith. Under the optimized conditions, five 5-nitroimidazoles could be baseline-separated by a simple isocratic elution and high pCEC mode within 11 min on the MIP monolith. The whole procedures demonstrated a good recovery, and the MIP monolith is robust enough after more than one month and 300 electrochromatographic runs. Compared with the present method for separation of nitroimidazole drugs, the proposed method shows its advantages in the simple of column preparation, environmental-friendly, low cost, fast analysis, and high efficiency. However, due to the low concentrations of 5-nitroimidazoles in some real analytical samples with environmental and biological origin, our

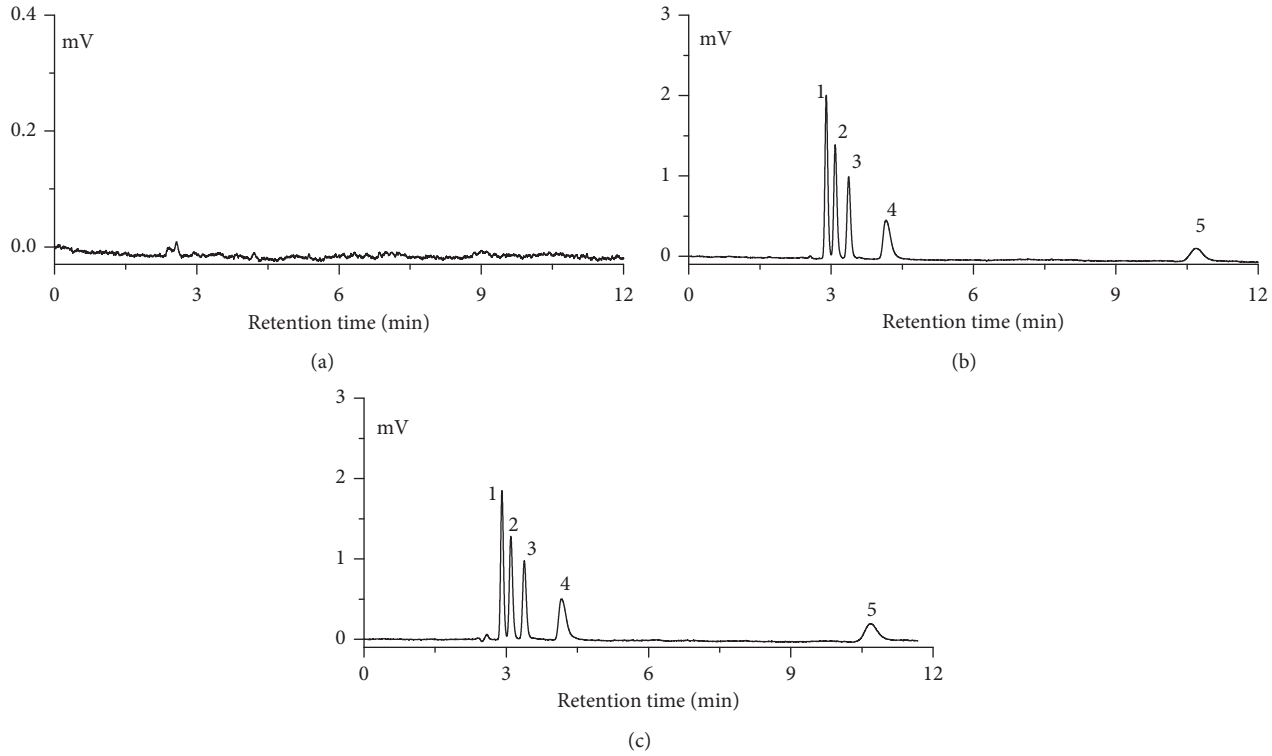


Figure 6: Separation of five 5-nitroimidazole drugs in the milk sample. Electrochromatographic conditions: pH 4.82; voltage: +20 kV. Other conditions and the order of peak can be seen in Figure 3. (a) Blank milk sample. (b) Spiked milk sample. (c) Standard sample.

future work will explore the combination of the present method with other sensitive detections or preconcentration.

Data Availability

The data used to support the findings of this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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