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RESEARCH PAPER

Potentiometric Sensor Based on Molecularly Imprinted Polymers for Rapid Determination of Clenbuterol in Pig Urine

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Abstract: A polymeric membrane ion-selective electrode for determination of clenbuterol is described. It is based on a molecularly imprinted polymer as an ionophore for molecular recognition, which can be synthesized by precipitation polymerization using clenbuterol hydrochloride as the template molecule. Under optimized conditions, the proposed membrane electrode exhibits Nernstian response to the protonated clenbuterol over the concentration range of 1.0×10^{-7} M to 1.0×10^{-4} M with a slope of 55.7 mV/dec., and a detection limit of 7.0×10^{-8} M. The MIP-based sensor shows excellent selectivity, rapid response time and satisfactory long-term stability. The potentiometric sensor has been successfully applied to the determination of clenbuterol in pig urine samples with recoveries between 98% and 107% and an analysis time of less than 3 min.

Key Words: Molecularly imprinted polymer; Potentiometric sensor; Clenbuterol

1 Introduction

Clenbuterol, ractopamine and salbutamol are commonly named “lean meat powders”, which can be used to increase animal growth rates and lean meat content. Among them, clenbuterol is the most widely used “lean meat powder” in China. Clenbuterol is a beta-adrenergic drug normally employed as a bronchial dilating agent for the treatment of pulmonary diseases, such as asthma. It was later discovered that clenbuterol can promote the growth of animal muscle and reduction of body fat when it is used as an additive in animal feed. However, it has been found that the residues of clenbuterol that accumulate in animal tissues can affect liver and heart functions and even cause death in persons who have eaten meat contaminated with clenbuterol[1]. Especially, the 2011 clenbuterol-tainted-meat incident in China led to a serious concern about clenbuterol. Therefore, it is highly desirable to develop analytical methods for the determination of clenbuterol in bio-samples.

In recent years, various techniques have been reported for clenbuterol detection, such as high-performance liquid chromatography (HPLC)[2], gas chromatography coupled with mass spectrometry (GC-MS)[3], gold immunochromatographic assay (GICA) and enzyme-linked immunosorbent assay (ELISA)[4]. Among these techniques, HPLC and GC-MS are the most used, but require expensive apparatus and tedious procedures. Although GICA is a relatively rapid method, it is susceptible to interferences and exhibits a high false-positive rate. ELISA is used mainly for qualitative rather than quantitative analysis. Nowadays, chemical sensors have attracted considerable attention because of their favourable portability, simple operation and suitability for on-site

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monitoring. With the potentiometric transduction mode, polymeric membrane ion selective electrodes (ISEs) have found widespread use in industrial, clinical and environmental analysis owing to their advantages such as good selectivity, low cost of preparation, rapidity and easiness of measurement and high reliability[5-7]. However, despite these advantages, none has been reported for determination of clenbuterol by using ISE.

Molecular imprinting is a unique technique used for preparing polymers with synthetic recognition sites having a predetermined selectivity for analytes of interest[8]. In recent years, molecularly imprinted polymers (MIPs) have gained wide acceptance as new molecular recognition materials in chemical sensors, since they are more stable, less costly and easier to produce compared to their biological counterparts, including antibodies and enzymes. Thus, MIP would be a promising alternative ionophore for potentiometric analysis in complex samples. In this paper, an ISE based on MIP as ionophore is described for determination of clenbuterol. It will be shown that the proposed potentiometric sensor enables the detection of clenbuterol in pig urine samples in a rapid, selective and sensitive way.

2 Experimental section

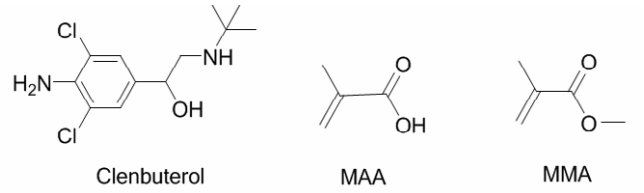
2.1 Instruments and reagents

Electromotive force (EMF) values were measured by a PXSJ-216 pH meter (Shanghai, China). Ultraviolet absorption spectrometric measurements were conducted with a Beckman DU-800 UV spectrophotometer. The molecularly imprinted polymer (MIP) was characterized by a field emission scanning electron microscopy (FE-SEM, Hitachi S-4800).

Clenbuterol hydrochloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. Methacrylic acid (MAA), methyl methacrylate (MMA), trimethylolpropane trimethacrylate (TRIM), divinylbenzene 80 (DVB 80), high-molecular-weight poly(vinyl chloride) (PVC), *o*-nitrophenyl octyl ether (*o*-NPOE), sodium tetrakis [3,5-bis(trifluoro-methyl) phenyl]borate (NaTFPB), tetrahydrofuran (THF) and ractopamine hydrochloride were purchased from Sigma-Aldrich. 2,2'-azobisisobutyronitrle (AIBN) was obtained from Tianjin Kermel Chemical Reagent Co., Ltd, China. Aqueous solutions were prepared with freshly deionized water (18.2 MΩ cm specific resistance) obtained with a Pall Cascada laboratory water system. A stock solution of 1×10^{-4} M clenbuterol was made by dissolving 31.4 mg of clenbuterol hydrochloride in 1 L of 5 mM Tris-HCl buffer solution of pH 7.4. Clenbuterol is a weak base with a pK_a of 9.6, and is readily protonated in aqueous solution at pH levels lower than 9.6. MAA, TRIM, DVB 80 and THF were distilled in vacuum prior to use. AIBN was re-crystallized from methanol. All other reagents were of analytical grade and used without any further purification.

2.2 Synthesis of MIPs

The clenbuterol MIP beads were synthesized by the precipitation method as described elsewhere[9]. The synthesis processes are as follows: the template clenbuterol (0.4 mmol), functional monomers MAA (2.5 mmol) and MMA (0.83 mmol), cross-linkers DVB 80 (0.4 mmol) and TRIM (1.0 mmol) and free-radical initiator AIBN (0.5 mmol) were dissolved in 40 mL of methanol in a 45-mL flask and sonicated for 10 min to maintain homogeneity. Then, the solution was purged with a gentle flow of N_2 for 10 min and sealed under N_2 atmosphere. Polymerization was carried out by placing the flask in an oil bath at 70 °C for 24 h. After polymerization, the template was removed by batch-mode solvent extraction with methanol/acetic acid (9:1, *V/V*) and methanol until no absorption of the methanol was observed at 245 nm. The resulting polymer was dried in vacuum overnight at 50 °C. Non-imprinted polymer (NIP) was prepared under identical conditions except for omission of the template clenbuterol. The structures of clenbuterol, MAA and MMA are shown in Scheme 1.



Scheme 1 Chemical structures of clenbuterol, MAA and MMA

2.3 Preparation of polymeric membranes and ISEs

The membrane contained MIP or NIP (5.0%, *w/w*), NaTFPB (1.0%, *w/w*), NPOE (62.7%, *w/w*) and PVC (31.3%, *w/w*). The components of each membrane (totaling 360 mg) were dissolved in THF (3.5 mL) and poured into a glass ring (i.d. 36 mm) fixed on a glass plate. Overnight evaporation of the solvent yielded membranes with a thickness of ~200 μm. For each electrode, a disk of 9-mm diameter was punched from the membranes and glued to a plasticized PVC tubing with THF. For measurements of clenbuterol, the internal filling and conditioning medium were 0.1 mM clenbuterol in 5 mM Tris-HCl buffer of pH 7.4, while for measurement of experimental selectivity coefficients, 0.01 M NaCl was used as the internal filling and conditioning medium. All the electrodes were conditioned in a solution identical to the inner filling solution for one day before measurement.

2.4 EMF measurements

All measurements of EMF were performed at 20–21 °C

using a PXSJ-216 pH meter with a saturated calomel electrode (SCE) as the reference electrode in the galvanic cell: SCE//sample solution/ISE membrane/inner filling solution/3.0 M KCl/AgCl/Ag. Clenbuterol standards were prepared by consecutively diluting 0.1 mM of clenbuterol with 5 mM Tris-HCl buffer of pH 7.4. Selectivity coefficients were determined by the separate solution method. All the EMF values were corrected for the liquid-junction potential according to the Henderson equation. The activity coefficient of ions, γ, was calculated from the modified Debye-Hükel equation.

3 Results and discussion

3.1 Characterization of MIP and NIP

The micrographs of the clenbuterol MIP and NIP beads prepared by the precipitation polymerization method were investigated by scanning electron micrography (SEM). As shown in Fig.1a, the MIP beads are spherical with a diameter distribution of 0.5–1.0 μm. As previously reported by our group[10], the uniform-sized beads can be well dissolved in the polymeric ISE membrane, which could cause more binding sites available in the membrane and lower membrane impedance. Indeed, the membrane ISE prepared with the uniform clenbuterol MIP beads showed a shorter response time and lower noise levels. The SEM images also indicate the NIP beads prepared with the same recipe have the similar morphological structure and particle size distribution (Fig.1b).

3.2 Optimization of Membrane Composition

The membrane composition has a profound impact on the sensing performance of polymeric membrane ISE. The proposed ion-selective membrane composition consists of MIP, cation-exchanger NaTFPB, plasticizer and PVC. Among them, MIP with binding cavities used as ionophore in the polymeric membrane selectively extracts clenbuterol molecules into the organic membrane phase via the strong hydrogen bonding between the carboxyl groups of poly(MAA-co-MMA) and the amino group of clenbuterol. The lipophilic salt NaTFPB can decrease the membrane resistance, reduce anion interference and improve selectivity of the electrode. However, it should be noted that NaTFPB is a cation exchanger that itself could induce a selective response if no or only an insufficient amount of ionophore is present. The plasticizer, the membrane solvent, not only dissolves ionophore and ion-exchanger and gives a homogeneous organic phase but also influences the dielectric constant (ε_r) of the membrane phase and the mobility of the ligands and their complexes. PVC is used as the membrane matrix, which provides for mechanical stability and elasticity. Table 1 shows the effect of membrane composition on the potential response of the MIP-based electrode. As can be seen, the response slope of the MIP-based membrane can be improved to be nearly theoretical with the lipophilic NaTFPB. Increasing the NaTFPB concentration to 1.0% shows optimal response towards clenbuterol. Further increase of the NaTFPB results in an elevation of detection limit. This elevation is attributed to the increase of the ion flux across the membrane with increasing ion-exchanger concentration. The sensitivity and linearity of the membrane electrode also depends on the amount of MIP, which determines the number of binding sites. It can be seen that the membrane exhibits a wider linear range upon increasing the amount of the MIP, probably because of the increase in the number of binding sites, which plays an important role in the sensitivity and linearity of the membrane electrode. However, at amounts above 5.0%, the ion-selective membrane becomes opaque, which suggests that part of the MIP may become insoluble and cannot be dispersed uniformly in the plasticized membrane. Such an insolubility problem might cause a low electrical conductivity and poor response behavior. In addition, the potential response of the MIP-based ISE is highly influenced by the membrane solvent. The change in plasticizer from polar *o*-NPOE (ε_r = 24.0) to apolar

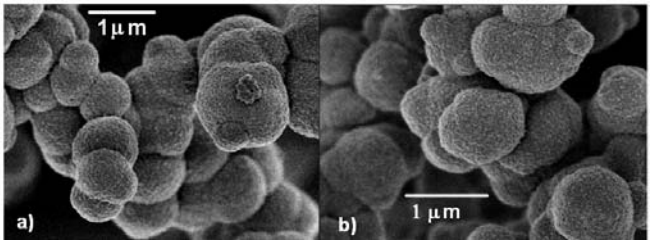


Fig.1 SEM images of obtained (a) clenbuterol-MIP and (b) NIP beads

Table 1 Influence of membrane composition on electrode response

PVC	Plasticizer (%, *w/w*)			NaTFPB	MIP	Linear range	Slope[a]
(%, *w/w*)	NPOE	DOS	DOP	(%, *w/w*)	(%, *w/w*)	(M)	(mV decade^{-1})
31.7	63.3	-	-	0	5	1×10^{-6}–1×10^{-5}	32.2 ± 1.0
31.3	62.7	-	-	1	5	1×10^{-7}–1×10^{-4}	55.7 ± 0.5
31.2	62.3	-	-	1.5	5	5×10^{-7}–1×10^{-4}	54.4 ± 0.5
33.0	66.0	-	-	1	0	1×10^{-6}–1×10^{-4}	55.8 ± 0.8
30.2	60.3	-	-	1	8.5	1×10^{-6}–1×10^{-4}	51.0 ± 0.4
31.3	-	62.7	-	1	5	5×10^{-7}–1×10^{-4}	56.3 ± 0.7
31.3	-	-	62.7	1	5	5×10^{-7}–1×10^{-4}	51.7 ± 0.5

[a] Average from three measurements ± standard deviation. NPOE, nitrophenyl octyl ether; DOP, dioctyl phthalate; NaTFPB, sodium tetrakis [3,5-bis(trifluoro-methyl) phenyl]borate.

DOS (ε_r = 4.8) and DOP (ε_r = 5.0) leads to a narrower linear range. This influence is probably due to the fact that the proposed ISE exhibits higher selectivity to clenbuterol cations with polar solvents. It has been found that the membrane with the composition 31.3t% of PVC, 62.7% of *o*-NPOE, 1.0% of NaTFPB and 5.0 % of MIP shows the best performance.

3.3 Effect of sample pH

The influence of sample pH on the potential response of MIP-based clenbuterol ISE was tested with clenbuterol of 0.1 mM in the range pH 1.0–11.0 adjusted with HCl and NaOH. The results are shown in Fig.2. It can be seen that the potential response is nearly constant in the range pH 7.0–8.5, but potential decreases appear at pH levels beyond this range. This is probably due to the fact that at pH > 8.5, clenbuterol exists mainly as a neutral molecule, which shows no potential response at ISE; but at pH < 7.0, clenbuterol can be hydrolyzed[11], which leads to the decrease in the amount of protonated clenbuterol ions.

3.4 Calibration curve

The potential response curves of MIP- and NIP-based membranes and the blank membrane (only with NaTFPB) are shown in Fig.3. The MIP-based membrane shows a near-Nernstian response of 55.7 mV/decade over the concentration range of 1.0×10^{-7}–1.0×10^{-4} M with a detection limit of 7.0×10^{-8} M. The better performance of MIP-based membranes is probably due to the specific interaction of protonated clenbuterol ions with the recognition sites of MIP in the polymeric membrane. However, for the NIP-based and blank membranes that exhibit a near-Nernstian response in a rather narrow concentration range, only the nonspecific interaction of the clenbuterol ions with the ion-exchanger occurs. Evidently, it can be demonstrated that the MIP is effective for specific recognition of the target ions.

3.5 Interference study

Since urine samples contain rather high concentrations of interfering ions, it is necessary to consider the interference of co-existing ions in urine samples. The selectivity of the MIP-based ISE was characterized by using Bakker's method to evaluate the influence of the discriminated ions[12]. The selectivity coefficients $K_{M,X}^{pot}$ for clenbuterol ions (M) over other cations (X) were estimated according to the Nicolskii-Eisenman equation[13]. Potentiometric selectivity coefficient values for the MIP- or NIP-based ISE are summarized in Table 2. It can be seen that the proposed electrode shows high selectivity for clenbuterol ions over most inorganic and organic amine cations normally found in urine. The logarithmic Nikolskii coefficients for clenbuterol ions over Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+ and ractopamine cations have been determined as –7.57, –5.94, –7.43, –7.78, –6.17 and –2.50, respectively. The selectivity coefficients of the proposed MIP membrane electrode over interfering ions such as Na^+ and K^+ are superior to those of the NIP membrane electrode because of the specific recognition of the MIP for the target ions.

3.6 Response time and long-term stability

The response time of the MIP-based membrane ISE is defined as the average time required for the sensor to reach 95% of the magnitude of the equilibrated potential signal after successively being immersed in a series of clenbuterol ion solutions, each having a 10-fold concentration difference. The dynamic potential response with time is shown in Fig.4, where the clenbuterol concentration is changed between 0.01 mM and 0.1 mM. It can be seen that the response of the MIP sensor is rapid (< 5 s) and fully reversible. Experiments also

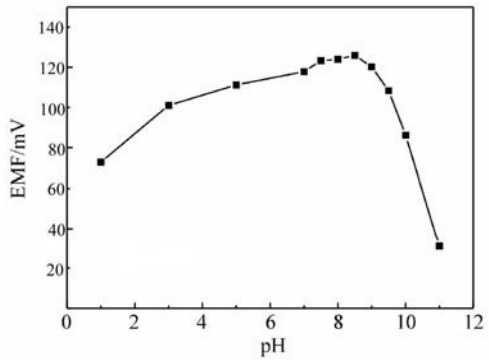


Fig.2 Effect of sample pH on electrode response of 0.1 mM clenbuterol

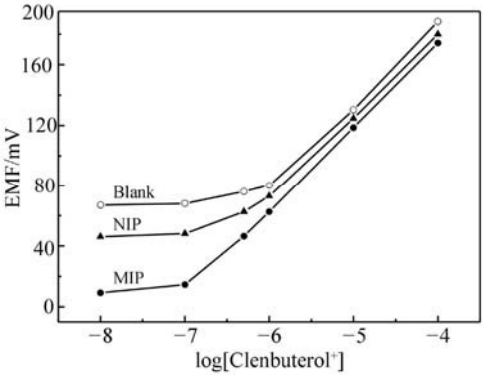


Fig.3 Calibration curves for clenbuterol-MIP, NIP and blank membranes

Table 2 Potentiometric selectivity coefficients $K_{M,X}^{pot}$ for the MIP-based clenbuterol ISE (n = 3)

Interfering ion, X	$\log K_{M,X}^{pot}$	Interfering ion, X	$\log K_{M,X}^{pot}$
Na^+	-7.57 (-6.28) [a]	Cu^{2+}	-7.35
K^+	-5.94 (-4.96) [a]	Zn^{2+}	-7.84
H^+	-6.86	Ractopamine$^+$	-2.50
Ca^{2+}	-7.43	Ethylenediamine^{2+}	-6.06
Mg^{2+}	-7.78	NH_4^+	-6.17

[a] Values in parentheses are selectivity coefficients for NIP membrane electrode.

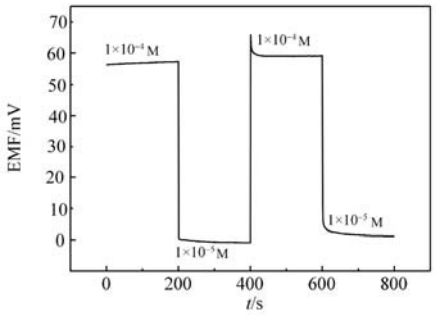


Fig.4 Dynamic potential response of MIP-based ISE

showed that the relative standard deviation (RSD) of the MIP-based clenbuterol ISE was 6.4% after the electrode was stored dry in the refrigerator (4 ºC) for one month.

3.7 Analysis of spiked urine samples

The proposed MIP-based ISE was used to analyze clenbuterol in spiked pig urine samples which were filtered through 0.22 μm-pore-size filters. The results are given in Table 3. It can be seen that the recoveries of urine samples vary from 98% to 107%, indicating that the present MIP-based sensor is can be used for reliable determination of clenbuterol in pig urine samples. The whole analysis time, including sample filtration, was less than 3 min. The proposed methodology has great promise for accurate and rapid determination of clenbuterol in pig urine samples.

4 Conclusions

A new polymeric membrane ion-selective electrode based on MIP for determination of clenbuterol has been established. The proposed sensor exhibits excellent characteristics, including wide dynamic range, high selectivity, short response time and low detection limits, which are required for accurate and rapid monitoring of trace clenbuterol in pig urine samples. The proposed MIP-based sensor is promising for use of detection of clenbuterol contamination in other biofluids such as blood, tissue and intracellular samples.

Table 3 Application of the proposed electrode to determination of clenbuterol in pig urine samples spiked with different amounts of clenbuterol

Sample	Concentration of clenbuterol (μg L^{-1})		Recovery (%)
	Amount added	Amount found[a]	
1	27.7	29.6 ± 1.6	107
2	83.1	84.7 ± 3.3	102
3	138.5	135.4 ± 4.5	98
4	166.2	171.2 ± 4.4	103

[a.] Average from three measurements ± standard deviation.

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