Development of a Biomimetic Enzyme-linked Immunosorbent Assay Method for the Determination of Methimazole in Urine Sample

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A fast and direct competitive biomimetic enzyme-linked immunosorbent assay (BELISA) method was developed for the determination of methimazole (MMZ) in urine sample based on a molecularly imprinted film as an artificial antibody. This is the first example to monitor methimazole with a direct competitive biomimetic enzyme-linked immunosorbent assay (BELISA) method. The imprinted film was directly synthesized on the well surface of MaxiSorp polystyrene 96-well plate and characterized. The results showed that it exhibited an antibody-like binding ability, rapid adsorption speed, high stability, which was particularly advantageous and suitable for BELISA development. The BELISA method established in this paper had a higher selectivity for MMZ than for the structurally related compounds and the IC_{50} (calculated as the concentration giving 50% inhibition of color development) and the detection limit values under optimized experimental conditions were 70 ± 4 μg L^{-1} and 0.9 ± 0.04 μg L^{-1}, respectively. The method was applied to the determination of MMZ in spiked urine sample with excellent recoveries ranging from 90% to 95%, and the imprinted film was able to be reused for 20 times without loss of sensitivity. The results obtained by BELISA correlated well with that obtained by the high performance liquid chromatography (HPLC) method.

Keywords: Methimazole; Molecularly imprinted film; BELISA; HPLC.

INTRODUCTION

MMZ (1-methyl-2-mercaptoimidazole, tapazole) (Fig. 1) is a kind of antithyroid drugs. Its action is to slow iodide integration into tyrosine and thus inhibits the production of thyroid hormones. So it is widely used in medicine for treatment of hyperthyroidism.[1] For it can promote the metabolism of protein, it has been applied illegally to animals' feed to obtain more lean meat. Although MMZ has been used in the treatment of hyperthyroidism, the uncontrolled introduction of this and other thyreostats into the human's food chain could have serious health implications.[2] MMZ may cause side effects such as the irritation of skin, impaired taste, olfaction, allergies or pharyngitis with fever, and in rare occasions, nephritis and liver cirrhosis.[3-4] Consequently, the use of MMZ in animal production has been prohibited in many countries including European Union.[5]

So far, various analytical methods in TLC,[6] GC-MS,[7-8] HPLC-UV[9-11] or MS[12-13] and CE[14-15] have been described for the effectively monitoring MMZ in kinds of samples including urine,[7-11] plasma,[6] tissues[9,12-13] and serum.[15] Flow injection analysis,[15] electrochemical detec-

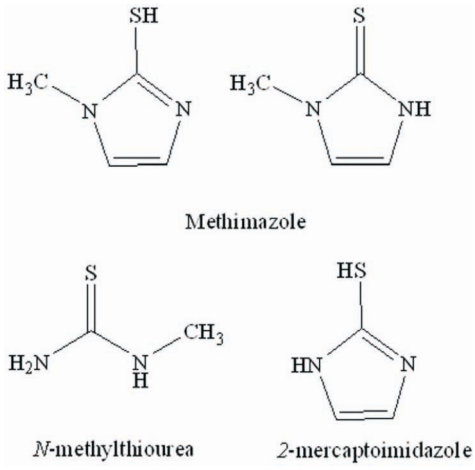

Fig. 1. Structures of MMZ, 2-mercaptoimidazole and *N*-methylthiourea.

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tion[16-18] and molecularly imprinted polymer (MIP)[19] for MMZ are also developed. Among all of these methods, HPLC is the most commonly used method because it is sensitive and selective and it could be provided at a low level of MMZ, but it is expensive and not suitable for extensively prevalence. It is very significant to develop a simple and inexpensive analytical method to monitor the residual quantity of MMZ in biological samples.

Enzyme-linked immunosorbent assay (ELISA) is sensitive and selective, and it can run many analytes simultaneously.[20] However, this method has many disadvantages such as the high cost, difficulty of the acquisition of antibody production, and the need of laboratory animals. Moreover, the production of antibodies for toxic substances or immunosuppressants is particularly difficult, because they have effects on metabolism and the immune system.[21] Some studies have attempted to design and synthesize artificial antibody to replace antibodies.

Molecular imprinting technology (MIT) has been proved to be an efficient method to produce functionalized materials which are used as antibodys to recognize the template from closely related compounds. MIP is inherent stability, low cost and ease of preparation. The MIP as antibodies for application in ELISA should be provided with appropriate wetting properties, a large surface area, and easily accessible binding sites. But if it is directly synthesized on the well surface of a MaxiSorp polystyrene 96-well plate, the traditional MIP is inhomogeneous and difficult to remove the template. Moreover it is hard to control the thickness of the MIP. The parameters affecting the performance of this BELISA method were discussed in detail. This developed method was applied to the analysis of MMZ in the urine of pig, and the results were validated by the HPLC method.

EXPERIMENTAL

Materials and Reagents

MMZ (99.7%) was purchased from Beijing Yanjing Pharmaceutical Co., Ltd. (Beijing, China). And pure standard of N-methylthiourea and 2-mercaptoimidazole (Fig. 1) were purchased from Sigma-Aldrich. Other chemicals used for the polymer synthesis were the solvent absolute alcohol (Concord Co., Tianjin, China) the functional monomer MAA (99%, Tianjin Chemical Reagent Research Institute, Tianjin, China) and the cross-linker EGDMA (98%, Sigma–Aldrich) and the free radical initiator AIBN (99%, Tianjin Kermel Chemical Reagents Co., Ltd. Tianjin, China). Dimethylformamide (DMF), N-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinate (NHS) and other organic chemicals used for hapten synthesis were purchased from Fluka. Horseradish peroxidase (HRP) was purchased from Boehringer-Mannheim (Germany). Reagent grade hydrogen peroxide and Tween-20 were purchased from Sigma (Sigma-Aldrich, USA). Maxisorp polystyrene 96-well plates were obtained from Nunc (Roskilde, Denmark). The thin layer chromatography (TLC) plate was purchased from Merck (Darmstadt, Germany).

HPLC-grade solvents used to prepare the LC-mobile phase were methanol purchased from Concord Co. (Tianjin, China) and doubly deionized water (DDW, 18.2 MΩ cm^{-1}) obtained from a Water Pro water system (Labconco Corp., Kansas City, MO, USA).

Solutions

5 × PBS: $Na_2HPO_4 \cdot 12H_2O$: 344 g, $NaH_2PO_4 \cdot 2H_2O$: 44.85 g, NaCl: 225 g, H_2O: 5 L, when used, diluted to 1 × PBS. PBS/T: PBS with 0.05% Tween-20, Substrate Solution: Color Reagent A (anhydrous sodium acetate: 8.2 g, β-cyclodextrin: 2.5 g, citric acid: 3.16 g, H_2O: 1 L, carbamide peroxide: 0.429 g), Color Reagent B (TMB: 0.05 g, DMSO: 5 mL). stop solution: 1.25 mmol L^{-1} H_2SO_4.

Instrumentation

HPLC system consisted of two LC-10ATVP pumps and a Shimadzu SPD-10AVP ultraviolet detector (Shimadzu, Kyoto, Japan). All separations were achieved on an analytical Hypersil-ODS column (4.6 mm × 250 mm long, Thermo) at a mobile flow rate of 1 mL min^{-1} at the room temperature. The detection wavelength and injection volume were 254 nm and 20 μL, respectively. Class-VP software was used to acquire and process spectral and chromatographic data. A Cary 50-Bio UV spectrophotometer (Bio-Rad, USA) and an ultrasonicator were also employed in the adsorption test (λ = 254 nm) and the preparation of HPLC mobile phase, respectively. Analysis of mass spectra (MS) was carried out on ESI mode on a Survegor-Lcce Advantage Max 10 mass spectrometer (Thermofinnigan, USA).

Immunoassay absorbance was read in dual-wavelength mode (450-650 nm) with a Labsystems 96-well plate reader (Helsinki, Finland).

Synthesis of the Molecularly Imprinted Film

The imprinted film was directly polymerized on the 96-well plate wells as follows: MMZ (0.25 g, 2.19 mmol)

was dissolved in 10 mL ethanol which was used as porogen in a 25 mL round-bottom flask, then added Functional monomer MAA (0.745 mL, 8.78 mmol), the mixture was sonicated for 15 min. After 15 min, the cross-linker EGDMA (0.415 mL, 2.19 mmol) was added to the mixture, sonicated for 20 min. At last the initiator AIBN (0.050 g, 0.304 mmol) was added to the mixture. When AIBN was dissolved, 200 μL of the mixture was placed in the wells of a 96-well plate. The plate was sealed and irradiated by UV irradiation for 12 h. After polymerization ended, the 96-well plate was extracted with 300 mL of methanol/acetic acid (3:1, v/v) for 24 h by an ultracted cleaner, the eluent was replaced by new methanol/acetic acid every 6 h. At last the plate was extracted by 300 mL of the methanol for 6 h to be free of MMZ, which was verified by detection of the methanol eluent using UV spectrophotometer. The plate was dried at room temperature.

For comparison, the non-imprinted film was prepared in the same manner in the absence of template molecule MMZ.

Under the condition of the water temperature in the ultracted cleaner below 50 ºC and methanol/acetic acid (3:1, v/v), the 96-well plate didn't melt.

Characterization of the Imprinted Film

To measure the adsorption capacity, the imprinted and non-imprinted wells of the 96-well plate were equilibrated with 200 μL of 5% methanol in PBS containing MMZ at various concentrations (10-120 mg L^{-1}). After the 96-well plates were shaken (300 r min^{-1}) for 60 min at room temperature. The supernatants were diluted and measured for the unextracted MMZ by a UV spectrometer at 254 nm, and the adsorption capacity (Q) was calculated.

Uptake kinetics of the imprinted films by 40 mg L^{-1} MMZ was evaluated. After shaking for 5, 10, 20, 40, 60, and 80 min at room temperature, the final concentration of supernatants was diluted and determined by UV spectrometry at 254 nm.

Synthesis of the hapten of MMZ

Briefly, 5.708 g MMZ was dissolved in 20 mL of freshly distilled tetrahydrofuran (THF), and then 1.875 g NaH (MMZ:NaH = 1:1.5, mole ratio) was added in batches. This step should be occurred in ice-bath. When there were no bubbles, 7.5 g bromoacetic acid was added. Finally, the resulting residue was dissolved in water, and the pH of the soultion was adjusted to 2 by the addition of HCl. After extraction by ethyl acetate (3 × 30 mL), all of the organic fractions were combined and evaporated to dryness under reduced pressure. ESI-MS: [M-H] 171, Fig. 2 ($C_6H_8O_2N_2S$, M 172).

Enzyme Conjugate Preparation

The process for the synthesis of the active ester was as Fig. 3.[22] To prepare the enzyme conjugates, 6.8 mg of NHS (0.06 mmol) and 24.8 mg of DCC (0.12 mmol) were added to the hapten of MMZ solution (10.15 mg of hapten of MMZ dissolved in 2.76 mL of dry DMF), and the mixture was stirred for 24 h protected form light at room temperature. After centrifugation, the supernatant was added slowly to 10 mg of HRP in 2 mL of 50 mmol L^{-1} K_2HPO_4 in ice-bath. The reaction solution was gently mixed by magnetic stirring bar and then left at 4 ºC overnight. The enzyme conjugated solution was then dialyzed against PBS.

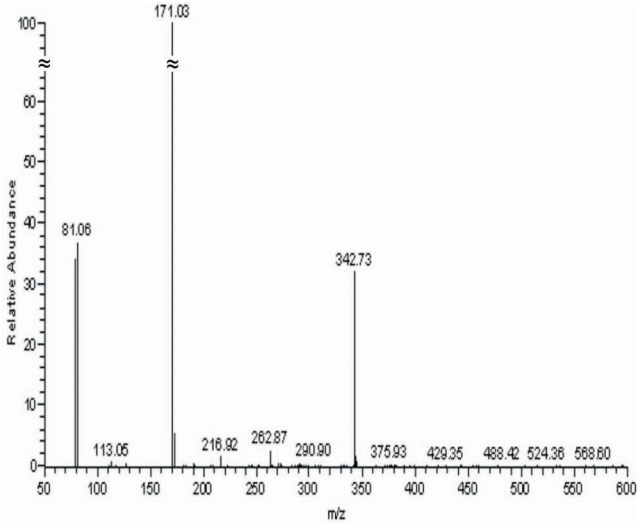

Fig. 2. Mass spectrum of the hapten of MMZ.

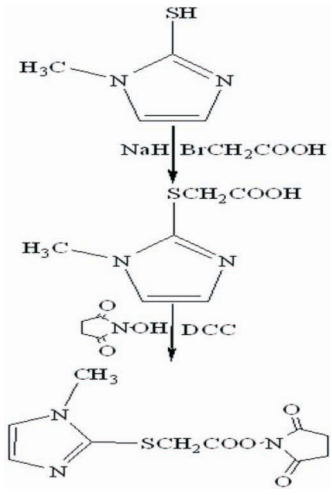

Fig. 3. Reaction scheme for the synthesis of MMZ hapten.

Accurately calculated the volume of enzyme conjugated, the same volume of glycerine was added to the solution, and stored at -20 ºC before use.

Standard Solution Preparation

For the construction of the calibration curve, six standard solutions containing MMZ in 5% PBS within the range of 0.6 μg L^{-1} to 60 mg L^{-1} were prepared freshly in glass tubes. The stock solution (1000 mg L^{-1} in methanol) of MMZ was diluted to 60 mg L^{-1}, and the solution of 60000 μg L^{-1} (in 5% PBS) was sequentially diluted to give 6000, 600, 60, 6, and 0.6 μg L^{-1}.

Direct Competitive BELISA Procedure

First, 100 μL of 5% PBS was added to the control and 200 μL blank wells, and 100 μL of standard solution or sample extracts was applied to the allocated wells. Then 100 μL of enzyme conjugate was immediately added to each well, except for the blank wells, and the mixture was incubated for 1.0 h. Following the washing with PBS/T solution five times, 150 μL of substrate solution was added to each well. The reaction was stopped after 30 min by adding 50 μL 1.25 mol L^{-1} H_2SO_4, and absorbances were recorded. Finally, the imprinted film was extracted with 300 mL of methanol/acetic acid (3:1, v/v) for 3.0 h by an ultrasonic cleaner, followed with 300 mL of methanol for 2.0 h, for the next BELISA procedure.

Preparation of Samples

The urine sample were filtered by 0.22 μm filters without any other pretreatment and stored in the precleaned glass bottles at 4 ºC prior to ELISA or HPLC analysis. To check the accuracy of the developed method, the samples for spiking were determined to be free of MMZ with HPLC. Briefly, 50, 100, or 150 μL of the standard MMZ solution (1 mg L^{-1}) was added to 5% methanol in urine.

Selectivity Studies

Cross-reactivity (CR) studies were carried out by measuring the competitive curves and selectivity properties for other chemically related compounds under the optimized conditions. The N-methylthiourea and 2-mercaptoimidazole were the competitors with similar structure and characteristics. Cross-reactivity was calculated as the percentage between the IC_{50} value (calculated as the concentration giving 50% inhibition of color development) for MMZ and the IC_{50} value for the interfering compound with the following equation:

$$CR\% = \{IC_{50}(MMZ)/IC_{50}(\text{cross-reacting compound})\} \times 100$$

Sample Analysis and HPLC Validation

The samples were determined using a HPLC at a mobile flow rate of 1.0 mL min^{-1} under isocratic conditions, and a mixture of methanol/water (90:10, v/v) was used as the mobile phase. Class-VP software was used to acquire spectral and chromatographic data. The injected sample size was 20 μL, and the UV detection wavelength was 254 nm. All of the analyses were carried out in triplicate.

RESULTS AND DISCUSSION

Characterization of the Novel Imprinted Film

The isothermal adsorptions of imprinted and non-imprinted films are plotted in Fig. 4. It was shown that the amounts of the template molecules extracted by the imprinted or non-imprinted film increased with increasing MMZ initial concentrations. And under the same experimental conditions, the adsorption capacity of the imprinted polymer at each concentration was higher than that of the non-imprinted polymer. And the adsorption capacity of the imprinted film (1.5 μg $well^{-1}$) was more than 1.5-fold that of the non-imprinted film (1.1 μg $well^{-1}$) at 120 μg L^{-1} concentration.

The adsorption kinetics curve should not changed with the initial MMZ concentration. We could see the adsorption capability was grown sharply under the concentration of 40 and 60 mg L^{-1} in Fig. 4, so uptake kinetics of MMZ by the imprinted film was examined at 40 mg L^{-1} concentration (Fig. 5). Results indicated that the prepared imprinted film had fast uptake kinetics, 68% of binding was obtained within a short period of 10 min, and the adsorption equilibrium was almost reached within 40 min.

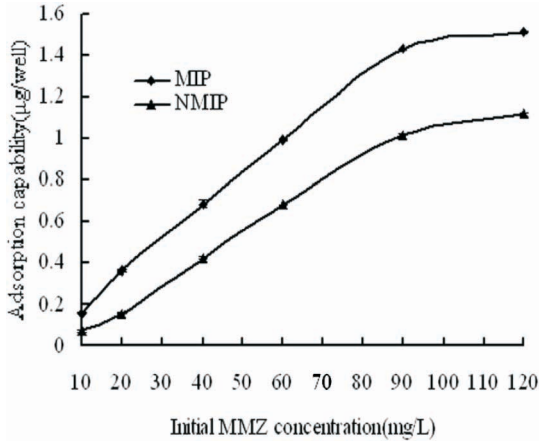

Fig. 4. Adsorption isotherms of the imprinted and non-imprinted films.

The rapid adsorption kinetics of the imprinted film is an obvious advantage for its application in the BELISA.

Hapten Selection and Synthesis

As previous studies have shown, the spatial arrangement, nature, and size of the enzyme conjugate must be tailored to the template structure to achieve efficient competition for the MIP binding sites. The labeled conjugate showing the best performance in a MIP-based assay was also the one providing the highest sensitivity in an immunoassay based on the same type of measurements.

A significantly different binding between a HRP-labeled conjugate and HRP to the imprinted film clearly indicated that hapten played an essential role in the specific recognition of MIP in the BELISA. MMZ (Fig. 1) contains one reactive sulfhydryl group, ideal for chemical modification to produce a hemisuccinate derivative with a terminal carboxyl group for conjugating to the enzyme.

The hapten was synthesized by reaction of the sulfhydryl group of MMZ with the bromoacetic acid (Fig. 3). The carboxylic acid hapten was converted to the active ester for the coupling of hapten to enzyme. The MMZ hapten ester obtained was compatible with enzymes, making an HRP-labeled conjugate possible.

Condition Optimization

The applicability of the developed BELISA method using the imprinted film as an artificial antibody for the determination of MMZ was evaluated. The preparing solvent and thickness of the imprinted film were optimized to achieve good sensitivity and precision. The solvent used in the preparation of standard solutions and samples can affect the BELISA. In order to investigate the influence on assay performance, the methanol solutions at 5, 10, and 15% in PBS were tested for their effects by comparing the standard curves. Methanol at 5% had higher assay sensitivity, and the sensitivity was reduced when the concentration of methanol was more than 5%. Thus, 5% methanol PBS solution was chosen as the preparing solvent for the routine analysis of MMZ in urine samples.

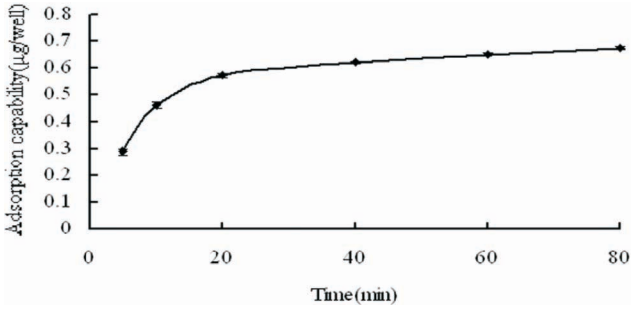

Fig. 5. Kinetic uptake plot of the imprinted films.

As previously reported for the immunoassay, different concentrations of immobilized antibodies in the 96-well plate well may have a direct effect on the sensitivity of the direct competitive ELISA.[21] The effect of the imprinted film thickness and polymerization time on the assay sensitivity was also investigated in the present study. Results indicated that when the plate was sealed and irradiated by UV irradiation for 12 h, lower IC_{50} values (70 ± 4 μg L^{-1}) and optimized competitive binding between MMZ and the HRP conjugate were observed.

Cross-Reactivity

The specificity of the developed BELISA was evaluated in the presence of other structurally related compounds. Cross-reactivity was calculated as the percentage between the IC_{50} value for MMZ and the IC_{50} value for the interfering compound, and the results are depicted in Table 1. The imprinted film had a higher selectivity for MMZ than other related compounds, and different CR were obtained with N-methylthiourea (1.1%), 2-mercaptoimidazole (1.3%). This may have resulted from the imprinting effect, the difference of the molecular interactions, and their structures. During the preparation of the imprinted film, the OH groups of MAA reacted with sulfhydryl groups of MMZ and the polyaniline complex formed. After removal of the MMZ, the imprinted cavities and specific binding sites for the sulfhydryl groups in a predetermined orientation were generated.

We also observed that the imprinted film had higher selectivity for 2-mercaptoimidazole than for N-methylthiourea, as the structure of 2-mercaptoimidazole was more closely related to the structure of MMZ than it (Fig. 1). Hence, the structure and the binding sites of the template molecules all play important roles in the recognition of the

Table 1. IC_{50} and cross-reactivity values for two competitors with the developed BELISA method (mean ± S.D., n = 3)

Compounds	IC_{50} (μg L^{-1})	CR (%)
MMZ	70 ± 4	100
N-methylthiourea	6500 ± 24	1.1
2-mercaptoimidazole	5400 ± 17	1.3

polymer and the competition reaction of BELISA.

Analytical Characterization

The normalized competition curves obtained using the imprinted film as the artificial antibody for MMZ standards at concentrations from 60000 μg L^{-1} to 0.6 μg L^{-1} in 5% PBS solution can be seen in Fig. 6. In the competitive assay format, free MMZ effectively competed with the HRP conjugate for the binding sites in the imprinted film surface (76% inhibitor). Under optimized experimental conditions, the IC_{50} of MMZ standard curves was 70 μg L^{-1}, and the limit of detection (LOD), calculated as the concentration of standard solution causing 15% inhibition of color development, was 0.9 μg L^{-1}.

Furthermore, the imprinted film had a reusability of 20 times of cycles, where the IC_{50} was 74 μg L^{-1} and IC_{15} was 0. 94 μg L^{-1}.

Results indicated that there was little loss of sensitivity. Thus, the cost per analysis of the BELISA method was drastically reduced, and it was really more suitable for rapid detection.

Sample Analysis and Accuracy of the BELISA

In an attempt to evaluate the suitability and applicability of the optimized assay for the measurement of real samples, the urine sample spiked at 50, 100, and 150 μg L^{-1} were determined (with three replicates for each concentration). The analytical data are shown in Table 2. A good correlation of results was observed with recoveries ranging from 90% to 95%, indicating that there was little matrix effect in the analysis of sample using the developed BELISA without any other sample treatment procedure except filtration. The accuracy of this BELISA method was validated

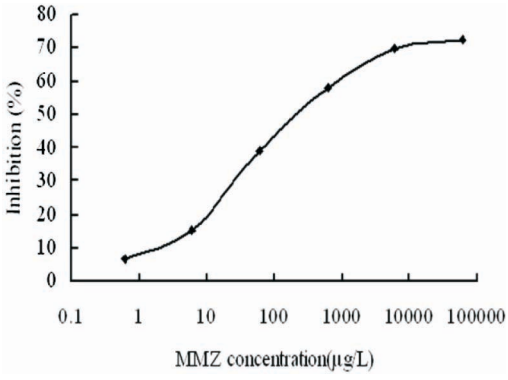

Fig. 6. MMZ BELISA standard curves using the imprinted film as the antibody.

Table 2. Analysis results of spiked MMZ in urine samples by the developed BELISA and HPLC methods (mean ± S.D., n = 3)

Spiked level (mg L^{-1})	HPLC (mg L^{-1})	BELISA (mg L^{-1})
0.050	0.047 ± 0.001	0.045 ± 0.002
0.100	0.095 ± 0.001	0.093 ± 0.007
0.150	0.143 ± 0.001	0.142 ± 0.002

by comparative analysis of the spiked samples with HPLC (Table 2), and no significant differences were observed between the results obtained by both methods.

CONCLUSIONS

There have described were various analytical methods in TLC, GC-MS, HPLC-UV or MS, CE, flow injection analysis and electrochemical detection for the effectively monitoring MMZ in kinds of samples, but these methods were too expensive or not suitable for extensively prevalence. Though the MIP has been used to monitor MMZ, there was no report about using it as the artificial antibody of ELISA. Now we developed a fast, direct competitive BELISA method using the imprinted film of controlled thickness as artificial antibody with the advantages of low-cost, time-saving, ease of preparation compared with traditional antibody. This BELISA method exhibited excellent performance in real sample analysis with a simple matrix dilution step, which has potential for being applied in the quantitative determination of MMZ. We also presented a methodology for the application of MIP in routine immunoassays, although its sensitivity is lower. With the development of MIT, the sensitivity and accuracy of MIP-based immunoassays would be improved, and they can provide an important analysis platform in the future.

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