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Molecularly imprinted polymer based microtiter chemiluminescence array for determination of phenothiazines and benzodiazepines in pork

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ABSTRACT

In this study, a molecularly imprinted polymer based chemiluminescence array capable of simultaneous determining phenothiazines and benzodiazepines was first reported. Two polymers were coated in different wells of the conventional 96-well microtiter plate as the recognition reagents, and the added analytes competed with a horseradish peroxidase-labeled bi-hapten conjugate to bind the recognition reagents. The light signal was induced by using a highly effective luminol-H_2O_2-IMP system. The assay procedure consisted of only one sample-loading step prior to data acquisition. Then, the array was used to determine 4 phenothiazines and 5 benzodiazepines in pork simultaneously. The limits of detection for the 9 drugs were in a range of 0.001–0.01 ng/mL, and the recoveries from the fortified blank pork were in a range of 63.5%–94.1%. Furthermore, the array could be reused for 8 times. The detection results for some real pork samples were consistent with an ultra performance liquid chromatography method.

Introduction

Phenothiazines (PZs) and benzodiazepines (BZs) are commonly used to treat psychotic diseases in human beings. When PZs and BZs are used as feed additives, they can reduce stress and promote the animal growth. However, their residues in foods of animal origin may induce orthostatic hypotension and dermatological reactions to the consumers. Therefore, it is important to detect the residues of PZs and BZs in foods of animal origin. By now, there have been many methods reported to determine their residues, such as high performance liquid chromatography [1,2], liquid chromatography mass spectrometry [3–5], and enzyme linked immunosorbent assay (ELISA) [6–13]. Among these methods, ELISA is the commonly used screening tool able because it is able to analyze large amount of samples in a single test. However, the previously reported ELISA methods can not determine PZs and BZs simultaneously, and can be used for only one time. Furthermore, the ELISA result is based on the colorimetric detection of the formed products during the oxidation of different substrates by H2O2, so the extremely weak signal maybe is not recorded, thus decreasing its sensitivity.

Chemiluminescence method (CL) is a type of rapid analytical method that has lower detection limit (pg/mL level) and wider linearity range than the conventional ELISA. This is because its result is based on the optical radiation during the process of chemical redox reaction. By now, CL-ELISA method has been used to determine many analytes [14], including chloramphenicol [15], sulfonamides [16], acrylamide [17], pesticides [18], estrogens [19], and chromotrope [20]. These CL-ELISA methods achieved higher sensitivity than the controlled ELISA methods, but they could only determine one or a group of analytes, and could be used for only one time. To the best knowledge of the authors, there has been no article reporting the use of CL-ELISA method for determination of PZs or BZs so far.

Molecularly imprinted polymer (MIP), also called “plastic antibody”, is a synthetic material that can recognize the specific analyte and can be reused for up to hundreds of times. In the past few years, MIPs have been used as recognition reagents to develop many CL methods for various analytes [21,22], including PZs [23,24]. Results showed that the selectivity and sensitivity of the conventional CL methods were significantly improved because the co-existed interferents were effectively eliminated by the MIP. Furthermore, these MIP-CL methods could be reused for different times. However, these methods required the tailor-made Y-type tube and the special dual-pump injection system, and could only be used to detect the samples one by one.

As discussed above, a method combining the merits of ELISA, MIP and CL is desirable (MIP-CL-ELISA). In two previous reports, the MIP particles were used as recognition reagent to develop two CL methods on the conventional 96-well microtiter plate [25,26]. However, the

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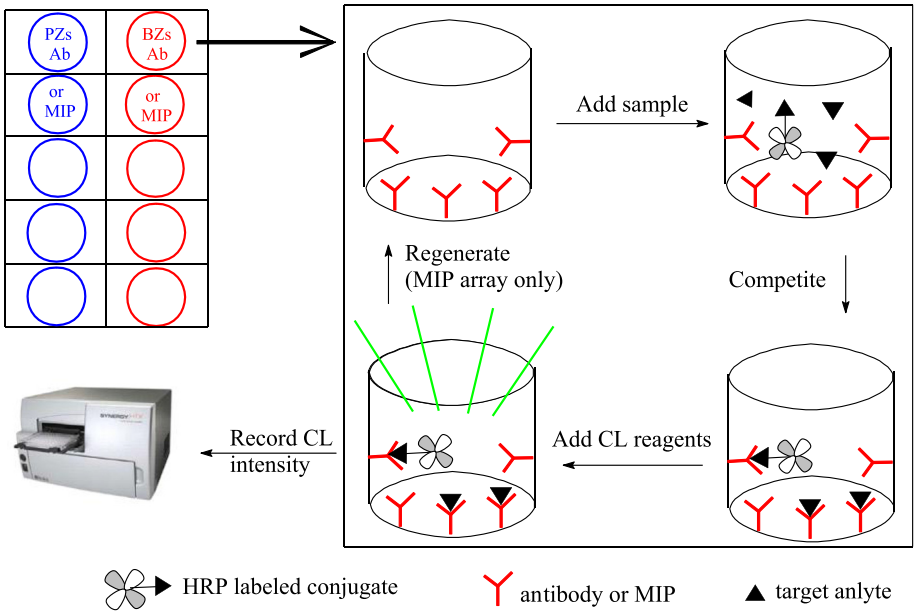

Fig. 1. The schematic representation of the two CL arrays.

high sensitivity of CL method was not demonstrated because the two methods used the common luminol-H_2O_2 reagents to induce CL reaction, and their detection limits were even lower than the controlled conventional ELISA methods. Furthermore, the two methods were only able to determine one analyte. To the best knowledge of the authors, there has been no article reporting the use of MIP-CL-ELISA method for determination of PZs or BZs so far.

In our recent reports, an antibody capable of recognizing 5 BZs [10], an antibody capable of recognizing 4 PZs [11], a MIP capable of recognizing 5 BZs, and a MIP capable of recognizing 4 PZs were obtained [27]. In the present study, the four reagents were used as the recognition elements to prepare a CL-ELISA array and a MIP-CL-ELISA array respectively on the conventional microtiter plate. The two arrays were performed at direct competitive mode by using a horseradish peroxidase-labeled bi-hapten conjugate. The light signal was induced by using the enhanced luminol-H_2O_2 CL system with 4-(imidazole-1-yl) phenol as the strong enhancer. Finally, the MIP-CL-ELISA array was used for simultaneous determination of the residues of PZs and BZs in pork. The results were confirmed with our recently reported ultra performance liquid chromatography method (UPLC) [27].

Materials and methods

Reagents and materials

Acepromazine (APZ), promethazine (PTZ), chlorpromazine (CPZ), perphenazine (PPZ), clonazepam (CLZ), diazepam (DZ), nitrazepam (NZ), oxazepam (OZ) and estazolam (EZ) were obtained from Dr. Ehrenstorfer (Ausburg, Germany). Horseradish peroxidase (HRP) and two enhancers (4-(imidazole-1-yl)phenol (IMP) and *p*-iodophenol (IOP)) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Poly(vinyl alcohol) (PVA) was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). Luminol was purchased from Acros (New Jersey, USA). Other chemicals were all at analytical grade from Beijing Chemical Company (Beijing, China). The opaque 96-well microtiter plate was purchased from Jingan Biotechnology Co., Ltd (Shanghai, China). The anti-clonazepam antibody and the anti-chlorophenothiazine antibody produced in our previous reports were called BZs antibody and PZs antibody respectively, and the MIPs based on clonazepam and (trifluoromethyl)phenothiazine were called BZs-MIP and PZs-MIP respectively in this study.

Solutions

The standard stock solutions of the nine analytes (10.0 μg/mL) were prepared with methanol respectively. Their working solutions (0.0005–100 ng/mL) were obtained by diluting the stock solutions with phosphate buffer-saline (PBS, pH 7.2: dissolving 0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO4, and 8.0 g NaCl in 1000 mL water). PBS containing 0.05% Tween (PBST) was used as washing buffer. The carbonate buffer (0.1 M, pH 9.6) was used as coating buffer. The substrate system for ELISA was prepared by adding 200 μL TMB in DMSO (1%, w/v) and 64 μL 0.75% H_2O_2 into 20 mL substrate buffer (0.1 M citrate solution, pH 5.5). The CL reagents including luminol, H_2O_2, IOP and IMP were prepared with 0.1 M Tris-HCl (pH 8.6) respectively.

Preparation of HRP-labeled conjugate

The bi-hapten conjugate was prepared by coupling the haptens of clonazepam [10] and chlorophenothiazine [11] to HRP in turn. Firstly, the clonazepam hapten was coupled to HRP by use of glutaraldehyde method. Briefly, 5 mg hapten dissolving in 3 mL DMF was added dropwise into 3 mL cold PBS containing 10 mg HRP (4 °C). Then 30 μL 25% glutaraldehyde was added, and the solution was stirred gently at 4 °C for 6 h. The solution was dialyzed in PBS for 3 days at 4 °C to obtain the interim conjugate.

Secondly, the chlorophenothiazine hapten was coupled to the interim conjugate by use of mixed anhydride method. Briefly, 5 mg hapten, 3 mL of DMF, 30 μL isobutyl chloroformate and 30 μL triethylamine were added into a glass jar to be stirred gently at 4 °C for 1 h. Then the solution was added dropwise into the interim conjugate solution to be stirred gently at 4 °C overnight. The solution was dialyzed in PBS for 3 days at 4 °C to obtain the HRP labeled bi-hapten conjugate. Finally, the two haptens, HRP and the bi-hapten conjugate were scanned on a UV spectrophotometer respectively to identify the conjugation. The coupling ratios of the two haptens to HRP were calculated according to the previous report [28].