Chinese Journal of Chemistry, **2004**, *22*, 708—713

Article

Different Biotinylation Strategies for Competitive Immunoassay of Estradiol

ZHAO, Jin-Fu(赵金富) WANG,Yong-Cheng(王永成) LI, Yuan-Zong(李元宗)
CHANG, Wen-Bao*(常文保)

Key Laboratory of Bioorganic Chemistry and Molecular Engineering, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

Study on biotinylation strategies for competitive immunoassay of estradiol (E_2) was carried out. Two types of competitive enzyme immunoassay (EIA) with Biotin-Avidin amplification system were established and optimized. The E_2-Biotin conjugate was used as a tracer in one assay, and biotinylated antibody was used as a tracer in the other. In both of EIAs, horseradish peroxidase-labelled Avidin (Avidin-HRP) was used with a spectrometric determination of enzyme activity. The precision, sensitivity and specificity were measured and compared. The results showed that although both were satisfactory in specificity, the EIA with hapten-Biotin showed to be superior to the EIA with biotinylated antibody in sensitivity and precision. The limit of detection of serum E_2 was 8 and 50 pg/mL with E_2-Biotin and biotinylated antibody as tracer, respectively.

Keywords estradiol, serum, EIA, Biotin, Avidin

Introduction

The assay of E_2 in serum or plasma has great value for clinical endocrinological investigation in women. Since serum E_2 levels are very low, the most commonly used method for determining serum E_2 in clinical study is radioimmunoassay (RIA) combined with extraction by an organic solvent, but this will produce organic and radioactive waste. For the replacement of RIA, recently several methods including enzyme immunoassay (EIA), time resolved fluorescence immunoassay (TrFIA), capillary electrophoresis immunoassay (CEIA), LC-MS-MS and GC-MS are developed for determining E_2.[1-14] In these methods, EIA is the most convenient and cheap one, which is very easily performed by an automatic analytical instrument. So its development is very fast. Several types of competitive EIAs using enzyme-labelled estrogens,[1,3] biotinylated eatrogens or antibodies[6-8] have been developed for direct determination of E_2 in serum or wastewater. In order to avoid the interference due to the effect of complex matrix and enhance the sensitivity, some assays were combined with extraction by an organic solvent, and the others developed with an amplification system.

The Biotin-Avidin amplification systems have long been used in immunoassay, and played a leading role for enhancing sensitivity and decreasing interference due to matrix effect. In the small-molecule competitive immunoassay, the main biotinylation strategies are biotinylated hapten or antibody.[6-8] The biotinylation of antibodies (including the secondary antibody) is simple and easy to carry out. But reactivities of hapten-protein conjugates are higher than those of haptens because a large molecule (*i.e.* enzyme-labelled hapten or hapten-protein conjugates) can bind to different sites of antibody at the same time.[9] The immunoreactivity of antibody can also be partially affected by biotinylation of antibody. These will decrease the sensitivity for the assay. On the other hand, the reactivities of labelled estrogens with antibody are not as the same as unlabelled estrogens because the derivatization procedure of estrogens can only keep the major antigenicity of estrogens. The labelled secondary antibody system is another amplification system. But Biotin-Avidin amplification system has shown to be better than the labelled secondary antibody system in affinity capacity and amplification ability, so the former has been always used to establish a more sensitive assay to analyze a low level analyte in a complex matrix. The optimized biotinylated strategy is very important for developing a competitive immunoassay to determine hapten accurately. To our knowledge the compared study on the different biotinylated strategies has not been reported before.

The aim of this work was to compare EIA with E_2-Biotin and EIA with biotinylated antibody in specificity, sensitivity and precision, in order to find out an optimized way for establishing a more sensitive and accurate method. For this purpose, two types of EIA for determination of E_2 were established and compared. The EIA with E_2-Biotin was based on competitive reaction of E_2-Biotin and E_2 for limited amount of immobilized

* E-mail: changwb@chem.pku.edu.cn; Tel.: 62754905; Fax: 62751708.
Received October 28, 2003; revised January 12, 2004; accepted March 1, 2004.
Project supported by the National Natural Science Foundation of China (No. 20175002).

anti-E_2 monoclonal antibody ($McAbE_2$). The EIA with biotinylated antibody was established on competitive reaction of E_2 and immobilized complete antigen (E_2-OVA) for limited biotinylated $McAbE_2$. In both systems, the biotin on the solid phase was quantitatively determined using Avidin-HRP as an indicator. The procedures of two EIAs are illustrated in Figure 1. The activity of the enzyme was determined by measuring absorbance at 450 nm with an automatic enzyme-kinetic analytical system.

Experimental

Materials and instrumentation

Bovine Serum Albumin (BSA, Mr 68000), Ovalbumin (OVA), estradiol, estrone, estriol, di-*tert*-butyldicarbonate, Biotin, 3,3',5,5'-tetramethylbenzidine (TMB), 1,3-diaminopropane, *iso*-butyl chloroformate, biotinamidocaproate *N*-hydroxysuccinimide ester (BNAS) and trifluoroacetic acid were obtained from Sigma (St. Louis, MO, USA). Horseradish peroxidase labelled Avidin (Avidin-HRP) and the second antibody (goat anti-mouse immunoglobins), were purchased from Huamei Co. (Beijing, China). The *anti*-E_2 monoclonal antibody ($McAbE_2$) was prepared by immunization of BALB/c mice with estradiol-3-carboxymethyloxime-BSA (E_2-3-CMO-BSA). 3-Biotinylaminopropylammonium trifluoroacetate was synthesized by acylation of 1, 3-diaminopropane according to previous reports.[15,16] Estradiol-3-carboxymethyl ether (E_2-3-CME) was prepared using E_2 and bromoacetic acid according to Dhar's method.[17] All the other reagents used were of analytical grade. 96 well microtiter plates (Tianjin plastic instrumental Co. Tianjin, China) were used for spectrometric detection. Spectrometric detection was performed using an automatic enzyme-kinetic analytical system (Tecan Instruments Co. Austria). Measurement wavelength and reference wavelength were set at 450 and 492 nm, respectively.

Buffers and substrate

De-ionized water was used throughout. The buffers used were prepared as follows: (A) coating buffer, 0.05 mol/L carbonate/bicarbonate buffer solution, pH＝9.6; (B) incubation buffer, 0.01 mol/L sodium phosphate-buffered saline (PBS), pH＝7.2—7.4; (C) washing buffer, buffer B with 0.05% Tween 20; (D) blocking buffer, buffer B with 0.8% glutin; (E) the enzyme buffer, buffer B with 4% calf serum; and (F) the substrate buffer, 0.1 mol/L phosphorate buffer (PB), pH＝6.0. The substrates for HRP were TMB.

Preparation of E_2-protein conjugate

E_2-3-CME was coupled to BSA and OVA by an activated ester method employing *N*-hydroxysuccinimide and dicyclohexylcarbodiimide as described by Mattox and Nelson.[18] The estimated binding ratio of E_2 to BSA and OVA was 18∶1 and 22∶1, respectively.[11] The products were dialyzed against 0.01 mol/L PBS and lyophilized, then stored at −20 ℃ before use.

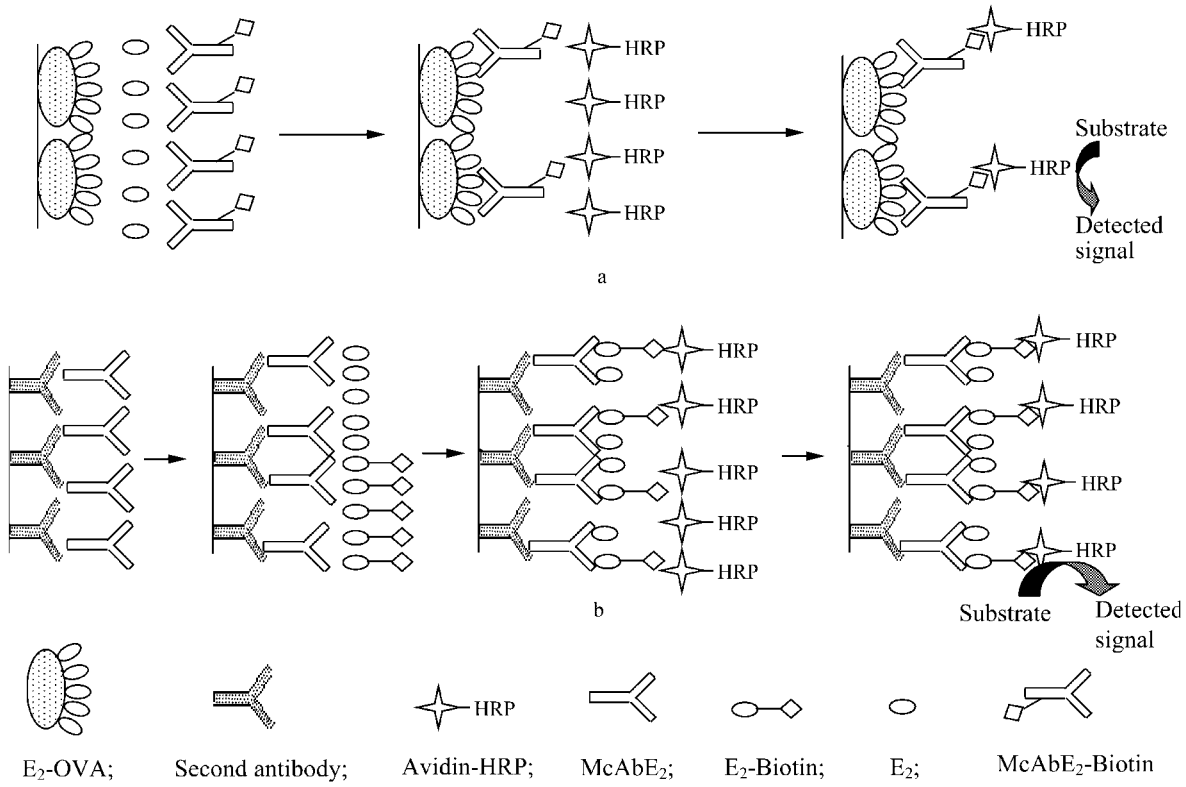

Figure 1 Illustration of two EIAs. (a) EIA with biotinylated antibody; (b) EIA with E_2-Biotin.

Preparation and purification of antibodies

$McAbE_2$ was prepared by immunization of mouse with E_2-3-CMO-BSA and purified by a modified *n*-caprylic acid-saturated ammonium sulfate method.[19] The $McAbE_2$ was essentially pure (>95%) as shown by CE.[20] The affinity constant K_{aff} was determined by Beatty's method,[21] which was 1.5×10^9 L/mol. The second antibody was also purified by that method.

Preparation of E_2-Biotin conjugate

The conjugation of E_2-3-CME to 3-biotinylaminopropylammonium trifluoroacetate was carried out by a mixed anhydride method described by Boudi and Fiet[16] with modification as follows: 0.36 g of E_2-3-CME (1.1 mmol) and 0.2 mL of triethylamine were dissolved in 3.0 mL of dioxane, and 0.14 mL of *iso*-butylchloroformate was added slowly with stirring. The reaction was continued at 5 ℃ for 30 min. Then a solution containing 0.37 g of 3-biotinylaminopropylammonium trifluoroacetate (1.0 mmol) and 0.3 mL of triethylamine in 1.0 mL of DMSO was added. After this, the mixture was stirred slowly at room temperature for 4 h and then was poured into 15 mL of water (4 ℃). The precipitate was washed with cold water and dried *in vacuo*, and the conjugate of E_2-Biotin was purified by column chromatography using CH_2Cl_2-MeOH (9/1, *V/V*) as eluent.

Preparation of biotinylated $McAbE_2$

5.0 mg of purified $McAbE_2$ was dissolved in 0.5 mL of 0.1 mol/L sodium carbonate buffer (pH=9.6). To this solution, 50 μL of 1.0 mg/mL BNAS in DMSO was added. This mixture was stirred slowly for 4 h at room temperature. Finally, the reaction solution was dialyzed against 0.01 mol/L PBS (pH=7.4) overnight. The conjugate of $McAbE_2$-Biotin was further purified by passing through a Sephadex G-25 column. The immunoactivity of purified $McAbE_2$-Biotin conjugate was measured by ELISA, and the solution was stored at 4 ℃ before use.

EIA procedures

Procedure for EIA with E_2-Biotin as tracer To each well of the plates precoated with second antibody, 150 μL of $McAbE_2$ solution (1500-fold diluted with PBS) was added, and incubated for 1 h at 37 ℃. The solution was discarded and the wells were washed three times with buffer C. 100 μL of the standards or unknown samples diluted with PBS (1∶1, *V*∶*V*) were added to the wells, and the plates were incubated for 30 min at 37 ℃. Then 50 μL of E_2-Biotin conjugate solution (2 ng/mL in PBS) was added and the plates were further incubated for 1 h at 37 ℃. The competitive immunoreaction was stopped by washing the plates three times with buffer C. Then, 150 μL of Avidin-HRP (200-fold diluted with enzyme buffer) was added and incubated for 30 min. After that, the plates were washed three times by washing buffer and twice by deionized water. Finally, 150 μL of substrate solution (1.5 μL of 30% H_2O_2 and 10 μL of 6 mg/mL TMB in 1 mL of PB[8]) was added to the wells and the enzyme reaction was kept in the darkness for 15 min at room temperature. The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4.

Procedure for EIA with biotinylated antibody To each well of the plates precoated with E_2-OVA, 100 μL of the standards or unknown samples diluted with PBS (1∶1, *V*∶*V*) were added. Then, 50 μL of $McAbE_2$-Biotin solution (1.8 μg/mL in PBS) was added and the plates were incubated for 1 h at 37 ℃. The following procedures were the same as competitive immunoassay with E_2-Biotin shown above.

Preparation of standards

In this assay, the steroids-free human serum obtained from healthy volunteers was used as a matrix for standards. The standards were prepared as follows: 0.9 mL of serum was added into 0.1 mL of ethanol solution of E_2 (1 μg/mL). The mixture was vortexed and serially diluted with steroid-free serum (10—10000 pg/mL). All the standards were left at 37 ℃ for 4 h, and stored at 4 ℃ overnight. The standards were stored at −20 ℃ before use. E_2-Biotin was also pretreated by this procedure.

Sample collection

Blood samples were obtained by venipuncture from normal women (age from 19 to 45) attending the clinic for consultations. Serum was analyzed with the routine CLIA kit directly. Then, serum was stored at −20 ℃ until analysis.

Data analysis

Non-specific blanks were subtracted from absorbance readings and A/A_0 (A is the absorbance reading at related dose of standards and A_0 is absorbance reading at 0 dose of standard) was calculated and plotted vs. log dose of the standards. Fitting the curves with a smoothed four parameters function enables evaluation of the sensitivity (represented by the slope of the curve), the response at 50% bound (the mid-point of the curve), and the responses at 0 and ∞ doses (the two asymptotes). The responses at 0 and ∞ doses provide information about the detectability and the non-specific signal of the assay, respectively.

Results and discussion

Optimization of reagents for EIAs

The competitive immunoassay involves the use of a limited concentration of a specific antibody and a labelled or immobilized antigen. The optimization of concentrations of antibody and labelled or immobilized antigen is very important to enhance the sensitivity and extend the working range for immunoassay. Therefore, these concentrations were always optimized firstly.

Optimization of reagents for EIA with E_2-Biotin as a tracer First of all, the E_2-Biotin titration

curves were performed at different concentrations of McAbE$_2$ and a constant concentration of second antibody. The results are shown in Figure 2. The initial optimal concentrations of E$_2$-Biotin and McAbE$_2$ were chosen from the curves. To further enhance the sensitivity of assay, the concentration of second antibody and Avidin-HRP was also optimized. Avidin-HRP was diluted with enzyme buffer for 200-fold, and the final optimal concentrations of second antibody, McAbE$_2$ and E$_2$-Biotin are 10, 2.5, and 1.0×10^{-3} μg/mL, respectively.

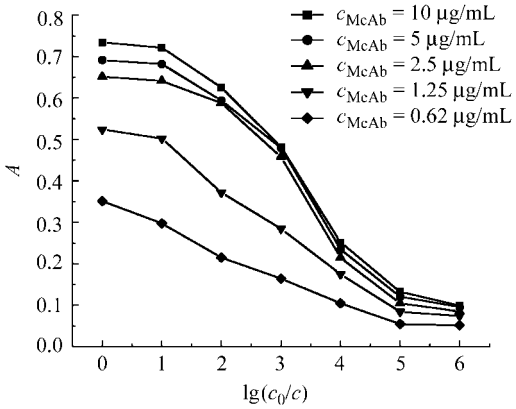

Figure 2 E$_2$-Biotin conjugate titration curves at different McAbE$_2$ concentrations from 10 to 0.62 μg/mL. *c* was the concentration of the E$_2$-Biotin conjugate ranging from 100 ng/mL to 10^{-4} ng/mL; c_0 was the primary concentration of the E$_2$-Biotin conjugate set at 100 ng/mL. The concentration of the second antibody was 10 μg/mL. *A* was absorbance at 450 nm.

Optimization of reagents for EIA with biotinylated antibody The titration curves of biotinylated McAbE$_2$ were carried out at different coating concentrations of E$_2$-OVA and the same concentration of Avidin-HRP, and the results are shown in Figure 3. The optimal concentrations of biotinylated McAbE$_2$ and E$_2$-OVA were 0.5 and 0.6 μg/mL, respectively.

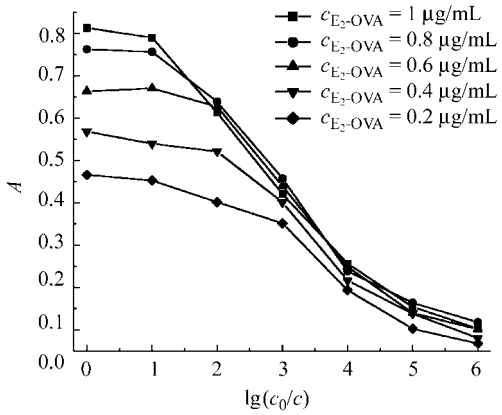

Figure 3 Biotinylated antibody titration curves at different coating concentrations of E$_2$-OVA from 1.0 to 0.1 μg/mL. *c* was the concentration of biotinylated antibody ranging from 600 μg/mL to 6×10^{-4} μg/mL; c_0 was the primary concentration of the biotinylated antibody set at 0.6 mg/mL. *A* was absorbance at 450 nm.

EIAs for serum E$_2$

Dose-response curve The dose-response curves for two assays were measured under optimized conditions discussed above, respectively. Figure 4 shows the representative dose-response curves of the mean standard value (n=8) and error bars of the individual points for two assays. The detection limit of the assay with E$_2$-Biotin and the assay with biotinylated antibody were 8 and 50 pg/mL (calculated as the concentration corresponding to twice of standard deviations below the mean of the zero standard measurements), respectively. The effective working ranges were 20—10000 and 80—6000 pg/mL, respectively. These results indicated that the assay with E$_2$-Biotin had wider effective working range and lower detection limit than that with biotinylated antibody. Furthermore, in order to measure lower concentration of hapten in complex matrix without any extraction procedure, the competitive immunoassay with biotinylation of hapten and Biotin-Avidin amplification was a more efficacious method.

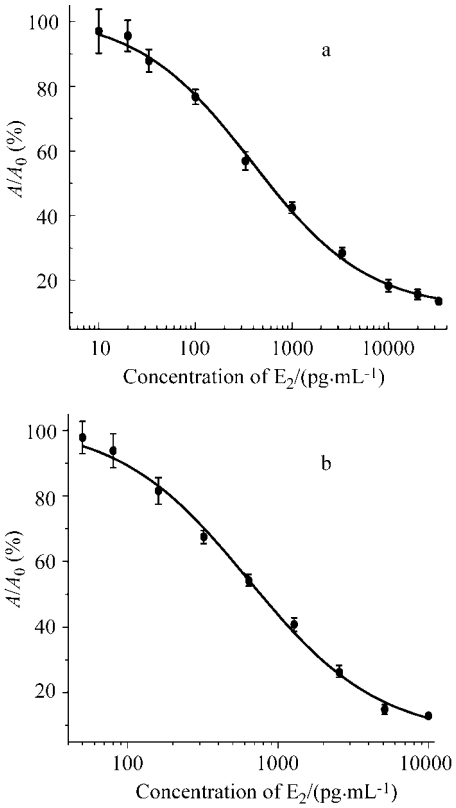

Figure 4 Calibration curves of the direct enzyme immunoassay for E$_2$ with E$_2$-Biotin (a) and with biotinylated antibody (b) as a tracer. The results shown are the mean of eight measurements, and the bars represent standard deviation of the data.

Precision The intra- and inter-assay precisions were calculated from three serum samples with endogenous E$_2$ levels ranging from 50—8500 pg/mL. The intra-assay precision was assessed from 8 replicate measurements for each assay. The inter-assay precision was estimated from 7 separate analyses. The results are

listed in Table 1. The CV values of EIA with E_2-Biotin are lower than the CV values of EIA with biotinylated antibody both in intra-assay and inter-assay. These results illustrate that the matrix effects have been reduced in EIA with E_2-Biotin, and the EIA with E_2-Biotin is also better than the EIA with E_2-HRP combined with an extraction by organic solvent.[12] Therefore, the quality of the assay would be greatly improved by the use of hapten-Biotin with amplification system.

Table 1 Precision of the direct EIA for E_2 with E_2-Biotin and biotinylated McAbE_2 as a tracer

EIA with E_2-Biotin		EIA with biotinylated McAbE_2	
E_2 concentration Mean $\pm$ SD/(pg·mL^{-1})	C.V./%	E_2 concentration Mean $\pm$ SD/(pg·mL^{-1})	C.V./%
Intra-assay			
69.8 $\pm$ 9.5	13.6	95.4 $\pm$ 18.6	19.5
725.4 $\pm$ 45.8	6.3	2474 $\pm$ 357	14.4
8529 $\pm$ 698	8.2	4905 $\pm$ 785	16.0
Inter-assay			
52.3 $\pm$ 6.9	13.2	67.5 $\pm$ 16.7	24.7
438.4 $\pm$ 42.9	9.8	653.3 $\pm$ 69.3	10.6
5059 $\pm$ 435	8.6	3496 $\pm$ 549	15.7

Accuracy Accuracy was evaluated by adding various amounts of E_2 (50—4500 pg/mL) to serum samples. The samples were measured simultaneously by the two types of EIA. The mean ($\pm$S.D.) recoveries were 95.5% $\pm$ 12% and 95.9% $\pm$ 18% for the EIA with E_2-Biotin and with biotinylated antibody, respectively. The results of recovery tests demonstrate that the accuracy by the EIA with E_2-Biotin was little better than that the EIA with biotinylated antibody.

Specificity for assays The cross reactivities of McAbE_2 with structurally related compounds in the two assays were almost the same: estradiol 100%, estrone 0.2%, estriol 0.5%, and not observed for progesterone, testosterone, and rostenedione (Maybe there are also other structurally related compounds to consider in order to realize a complete study of cross-reactivity, but these compounds represent the most similar compounds related to the structure of E_2. Therefore, we just tested these compounds). The results were measured and calculated by 50%-D method (Percent cross-reaction: [E_2 (pmol) that leads to 50% binding inhibition/substance (pmol) that leads to 50% binding inhibition] $\times$ 100).[15] These results showed that in the effective working range for measuring E_2, this assay was free of interference from E_1, E_3 and other steroids existing in serum samples and satisfied the needs for determination of serum E_2.

Analytical application Samples from patients were analyzed by the EIA with E_2-Biotin without any pretreatment. The feasibility and repeatability of the method were examined from the analysis of ten serum samples and the results are listed in Table 2. Compared with the results from CLIA, these results confirmed that the EIA with E_2-Biotin showed good performances in both the feasibility and repeatability of the assay.

Conclusion

In this work, we compared different biotinylation strategies for competitive immunoassays of estradiol. The two types of EIAs with Biotin-Avidin amplification were established and compared. The results have demonstrated that the EIA with E_2-Biotin is superior to EIA with biotinylated antibody in terms of sensitivity, precision, kinetics, and working range of E_2. So the competitive immunoassay with hapten-Biotin could be an interesting method with high performance to apply for measuring lower-level haptens in a complex matrix without extraction.

Although competitive immunoassay with hapten-Biotin could enhance the sensitivity of the assay and extend the effective working range, the full-procedure for the synthesis of hapten-Biotin is still an obstacle to

Table 2 Analytical data corresponding to the analysis of E_2 in serum

Sample number	Concentration[a] detected/(pg · mL^{-1})	R.S.D[a] (n=6)/%	Concentration[b] detected/(pg · mL^{-1})	R.S.D[b] (n=6)/%
1	215	5.7	213	4.5
2	142	10.5	132	6.2
3	239	8.4	234	5.2
4	302	6.9	289	7.9
5	157	8.8	150	8.4
6	168	4.5	165	9.1
7	364	6.2	368	5.3
8	189	12.2	197	7.2
9	197	7.9	202	5.1
10	146	10.2	141	8.9

[a] Results from the EIA with E_2-Biotin; [b] results from CLIA.

perform. If the synthesis of hapten-Biotin could be achieved by a simple procedure, the competitive immunoassay with hapten-Biotin could be an innovative method to use for haptens monitoring in complex matrix.

Acknowledgements

The authors thank Monoclonal Laboratories, Department of Biology, Peking University, China for their technical supports of monoclonal antibody culture.

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(E0310281 LI, L. T.)