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Highly Sensitive Detection of Clenbuterol in Urine Sample by Using Surface Plasmon Resonance Immunosensor

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Abstract

This study proposed the filtration method for removal of inhibitors from real urine samples for immunoassay without centrifuge. Although the inhibitors could not be removed by the physical filtration, the carboxyl group terminated silica effectively removed the inhibitors. In a low pH, antibody formed aggregation due to the protonation. We propose to adjust pH of the sample solution by adding a phosphate buffer solution (pH 7.5). As a result of pretreatment, the SPR immunosensing achieved the SPR signal of 45 mdeg and a low limit of detection with 100 ppq (100 fg mL^{-1}).

Key words: Clenbuterol, SPR immunosensing, indirect competitive inhibition, inhibitor determination, urine

1. Introduction

β-agonists are used as medicine for pulmonary diseases and asthma [1]. These drugs are also used as growth-promoting agents to stimulate the growth of muscular tissues, when they administrated in amounts above the therapeutic level. Among other family members of β-agonists, clenbuterol has been reported as the most often illegally used by athletes and meat producer [2-4]. The residue of clenbuterol remained in meat tissue. After accumulation in body it may causes cardiac disease, nausea, fever and specific cell deaths [1,5]. At present, the use of clenbuterol in animal feed is prohibited in the United States, China and most of EU countries. However, the misuse of clenbuterol in the animal farm and in professional athletes has been often reported [3]. In addition, food poisoning in terms of clenbuterol is reported in many countries such as Mexico, Portugal, China, France, Spain, Italy and the United states [6]. The limit of detection of clenbuterol residue set by the EU is 0.1 μg/kg for muscle, 0.5 μg/kg for liver and kidney, and 0.05 μg/kg for bovine milk [7]. Since the excretion rate in urine is 22-49% after the oral administration of clenbuterol [8], urine is generally used for evaluation of clenbuterol.

In market, gas chromatography/mass spectrometry (GC/MS) and liquid chromatography with mass spectrometry (LC-MS), enzyme linked emmunosorbent-based assay (ELISA), and immmunochromatographic immunoassay are the main analytical methods for monitoring clenbuterol [9-11]. All methods request a use of centrifuge to remove the inhibitors from real urine sample. In the protocols of LC-MS [12] and GC-MS [13, 14], urine sample needs to be centrifuged at 3000 g for 10 min after the enzymatic hydrolysis. ELISA [15, 16] also requests the centrifuge at 4000 g for 5 min after dilution. Here, veterinary needs to perform the diagnosis in paddock of

cow. Further, they have at least 100 samples each site. Therefore, veterinary requests the development of simple pretreatment method without centrifuge.

Immunoassay is one of highly selective detection method for a specific analyte. Among immunoassay formats, indirect competitive inhibition immunoassay is designed for a small analyte [17-24]. Combination with the most sensitive mass transducer, Surface Plasmon Resonance (SPR) sensor, achieved a highly selective and a highly selective detection [17-28]. So far, we have reported the detection of clenbuterol by this immunoassay [22-24]. However, our SPR immunosensor faced the challenge, when it was applied to real sample analysis. It was found that the SPR signal loss was approximately 65% due to non-specific adsorption for urine sample compared to the ideal buffer solution. The SPR signal of non-specific adsorption was more than 30 mdeg. It is known that urine sample contains a lot of large and small molecules such as proteins, fats, vitamins, hormones, metabolites, minerals etc [30]. Those entities can block the reaction sites of receptor and biorecognition molecules in immunoassay. Aiming to eliminate inhibitors from urine sample, Johasson et al. employed molecular weight cut-off filtration process [30]. Therefore, an efficient sample pretreatment is studied in this work.

We proposed here the pretreatment method using just a filtration. Physical filtration (molecular weight cut-off filtration) and chemical filtrations are compared by the SPR immunosensing. The primary and secondary immunoreactions are employed here in order to amplify the signal, because we thought that a large SPR signal change can reduce the effect of non-specific adsorption. In our past reports, the detections of clenbuterol in pico gram order were achieved in PBS solution. However, the signal change was less than 10 mdeg [22-24]. In order to obtain more signal amplification in the secondary immunoreaction, secondary antibody labeled with 40

nm AuNP is used. In previous work, 25 ppq of detection limit of clenbuterol and more than 40 mdeg of SPR signal change were achieved by this system in PBS solution [31]. Therefore, it is expected that this system with filtration has a high possibility to achieved the detection limit enough to be used in paddock even though a minor nonspecific adsorption occurred on the sensor surface.

2. Experimental

2.1 Materials

Ethanol, potassium dihydrogen phosphate and ammonium chloride were all obtained from WAKO, Japan, respectively. Sodium dihydrogen phosphate, disodium hydrogen phosphate and ethanolamine were purchased from Kanto Chemical Co. Inc., Japan, respectively. Anti-mouse IgG (H+L) and anti-mouse IgG (H+L) labeled with Au- nanoparticle (40 nm, 2.14 x 10^{11} particle/ mL) were obtained from Jacson Immunoresearch Lab. Inc. and Cytodiagonestic, Canada, respectively. DSP (dithiobis succinimidyl propionate) and phosphate buffer saline (PBS, pH 7.4) and Clenbuterol hydrochloride (Clb) were obtained from Thermos Scientific, USA. Sodium sulfite and methanol were supplied by Dojindo, Japan and Nakali Tesque Inc. Kyoto, Japan. Sodium hydroxide and matching fluid with refractive index 1.518 were supplied by Junsei Chemical Co., Ltd., Japan, and Cargille Labs, USA, respectively. All chemical were reagent grade. Buffer solution was prepared using deionized water (18.2 MΩ cm) was produced from a Millipore Milli-Q purification system.

2.2. Preparation of Au substrates

BK7 (refractive index = 1.515) microscopic glass chips (20 mm × 13 mm × 0.7^{t} mm from Matsunami Glass Ind., Ltd.) were immersed and sonicated in soap water (10% Contrad. 70 detergent; Fisher Scientific, USA) for

cleaning. After this process, the glass chips were rinsed using copious amounts of Mill-Q water and dried by pure nitrogen gas. Au sensor chip (Au thickness: Ca. 50 nm) was prepared by sputtering. During sputtering the chamber pressure was 2.0–2.5 Pa. Then the chips were immediately taken into SPR chamber.

2.3. Surface plasmon resonance measurements

SPR analyzer (SPR-670, Nippon Laser Electronics, Japan) was used for SPR measurements. The Kretschmann typed SPR instrument was equipped with computer controlled flow-injection system. The gold chip was attached on the semi-cylindrical prism (refractive index = 1.515) of the SPR analyzer using a refractive index matching liquid. A beam of red light (670 nm) was emitted from Ni–Cd laser and passed through the prism. The light was incident on the backside of the gold-film deposited glass slide, and the reflected light intensity was recorded as a function of the incident angle using a CCD camera. Incident angle for which the reflected light intensity is minimum, called SPR angle, was monitored. Since many organic compounds have a refractive index higher than that of aqueous solution, adsorption of an organic compound on the sensor surface generally increases its SPR angle. All SPR experiments were conducted in an air-conditioned room (25 ± 1 °C).

2.4. Fabrication of immunosurface

Indirect competitive inhibition immunoassay uses antigen-immobilized sensor surface. The sensor surface was prepared according to the past reports [22-24]. The SPR sensogram of the immunosurface fabrication process were illustrated in S1. In briefly, we would like to introduce the process. At first, the sensor surface prepared upon injection of 5 mM DSP in methanol onto freshly prepared Au-chip (Ca. 50 nm). In this step, disulfide bond of DSP was cleaved and succinimidyl terminated propenthiol monolayer was formed onto the sensor chip. After 40-min period of

injection, the running solution was changed from methanol to phosphate buffered saline (PBS). After established baseline, clenbuterol (1000 ppm) in PBS solution is injected and clenbuterol attached onto the sensor surface through amide coupling reaction. Finally, ethanolamine (1000 ppm) in PBS solution was injected in order to block unreacted N-Hydroxysuccinimide group. Therefore, the sensor surface is composed of clenbuterol-propenthiol and ethanolamine-propenthiol. The maximum SPR angle shift obtained for covalent immobilization of clenbuterol on the succinimidyl terminated propenthiol monolayer was 21.8 (±2.3) mdeg (millidegree), and it corresponds to 7.87 (±0.83)× 10^{-11} mol cm^{-2}. Similarly, 3.80 (±0.60)× 10^{-11} mol cm^{-2} and 1.59 (±0.034) × 10^{-10} mol cm^{-2} for clenbuterol and ethanolamine, respectively. All the solutions were injected at a flow rate 5 μL min^{-1} and the loop volume 200 μL.

2.5 Immunoassay format

Because clenbuterol is a small molecule (277 g mol^{-1}), indirect competitive inhibition immunoassay was used. In briefly, the protocol is explained here. Primary immunoreaction was carried out by injecting a premixed solution that contains 1 ppm anti-clenbuterol mouse IgG (primary Ab) and a wide range concentration (0.01 ppt – 100 ppb). After injection the premixed solution, the sensor surface was washed with running buffer (PBS+10% ethanol) solution. Subsequently, 1 ppm of anti-mouse polyclonal Ab labeled with AuNP (Ab_2-AuNP conjugate, 40 nm) solution was injected. The secondary Ab_2-AuNP conjugate binds with the primary Ab onto the sensor surface.

2.6 Urine sample preparation:

Urine samples of cow were collected from cows (8 different cows) in the Field Science Center for Northern Biosphere, Hokkaido University, Japan. 10 mL of

collected urine was taken into 5 mL centrifuge tube. Then, 10 μg of clenbuterol is added to 5 mL urine sample, and the concentration of urine sample was adjusted to 2 ppm. Another 5 mL Urine was used as blank (for control experiment).

In the pretreatment process, physical and chemical filtration was carried out. For physical filtration, Minisart syringe filter (pore size 0.2 μm) along with a pocket pump (Yabegawa Co. Lid, Japan) was used. The filtration process is represented in Fig.1 (a). For the chemical filtration, monospin filter is studied. Monospin filter (monolithic silica spin column) has recently become commercially available. Three different functional groups terminated Monospin C18, Monospin amide and monospin CBA (Fig.1 (b) were used. The filtration process is represented in Fig. 1 (c). After that, the pH of urine samples (both spiked and blank sample) was adjusted to 7.4 by 2-fold dilution using PBS solution. Finally, 0.01 ppt to 100 ppb clenbuterol in urine sample were prepared through a serial dilution using blank sample as a solvent. The sample preparation flow chart is summarized in the Fig. 2.

3 Results and Discussion

3.1 Comparison of filters

The SPR sensorgrams of the urine and PBS solution with the physical filtration (Minisart, molecular weight cut-off filtration) are shown in Fig. 3 (a). Urine and PBS solution was premixed with antibody (anti-clenbuterol monoclonal Ab, 2 ppm) in PBS buffer in the ratio of 1to 1. Once a stable base line achieved, the premixed solution was injected onto the sensor surface in a flow rate of 100 μL min^{-1}. The SPR signal was initially decreased due to difference of dielectric constant of running buffer and the pre-mixed solution. During the immunoreaction, SPR signal increased due to the binding of antibody to the sensor surface. After 2 min injection,

the SPR signal reached at a higher level compared to the base level in running buffer solution. Then the running buffer solution flowed over the sensor surface. Most of nonspecific adsorbed species were usually washed away in this process. However, the signal of urine (35 mdeg.) was much higher than that of PBS solution (7.1±1.2 mdeg). It considered that this difference was caused by the non-specific adsorption. Therefore, it is concluded that the physical filtration cannot remove the non-specific adsorbed species from urine.

SPR signal changes of chemical modified filters (monospins) and molecular weight cut-off filtration (minisart) were compared as shown in Fig. 3(a) and (b). The signal change was calculated from the SPR signal before and after sample injection. The difference from 7.1 mdeg indicates the amount of non-specific adsorption. For example, the largest SPR angle shift with a magnitude of 35 mdeg was observed in minisart filter. Amount of the non-specific binding is calculated to 27.9 mdeg. The amount of non-specifically adsorbed protein is 27.9 ng cm^{-2}.

On the other hand, the results of monospin filtration show comparable values to the ideal condition. Monospin filters are chemically modified with octadecyl, amide and carboxyl groups (monospin C18, monospin amide and monospin CBA, respectively). In monospin C18, a hydrophobic-hydrophobic interaction between protein molecules of urine sample and octedecyl group works for the removal of protein. In other monospins, amide and carboxyl functional groups of monospin filters uses an affinity with the functional groups of small protein for filtration. Monospin amide and monospin CBA depicted the less matrix effect compared to the monospin C18. In particular, CBA showed the best filtration performance. Probably, the amine group of inhibitor has positive charge in pH 7.4. Therefore, monospin CBA was used for the following experiment.

3.2 pH effect on secondary immunoassay

pH of cow urine is highly dependent on physical condition such as pregnancy, illness and feed. The range of pH of urine is reported to 4.6 to 7 in human sample, and the average pH of beef cattle and daily cow are 7.27 and 8.10, respectively [32]. In this study, the observed average pH was 8.15±0.10 (n=7). Therefore, pH range was set up 4.5 to 8.5 with an interval of 1.0 in this experiment.

In order to examine the pH effect by SPR immunosensing, pH-adjusted PBS solution was used. Typical sensorgrams of pH 4.5 and pH 8.5 are shown in Figure 4. Initially the sensor surface was equilibrated with running buffer (PBS solution, pH 7.4) and the baseline was established. And then, the running solution was switched to the primary antibody (1 ppm) in pH-adjusted PBS solution. The observed angle shift with a magnitude of 12.2±1.8 mdeg was obtained, whereas it was 4.2±0.3 mdeg for pH 8.4. The obtained result indicated that approximately 3-times higher amount of primary Ab was adsorbed onto the sensor surface from an acidic solution compared to the pH 8.4. The similar behavior was found for secondary immunoreaction. The angle shifts with respect to pH were summarized in Fig. 5. The total SPR angle shift was calculated from sum of primary immunoreaction and secondary immunoreaction. Interestingly, the angle shift was dramatically increased when pH down from 5.5. These results clearly demonstrated when antibody (both the primary and Ab_2-AuNP conjugate) mixed with lower pH buffer solution, due to protonation of charge of antibody surface being changed (pI value of IgG is 6.4-7.6). Therefore, the inter antibody-antibody interaction resulting aggregation, consequently mass density increased accordingly. In other words, the antibodies might be aggregated due to the positive charge of antibody in acidic solution. As a result, total mass of antibody aggregation increases with increasing SPR signal shift.

In conclusion, pH of urine sample needs to be adjust to pH 7.4 by mixing with PBS solution (pH 7.4) in the ratio of 1 to 1. Otherwise, pH highly affects the immunoreaction.

3.3 Inhibition effect:

Aiming to determine of inhibitors, artificial urine sample was prepared according to Japan Industry standard (JIS T3214). The composition of artifical urine is listed in Table S-1. pH of artificial urine was 6.83. Hence, the pH of artificial urine was adjusted with PBS solution (pH 7.4). And then, artificial urine was filtered by monospin CBA. SPR sensorgrams were illustrated in Fig. 6. The premixed solution was prepared by adding 1 ppm primary Ab into filtered and without filtered of artificial urine sample, respectively. The SPR response of the artificial urine without filtration (4.2±0.3 mdeg.) was almost the same as that with filtration (4.5±0.6 mdeg.). There is no significant difference in those angle shifts. Therefore, it is concluded that there is no inhibitor in the artificial urine. Difference between artificial urine and real urine is proteins and sugars. In other words, the SPR immunosensor cannot be used for real urine analysis without pretreatment, even though a good sensing performance is obtained in artificial urine.

3.4 Clenbuterol detection:

For detection of clenbuterol in real urine sample, indirect competitive inhibition immunoassay was used. Antibodies with unoccupied binding sites are quantified through their ability to bind to analyte immobilized onto sensor surface. Because the primary Ab could not generate sufficient SPR signal, amplification step using secondary Ab labeled with Au NP was carried out in secondary immunoreaction.

Figure. 7. shows a representative sensorgram for the secondary immunoreaction followed by primary immunoreaction. 1 ppm-anti-clenbuterol Ab in urine solution was injected at 100 s. After the primary immunoreaction, 1 ppm Ab_2-AuNP (40 nm) was injected. The SPR signal is increased due to binding of primary Ab onto sensor surface. After 120 s injection of secondary Ab labeled with AuNP (40 nm), the sensor surface was washed with the running buffer solution. Then the sensor surface was regenerated with 0.1 M NaOH solution. In the regeneration process, the primary An and secondary Ab-AuNP were detached from the sensor surface. The sensor surface can be used again for the next sample. One detection cycle takes only 1000 s.

Thus, the same sensor surface was used for the premixed solution containing different concentration (0.01 ppt-100 ppb) of clenbuterol spiked urine sample. The obtained angle shifts before and after injection of the premixed sample solution were plotted with respect to the concentration of clenbuterol in the range of 0.01 ppt to 100 ppb in Figure 8. Those plots show the sigmoid typed calibration curves, because this angle shift is caused by the binding event of unreacted antibody. The amount of unreacted antibody is decreased, as the clenbuterol concentration is increasing.

They were almost comparable for urine solution to PBS solution. The limit of detection at 85 % of blank signal was estimated to 0.1 ppt in urine sample using secondary Ab labeled with AuNP, while a 3-times lower LOD (0.03) was estimated in the PBS solution. They were in the same range considering error bar (n=7). The obtained LOD is four orders better than the maximum allowed level of clenbuterol set by the EU commission. Therefore, it is concluded that this protocol is applicable for the real urine sample analysis. It is considered as a fast and sensitive tool for the detection of clenbuterol in the urine sample.

4. Conclusion

In this study, we optimized the pretreatment of real urine of cow for SPR immunosensing. It was found that urine contained inhibitor for the immunoreaction. This inhibitor could not be removed by physical filtration but chemically modified porous silica filter. Among the chemically modified silica filters, carboxyl group terminated silica showed the highest filtration of inhibitor. In addition, pH dependence was also examined. It was noticed that the SPR angle shift increased with decreasing pH. This behavior implied the formation of aggregation of antibody in a low pH due to the protonation of antibody. Therefore, pH adjustment is necessary to control the immunoreaction for real sample analysis.

By comparison with artificial urine regulated by Japan Industrial Standards, it was found that the inhibitor of immunoreaction was not contained in the artificial urine.

Our proposed immunosensing with pretreatment produced the SPR signal of 45 mdeg and a low limit of detection with 0.1 ppt. It was concluded that this system can be used for real urine sample analysis without centrifuge. We expect that this system will be widely used in paddock.

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Figure 1

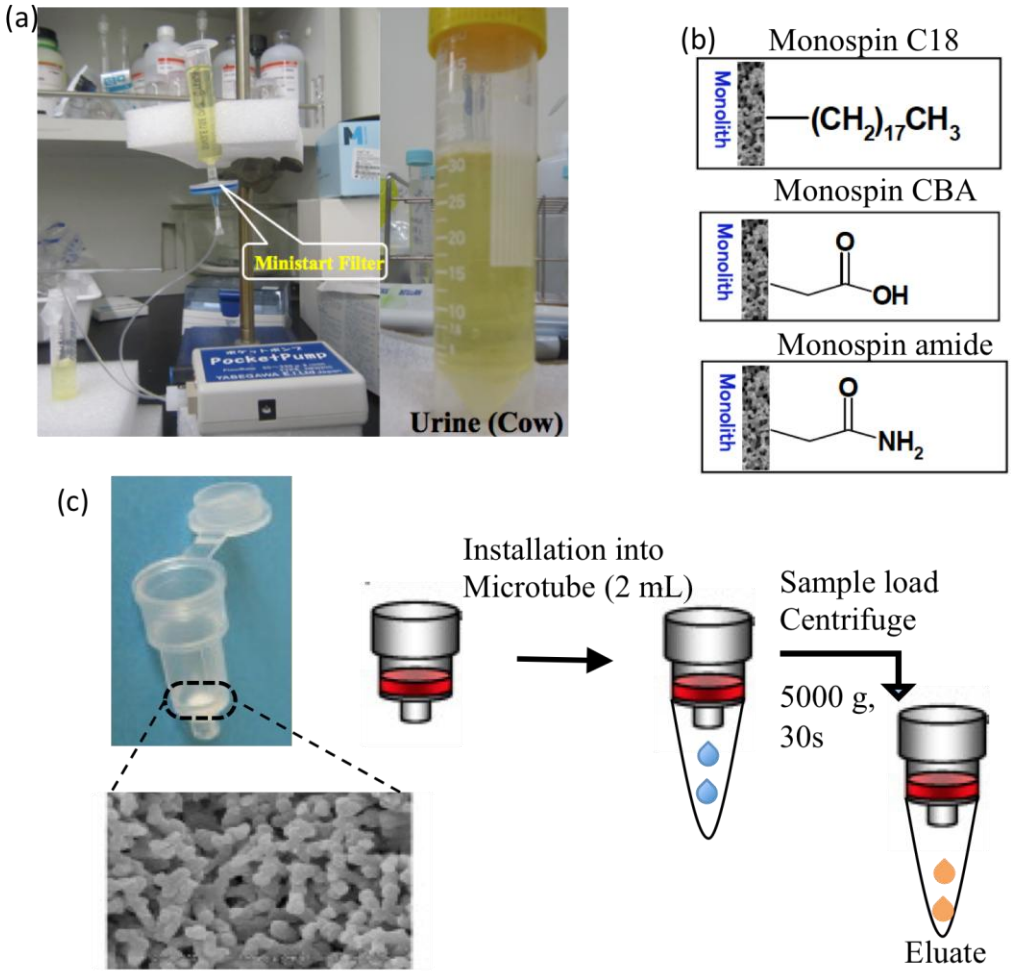


Fig. 1. Summery of filtration procedure (a) physical filtration using minisart syringe filter, (b) Chemical structure of monospin filter (c) Chemical filtration mino-spin filter for clenbuterol spiked urine sample.

Figure 2

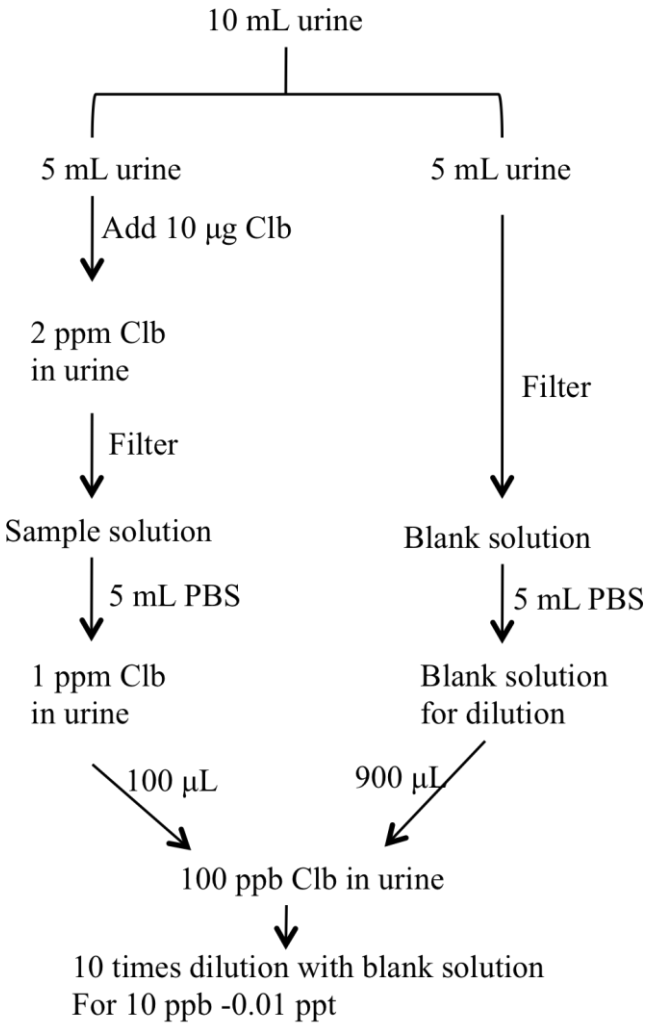


Fig. 2. Clenbuterol spiked urine sample preparation process.

Figure 3

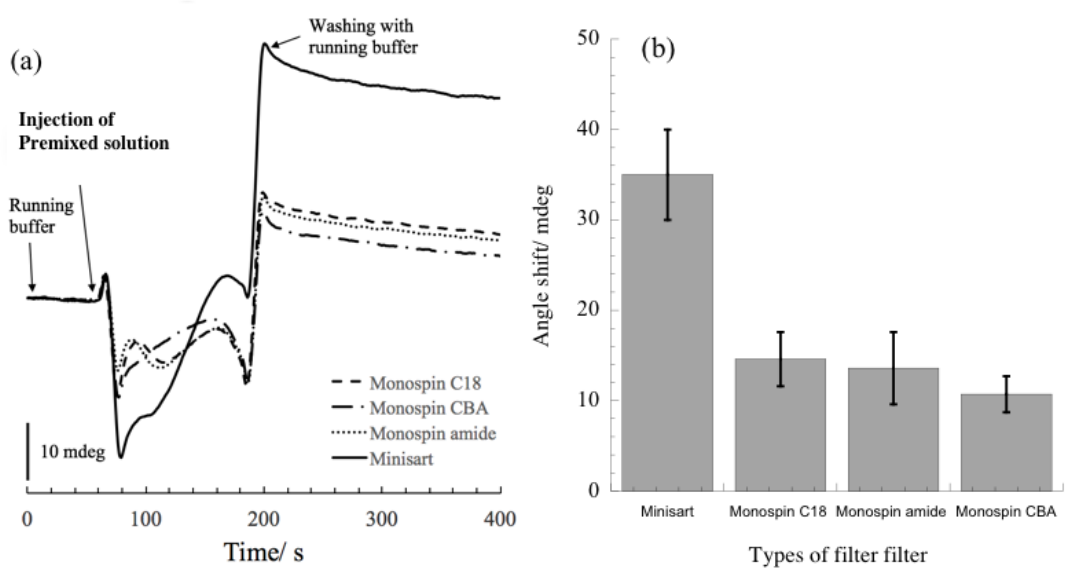


Fig. 3. Comparison of SPR signal change; (a) Sensorgrams of premixed (real urine sample + 1 ppm clenbuterol antibody) solution using different filter. The flow rate was 100 μL min^{-1}. (b) Comparison of signal change for different filter.

Figure 4

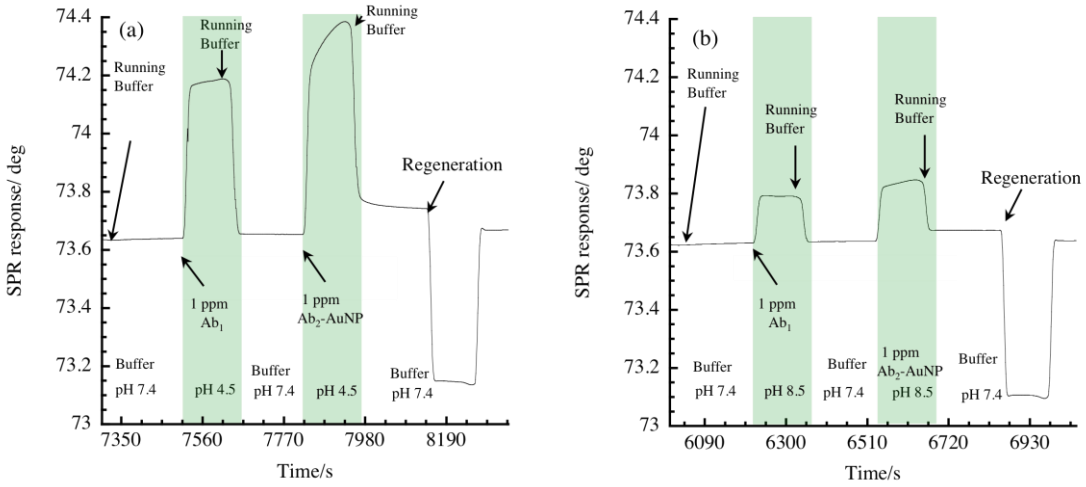


Fig.4. SPR response of primary and secondary immunoreactions (a) pH 4.5 (b) pH 8.5. The flow rate was 100 μL min^{-1}.

Figure 5

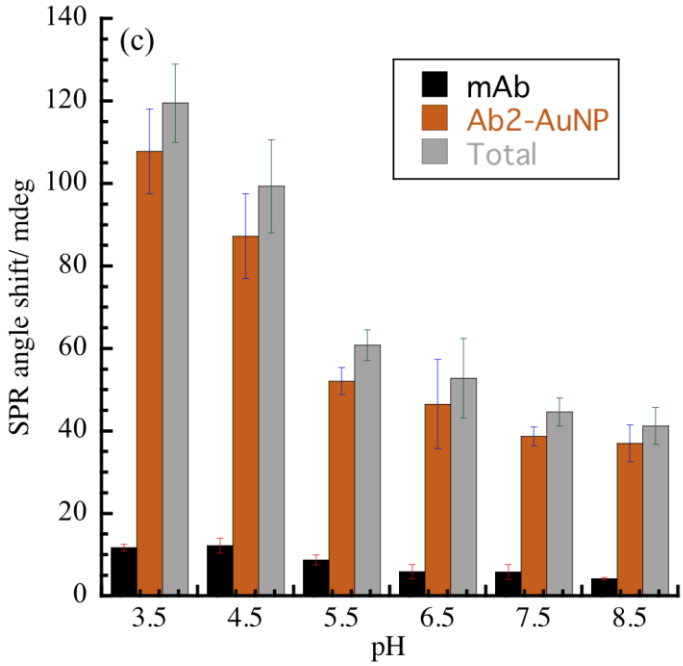


Fig.5. Effect of pH on SPR angle shift.

Figure 6

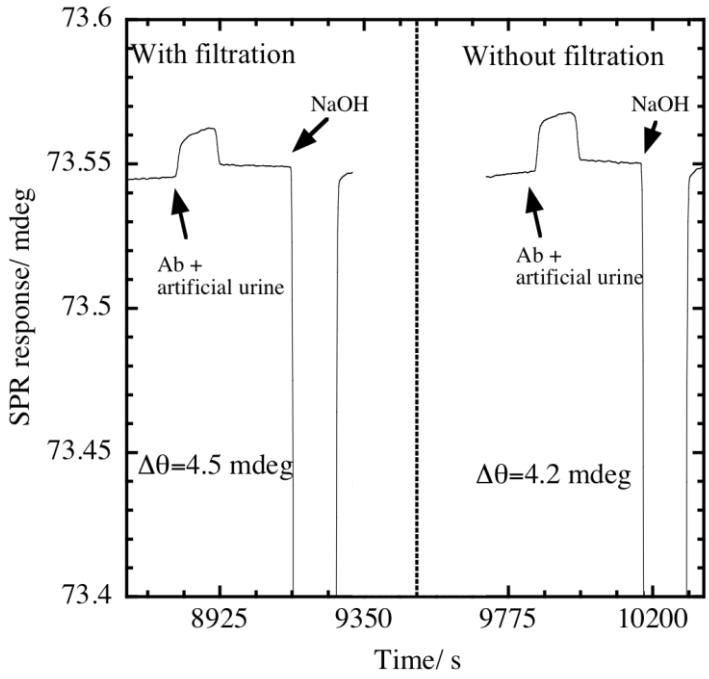


Fig. 6. Comparison of SPR response for filtered and without filtered artificial urine sample by using primary immunoreaction. The flow rate was 100 μL min^{-1}.

Figure 7

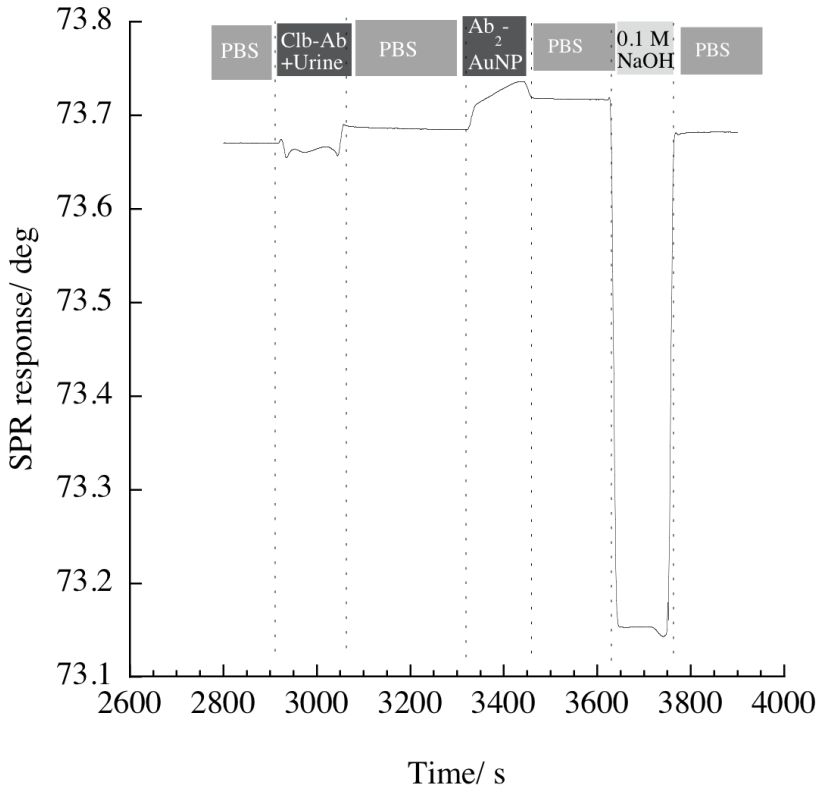


Fig. 7. SPR sensogram represents the angle change for primary and secondary immunoreaction. Urine sample was filtered using monospin amide. The flow rate was 100 μL min^{-1}. 0.1 M NaOH was the regeneration solution.

Figure 8

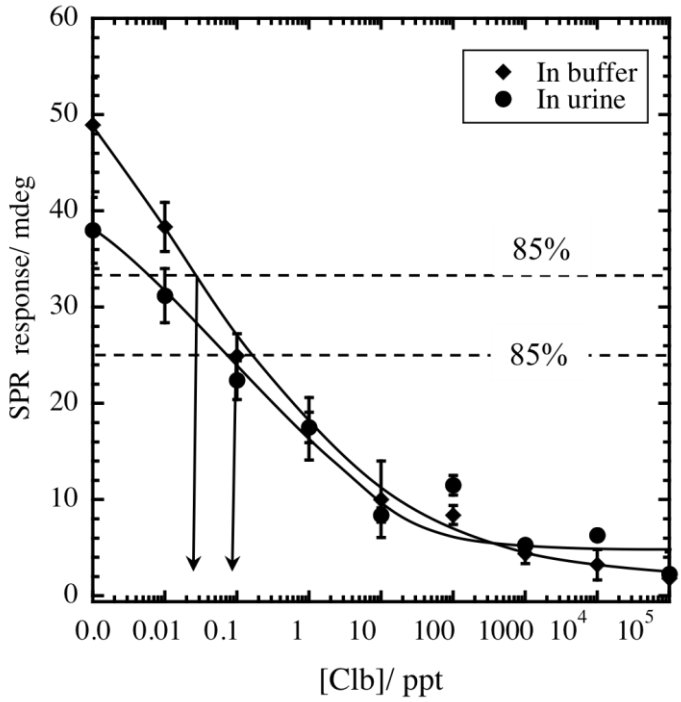


Fig. 8. Comparison of calibration plot performed in ideal PBS buffer solution and in urine filtered with monospin CBA.